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paper

PROBIOTICS IN ANIMAL NUTRITION

Production, impact and regulation

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Contents

Preface

This report presents a unique and exhaustive review of the state-of-knowledge on the use of probiotics in various animal production systems, and their impact on animal productivity. It focuses specifically on definitions, production, mechanisms of action, applications in diverse animal production systems, effects, safety and potential public health risks of probiotics. Also covered are the labelling of probiotic products and global regulatory status of probiotics in animal feed.

The need for this review was prompted by the lack of comprehensive, science-based, and consolidated information on the impact of probiotics on monogastric and ruminant animals. Given that the consumption of animal products has increased at a high pace in the last two decades and is likely to increase substantially, especially in developing countries, there is increasing pressure on the livestock sector to produce more with limited resources. Two of the most important objectives for using probiotics in animal feed are to maintain and improve the performance of the animal, and prevent and control enteric pathogens. In the context of the growing concern with the sub-therapeutic use of antibiotic growth promoters in animal feed and greater appreciation of the role of the microbial ecology of the gastro-intestinal tract in determining animal productivity, increasing numbers of probiotic products are being developed and used in animal nutrition.

The report references over 250 publications on a large number of probiotics being evaluated, and highlights those that have promise, given their demonstrated effectiveness. Knowledge gaps have also been identified.

This in-depth assessment will inform those that are interested in identifying and designing interventions that increase productivity of the livestock sector. It will also help to identify research and development priorities in the area. The current report would also give an impetus to the development of new probiotics having consistent long-term effects that could possibly be used in feed in place of antibiotic growth promoters.

Harinder P.S. Makkar
Editor

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Introduction

The world's population is expected to reach more than 9 billion by 2050, imposing food security challenges particularly for developing countries. Moreover, economic growth has increased the demand for livestock products putting pressure on the livestock sector to produce more with limited resources. Nevertheless, the livestock sector is one of the fastest growing agricultural sectors contributing about 40 percent of the global value of agricultural production (Bruinsma, 2003), supporting the livelihoods and food security of almost 1.3 billion people. This expansion poses issues regarding: the most efficient use of resources to produce food for humans; effects of land conversion and more intensified use on conservation of environmental services and biodiversity; effects of ruminant methane production on climate change; and effects of climate change-induced temperature rise on animal production.

Livestock provide a major source of disposable income for disadvantaged and marginal populations in developing countries, and livestock provides a major entry point to fight against rural poverty (Randolph *et al.*, 2007; Smith *et al.*, 2013). In addition to being a good source of income and nutrition, livestock provide draught power and manure for use as fuel and fertilizer. Also livestock enterprises can offer inflation-proof animal assets for insurance and financing (Sansoucy *et al.*, 1995; Ehui *et al.*, 1998). Intensive production systems are playing an increasingly important role in the livestock sector worldwide, but this increases the need to ensure that animal welfare issues are appropriately considered.

Despite the benefits to many of increased livestock production, this has created two major public health issues. First, sub-therapeutic use of antibiotics as growth promoters in animal feed has evoked widespread concern, with their use banned in many countries, including the European Union (EU), due to the potential to develop antibiotic resistance in microbial populations associated with human and animal diseases. Second, some of the foodborne zoonotic diseases like salmonellosis, campylobacteriosis and pathogenic *Escherichia coli* infection, among others, are serious public health concerns around the world and can cause serious economic loss.

Probiotics (or direct fed microbials) are becoming increasingly popular as one of the alternatives to Antibiotic Growth Promoters (AGP). The most important objectives for using probiotics in animal feed are to maintain and improve the performance (productivity and growth) of the animal and prevent and control enteric pathogens. In the context of the growing concern with the sub-therapeutic use of AGP in animal feed and greater appreciation of the role of the microbial ecology of the gastro-intestinal tract (GIT) in determining animal productivity, increasing numbers of probiotic products are being developed and used in animal nutrition.

Feed additives which can successfully substitute for antibiotic growth promoters (AGP) will provide significant benefits for animal production systems. To realize their potential requires a holistic and systems-based approach to improving production efficiency.

Scope of the document

The scientific background for the effects, safety and regulation of probiotics used in animal feed is the focus of this review. It is not a meta-analysis of the effects of probiotics. Due to variation in the genera, species and strains of micro-organisms, animal species, age, husbandry practices, dose rate and duration of application, it is not possible to complete a meaningful meta-analysis.

This document provides information about probiotics, their mode of action, effects in different categories of livestock, safety and risk associated with their use in animal nutrition and the global regulatory situation. Mention of any commercial product in this document does not mean the endorsement of such products by the authors or the Food and Agriculture Organization of the United Nations.

Probiotics: definition and classification

DEFINITION

The term 'probiotics' was first used by Lilly and Stillwell (1965) to designate unknown growth promoting substances produced by a ciliate protozoan that stimulated the growth of another ciliate. The term now covers a much broader group of organisms. Parker (1974) defined probiotics as "organisms and substances which contribute to intestinal microbial balance" thus including both living organisms and non-living substances. Fuller (1989) was critical of the inclusion of the word 'substances' and redefined probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance".

The joint Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) Working Group defined probiotics as "live micro-organisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). This definition is widely accepted and adopted by the International Scientific Association for Probiotics and Prebiotics (Hill *et al.*, 2014).

The FAO and WHO definition of probiotics as "live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host" is the most widely accepted.

CLASSIFICATION

There is an array of micro-organisms used as probiotics, which can be classified as follows.

1. Bacterial vs Non-bacterial probiotics: With the exception of certain yeast and fungal probiotics, most of the micro-organisms used are bacteria. Examples of bacterial probiotics are several species of *Lactobacillus* (Mookiah *et al.*, 2014), *Bifidobacterium* (Khaksar, Golian and Kermanshahi, 2012; Pedroso *et al.*, 2013), *Bacillus* (Abdelqader, Irshaid and Al-Fataftah, 2013), and *Enterococcus* (Mountzouris *et al.*, 2010). Non-bacterial (yeast or fungal) probiotics include *Aspergillus oryzae* (Daskiran *et al.*, 2012; Shim *et al.*, 2012), *Candida pintolopesii* (Daskiran *et al.*, 2012), *Saccharomyces bourlardii*, (Rahman *et al.*, 2013), and *Saccharomyces cerevisiae* (Bai *et al.*, 2013).
2. Spore forming vs Non-spore forming probiotics: Although non-spore forming *Lactobacillus* and *Bifidobacterium* strains predominated initially, spore forming bacteria are now used, e.g. *Bacillus subtilis* (Alexopoulos *et al.*, 2004a) and *Bacillus amyloliquefaciens* (Ahmed *et al.*, 2014).

3. Multi-species (or multi-strain) probiotics vs Single-species (or single-satrain) probiotics: The microbial composition of probiotic products ranges from a single strain to multi-strain or species compositions (Table 1). Examples of multi-species probiotics are PoultryStar ME (contains *Enterococcus faecium*, *Lactobacillus reuteri*, *L. salivarius* and *Pediococcus acidilactici*) (Giannenas *et al.*, 2012); PrimaLac (contains *Lactobacillus* spp., *E. faecium,* and *Bifidobacterium thermophilum*) (Pedroso *et al.*, 2013); and Microguard (contains various species of *Lactobacillus, Bacillus, Streptococcus, Bifidobacterium* and *Saccharomyces* (Rahman *et al.*, 2013). Single-species probiotics include Bro-bio-fair (*Saccharomyces servisia*) (Abdel-Raheem, Abd-Allah and Hassanein, 2012) and Anta Pro EF (*E. faecium*) (Abdel-Raheem, Abd-Allah and Hassanein, 2012).
4. Allochthonous probiotics vs Autochthonous probiotics: The micro-organisms used as probiotics which are normally not present in the GIT of animals are referred to as allochthonous (e.g. yeasts), while the micro-organisms normally present as indigenous inhabitants of the GIT are referred to as autochthonous probiotics (e.g. *Lactobacillus* and *Bifidobacterium*).

Micro-organisms used in probiotics

Many commercial products use multi-strain probiotics, although the benefits of using more than one strain or species in a single product has not been clearly established (Zhao *et al.*, 2013). Micro-organisms that have been used as probiotics in animal feed are listed in Table1.

TABLE 1
Micro-organisms used as probiotics in animal diets

Species	Strain	Commercial products containing the species	References
ASPERGILLUS			
oryzae	–	–	Daskiran *et al.*, 2012; Shim *et al.*, 2012
niger	–	–	Seo *et al.*, 2010
BACILLUS			
amylolique-faciens	CECT 5940 H57	Ecobiol Norel Animal Nutrition, Madrid, Spain	Ortiz *et al.*, 2013
toyonensis	BCT-7112	Toyocerin Rubinum S.A., Barcelona, Spain	Taras *et al.*, 2005; Kantas *et al.*, 2015
coagulans	ATCC 7050 ZJU0616	–	Adami and Cavazzoni, 1999; Hung *et al.*, 2012
licheniformis	DSM 5749	Microguard, PeterLab Holdings, Negeri Sembilan, Malaysia, LSP 122 Alpharma, Vega Baja, Puerto Rico, BioPlus 2B Chr Hansen, Hørsholm, Denmark, Probios Chr Hansen, Hørsholm, Denmark, BioPlus YC Evonik Industries, Essen, Germany	Alexopoulos *et al.*, 2004a; Rahman *et al.*, 2013
megaterium	–	Microguard, PeterLab Holdings, Negeri Sembilan, Malaysia	Rahman *et al.*, 2013
mesentricus	–	Microguard, PeterLab Holdings, Negeri Sembilan, Malaysia	Rahman *et al.*, 2013
polymyxa	–	Microguard, PeterLab Holdings, Negeri Sembilan, Malaysia	Rahman *et al.*, 2013
subtilis	588, CA #20, DSM 17299, PB6, ATCC-PTA 6737, DSM 5750	GalliPro Evonik Industries, Essen, Germany, Microguard, PeterLab Holdings, Negeri Sembilan, Malaysia, Super-CyC Choong Ang Biotech Co. Ltd., Gyeonggy, South Korea, CloSTATTM Kemin Industries Inc., Des Moines, USA, MicroSource S Agtech Products Inc., Waukesha, USA, BioPlus 2B Chr Hansen, Hørsholm, Denmark, Probios Chr Hansen, Hørsholm, Denmark, BioPlus YC Evonik Industries, Essen, Germany, Enviva Pro DANISCO Animal Nutrition, Wiltshire, UK, Probion Woogene B&G Co. Ltd., Seoul, South Korea	Alexopoulos *et al.*, 2004a; Davis *et al.*, 2008; Rahman *et al.*, 2013; Afsharmanesh and Sadaghi, 2014
BREVIBACILLUS			
laterosporus	–	–	Hashemzadeh *et al.*, 2013

(Continued)

TABLE 1
Micro-organisms used as probiotics in animal diets *(Continued)*

Species	Strain	Commercial products containing the species	References
BIFIDOBACTERIUM			
animalis	503, DSM 16284	PoultryStar ME BIOMIN GmbH, Getzersdorf, Austria, Probios Chr Hansen, Hørsholm, Denmark	Mountzouris *et al.*, 2010; Giannenas *et al.*, 2012; Wideman *et al.*, 2012
bifidium	–	PrimaLac Star Labs, Inc., Clarksdale, USA, Protexin International Animal Health Products, Huntingwood, Australia	Haghighi *et al.*, 2008; Daskiran *et al.*, 2012; Landy and Kavyani, 2013
bifidus	–	Microguard PeterLab Holdings, Negeri Sembilan, Malaysia	Rahman *et al.*, 2013
thermophilus	–	PrimaLac Star Labs, Inc., Clarksdale, USA,	Khaksar, Golian and Kermanshahi, 2012; Pedroso *et al.*, 2013
longum	–	–	Seo *et al.*, 2010
pseudo-longum	–	–	Seo *et al.*, 2010
lactis	–	–	Seo *et al.*, 2010
CANDIDA			
pintolepesii	–	Protexin Probiotics International Ltd., Lopen Head, Somerset, UK	Daskiran *et al.*, 2012
CLOSTRIDIUM			
butyricum	–	Probion Woogene B&G Co. Ltd., Seoul, South Korea	Zhang *et al.*, 2012; Zhao *et al.*, 2013; Zhang *et al.*, 2014a
ESCHERICHIA			
coli	Nissle 1917	–	Hashemzadeh *et al.*, 2013
ENTEROCOCCUS			
faecium	589, NCIMB 11181, E1708, DSM 10663, NCIMB 10415, DSM 16211, DSM 3530, HJEF005	All-Lac Alltech Inc., Nicholasville, USA, PoultryStar ME BIOMIN GmbH, Getzersdorf, Austria, PrimaLac Star Labs, Inc., Clarksdale, USA, Protexin International Animal Health Products, Huntingwood, Australia, Pro-Soluble Probiotics International Protexin Ltd., Somerset, UK, Anta Pro EF Dr. Eckel GmbH, Niederzissen, Germany, Biomin IMBO BIOMIN GmbH, Getzersdorf, Austria, Probios Chr Hansen, Hørsholm, Denmark UltraCruz Santa Cruz Animal Health, Paso Robles, USA	Mountzouris *et al.*, 2010; Giannenas *et al.*, 2012; Khaksar, Golian and Kermanshahi, 2012; Wideman *et al.*, 2012; Abdel-Rahman *et al.*, 2013; Cao *et al.*, 2013; Chawla *et al.*, 2013; Landy and Kavyani, 2013; Pedroso *et al.*, 2013; Zhao *et al.*, 2013
faecalis	–	–	Seo *et al.*, 2010
LACTOBACILLUS			
thermophilus	–	All-Lac Alltech Inc., Nicholasville, USA	Pedroso *et al.*, 2013
acidophilus	–	Probios Chr Hansen, Hørsholm, Denmark, Microguard PeterLab Holdings, Negeri Sembilan, Malaysia, Protexin International Animal Health Products, Huntingwood Australia, UltraCruz Santa Cruz Animal Health, Paso Robles, USA, PrimaLac, Avian PAC Soluble, Probion Woogene B&G Co. Ltd., Seoul, South Korea	Morishita *et al.*, 1997; Haghighi *et al.*, 2008; Daskiran *et al.*, 2012; Khaksar, Golian and Kermanshahi, 2012; Shim *et al.*, 2012; Rahman *et al.*, 2013; Zhang *et al.*, 2014a

(Continued)

TABLE 1
Micro-organisms used as probiotics in animal diets *(Continued)*

Species	Strain	Commercial products containing the species	References
brevis	I 12, I 211, I 218, I 23, I 25	–	Mookiah *et al.*, 2014
bulgaricus	–	Microguard, PeterLab Holdings, Negeri Sembilan, Malaysia, Protexin International Animal Health Products, Huntingwood, Australia	Daskiran *et al.*, 2012; Rahman *et al.*, 2013
casei	CECT 4043	PrimaLac Star Labs, Inc., Clarksdale, USA, , Probios, UltraCruz Santa Cruz Animal Health, Paso Robles, USA	Fajardo *et al.*, 2012; Khaksar, Golian and Kermanshahi, 2012; Landy and Kavyani, 2013
delbrueckii subspecies bulgaricus	–	Protexin International Animal Health Products, Huntingwood, Australia	Daskiran *et al.*, 2012
farciminis	–	Enviva MPI DANISCO Animal Nutrition, Wiltshire, UK	–
fermentum	JS	JSA-101 Gold Well-being LS Co. Ltd., Gangwon, Korea	Bai *et al.*, 2013
gallinarum	I 16, I 26, LCB 12	–	Ohya, Marubashi and Ito, 2000; Mookiah *et al.*, 2014
jensenii	–	–	Sato *et al.*, 2009
paracasei	–	–	Bomba *et al.*, 2002
plantarum	–	Microguard PeterLab Holdings, Negeri Sembilan, Malaysia, Protexin International Animal Health Products, Huntingwood, Australia, UltraCruz Santa Cruz Animal Health, Paso Robles, USA, Probios Chr Hansen, Hørsholm, Denmark	Daskiran *et al.*, 2012; Rahman *et al.*, 2013
reuteri	514, C 1, C10, C16, DSM 16350, DSM 16350	PoultryStar ME BIOMIN GmbH, Getzersdorf, Austria	Mountzouris *et al.*, 2010; Giannenas *et al.*, 2012; Wideman *et al.*, 2012; Mookiah *et al.*, 2014
rhamnosus	–	Protexin International Animal Health Products, Huntingwood, Australia, Enviva MPI DANISCO Animal Nutrition, Wiltshire, UK	Daskiran *et al.*, 2012; Hashemzadeh *et al.*, 2013
lactis	–	Probios Chr Hansen, Hørsholm, Denmark	
salivarius	DSM 16351, I 24	FloraMax-B11 Pacific Vet Group, Fayetteville , USA, PoultryStar ME BIOMIN GmbH, Getzersdorf, Austria	Mountzouris *et al.*, 2010; Biloni *et al.*, 2013; Mookiah *et al.*, 2014
sobrius	–	–	Konstantinov *et al.*, 2008
LACTOCOCCUS			
lactis	CECT 539	–	Fajardo *et al.*, 2012
MEGASPHAERA			
elsdenii	–	–	Seo *et al.*, 2010
PEDIOCOCCUS			
acidilactici	DSM 16210	All-Lac Alltech Inc., Nicholasville, USA, PoultryStar ME BIOMIN GmbH, Getzersdorf, Austria	Mountzouris *et al.*, 2010; Wideman *et al.*, 2012; Pedroso *et al.*, 2013
parvulus	–	FloraMax-B11 Pacific Vet Group, Fayetteville, USA	Biloni *et al.*, 2013

(Continued)

TABLE 1
Micro-organisms used as probiotics in animal diets *(Continued)*

Species	Strain	Commercial products containing the species	References
PREVOTELLA			
bryantii	–	–	Seo *et al.*, 2010
PROPIONIBACTERIUM			
shermanii	–	–	Seo *et al.*, 2010
freudenreichii	–	–	Seo *et al.*, 2010
acidipropi-onici	–	–	Seo *et al.*, 2010
jensenii	–	–	Seo *et al.*, 2010
SACCHAROMYCES			
bourlrdii	–	Microguard, PeterLab Holdings, Negeri Sembilan, Malaysia	Rahman *et al.*, 2013
cerevisiae	KCTC No.7193	JSA-101 Gold, Super-CyC Choong Ang Biotech Co. Ltd., Gyeonggi, South Korea	Shim *et al.*, 2012; Abdel-Rahman *et al.*, 2013; Bai *et al.*, 2013
servisia	–	Bro-biofair Vitality Co., Egypt	Abdel-Raheem, Abd-Allah and Hassanein, 2012
STREPTOCOCCUS			
faecalis	–	–	Haghighi *et al.*, 2008
faecium	–	Microguard PeterLab Holdings, Negeri Sembilan, Malaysia, Avian PAC Soluble Loveland Industries Inc., Colorado, USA	Morishita *et al.*, 1997; Rahman *et al.*, 2013
gallolyticus	TDGB 406	–	Kumar *et al.*, 2014
salivarius subsp. thermophilus	–	Protexin International Animal Health Products, Huntingwood, Australia	Daskiran *et al.*, 2012
bovis	–	–	Seo *et al.*, 2010

Manufacture of probiotics

SELECTION OF MICROBIAL STRAINS

In addition to being non-pathogenic to animals, micro-organisms used as probiotics are selected on the basis of their survival in the gastro-intestinal environment and ability to withstand low pH and high concentrations of bile acids. In addition, the chosen strain should tolerate the manufacturing, transportation, storage and application processes, maintaining its viability and desirable characteristics (Collins, Thornton and Sullivan, 1998). The capacity of potential probiotic micro-organisms to withstand the gastro-intestinal environment can be tested *in vitro* by challenging with low pH (Hood and Zoitola, 1988; Collado and Sanz, 2006). The capacity to tolerate an acidic environment and bile varies among strains (Mishra and Prasad, 2005). Another desirable characteristic is the ability to adhere to the intestinal epithelium, enabling the probiotic strain(s) to colonize the intestine (Guarner and Schaafsma, 1998). In addition, ability to grow rapidly on inexpensive media is a requisite (Collins, Thornton and Sullivan, 1998) for economically viable production.

Spore forming bacteria, particularly from the genus *Bacillus*, are increasingly being used as probiotics. *Bacillus* spores are resistant to physical and environmental factors, such as heat, desiccation and UV radiation (Mason and Setlow, 1986; Nicholson *et al.*, 2000; Setlow, 2006; Cutting, 2011) enabling them to maintain their viability during feed pelleting, storage and handling. *Bacillus lavolacticus* DSM 6475, and two species (total four strains) of *Sporolactobacillus* (*Sp. Inulinus* and *Sp. laevus*) were resistant to pH 3, and *B. racemilacticus* and *B. coagulans* were tolerant of bile (Hyronimus *et al.*, 2000).

FERMENTATION

Fermentation techniques are used either to produce microbial cells in large quantity or to produce extracellular microbial products (e.g. food-grade lactic acid), enzymes, amino acids, vitamins and other pharmaceutical compounds.

Animal studies have used probiotics cultured in the laboratory (Zhou *et al.*, 2010; Shim *et al.*, 2012), or commercially available probiotics. Scaling up from the laboratory to a commercial product is not a trivial process, and quality control is paramount for a beneficial product outcome.

Growth media

Micro-organism-specific growth media, either synthetic or dairy based, are generally used to grow probiotics in an economically viable way (Muller *et al.,* 2009). Approximately 30% of the total cost of fermentation is media cost (Rodrigues, Teixeira and Oliveira, 2006). Dairy based media have been preferred for production of human probiotics, with the use of dairy-based foods such as yoghurt as the carrier. Some countries have legal requirements preventing the use of synthetic media for the production of human probiotics (Muller *et*

al., 2009), but there are no such restrictions for fermentation media for the production of probiotics for animal use.

Use of pure chemical substrates as carbon sources (Javanainen and Linko, 1995; Xiaodong, Xuan and Rakshit, 1997) for fermentation generally results in high quality products. However, agricultural and other industrial by-products are preferred substrates for fermentation because of reduced cost (Hofvendahl and Hahn-Hägerdal, 2000). For example, popular substrates for industrial fermentation are whey (Timmer and Kromkamp, 1994; Øyaas *et al.*, 1996), molasses (Montelongo, Chassy and McCord, 1993; Göksungur and Güvenç, 1997) and starch (Xiaodong, Xuan and Rakshit, 1997). Similarly, yeast extract and peptone are popular nitrogen sources in fermentation media (Chiarini, Mara and Tabacchioni, 1992). Yeast extract can be replaced with cheaper agricultural products (e.g. lentil flour) as nitrogen sources (Altaf *et al.*, 2006). Feed grade vegetable proteins and food grade carbohydrates have also been used for production of commercial probiotics (EFSA, 2008). However, media information is not available for most commercial probiotics.

The ideal growth medium that maximizes microbial growth can be very complex and expensive (Muller *et al.*, 2009). Different probiotic strains generally require different media.

Growth conditions

Temperature and pH affect fermentation growth rates, which are species and strain dependent. Optimum temperatures for *Lactobacillus* strains varies between 25°C and 45°C (Hofvendahl and Hahn-Hägerdal, 2000). Similarly, optimal pH for the growth of probiotics also varies with microbial species and strain. In some cases, pH is set at the beginning of fermentation and allowed to drift (often decreasing due to the production of acids) while fermentation proceeds, while in other cases pH is kept fairly constant by adding buffer (Hofvendahl and Hahn-Hägerdal, 2000; Muller *et al.*, 2009).

Fermentation methods

Probiotics can be produced by either batch or continuous fermentation. In batch fermentation, all of the substrate (sterilized) and the inoculum are mixed together in the fermenter at the beginning and kept at the optimum temperature for the growth of the probiotic. In fed-batch fermentation, limiting nutrients can be added during the fermentation. The reduction of pH in the fermentation medium, to the level where it inhibits the rate of microbial growth, is one of the challenges with batch fermentation and is generally managed by adding a base or a buffer to the medium to maintain pH (Muller *et al.*, 2009). After completion of the fermentation process, which is generally determined by measuring the concentration of probiotic in the fermenter, cells are recovered by centrifugation or filtration (Champagne, Gardner and Lacroix, 2007). Obtaining a high cellular concentration while maintaining low viscosity is an important objective in optimizing the batch fermentation process, as high viscosity hinders the recovery of cells from the growth medium (Champagne, Gardner and Lacroix, 2007). For spore-forming bacteria, vegetative cells are induced to sporulate, generally by limiting nutrient availability, before harvesting. Reduction of pH is another method of triggering sporulation.

With continuous fermentation, fresh growth medium is continuously added to the culture while bacterial cells and any inhibitory substances produced during fermentation are

continuously removed so that continuous production of the probiotic can be maintained (Lamboley *et al.*, 1997; Muller *et al.*, 2009). Genetic drifts due to mutation(s) or to contamination with other bacteria occurring during the fermentation process are issues with continuous fermentation. Batch fermentation has been preferred because it is less costly than continuous fermentation (Muller *et al.*, 2009).

Doleyres, Fliss and Lacroix (2004) developed a two-stage fermentation system as used in yoghurt production. In their laboratory trial, the inoculum strain(s) was immobilized as a pure culture in carrageenan/locust bean gel beads, which then released bacteria at a controlled rate into the linked, continuous fermentation reactor to produce probiotics containing the required ratio of *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* MD and *B. longum* ATCC 15707 cells, but this ratio could not be maintained.

DRYING

After fermentation the bacterial and yeast cells are usually dried for ease of transport and storage thus avoiding any need for specialized facilities for storage and transport of liquid inoculants or frozen cells. Probiotic micro-organisms are generally dried by freeze drying or spray drying (Muller *et al.*, 2009), but vacuum drying and fluidized bed drying are also used. Maintaining cell viability during drying is critical for successful probiotic production (Meng *et al.*, 2008).

Freeze drying

A two-step process of freezing and drying is used. The bacteria are first frozen by using liquid nitrogen or dry ice, or refrigerated at -20°C and then dried under high vacuum to reduce the moisture level to 4% or below (Ananta *et al.*, 2004). The freezing process should be fast enough to avoid the formation of ice crystals inside the cell (Mazur, 1976). Although this is the best method to dry bacteria, in terms of maintaining viability, the high cost associated with the process often hinders its application (Chávez and Ledeboer, 2007).

Similarly, yeast cultures have also been preserved and stored by freeze drying (Kawamura *et al.*, 1995). A modification of the standard freeze drying method involving evaporative cooling can preserve yeast cells for 30 years (Bond, 2007). In this method, a centrifugal head connected with a freeze dryer is used to initially dry the yeast culture mixed with lyoprotectant, followed by secondary drying under vacuum using phosphorus pentoxide as a desiccant. Dehydration of yeast cells with successive reduction in pressure is a feasible alternative to freeze-drying (Rakotozafy *et al.*, 2000).

Spray drying

Fine droplets of probiotic culture, atomized by spraying through a heated nozzle, are sprayed into the drying chamber against hot air (Masters, 1972; Knorr, 1998). The micro-organisms (bacteria or yeast) are dried during the process and collected at the bottom of the chamber (Masters, 1972). The exposure to the high temperature during drying can kill a significant proportion of the vegetative cells, so this is a major constraint (Elizondo and Labuza, 1974). However, the technique is popular because of the low cost of drying for the bulk production of probiotics. It is more suitable for drying spores as the probiotic product.

Probiotic micro-organisms are generally produced by a fermentation process with species- and strain-specific temperature and pH, and mostly dried by a freeze-drying or spray drying process. Growth in inexpensive media is important for commercial production. Probiotics for animal nutrition need to maintain their viability during manufacturing, storage and handling, and quality control is needed to ensure this. Probiotics are selected to presumably withstand the gastro-intestinal environment and adhere to the intestinal epithelium.

Mode of probiotic action

Different probiotics exert their effects through various mechanisms not yet fully understood and presumed to be due to their action either in the gastro-intestinal lumen or the wall of the GIT. Although probiotics are being promoted as a substitute for AGP, the mechanism of action of these feed additives appears to be different (Fajardo *et al.*, 2012).

Probiotics help to prevent and control gastro-intestinal pathogens and/or improve the performance and productivity of production animals through various mechanisms. Closely related strains may differ in their mode of action (Fioramonti, Theodorou and Bueno, 2003; Roselli *et al.*, 2007; Lodemann, 2010). There are increasing numbers of spore forming bacterial strains being used as probiotics. A small proportion of ingested spores is believed to germinate in the intestine of animals (Casula and Cutting, 2002; Tam *et al.*, 2006). However, It is not clear whether the germinated spores or the spores in its ingested form exert beneficial effects on the host. Major mechanisms of action proposed for probiotics are considered in the following sections.

MODIFICATION OF THE MICROBIAL POPULATION OF THE GIT: PROMOTING FAVOURABLE GIT MICROFLORA

Maintaining gut health in animals, particularly in the context of AGP being gradually phased out, through the manipulation of diet is crucial to maintain or improve the performance of production animals (Choct, 2009). One of the major determinants of a healthy GIT is the composition of the microbial population. Probiotics can change the microbial population dynamics in the GIT eventually creating a more favourable microbial population due to a shift in the balance of beneficial and harmful microbes (Mountzouris *et al.*, 2007; An *et al.*, 2008; Mountzouris *et al.*, 2009). Healthy microbial populations in the GIT are often associated with enhanced animal performance, reflecting more efficient digestion and improved immunity (Niba *et al.*, 2009; Hung *et al.*, 2012). The reduction in pathogenic micro-organisms in the GIT may be attributable to the production of antimicrobial substances such as bacteriocins (Shim *et al.*, 2012) and adhesion of the probiotic microbes to the intestinal epithelium, thereby excluding pathogens competitively or by inducing immune system response.

The most common modulation of the GIT microflora by probiotics (for example in chickens) is an increase in the populations of *Lactobacillus* and *Bifidobacteria* (Vahjen, Jadamus and Simon, 2002; Mountzouris *et al.*, 2010; Zhang *et al.*, 2011; Hung *et al.*, 2012; Khaksar, Golian and Kermanshahi, 2012; Shim *et al.*, 2012; Yang *et al.*, 2012a; Abdelqader, Irshaid and Al-Fataftah, 2013; Cao *et al.*, 2013; Landy and Kavyani, 2013; Mookiah *et al.*, 2014; Zhang *et al.*, 2014a) while populations of coliforms particularly *Escherichia coli* (Mountzouris *et al.*, 2010; Samli *et al.*, 2010; Hung *et al.*, 2012; Khaksar, Golian and Kermanshahi, 2012; Shim *et al.*, 2012; Yang *et al.*, 2012a; Abdelqader, Irshaid and Al-Fataftah, 2013; Cao *et al.*, 2013; Landy and Kavyani, 2013; Mookiah *et al.*, 2014; Zhang *et al.*, 2014b) and *Clostridium* spp. (Shim *et al.*, 2012; Yang *et al.*, 2012a; Abdelqader, Irshaid and Al-Fataftah, 2013; Cao *et al.*, 2013) decrease. This pattern of modification of the GIT microflora occurs

with all the common types of bacteria used as probiotics, such as lactic acid bacteria (LAB) (Mountzouris *et al.*, 2010; Cao *et al.*, 2013; Mookiah *et al.*, 2014), spore forming bacteria (*Bacillus* spp.) (Shim *et al.*, 2012; Abdelqader, Irshaid and Al-Fataftah, 2013) and clostridial bacteria (*C. butyricum*) (Zhang *et al.*, 2011; Yang *et al.*, 2012b), and with both Gram positive and Gram negative strains (Hashemzadeh *et al.*, 2013). In contrast, dietary supplementation of broiler diet with a commercial probiotic containing *S. cerevisiae* did not affect total aerobic microbes, lactose faecal coliforms, *Lactobacillus*, and *E. coli* in the content of all intestinal sections (duodenum, jejunum, ileum and caeca) at day 21 (Abdel-Raheem, Abd-Allah and Hassanein, 2012). At day 42 only the population of *Lactobacillus* in the duodenum was increased significantly without change in the population of all other measured microbes (as mentioned above) in all intestinal sections. However, the probiotic induced increased body weight by 9%, feed intake by 3% and feed conversion ratio (FCR) by 6%.

Lactobacilli and bifidobacteria produce proteins or polypeptide bacteriocins which reduce the growth of closely related bacterial species (Yildirim and Johnson, 1998; Kawai *et al.*, 2004), which may reduce the number of harmful micro-organisms in the GIT.

Lactobacillus adheres to the ileal epithelial cells of chickens (Jin *et al.*, 1996). This may competitively exclude pathogenic micro-organisms from the GIT (Mookiah *et al.*, 2014). In addition, these bacteria produce short chain fatty acids (SCFAs) such as acetic and lactic acid, which can inhibit harmful microbes in the GIT (Watkins, Miller and Neil, 1982; Jin *et al.*, 1996; Mookiah *et al.*, 2014).

Probiotics may increase the population of beneficial micro-organisms including lactobacilli and bifidobacteria which then inhibit growth of harmful micro-organisms by producing inhibiting substances (bacteriocins and/or organic acids) and by competitive exclusion.

However, because only a small proportion of the microbial flora in the GIT can be cultured, modern DNA sequencing methods are required to delineate the effects of probiotics on the animals GIT microbiome. In a probiotic dose response study, Mountzouris *et al.* (2010) showed that improvement in the growth rate of chickens occurred without a significant change in the populations of microbes in the GIT assessed using culture based techniques. Inclusion of a multi-strain commercial probiotic (PoultryStar ME) in poultry feed at 10^8 cfu/kg enhanced the growth rate of broiler chickens without an observable effect on caecal microflora composition. Increasing the concentration of the probiotic in feed to 10^9 cfu/kg, however, altered the caecal microbial populations, reducing coliform.

Two important points about the effects of probiotics in gastro-intestinal microbial ecology are: first, there appear to be species-specific effects (discussed later) of probiotics on GIT microflora; and second, traditional culture-based techniques applied in most of the studies are not able to adequately reflect the actual GIT microbial population. As traditional culture-based techniques are extremely limited in their ability to distinguish changes in microbial ecosystems, the application of modern molecular identification and sequencing techniques are required to provide insight into the effects of probiotics on the GIT microbiota.

INCREASE IN DIGESTION AND ABSORPTION OF NUTRIENTS

Improvements in productivity of animals due to probiotics can be associated with an increase in digestion and absorption of nutrients. The response in broiler chickens to dietary supplementation with *L. bulgaricus* varied with level of probiotic provided. At a rate of 2 $\times 10^6$ cfu/g there was no significant effect on digestibility of crude protein or fat, but at 6 $\times 10^6$ cfu/g and 8 $\times 10^6$ cfu/g there was a significant increase, ranging from 7 to 11% for protein and 6.5 to 13.4% for fat, with 7.9 to 11.7% increase in weight gain (Apata, 2008). In another study, although supplementation of broiler diet a commercial probiotic (AgiPro A100) increased digestibility of dry matter (DM) by 12.4% at day 42 (Li *et al.*, 2008) weight gain, average daily gain, feed intake and FCR were not significantly affected. Similarly, probiotics increased the apparent ileal digestibility of essential amino acids, with 5% improvement in body weight gain (Zhang and Kim, 2014) and improved the bioavailability of calcium in chicken (Chawla *et al.*, 2013).

Increased digestibility of nutrients in diet may be due to increased enzyme activity in the intestine due to probiotics. *Lactobacillus* probiotics altered the digestive enzyme activity in the GIT of poultry and pigs. Amylase activity in the small intestine of poultry increased by 42% in response to *L. acidophilus* supplied at a rate of 2 $\times 10^6$ cfu/g of maize-soybean-based diet (Jin *et al.*, 2000). However, there was no change in proteolytic and lipolytic activity. This improvement in amylase activity was associated with a 4.6% increase in body weight gain and 5% improvement in feed use efficiency. Similarly, sucrose, lactase and amylase but not peptidase activity in the small intestine of pre-weaned pigs increased in response to a commercial probiotic (Probios) containing *L. plantarum, L. acidophilus, L. casei and E. faecium* (Collington, Parker and Armstrong, 1990).

Spore forming bacteria, like *Bacillus amyloliquefaciens,* produce extracellular enzymes including α-amylase, cellulase, proteases and metalloproteases (Gould, May and Elliott, 1975; Gangadharan *et al.*, 2008; Lee *et al.*, 2008) which could increase nutrient digestion.

Increased enzyme activity in the GIT of animals supplemented with probiotics could be due to either production of enzyme by the probiotic itself or induced change in the microbial population and thence enzyme production.

Probiotics increased the height of intestinal villi and villus height:crypt ratio in poultry (see later) (Biloni *et al.*, 2013; Jayaraman *et al.*, 2013; Afsharmanesh and Sadaghi, 2014), thus increasing the surface area for nutrient absorption.

PRODUCTION OF ANTIMICROBIAL SUBSTANCES

Some probiotics produce antimicrobial substances that may inhibit growth of pathogenic micro-organisms in the intestine.

Many bacterial species, including lactic acid bacteria (LAB) (Klaenhammer, 1988; Nes *et al.*, 1996; Flynn *et al.*, 2002), bifidobacteria (Cheikhyoussef *et al.*, 2008) and bacillus (Hyronimus, Le Marrec and Urdaci, 1998; Le Marrec *et al.*, 2000), can produce several types of thermostable bacteriocins (Cotter, Hill and Ross, 2005) which have antimicrobial activity against a range of potential pathogens of animals including *Bacillus, Staphylococcus, Enterococcus, Listeria*, and *Salmonella* species (Flynn *et al.*, 2002; Corr *et al.*, 2007; Rea *et al.*, 2007). Corr and colleagues (2007) demonstrated that the probiotic *L. salivarius* strain UCC118 produced a broad spectrum bacteriocin, Abp118, which protected mice against

pathogenic *Listeria monocytogenes*. A mutant of the same probiotic unable to produce bacteriocins did not protect the mice, confirming bacteriocins were the active agent.

Bacteriocin produced by LAB (for example Nisin) inhibits the growth of pathogenic micro-organisms by inhibiting cell wall synthesis, with the formation of pores in the bacterial surface (Wiedemann *et al.*, 2001; Hassan *et al.*, 2012). To achieve this, the bacteriocin binds the cell wall precursor, lipid II, forming a complex which can form a pore in the bacterial cell membrane leading to the death of the bacterium (Wiedemann *et al.*, 2001; Bierbaum and Sahl, 2009).

Many probiotic bacteria, especially LAB producing SCFAs, particularly lactic and acetic acids, can inhibit pathogenic bacteria (Commane *et al.*, 2005; Fayol-Messaoudi *et al.*, 2005). SCFAs reduce the pH in micro-environments within the intestinal lumen and can then be taken up by GIT microbes in broiler chickens, reducing their intracellular pH to a lethal level for some bacteria (Daskiran *et al.*, 2012).

Probiotic bacteria produce other antimicrobial compounds that may inhibit harmful microbes in the GIT. Brashears, Reilly and Gilliland (1998) found that *Lactobacillus lactis* strains, when inoculated in refrigerated raw chicken meat inoculated with *E. coli* 0157: H7 inhibits the growth of *E. coli* 0157: H7 due to production of hydrogen peroxide. Does *Lactobacillus* produce hydrogen peroxide in the gastro-intestinal environment? *B. subtilis* PB6, a bacterial strain isolated from the GIT of chickens produces a heat stable anticlostridial factor, which inhibited *Clostridium perfringens*, the causative agent of necrotic enteritis in poultry, *in vitro* as well as *Clostridium difficile*, *Streptococcus pneumoniae, Campylobacter jejuni,* and *Campylobacter coli* (Teo and Tan, 2005). Similarly, *B. amyloliquefaciens,* a probiotic that improved performance of broiler chickens (Ahmed *et al.*, 2014; Lei *et al.*, 2015), produces several antimicrobial cyclic lipopeptide compounds (e.g. surfactin, fengycin, bacillomycin D, iturin A) (Sun *et al.*, 2006; Ongena and Jacques, 2008; Chen *et al.*, 2009; Arrebola *et al.*, 2010) and polyketides (e.g. macrolactin, difficidin, bacillaene, chlorotetaine) (Rapp *et al.*, 1988; Chen *et al.*, 2009) which antagonise the growth of plant pathogens (Chen *et al.*, 2009).

ALTERATION IN GENE EXPRESSION IN PATHOGENIC MICRO-ORGANISMS

Bacteria communicate cell to cell through the secretion of chemical signals, called auto-inducers, which affect the behaviour of bacteria (Miller and Bassler, 2001; Waters and Bassler, 2005). This process of bacterial communication, called quorum sensing, is also used for communication between bacteria and their host (Hughes and Sperandio, 2008).

Probiotics may affect quorum sensing in pathogenic bacteria, thus influencing their pathogenicity. Extracellular secretion of a chemical signal (autoinducer-2) by human enterohaemorrhagic *E. coli* serotype O157:H7 was substantially inhibited by fermentation products from *L. acidophilus* La-5, resulting in the suppression of the virulence gene (LEE – locus of enterocyte effacement) expression *in vitro*. This disrupts quorum sensing and eventually prevents GIT colonization by *E. coli* serotype O157:H7 in the GIT (Medellin-Peña *et al.*, 2007).

IMMUNOMODULATION

The GIT component of the immune system protecting the host from the different types of antigens in the lumen of the GIT is affected by probiotics. Both innate and adaptive immunity are affected by probiotics.

Improvement in innate gut immunity through restitution of intestinal barrier function

Epithelial cells in the gastro-intestinal mucosa create a selectively permeable barrier between the intestinal lumen (which contains harmful substances such as foreign antigens, micro-organisms and toxic materials, as well as beneficial nutrients) and the internal environment of the body (Blikslager *et al.*, 2007; Groschwitz and Hogan, 2009). This barrier is the first line of defence against the microbes in the GIT (Baumgart and Dignass, 2002; Peterson and Artis, 2014). It has a combined defence function, incorporating anatomical structures, immunological secretions consisting of mucus, immunoglobulins, e.g. IgA, antimicrobial peptides, and the epithelial junction adhesion complex (Baumgart and Dignass, 2002; Ohland and MacNaughton, 2010). Disease conditions which cause immunological disturbances disrupt this barrier (Turner, 2009), inducing inflammation of the intestinal wall, and intestinal disorders (Hooper *et al.*, 2001; Sartor, 2006).

Probiotic formulations prevent chronic inflammation of the GIT through stimulation of innate immunity in the gastro-intestinal epithelium (Galdeano and Perdigon, 2006; Pagnini *et al.*, 2010). For example, a high dose (50 $\times 10^9$ cfu/day) of a probiotic formulation (VSL#3) containing four strains of lactobacilli (*L. casei, L. plantarum, L. acidophilus* and *L. delbrueckii* subspecies *bulgaricus*); three strains of bifidobacteria (*Bi. longum, Bi. breve* and *Bi. infantis*); and one strain of streptococcus (*S. salivarius* subspecies *thermophilus*), when fed to senescence-accelerated-prone mice for six weeks either completely prevented ileitis or significantly reduced the severity of inflammation (Pagnini *et al.*, 2010). Although this probiotic formulation was found to prevent ileitis, it was ineffective in treating the inflammation when administered to older mice that had already developed ileitis (Pagnini *et al.*, 2010).

Experiments in animal models have shown that improvement in intestinal barrier function by probiotics is due to a reduction in the permeability of the intestinal epithelium. Translocation of intestinal microbes out of intestinal sites and into sites such as the liver, spleen and mesenteric lymph nodes decreased in mice with induced colitis and pre-treated with *Lactobacillus* probiotics (Mao *et al.*, 1996; Pavan, Desreumaux and Mercenier, 2003; Llopis *et al.*, 2005). Translocation of enterotoxigenic *E. coli* to mesenteric lymph nodes was reduced in post-weaning piglets with dietary supplementation of probiotic *P. acidilactici* compared with the control group after enterotoxigenic *E. coli* challenge (Lessard *et al.*, 2009).

Generally, timing of probiotic treatment is very important in maintaining intestinal barrier function. Administration of probiotics before the infectious or pathogenic agent is introduced experimentally, or before the pathogens enter the GIT and multiply naturally, is the most effective time for probiotic introduction (Lodemann, 2010).

Stimulation or suppression of immune response

The immune response in the host should be sometimes stimulated (for example infection and immunodeficiencies) while it should be supressed in some other cases (for example allergy and autoimmune diseases) based on the clinical condition (Borchers *et al.*, 2009). Diets containing probiotics could modulate the host immune response.

The responses are complicated as they vary with the probiotic strain or species, with the dose level, and may differ in their effect pre- and post-weaning, and whether the antigen is a bacterium, such as *Salmonella,* or a virus, such as the human rotavirus.

The pattern of immune-response-related blood plasma cells can vary between the ileum and jejunum lymph tissue. Probiotics can affect the expression of the anti-inflammatory cytokine or cell signalling proteins, and may do so differentially depending on the cytokine. Can probiotics "prime" the immune system in commercial operations to support response to animal and/or human bacterial and viral disease antigens and reduce their shedding in faeces? These are very complicated responses and the variation between probiotic strains means that there is no general "story" about the way probiotics might affect the immune system.

However, the significant outcome is that probiotic microbes can modulate the immune system and response to pathogen antigens, and a systems-based approach is required to address the response to a probiotic in terms of host disease susceptibility, shedding of pathogens (both human and/or porcine), growth and feed use efficiency, as a guide to what probiotic a producer might wish to use. It may depend on what is the dominant factor needing to be addressed in the production system. With increasing community (and regulatory) pressure to reduce antibiotic use in commercial animal production, modulation of the immune system by probiotics is a major potential benefit to be factored into production systems.

Several studies have demonstrated immunostimulatory effects of probiotics. Bai *et al.* (2013) demonstrated that a probiotic containing *L. fermentum* and *S. cerevisiae* stimulated the intestinal T-cell immune system, indicated by increased production of CD3+, CD4+ and CD8+ T-lymphocytes in the GIT of broiler chickens. Expression of CD3+, IL-2 and IFN-γ genes was significantly greater in the small intestine of neonatal chicks (day 3 and 7) fed with probiotics *L. jensenii* TL2937 and *L. gasseri* TL2919 than in the control without probiotics (Sato *et al.*, 2009). Dalloul *et al.* (2003) found similar effects of probiotics on the intestinal immune system of broiler chickens treated with a commercial probiotic product (Primalac) containing *L. acidophilus, L. casei, E. faecium* and *Bi. bifidium* and infected with coccidia oocysts, the response being an increased population of intestinal intraepithelial lymphocytes (IEL) compared with control birds not given the probiotic. An increase in expression of CD3+, CD4+, CD8+ and $\alpha\beta$TCR (T Cell Receptor - a double chain glycoprotein on the surface of the T cell) was observed. Probiotic *B. cereus* var. *toyoi* also caused significant increase in the intraepithelial population of CD8+ T cells in intestine of piglets (Scharek *et al.*, 2007). Similarly, administration of probiotic *E. faecium* to broiler chickens challenged with *E. coli* resulted in increased concentrations of cytokines (IL-4 and TNF-α) and IgA in the small intestinal mucosa (Cao *et al.*, 2013).

Probiotics also increase serum immunoglobulin levels. A multi-strain probiotic containing *L. acidophilus, B. subtilis* and *C. butyricum* increased serum levels of IgA and IgM in chickens (Zhang and Kim, 2014). Likewise, addition of a commercial product (Gallipro) containing *B. subtilis* to broiler chicken diets increased the antibody response to sheep red blood cells administration (Afsharmanesh and Sadaghi, 2014). Antibody titre against the common poultry diseases Newcastle Disease, Infectious Bronchitis and Infectious Bursal Disease was increased by the use of probiotic product Primalac (Landy and Kavyani, 2013).

In the piglets, probiotic *L. fermentum* I5007 modulated immune function in piglets by enhancing T cell differentiation and upregulating ileum cytokine expression (Wang *et al.*, 2009). Similarly, probiotic containing *P. acidilactici* and *S. cerevisiae* subsp. *boulardii* increased T cells in ileum and IgA secretion in post-weaning piglets challenged with enterotoxigenic *E. coli* (Lessard *et al.*, 2009).

In contrast, some studies have shown immunosuppressive action of probiotics in the host. *E. faecium* NCIMB 10415 had an immunosuppressive effect, delaying early immune response to antigens in post-weaning piglets (Siepert *et al.*, 2014). *E. faecium* NCIMB 10415 reduced proliferation of blood mononuclear cells in response to *Salmonella* serovar *typhimurium* DT104 antigen during 1 to 3 days post-infection, followed by a similar proliferative response with or without the probiotic 7 days post-infection (Siepert *et al.*, 2014). Similarly, expression of intestinal immune-associated genes, especially during the post-weaning period, were reduced (Siepert *et al.*, 2014). In the post-weaning period, expression of IL-8, IL-10 and CD86 (cluster of differentiation 86) genes in ileal Peyer's patches was significantly reduced in probiotic-treated piglets. In contrast, probiotic caused increased expression of IL-10 gene and CTLA4 (T cell inhibitory molecule) in Jejunal Peyer's patches in the post-weaning period. Blood serum inflammation-related cytokines IL-6 and IL-8 were not affected by the probiotic.

In an earlier study, supplementation of piglet diet with the same probiotic strain (*E. faecium* NCIMB 10415) had no effect on the lymphocyte populations in the jejunal Peyer's patches (Scharek *et al.*, 2005). The serum level of immunoglobulin IgG was reduced in probiotic-treated piglets during the post-weaning period (28-56 days) but was not affected in the pre-weaning period (Scharek *et al.*, 2005).

In another study, oral administration of *L. brevis* ATCC 8287 at the high dose rate of 10^{10} cells per animal per day to weaned piglets reduced expression of IL-4, IL-6 and TGFβ1 genes in the ileum, and increased expression of IL-4 and IL-6 genes in the jejunum, caecum and colon (Lähteinen *et al.*, 2014). However this change in cytokine gene expression in the intestine did not change the systemic humoral immune response. Levels of serum immunoglobulins IgA and IgG were the same in control and probiotic-treated piglets.

Drenching of *L. acidophilus* strain NCFM at low dose rates (up to 10^6 cfu/dose × 5 doses) significantly increased the population of the antiviral interferon IFN-γ producing T cells and reduced the regulatory T cells and production of TGFβ1 and IL-10 in intestinal lymphoid tissue of gnotobiotic piglets compared with untreated animals (Wen *et al.*, 2012). In contrast, the same probiotic when administered at a high dose rate (up to 10^9 cfu/dose × 14 doses) increased regulatory T cells.

Such dose-dependent responses could be one of the reasons for variable results in different studies and with different probiotics. The gastro-intestinal microbial profile of the host also could influence the immune response of the host against specific probiotic (Borchers *et al.*, 2009).

COLONIZATION RESISTANCE

The GIT of neonatal animals and birds reared naturally are colonized with micro-organisms, generally originating from the adult (mother). These micro-organisms provide protection from enteric pathogens. Intensification of animal agriculture has reduced the opportunity for natural colonization of the GIT, making animals more susceptible to intestinal pathogen challenge. Probiotics could mimic natural colonization in neonates, or colonize adult animals, preventing pathogenic organisms from colonizing the intestinal mucosa.

Certain strains of *Lactobacillus* and *Bifidobacterium* possess hydrophobic surface layer proteins which help the bacteria to non-specifically adhere to the animal cell surface

(Coconnier *et al.*, 1992; Bernet *et al.*, 1994; Hudault *et al.*, 1997; Tuomola and Salminen, 1998; Bibiloni *et al.*, 2001; Johnson-Henry *et al.*, 2007). Such adhesion of probiotic bacteria to the intestinal epithelium covers the receptor binding sites, preventing pathogenic micro-organisms like *E. coli* O157:H7, *Salmonella,* etc., from attaching to the epithelium (Bernet *et al.*, 1994; Hudault *et al.*, 1997; Johnson-Henry *et al.*, 2007).

There are several proposed modes of action of probiotics. Some of these mechanisms are associated with the inhibition of enteric pathogenic micro-organisms, while others are responsible for improved animal performance. Different probiotics may have similar mode(s) of action, while a specific strain could function through multiple mechanisms. For example, several probiotic strains have similar effects on the gastro-intestinal microbial population. However, modes of action of specific probiotics are generally not understood. In most of the studies about effects of probiotics on performance, the exact mode of action of probiotics is not fully understood. Because closely related probiotic micro-organisms appear to have different modes of action, mechanisms need to be studied on a case-by-case basis. Effects of probiotics are the outcome of interaction between host and probiotic micro-organism. Therefore, further studies on host-microbes interaction could elucidate the probiotic mode of action. The rapid advances in molecular methods and DNA sequencing used to study microbial ecology will greatly facilitate our understanding of the way probiotics work.

Probiotic application in different livestock production systems

PROBIOTICS IN POULTRY NUTRITION

Poultry are the cheapest source of animal protein, contributing significantly to supplying the growing demand for animal food products around the world (Farrell, 2013). The consumption and trade in poultry products is increasing rapidly as the human population increases, making it the second largest source of meat after pork (FAO, 2014).

Probiotics can improve broiler chicken growth rates (Afsharmanesh and Sadaghi, 2014; Mookiah *et al.*, 2014; Zhang and Kim, 2014; Lei *et al.*, 2015) and control or prevent enteric diseases, including; salmonellosis (Haghighi *et al.*, 2008; Tellez *et al.*, 2012; Biloni *et al.*, 2013), necrotic enteritis (Jayaraman *et al.*, 2013) and coccidiosis (Dalloul *et al.*, 2003). However the outcomes from probiotic use are not consistent.

Growth rate

Probiotics have enhanced the growth rate in broilers better than AGP (avilamycin) (Zhang and Kim, 2014) and other substitutes for AGP, such as phytochemicals (e.g. essential oils) (Khaksar, Golian and Kermanshahi, 2012). However, the general applicability of the probiotic approach as an alternate for AGP is not yet well established.

Probiotics ranging from non-spore forming LAB to spore formers and yeast have been evaluated for their potential to improve growth rates in commercial poultry production (Shim *et al.*, 2012; Bai *et al.*, 2013; Afsharmanesh and Sadaghi, 2014). In many cases the improvement in growth rate in the probiotic treated birds was associated with increased feed intake (Abdel-Raheem, Abd-Allah and Hassanein, 2012; Landy and Kavyani, 2013; Lei *et al.*, 2015) and improved feed use efficiency (Mountzouris *et al.*, 2010; Shim *et al.*, 2012; Zhang and Kim, 2014) compared with untreated birds. Therefore, increased digestibility of feed resulting in improved feed use efficiency could be one of mode of actions for improved growth rate. Also, the differences in performance between treated and untreated birds may be due to a change in microbial populations in the GIT resulting increased production of SCFA and immuno-modulation (Zhao *et al.*, 2013). Increased growth rate has also been associated with increased villus height, which increases absorption of nutrients from the intestine.

In contrast, some probiotics did not improve growth in broilers (Fajardo *et al.*, 2012; Hung *et al.*, 2012; Zhao *et al.*, 2013) even with the same species of probiotic microbe. For example, Cao *et al.* (2013) found that *E. faecium* (HJEF005) at 10^9 cfu/kg of feed improved growth rate in male Cobb broilers challenged with *E. coli,* while Zhao *et al.* (2013) using a different strain (LAB 12 – CGMCC 4847), fed at the rate of 2×10^9 cfu/kg of feed to male Ross broilers, found no growth effect. Use of different broiler breeds in these two studies or different probiotic strains could be the reason for contrasting results. Recent studies sug-

gested that probiotics could be more effective when used with pre-biotics (Mookiah *et al.*, 2014). "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastro-intestinal microflora that confers benefits upon host wellbeing and health" (Gibson *et al.*, 2004).

Effects of probiotics on growth in poultry are detailed in Table 2.

One of the interesting observations from probiotic feeding trials in poultry is that some promote growth in the starter (early) phase (Bai *et al.*, 2013) while others affect the grower-finisher (later) phase (Shim *et al.*, 2012; Abdel-Rahman *et al.*, 2013; Chawla *et al.*, 2013) (Table 2). Other studies found improved growth throughout the broiler production cycle (Cao *et al.*, 2013; Landy and Kavyani, 2013; Rahman *et al.*, 2013; Mookiah *et al.*, 2014) (Table 2). The underlying reason for this difference is not known, but presumably relates to the dynamics of the gut microbiota. Whether different probiotics should be used in particular growth periods, i.e. *choosing the right probiotic for the right time*, remains to be determined.

Many strains of probiotic microbes improve the growth rate of poultry, but results can be inconsistent.

Feed intake and feed efficiency

As feed is the largest cost in poultry production, small improvements in feed use efficiency have a significant economic impact. The improvement in performance and productivity of poultry due to the use of probiotics in feed has been attributed to increased feed intake and improved feed efficiency (Shim *et al.*, 2012) but this is not always the outcome. Probiotics can:

- Increase feed intake without significant improvement in feed conversion ratio (FCR) (Afsharmanesh and Sadaghi, 2014)
- Improve FCR without significant difference in feed intake (Mountzouris *et al.*, 2010; Shim *et al.*, 2012; Zhang *et al.*, 2012; Zhang and Kim, 2014) and
- Increase feed intake along with significant improvement in FCR (Landy and Kavyani, 2013).

In contrast, Hung *et al.* (2012) found that dietary use of the probiotic *B. coagulans* reduced the average daily feed intake by 8% in the broiler grower-finisher phase (days 22–42) with reduction in FCR by 10%. Similarly, Amerah *et al.* (2013) administered a commercial probiotic (Enviva Pro 202 GT; Danisco Animal Nutrition, Marlborough, UK) containing three strains of *B. subtilis* (strains (BS8, 15AP4 and 2084) during grower/finisher phase of a 42-day feeding trial and found a reduction in feed intake of 2% along with reduction in FCR of 2.7%. Similarly, Mookiah *et al.* (2014) found a reduction in feed intake of 5.6% during the starter phase (1–21 days) in birds treated with a multi-strain probiotic containing 11 *Lactobacillus* strains (*L. reuteri* C1, C10 and C16; *L. gallinarum* I16 and I26; *L. brevis* I12, I23, I25, I218 and I211, and *L. salivarius* I24). However, FCR was improved in both starter (by 7.3%) and finisher phase (by 12%).

TABLE 2
Probiotic effects on performance of poultry

Micro-organisms	Commercial products*	Growth rate/Final body weight			Feed intake	Feed Conversion Ratio			Histomorphology		References
		Pre-starter - starter phase	Grower-finisher phase	Over all *(lifetime)*		Pre-starter - starter phase	Grower-finisher phase	Over all *(lifetime)*	Villus height	Villus heightcrypt depth radio	
B. subtilis	GalliPro PrimaLac	NS	_	S (+)	S (+)	NS	_	_	S (+)	S (+)	Afsharmanesh and Sadaghi, 2014
B. subtilis	Super-CyC	NS	S (+)	S (+)	_	NS	_	_	_	_	Abdel-Rahman *et al.*, 2013
E. faecium	Anta Pro EF	NS	S (+)	S (+)	_	NS	_	_	_	_	Abdel-Rahman *et al.*, 2013
L. fermentum *S. cerevisiae*	JSA -101 Gold	S (+)	NS	_	S (+)	S (-)	NS	NS	_	_	Bai *et al.*, 2013
L. salivarius *P. parvulus*	FloraMax-B11	NS	_	_	_	_	_	_	S (+)	NS	Biloni *et al.*, 2013
E. faecium	_	NS	S (+)	_	_	_	_	_	_	_	Chawla *et al.*, 2013
B. coagulans	_	NS	NS	NS	NS	S (-)	S (-)	S (-)	NS	NS	Hung *et al.*, 2012
B. coagulans	_	_	_	S(+)	_	_	_	S (-)	_	_	Zhou *et al.*, 2010
L. acidophilus, *B. subtilis* *S. cerevisiae* *A. oryzae*	_	NS	S (+)	S (+)	NS	S (-)	S (-)	S (-)	_	_	Shim *et al.*, 2012
L. reuteri *E. faecium* *Bifidobacterium animalis* *Pediococcus acidilactici* *L. salivarius*	PoultryStar ME	NS	S (+)	S (+)	NS	NS	S (-)	S (-)	_	_	Mountzouris *et al.*, 2010
C. butyricum	_	NS	S (+)	S (+)	S (+)	NS	NS	NS	_	_	Zhao *et al.*, 2013
E. faecium	_	NS	NS	NS	NS	NS	NS	NS	_	_	Zhao *et al.*, 2013

(Continued)

Micro-organisms	Commercial products*	Growth rate/Final body weight			Feed intake	Feed Conversion Ratio			Histomorphology		References
		Pre-starter - starter phase	Grower-finisher phase	Over all *(lifetime)*		Pre-starter - starter phase	Grower-finisher phase	Over all *(lifetime)*	Villus height	Villus height:crypt depth ratio	
L. acidophilus, *B. subtilis* DSM 17299, *C. butyricum*	Probion	NS	S (+)	S (+)	NS	NS	S (-)	NS	_	_	Zhang and Kim, 2014
L. acidophilus *L. bulgaricus* *L. plantarum* *S. faecium* *Bi. bifidus* *B. subtilis* *B. licheniformis* *B. megaterum* *B. mesentricus* *B. polymyxa* *S. bourlrdii*	Microguard	S (+)	S(+)	S(+)	_	_	_	_	_	_	Rahman et al., 2013
E. faecium		S (+)	S (+)	S (+)	_	_	_	_	S (+)	S (+)	Cao et al., 2013
S. cerevisiae	Bro-bio-fair	_	_	S (+)	S (+)	_	_	S (-)	S (+)	S (+)	Abdel-Raheem, Abd-Allah and Hassanein, 2012
L. plantarum *L. delbrueckii* subsp. *bulgaricus* *L. acidophilus* *L. rhamnosus* *Bi. bifidum* *S. salivarius* subsp. *thermophilus* *E. faecium* *A. oryzae* *C. pitolepesii*	Protexin	NS	NS	NS	NS	NS	NS	NS	_	_	Daskiran et al., 2012
L. casei subsp. *casei* CECT 4043		S (-)	_	NS	NS	NS	_	NS	_	_	Fajardo et al., 2012
L. lactis subsp. *lactis* CECT 539		S (-)	_	NS	S (-)	NS	_	NS	_	_	Fajardo et al., 2012

(Continued)

Micro-organisms	Commercial products*	Growth rate/Final body weight			Feed intake	Feed Conversion Ratio			Histomorphology		References
		Pre-starter - starter phase	Grower-finisher phase	Over all *(lifetime)*		Pre-starter - starter phase	Grower-finisher phase	Over all *(lifetime)*	Villus height	Villus height:crypt depth ratio	
L. acidophilus *L. casei* *E. faecium* *Bi. bifidium*	Primalac	S (+)	S (+)	S (+)	S (+)	S (-)	S (-)	S (-)	_	_	Landy and Kavyani, 2013
11 Lactobacillus strains (*L. reuteri* C 1, C 10 and C 16; *L. gallinarum* I 16 and I 26; *L. brevis* I 12, I 23, I 25, I 218 and I 211, and *L. salivarius* I 24)		S (+)	S (+)	S (+)	NS	S (-)	S (-)	S (-)	_	_	Mookiah et al., 2014
B. amyloliquefaciens		NS	S (+)	S (+)	S (+)	S (-)	S (-)	S (-)	S (+)	S (+)	Lei et al., 2015
B. amyloliquefaciens		S (+)	S (+)	S (+)	S (+)	S (-)	NS	S (-)	_	_	Ahmed et al., 2014

Notes: S (+) = significantly increased, S (-) = significantly decreased, NS = non-significant, - = not studied, * Details (manufacturer, city and country) of commercial products are given in table 1).

The effect of probiotics on feed intake and feed use efficiency may be growth phase dependent. Some probiotics had no effect on feed intake and FCR during the starter phase while feed intake increased during the grower-finisher phase or *vice versa* (Giannenas *et al.*, 2012; Chawla *et al.*, 2013; Afsharmanesh and Sadaghi, 2014; Mookiah *et al.*, 2014).

Many probiotics have positive effects on feed intake and feed use efficiency. However, as with other effects of probiotics, the impact on feed intake and feed use efficiency has not been consistent across studies or with different probiotics.

Carcass yield and quality

Few studies have examined the effects of probiotics on carcass yield and quality in poultry. Marketable carcass yield or ready-to-cook quantity of carcass at day 42 was increased concurrently with increased growth rate and improved feed use efficiency with the use of the commercial probiotic Anta Pro EF containing *E. faecium* DSM 10663 NCIMB 10415 (in drinking water) and Super-CyC , a mix of the spore-forming bacterium *B. subtilis* and a yeast *S. cerevisiae* KCTC 7193 (in feed) (Abdel-Rahman *et al.*, 2013). Anta Pro EF (*E. faecium)* in drinking water at the rate of 2 g per 100 birds per day increased ready-to-cook carcass weight and overall body weight gain at day 42 (Abdel-Rahman *et al.*, 2013). In contrast, Afsharmanesh and Sadaghi (2014) did not find any difference in carcass yield, growth rate and feed use efficiency of birds at day 42 treated with a commercial probiotic (GalliPro) containing *B. subtilis*.

Water holding capacity of poultry meat was increased (reduced drip loss) in birds fed with the probiotic *B. coagulans* (Zhou *et al.*, 2010). The tenderness of the meat was also improved in probiotic treated birds in the same study using a local breed of meat-type chicken in China. In contrast, Zhang *et al.* (2005), using another probiotic (*S. cerevisiae*), found no improvement in tenderness in breast meat of commercial broilers. However, both the probiotics had positive effects on growth rate and FCR.

Zhao *et al.* (2013) found differences in meat quality of Ross broiler chicks between two different probiotics. The intramuscular fat content in breast muscle was increased by 3.6% (1.99 vs 1.92 mg/g) in birds treated with probiotic *C. butyricum,* while there was no effect with the probiotic *E. faecium*.

The effect of probiotics on the relationship between carcass quality and yield is unclear – is it due to an effect on muscle or due to improved growth performance *per se*? The inconsistencies in the response may be due to the differences in probiotic strains and/or the breed of birds used.

The effects of probiotics on carcass quality and yield are inconclusive.

Nutrient Digestibility

The apparent ileal digestibility (AID) of essential amino acids was improved in birds fed

a maize-soybean-based diet supplemented with a low dose (1 to 2 $\times 10^2$ cfu/g) of a multi-strain commercial probiotic (Probion) containing *L. acidophilus, B. subtilis* and *C. butyricum* (Zhang and Kim, 2014). All essential amino acids, except histidine and phenylalanine, had improved AID in treated birds compared with control birds, but there was no effect of probiotics on digestibility of DM, nitrogen and energy. However, Li *et al.* (2008), found an increase in the apparent digestibility of DM, energy, CP, Ca, P and amino acids in male broilers fed maize-soybean-based diet supplemented with commercial probiotic (AgiPro A100) containing yeast and other microbes. Interestingly, digestibility of nutrients in the grower-finisher phase was higher than in the starter phase. Apata (2008) also found that the probiotic *L. bulgaricus* could improve apparent ileal digestibility of DM and CP in broiler chicken fed a maize-soybean-based diet. Similarly, Chawla *et al.* (2013) found the probiotic *E. faecium* increased blood calcium levels in Vencobb broiler chicks, indicating improved bioavailability. Different strains of probiotic microbes produce different enzymes, and understanding the effects these might have on different feed ingredients would help understanding of the way probiotics might "work" for animal production.

Probiotics can improve nutrient digestibility in poultry, but the interaction with different feedstuffs used in poultry diets is little understood at present.

Intestinal histomorphology

The structure of the intestinal mucosa is an important determinant of intestinal function (digestive and absorptive) affecting growth performance of poultry. Generally, increase in villus height and villus height:crypt ratio increases the absorption of nutrients due to a larger surface area (Afsharmanesh and Sadaghi, 2014).

Probiotics in poultry diets can affect the histology of the intestinal mucosa. The villus height and the villus:crypt ratio in the intestinal mucosa were increased by *B. subtilis* (Jayaraman *et al.*, 2013; Afsharmanesh and Sadaghi, 2014), *B. coagulans* (Hung *et al.*, 2012), the lactic acid producing bacteria *L. salivarius*, *P. parvulus* (Biloni *et al.*, 2013) and *E. faecium* (Abdel-Rahman *et al.*, 2013; Cao *et al.*, 2013).

Villus height in probiotic (*B. coagulans* ATCC 7050)-treated birds was greater than in birds treated with an AGP (zinc–bacitracin) when measured at 6 weeks age (Hung *et al.*, 2012). Similarly, the probiotic *B. subtilis* PB6 reconstituted the normal structure of chicken intestinal villi distorted and damaged by necrotic enteritis caused by *Cl. perfringens* (Jayaraman *et al.*, 2013).

Some probiotics affect intestinal histomorphology favourably.

Control or prevention of enteric pathogens

The public health risk from zoonotic pathogens of poultry like *Salmonella* and *Campylobacter* and antibiotic resistance is increasing with intensification of the poultry industry in developing countries and imprudent use of antibiotics in animal production systems (van den Bogaard and Stobberingh, 2000; Singer *et al.*, 2003). In addition, other enteric diseases of poultry, like necrotic enteritis and coccidiosis, cause huge economic losses to the industry (Williams, 1999; Bera *et al.*, 2010; Skinner *et al.*, 2010). The change in the poultry

production systems which result in delayed colonization of the gastro-intestinal mucosa by healthy microflora may be one of the reasons for the increasing incidence of enteric pathogens (Crhanova *et al.*, 2011). The virtually sterile environment immediately post-hatch makes it possible for opportunistic pathogens to colonize the intestine (Flint and Garner, 2009). Probiotics may prevent or control such enteric pathogens.

Salmonellosis

Salmonellosis in poultry is a significant food safety issue as the pathogen causes a major foodborne illness in humans. Successful use of undefined gastro-intestinal culture for the prevention and control of *Salmonella* infection in chicken by Nurmi and Rantala (1973) led to many studies about use of gastro-intestinal culture and probiotics to control *Salmonella* in poultry (Lloyd, Cumming and Kent, 1977; Snoeyenbos, Weinack and Smyser, 1979; Bolder *et al.*, 1992). Competitive exclusion between pathogenic and non-pathogenic ingested bacteria was believed to be the mechanism preventing infection in earlier studies.

Probiotics are emerging as an alternative *Salmonella* control method which also addresses the increasing concern about antibiotic resistant strains of *Salmonella* (Tellez *et al.*, 2012). Haghighi *et al.* (2008) demonstrated that probiotics could reduce caecal colonization by *Salmonella* by several fold (1.2 to 3.0 $\log_{10}$) depending on probiotic dose. With a single application at dose rates of 1×10^5 and 1×10^6 cfu of a commercial probiotic product containing *L. acidophilus, Bi. bifidum,* and *S. faecalis*, the larger dose rate caused a larger reduction in the caecal *Salmonella* population.

The protection against *Salmonella* colonization appeared linked to a change in cytokine expression (IFN-γ and IL-12) in gut-associated lymphoid tissue. Some probiotics produce SCFA in the caeca in sufficient amounts to inhibit *Salmonella enterica* serovar *enteritidis* (Argañaraz-Martínez *et al.*, 2013). By using an *in vitro* test, Argañaraz-Martínez *et al.* (2013) demonstrated that SCFA production in the caeca of chickens treated with *Propionibacterium acidipropionici* LET 105 was 30% greater than in the control birds. This probiotic also competed with *Salmonella* for adhesion to the intestinal mucosa (Argañaraz-Martínez *et al.*, 2013). Probiotics also reduced the spread of *Salmonella* from infected to healthy birds. Transmission of *Salmonella* infection within the flock (horizontal transmission) was slower with a probiotic containing *L. salivarius* and *Pediococcus parvulus* (Biloni *et al.*, 2013).

Campylobacteriosis

Campylobacteriosis is an important zoonotic disease of poultry caused by *Ca. jejuni. In vitro* experiments with probiotic bacterial strains *(E. faecium, P. acidilactici, L. salivarius* and *L. reuteri)* isolated from the GIT of healthy chickens showed that they could inhibit growth of *Ca. jejuni* on agar plates (Ghareeb *et al.*, 2012). The result was confirmed *in vivo* with broiler chickens. Inhibition of growth *in vitro* suggests production of a growth inhibiting factor by probiotics. Similarly, the commercial probiotic Primalac (containing *Lactobacillus*, *Bifidobacterium* and *Enterococcus*) reduced the prevalence of *Campylobacter* infection in broiler chickens (Willis and Reid, 2008). Morishita *et al.* (1997) had earlier demonstrated that oral administration (via drinking water) of a commercial probiotic containing a mixture of *L. acidophilus*

and *S. faecium*, to broiler chickens, during the first 3 days of life, reduced the shedding of *Campylobacter* by 70% in artificially infected birds and decreased the intestinal colonization by *Campylobacter* by 27%.

Necrotic enteritis

Necrotic enteritis (NE) caused by *Cl. perfringens* is an economically important disease in poultry due to the high prevalence of losses (McDevitt *et al.*, 2006; Hermans and Morgan, 2007), causing significant economic loss to the industry worldwide (Van der Sluis, 2000; Timbermont *et al.*, 2011).

Administration of *B. subtilis* (strain PB6) to broiler chickens artificially infected with *Cl. perfringens* reduced the severity of intestinal lesions and significantly reduced the number of pathogen cells in the GIT (Jayaraman *et al.*, 2013). *B. subtilis* strain PB6 produces a heat resistant and anticlostridial factor which could be used to control clostridial infections caused by *Cl. perfringens* and *Cl. difficile* (Teo and Tan, 2005).

Coccidiosis

Coccidiosis is the most important protozoan parasitic disease of poultry due to its ubiquitous nature, high rate of resistance to anticoccidial drugs and severe economic consequences for infected flocks (Williams, 1999). The disease is caused by different species of *Eimeria* protozoa that colonize different sections of the GIT. Studies evaluating the effects of probiotics on coccidiosis gave inconclusive results (Dalloul *et al.*, 2003; Lee *et al.*, 2007). However, Giannenas *et al.* (2012) found a reduction in coccidiosis by using probiotics based on *E. faecium, B. animalis, L. reuteri* and *B. subtilis,* either singly or in combination.

The probiotics were thought to maintain intestinal health in infected birds and significantly reduce the shedding of oocysts from infected birds, thereby reducing the spread of disease (Dalloul *et al.*, 2003; Giannenas *et al.*, 2012).

Probiotics could be a potential alternative to antibiotic feed additives to manage the enteric pathogen load in poultry, by reducing intestinal colonization and spread of common zoonotic and other enteric pathogens.

Egg production and quality

While probiotics can affect the production, feed use efficiency and quality of eggs in laying hens, these effects have been very inconsistent (Table 3). Studies showing increase in egg production with supplementation of diets with probiotics (Kurtoglu *et al.*, 2004; Yörük *et al.*, 2004; Xu *et al.*, 2006; Gallazzi *et al.*, 2009), contrast with those showing no effect on egg production (Asli *et al.*, 2007; Salma *et al.*, 2007; Dizaji and Pirmohammadi, 2009; Capcarova *et al.*, 2010; Mikulski *et al.*, 2012). Similarly, there are variable effects of probiotics on feed use efficiency in laying hens. One of the most promising effects of probiotics on egg quality is the consistent reduction of cholesterol in egg yolk. Yolk cholesterol has been reduced by lactic acid bacteria (Haddadin *et al.*, 1996; Panda *et al.*, 2003), *Bacillus* spores (Kurtoglu *et al.*, 2004) and yeast (Yousefi and Karkoodi, 2007).

PROBIOTICS IN PIG NUTRITION

Although banned in some areas, including the EU, sub-therapeutic use of antibiotics in feed to prevent diarrhoea and improve performance is still common in the swine industry. Therefore, substitution of AGPs with probiotics to address the issue of antibiotic resistance is critical in pig production. For monogastrics, this substitution has been more extensively studied in poultry than in pigs.

As with other livestock, it is difficult to make generalizations because of the variation in the micro-organisms used, doses, duration of treatment and husbandry practices (Kenny *et al.*, 2011).

Growth rate and feed use efficiency

Several probiotics have been used to enhance the performance of pigs (Table 4). In a large-scale experiment in a high performing commercial setting, the commercial probiotic product BioPlus 2B containing *B. subtilis* and *B. licheniformis* was a viable substitute for AGPs (neomycin, oxytetracycline, tylosine, etc.) without a decrease in weaned pig performance; and with no increase in production costs (Kritas and Morrison, 2005). BioPlus 2B also improved weight gain by up to 8% and feed use efficiency by up to 10% in grower and finisher pigs in a dose-dependent manner (Alexopoulos *et al.*, 2004b). For doses of

TABLE 3
Probiotic effects on egg production and quality

Micro-organism	Egg production	FCR *(feed weight/egg weight)*	Quality of egg					References
			Weight	Egg shell thickness	Yolk cholesterol	Albumin viscosity *(Haugh unit)*	Specific gravity	
L. acidophilus D2/CSL	S (+)	S (-)	NS	NS	-	S (+)	S (+)	Gallazzi *et al.*, 2009
P. acidilactici	NS	S (-)	S (+)	-	S (-)12%	-	S (+)	Mikulski *et al.*, 2012
R. capsulatus	NS	NS	-	NS	S (-)26%	NS	-	Salma *et al.*, 2007
L. plantarum, L. bulgaricus, L. acidophilus, L. rhamnosus, B. bifidum, S. hermophilus, E. faecium, A. oryzae C. pintolopessi	NS	-	-	NS	NS	NS	-	Asli *et al.*, 2007
S. cerevisiae	NS	-	-	NS	NS	NS	-	Asli *et al.*, 2007
B. licheniformis B. subtilis	S (+)	S (-)	NS	-	S (-)38%	-	NS	Kurtoglu *et al.*, 2004

(Continued)

TABLE 3
Probiotic effects on egg production and quality *(Continued)*

Micro-organism	Egg production	FCR *(feed weight/egg weight)*	Quality of egg					References
			Weight	Egg shell thickness	Yolk cholesterol	Albumin viscosity *(Haugh unit)*	Specific gravity	
Lactobacillus spp., *Bifidobacterium* spp., *Streptococcus* spp., *Enterococcus* spp.	S (+)	S (-)	NS	-	-	-	-	Yörük *et al.*, 2004
L. acidophilus, L. casei, Bi. bifidum, A. oryzae, S. faceium Torulopsis spp.,	S (+)	NS	NS	S {+)	S (-)14%	-	-	Panda *et al.*, 2003
E. faecium	NS	-	NS	-	-	-	-	Capcarova *et al.*, 2010
S. cerevisiae (strain NCYC sc 47)	NS	S (+)	S (-)	-	-	-	-	Dizaji and Pirmohammadi, 2009
B. subtilis (CH201) *B. lichenioformis* (CH200)	NS	S (+)	S (-)	-	-	-	-	Dizaji and Pirmohammadi, 2009
L. acidophilus	S (+)	S (+)	-	NS	S (-)	-	-	Haddadin *et al.*, 1996
S. cerevisiae	NS	NS	NS	S +)	-	-	-	Hassanein and Soliman, 2010
E. faecium	NS	S (-)	NS	NS	NS	S (-)	-	Hayirli *et al.*, 2005
B. subtilis B. lichenioformis	NS	NS	NS	NS	S (-)	NS	-	Mahdavi *et al.*, 2005
B. subtilis	S (+)	S (-)	NS	-	-	-	-	Xu *et al.*, 2006
S. cerevisiae	NS	NS	NS	NS	S (-)	-	-	Yousefi and Karkoodi, 2007
L. plantarum, L. delbrueckii subsp. bulgaricus L. acidophilus L. rhamnosus Bi. bifidum S. salivarius subsp. thermophilus E. faecium A. oryzae C. pitolepesii	NS	S (-)	NS	-	-	-	NS	Balevi *et al.*, 2001

Notes: S (+) = significantly increased; S (-) = significantly decreased; NS = non-significant; - = not studied.

TABLE 4
Probiotic effects on performance of pigs

Micro-organisms	Growth rate (ADG)	FCR	Feed intake	Age group	Reference
B. subtilis *C. butyricum*	S (+)	S (-)	NS	Growing-finishing pigs	Meng *et al.*, 2010
L. acidophilus, *S. cerevisae* *B. subtilis*	S (+)	NS	NS	Growing pigs	Chen *et al.*, 2005
L. plantarum ATCC 4336, *L. fermentum* DSM 20016 *E. faecium* ATCC 19434	S (+)	NS	-	Weaned piglets	Veizaj-Delia *et al.*, 2010
E. faecium EK13	NS	-	-	Newborn piglets	Strompfova *et al.*, 2006
*Bi. longu*m (AH1206)	NS	NS	-	Neonatal piglets	Herfel *et al.*, 2013
B. licheniformis	S (+)	S (-)	-	Weaned piglets	Kyriakis *et al.*, 1999
B. subtilis *B. licheniformis*	S (+)	S (-)	NS	Growing pigs	Kritas *et al.*, 2000
B. subtilis *B. licheniformis*	S (+)	S (-)	NS	Grower finisher pigs	Alexopoulos *et al.*, 2004b
B. subtilis MA139	NS	S (-)	NS	Weaned piglets	Guo *et al.*, 2006
Bacillus toyonensis	S (+)	S (-)	S (+)	Weaning piglets	Kantas *et al.*, 2015
B. licheniformis *B. subtilis*	NS	S (-)	NS	Growing-finishing pigs	Davis *et al.*, 2008
S. cerevisiae subsp. *boulardii* CNCM I-1079	-	S (-)	-	Weaned piglets	Le Bon *et al.*, 2010

Notes: S (+) = significantly increased; S (-) = significantly decreased; NS = non-significant; - = not studied; ADG = average daily gain; FCR = feed conversion ratio.

0.64, 1.28 and 1.92 $\times 10^6$ cfu/g of feed, the daily gain increased with dose rate. Guo *et al.* (2006) also found *B. subtilis* MA139 effective in significantly improving FCR. Kyriakis *et al.* (1999) reported that average daily gain in post-weaning piglets was increased by 99% over a period of 28 days when the piglets' diet was supplemented with spores of *B. licheniformis* at the rate of 10^7 spores per gram of feed. Feed use efficiency was improved by 24%. In a recent study, the commercial probiotic product Toyocerin containing *Bacillus toyonensis* given to post-weaning piglets at the rate of 1.24 $\times 10^6$ cfu per gram of feed improved average daily gain by 5% over the 42-day experimental period (Kantas *et al.*, 2015). Average daily feed intake was increased by 1.7% and feed use efficiency was improved by 4.7% over the same period. In contrast, another commercial probiotic product MicroSource S (Agtech Products Inc.) containing *B. licheniformis* and *B. subtilis* did not improve growth rate or feed intake (Davis *et al.*, 2008) when fed at the very high dose rate of 1.47 $\times 10^8$ cfu/g feed but did improve feed use efficiency by 3%.

Supplementation of weaned pigs with 2 $\times 10^9$ cfu/kg feed with *S. cerevisiae* subsp. *boulardii* CNCM I-1079 for 6 weeks, followed by 1$\times 10^9$ cfu/kg feed of *P. acidilactici* CNCM MA 18/5 M for 3 weeks significantly improved the FCR without affecting intestinal structure (villus height, crypt depth, goblet cell number and thickness of the mucus layer) (Le Bon *et al.*, 2010). In contrast, Van Heugten, Funderburke

and Dorton (2003) did not observe any positive responses in growth or nutrient digestibility when *S. cerevisiae* SC47 was added to a pig diet at a rate of 1.6 $\times 10^7$ cfu/g of feed.

Similarly, probiotic *L. sobrius* DSM 16698 was effective in improving average daily gain by 74%, with 6% increase in feed intake in piglets infected with enterotoxigenic *E. coli* and also fed the probiotic at the high rate of 10^{10} cfu/animal/day (Konstantinov *et al.*, 2008). In another experiment, final body weight was not improved when *L. amylovorus* and *E. faecium* were fed at the rate of 3 $\times 10^8$ cfu/animal/day (Ross *et al.*, 2010). However feed intake was significantly reduced with improvement in feed use efficiency by 15% to 42% during different periods of the experiment. Likewise, application of *E. faecium* to primiparous sows at 5 $\times 10^8$ cfu/kg feed, increased feed intake and improved reproductive performance (Böhmer, Kramer and Roth-Maier, 2006).

Use of different strains and doses of micro-organisms and differences in husbandry practices (nutrition, housing, etc.), and age of pigs and feed type may explain contrasting results with the same probiotic micro-organisms.

Probiotics can enhance the growth of pig but with less consistent results than for poultry.

Health

Adding a commercial probiotic containing *B. licheniformis* and *B. subtilis* spores (BioPlus 2B) to the diet of weaned, grower and finisher pigs at the rate of 0.64 to 1.28 $\times 10^6$ cfu/g feed significantly reduced morbidity and mortality (Alexopoulos *et al.*, 2004b). The same combination of probiotics when fed to pregnant sows from two weeks prior to expected farrowing date and during lactation improved the performance of the litter, with reduced piglet diarrhoea, reduced pre-weaning mortality and increased body weight at weaning (Alexopoulos *et al.*, 2004a). Decreased weight loss in sows during lactation and production of milk with higher fat and protein content were suggested reasons for the improved health and performance of the piglets.

Probiotics inhibits the adhesion of enteric pathogens in intestinal mucosa. *Bi. lactis* Bb12 and *L. rhamnosus* LGG individually or in combination inhibited adhesion of pathogens (*Salmonella, Clostridium* and *E. coli*) to the intestinal mucosa collected from young healthy pigs in an *in vitro* experiment (Collado, Grzeskowiak and Salminen, 2007). Adhesion of pathogens was measured by using radioactively labelled micro-organisms and measuring radioactivity before and after adhesion to the intestinal mucosa. However, Szabo *et al.* (2009) found that *E. faecium* NCIMB 10415 treatment did not improve the clinical signs in pigs experimentally infected with *S. enterica* serovar *typhimurium* DT104.

Post-weaning diarrhoea, caused mainly by enterotoxigenic *E. coli,* is one of the major health problems in swine worldwide, causing substantial economic losses due to mortality, reduced growth rate and associated veterinary costs (Fairbrother, Nadeau and Gyles, 2005). Probiotics reduced the incidence and severity of post-weaning diarrhoea in pigs. Supplementation of weaned piglet diets with *B. licheniformis* spores at the rate of 10^6 and 10^7 cfu/g of feed significantly reduced post-weaning diarrhoea and associated mortality

(Kyriakis *et al.*, 1999). Performance of piglets fed the higher dose (10^7 cfu/g) of probiotics was better than those fed the lower dose. In another study, the incidence of post-weaning diarrhoea decreased following the addition of *B. toyonensis* to the diet of pregnant sows from 90 days before farrowing to 28 days postpartum and in the diet of piglets from days 15 to 56 (Taras *et al.*, 2005). Kantas *et al.* (2015) also demonstrated the beneficial effects of *B. toyonensis* (commercialized as Toyocerin) to reduce the enteric pathogen load and diarrhoea in post-weaning piglets.

Probiotics reduced intestinal colonization by pathogenic *E. coli* and prevented or reduced the severity of the intestinal infection. The level of enterotoxigenic *E. coli* in the ileum of experimentally infected piglets after weaning was significantly lowered by treating with *L. sobrius.* (Konstantinov *et al.*, 2008). *L. paracasei* mixed with maltodextrin also reduced intestinal colonization by *E. coli* in piglets raised in an apparently sterile environment (Bomba *et al.*, 2002). Similarly, translocation of pathogenic *E. coli* to mesenteric lymph nodes was reduced in pigs treated with *P. acidilactici* and *S. cerevisiae* subsp. *boulardii* and then challenged with pathogenic *E. coli* (Lessard *et al.*, 2009). Positive effects on intestinal barrier function may be the possible mode of action for these probiotic effects.

Le Bon *et al.* (2010) found a dramatic reduction in the level of *E. coli* after four weeks of treatment with *S. boulardii* and *P. acidilactici* in weaned piglets. Similarly, *E. faecium* added to pig diets controlled post-weaning diarrhoea and mortality due to *E. coli* infection (Underdahl, Torres-Medina and Dosten, 1982; Taras *et al.*, 2006; Zeyner and Boldt, 2006).

Probiotics can be effective in reducing post-weaning diarrhoea in piglets and morbidity and mortality in pigs.

GIT microbial population

A single large dose (5×10^9 or 5×10^{10}) of *L. plantarum* (DSMZ 8862 and 8866) given to piglets one week before weaning or at weaning resulted in a significant change in the microbial population of the small and large intestines (Pieper *et al.*, 2009). However, the observations were only made at 2 weeks post treatment and did not explore the long-term effects of the single administration. In another study, the probiotic *L. paracasei* mixed with fructo-oligo-saccharides increased populations of *Lactobacillus* spp., *Bifidobacterium* spp., total anaerobes and total aerobes, and decreased *Clostridium* and *Enterobacterium* in faeces of weanling pigs (Bomba *et al.*, 2002). Similarly, *S. cerevisiae* and *P. acidilactici* produced a temporary (about two-week) reduction in the population of *E. coli* and other coliforms in pig faeces after application of probiotics for four weeks at 2×10^9 cfu/kg feed (Le Bon *et al.*, 2010). However in other trials, inclusion of a yeast probiotic (*S. cerevisiae*) did not change the populations of *E. coli, Streptococcus, Lactobacillus* and total culturable yeast in the GIT, as it did in some earlier feeding trials (Mathew *et al.*, 1998; Li *et al.*, 2006). Nevertheless, pigs fed the probiotics performed better in terms of body weight gain and feed use efficiency in these experiments. Enhancement in performance in probiotic fed animals is apparently not necessarily associated with a change in the gastro-intestinal micro-

bial population that can be cultured. However, sequencing of the GIT microbial DNA indicates that the microbiome diversity is dominated by microbial species that have not yet been cultured.

In pigs probiotics increased lactic acid bacteria and decreased *Clostridium*, *E. coli* and *Enterobacterium* spp. in the GIT.

PROBIOTICS IN RUMINANT NUTRITION

The rumen has a complex microbial ecology, where polysaccharides and protein ingested by the host are degraded by rumen micro-organisms, resulting in the synthesis of SCFAs and microbial protein, which are used by the host as energy and protein sources. There is increasing international interest in manipulating the rumen ecosystem to increase the efficiency of the ruminal fermentation processes to improve animal productivity and reduce unwanted by-products, such as methane.

Yeast (*S. cerevisiae*) is a commonly used probiotic in ruminants (Chaucheyras-Durand, Walker and Bach, 2008), affecting mainly the microbial population dynamics in the rumen and the breakdown of nutrients. Lactic acid-producing bacteria are another important group of probiotics.

Apart from the use of probiotics in formulated animal feed, beneficial bacteria used as silage inoculants may also have a probiotic effects in the rumen (Weinberg *et al.*, 2004). However, this response depends on the survival of the silage inoculant in the silage as the pH drops.

Milk yield

Probiotics can improve the milk yield in dairy animals. Milk yield was increased by 2.3 litre per cow per day following dietary supplementation with 5 $\times 10^9$ cfu of *E. faecium* and 2 $\times 10^9$ yeast cells (*S. cerevisiae*) per cow per day (Nocek and Kautz, 2006). Weiss, Wyatt and McKelvey (2008) found that dairy cattle fed the probiotic *Propionibacterium* strain P169 had the same milk production as control animals, but with decreased feed consumption, resulting in 4.4% increase in energy efficiency. Dietary supplementation with a combination of *L. acidophilus* NP51 and *P. freudenreichii* NP24 (4 $\times 10^9$ cfu/animal/day) resulted in a 7.6% increase in average daily milk yield in Holstein cows (Boyd, West and Bernard, 2011). Average milk yield per day increased by *ca.* 14% compared to non-treated, lactating Saanen dairy goats receiving *S. cerevisiae* at the rate of 4 $\times 10^9$ cfu/day/animal (Stella *et al.*, 2007).

Desnoyers *et al.* (2009) undertook a quantitative meta-analysis of 110 papers, 157 experiments and 376 treatments, studying the effects of yeast probiotics (containing at least one strain of *S. cerevisiae*) in ruminants (cattle, goats, sheep and buffaloes) on feed intake, milk production and rumen fermentation. Supplementation with live yeast probiotics increased milk yield by about 1.2 g/kg body weight. DM intake by the animals was increased by 0.44 g/kg of body weight. Overall the effect on milk yield was significant, but the results were highly variable and the economic benefits were

not analysed. There was no effect on milk protein content. A similar meta-analysis by Poppy *et al.* (2012) concluded that commercial probiotics containing *S. cerevisiae* increased milk yield by 1.18 kg/day, fat-corrected milk by 1.61 kg/day, and energy-corrected milk by 1.65 kg/day. Similarly, dietary supplementation of *S. cerevisiae* increased milk fat yield by 0.06 kg/day and milk protein yield by 0.03 kg/day. DM intake was increased by 0.62 kg/day during early lactation and 0.78 kg/day during late lactation. Increased feed intake together with improved microbial digestion (see later) of feed could be the possible mode of action for improved animal performance.

In contrast, Krishnamoorthy and Krishnappa (1996) found no differences in DM intake, body weight gain, milk yield and milk composition when yeast was added in a diet based on finger millet (*Eleusine coracana*) straw for lactating crossbred cattle.

Growth

Probiotics can increase the weight gain of ruminants. For example, a probiotic containing a mixture of micro-organisms (*L. reuteri* DDL 19, *L. alimentarius* DDL 48, *E. faecium* DDE 39 and *Bi. bifidium* DDBA) isolated from a healthy goat, when fed to goats for eight weeks, commencing at 75 days of age, resulted in improvement in average body weight by 9% (Apás *et al.*, 2010).

Similar improved growth rate was obtained with a yeast-based commercial probiotic containing *S. cerevisiae* given to growing dairy heifers (Ghazanfar *et al.*, 2015). *B. amyloliquefaciens* strain H57 when fed to pregnant White Dorper ewes on a palm kernel-based diet, increased DM intake and live weight gain during pregnancy, followed by better performance of the lambs during early lactation (Le *et al.*, 2014; McNeill *et al.*, 2016). The same strain of *B. amyloliquefaciens* when fed to dairy calves at the rate of 3.16×10^8 cfu per kg dietary DM from week 4 to 12 improved growth rate by 39% (551 vs 767 g/day), increased feed use efficiency by 14% (2.5 vs 2.9 kg milk + starter DM/kg weight gain) (Le *et al.*, 2016). Likewise, a novel bacterial strain isolated in Australia, *P. jensenii* 702, significantly enhanced weight gain in Holstein calves by 25% during the pre-weaning period and by 50% during the weaning period (Adams *et al.*, 2008).

Frizzo *et al.* (2011), based on meta-analysis of 21 publications between 1985 and 2010, concluded that lactic acid probiotic bacteria in comparisons with and without *L. acidophilus, L. plantarum, L. salivarius, E. faecium, L. casei/paracasei* or *Bifidobacterium* spp., increased body weight gain (standardized mean difference = 0.22822, 95% confidence interval = 0.1006 to 0.4638) and improved feed use efficiency (standardized mean difference = -0.8141, 95% CI = –1.2222 to –0.4059) in young calves compared with control groups when probiotics were added to milk replacer, but were ineffective when added to whole milk. In contrast, some studies have reported no effect on calf growth when the diet was supplemented with *L. acidophilus* (Abu-Tarboush, Al-Saiady and El-Din, 1996; Cruywagen, Jordaan and Venter, 1996), a mixture of *L. acidophilus* and *Streptococcus faecium* (Higginbotham and Bath, 1993), a mixture of *L. acidophilus* and *L. plantarum* (Abu-Tarboush, Al-Saiady and El-Din, 1996), *B. subtilis* (Galina *et al*., 2009), or a mixture of *L. acidohilus*, *L. lactis* and *B. subtilis* (Galina *et al*., 2009).

Quality control of the probiotics strain production and subsequent shelf viability is a critical component of trials assessing the affect they have when fed, and often in nutrition trials this is inadequately dealt with and could be a reason for the variability in animal response between trials.

Nutrient digestibility

The improvement in performance by ruminants is often associated (at least partially) with improvement in nutrient digestibility. A combination of *L. acidophilus* NP51 and *P. freudenreichii* NP24 improved the digestibility of crude protein, neutral detergent fibre and acid detergent fibre in lactating Holstein cows resulting in increased milk production per day by 7.6% without increase in dry matter intake (DMI) (Boyd, West and Bernard, 2011) and it was suggested that this was due to a change in the rumen microbial ecosystem. Similarly, supplementation of dairy cows with Probios TC containing 2 strains of *Enterococcus faecium* at the rate of 5×10^9 cfu per day as well as 2×10^9 viable yeast cells per day from 21 days prior to expected calving date through to 10 weeks postpartum, increased milk production by 2.3 kg per cow per day, with no difference in 3.5% fat corrected milk. The *E. faecium* strains were thought to act by producing lactic acid, which supported a rumen microbial population, which increased ruminal digestion of roughages in the maize silage and haylage diet, as well as increasing DMI (Nocek and Kautz, 2006). In contrast, Hristov *et al.* (2010) found no improvement in digestibility of maize-silage-based diet from supplementation with a yeast (*S. cerevisiae*) probiotic in Holstein cows. Although the yeast supplementation increased ruminal microbial protein synthesis, there were no differences in DMI, milk yield and milk composition.

Based on a meta-analysis of papers published on the effects of yeast probiotics in all ruminant species reared for milk or meat, Desnoyers *et al.* (2009) found much variability in response, with an overall average increase in DMI of 0.44 g/kg body weight and total tract organic matter digestibility by 0.8%, effects too small to warrant probiotic addition. However particular strains, increasing levels of inoculum addition, and feed compositions with a larger proportion of concentrates, have produced a better response than this average. Improvement in microbial digestion of feed may be either due to production of enzymes by probiotics or alterations in rumen microbial ecology.

Probiotics improve productivity, increase milk yield, induce better nutrient digestion and enhance growth rate in ruminants.

Health

Apart from their use in improving the performance of ruminant animals, probiotics have been effective in improving animal health. Apas *et al.* (2010) demonstrated that a probiotic containing *L. reuteri* DDL 19, *L. alimentarius* DDL 48, *E. faecium* DDE 39 and *Bi. bifidum* DDBA (at a ratio of 1:1:1:1), isolated from the faeces of healthy goats, when fed to weaned goats (dose rate 2×10^9 cfu/animal/day) reduced the number of pathogenic bacteria (*Salmonella* and *Shigella*) in faeces.

Rumen acidosis

The pH of the rumen may drop below the optimum range following consumption of a diet with a high proportion of non-structural carbohydrates (starch) and/or decreased proportion of fibre (Duffield *et al.*, 2004); SCFAs accumulate and unbalance the buffering capacity of the rumen (Plaizier *et al.*, 2008). The condition is referred to as subacute ruminal acidosis (SARA) when the pH drops below 5.6 and remains between 5.2 and 5.6 for at least 3 hours per day (Gozho *et al.*, 2005). This condition is economically very important as milk production by the suffering animal is reduced due to loss of appetite, diarrhoea, dehydration, debilitation, impaired rumen motility and impaired fibre digestibility (Duffield *et al.*, 2004; Plaizier *et al.*, 2008). Lactic acidosis is the more severe form of ruminal acidosis where the pH drops below 5.2 due to accumulation of lactate (Owens *et al.*, 1998).

Probiotics are effective in preventing or treating ruminal acidosis. Application of *Propionibacterium* P63, *L. plantarum* strain 115 and *L. rhamnosus* strain 32 to the rumen directly via a rumen cannula at the rate of 1×10^{11} cfu/animal/day – a very high dose – was effective in stabilizing rumen pH and preventing acidosis artificially induced by three days of concentrate challenge (wheat, maize or beet pulp) in sheep (Lettat *et al.*, 2012). It was hypothesized that stability in ruminal pH was achieved by the probiotics modulating rumen microbes so that their capacity to hydrolyse cellulose was increased and lactic-acid producing bacteria were inhibited. Similarly, the lactate-utilizing bacterium *Megasphaera elsdenii* (Prabhu, Altman and Eiteman, 2012) was effective in preventing lactic acid accumulation during *in vitro* fermentation (Kung and Hession, 1995). Klieve *et al.* (2003) demonstrated that the probiotic *M. elsdenii* strain YE34 could be established in the rumen of cattle fed high-grain diets, inducing the establishment of lactic acid-utilizing bacteria some 7–10 days earlier than in non-inoculated cattle. Interestingly, ruminants fed high–grain diet (barley) have *Ruminococcus bromii* as a dominant bacterial population in the rumen and this bacterium has been suggested as a potential probiotic to enhance the efficiency of starch utilization in grain-fed cattle (Klieve *et al.*, 2007). Similarly, yeast *S. cerevisiae* decreased the lactic acid concentration in the rumen of lactating Holstein cows (Marden *et al.*, 2008), which may prevent ruminal acidosis (Thrune *et al.*, 2009). In contrast, Hristov *et al* (2010) found no effect of *S. cerevisiae* culture, containing metabolites of yeast fermentation, on ruminal fermentation.

Even though probiotics were found effective in preventing rumen acidosis, it has been difficult to establish stable populations of potential probiotics in the rumen. Chiquette *et al.* (2007) tried to establish *Ruminococcus flavefaciens* NJ by adding the bacterium with the probiotic yeast *S. cerevisiae,* hoping it would stabilize ruminal conditions to favour the establishment of the inoculated bacteria. Similarly, *Ruminococcus bromii* YE282 was inoculated with *Megasphaera elsdenii* YE34 as an alternative starch-utilizing bacterium in steers (Klieve, McLennan and Ouwerkerk, 2012). There was no effect on acidosis and only *M. elsdenii* YE34 established in the rumen environment. However, Jones and Megaritty (1986) successfully introduced and established an exogenous microbe *Synergesties jonesii* (Allison *et al.*, 1992) in the rumen of goat and subsequently cattle (Pratchett, Jones and Syrch, 1991; Jones, Coates and Palmer,

2009) which at the time was believed to prevent toxicity due to the amino acid mimosine when leaves of the leguminous shrub *Leucaena* are used as fodder. But another mechanism may be a buildup of tolerance to mimosine and its toxic breakdown product 3,4-dihydroxypyridine and its detoxification in the liver (Halliday *et al.*, 2013).

Reduced shedding of* E. coli *O157:H57

E. coli O157:H57, the Shiga-toxin-producing *E. coli*, is an important zoonotic pathogen causing haemorrhagic diarrhoea and haemolitic uraemic syndrome (HUS), which can result in acute kidney failure in children (Karmali, Gannon and Sargeant, 2010). Contamination of animal products (meat, milk, egg) from infected animals with this pathogen is a serious public health issue. Wisener *et al.* (2014) undertook a meta-analysis of the effect of probiotics in reducing the shedding of *E. coli* O157:H57 in beef cattle and found both the long- (>90 days) and short-(<90 days) term applications were effective. The combination of *L. acidophilus* and *P. freudenreichii* was the most effective probiotic treatment, while a dose rate of 10^9 cfu/animal/day was more effective than lower dose rates. Earlier studies had also found that a combination of *L. acidophilus* and *P. freudenreichii* significantly reduced faecal shedding of O157 in cattle (Sargeant *et al.*, 2007).

Similarly, Ohya, Marubashi and Ito (2000) developed a probiotic containing *S. bovis* LCB6 and *L. gallinarum* LCB 12, isolated from adult cattle, that was effective in eliminating the shedding of O157. They postulated that a significantly increased concentration of SCFA, particularly acetic acid, in the GIT could be the reason for the inhibition of O157.

Calf scours

Stress in young calves frequently leads to scours or diarrhoea and weight loss. The stressors are often animal husbandry practices, including weaning, vaccination, dehorning, castration, tagging, etc., or high temperatures. In addition, the rumen and its microbial population are not fully-developed and functional in the early days of life.

Probiotics can reduce such problems in young calves, but results were variable. The effect of the probiotic *L. acidophilus* in reducing the incidence of diarrhoea in young dairy calves was reported as early as 1977 (Bechman, Chambers and Cunningham, 1977). Other studies using LAB probiotics, also obtained a reduced incidence of diarrhoea in calves (Abe, Ishibashi and Shimamura, 1995; Abu-Tarboush, Al-Saiady and El-Din, 1996; Jatkauskas and Vrotniakiene, 2010). Similarly, the incidence of diarrhoea per calf, the duration of each event of diarrhoea and total number of days of diarrhoea in dairy calves from weeks 4 to 12 raised in sub-tropical summer was significantly reduced by dietary supplementation of *B. amyloliquefaciens* strain H57 (Le *et al.*, 2016). In contrast, Cruywagen, Jordaan and Venter (1996) found no reduced incidence of diarrhoea when young dairy calves were fed *L. acidophilus* with milk replacer at the rate of 10^8 cells per animal per day. However, the probiotic did prevent weight loss in the treated calves, while the control calves lost weight. Riddell *et al.* (2010) also found no effect on the incidence and duration of diarrhoea in young calves from feeding with milk replacer the commercial probiotic (Bioplus 2B) containing *B. licheniformis* (DSM 5749) and *B. subtilis* (DSM 5750). Stress in animals causing dysbiosis or microbial imbalance in the GIT may be needed for the probiotic to benefit calf health.

Probiotics can reduce diseases of ruminants particularly those related to the disturbance of rumen pH (e.g. acidosis), calf scours and pathogenic *E. coli*. Probiotics are believed to stabilize ruminal pH by modulating rumen microbes. Lactate utilizing bacteria (e.g. *Megasphaera elsdenii*) could potentially be used to prevent the accumulation of lactic acid in the rumen. However, the establishment of such micro-organisms in the rumen is difficult. Similarly, probiotics are effective in reducing the incidence of calf scours by preventing ruminal dysbiosis. Probiotics are also effective in reducing the faecal shedding of the shiga-toxin producing *E. coli* O157:H57. However, these responses to use of probiotics are highly variable and reflect differences in micro-organisms (species, strains) used as probiotics and differences in animal husbandry practices (nutrition, housing etc.).

Rumen fermentation

The meta-analysis of the application of yeast probiotics (containing at least one strain of *S. cerevisiae*) in ruminants by Desnoyers *et al.* (2009) demonstrated that live yeast significantly increased rumen concentrations of SCFA and increased rumen pH, but the results were highly variable. Although yeast supplementation moderately decreased rumen lactic acid concentration, there was no effect on the acetate to propionate ratio. However, the effect of yeast supplementation on rumen fermentation varied with the proportion of concentrate in the diet. In general, "The positive effect of yeast supplementation on rumen pH increased with the percentage of concentrate in the diet and with the Dry Matter Intake (DMI) level" (Desnoyers et al., 2009).

Similarly, yeast probiotics increased the concentration of SCFA with increased CP concentration and DMI (Desnoyers *et al.*, 2009). The higher the proportion of concentrate and neutral detergent fibre in the diet, the better the digestibility of organic matter resulting from the live yeast supplementation (Desnoyers *et al.*, 2009).

It has been postulated that yeast-based probiotics in ruminants increase the number of cellulolytic bacteria, which affects the microbial fermentation, resulting in higher cellulose degradation and increased microbial protein production (Dawson, Newman and Boling, 1990; Newbold, 1996; Chaucheyras-Durand, Walker and Bach, 2008).

Using quantitative real-time PCR, Ding *et al.* (2014) demonstrated that *S. cerevisiae* increased the total number of rumen bacteria in crossbred steers fed alfalfa mixed with concentrates, but the number of rumen fungi and protozoa did not change. The percentage of *Selenomonas ruminantium*, a lactate-utilizing bacterium, increased, while the percentage of *Ruminobacter amylophilus,* a starch-degrading bacterium, decreased.

Probiotics with roughage-based diet

Most ruminant animal production occurs on low quality roughage, and the improvement of digestibility with the use of probiotics is of much interest, even though at present it is only animals fed high quality diets where probiotics could be readily applied.

Yeast probiotics can increase the population of cellulolytic bacteria in the rumen (Harrison *et al.*, 1988; Dawson, Newman and Boling, 1990), which may result in an increased

rate of fibre digestion and increased microbial protein turnover, hopefully improving animal performance (Newbold, 1996). However, increase in cellulolytic bacteria may not always result in increased fibre digestion, as their activity depends on rumen pH (Russell and Wilson, 1996). Dawson, Newman and Boling (1990) found an increase in the population of cellulolytic bacteria in the rumen of Jersey steers, when a high-roughage-based diet was supplemented with either *S. cerevisiae* or a combination of *S. cerevisiae*, *L. acidophilus* and *E. faecium.*

The effects of yeast on rumen fermentation in animals with roughage-based diet are variable. Dietary inclusion of *S. cerevisiae* and/or *Armillaria heimii* (white rot fungus) in sheep increased DMI, metabolizable energy intake and digestibility of neutral detergent fibre (Mpofu and Ndlovu, 1994). Potentially digestible neutral detergent fibre, crude protein and dry matter of alfalfa hay, maize stover and coffee hull fed to fistulated Holstein steers was increased with the supplementation of *S. cerevisiae* (Roa *et al.*, 1997). In contrast, addition of yeast to cattle fed a high-fibre (barley straw-based) diet (Moloney and Drennan, 1994) or high grain diet (Mir and Mir, 1994) did not affect the digestibility of dry matter and neutral detergent fibre, and decreased the digestibility of crude protein. Supplementing a sugar cane tops-based diet for sheep with yeast did not improve rumen fermentation and digestibility, although rumen pH decreased (Arcos-García *et al.*, 2000).

Although probiotics, especially Saccharomyces cerevisiae can improve digestibility of low quality roughage by ruminants, the results are inconsistent. Further study with a wider selection of well characterized probiotic micro-organisms (including bacteria) and animal feed constituents, including non-conventional feed resources such as agricultural by-products, is needed to assess the benefits.

Safety of probiotics and potential public health risks

The safety of probiotics is discussed in general terms and is not specific to those used in animal feed. The possibility of probiotics used in animal feed entering the human food chain cannot be ruled out. However, there is very little information available about the risk of human food "contamination" with probiotics used in animals.

The microbial genera and species used as probiotics in animal feed are generally considered safe. The most serious risk posed by probiotic microbes in feed are, first, transfer of antibiotic resistance due to the presence of transmissible antibiotic resistance genes/determinants in some probiotic bacteria; and second, infections from the probiotic micro-organisms and presence of enterotoxins and emetic toxins in probiotic bacteria.

Most publications relating to probiotics deal with their efficacy rather than safety. Most of the information about the safety of probiotics is based on *Lactobacillus* and *Bifidobacterium* (Hempel *et al.*, 2011; Shanahan, 2012). Therefore more research is required in relation to the safety of probiotics.

Shanahan (2012) highlights the limitations of claims made about the safety of probiotics in general, and especially the safety of any particular probiotic. According to Shanahan (2012):

- Safety assessment and information on a particular probiotic strain cannot be generalized to similar probiotics (even with a different strain of the same species), as each probiotic requires safety and risk assessment on a case-by-case basis.
- The adverse effects and the severity of the effects of a probiotic could be context specific and depend on the susceptibility (immunity) and physiological state of the host (animal or human). Therefore, probiotic strains deemed to be safe in certain conditions may not be safe in other conditions. For example, the prematurely born and immunologically compromised host could be at greater risk than the host born at term.
- No probiotic can be regarded as 100% safe or with zero risk, as is the case with drugs.
- Public awareness about the risk from probiotics is limited, and there is a need for proper risk benefit analysis and communication of this to the user/consumer of the probiotics.

The contamination of probiotics with unwanted microbes or substances is an important safety and quality issue as with the safety and quality of probiotic micro-organisms *per se*. Sometimes, hazards associated with contaminants may be a more important issue than the specific quality of the probiotics. In 2010, the Agency for Healthcare Research and Quality under the U.S. Department of Health and Human Services, conducted a systematic study of published data and information on the safety of probiotics. The study concluded that

"there is a lack of assessment and systematic reporting of adverse events in probiotic intervention studies, and interventions are poorly documented" (Hempel *et al.*, 2011).

Although there are many publications on the safety of probiotics, the evidence available is not enough to address all the safety issues and precludes a declaration of probiotics as universally safe or unsafe (Hempel *et al.*, 2011).

Although micro-organisms used as probiotics in animal feed are generally safe, some of the bacterial species and/or strains pose risks mainly by transmission of antibiotic resistance to pathogenic microbes, or production of enterotoxins (Anadón, Martínez-Larrañaga and Martínez, 2006).

RISK ASSOCIATED WITH PROBIOTICS

Although micro-organisms used as probiotics in animal feed are relatively safe, precautions should be taken to protect animals, humans and the environment from potentially unsafe micro-organisms. Theoretically, risks associated with the use of probiotics in animal feed are as follows (Marteau, 2001; FAO/WHO, 2002; Doron and Snydman, 2015):

- Infection (gastro-intestinal or systemic) of the animal fed the probiotic.
- Infection (gastro-intestinal or systemic) of the consumers of animal products produced by animals fed probiotics.
- Transfer of antibiotic resistance from probiotics to other pathogenic micro-organisms.
- Release of infectious micro-organisms or noxious compounds to the environment from the animal production system.
- Infection (gastro-intestinal or systemic) of the handlers of animal or animal feed.
- Skin and/or eye and/or mucus membrane sensitisation in the handlers of probiotics.
- Detrimental metabolic or toxic effects in the host due to the production of toxins by the micro-organisms contained in probiotics.
- Hyper-stimulation of the immune system in susceptible hosts.

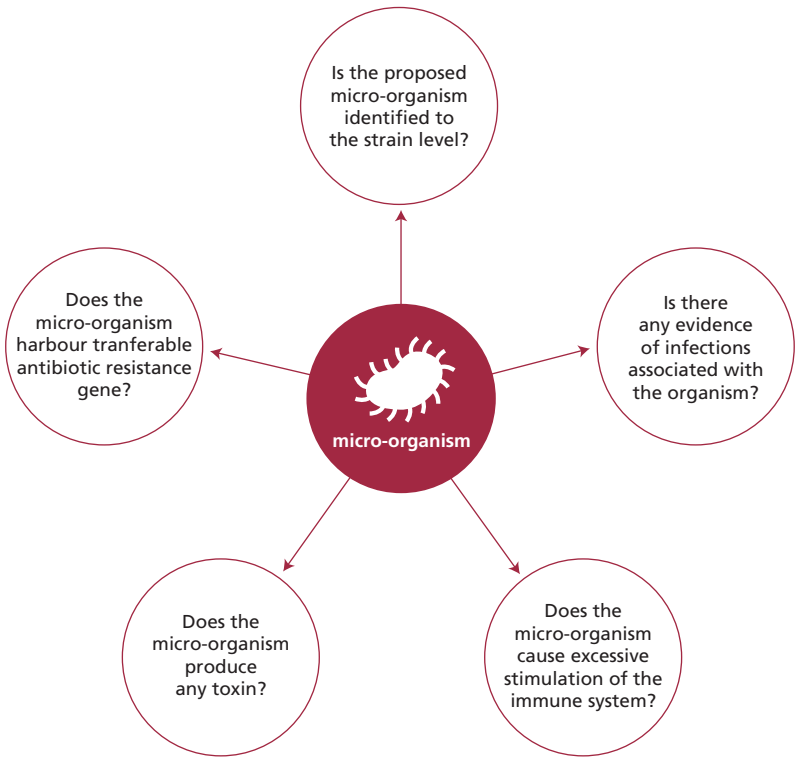
Assessment of risk

The micro-organisms considered for use as probiotics in animal diets should be assessed against the above-mentioned risks. The micro-organism under consideration need to be identified to strain level (Figure 1). The particular strain of micro-organism should not have been associated with any infection in humans or animals. Similarly, the putative probiotic should not harbour transferable antibiotic resistance genes. Micro-organisms which either produce toxins or cause hyper-stimulation of the immune system in the host are generally not suitable for probiotics.

Qualified Presumption of Safety (QPS): European approach for the assessment of the safety of probiotics

In 2002, a group of scientists, consisting of members of the former Scientific Committees on Animal Nutrition, Food and Plants of the European Commission, developed the concept of QPS to address a need for a tool which selectively prioritizes the assessment of risk of the use of a particular micro-organism in food and feed (EFSA, 2007). The European Food Safety Authority (EFSA) has been using this concept since 2007 as a generic risk assessment tool to assess the safety of a micro-organism intended to deliberately enter the food chain. According to this concept, if micro-organisms of certain predetermined taxonomic groups either do not pose any safety risk or the risk can be clearly defined and eliminated,

FIGURE 1
Major questions to be addressed when assessing the safety of micro-organisms being considered for use as probiotics in animal feed



Source: Authors.

the group can be designated as a group with QPS status. Any particular micro-organism intended to be introduced into the food chain, which can be unequivocally identified and have QPS status, may not be the subject of a detailed pre-market safety assessment other than satisfying predetermined specific qualifications (EFSA, 2007). Thus, resources (time and money) could be prioritized to those micro-organisms that do not fulfil the above-mentioned qualifications and have an uncertain risk status, thus avoiding the need to investigate micro-organisms with proven safety. Micro-organisms not listed as having QPS status would undergo a detailed pre-market safety assessment. QPS status is only given to micro-organisms but not to any product containing such micro-organism (EFSA, 2007). QPS status is maintained up to the species level.

Safety assessment of a particular micro-organism or a taxonomic group to decide QPS status is usually done on the basis of four pillars of QPS assessment (EFSA, 2007) as outlined in Figure 2. EFSA has listed more than 100 species of micro-organisms under QPS status; which are broadly categorized into (i) Gram-positive non-sporulating bacteria, (ii) *Bacillus* species and (iii) yeasts.

FIGURE 2.
Assessment of a micro-organism or a taxonomic unit to assign QPS status

Source: Authors.

Probiotic use is not without risk. Probiotics could be responsible for a range of hazards in animal health, human health and the environment, ranging from mild reactions to serious, life-threatening infections. Moreover, information about safety of one particular micro-organism should not be applied to other closely related micro-organisms. Present levels of information are not sufficient to declare any group of probiotics 100% safe. Therefore, risk assessment on a case-by-case basis is recommended.

SAFETY OF MICROBIAL GENERA COMMONLY USED AS PROBIOTICS

Lactobacillus and *Bifidobacterium*

Lactobacillus and *Bifidobacterium* are probably the safest micro-organisms used as probiotics because, first, these micro-organisms have been safely used traditionally in various fermented food (Shortt, 1999); second, these micro-organisms are naturally present in the GIT and other sites in humans (Human Microbiome Project Consortium, 2012; Huse *et al.*, 2012) and animals (Yeoman *et al.*, 2012; Yeoman and White, 2014) in large quantities; and third, infections associated with these micro-organisms are extremely rare (Gasser, 1994; Saxelin *et al.*, 1996). *L. acidophilus* and *L. bulgaricus* have been categorized as "Generally Regarded as Safe" by the US Food and Drug Administration (FDA) (US-FDA, 2013). Nevertheless, LAB have been reported to cross the intestinal mucosal barrier, resulting in bacteraemia and inflammation of the heart muscle (endocarditis) in susceptible people with compromised immunity (Soleman *et al.*, 2003; Cannon *et al.*, 2005; De Groote *et al.*, 2005; LeDoux, LaBombardi and Karter, 2006). However, the chance of this happening is extremely rare and reported to be less than 1 per 10^6 (Sanders *et al.*, 2010). These rare incidences of lactobacillaemia can be very serious or even fatal (Saxelin *et al.*, 1996; Husni *et al.*, 1997).

In a small number of cases, incidences of endocarditis and other internal infections characterized by internal inflammatory lesions (e.g. liver abscess) were reported to be associated with the consumption of large quantities of dairy products containing *L. rhamnosus* GG as a probiotic (Rautio *et al.*, 1999; Cannon *et al.*, 2005). However, the nature of risk from the probiotics used in animal diets and those from human food could be entirely different.

It is often difficult to define the clinical significance of the occurrence of *Lactobacillus* in clinical specimens, as mostly the infection is opportunistic due to compromised immunity of the host (EFSA, 2007). Therefore, safety assessment tools may not be able to exclude these types of opportunistic infections (EFSA, 2007). There are 35 species of *Lactobacillus* included in the EFSA QPS list (EFSA BIOHAZ Panel, 2013). *L. plantarum* KKP/593/p and *L. rhamnosus* KKP 825 were the latest addition to be authorized as safe to use as feed additive for chickens (EFSA FEEDAP Panel, 2016).

In the context of *Lactobacillus* taxonomy being updated with advances in knowledge from molecular biology, some of the previous claims about *Lactobacillus* and its aetiology in clinical disease may have been wrongly reported due to misidentification of the causative agent as *Lactobacillus* (Salminen *et al.*, 2002; Bernardeau *et al.*, 2008).

Like *Lactobacillus*, *Bifidobacterium* is also another safe choice as probiotic bacteria. They are very rarely associated with infections in healthy hosts. *Bifidobacterium adolescentis, Bi. animalis Bi. bifidum, Bifidobacterium breve* and *Bi. longum* have been given QPS status by EFSA (EFSA BIOHAZ Panel, 2013). However, incidences of bifidobacteria associated with infections have been reported in immunocompromised hosts (Ohishi *et al.*, 2010; Jenke *et al.*, 2011; Barberis *et al.*, 2012).

Lactobacillus and *Bifidobacterium* species are generally considered the safest choice as probiotics. Nevertheless, some very rare cases of infections (e.g. endocarditis, lactobacillaemia) have been reported in immunocompromised people.

Bacillus

Spore-forming bacteria, particularly various species from the genus *Bacillus,* are becoming increasingly popular as probiotics for use in animal feed, due to their robustness in withstanding high temperatures making them easier to handle during manufacture, storage and transportation of feed. EFSA has identified 13 *Bacillus* species with QPS status, including *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. coagulans* and *B. megaterium,* which are used in probiotics for animal feed (EFSA BIOHAZ Panel, 2013). These *Bacillus* species were identified as safe mainly due to an absence of enterotoxins and emetic toxins (EFSA BIOHAZ Panel, 2013).

The use of spore-forming bacteria as probiotics is not risk-free, as some *Bacillus* species (e.g. *B. anthracis, B. cereus, B. thuringiensis,* etc.) are pathogenic in humans and animals (Damgaard *et al.*, 1997; Hernandez *et al.*, 1998; Little and Ivins, 1999; Kotiranta, Lounatmaa and Haapasalo, 2000.; Raymond *et al.*, 2010). Although there is detailed information about the pathogenicity of *B. anthracis* and *B. cereus*, there is no evidence for pathogenic effects for other endospore-forming bacteria.

B. cereus produces the emetic toxin cereulide and enterotoxins haemolysin BL (Hbl) and non-haemolytic enterotoxin (Nhe) and cytotoxin K (CytK), which cause serious illness in humans (Granum and Lund, 1997; Schoeni and Lee Wong, 2005). From *et al.* (2005) screened 333 strains from various species of *Bacillus* to investigate the production of enterotoxins and emetic toxins. Eight *Bacillus* strains belonging to *B. subtilis, B. mojavensis, B. pumilus* and *B. fusiformis* were found to produce cytoxic and emetic toxins. In addition, some *Bacillus* species, such as *B. cereus,* has been reported to cause mastitis in cattle (Parkinson, Merrall and Fenwick, 1999) and *B. licheniformis* was associated with abortion in cattle (Agerholm *et al.*, 1997).

Some *Bacillus* species used as probiotics (e.g. *Bacillus subtilis*) produce cytotoxic and emetic toxins. Therefore, detailed safety studies are recommended for these microbial strains before use as probiotics.

Enterococcus

In spite of several examples of beneficial effects of *Enterococcus* probiotics in animals and humans and a long history of safe use, these bacteria have been associated with several infections in humans and the presence of transferable antibiotic resistance determinants (Franz, Holzapfel and Stiles, 1999; Franz *et al.*, 2003; 2011). *Enterococcus* species, particularly *E. faecalis* and *E. faecium,* are associated with community- and hospital-acquired infections, and were amongst the most prevalent causes of hospital acquired infections in the 1990s (Spera and Farber, 1992). Several virulence factors from *Enterococccccus* have been identified and are associated with either colonization, invasion or production of pathological lesions (Franz *et al.*, 2011). These bacteria are also opportunistically associated with urinary tract infection, endocarditis and enterococcal bacteraemia in humans (Morrison, Woodford and Cookson, 1997). There are many commercial probiotic products available on the market, which contain *Enterococcus* bacteria (Mountzouris *et al.*, 2010; Khaksar, Golian and Kermanshahi, 2012; Wideman *et al.*, 2012; Abdel-Rahman *et al.*, 2013; Landy and Kavyani, 2013). Due to the widespread prevalence of enterococcal infections and proven virulence of the bacteria, EFSA has not given this genus QPS status, thus requiring safety assessment on a case-by-case basis (EFSA BIOHAZ Panel, 2013).4.575

Enterococcus bacteria are associated with community- and hospital-acquired infections and therefore stringent safety evaluations are required before use as probiotics.

ANTIBIOTIC RESISTANCE ASSOCIATED WITH PROBIOTICS

The emergence of multi-drug resistant pathogens is now one of the greatest threats to public health around the world (Sengupta, Chattopadhyay and Grossart, 2013). Although the initial emergence of antibiotic resistance is believed to be the out-

come of evolution, imprudent use of antibiotics is believed to be the major cause of widespread antibiotic resistance (Davies and Davies, 2010; Laxminarayan *et al.*, 2013). Antibiotic resistance genes are generally present in plasmids, transposons and integrons of bacteria and can transfer from one bacterium to another (intra- or inter-species) by mechanisms of horizontal gene transfer (Alekshun and Levy, 2007; van Reenen and Dicks, 2011; Santagati, Campanile and Stefani, 2012; Blair *et al.*, 2015). Transposons are the most important mobile element in the bacterial cell and responsible for inter-species transfer of antibiotic resistance genes (Wozniak and Waldor, 2010). The nature of antibiotic resistance determinants is more important than antibiotic resistance *per se* because all of the determinants of antibiotic resistance may not be transferrable (Davies and Davies, 2010).

Although resistance to antifungal drugs in pathogenic fungi is becoming a problem of increasing importance (Pfaller and Diekema, 2004; Morschhäuser, 2010), the mechanism of transfer of resistance determinants in fungi differs from antibiotic resistance in bacteria (Anderson, 2005). In fungi, horizontal transfer of drug-resistance genes (and other genes) does not take place easily, particularly among divergent taxa (Anderson, 2005). Therefore, there is no evidence regarding the risk of transfer of antifungal resistance from yeast probiotics.

The GIT of animals contain a complex microbial ecosystem with diverse and large numbers of micro-organisms. Proximity of bacteria to each other in complex microbial ecosystem like the intestine can favour the transfer of genetic material, including antibiotic resistance genes from non-pathogenic to pathogenic micro-organisms (Aarts and Margolles, 2015). The possibility of the transfer of antibiotic resistance genes to potential enteric pathogens in the GIT cannot be excluded (Aarts and Margolles, 2015). Therefore, if a bacterium intended to be used as an animal probiotic is harbouring transferable antibiotic resistance genes, this could be a medium for transfer of antibiotic resistance to the environment and humans (González-Zorn and Escudero, 2012).

Antibiotic resistance in *Lactobacillus*

Although *Lactobacillus* spp. are considered one of the safest bacteria used as probiotics, many species of these bacteria harbour one or more antibiotic resistance genes (Mathur and Singh, 2005; Ammor, Florez and Mayo, 2007; Gueimonde *et al.*, 2013). The possibility of horizontal transfer of these antibiotic resistance genes and their association with mobile elements (plasmids, transposons and integrons) has not been extensively studied. Nevertheless, some of the foodborne species of *Lactobacillus* have antibiotic resistance genes, which are capable of being transferred horizontally to pathogenic bacteria and are associated with mobile elements (Table 5) (Tannock *et al.*, 1994). Some *Lactobacillus* species have acquired antibiotic resistance genes from other Gram positive bacteria (Shrago, Chassy and Dobrogosz, 1986; Tannock, 1987).

The *Lactobacillus* species reported to harbour transferable antibiotic resistance genes, are components of some commercial probiotics (Mountzouris *et al.*, 2010; Daskiran *et al.*, 2012; Bai *et al.*, 2013; Biloni *et al.*, 2013; Mookiah *et al.*, 2014). However, the presence of such elements in those particular probiotic strains has not been

TABLE 5

***Lactobacillus* species with antibiotic resistance genes capable of horizontal transfer**

Species	Source	Antibiotic resistance gene(s)	Associated mobile elements	Reference
L. brevis	Dairy	tet(M)	Not known	Nawaz et al., 2011
L. fermentum	Dairy	erm(B), msrC, erm(C), erm(T), tet(K), tet(L)	Plasmid, transposon	Gfeller et al., 2003 Nawaz et al., 2011 Thumu and Halami, 2012
L. paracasei	Dairy	tet(M)	Tn916	Devirgiliis et al., 2009
L. plantarum	Dairy, Vegetables	tet(M), erm(B), tet(W), tet(L)	Plasmid	Nawaz et al., 2011 Feld et al., 2009 Thumu and Halami, 2012
L. salivarius	Fermented food, Vegetables	erm(B), tet(W), tet(M), tet(O), tet(L)	Not known	Nawa et al., 2011 Thumu and Halami, 2012
L. reuteri	Fermented food, Poultry	erm(B), Cat-TC, tet(W)	Plasmid	Lin et al., 1996 Thumu and Halami, 2012

established. Tetracycline resistance genes (*tet*) are the most frequent in *Lactobacillus* (Ammor *et al.*, 2008c) while aminoglycoside resistance genes and β-lactam resistance genes (blaZ) are least frequent (Aquilanti ***et al.***, 2007).

Antibiotic resistance in *Bifidobacterium*

Some species of *Bifidobacterium* demonstrate phenotypic antibiotic resistance characters and have associated antibiotic resistance genes (Ammor *et al.*, 2008b) but most are not associated with mobile elements and thus are non-transferable. These bacteria are therefore suitable for use in the food chain as probiotics in animal feed (Flórez *et al.*, 2006; Kazimierczak *et al.*, 2006; Ammor *et al.*, 2008a; Van Hoek *et al.*, 2008). However, several species and strains of *Bifidobacterium*, including *B. longum* and *B. animalis* subsp. *lactis* harbour the antibiotic resistance gene tet(W), which is capable of intra-species transfer among *Bifidobacterium* (Gueimonde et al., 2013; Aarts and Margolles, 2015).

Antibiotic resistance in *Bacillus*

Antibiotic resistance has frequently been reported in *Bacillus. B. subtilis,* a frequently used probiotic can harbour conjugative transposons (e.g. Tn5397), which can transfer resistance to tetracycline encoded by the *tet(M)* gene (Mullany *et al.*, 1990; Roberts *et al.*, 1999). Phelan *et al.* (2011) reported another transferable tetracycline resistance gene, *tet(L),* in a *Bacillus* sp. encoded by a plasmid. *B. subtilis* can contain the macrolide-lincosamide-streptogramin B (MLS) resistance determinants on a plasmid (Monod, DeNoya and Dubnau, 1986). Macrolides are a very important class of antibiotics widely used

to control human and animal infections. The MLS determinant is homologous to the *erm(C)* gene, one of 19 analogous *erm* resistance genes (Monod, DeNoya and Dubnau, 1986). The most prevalent antibiotic resistance gene is *erm(D)* which encodes the determinants for the resistance to MLS (Gryczan *et al.*, 1984; EFSA, 2007). However, transferability of the determinants encoded by this gene has not been confirmed (EFSA, 2007).

Transfer of antibiotic resistance genes to potential pathogenic micro-organisms is one of the serious risks associated with probiotics, as many bacterial species used as probiotics harbour transferable antibiotic resistance genes. Therefore, stringent quality assurance measures are recommended in this regard using microbes as probiotics only with proven absence of transferable antibiotic resistance genes. *Lactobacillus*, *Bacillus* and *Enterococcus* present greater risk, as many species of these genera have transferable antibiotic resistance genes, while *Bifidobacteria* carry less risk as most of the resistance genes in these bacteria are non-transferable. However, the status of antibiotic resistance genes in microbial strains used as probiotics has not been determined. Presence of antibiotic resistance genes may not be a serious issue if such genes are intrinsic in chromosomes and not transferable. Nevertheless, precautions should be taken to avoid microbes with acquired genes being used as probiotics.

Labelling of probiotics used in animal feed

Labels in the packaging of commercial probiotic products should provide information about content, positive effects of the products, date of expiry, dose rates, contra-indications (if any), etc. However, commercial probiotics are often inadequately or incorrectly labelled. Weese (2003) suggested that an ideal probiotic label "should state the organisms that are present to the strain level, correctly spell and identify the contents, state the number of live organisms, and guarantee that the stated number would be present at the time of expiry".

Another piece of essential information that should be present on the label is the dose rate to be used for different categories of animals. This was often neglected on the labels (Weese, 2003).

Few studies have examined the quality and authenticity of probiotic labelling. Weese and Martin (2011) found that the labelling of commercial probiotics was very poor. The common errors in the labelling were failing to mention specific names of micro-organisms in the product, failing to give number of viable micro-organisms in the product, giving conflicting information, not mentioning expiry date, and misspelling the microbial name (Weese, 2003; Weese and Martin, 2011).

On labels of commercial probiotics produced for humans and animals, and marketed through health food stores, pharmacies, grocery stores, companion pet stores and veterinary clinics, some manufacturers use vaguely descriptive terms like "dried lactobacillus," "lactobacillus cultures", "probiotic cultures", "fermentation products" etc., instead of specific names of the micro-organisms in the product (Weese, 2003). Although a significant proportion of the commercial products included the name of the micro-organism(s) on the label, only eight out of twenty five (32%) products studied in Canada had a label with the correct names of micro-organisms and the number of viable organisms in the product (Weese and Martin, 2011). A significant number of producers misspelled the name of microbes, including using obsolete names and even listing the names of microbes which did not exist (Weese, 2003). Very few of the products were reported to have labels with the name of micro-organisms to the strain level (Weese, 2003).

Similarly, not all products had information about the number of viable micro-organisms, and even if the information was present it was not clear whether the specified quantity was at the time of manufacture or at the time of expiry (Weese, 2003). More serious was the problem that only four out of 15 (27%) products which mentioned the quantity of viable micro-organisms, actually met their claimed quantity. Ironically, there was even a product with no viable micro-organisms at all, despite claiming to have 14 million cfu/capsule. Only a small proportion (8%) of the studied products had both a satisfactory label and the quantity of viable bacteria as claimed on the label (Weese and Martin, 2011).

The most serious probiotic labelling errors occurred from wrong information, such as labelling the product as yeast instead of *Lactobacillus* sp., or claiming to have bacteria present that were not detected in the product, or claiming to have more bacteria than were actually present in the products (Weese, 2002; Lata *et al.*, 2006). Inclusion of micro-organisms with no proven probiotic effects and inclusion of potentially pathogenic micro-organisms in commercial products were other serious issues noted (Weese, 2002).

The objectives of probiotic labelling should be to provide the users with all necessary information to properly handle, store, transport and use the products, with necessary precautions to minimize hazards associated with the product. The label should be in a language understandable to the intended users. Probiotics with labels only in the English language are commonly marketed in developing countries, where the users may not understand English. Therefore, labels should be tailored to the intended audiences. The label should also assist in making an informed choice by end users.

Global regulatory status of probiotics in animal feed

The advancement in the knowledge of GIT microbial ecology and the mechanism(s) of probiotic action increases the possibility of the introduction of new probiotics. There is therefore increasing interest in the regulation of these products to protect human health, animal health and the environment. It is also important that the claims made by the manufacturers of probiotics are correct and consumers are appropriately protected.

Unlike other feed additives, probiotics have certain distinctive attributes. Probiotics are living organisms, can be inactivated in the GIT, and may interact with the genetics of the host animals. These factors require probiotics to be regulated more stringently than other feed additives (Hoffmann *et al.*, 2013). Moreover, there is a fine line between whether a probiotic is treated as a feed additive or a therapeutic agent. This affects the way in which the probiotic is regulated.

There are no studies on the release of probiotics into the environment either from animal manures or from other sources in their production and use.

CODEX ALIMENTARIUS COMMISSION

Codex Alimentarius Commission (CAC), originally established by FAO and WHO to develop food safety guidelines, has defined a feed additive in *Code of practice on good animal feeding* - CAC/RCP 54-2004 as "any intentionally added ingredient not normally consumed as feed by itself, whether or not it has nutritional value, which affects the characteristics of feed or animal products" (CAC, 2004), which includes micro-organisms, enzymes, acidity regulators, trace elements and vitamins. Therefore, "code of practice on good animal feeding" is the relevant code of CAC to follow as guidelines for the production, processing, storage, transport and distribution of probiotics by member states, in addition to their national legislation, to regulate probiotics.

UNITED STATES FOOD AND DRUG ADMINISTRATION

The United States Food and Drug Administration (US FDA) is the primary authority within the US Department of Health and Human Services with a mandate to regulate and oversee the use of foods, medicines (both prescription and over-the-counter drugs), vaccines, veterinary products, dietary supplements, etc. All products under the jurisdiction of the FDA are regulated by one of its six centres. based on the category of the products according to the intended use, generally as stated by the manufacturers. Hence, intended use is more important than the contents of the products in determining the nature of regulation of the products.

All livestock feeds, pet foods, veterinary drugs and devices and veterinary biologicals are regulated by the Center for Veterinary Medicine (CVM) under the FDA. The CVM regulates the safety, effectiveness, labelling and distribution of the products under its jurisdiction. In the case of any ambiguity or when there is confusion about which product should be regulated by which centre, the Office of Combination Products (OCP) under the FDA provides guidelines.

Similarly the Federal Trade Commission regulates the advertising and marketing of the products and may have a role in certain aspects of probiotic regulation.

The FDA uses the phrase Direct-fed Microbial (DFM) products for probiotics used in animal feed. The FDA guidance document (CPG Sec. 689.100) has defined DFMs as "products that are purported to contain live (viable) micro-organisms (bacteria and/or yeast)" (US-FDA, 2015). This FDA guideline has approved the micro-organisms listed in the official publication of the Association of American Feed Control Officials (AAFCO) that can be used in DFM (Table 6). Products marketed solely as silage additives are not regulated as DFM. For regulatory purposes, DFMs are considered either as fermentation products or yeast products.

The regulation of probiotics by FDA is chiefly determined by the intended use or claim of the product (Table 7). It could be either food/feed or drug or both, and regulated accordingly. The probiotics with the following claims are categorized as "new animal drugs" and regulated as a drug and need an approved new animal drug application (US-FDA, 2015).

- Cure, mitigation, treatment or prevention of diseases.
- Affect the structure or function of the body.

The approved micro-organisms listed in the publication of the AAFCO when marketed as DFM without any therapeutic or structure/function claims are categorized as food and regulated accordingly. The products categorized as food are monitored by the respective State Government rather than FDA unless these products have any safety issue (US-FDA, 2015). However, if the marketed micro-organisms are not listed by AAFCO and have no therapeutic or structure/function claims, the product is categorized as a food additive and regulated accordingly.

TABLE 6
Micro-organisms in the official list of AAFCO that are suitable for use in animal feed

Aspergillus niger	*Bifidobacterium thermophilum*	*Pediococcus acidilacticii*
Aspergillus oryzae	*Lactobacillus acidophilus*	*Pediococcus cerevisiae (damnosus)*
Bacillus coagulans	*Lactobacillus brevis*	*Pediococcus pentosaceus*
Bacillus lentus	*Lactobacillus buchneri* (cattle only)	*Propionibacterium acidpropionici (cattle only)*
Bacillus licheniformis	*Lactobacillus bulgaricus*	*Propionibacterium freudenreichii*
Bacillus pumilus	*Lactobacillus casei*	*Propionibacterium shermanii*
Bacillus subtilis	*Lactobacillus cellobiosus*	*Saccharomyces cerevisiae*
Bacteroides amylophilus	*Lactobacillus curvatus*	*Enterococcus cremoris*
Bacteroides capillosus	*Lactobacillus delbruekii*	*Enterococcus diacetylactis*
Bacteroides ruminocola	*Lactobacillus farciminis* (swine only)	*Enterococcus faecium*
Bacteroides suis	*Lactobacillus fermentum*	*Enterococcus intermedius*
Bifidobacterium adolescentis	*Lactobacillus helveticus*	*Enterococcus lactis*
Bifidobacterium animalis	*Lactobacillus lactis*	*Enterococcus thermophilus*
Bifidobacterium bifidum	*Lactobacillus plantarum*	*Yeast*
Bifidobacterium infantis	*Lactobacillus reuterii*	
Bifidobacterium longum	*Leuconostoc mesenteroides*	

Source: Pendleton, 1998

TABLE 7
Regulation of Direct Fed Micro-organisms (Probiotics) by FDA

Product	Intended use/Claim	Legal status	Regulated as	Regulated by
DFM	Cure, mitigate, treatment or prevention of disease	New animal drug	Drug	FDA
	Affect the structure and function of the body	New animal drug	Drug	FDA
	Without any therapeutic or structure/function claim (micro-organisms listed in AAFCO official publication)	Food	Food	State government
	Without any therapeutic or structure/function claim (micro-organisms not listed in AAFCO official publication)	Food additives	Food additives	FDA

Generally regarded as safe (GRAS)

The US Food and Drug Administration's Center for Veterinary Medicine (CVM) has a Generally Recognized as Safe (GRAS) notification programme for ingredients in animal feed. According to this programme "any substance that is intentionally added to food" is exempt from regulation as a food additive if the substance is GRAS. A food additive could get GRAS status either through scientific justification or based on a long history of safe use of the product in animal feed (before 1958).

EUROPEAN FOOD SAFETY AUTHORITY (EFSA)

The EU applies a very strict regulation for the assessment of probiotics, for which manufacturers of the probiotics should provide evidence of the identity, safety and efficacy of the product, which is assessed by a scientific committee of experts (European Commission, 2003). Probiotic products can only be marketed following assessment and approval from the scientific committee and authorization under EU regulation (EC) No. 1831/2003 on additives for use in animal nutrition. The manufacturers should follow use and labelling conditions to market the product as authorized by the European Commission.

Regulation (EC) No. 1831/2003 of the European Parliament and the Council of 22 September 2003 on additives for use in animal nutrition has classified feed additives into 5 categories: (a) technological additives; (b) sensory additives; (c) nutritional additives; (d) zootechnical additives; and (e) coccidiostats and histomonastats (European Commission, 2003). Although the word 'probiotics' is not used in the regulation, "micro-organisms or other chemically defined substances, which when fed to animals, have a positive effect on the gut flora" are categorized as "gut-flora stabilizers", a functional group under zootechnical additives. Therefore, probiotics in animal feed are regulated as zootechnical additives in the EU. Regulation 1831/2003 legislates the authorization, use, monitoring, labelling and packaging of feed additives.

In April 2008, the EU published Commission (EC) No. 429/2008, giving detailed rules for the implementation of Regulation (EC) No. 1831/2003, which details procedures for authorization of new probiotics entering the EU (Europen Commission, 2008), as outlined in Figure 3. Authorization granted according to this legislation is valid for 10 years and should be renewed thereafter.

FIGURE 3
Pathway for the authorization of new probiotics in the European Union as per Regulations (EC) No. 1831/2003 and 429/2008.

Source: Authors.

REGULATION OF PROBIOTIC LABELLING

The EU Regulation (EC) No 1831/2003 on additives for use in animal nutrition covers labelling of probiotics. According to this legislation, it is illegal to sell feed additives (including probiotics) without clearly labelling the products with (a) specific name and functional group of the additives (b) name and address of the business responsible for the product (c) net weight or net volume (in case of liquid) (d) approval number to establish and operate the establishment or the intermediary pursuant (where appropriate) (e) instructions for use including the species and categories of animal (f) date of manufacture with batch number (European Commission, 2003). In addition to these general requirements for feed additives, probiotics should have the following specific information on their label:

> "the expiry date of the guarantee or the storage life from the date of manufacture, the directions for use, the strain identification number, and the number of colony-forming units per gram" (European Commission, 2003).

In 1987, a joint exercise by FDA, Association of American Feed Control Officials (AAFCO) and National Feed Ingredients Association (later merged with the American Feed Industry Association) consensually agreed to include the phrase "contains a source of live (viable), naturally occurring micro-organisms" followed by the name of the micro-organisms in the product with the content guarantee, as colony-forming units per gram on the label of commercial probiotic products (DFM) to be used in animal feed (Pendleton, 1998). Before this decision, probiotics had been labelled and regulated as commercial feed in accordance with the AAFCO regulations and the label had to contain guarantees for protein, fat and fibre, which was obviously not relevant to the product (Pendleton, 1998).

The classification and marketing of probiotics as feed additives in most countries may result in the regulation and quality control of probiotics not being as stringent as that of veterinary drugs (Weese, 2003). This may result in probiotic labelling errors being overlooked.

Confusion and ambiguity prevail with regard to the regulation of probiotics in most countries. Approaches to risk assessment and level of stringency to authorize novel probiotics varies among nations. A global approach and guidelines to classify and regulate probiotics and assess risk could be effective in harmonizing regulations and protecting public health.

Conclusion

Increasing intensification of animal agriculture with consequent imprudent use of antibiotic growth promoters poses risks to human and animal health in terms of increasing antibiotic resistance in pathogenic micro-organisms. Live micro-organisms have been studied and used as probiotics for a long time, and as an alternative to antibiotic growth promoters in animal production. Several probiotics have been found effective in improving animal performance and preventing disease and the spread of the enteric pathogens in both monogastric and ruminant livestock industries.

With the advancement in knowledge in gastro-intestinal microbial ecology and mode of action of probiotics, the number of probiotic products available for use in animal nutrition is increasing. However, the micro-organisms used as probiotics and their efficacy are highly variable. There are many promising effects of probiotics on animal performance and health. However, the major limitation for the widespread and sustainable use of probiotics is the uncertainty in the reproducibility of effect, with a wide range of probiotic species, livestock species and husbandry practice highlighting the complexity of the interactions in animal production systems. Study about the effects of a particular microbial strain on variety of animal species, age groups, growth condition and diet types may help to identify the condition in which the probiotics could work. Although the use of probiotics could be a potentially viable solution to address the issue of increasing antibiotic resistance, it requires much further study on the effect, mechanism of action and safety of probiotics, to obtain consistent effects and a similar economic benefit to AGPs.

The claims made by commercial probiotic producers are often difficult to substantiate due to variation in animal species and husbandry practices and lack of scientific publications regarding the product. It is not possible to generalize the mechanism of action of probiotics. As the effects of probiotics in a host is the outcome of interaction between the host and the probiotic micro-organism, further studies should be focused on host-probiotic interactions to elucidate the mode of action. Although generally considered safe, there is little evidence that probiotics are absolutely safe and it has been agreed that "zero risk does not exist" (Marteau, 2001). Therefore, uncertainty would always exist about the efficacy and safety of probiotics. Studies about the minimum required dose of particular probiotic to achieve intended benefits and maximum dose rate which could be used without any adverse effects on the host help to assure the benefits and minimize the risk.

Further studies are also required to determine whether the probiotics used in animal nutrition enter the human food chain and how they affect human health. Information about specific precautions concerning handling by particularly vulnerable populations, such as immunocompromised people, or use in such hosts may further help to reduce any risk.

The stringencies of the regulations on the use of probiotics in animal agriculture vary, even in developed countries. Regulation of probiotics in the EU based on the assessment by a scientific committee of experts reviewing identity, safety and efficacy of the probiotic micro-organisms is exemplary.

The issue of maintaining safety and efficacy of probiotics could be more serious in developing countries where institutions that can do research on the efficacy of such probiotics and regulate the proper use of probiotics, are often in need of strengthening and capacity building. Therefore, focusing on relevant research for identification of risk associated with probiotics together with capacity building of competent regulation authority are important aspects to protect both public and animal health.

Bacterial genera commonly used as probiotics have been found to harbour antibiotic resistance genes on mobile genetic elements capable of transferring to potential enteric pathogens. Using microbial strains as probiotics only with proven absence of transferable antibiotic resistance genes could minimize this serious safety risk. Similar precautions should be carried out while using microbes with acquired resistance genes.

Therefore, international guidelines for the production, marketing and use of probiotics in animal nutrition are essential, especially with increasing globalisation. Such guidelines would help prevent the use of inappropriate micro-organisms as probiotics and maintain the efficacy of probiotics in achieving the targeted benefits. Such guidelines would assist institutions involved in the production, marketing and regulation of probiotics and protect public health. Such guidelines should also give detailed instructions for the analysis of the risk associated with probiotics intended for use in animal production.

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This document presents a unique and exhaustive review of the state-of-knowledge on the use of probiotics in diverse livestock production systems, and their impact on animal productivity. It focuses specifically on definitions, production, mechanisms of action, applications, effects, safety and potential public health risks of probiotics. In addition the labelling of probiotic products and global regulatory status of probiotics in animal feed is also covered. This publication will inform those that are interested in identifying and designing interventions for increasing animal productivity. It would also give an impetus to the development of new probiotics having consistent long-term effects that could possibly be used in feed in place of antibiotic growth promoters.

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