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**Direct-Fed Microbials (DFMs) in horses and poultry:
effects on digestibility, nutritional value of animal
products and animal health.**

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CHAPTER 1

General Introduction

1. General Introduction

The ban on the use of antibiotics as feed additives in animal nutrition has led to a worldwide search and implementation of alternative strategies aimed to preventing the growth of pathogenic bacteria in farm animals, to maintain their health and performance. The first barrier against pathogenic bacteria is the feed itself.

At the same time as making gains in production and efficiency, the industry has had to maximize the health and well being of the animals and minimize the impact of the industry on the environment. The use of feed additives has been an important part of achieving this success. The diet of animals and humans contains a wide variety of additives.

Feed additives are products used in animal nutrition for purposes of improving the quality of feed and the quality of food from animal origin, or to improve the animals' performance and health, e.g. providing enhanced digestibility of the feed materials. Feed additives may not be put on the market unless authorization has been given following a scientific evaluation demonstrating that the additive has no harmful effects, on human and animal health and on the environment.

According to Regulation (EC) No 1831/2003 and No 767/2009, only additives that have been through an authorization procedure may be placed on the market. Authorizations are granted for specific animal species, specific conditions of use and for ten years periods.

Additives may be classified into the following categories:

- ✓ **Technological additives;** This classification refers to a group of additives, which influences the technological aspects of the feed. This does not directly influence the nutritional value of the feed but may do indirectly by improving its handling or hygiene characteristics, for example. (e.g. preservatives, antioxidants, emulsifiers, stabilizing agents, acidity regulators, silage additives)
- ✓ **Sensory additives;** This refers to a group of additives which improve the palatability of a diet by stimulating appetite, usually through the effect these products have on the flavour or colour of the diet. (e.g. flavours, colorants)
- ✓ **Nutritional additives;** Such additives supply specific nutrient required by the animal for optimal growth. (e.g. vitamins, minerals, amino acids, trace elements)
- ✓ **Zootechnical additives;** These additives improve the nutrient status of the animal, not by providing specific nutrients, but by enabling more

efficient use of the nutrients present in the diet. (e.g. digestibility enhancers, gut flora stabilizers)

- ✓ **Coccidiostats and histomonostats;** These products are used to control intestinal health of poultry through direct effects on the parasitic organism concerned. They are not classified as antibiotics.

The European Food Safety Authority (EFSA) is responsible for conducting the evaluation of the data submitted requesting authorisations. After a favourable opinion of the EFSA, the Commission prepares a draft regulation to grant authorisation, following the procedure involving Member States within the Standing Committee on the Food Chain and Animal Health – Animal Nutrition. Any additives used in feed must be approved for use and then used as directed with respect to inclusion levels and duration of feeding. Within each one of these classes of additives there can be dozens of specific additives manufactured and distributed by a wide variety of companies. Again, all ingredients and additives must be noted on the label and their use and inclusion levels meet the standards as defined by law.

All animals need to be well fed and healthy if they are to grow to their potential. The nutrition of an animal is therefore of great importance if this is to be achieved in practice. Much of the nutrition of farm animals is derived from the major feed ingredients such as corn and soybean meal, but if these were the only ingredients then the animal would not grow particularly well and would likely become deficient in some essential nutrients.

In the case of animals in the wild, such deficiencies are either tolerated or moderated by selection of a wide variety of dietary ingredients (many of which are only available on a seasonal basis). In modern-day farming, the nutritional requirements of farm animals are well understood and all requirements can be met through direct dietary supplementation of the limiting nutrients in concentrated form. Nutritional quality of a feed is influenced not only by nutrient content but also by many other aspects such as, feed presentation, hygiene, content of anti-nutritional factors, digestibility, palatability and effect on intestinal health to name a few. Feed additives provide a mechanism by which such dietary deficiencies can be addressed which benefits not only the nutrition and thus the growth rate of the animal concerned, but also its health and welfare.

1.1. Probiotics

The name probiotic comes from the Greek “pro bios” which means “for life”. The origin of cultured dairy products dates back to the dawn of civilization; they are mentioned in the Bible and the sacred books of Hinduism. At the beginning of the 20th century the main functions of gut flora were completely unknown. Ilya Ilyich Metchnikoff, the Nobel Prize winner in Medicine in 1908, at the Pasteur Institute linked the health and longevity to ingestion of bacteria present in yoghurt (Metchnikoff *et al.* 1910). He believed that the constitution of the human body presented several disharmonies inherited from primitive mammals, such as body hair, wisdom teeth, stomach, vermiform appendix, caecum, and large intestine. In 1907, he postulated that the bacteria involved in yoghurt fermentation, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, suppress the putrefactive type fermentations of the intestinal flora and that consumption of these yoghurts played a role in maintaining health. Indeed, he attributed the long life of Bulgarian peasants to their intake of yoghurt containing *Lactobacillus* species (Metchnikoff *et al.* 2004). In particular, he reported that the large intestine, useful to mammals in managing rough food composed of bulky vegetables, is useless in humans. Moreover, it is the site of dangerous intestinal putrefaction processes which can be opposed by introducing lactobacilli into the body, displacing toxin producing bacteria, promoting health, and prolonging life (Del Piano *et al.* 2006).

Tissier's discovery of bifidobacteria in breast-fed infants also played a key role in establishing the concept that specific bacteria take part in maintaining health. In 1906, Tissier reported clinical benefits from modulating the flora in infants with intestinal infections (Tisser *et al.* 1906). At the time, many others were sceptical about the concept of bacterial therapy and questioned in particular whether the yoghurt bacteria (*L. bulgaricus*) were able to survive intestinal transit, colonize and convey benefits (Kulp *et al.* 1924). In the early 1920s, *L. acidophilus* milk was documented to have therapeutic effects, in particular, a settling effect on digestion (Cheplin *et al.* 1922). It was believed that colonization and growth of these microorganisms in the gut were essential for their efficacy, and therefore, the use of intestinal isolates was advocated. In Japan in the early 1930s, Shirota focused his research on selecting the strains of intestinal bacteria that could survive passage through the gut and on the use of such strains to develop fermented milk for distribution in his clinic. His first product containing *L. acidophilus* Shirota (subsequently named *L. casei* Shirota) was the basis for the establishment of the Yakult Honsha Company (1998).

Only at the end of the century, it became clear that intestinal microflora had several functions, including metabolic, trophic and protective ones (Guarner *et al.* 2003). Metabolic functions are primarily characterized by the fermentation of

non-digestible dietary residue and endogenous mucus, savings of energy as short chain fatty acids, production of vitamin K, and absorption of ions. Trophic functions are based on the control of epithelial cell proliferation and differentiation, and development and homeostasis of the immune system. Finally, protective functions are connected with the barrier effect and protection against pathogens (Del Piano *et al.* 2006). The health benefits derived from the consumption of foods containing *Lactobacillus acidophilus*, *Bifidobacterium* and *L. casei* are now well documented. *Streptococcus thermophilus* and *L. delbrueckii ssp. bulgaricus* are yoghurt starter cultures, which offer some health benefits; however, they are not natural inhabitants of the intestine. Therefore, for yoghurt to be considered as a probiotic product, *L. acidophilus*, *Bifidobacterium* and *L. casei* are incorporated as dietary adjuncts. Thus, the normal practice is to make a product with both starter organisms, e.g. *S. thermophilus* and *L. delbrueckii ssp. bulgaricus*, and one or more species of probiotic bacteria (Shah 2007).

The guidelines that stipulate what is required for a product to be called a probiotic were published by FAO/ WHO in 2002 (FAO/WHO 2002). They require that strains be designated individually, speciated appropriately and retain a viable count at the end of their shelf life in the designated product formulation that confers a proven clinical end-point. The probiotic definition requires that the efficacy and safety of probiotics be verified and thus, assessment of this constitutes an important part of their characterization for human use (Isolauri *et al.* 2004).

Over the years the word probiotic has been used in several different ways. It was originally used to describe substances produced by one protozoan which stimulated by another (Lilly *et al.* 1965), but it was later used to describe animal feed supplements which had a beneficial effect on the host animal by affecting its gut flora (Parker 1974). Crawford (1979) defined probiotics as “a culture of specific living microorganisms (primarily *Lactobacillus spp.*) that implants in the animal to ensure the effective establishment of intestinal populations of both beneficial and pathogenic organisms”. Fuller (1989) later gave a unique definition of probiotics as “a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance”. The US National Food Ingredient Association presented, probiotic (direct fed microbial) as a source of live naturally occurring microorganisms and this includes bacteria, fungi and yeast (Miles *et al.* 1991).

According to the currently adopted definition by FAO/WHO, probiotics are: "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). More precisely, probiotics are live microorganisms of non-pathogenic and nontoxic in nature, which when administered through the digestive route, are favourable to the host's health (Guillot 1998). Despite these numerous theoretical definitions, however, the

practical question arises whether a given microorganism can be considered to be a probiotic or not. Some strict criteria have been proposed. Havenaar *et al.* (2002), for example, proposed the following parameters to select a probiotic: total safety for the host, resistance to gastric acidity and pancreatic secretions, adhesion to epithelial cells, antimicrobial activity, inhibition of adhesion of pathogenic bacteria, evaluation of resistance to antibiotics, tolerance to food additives and stability in the food matrix. The probiotics in use today have not been selected on the basis of all these criteria, but the most commonly used probiotics are the strains of lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus* (*S. thermophilus*); the first two are known to resist gastric acid, bile salts and pancreatic enzymes, to adhere to colonic mucosa and readily colonize the intestinal tract (Fioramonti, 2003).

Their use was linked with a proven efficacy on the gut microflora resulted in improved health status. Two main mechanism of action have been suggested and are summarized as follows: (a) nutritional effect, characterized by reduction of metabolic reactions that produce toxic substances, stimulation of indigenous enzymes and production of vitamins and antimicrobial substances; and (b) health or sanitary effect, distinguished by increase in colonization resistance, competition for gut surface adhesion and stimulation of the immune response (Guillot, 2003) the last effect acting as 'bio-regulators of the gut microflora' and reinforcing the host natural defences. The probiotics would have therefore a role on the balance of gut microflora increasing the resistance to pathogenic agents, both through a strengthening of the intestinal barrier and stimulating directly the immune system. A list of the probiotic species for studies or application in animal feed in shown in **Table 1**.

The characteristics of good probiotics are:

- The culture should exert a positive effect on the host. It should be acid resistant, bile resistant and contain minimum 30×10^9 CFU (colony forming unit) per gram.
- The culture should possess high survival rate and multiply faster in the digestive tract. It should be strain specific.
- The culture microorganisms should neither pathogenic nor toxic to the host.
- The adhesive capability of microorganisms must be firm and faster.
- Be durable enough to withstand the duress of commercial manufacturing, processing and distribution so that can be delivered alive to the intestine.
- The cultured microorganisms should possess the ability to reduce the number of pathogenic microorganisms in intestine. (Choudhari *et al.* 2008)

Table 1. List of probiotics studied for application in animal feed.

Genus	Species
<i>Bifidobacterium</i>	<i>B. animalis</i> subsp. <i>animalis</i> (<i>B. animalis</i>) <i>B. lactis</i> subsp. <i>lactis</i> (<i>B. lactis</i>) <i>B. longum</i> subsp. <i>longum</i> (<i>B. longum</i>) <i>B. pseudolongum</i> subsp. <i>pseudolongum</i> <i>B. thermophilum</i>
<i>Enterococcus</i>	<i>E. faecalis</i> (<i>Streptococcus faecalis</i>) <i>E. faecium</i> (<i>Streptococcus faecium</i>)
<i>Lactobacillus</i>	<i>L. acidophilus</i> <i>L. amylovorus</i> <i>L. brevis</i> <i>L. casei</i> subsp. <i>casei</i> (<i>L. casei</i>) <i>L. crispatus</i> <i>L. farmicinis</i> <i>L. fermentum</i> <i>L. murinus</i> <i>L. plantarum</i> subsp. <i>plantarum</i> (<i>L. plantarum</i>) <i>L. reuteri</i> <i>L. rhamnosus</i> <i>L. salivarius</i> <i>L. amylovorus</i> (<i>L. sobrius</i>)
<i>Lactococcus</i>	<i>L. lactis</i> subsp. <i>cremoris</i> (<i>Streptococcus cremoris</i>) <i>L. lactis</i> subsp. <i>Lactis</i>
<i>Leuconosto</i>	<i>L. citreum</i> <i>L. lactis</i> <i>L. mesenteroides</i>
<i>Pediococcus</i>	<i>P. acidilactici</i> <i>P. pentosaceus</i> subsp. <i>Pentosaceous</i>
<i>Propionibacterium</i>	<i>P. freudenreichi</i>
<i>Streptococcus</i>	<i>S. infantarius</i> <i>S. salivarius</i> subsp. <i>Salivarius</i> <i>S. thermophilus</i> (<i>S. salivarius</i> subsp. <i>thermophilus</i>)
<i>Bacillus</i>	<i>B. cereus</i> (<i>B. cereus</i> var. <i>toyoi</i>) <i>B. licheniformi</i> <i>B. subtilis</i>
<i>Saccharomyces</i>	<i>S. cerevisiae</i> (<i>S. boulardii</i>) <i>S. pastorianus</i> (<i>S. carlsbergensis</i>)
<i>Kluyveromyces</i>	<i>K. fragili</i> <i>K. marxianus</i>
<i>Aspergillus</i>	<i>A. oryza</i> <i>A. niger</i>

1.2. Probiotics history and legal basis in the EU in feed

The microbial feed additives were covered by the Council Directive 70/524/EEC of 23 November 1970 concerning additives in feedingstuffs (OJ No. L 270, 14.12.1970). The Directive 70/524/EEC was amended five times; the last amendment was by the Council Directive 96/51/EC of 23 July 1996 (OJ No. L 235, 17.9.1996). In 2003, these Directives were repealed by the new Regulation (EC) No. 1831/ 2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (OJ No. L 268, 18.10.2003) which sets out the rules for its authorisation, use, monitoring, labelling and packaging. In the Regulation (EC) No. 1831/2003, the micro-organisms are included in the category “zootechnical additives” and as functional group within the “gut flora stabilisers” defined as micro-organisms or other chemically defined substances, which, when fed to animals, have a positive effect on the gut flora. Under this Regulation, specific labelling requirements are needed for micro-organisms such as the expiry data of the guarantee or storage life from the data of manufacture, the directions for use, the strain identification number, and the number of CFU per gram. In the EU, there is a positive list of the micro-organisms to be included in market products; this list contains nine columns which describe the following specifications: (1) EU number, (2) additive, (3) chemical formula, description, (4) species or category of animal, (5) maximum age, (6) minimum content (colony forming units per kilogram, CFU/kg) in the complete feeding stuffs, (7) maximum content (CFU/kg) in the complete feeding stuffs, (8) other provisions, and (9) period of authorisation. With respect to the guidelines for the assessment of microorganisms, the Council Directive 87/153/EEC of 16 February 1987 established the composition of the submission dossier for all feed additives (OJ No. L 64, 7.3.1987). The Commission has updated these guidelines in 1994 introducing specific requirements for enzymes and micro-organisms (Commission Directive 94/40/EC, OJ No. L 208, 11.8.1994). In 2001, the Directive 87/153/EEC was amended by the Commission Directive 2001/79/EC of 17 September 2001 fixing guidelines for the assessment of additives in animal nutrition (OJ No. L 267, 6.10.2001). This Regulation shall contain specific guidelines for the authorisation of feed additives (Article 7 of the Regulation). For that, the scientific panel on additives and products or substances used in animal feed (FEEDAP Panel) of the EFSA has worked in the last time on specific guidelines regarding the additive categories including the micro-organisms using as background the adopted previous guidelines provide by Council Directive 87/153/EEC. These guidelines will be submitted to the Health and Consumer Protection Directorate-General of the European Commission for its

consideration and establishment. In the meantime, Scientific Committee on Animal Nutrition (SCAN) (European Commission, 2001a,b) has published its opinion concerning guidelines for the assessment of additives in feedingstuffs, Part II: enzymes and micro-organisms. The guidelines impose the layout of the submission dossiers based on six sections: (1) summary of the data in the dossier; (2) identity, characterisation, and conditions of use of the additive. Methods of control; (3) studies concerning the efficacy of the additive, (4) studies concerning the safety of the use of the additive; (5) form of monograph; (6) form of identification note.

The 13 July 2009 was born the Regulation (EC) No 767/2009 of the European Parliament and of the Council on the placing on the market and use of feed, amending Regulation (EC) No 1831/2003 and repealing Council Directive 79/373/EEC, Commission Directive 80/511/ECC, Council Directives 82/471/EEC, 83/228/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC. The objective of this Regulation, in accordance with the general principles laid down in Regulation (EC) No 178/2002, is to harmonise the conditions for the placing on the market and the use of feed, in order to ensure a high level of feed safety and thus a high level of protection of public health, as well as to provide adequate information for users and consumers and to strengthen the effective functioning of the internal market.

1.3. Selection of probiotics for animal feed production

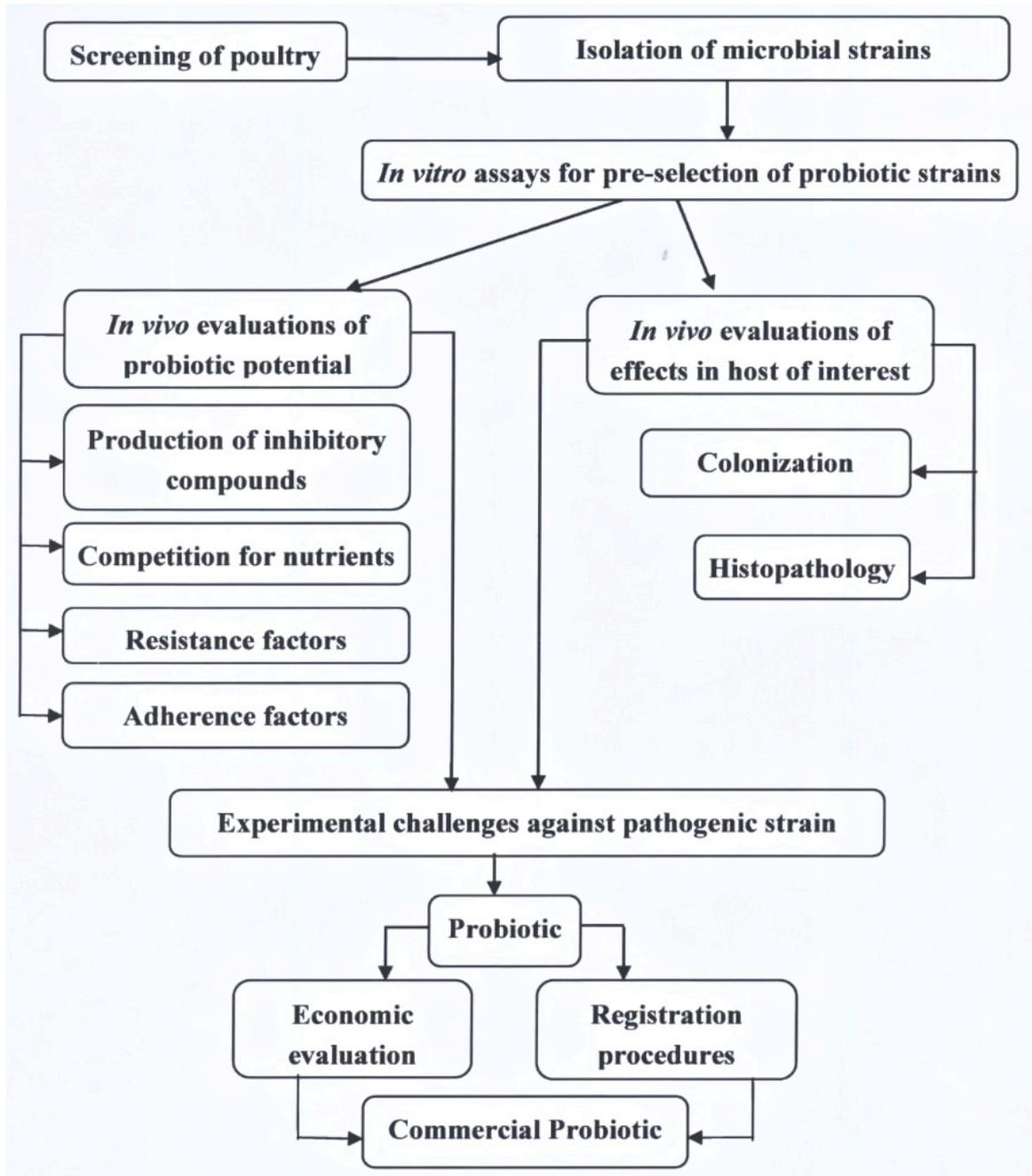
A summary of conventional criteria that can be used for the selection of microbial strains to be used as probiotics includes the following properties (Fuller, 1992 and Reuter, 2001):

- ✓ *Biosafety*: the strains of microorganisms should be Generally Recognized As Safe (GRAS microorganisms), for example, *Lactobacillus* species or some *Bifidobacterium* and *Streptococcus* (*Enterococcus*) species;
- ✓ *The choice of the origin of the strain*: probiotics should preferentially originate from the target animal microflora. This choice is determined by the specific purpose of the application of the probiotics (e.g. location specificity or requirement for colonization). The strains should be properly isolated and identified before use;
- ✓ *Resistance to in vivo/vitro conditions*: after administration of the probiotic, the microorganisms should not be killed by the defence mechanisms of the host and they should be resistant to the specific conditions occurring in the body. They should be resistant to the pH, bile and pancreatic juice conditions;

- ✓ *Adherence and colonization of intestinal epithelium/tissue*: factors that affect colonization should be considered. These should include the resistance of bacteria themselves, the effect of gastrointestinal environment (ingredient, pH, bile, salt, etc.) on colonization, the existing microbes that exert interacting factors (probiotics-host-microflora interactions);
- ✓ *Antimicrobial activity/antagonisms to pathogens*: lactic acid bacteria, which are frequently used as probiotics, have a number of antagonistic properties which operate by decreasing pH by the production of lactic acid, consumption of available nutrients, decreasing the redox potential, production of hydrogen peroxide under aerobic conditions, production of specific inhibitory components, such as bacteriocines, and which would help to protect against pathogenic organisms. This is important for the probiotics to be effective;
- ✓ *Stimulation of immune response*;
- ✓ *Viability/survival and resistance during processing* (e.g. heat tolerance or storage).

The choices of mono or multi strains, beneficial systematic effects (e.g. prebiotic-symbiotic system) and other properties such as oxygen tolerance, selective stimulation of beneficial bacteria and suppression of harmful bacteria are also considered (Rosset *et al.* 2005). The perceived desirable traits for selection of functional probiotics are many. The probiotic bacteria must fulfil the following conditions: it must be a normal inhabitant of the gut, and it must be able to adhere to the intestinal epithelium to overcome potential hurdles, such as the low pH of the stomach, the presence of bile acids in the intestines, and the competition against other micro-organisms in the gastro-intestinal tract (Nurmi *et al.* 1983, Chateau *et al.* 1993). The tentative ways for selection of probiotics as biocontrol agents in the poultry industry are illustrated in **Figure 1**. Many *in vitro* assays have been developed for the pre-selection of probiotic strains (Ehrmann *et al.* 2002, Koenen *et al.* 2004). The competitiveness of the most promising strains selected by *in vitro* assays was evaluated *in vivo* for monitoring of their persistence in chickens (Garriga *et al.* 1998). In addition, potential probiotics must exert its beneficial effects (*e.g.*, enhanced nutrition and increased immune response) in the host. Finally, the probiotic must be viable under normal storage conditions and technologically suitable for industrial processes (*e.g.*, lyophilized).

Figure 1. Diagram for selection of probiotics in the poultry industry (modified from Garriga *et al.* 1998, Klaenhammer *et al.* 1999, Morelli 2000, Ehrmann *et al.* 2002, Koenen *et al.* 2004,).



1.4. Probiotics applications in animal production

Several mechanisms have been proposed to explain the effects of probiotics and it is likely that the positive results reported in the different animal studies are due to a combination of some, if not all, of these. The metabolic activities of the probiotic strains and survivability throughout the gut appear to be of great importance for an optimal efficacy (Chaucheyras-Durand *et al.* 2008). Effects are also greatly dependent on the strain used (Newbold *et al.* 1995).

Probiotic is a generic term, and products can contain yeast cells, bacterial cultures, or both that stimulate microorganisms capable of modifying the gastrointestinal environment to increase health status and improve feed efficiency (Dierck 1989). Administration of probiotic strains separately and in combination significantly improved feed intake, feed conversion rate, daily weight gain and total body weight in chicken, pig, sheep, goat, cattle and equine (Chiofalo *et al.* 2004; Bontempo *et al.* 2006; Casey *et al.* 2007; Stella *et al.* 2007, Torres-Rodriguez *et al.* 2007, Quarantelli *et al.* 2008, Agazzi *et al.* 2009).

Many studies have reported the effects of yeast supplementation on hindgut digestion and microbial population in horses.

Research indicates that adding yeast culture to the diet of horses can improve nutrient digestibility (Switzer *et al.* 2003), increase microbial populations (Medina *et al.* 2002, Lattimer *et al.* 2005), and maintain cecal pH (Medina *et al.* 2002, Hall *et al.* 2005). However, other reports observed no improvement in nutrient digestibility when yeast culture was supplemented to horses in vivo (Webb *et al.* 1985, Glade *et al.* 1986) and in vitro (Lattimer *et al.* 2005).

Mechanisms by which probiotics improve feed conversion efficiency include alteration in intestinal flora, enhancement of growth of nonpathogenic facultative anaerobic and gram positive bacteria forming lactic acid and hydrogen peroxide, suppression of growth of intestinal pathogens, and enhancement of digestion and utilization of nutrients (Yeo *et al.* 1997). Therefore, the major outcomes from using probiotics include improvement in growth (Yeo *et al.* 1997, Bontempo *et al.* 2009), reduction in mortality (Kumprecht 1998), and improvement in feed conversion efficiency (Yeo *et al.* 1997). These results are consistent with previous experiment of Tortuero and Fernandez (1995), who observed improved feed conversion efficiency with the supplementation of probiotic to the diet.

It is clearly evident from the result of Kabir *et al.* (2004) that the live weight gains were significantly ($P < 0.01$) higher in experimental birds as compared to control ones at all levels during the period of 2nd, 4th, 5th and 6th weeks of age, both in vaccinated and nonvaccinated birds. This result is in agreement with many investigators (Khaksefidi *et al.* 2006, Timmerman *et al.* 2006, Willis *et al.* 2007, Rasteriro *et al.* 2007, Nayeypor *et al.* 2007, Mountzouris *et al.* 2007, Apata 2008,

Awad *et al.* 2009, Sahin *et al.* 2009, Ashayerizadeh *et al.* 2009, Ferroni *et al.* 2009) who demonstrated increased live weight gain in probiotic-fed birds. On the other hand, Lan *et al.* (2003) observed higher ($P < 0.01$) weight gains in broilers subjected to two probiotic species. Huang *et al.* (2004) demonstrated that inactivated probiotics, disrupted by a high-pressure homogenizer, have positive effects on the production performance of broiler chickens when used at certain concentrations. In addition, Torres-Rodriguez *et al.* (2007) reported that administration of the selected probiotic (FM-B11) to turkeys increased the average daily gain and market body weight, representing an economic alternative to improve turkey production. However, Karaoglu and Durdag (2005) used *Saccharomyces cerevisiae* as a dietary probiotic to assess performance and found no overall weight gain difference.

Probiotics have been shown to be involved in protection against a variety of pathogens in chicken including *Escherichia coli* (Chateau *et al.* 1993), *Salmonella* and *Campylobacter* (Stern *et al.* 2001), *Clostridium* and *Eimeria* (Dalloul and Lillehoj 2005). Probiotics tended to improve the health status and fertility of sows (Alexopoulos *et al.* 2004), reduce the adhesion of porcine enteropathogenic *E. coli* and invasion of *Salmonella typhimurium* with epithelial cells *in vitro* (Kleta *et al.* 2006). A *Pediococcus acidilactici* based probiotic effectively enhanced the resistance of birds and partially protected against the negative growth effects associated with coccidiosis (Lee *et al.* 2007).

Probiotic can exhibit antibacterial activity against fish pathogenic bacteria (Sugita *et al.*, 2002) and could reduce mortality of fish challenged with a virulent strain of *Aeromonas salmonicida* (Nikoskelainen *et al.* 2001). Probiotic can also alter the balance of gastrointestinal microflora in healthy cats (Marshall-Jones *et al.* 2006) and were shown to be effective in preventing antibiotic associated diarrhoea (Hawrelak *et al.* 2005).

Some probiotics produce nutrients and growth factors, which are stimulatory to beneficial microorganisms of the gut microbiota. Recently Yaman *et al.* (2006), Mountzouris *et al.* (2007) and Higgins *et al.* (2007) demonstrated that probiotic species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* have a potential effect on modulation of intestinal microflora and pathogen inhibition.

In addition to interacting and stimulating other microorganisms, probiotics also interact with the host, by influencing the immune response (Delcenserie *et al.*, 2008), or producing components able to positively affect mucosa development or the metabolism of the host's intestinal cells (Johnson-Henry *et al.*, 2008).

The indigenous intestinal bacteria inhibit pathogens by competition to colonization sites and nutritional source and production of toxic or stimulation of the immune system (Paravez *et al.*, 2006). These mechanisms are not mutually exclusive and inhibition may comprise one, or all of these mechanisms. The

variation in efficacy of probiotics under different conditions may be attributable to the probiotic preparation itself or may be caused by external conditions. Probiotics can significantly protect mice against infection with the invasive food borne pathogen *Listeria monocytogenes* and *Salmonella typhimurium* and protect pigs against diarrhoea (Corr *et al.*, 2007). The protection included a ten-fold increase in survival rate, significantly higher post-challenge feed intake and weight gain and reduced pathogen translocation to visceral tissues (Shu *et al.*, 2000).

Probiotic reduced gastric inflammation and bacterial colonization in *Helicobacter pylori*-infected animals (Johnson-Henry *et al.*, 2005) and induced an inflammatory response in feedlot steers fed high-grain diets (Emmanuel *et al.*, 2007). The improvement in the immune system may be by three different ways: (a) enhanced macrophage activity and enhanced ability to phagocytose microorganism or carbon particles; (b) increased production of antibodies usually of IgG & IgM classes and interferon (a nonspecific antiviral agent) and; (c) increased local antibodies at mucosal surfaces such as the gut wall (usually IgA). Probiotic in the organism of a healthy animal stimulates non-specific immune response and enhances the system of the immune protection (Ceslovas *et al.*, 2005). Probiotic increased intestinal IgA secretion both in sows and piglets and elevated IgG and IgM levels in turkey (Cetin *et al.*, 2005). The effect of intestinal IgA secretion could be related to a more successful mucosal defense which in turn led to a lower level in systemic IgG production in piglets after weaning (Scharek *et al.*, 2007). Furthermore, administration of probiotic results in beneficial systemic and immunomodulatory effects in cats (Marshall-Jones *et al.*, 2006). Kabir *et al.* (2004) evaluated the dynamics of probiotics on immune response of broilers and they reported significantly higher antibody production in experimental birds as compared to control ones.

The manipulation of gut microbiota via the administration of probiotics influences also the development of the immune response (McCracken *et al.* 1999). The exact mechanisms that mediate the immunomodulatory activities of probiotics are not clear. However, it has been shown that probiotics stimulate different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of the immune response (Maassen *et al.* 2000, Christensen *et al.* 2002, Lammers *et al.* 2003,). Probiotics, especially lactobacilli, could modulate the systemic antibody response to antigens in chickens (Kabir *et al.* 2004, Huang *et al.* 2004, Koenen *et al.* 2004, Haghghi *et al.* 2005, Mathivanan *et al.* 2007, Apata *et al.* 2008).

It is believed by most investigators that there is an unsteady balance of beneficial and non-beneficial bacteria in the tract of normal, healthy, non-stressed poultry. When a balance exists, the bird performs to its maximum efficiency, but if stress is imposed the beneficial flora, especially lactobacilli, have a tendency to decrease in numbers and an overgrowth of the non beneficial ones seems to occur. This

occurrence may predispose frank disease, diarrhoea, or be subclinical and reduce production parameters of growth, feed efficiency, etc. The protective flora, which establishes itself in the gut is very stable, but it can be influenced by some dietary and environmental factors. The three most important are excessive hygiene, antibiotic therapy and stress. In the wild, the chicken would receive a complete gut flora from its mother's faeces and would consequently be protected against infection. However, commercially reared chickens are hatched in incubators which are clean and do not usually contain organisms commonly found in the chicken gut. There is an effect of shell microbiological contamination, which may influence gut microflora characteristics. Moreover, also HCl gastric secretion, which starts at 18 days of incubation, has a deep impact on microflora selection.

Therefore, an immediate use of probiotics supplementation at birth is more important and useful in avian species than in other animals. The chicken is an extreme example of a young animal which is deprived of contact with its mother or other adults and which is, therefore, likely to benefit from supplementation with microbial preparations designed to restore the protective gut microflora (Fuller, 2001).

1.5. Selenium in animal feed

Incorporation of selenium into the diets of all animals is required for maintenance of health, growth, and physiological functions (Scott *et al.*, 1982). It was discovered in 1817 by Berzelius (Levander, 1986; Sunde, 1997), and for many years, Se was thought to be toxic to animals. However, in 1957, Se was reported to prevent liver necrosis in rats (Schwarz and Foltz, 1957), which established Se as a dietary essential nutrient. Since then, Se has been identified to be an integral part of over 30 distinct selenoproteins, including the enzyme, glutathione peroxidase (Sunde, 1997 and Arthur, 2000). The glutathione peroxidases are a group of antioxidant enzymes that are essential for protection of the cells of the body from peroxidative and free-radical damage (Sunde, 1997; Arthur, 2000). These enzymes are unique because Se is required in the form of selenocysteine (Rotruck *et al.*, 1973, Sunde, 1997, Arthur, 2000). Selenium also is necessary in the diets of poultry to protect from exudative diathesis and pancreatic fibrosis, which are two common conditions in poultry caused by Se deficiency (Cantor, 1975a,b). Despite the establishment of a dietary need for Se, it is still considered to be the most toxic dietary essential trace mineral.

The dietary requirement of poultry for Se often can be met by natural feedstuffs in the diet, but these feedstuffs vary widely in Se concentration depending on the region that they are grown. Therefore, it is common practice in poultry production to supplement diets with Se. One of the most common supplements used is sodium selenite (SS), an inorganic form of Se.

Currently it is well established that the biological function of Se in body is mediated via specific selenoproteins/selenoenzymes, hydrogen selenide and methylated Se-compounds, respectively. About 30 specific selenoproteins have been identified but only half of them are functionally described (Behne and Kyriakopoulos, 2001). Contemporary knowledge of the major biological functions of Se can be briefly listed as follows:

- ✓ Antioxidant to prevent oxidative stress
- ✓ Proper thyroid function
- ✓ Maintenance of cellular redox status
- ✓ Reduction of oxidized ascorbic acid, which in turn can recycle tocopheroxyl to tocopherol
- ✓ Development and maintenance of immunocompetence
- ✓ Detoxification of heavy metals and some xenobiotics
- ✓ Anticancerogenic effects of some methylated Se-compounds

More detailed recent information on the biological functions of Se can be found in McKenzie *et al.* (1998), Arnér and Holmgren (2000), Rayman (2000) and Schrauzer (2000), Arthur *et al.* (2003), Kohrle (2005).

In the form of selenocysteine, Se is integral part of the functional units of all selenoenzymes known so far, the most prominent being the glutathione peroxidases (GPX), the iodothyronine deiodinases (ID), the thioredoxin reductases and a selenophosphate synthetase. Selenoenzymes are part of the antioxidant defence system in the body and are involved in thyroid hormone metabolism, in spermatogenesis, and probably in other as yet unidentified processes. The activity of these selenoenzymes depends on adequate Se intake, defining this trace element as essential nutrient. In recent years, low Se status in humans has been associated indeed with a number of pathologic conditions (Kohrle J. *et al.* 2000, Rayman M.P. *et al.* 2000), such as Keshan disease, certain cancers, a myxedematous form of cretinism, atherosclerosis, reduced immune function and reduced male fertility, suggesting an important role of Se in human health. Moreover, low Se intake usually combined with a low vitamin E status is known to cause serious Se deficiency diseases in many animal species.

Exudative diathesis, pancreatic fibrosis, and impaired reproduction are observed if the Se level in the diet is deficient. Exudative diathesis and pancreatic fibrosis, which are discussed in detail below, have a major difference in the form of Se needed to alleviate their deficiency signs.

Reproductive impairment, on the other hand, does not seem to be specific in the form of Se needed to alleviate its deficiency signs (Underwood and Suttle, 1999). Exudative diathesis is characterized by a general oedema due to atypical permeability of the capillary walls (Underwood and Suttle, 1999). It first appears on the breast, wing, and neck as greenish-blue discoloration due to fluid accumulation under the ventral skin. Abnormal growth rate and high mortality are common in flocks with exudative diathesis, and Hartley and Grant (1961) indicated that this condition usually occurs between 3 and 6 weeks of age. Noguchi *et al.* (1973a) reported that either Se or vitamin E could prevent exudative diathesis. In a subsequent study, Noguchi *et al.* (1973b) reported that dietary Se is directly related to GPX-3 activity and the prevention of exudative diathesis. Selenium in the form of SS or selenocysteine provides the most protection from exudative diathesis (Cantor *et al.* 1975a,b).

Pancreatic fibrosis results from a severe Se deficiency in poultry, and it causes atrophy of the pancreas, as well as poor growth and feathering (Thompson and Scott, 1969). Bunk and Combs (1980) reported that appetite depression associated with this condition is negated within hours of Se supplementation. Furthermore, Noguchi *et al.* (1973a) indicated that the pancreatic lesions, which become apparent by 6 days of age, return to normal within 2 weeks after the onset of Se supplementation. High dietary vitamin E cannot alleviate this condition as pancreatic fibrosis results in a secondary vitamin E deficiency due to impaired formation of lipid bile micelles, which are necessary for the absorption of vitamin E (Thompson and Scott, 1969). Selenium in the form of

selenomethionine (SM) protects poultry from pancreatic fibrosis more efficiently than sodium selenite (SS) or selenocysteine (Cantor *et al.* 1975a,b).

Impaired reproduction in females also can result from Se deficiency. Cantor and Scott (1974) reported that egg production and hatchability were reduced in laying hens fed diets with reduced levels of Se, and Latshaw *et al.* (1977) indicated that hatchability was the most sensitive criteria of Se deficiency in hens. Furthermore, Jensen (1968) reported that low dietary Se impaired fertile egg hatchability and chick viability in Japanese quail.

In ruminants, lack of dietary Se and vitamin E causes nutritional muscular dystrophy also known as white muscle disease, which is characterized by necrosis of cardiac and skeletal muscles and elevated creatine kinase (CK) activity (McMurray C.H *et al.* 1983). The conditions are reversible to some extent by supplementing the affected animals with Se or vitamin E or both (Combs G.F. *et al.* 1986, Whanger P.D. *et al.* 1976).

The variety and severity of clinical signs in Se deficiency within a species is far from being homogeneous (McMurray C.H *et al.* 1983) and it is not surprising that the pattern of pathology in Se deficiency diseases differs considerably between species (Combs G.F. *et al.* 1986). Nevertheless, it is noteworthy that there also exist distinct similarities in Se-deficient animals of different species.

For example, with regards to vascular lesions, it was reported (McMurray C.H *et al.* 1983) that the pathology in one calf affected with white muscle disease resembled dietetic microangiopathy (mulberry heart disease) seen in pigs, whereas in another calf changes occurred similar to exudative diathesis in chickens. Until the supplementation of farm animal diets with Se becoming common practice, these deficiency disorders frequently occurred in many areas of the world, where soil Se content is low, causing substantial economic losses. Because both vitamin E and Se are potent antioxidants, it is believed that increased peroxidative challenge due to deficiency of Se or vitamin E or both is involved in the pathogenesis of these degenerative diseases.

1.6. Use of selenium for poultry

Selenium is a dietary essential nutrient for poultry. The Se requirement for the laying hen ranges from 0.05 to 0.08 ppm depending on daily feed intake while the broiler's requirement is 0.15 ppm (NRC, 1994). Natural feedstuffs often will meet these requirements, but as previously mentioned, there is considerable variation in Se content of natural feedstuffs. Therefore, it is common practice in the poultry industry in the U.S. to supplement the diet with some form of Se. The maximum level of Se supplementation allowed in poultry diets is 0.30 ppm (NRC, 1994; AAFCO, 2003). This supplementation has historically come from

inorganic sources of Se, primarily SS, but in 2000, the FDA approved the use of SY. There have been several reports comparing the use of organic Se with inorganic Se in broilers and laying hens, which will be discussed below.

1.7. Selenium in broilers diet

The response to dietary Se supplementation has been somewhat variable. Several researchers reported that Se supplementation increased growth performance (Thompson and Scott, 1969; Bunks and Combs, 1980; Cantor *et al.*, 1982; Echevarria *et al.*, 1988b) while several others have reported no effect (Miller *et al.*, 1972; Shan and Davis, 1994; Edens *et al.*, 2001; Spears *et al.*, 2003). Only Echevarria *et al.* (1988a) reported a negative effect of Se on growth performance, and they were feeding very high levels of SS (3, 6, or 9 ppm), which could be toxic to 17 broilers. None of the researches has reported a difference in growth performance due to source (organic versus inorganic).

The results of Se supplementation on tissue Se concentrations are fairly consistent when diets are supplemented with Se. There are several reports of Se supplementation increasing breast, liver, or plasma Se levels (Scott and Thompson, 1971; Cantor *et al.*, 1982; Echevarria *et al.*, 1988a,b; and Spears *et al.*, 2003). Furthermore, Cantor *et al.* (1982) and Spears *et al.* (2003) both indicated that organic Se increased tissue Se levels more than inorganic Se or a diet with no supplemental Se.

The published results on plasma glutathione peroxidase 3 (GPX-3) activity are variable. Cantor *et al.* (1982) and Spears *et al.* (2003) both reported that plasma GPX-3 activity was increased when diets were supplemented with Se, regardless of source. However, in a second trial, Spears *et al.* (2003) indicated that plasma GPX-3 was increased more by SS supplementation than by SM. Only Cantor *et al.* (1975) indicated no differences in plasma GPX-3 when broilers were fed SS, SM, or no supplemental Se.

1.8. Selenium in layers diet

The effects on supplemental Se in diets for laying hens are relatively consistent. Several researchers have indicated no difference in daily egg production due to Se supplementation or source (Cantor and Scott, 1974, Latshaw and Osman, 1975, Ort and Latshaw, 1978, Cantor *et al.*, 2000; Patton, 2000).

The research on whole egg Se concentration when diets are supplemented with Se is large and relatively consistent. The increase in whole egg Se when diets are supplemented with Se has been reported by several authors and is very consistent (Cantor and Scott, 1974; Latshaw and Osman, 1975; Ort and

Latshaw, 1978; Latshaw and Biggert, 1981; Martello and Latshaw, 1982; Swanson, 1987; Davis *et al.*, 1996; and Cantor *et al.*, 2000). Several reports also have indicated that yolk or white Se concentrations are increased depending on Se source, but these reports are slightly variable. Latshaw and Osman (1975), Martello and Latshaw (1982), Swanson (1987), and Davis *et al.* (1996) reported that eggs from hens fed diets supplemented with selenomethionine (SM) had higher Se in the white than those fed SS or selenocysteine. Ort and Latshaw (1978) indicated that the Se level of yolks was greater when hens were fed SS, but Swanson (1987) and Davis *et al.* (1996) indicated that SM increased yolk Se more than SS. Latshaw and Biggert (1981) and Cantor *et al.* (2000) reported that whole egg, egg white, and egg yolk Se levels were greater in hens fed SM compared with those fed SS.

1.9. Selenium-enriched yeast in the diet of laying hens

Selenium is required for maintenance of health, growth, and physiological functions. Traditionally, Se has been added to poultry diets via inorganic sources, such as sodium selenite (Na_2SeO_3).

Research has shown that organic Se is more bioavailable than Se in sodium selenite (Cantor *et al.*, 1982). Therefore, organic sources of Se, such as Se yeast (SY), have been explored as an alternative to inorganic supplementation (Payne, *et al.*, 2005, Invernizzi *et al.* 2007). The use of organic Se results in less Se being transferred to the environment through feces, and more Se is deposited into body tissues and eggs. Previously, eggs have been very useful in studying the absorption of various Se compounds (Latshaw and Osman, 1975).

Se-yeast is a product derived from the fermentation of specific strains of yeast incubated in high selenium levels during their growth phase. Being biochemically similar to sulphur, Se replaces the sulphur molecule in the normal biosynthetic pathways of the yeast cell and is absorbed actively across the intestine by the same amino acid carrier (Kim and Mahan, 2003).

The Se-enriched eggs are produced using Se-enriched yeast (SY) as a major source of Se for laying hens at a level of 0.3-0.5 mg Se/kg in feed (Fisinin *et al.*, 2008). However, the production process of Se-enriched yeast requires complex and high technology (Suhajda *et al.*, 2000; Ouerdane *et al.* 2008). A common yeast species, *Saccharomyces cerevisiae*, in SY acts as a Se biotransformation vector, (Suhajda *et al.* 2000) and this strain of yeast, as well as some others (e.g., *Saccharomyces boulardii* and *Candida utilis*), can also serve as probiotics (Agarwal *et al.* 2000, Yang *et al.* 2009). Besides these yeast strains, there may be other microorganisms that also have the ability to transform inorganic Se to organic forms. Numerous applied studies on SY have been conducted in broilers and

laying hens, and the results has shown that dietary supplementation of SY resulted in better production efficiencies. (Surai *et al.* 2000, Leeson *et al.* 2008). Latshaw and Biggert (1981) reported that whole egg, egg white, and egg yolk Se levels were 44%, 79%, and 15% greater, respectively, in hens fed organic selenium compared with those fed SS; this was confirmed by results obtained by Cantor *et al.* (2000), who used the same range of supplementation.

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CHAPTER 2

Objectives

2. Objectives

The objective of this thesis was to evaluate the effect of different Direct-Fed Microbials (DFM) in horses and poultry, on digestibility and nutritional value of animal products and animal health. To achieve this objective, three different trials were designed; in the first one the effects of live yeast on apparent digestibility in horses were investigated (Chapter 3), the second one studied the effects of the inclusion of a probiotics mix in broiler chickens infected with *Eimeria spp.* (Chapter 4) and the last examined the effects of inclusion of selenium-enriched yeast in the diet of laying hens on production performances, health parameters, eggshell quality, and selenium tissue deposition (Chapter 5).

CHAPTER 3

Evaluation of the effects of live yeast supplementation on apparent digestibility of high-fiber diet in mature horses using the acid insoluble ash marker modified method

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3. Evaluation of the effects of live yeast supplementation on apparent digestibility of high-fiber diet in mature horses using the acid insoluble ash marker modified method.

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3.1. Abstract

The objective of this study was to evaluate the effects of live yeast (LY) in a high-fiber diet on nutrients digestibility in mature horses. Six Italian Standardbred mares (weight: 544 ± 14 kg; age: 15.30 ± 3.9 years) in two-period crossover design were fed a basal diet (2.5% body weight [BW]) in a 70:30 forage:concentrate ratio with (LY) or without (CTR) the administration of 4.6×10^{10} colony forming unit (CFU)/d of *Saccharomyces cerevisiae* (MUCL 39885). An adaptation to the diet of 14 days, and an 18-day administration phase, with fecal collection in the last 3 days were performed for each period. Yeast was top-dressed twice a day during the concentrate meal (12:30 AM and 09:00 PM). Change in BW was measured at the beginning of each experimental phase and the diet adjusted accordingly, and individual feed intake was recorded daily. Concentrate samples were collected at the beginning of each confinement period and individual hay samples were obtained for each confinement day 38 hours before fecal collection. No influence of LY was observed on BW change ($P = .64$), feed intake ($P = .48$), hay intake ($P = .48$), or concentrate intake ($P = .47$). *S. cerevisiae* supplementation improved apparent digestibility of dry matter (64.5% vs. 60.1%, $P = .03$), organic matter (66.1% vs. 61.6%, $P = .04$), neutral detergent fiber (42.5% vs. 35.9%, $P = .04$), and acid detergent fiber (36.5% vs. 28.0%, $P = .03$) with a positive trend on crude protein ($P = .08$). In the present study, the administration of LY to horses significantly improved the digestion of the fiber fractions of the diet.

3.2. Introduction

During the last two decades, many articles have reported the effects of yeast supplementation on hindgut digestion and microbial population in horses (Morgan *et al.* 2007, Medina *et al.* 2002, Jouany *et al.* 2008, Lattimer *et al.* 2007). In field trials, some stimulatory effects and beneficial alterations of microbial population have been outlined, but variable and sometimes inconsistent evidence were also found. Generally, the positive effects observed were referred to an increased availability of nutrients in the diet (Godbee *et al.* 2003), whereas cellulolytic microbial population may or may not be increased (Medina *et al.* 2002, Lattimer *et al.* 2007, Moore *et al.* 1994).

However, *S. cerevisiae* supplementation in the diet of horses has been found to decrease the accumulation of lactate, and subsequently the pH; these changes led to an increased activity of the lower-gut cellulolytic bacterial populations in the horse (Jouany *et al.* 2008). The positive changes in the gut environment may decrease susceptibility to colic pain or laminitis (Jouany *et al.* 2008). Alternatively, the lack of response of liveyeast (LY) found in some in vivo or in vitro studies Webb *et al.* 1985, and Glade *et al.* 1986) can be related to the dosage applied, the yeast strain supplemented, and also time and sampling procedures adopted for diet and feces collection (Goachet *et al.* 2009). Among the in vivo digestibility assessments methods, total collection, considered the most accurate, can be replaced by naturally occurring indigestible markers such as acid insoluble ash (AIA) Bergero *et al.* 2005. The use of AIA as an internal marker for the apparent digestibility of nutrient in horse has been adopted by many authors previously (Sutton *et al.* 1977, Frap *et al.* 1982, Cuddeford *et al.* 1990, Miraglia *et al.* 1999, and Peiretti *et al.* 2003), leading to similar (Peiretti *et al.* 2006 and Almeida *et al.* 2001) or different (Peiretti *et al.* 2003) results when compared with total collection method. These variations among the two methods could be related to many factors concerning the increasing rates of concentrate inclusion in the diet (Peiretti *et al.* 2003), the percentage of recovery of AIA in the feces (O'Connor-Robison *et al.* 2007), the contamination with soil while collecting the samples from the ground (Pagan *et al.* 1998), the duration of the collection period (Goachet *et al.* 2009), and the number of hay and concentrate chemical analyses performed. With regard to the latter point, works concerning the evaluation of digestibility by total fecal collection or by different internal markers are usually based on a single hay sample for each day of fecal collection, or else the amount of analyzed hay samples is unclear (Morgan *et al.* 2007, Jouany *et al.* 2008 and O'Connor-Robison *et al.* 2007). Moreover, total mean retention time (MRT) has been reported to vary widely depending on the forage:concentrate ratio in the diet and exercising/resting activity of the horse,

with a mean value of 38.7 hours for nonexercised horses (Pagan *et al.* 1998); thus, sampling diets and feces on the same day means that the sampled feces are not derived from the samples of hay collected.

Therefore, the purpose of this study was to evaluate the effects of LY supplementation on nutrient digestibility in mature horses fed a high-fiber diet.

3.3. Materials and Methods

3.3.1 Horses and Dietary Treatments

Care, handling, and sampling of animals defined in the present study were approved by the University of Milan Animal Care and Use Committee. Six Italian Standardbred mares from “Centro Ippico La Fornace” (Zelo Buon Persico, Lodi, Italy) were used in a two-period crossover design with two treatments. Horses ranged in age from 11 to 23 years (average age, 15.3 ± 3.9 years) and weighed between 490 and 559 kg (mean body weight [BW], 544 ± 14 kg). BW was assessed by an electronic scale ($2.25 \times 1.11 \times 1.62$ m³, maximum weight: 3,000 kg, sensitivity: 1 kg; Bottaro Sist. di Pes. Ind. sas, Grassobbio, BG, Italy). Vaccination and deworming practices were consistent with farm protocol. Horses were vaccinated annually for tetanus, Western equine encephalitis, Eastern equine encephalitis, West Nile, influenza, equine herpes virus (EHV-1, EHV-4), strangles, and rabies. The trial consisted of two different periods named period 1 (P1) and period 2 (P2) of 35 days each, including 14 days adaptation period to the diet, 18 days of supplementation with the test material, and 3 days of fecal collection. P1 and P2 schedules were modified with longer adaptation to the maintenance diet and administration of LY, than those in the studies conducted by Morgan *et al.* 2007, so as to better acclimatize the horses, while the 3-day fecal collection period was maintained. All horses were kept in single boxes. During the first 32 days of each period, horses were allowed to exercise daily for 2 hours, whereas in the last 3 days of each period experimental animals were confined 24 hours a day. During the 2 hours daily turnout, every horse was free to move into a yard with sand so as to avoid any other FI except the experimental diet. In both P1 and P2, the horses were constantly maintained on shavings in the boxes to avoid any nutrient ingestion other than the experimental diets. All horses received an individual basal diet consisting of first cut grass/legume mixed hay in a 1:1 ratio and concentrate (Plurificoc gran mix 40, Morando spa, Andezeno, TO, Italy; containing flaked corn, barley, oat, soybean, beet pulps, carob, flavors, cane molasses, salt). Each horse was fed approximately 2.5% of BW daily as reported by the National Research Council (2007), at a 70:30 (dry matter [DM] basis) forage:concentrate ratio. The same batches of hay and concentrate were used during the whole trial period. Horses

were fed individually four times per day with hay and concentrate separately (08:30 AM and 05:00 PM for hay, and 12:30 AM and 09:00 PM for concentrate), with free access to fresh water. A four-meal daily feeding program was chosen to maintain the feeding behaviours of the experimental animals to the previously adopted routine in the Center. Although National Research Council guidelines suggest the use of only hay for maintenance diets and the administration of grains could increase MRT (Pagan *et al.* 1998), concentrate was introduced in the diet to mimic a common standard high-fiber diet, in which an inclusion of grains is often adopted. Consumption of concentrate and hay was recorded for each 24-hour period during the whole experiment. Individual weighted single bags for each meal of hay and concentrate were provided for each horse in the two groups for the whole trial. BW was measured at 0, 15, 33, and 36 days for each period. Before the beginning of the study, horses were blocked by age and BW and were randomized within blocks to each treatment group. Treatment consisted of the following: (1) administration of basal diet without the supplementation of LY (CTR); (2) administration of basal diet with the supplementation of LY. LY (Biosprint[®], *Saccharomyces cerevisiae* MUCL 39885, Prosol, Madone, Bergamo, Italy) added to a micronized oat was top-dressed twice a day on the concentrate (at 12:30 AM and 09:00 PM) at the rate of 25 g per meal (50 g per horse per day, 4.6×10^{10} CFU/d). At the same meals, CTR horses received 50 g of micronized oat without LY. All horses were fed by individual feeders so as to allow any soil contamination that can partially influence AIA determination.

3.3.2 *Sample Collection*

During each 3-day collection period, horses were fitted with fecal collection harnesses (Equisan Marketing Pty. Ltd., South Melbourne, Victoria, Australia) and housed in individual stalls (3.50×3.50 m²) with ad libitum access to water. Fecal samples were removed from the collection harnesses after every 6 hours. Individual 500 g of fecal samples were obtained from daily homogenized total fecal collection for each horse, frozen, and stored (-20°C) for subsequent analytical determinations. At the beginning of each fecal collection period, a 500 g sample of concentrate was collected and stored (-20°C) for future analyses. Two samples of mix hay (morning and evening feeding) for each horse were collected approximately 38 hours before the first fecal collection day: this procedure was performed also for the second and third fecal collection day. A unique individual 200 g fresh matter daily hay sample was then provided with a 1:1 ratio and frozen for subsequent analytical determinations, similar to that done for the concentrate. This kind of procedure for hay samples was adopted

to fit diet and fecal chemical composition adequately on the basis of the previously obtained results on MRT (Jouany *et al.* 2008, Pagan *et al.* 1998, Drogoul *et al.* 2000) in the whole hindgut, and considering that the experimental diet adopted in the present study had a forage to concentrate ratio of 70:30.

3.3.3. *Preparation of Samples for Chemical Analyses*

Hay, concentrate, and fecal samples were analyzed for DM, organic matter (OM), crude protein (Kjeldahl N \times 6.25), ether extract, ash, and AIA content in accordance with the Association of Official Agricultural Chemists (AOAC 2000) protocols. All samples were analyzed for neutral detergent fiber (NDF) and acid detergent fiber (ADF) content according to procedures described by Van Soest *et al.* 1991. Chemical composition of the concentrate and hay used in the trial is listed in **Table 1**. AIA content was used as an internal marker to determine the apparent digestibility of the experimental diets as reported by Van Keulen and Young (Van Keulen *et al.* 1977).

3.4. **Statistical Analysis**

All data were analyzed using a PROC MIXED procedure of SAS (SAS/STAT, Version V9.1, SAS Inst Inc., Cary, NC, USA) with treatment (CTR, LY), day of sampling (1,2,3), period (1,2), and horses (1-6) in the model statement. In this study, horse was considered the experimental unit for all the collected fecal samples. Least square means and standard errors were obtained according to treatment effects on the tested parameters. Data were analyzed for two treatments (CTR, basal diet without LY supplementation; LY, basal diet with yeast supplementation). Probabilities of $\leq .05$ were considered statistically significant, and probably values between .05 and .10 were considered to be trends toward significance.

3.5. Results and Discussion

3.5.1. *Chemical Composition of Hays, Feed Intake, and Live Body Weight*

No behavioural differences between supplemented and nonsupplemented horses were evidenced for each experimental period. Mean chemical composition of hay is reported in **Table 1**. Although experimental horses were fed at restricted levels, some refusals were found in both experimental groups for hay and concentrate but, at a very small level. No differences were evidenced in total feed intake (FI) expressed as a percentage of BW when *S cerevisiae* was fed to mature horses ($P = .48$) (**Table 2**). In the same way, voluntary hay ($P = .48$) or concentrate ($P = .47$) intake was not different among horses receiving basal diet or diet supplemented with LY. These findings are in accordance with the results obtained by Morgan *et al.* (2007), who did not find any effect of yeast supplementation in concentrate and hay intake expressed as a percentage of BW. In that study, increased FI was associated more with low-quality hay than *S cerevisiae* supply, probably because of the lower nutritional content of the material. Otherwise total FI in the present study is in contrast with the results reported by Jouany *et al.* (2008), who evidenced an increase in FI (approximately 2.05% vs. 2.00% BW) in horses fed high-fiber diets and supplemented with *S cerevisiae*. The author stated that these differences were mainly because of some hay refusals by horses in control group. BW of experimental horses was found to be similar at the beginning and at the end of the experiment (**Table 3**); moreover no effect of the administration of *S cerevisiae* was evidenced for the single period, phase, or the interaction between them as previously reported in other studies (Morgan *et al.* 2007). During the whole trial period, all animals in both groups gained approximately 1.7% of initial BW, and there were no obvious signs of digestive or metabolic disorders. The observed increase in BW in both experimental groups is much lower than the range of 10% BW evidenced by Jouany *et al.* (2008). These discrepancies in BW gain could be because of the fact that the horses used in the present trial were allowed to move during adaptation, with a 3-day confinement period, whereas horses used by Journey *et al.* (2008) had less possibility to move because they were fistulated and had a longer fecal collection period.

3.5.2. *Effect of S. cerevisiae on Apparent Digestibility of Nutrient*

Positive effects of the administration of *S cerevisiae* were evidenced during the trial on apparent digestibility of nutrients (**Table 4**). DM and OM digestibility were improved in horses fed LY by 7.3% and 8.7%, respectively. These positive

findings in LY horses are in accordance with results previously reported with the use of AIA as internal markers supplying (Miraglia *et al.* 1999), and later confirmed by the study of Jouany *et al.* 2008. Crude protein and crude fat digestibility were not influenced by the treatment at any time, although protein apparent digestibility had a tendency to be higher during the fecal sampling periods in LY group ($P = .08$). On the basis of the statistical analysis, there was a strong positive effect of the treatment on the apparent digestibility of the fibrous fractions such as NDF ($P = .04$) and ADF ($P = .03$) in horses fed 4.6×10^{10} CFU/d of LY. No negative values on apparent digestibility of fibrous fractions were observed in both experimental groups. The fibrous fractions digestibility was within the ranges reported (Lattimer *et al.* 2007, Miraglia *et al.* 1999). As in the present study, generally a positive effect of *S cerevisiae* on ADF digestibility of horse has been reported previously (Medina *et al.* 2002, Glade *et al.* 1991, Kim *et al.* 1991), although some works showed no effect of yeast (Glade *et al.* 1986, Hall *et al.* 1990). Although the microbial breakdown of the plant material occurs at the beginning of the digestive tract in ruminants and at the end of the digestive tract in horses, the mechanisms involved in the digestion of cellulosic material are very similar in their basic approach in both species. These data underline that large similarities exist between the rumen and the hindgut of horses with regard to the effect of LY on the digestive microbial populations (Jouany *et al.* 2009). Therefore, LY can be used in horses to balance and stabilize the digestive microbial ecosystem as they are in ruminants. As reported in a previous study (Medina *et al.* 2002, Jouany *et al.* 2008), the possible mechanism that can explain the positive results observed in the present trial is that a large part of ingested LY can survive during transit through the digestive tract of horses to the cecum and colon resulting in an increase in the digestibility of the fiber fractions. Although most of published works on LY administration in horses suggest that *S cerevisiae* is not able to colonize the cecum and the colon, the positive effects observed could be related to the viable yeast present in the intestinal ecosystem (Nagaraja *et al.* 1997). Medina *et al.* (2002) found that the concentration of yeast cells in the cecum remained at the level close to that initially supplemented to horses; these data suggest that the positive effects on fiber fractions could be because of the increased activities of yeast cells and bacteria involved in the digestion of cellulosic material rather than in increased bacterial biomass (Jouany *et al.* 2008).

In accordance with this mechanism of action, the administration of *S cerevisiae* significantly modified pH, concentrations of lactic acid ammonia and (acetate + butyrate)/propionate ratio with high-fiber diets (Medina *et al.* 2002). Moreover, the molar percentage of acetate was increased in the cecum and in the colon with yeast supplementation, suggesting an increased fibrolytic activity in the hindgut of horses supplemented with *S cerevisiae*,

although no increase in cellulitic bacteria population was observed (Medina *et al.* 2002). Increased apparent digestibility of nutrient in the present study was expected to increase dietary energy intake but, because of the lack of differences in behaviors and BW between control and treated horses, currently it is not possible to support this hypothesis: further investigations are needed to ascertain the positive effect of LY administration over dietary energy availability.

3.5.3. *Factors Affecting Apparent Digestibility of Nutrient*

The generally higher digestion levels of nutrients evidenced during the present trial with respect to previous works (Morgan *et al.* 2007, Jouany *et al.* 2008, Miraglia *et al.* 1999) could be because of several factors. Most of the published data present great variability in apparent digestibility rates for all investigated nutrients despite the fecal collection methods adopted (Miraglia *et al.* 1999). Thus, the composition of the diet in terms of forage:concentrate ratio seems to be partly responsible for these variations (Lattimer *et al.* 2007). Nevertheless, the quality of feed itself and the feeding strategies could influence the digestibility coefficients (Medina *et al.* 2002, Jouany *et al.* 2008). By contrast, the use of AIA is generally reported to over-estimate the digestion rates, depending on the collection period adopted and the fecal recovery of AIA (Goachet *et al.* 2009). Goachet *et al.* (2009) evidenced how digestibility coefficients were higher with AIA method than total fecal collection on a 5-day collection period, but this difference among the two methods was lower for a 3-day collection period. Although these results are in contrast with the studies by others (Miraglia *et al.* 1999), who evidenced no differences for AIA or total fecal collection, it can be speculated that a shorter 3-day sampling period with AIA method is most comparable with results obtained from total fecal collection. In the present study, a 118% fecal recovery of AIA was evidenced in accordance with data by Goachet *et al.* 2009. Such a high recovery rate can partially explain higher digestibility values evidenced in both groups probably because of either an under-estimation of the ingested concentration of AIA or an artificial increase in fecal AIA concentration. Moreover, forage:concentrate ratio has been reported to influence the digestibility coefficients of nutrient in the diet of horses. Variations in NDF/starch ratio of the diet alter the hindgut ecosystem in the cecum and colon as a result of changes in dietary carbohydrate sources (Medina *et al.* 2002). The amount of starch administered is a factor that could explain in some cases the negative interactions with cellulolysis and inhibition of the degree of cellulolysis, (Jouany *et al.* 2008). Furthermore, the technological processes applied to grains can influence the enzymatic digestion of starch in the small intestine with consequent alterations in the amount of starch reaching

the large intestine and interacting with fiber digestion (Julliand *et al.* 2006). Currently, most of the works have been performed by adopting two (Medina *et al.* 2002, Jouany *et al.* 2008) or three (Rosenfeld *et al.* 2006) meals per day, but, to the authors' knowledge, only Goachet *et al.* 2009 adopted a four-meals per day strategy. Further investigations are necessary to determine the effect of this kind of feeding practice. All the presented factors can significantly affect the MRT. A mean value of 38.7 hours for nonexercised horses was found (Pagan *et al.* 1998), with a minimum of 30.8 hours for an all-forage diet and a maximum of 43.4 hours with a 42% concentrate inclusion in the diet. A larger variability in a chopped-hay diet was instead evidenced later (Drogoul *et al.* 2000), ranging from 27.7 hours to 46.7 hours, depending on the marker used. Increasing MRT with increasing levels of concentrate in the diet was later confirmed by Jouany *et al.* 2008, who found a 3.5 hours higher MRT in horses fed high starch diets than animals fed a high fiber-containing diet. In this case, MRT in the whole hindgut ranged between 31.7 and 35.0 hours.

3.6. Summary

The administration of *S cerevisiae* to mature horses fed high-fiber diet increased apparent nutrient digestion rate for most of the investigated parameters considered for this trial. The apparent digestion rates of DM and OM were significantly improved in treated animals as compared with control subjects, but the most relevant difference among experimental groups was evidenced by a positive effect of the of LY over the fibrous fractions such as NDF and ADF.

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3.8. Tables and figures

Table 1 Chemical composition of concentrate, hay, and diets used in experimental mature horses (%DM)

Chemical composition	Concentrate ^a	Hay ^b	Diets (Calculated) ^c	
			CTR	LY
DM %	90.43	89.79	90.10	89.75
OM %	85.13	83.12	83.79	83.57
CP %	13.06	9.75	10.77	10.23
EE %	2.91	1.08	1.60	1.62
NDF %	33.04	64.19	54.40	53.20
ADF %	18.34	46.20	37.72	37.68
Ash %	5.30	6.67	6.13	6.19
AIA %	0.47	0.45	0.48	0.44

^aContaining flaked corn, barley, oat soybean, beet pulps, carob, flavpr, cane molasses, salt.

^bFirst cut grass/legume mixed hay in 1:1 ratio.

^cDiet designation represent the presence of 50 g/head/d of a supplement containing micronized oat an *S. cerevisiae* (4.6×10^6 CFU/d) (LY= yeast culture) or 50 g/head/d of micronized oat without any additions of yeast (CTR= control) in the diet of experimental animals; diets composed of 70:30 hay concentrate ratio.

Table 2 Feed intake (FI) of diet, concentrate intake and hay intake, and mean body weight (BW) of mature horses fed *S cerevisiae*^a

Feed intake	Group		SEM	P-Value
	CTR	LY		
FI, %BW^b	2.27	2.28	0.02	0.48
Concentrate intake, %BW	0.71	0.71	0.02	0.47
Hay Intake, %BW	1.56	1.57	0.01	0.48

^aLY, horses fed live yeast (*S cerevisiae*); CTR, horses fed basal individual diet.

^bEach mean represent 6 individually fed horses.

Table 3 Body weight (BW) during the different experimental phases for each trial periods of mature horses fed *S cerevisiae*

Phase	Period 1			Period 2			End	SD	P-Value ^b
	1	2	3	1	2	3			
Treat. ^a									
CTR	534	535	539	552	553	553	550	14	0.66
LY	536	548	548	544	544	539	538		

^aBody weight of experimental horses fed (LY) or not (CTR) *S cerevisiae* are expressed as kg ± SEM.

^bProbability are referred to treatment (T), phase (Ph), period (P), and interaction between T x Ph x P.

Table 4 Apparent digestibility of the high-fiber diets containing 70:30 hay-concentrate ratio with or without the administration of *S cerevisiae* in the mature horses ^a

Item ^a	Group		SEM	SD ^b	Min ^c	Max ^d	P-Value		
	CTR	LY					Treatment	Day of sampling	Interaction ^e
DM%	60.1 ^f	64.5 ^g	1.6	8.2	48.5	78.4	0.03	0.68	0.95
OM%	61.6 ^f	66.1 ^g	1.8	9.7	48.1	95.1	0.04	0.83	0.45
CP%	77.0	79.1	1.7	6.7	67.5	88.3	0.08	0.18	0.69
EE%	39.9	45.2	5.0	19.1	-0.9	80.6	0.34	0.38	0.59
NDF%	35.9 ^f	42.5 ^g	2.6	11.2	19.1	64.1	0.04	0.64	0.87
ADF%	28.0 ^f	36.5 ^g	3.1	13.6	8.6	59.2	0.03	0.73	0.90

^aEach mean represents six individually fed horses.

^{b,c,d}Standard deviation (SD), Minimum (Min), and Maximum value (Max) refer to all apparent digestibility coefficients over the whole experimental horses in P1 and P2.

^eInteraction between yeast supplementation and day of sampling.

^{f,g}Means within a row lacking a common superscript differ ($P \leq 0.05$) for treatment effect.

CHAPTER 4

Effect of the inclusion of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum* in the ration of broiler chickens infected with *Eimeria spp.* on growth performance, health status, and intestinal morphology.

4. Effect of the inclusion of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum* in the ration of broiler chickens infected with *Eimeria spp.* on growth performance, health status, and intestinal morphology.

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4.1. Abstract

The aim of the study was to investigate the effects of the inclusion of a probiotics mix (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum*) on growth performance, health status, and intestinal morphology of broiler chickens infected with *Eimeria spp.* for a complete production cycle from hatching to slaughter. A total number of 900 Hubbard male chickens, 1d old and coming from the same hatchery, were divided in 18 pens located in 3 identical rooms (6 pens per room).

At the beginning of the trial, the animals were homogeneously distributed by body weight to 3 experimental dietary treatments consisting of a basal diet plus: PC= probiotics 0.05% (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum*) + a coccidiostat; C= a coccidiostat; P= probiotics 0.1%. On the second week of life each box of the experimental groups was challenged with 400g of litter containing 2.5×10^5 oocysts of *Eimeria spp.* homogeneously distributed.

Considering the trial divided in two periods: no challenge (first two weeks) and challenge (3 to 8 weeks), in the first two weeks of the trial, birds fed the diets containing probiotics (PC and P) had significantly higher body weight (BW), average daily gain (ADG) and feed intake (FI) compared to animals fed the diet containing only a coccidiostat (C) and the feed conversion ratio (FCR) was significantly lower in the groups fed the diets containing probiotics (P and PC).

During the challenge period BW was significantly higher in birds fed the diets containing coccidiostat (C and PC), while no differences were observed for FCR, ADG and FI. The administration of probiotics plus coccidiostat (PC) positively affected the number of intestinal fold goblet cells (F) in the duodenum and ileum. In the ileum of PC group the lowest values for gland depth (G) were

assessed, while F:G ratio was lower compared with C group. Fecal count of *Eimeria spp* did not show any statistical difference between the three experimental groups at the end of the trial.

The results of this work show that the administration of probiotics (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum*) in normal farming conditions had positively influenced the growth performance of broiler chickens.

Subsequently in challenge period no differences were detected between broilers fed either probiotics and/or coccidiostat considering; growth performance and fecal count of *Eimeria spp*, these results suggest probiotics may have positive effects administered alone or in combination with coccidiostats in broilers chickens infected with *Eimeria spp*.

4.2. Introduction

Feed additive antibiotics have been used as growth promoters and control disease in animals, for more than 50 years in the feed industry all over the world. However, antibiotic use tends to produce antibiotic resistance and residues in animal products.

The indiscriminate use of antibiotic growth promoters (AGP) led to emergence of resistance in pathogenic bacteria, and as result, the European Union has banned the addition of several antibiotics in broiler feeds since 2006, and other countries should follow this trend soon.

Considering the inevitable ban of antibiotic growth promoters, and aiming at maintaining the productivity levels achieved by modern poultry production, research has been focused to find efficient alternatives to the use of antibiotic growth promoters. In this context, microbial products, such as probiotics, may be one of such alternatives.

Recently, beneficial effects of probiotics on broiler I) performance (Mountzouris *et al.*, 2007; Vicente *et al.*, 2007; Apata, 2008), II) nutrient digestibility (Apata, 2008; Li *et al.*, 2008), III) modulation of intestinal microflora (Mountzouris *et al.*, 2007; Teo and Tan, 2007; Yu *et al.*, 2008), IV) pathogen inhibition (Higgins *et al.*, 2008; Vicente *et al.*, 2008; Mountzouris *et al.*, 2009), and V) immunomodulation and gut mucosal immunity (Chichlowski *et al.*, 2007; Teo and Tan, 2007) have been reported.

In particular many probiotic species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* have a beneficial effect on broiler performance (Willis *et al.* 2008, Apata *et al.* 2008, Awad *et al.* 2009, Sahin *et al.* 2009, Ashayerizadeh *et al.* 2009), modulation of intestinal microflora and pathogen inhibition (Mountzouris *et al.* 2007, Kabir *et al.* 2005, Yaman *et al.* 2006, Higgins, *et al.* 2007). Enhancement of colonization resistance and/or direct inhibitory effects against pathogens are important factors by which probiotics have reduced the incidence and duration of diseases. The mode of action of probiotics in poultry includes: (I) maintaining normal intestinal microflora by competitive exclusion and antagonism (Schneitz 2005, Kizerwetter-Swida *et al.* 2009), (II) altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production (Jin *et al.* 2000, Yoon *et al.* 2004), (III) improving feed intake and digestion (Awad *et al.* 2006), and (IV) stimulating the immune system (Nayebpor *et al.* 2007, Apata 2008, Mathivanan *et al.* 2007, Brisbin *et al.* 2008).

Among all, coccidiosis of chickens is one of the most economically important disease affecting the poultry industry and is caused by seven species of intracellular protozoan parasites of the genus *Eimeria*. *Eimeria* typically invades

cells of the intestinal epithelium and causes destruction of the infected cells resulting in reduction of feed conversion, body weight gain, and increased morbidity and mortality (Min *et al.*, 2004; Morris and Gasser, 2006; Mc Donald and Shirley, 2009). Therefore, several studies have been undertaken to identify various dietary supplements and probiotics to control *Eimeria* infections (Dalloul *et al.*, 2003, Jang *et al.* 2007, Molan *et al.* 2009, Nweze and Obiwulu 2009, Dongjean *et al.* 2010). Dietary supplementation with a *Bacillus* in coccidiosis-affected poultry showed to improve feed conversion (Kyung *et al.*, 2010).

The aim of the study was to investigate the effect of the administration of *Bacillus coagulans*, *B.subtilis* and *Cl. butyricum* on growth performance, health status, and intestinal flora of broiler chickens infected with *Eimeria spp.* for a complete production cycle from hatching to slaughter.

4.3. Materials and methods

4.3.1. *Animals and Experimental Design*

A total number of 900 Hubbard male chickens, 1d old and coming from the same hatchery, were divided in 18 pens located in 3 identical rooms (6 pens per room). At the beginning of the trial, the animals were homogeneously distributed by body weight to 3 experimental dietary treatments consisting of a basal diet plus: PC= probiotics* 0.05% (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum*) plus a coccidiostat**; C= a coccidiostat; P= probiotics 0.1%. Before the beginning of the trial all animals received a vaccination program according to the procedure adopted by the hatchery.

The three groups of animals were reared following the breeding practices as reported by the genotype guidelines (HUBBARD Management Guide).

On the second week of life each pen of the experimental groups was challenged with 400g of litter containing 2.5×10^5 oocysts of *Eimeria spp.* homogeneously distributed. To ensure a similar microclimate, animals were housed in 3 identical rooms located in the same building. The chicken feed was produced in two steps, first for control groups and then for treated groups. In order to avoid contamination with the experimental product, the different feeds were kept in the facility in separate silos.

The 18 pens had the same area of 5m^2 and animals density of 10.0 animals/ m^2 (50 animals per pen), to ensure the same housing conditions.

Pens were bedded with shavings of white wood, and chickens had the same light cycle and temperature. Room climate programs considered a temperature equal to 33°C under the brooder and 30°C in the living area with a humidity of 60%, and a ventilation of $1\text{m}^3/\text{kg BW}$ until day 21. From day 22 of age until 35 the experimental rooms had 26°C under the brooder, 23°C in the living area, 65% humidity and a ventilation of $3.4\text{m}^3/\text{kg BW}$. From 36 to slaughtering room temperature was maintained at 19°C with 65% humidity and a ventilation of $3.4\text{m}^3/\text{kg BW}$. Lighting program was based on a 24h-on for the first 5 days decreasing until 12h-on/12h-off at 21d of life.

Chickens had free access to drinking water at all times and the experimental diets were available *ad libitum*. The experimental diets were based on corn, soybean meal, corn gluten meals, extruded soybean meal and animal fat (Table.1 and Table.2). During the trial the feed was sampled at each change of ration depending on growing stage and analysed for DM, CP, EE, ash, Ca, total P, Lys and Met content.

***Probion**[®], by WooGene B&G Co., Ltd.

** **Avatec**[®] 150G, by Pfizer.

4.3.2. *Growth performance*

Individual body weight (BW) of chickens was recorded weekly from the beginning of the trial (day 0) with an electronic weighing scale (sensitivity 1g), and average daily gain (ADG) was calculated.

Feed intake (FI) per pen was recorded daily and feed conversion rate (FCR) was calculated weekly. Health status and mortality was recorded daily.

At slaughterhouse hot carcass weight was registered.

4.3.3. *Coccidia quantitative analysis*

On 10 pools of faeces from each group at end of the trial (30 pools in total) quantitative presence of coccidia was analysed using the procedure FLOTAC: new multivalent techniques for qualitative and quantitative copromicroscopic diagnosis of parasites in animals and humans (Cringoli *et al.* 2010).

4.3.4. *Intestinal morphology (analysis performed on two groups C and PC)*

Six experimental chickens for each C or PC groups were selected at slaughtering for histomorphological analysis and measurements of the small and large intestine. The entire intestinal tracts were removed, and the distal ileum (2 cm prior to the opening into the caecum) was collected from each animal and promptly fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) pH 7.4 for 24 h at 4°C. The specimens were then dehydrated in graded alcohols, cleared with xylene and embedded in paraffin. After dewaxing and re-hydration, serial microtome sections (4 µm thick) were stained with haematoxylin and eosin and examined to assess micro anatomical structure and determine villus height (VH), crypt depth (CD), VH/CD ratio, number of goblet cells, number and sizes of cecal tonsils.

4.4. Statistical analysis

BW, ADG, FI and FCR were analysed by a MANOVA procedure for repeated measures of SAS/STAT, (Version V8, 1999, SAS Inst, Inc., NC, U.S.A.) including the fixed effect of the treatment, day, and the interaction treatment x day and considering the box as the experimental unit.

Carcass weight was analysed by a General Linear Model (GLM) procedure of (SAS/STAT, Version V8, 1999, SAS Inst, Inc., NC, U.S.A.) including the fixed effect of the treatment and considering the group as the experimental unit. The significance was declared at $P \leq 0.05$.

Histometric data (villus height, crypt depth, V:C ratio) were analysed by ANOVA using the mixed procedure of the SAS package. The model included treatment as fixed effect and the chicken as random effect. The data were presented as least squared means \pm SE. Differences between least squared means were analysed by orthogonal contrast and considered significant at $P \leq 0.05$.

4.5. Results

Results of growth performance are summarized in Table 3 and Table 4.

In the first two weeks of the trial, birds fed the diets containing probiotics (P and PC) had significantly higher body weight (BW), average daily gain (ADG) and feed intake (FI) compared to animal fed the diets containing only coccidiostat (C) and the feed conversion ratio (FCR) was significantly lower in the groups fed the diets containing probiotics (P and PC).

During the challenge period BW was significantly higher in birds fed the diets containing coccidiostat (C and PC), while no differences were observed about FCR, ADG and FI.

At slaughtering no differences were observed on carcass weight between the experimental groups (Table 3).

The administration of probiotics plus coccidiostat (PC) positively affected the number of intestinal fold goblet cells (F) in the duodenum and ileum.

In the ileum of PC group the lowest values for gland depth (G) were assessed, while F:G ratio was lower compared with C group (Table 5).

Fecal count of *Eimeria spp* did not show any statistical difference between the three experimental groups at the end of the trial (Table 6).

4.6. Discussion

4.6.1. *Individual body weight (BW), average daily gain (ADG), carcass weight, feed conversion rate (FCR) of the chicken broilers of the three experimental groups.*

In the first two weeks before *Eimeria spp.* challenge (Table 4), the beneficial effects of a probiotic product on broiler growth performance parameters are in agreement with previous studies (Mountzouris et al., 2007, Awad *et al.* 2009, Bansal *et al.* 2011). The major outcomes from using probiotics include improvement in growth (Yeo *et al.* 1997), and improvement in feed conversion rate (Yeo *et al.* 1997); however in contrast, has been observed that supplementation with probiotics has no effect on the performance of broiler chicks (Patidar *et al.* 1999, Kumprechtova *et al.* 2000). Also, with regard to the feed conversion rate the results are in contrast, some studies reported that probiotics supplementation in chickens can improve the FCR (Jagdish *et al.* 1993, Yeo *et al.* 1997) while others suggested no such effect on feed conversion ratio (Ergun *et al.* 2000, Panda *et al.* 2000).

The results obtained in the present trial on growth performance of broilers evidenced increased body weight at the end of the trial for animals fed probiotic plus coccidiostat compared to broilers fed probiotics alone, but no differences were detected respect to body weight of animals fed coccidiostat alone.

Average daily gain (ADG), feed intake (FI) and feed conversion rate (FCR) evidenced different significant trends during the challenge period (3-8 wks) of the trial for all the experimental groups with weekly significant variation in the performance, but means valued overall the trial period did not evidence any effect of the adopted dietary treatment.

Vanderhoof (2001) reviewed the concept of probiotics as a viable therapeutic modality in the treatment of gastrointestinal disease. The antibiotics used for the hope of growth stimulation affect the gut microflora, which results in the reduction of the resistance to infection caused by certain bacteria.

Sub-therapeutic antibiotic dosages not only influence intestinal microbial populations and activities but also affect animal metabolism and specifically alter intestinal function (Anderson *et al.*, 2000). With the use of sub-therapeutic antibiotics, the intestinal pathogenic micro-flora creates resistance and useful microflora assisting digestive process is damaged. The probiotic supplementation helps and repairs the deficiencies in the gut flora and the balanced intestinal microbiota enhancing resistance to infection and reduction (Blecha, 2000; Soderholm and Perdue, 2001).

In monogastrics, the production of organic acids (lactic or acetic acid) by bacterial probiotics can help to decrease the gut pH, creating more favourable ecological conditions for the resident microbiota with a decreased risk of pathogen colonisation (Servin, 2004). The release of antimicrobial peptides, such as bacteriocins, that inhibit the growth of pathogenic bacteria, or production of enzymes able to hydrolyse bacterial toxins (Buts, 2004) has been demonstrated. Some strains can competitively exclude pathogenic bacteria through their higher affinity for nutrients or adhesion sites (La Ragione *et al.*, 2003, 2004). Enhancements of colonization resistance and/or direct inhibitory effects against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Fecal count of *Eimeria spp* did not show any statistical difference between the three experimental groups. The development and use of probiotics for poultry is based on the knowledge that the gut flora is involved in resistance to enteric infections, where it has been shown to be involved in protection against a variety of pathogens including *Eimeria* (Dalloul *et al.* 2005). Despite the fact that several studies have shown disease prevention or immune enhancement resulting from oral administration of probiotics (La Ragione *et al.* 2004, Koenen *et al.* 2004), few studies are available on their specific effects on the gut defence mechanisms in chickens. Dalloul *et al.* (2003) showed increased resistance in *Lactobacillus*-treated broilers to *Eimeria*, as manifested by reduced shedding of faecal oocysts.

4.6.2. *Histometry of Duodenum, Ileum and Cecum.*

In our study, the administration of probiotics plus coccidiostat positively affected the number of intestinal fold goblet cells (Table 5).

These results are in agreement with Samanya *et al.* (2002) that reported birds fed dietary *B. subtilis* for 28 days had a tendency to greater growth performance and pronounced intestinal histologic parameters, such as prominent villus height, extended cell area and consistent cell mitosis, than the controls.

Edens *et al.* (1997) reported an increase in goblet cell numbers and mucus secretion in the intestine of chickens fed probiotic. The epithelial surface is covered with a mucous gel secreted by epithelial goblet cells that acts as a protective barrier against harmful intraluminal components.

The presence of this mucous layer prevents bacterial translocation because to cause damage, gut pathogens must pass through this mucous layer before adhering to and invading the epithelial cells.

Many studies indicate that probiotics have few effects on the main physiological functions of the gastrointestinal tract, which are digestion, absorption and

propulsion, but the main action of probiotics can be summarized as a reinforcement of the intestinal mucosal barrier against deleterious agents.

Experimental data indicate that some probiotics reduce pathological alterations in paracellular permeability to large molecules or bacteria, stimulate mucosal immunity, display a trophic action on the mucosa, reduce mucus degradation and interact with mediators of inflammation (Fioramonti et al., 2003).

The possible protective effects of probiotics on intestinal permeability have raised considerable interest, because a loss of barrier function (increase of permeability) is involved in the pathogenesis of many gastrointestinal diseases such as intestinal infections with pathogenic, inflammatory bowel diseases, and even sepsis.

4.7. Conclusions

The results of this work show that the administration of probiotics (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum*) in normal farming condition had positively influenced the growth performance of broiler chickens.

Subsequently in challenge period no differences were detected between broilers fed either probiotics and/or coccidiostat considering growth performance and fecal count of *Eimeria spp* these results suggest probiotics may have positive effects administered alone or in combination with coccidiostats in broilers chickens infected with *Eimeria spp*.

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4.9. Tables and figures

Table 1. Composition and expected chemical analysis of administered feeds during the trial

	0-20d	21-40d	41d- slaughtering
Composition (%as fed)*			
Corn meal	59.18	62.18	64.93
Soybean meal (47%CP)	29.00	25.50	22.00
Corn gluten meal (57%CP)	3.00	2.00	2.00
Extruded soybeans	3.00	3.00	3.00
Animal fat	2.00	3.00	3.75
Dicalcium phosphate	1.90	1.70	1.70
Calcium carbonate	1.03	1.10	1.10
Wheat bran	0.20	0.20	0.20
Soybean oil	---	0.50	0.50
Sodium chloride	0.20	0.30	0.30
DL-methionine	0.15	0.15	0.15
Mineral premix	0.13	0.13	0.13
Sodium bicarbonate	0.10	0.05	0.05
Choline chloride (75%)	0.07	0.10	0.10
Coccidiostat	---	---	---
L-Lysine	0.04	0.10	0.10
Expected chemical analysis (% as fed)			
DM	88.19	88.33	88.40
CP	21.40	19.50	18.08
EE	5.28	6.82	7.64
CF	2.57	2.47	2.38
Ash	5.76	5.51	5.34
Lysine	1.13	1.07	0.97
Methionine	0.50	0.47	0.45
Ca	0.98	0.94	0.94
P tot	0.72	0.66	0.65

* coccidiostat was included in the mix at a rate of 0.60% subtracting an equal amount of corn meal.

Table 2. Analysed chemical composition during the experimental period.

	Phase		
	1 (0-20d)	2 (21-40d)	3 (41-56d)
(%DM)			
DM	93.53	92.83	92.91
CP	21.57	19.40	17.93
EE	5.48	7.24	7.59
Ash	5.55	5.42	4.90
NDF	13.66	17.62	13.16
Lysine	1.00	0.95	0.90
Methionine	0.45	0.38	0.39
Ca	0.90	0.85	0.87
P tot	0.72	0.76	0.74

Table 3. Body weight (BW) and average daily gain (ADG) of broiler fed either probiotics and/or coccidiostat before (weeks 1 and 2 of life) and after (weeks 3 to 8 of life) challenge with coccidia.

Item*	Week	Group			SEM	P		
		C ¹	P ²	PC ³		Group	Week	G.x W.
BW (g)								
	No challenge							
	0	52.89	51.02	51.75	2.86	<0.01	<0.01	<0.01
	1	155.90 ^B	165.48 ^A	161.40				
	2	370.74 ^C	400.92 ^A	382.74 ^B				
	Challenge							
	3	707.27	726.83	725.17	22.03	<0.01	<0.01	<0.01
	4	1085.03	1109.75	1100.47				
	5	1682.60	1650.81	1667.86				
	6	2185.43 ^A	2090.52 ^B	2202.89 ^A				
	7	2768.61 ^A	2616.21 ^B	2771.12 ^A				
	8	3101.11 ^a	3039.53 ^b	3128.42 ^a				
ADG (g/day/bird)								
	No challenge							
	0-1	14.72 ^{Bb}	16.35 ^A	15.66 ^a	0.44	<0.01	<0.01	0.07
	1-2	30.70 ^{Bb}	33.63 ^A	31.62 ^a				
	0-2	22.70 ^c	25.00 ^a	23.64 ^b	0.35	<0.01	--	--
	Challenge							
	2-3	48.07	46.56	48.92	3.18	0.22	<0.01	<0.01
	3-4	53.97	54.70	53.61				
	4-5	85.36 ^a	77.30 ^b	81.05				
	5-6	71.83 ^A	62.81 ^B	76.43 ^A				
	6-7	72.90 ^a	65.71 ^b	71.02				
	7-8	55.42 ^B	70.55 ^A	59.54 ^B				
	3-8	64.60	62.93	65.10	1.03	0.13	--	--
Carcass Weight (g)								
		2188.59	2124.81	2155.95	36.25	0.39		

¹ C=basal diet plus coccidiostat;

² P= basal diet plus probiotics (0.1%);

³ PC=probiotics 0.05% (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. B. butyricum*) plus coccidiostat.

Table 4. Feed intake (FI) and feed conversion rate (FCR) of broiler fed either probiotics and/or coccidiostat before (weeks 1 and 2 of life) and after (weeks 3 to 8 of life) challenge with coccidia.

Item*	Week	Group			SEM	P		
		C ¹	P ²	PC ³		Group	Week	G.x W.
FI								
(g/birds/day)	No challenge							
	0-1	19.05	18.91	18.10	0.73	<0.01	<0.01	0.07
	1-2	50.31 ^{AB}	52.46 ^A	48.92 ^B				
	Mean	34.68 ^a	35.69 ^A	33.51 ^{Bb}				
	Challenge							
	2-3	77.26	79.46	81.80	2.70	0.24	<0.01	<0.01
	3-4	99.87	99.34	100.15				
	4-5	142.39	138.33	144.58				
	5-6	160.98 ^a	151.10 ^b	156.33 ^{ab}				
	6-7	166.37	161.54	166.39				
	7-8	155.18 ^B	179.66 ^{Aa}	170.38 ^{Ab}				
	Mean	133.67	134.91	136.61				
FCR								
	No challenge							
	0-1	1.31 ^A	1.16 ^B	1.16 ^B	0.03	<0.01	<0.01	0.02
	1-2	1.65 ^a	1.57 ^b	1.57 ^b				
	Mean	1.48 ^A	1.36 ^B	1.37 ^B				
	Challenge							
	2-3	1.62	1.71	1.69	0.08	0.54	<0.01	0.02
	3-4	1.87	1.81	1.89				
	4-5	1.68	1.81	1.79				
	5-6	2.25 ^{AB}	2.43 ^A	2.05 ^B				
	6-7	2.30 ^b	2.53 ^a	2.38 ^{ab}				
	7-8	2.82 ^a	2.60 ^{Bb}	2.89 ^A				
	Mean	2.09	2.15	2.12				

¹ C=basal diet plus coccidiostat;

²P= basal diet plus probiotics (0.1%);

³PC=probiotics 0.05% (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. B. butyricum*) plus coccidiostat.

Table 5. Histometry of Duodenum, Ileum and Cecum districts of the chicken broilers of CI and PI groups.

Item*		Group		SEM	P
		C ¹	PC ²		
Duodenum					
Intest. fold (F) height	µm	1736.57	1790.42	42.87	0.38
Intest. gland (G) depth	µm	311.79	298.23	8.44	0.26
F:G ratio		5.71	6.16	0.24	0.19
Intest. fold goblet cells	(n/mm ²)	1465.94 ^b	1806.10 ^a	117.61	0.05
Intest. gland goblet cells	(n/mm ²)	2119.27	2111.17	138.33	0.97
Ileum					
Intest. fold (F) height	µm	1033.29	1035.87	23.68	0.94
Intest. gland (G) depth	µm	265.73 ^A	236.35 ^B	6.82	<0.01
F:G ratio		3.97 ^B	4.46 ^A	0.14	0.02
Intest. fold goblet cells	(n/mm ²)	2167.87 ^B	2640.32 ^A	116.05	<0.01
Intest. gland goblet cells	(n/mm ²)	2813.1	3010.18	147.28	0.35
Cecum					
Mucosa thickness	µm	372.93	370.07	12.35	0.87
Goblet cells	(n/mm ²)	629.03	726.22	37.54	0.07
Lymphatic follicles	(n/mm ²)	6.81	6.6	0.42	0.72
Lymphatic follicles area	(µm ²)	26943	23438	4101.21	0

²P= basal diet plus probiotics (0.1%);

³PC=basal diet plus probiotics 0.05%;

Table 6. Fecal count of *Eimeria Spp.* of the chicken broilers of the three experimental groups at end of the trial.

Item*	Week	Treatment			SEM	P
		C ¹	P ²	PC ³		
<i>Eimeria spp</i> (log ¹⁰)						
	8	4.37	5.11	4.78	0.27	0.14

¹ C=basal diet plus coccidiostat;

²P= basal diet plus probiotics (0.1%);

³PC=probiotics 0.05% (10⁸ cfu/g of *Bacillus coagulans*, *B.subtilis* and *Cl. Butyricum*) plus coccidiostat.

CHAPTER 5

Effects of inclusion of selenium-enriched yeast in the diet of laying hens on production performances, health parameters, eggshell quality, and selenium tissue deposition

Paper submitted to: Animal.

5. Effect of inclusion of selenium-enriched yeast in the diet of laying hens on production performance, health parameters, eggshell quality, and selenium tissue deposition.

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5.1. Abstract

Forty-eight ISA Brown laying hens, each 22 weeks of age, were utilized for an 8-week trial to evaluate the bioavailability of ingested selenium yeast and its effects on egg-laying performance, eggshell quality, health parameters and tissue Se distribution. Animals were divided into 3 treatment groups: the control group (C) received a basal diet containing 0.11 mg of Se /kg DM; the inorganic Se group (SS) received the control diet plus sodium selenite at 0.4 mg/kg DM; and the Se-yeast group (SY) received the control diet plus selenium yeast at 0.4 mg/kg DM. Feed intake, egg mass ratio, and production performances were not affected by supplementation with Se, regardless of the Se source. Only egg weight was higher ($P < 0.05$) for hens with diets supplemented with Se. Eggshell weight was improved in both Se-supplemented groups, whereas breaking strength was increased by the administration of SY. Breast muscle, liver and skin Se level was higher in the SY group than in the C group, whereas the Se level did not differ between the SS and C groups. Kidney selenium content was statistically significant only for SS animals. Eggs from SY hens had higher Se levels than those from SS hens. Results indicated that 0.4 ppm of selenium from SS or SY did not affect hens' blood metabolites; nor did it affect laying performance, other than resulting in heavier eggs. Se and particularly its organic form improved eggshell quality. The source of selenium influenced the Se distribution in hen tissues. Indeed, a higher Se level was detected in eggs and breast muscle of hens fed SY. This might be related to the greater bioavailability of organic selenium sources when compared to inorganic sources.

5.2. Introduction

Selenium (Se) is an essential trace element for animals and humans. It is required for growth, maintenance of health, and for physiological functions. In particular, it is essential for the proper functioning of the antioxidant enzyme glutathione peroxidase, which protects the cell by destroying free radicals (Rotruck *et al.*, 1973). Laying hens' requirements for Se range from 0.05 to 0.08 ppm, depending on daily feed intake (National Research Council, 1994). Se exists in nature in inorganic and organic forms. Traditionally, Se has been added to poultry diets from inorganic sources, such as sodium selenite (Na_2SeO_3). A typical corn soybean meal diet can satisfy Se requirements, but the Se content of this feed is highly dependent on the amount of Se in the soil in which it was grown, which varies from region to region and also depends on the ability of the plants to take up and accumulate the element (Dumont *et al.*, 2006).

Organic sources of Se, such as Se yeast, have been explored as an alternative to inorganic supplementation (Payne *et al.*, 2005; Utterback *et al.*, 2005; Schrauzer, 2006). The use of organic Se results in less Se being passed on to the environment through excrement and more Se being deposited into body tissue and eggs. Cereals and forage crops convert Se mainly into selenomethionine (Se-Met) and incorporate it into protein in competition with methionine (Met), as tRNA^{Met} does not discriminate between Met and Se-Met (Schrauzer, 2003). In the same manner, *Saccharomyces cerevisiae* may assimilate up to 3000 μg of Se/g (Schrauzer, 2003), the major product of which is Se-Met, which is incorporated into yeast proteins or is physically associated with macromolecules, especially as cell-wall constituents (Polatajko *et al.*, 2004).

As a rule, the replacement of Met by Se-Met does not significantly alter protein structure, but may influence the activity of enzymes if Se-Met replaces Met in the vicinity of active sites (Schrauzer, 2003). Selenomethionine is the form of Se that enters the food chain of higher animals and humans because they have no efficient mechanisms for Met synthesis.

For this reason, selenomethionine fulfills the criteria of an essential amino acid (Schrauzer, 2006). However, selenocysteine (SeCys) represents the functional core of selenoproteins, being together with Se-Met the majority of Se in body tissues and fluid. Phipps *et al.* (2008), observed in dairy cows higher concentrations of SeCys than Se-Met in blood and the opposite in milk.

Several supplementation studies have indicated that Se from Se yeast exhibits greater bioavailability than that from inorganic Se (Dumont *et al.*, 2006; Schrauzer, 2006), and that increased Se levels are maintained for a longer period after supplementation has ceased.

Many studies have shown that the concentration and form of Se in hens' diet will influence Se content and distribution in eggs (Cantor and Scott, 1974;

Cantor *et al.*, 2000). For this reason, studies conducted on laying hens have been very useful for studying the absorption of various Se compounds (Latshaw and Biggert, 1981). Many studies have been published comparing the effects of supplementation with sodium selenite (SS) with those of Se-Met or selenium yeast (SY) in laying hens, and the results from these experiments are consistent. The whole-egg Se concentration is increased by SS, Se-Met, or SY supplementation as dietary levels increase, but Se-Met and SY supplements have been reported to increase egg Se more than does SS supplementation (Davis *et al.*, 1996; Cantor *et al.*, 2000). Arnold *et al.* (1973) reports an increase in whole-egg Se content when hens are fed 8 ppm of SS, as compared with hens fed diets containing no or 2 ppm of SS and no differences between the latter two groups. Davis *et al.* (1996) shows that both SS and Se-Met fed at 2 ppm will increase yolk Se concentrations in comparison with a diet not supplemented with Se. A study conducted by Jiakui and Xiaolong (2004) analyzed the effect of organic and inorganic sources of selenium on selenium content in blood, liver, and kidneys and showed that the metabolic route of Se from an organic source is similar to that of Se from an inorganic source.

Most papers comparing Se supplementation from inorganic sources (SS) with that from organic sources (Se-Met, SY) have thus far investigated very specific consequences; very few have investigated at the same time the effect of SY supplementation in hens' diets on the accumulation and distribution of Se in organs, on hen laying productivity and health, and on shell stiffness.

The aim of this study was to investigate the effects of Se yeast supplementation in laying hens starting at age 22 weeks and continuing for an additional 8 weeks. The effects investigated were: laying productivity and health, eggshell quality, as well as Se content in the serum, egg, breast muscle, kidneys, skin and liver.

5.3. Materials and methods

5.3.1. *Animals and Experimental Design*

Forty-eight ISA Brown Warren laying hens from the same stock with homogenous genetics and initial live weights were randomly distributed into 3 experimental groups of 16 hens each and housed 2 birds per cage in 34x40x45 cm cages. Each experimental group consisted of 8 groups of 2 hens each in order to obtain 8 replicates, and 3 dietary treatments were assigned: (1) control (group C, n. = 16, BW 1638±149 g) fed the basal diet containing 0.11 mg of Se/kg; (2) inorganic Se (group SS, n. = 16, BW 1519±261 g) fed the control diet plus sodium selenite at 0.4 mg Se/kg; and (3) organic Se (group SY, n. = 16, BW 1613±152g) fed the control diet plus selenium yeast at 0.4 mg Se/kg. The selenium yeast (Alkosel R397 (EU n°3b8.11), Lallemand SAS, Blagnac, F-31702, France)

was a commercial product containing 2000 ppm of total selenium with 98% organic selenium; 65-75% of the organic selenium was composed of selenomethionine. Animal care and treatment were in accordance with the European Community 86/609/EE guidelines (EEC, 1986) approved by the Italian Ministry of Health. Birds were housed in the same shed, environmental conditions were set according to the ISA Warren layer management guide, animals were fed ad libitum, and the feed was formulated to meet nutritional requirements according to the ISA Warren management manual (ISA SAS, 2005).

From 17 weeks to 22 weeks of age, hens were fed the C diet without any Se supplementation; the experimental period started at age 22 weeks and continued for 8 weeks. At the beginning of the trial and for the entire length of the experiment, control and treated feeds from the same batch were sampled and analysed for DM, CP, EE, NDF, ash, Ca, P, lysine, and Se content.

Feed composition and the analysed chemical composition of the experimental diets are reported in Tables 1 and 2.

Beginning at 22 weeks of age until the end of the experimental period, feed intake per cage was evaluated weekly, and the feed conversion ratio was calculated during the laying period as weekly feed intake per cage over weekly produced egg mass per cage.

Daily egg number per cage as well as egg weight were recorded at the same time of day (ranging from 17:00 to 19:00 h), and daily laying rate per cage was calculated as the ratio between the number of eggs produced and an ideal number of eggs (2 eggs per cage per day).

The selenium content in the eggs was analysed at 0, 18, 36, and 56 days from the beginning of the trial. Two eggs per replicate were collected and refrigerated.

Subsequently, the 2 eggs from each replication were cracked, the shells discarded, and the fluid content homogenized and frozen pending Se analysis. Thus, a total of 96 samples were analysed (8 replicates x 4 sampling times x 3 treatment groups). The dry matter of the eggs was also determined.

Following the same sampling schedule, eggshell quality was assessed in 4 eggs per replicate, collected 2 days prior to each sampling day.

At the end of the trial, 8 animals per group were anesthetized and then decapitated, and the selenium content in breast muscle, liver, kidneys, and skin was analysed; results are expressed on a dry matter basis.

A necropsy was performed on the slaughtered animals via macroscopic observation.

Two sets of blood samples were collected before slaughtering from 8 animals per group to determine: selenium, glucose, total protein, albumin, cholesterol, alanine transaminase (ALAT) and aspartate transaminase (ASAT), bilirubin, alkaline phosphatase (ALP) content, and serum glutathione peroxidase activity (GSH-Px).

Blood from the jugular vein was drawn into two 10-mL vacuum tubes. Blood samples were subsequently centrifuged ($1,400 \times g \times 10$ minutes), and 2 serum aliquots from each tube were collected and frozen (-20°C).

5.3.2. *Eggshell Quality Analyses*

The length and breadth (mm) of each egg were measured, and a shape index was calculated ($\text{SI} = \text{egg length}:\text{breadth}$). Shell weight (g) was measured after washing the shells and drying them overnight at 80°C . Eggshell percentage, eggshell index, and egg surface area were calculated as described by Mabe *et al.* (2003). Eggshell thickness (without shell membranes) was measured at 3 positions (top, middle, and bottom) using a micrometre (Digimatic 0-25 mm 0.001 mm, Mitutoyo corp., Kanagawa, Japan). Eggshell mechanical stiffness (N/mm) and breaking strength (N) were measured by quasi-static compression using a testing machine (model 5542, Instron, Norwood, MA, USA) fitted with a 500-N load cell and equipped with a food texture fixtures compression anvil (catalog number 2830-009, Instron, Norwood, MA, USA). Breaking strength was measured as the maximum force required to fracture each egg at a compression speed of 5 mm/min. Static stiffness was calculated as a linear slope of the force deformation curve resulting from the load applied up to 10 N at a compression speed of 5 mm/min on the equator of each egg. The elastic modulus (N/mm^2) and fracture toughness ($\text{N}/\text{mm}^{3/2}$) of each egg were estimated using formulae developed by Bain (1990) and described by Mabe *et al.* (2003).

5.3.3. Selenium Analyses

To determine selenium levels in the blood by inductively coupled plasma atomic emission spectrometry (ICP-AES), serum sample solutions were prepared by acid digestion in an open system in order to eliminate spectral interference caused by carbon, as described by Machát *et al.* (2002). Briefly, 2 mL of each serum sample were heated in 8 mL of HNO₃ (65%) (Baker Instra-Analyzed) at 145 °C for 6 h. After cooling, 2 mL of H₂O₂ (30%) was added to the solution until light yellow colour development, and samples were heated again until evaporation. The serum reference material (Seronorm™ Trace Element Serum, Sero AS, Norway) with a certified selenium content of 0.136 mg/L was used to test the accuracy and precision of the analytical procedure. For liver and kidney samples, a closed-vessel microwave (MARS 5, CEM Corp., Matthews, NC) mineralization procedure was performed. Briefly, liver aliquots were weighed (0.8 – 0.9 g) in Teflon vessels, and then 10 mL of HNO₃ (65%) was added. The vessels were sealed tightly and kept in the microwave for 25 minutes under 600 W of microwave power at 210 °C, and 170 psi. After digestion and cooling down to room temperature, the samples were carefully transferred to glass tubes; 2 mL of HNO₃ (65%) was added to each sample, which was then heated at 105°C until complete evaporation. Serum, liver, and kidney samples were finally resuspended in 2 mL of HNO₃ (5%) prior to determination of selenium by ICP-AES. An ICP emission spectrometer (OPTIMA 3300 XL, Perkin-Elmer Corp., Waltham, MA) with a standard axial torch was used. The instrument was optimized to obtain the maximum signal-to-background ratio and minimum relative standard deviation (RSD) of signal and background. The most sensitive line Se 196.026 nm was used. In all digestates, the selenium concentration (Seronorm™ included) was determined using a calibration curve constituted by standard solutions (0-0.5 mg/L) of 100 ppm inorganic Selenium (AccuTrace™, AccuStandard Inc., New Haven, CT).

Prior to analysis, skin, muscle, eggs, and diet aliquots were submitted to a closed-vessel acid digestion protocol. Samples were treated with a hydrated mixture of MgO: Mg (NO₃)₂ at a 1:10 ratio and digested at low temperatures on a hot plate until fully dry. Afterwards, the samples were placed in a muffle furnace at 500 °C for 1 hour in order to remove the entire organic matrix. The residues were mixed with an acid solution and treated to reduce selenium to the IV form. An atomic absorption spectrophotometer Perkin Elmer 4100 ZL with hydride generator Perkin Elmer FIAS 100 was used (Perkin-Elmer Corp., Waltham, MA). The instrument was optimized to obtain the maximum signal-to-background ratio and the minimum relative standard deviation (RSD) of signal and background.

The most sensitive line Se 192 nm was used. Selenium amount was determined using a calibration curve obtained from standard solutions at 3 levels.

5.3.4. *Serum Analyses*

Serum samples were analysed using a Synchron CX5[®] Delta chemistry analyser (Beckman Coulter, Brea, CA, USA) in order to determine ALAT, ASAT, albumin, total protein, glucose, ALP, and cholesterol content.

GSH-Px activity was measured using a commercial assay kit from Cayman Chemical Company (Ann Arbor, MI 48108, USA) adapted for a spectrophotometer. The activity was assayed in a 190 μ L reaction mixture containing 100 μ L of assay buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA), 50 μ L of co-substrate mixture containing NADPH, glutathione, and glutathione reductase, and 20 μ L of cumene hydroperoxide as the starter reactive. The decrease in absorbance caused by the reduction of hydroperoxide by GSH-Px was monitored at 340 nm. GSH-Px activity was calculated using an extinction coefficient for NADPH at 340 nm of 0.00622 μ M/cm.

5.4. **Statistical analyses**

The experimental design foresaw 3 dietary treatments assigned randomly to 3 groups of 16 hens each. One cage (2 hens) represented the unit for statistical analysis. Data relative to feed consumptions, egg selenium content, egg weight, feed:egg mass ratio, egg production, and eggshell quality were analysed by an ANOVA using a MIXED procedure of SAS for repeated measures (SAS Institute, 2006). The model represented the effects of selenium source, treatment day, and their interaction, the random effect of animals nested within treatment, and the residual error. The model applied was

$$Y_{ij} = \mu + T_i + D_j + (T \times D)_{ij} + e_{ij}$$

where Y_{ij} = dependent variable feed consumptions, egg selenium content, egg weight, feed:egg mass ratio, egg production, and eggshell quality parameters; μ = general mean; T_i = effect of selenium source; D_j = effect of day of sampling; $(T \times D)_{ij}$ = effect of the interaction between treatment and time; e_{ij} = casual effect of each observation.

Data obtained for blood parameters and selenium tissue deposition were analysed by ANOVA using a General Linear Model of SAS (SAS Institute, 2006). Significance was designated at $P < 0.01$ and $P < 0.05$.

5.5. Results

Results of hen productivity are summarized in Table 3. Average daily feed intake (ADFI), feed:egg mass ratio (feed intake over egg mass), egg production, and egg mass produced by hens were not affected by the Se source. Egg weight was higher in Se-supplemented hens compared with unsupplemented hens ($P < 0.05$). Eggshell weight and egg surface area were both affected by Se supplementation independently from the source (Table 4) ($P < 0.05$). The eggshell index was higher in the SY group when compared with the control group ($P < 0.05$), and breaking strength was also higher in the organic Se-supplemented hens when compared with both the inorganic Se and non-supplemented hen groups ($P < 0.01$). Resulting eggshell percentage, thickness, stiffness, elastic modulus, and fracture toughness were not statistically different among the 3 treatment groups. Total egg Se content did not differ among the experimental groups when hens were fed an unsupplemented diet during the adaptation period or at the beginning of the trial (**Figure 1**).

Eggs from hens fed Se supplements had greater Se content (0.94 ppm for the SS group and 1.38 ppm for the SY group vs. 0.54 ppm for the C group; $P < 0.01$) than those from hens fed the basal diet. Eggs from hens fed the SY diet possessed 46.81% higher Se content ($P < 0.01$) than those from hens fed the SS diet (**Figure 1**).

Analysis of serum selenium contents revealed higher concentrations in the SY group in comparison with the C ($P < 0.01$) and SS ($P < 0.05$) groups, even if Se serum content was increased when SS was added to the basal diet (**Figure 2**).

As shown in Table 5, breast muscle selenium content in hens fed organic Se was significantly higher than that in hens fed the unsupplemented or inorganic selenium-supplemented diet.

Dietary organic selenium supplementation significantly increased liver selenium content when compared with the C (1.84 ppm vs. 1.36 ppm; $P < 0.05$). No difference in liver selenium content was detected when the hens' diet was unsupplemented or supplemented with inorganic selenium. SY addition significantly increased ($P < 0.01$) skin selenium content compared with the C diet. No difference in skin selenium content was observed when the diet was unsupplemented or supplemented with inorganic selenium.

Kidney selenium content was higher in Se-supplemented hens compared with unsupplemented hens, but the difference achieved statistical significance only for SS animals (1.45 ppm vs. 0.9 ppm; $P < 0.05$).

The results of the serum biochemical analyses are shown in Table 6. Inorganic or organic supplementation of Se did not affect plasma total protein, albumin, cholesterol, total bilirubin, ALAT, ASAT, ALP, or glucose. The serum

glutathione peroxidase concentration was significantly increased ($P < 0.05$) when Se was added to the diet, independent of the source.

5.6. Discussion

5.6.1. *Laying hens performances*

Supplementation with SY or SS did not influence feed intake and feed efficiency, in agreement with Payne *et al.* (2005); however in contrast, they did observe an increase in feed intake among hens fed the basal diet compared to those supplemented with Se.

Our results for egg production agree with those of Cantor *et al.* (2000), Paton *et al.* (2001), and Payne *et al.* (2005), who reported no difference in egg production when hens were fed a diet supplemented with selenium selenite or organic selenium. In a recent experiment no differences in egg production were observed in the first 8 weeks of the trial, whereas in the last 8 weeks SY increased egg production compared to control and SS (Pavlović *et al.*, 2009). Cantor and Scott (1974) already reported an increase in egg production in hens fed a supplemented Se diet compared to an unsupplemented diet. A probable reason explaining the lack of difference in egg production when Se was added to the basal diet is that our control diet was only partially deficient in Se, while the dietary Se level of the basal diet (0.02 ppm) of Cantor and Scott (1974) was clearly deficient.

5.6.2. *Egg weight and eggshell quality*

In contrast with several studies (Utterback *et al.*, 2005; Chantiratikul *et al.*, 2008), supplementation with Se led to production of heavier eggs. However, our results are in agreement with data obtained by Rutz *et al.* (2003) showing in layers that selenium supplementation improves egg weight. Furthermore, Skřivan *et al.* (2006), in agreement with our results, observed the production of heavier eggs in hens supplemented with organic selenium in comparison with either C or SS-supplemented hens. Similarly, eggshell weight and egg surface area in the SS and SY groups were higher than in the C group. The shape index was higher in the SY group than in C, whereas eggshell percentage was equal between treatment groups, indicating that egg and shell weight increased proportionally. In agreement with Renema (2004), Se supplementation and, in particular, supplementation with SY resulted in the greatest positive changes in eggshell quality. However, our results are in contrast with those reported by Pavlović *et*

al., (2010) where either the source or the level of Se did not affect eggshell quality.

Breaking strength results contrasted with earlier literature (Cantor and Scott, 1974; Combs and Scott, 1979), although it should be noted that Cantor and Scott (1974) tested much lower concentrations of SS than we did. More recent studies seem to confirm our results on egg shell breaking strength (Paton *et al.*, 2000; Siske *et al.*, 2000; Golubkina and Papazyan, 2006), corresponding to a high Se concentration in the shell and shell membrane, which increased in particular with organic supplementation, suggesting that the high Se concentration could be the reason for increased shell strength.

Eggshell thickness did not differ between treatment groups, an outcome consistent with results shown by Arnold *et al.* (1973), who added 2 and 8 ppm of sodium selenite to the hens' diet, and Chantiratikul *et al.* (2008), who added 0.3, 1 and 3 ppm of both sodium selenite and a chelated type of Se source as zinc-Lselenomethionine.

5.6.3. *Selenium in eggs*

When hens received a diet without any Se supplementation, the Se content of eggs was similar in the 3 experimental groups, but when Se was added as selenium yeast or in an inorganic form, the Se egg content doubled and increased by 74%, respectively. SY increased Se egg content 47% more than did SS. Several authors have reported that organic Se supplementation of the diet with SY is more effective than a SS diet for increasing the Se content of eggs (Payne *et al.*, 2005; Pan *et al.*, 2007). The Se content of the basal diets used in the aforementioned works ranged from 0.02 to 0.16 ppm, inclusive of the level used in the present study (0.11 ppm).

Many studies were conducted on whole egg Se concentration when diets were supplemented with Se (Cantor and Scott, 1974; Latshaw and Biggert, 1981; Cantor *et al.*, 2000).

Latshaw and Biggert (1981) reported that whole egg, egg white, and egg yolk Se levels were 44%, 79%, and 15% greater, respectively, in hens fed organic selenium compared with those fed SS; this was confirmed by results obtained by Cantor *et al.* (2000), who used the same range of supplementation.

The main reason for the increased Se deposition in eggs by SY is that the majority of Se in SY is selenomethionine, a Se analogue of methionine (Kelly and Power, 1995). The other organic Se components have not yet been clearly identified, but act as Se-Met precursors; although there is some recent evidence (Polatajko *et al.*, 2005) that selenomethionine represents 80% of the organic selenium in Se yeast. Se-Met is deposited in the egg to a greater extent than is

selenium selenite, and is actively absorbed and incorporated into eggs as effectively as methionine (Combs and Combs, 1986).

5.6.4. *Blood chemistry and selenium content*

Supplementation of Se increased the serum Se concentration, but the difference was statistically significant only when Se yeast was included in the diet. Jiakui and Xiaolong (2004) and Petrovič *et al.* (2006) observed a higher blood selenium content when inorganic or organic selenium was added to a hen's diet, while Scott and Thompson (1971) reported an increase in blood Se concentrations when Se was provided in an organic form compared with sodium selenite.

The serum glutathione peroxidase concentration was significantly higher when Se was added to the basal diet, but no differences due to the form of selenium delivered by the diet were observed. Our results are in accordance with those observed by Petrovič *et al.* (2006). GSH-Px mRNA is regulated by absorbed selenium during a post-transcriptional step (Toyoda *et al.*, 1990; Petrovič *et al.*, 2006). Our observation that the form of selenium did not influence the serum GSH-Px activity is in agreement with the results obtained by Petrovič *et al.* (2006) and Kuricová *et al.* (2003), and can be explained by the fact that all Se sources have to be split into H₂Se before selenocysteine is synthesized *de novo* for incorporation into an active center of selenoenzymes (Schrauzer, 2003; Petrovič *et al.*, 2006). All other blood metabolites investigated were within the normal range for laying hens.

5.6.5. *Selenium in tissues*

SY has a higher selenium bioavailability than inorganic Se sources (Yoshida *et al.*, 2002); this means that when SY is administered to hens, an increase in the Se content of tissues is expected. Indeed, we observed that breast muscle Se content was higher in SY-supplemented hens compared with SS-supplemented animals. Petrovič *et al.* (2006), Pan *et al.* (2007), and Leeson *et al.* (2008) observed an increased Se concentration in the breast muscle of hens fed selenium yeast when compared to hens fed sodium selenite. According to Petrovič *et al.* (2006), the muscle tissue of birds fed salinized yeast becomes the most significant site of selenium deposition, since striated muscle mass represents about 52-56% of body weight in poultry.

It has been suggested that selenomethionine deposited in the muscle tissue of an animal fed with Se yeast may account for more than 50% of the total selenium in

the body (Daniels, 1996). The possible benefit of selenomethionine being deposited in body tissues is that it may serve as quantitatively important storage (Oster *et al.*, 1988), capable of releasing Se during episodes of an insufficient dietary selenium supply (Zuberbuehler *et al.*, 2006). The portion of selenomethionine absorbed from the digestive tract that is not immediately used for synthesis of specialized selenoproteins is incorporated non-specifically into the structural proteins of muscle, gizzard, heart, and other organs. Selenomethionine substitutes for the common amino acid methionine, which contains sulfur instead of selenium (Schrauzer, 2003). In this way, muscle tissue becomes the most significant site of selenium deposition in the form of selenomethionine when using organic Se dietary supplementation in animals. The intensive uptake of selenomethionine by muscle proteins is also very important for the increased transport of Se from hens to eggs and embryos, for the subsequent development of chicken immunocompetence, and for the overall health of the birds (Surai, 2000).

Pan *et al.* (2007) and Leeson *et al.* (2008) reported that either SS or SY supplementation increased the selenium concentration in hens' liver, but that this effect was higher when organic selenium was used compared with inorganic selenium. In accordance with these authors, we observed an increase of liver selenium concentration when Se was supplemented, but the difference compared with unsupplemented hens was statistically significant only in SY-supplemented hens.

The selenium concentration in skin follows the same pattern exhibited by the liver Se concentration. No recent data are available on the effect of the source of Se on Se content in skin: Scott and Thompson (1971) reported an increase in Se content in the skin of chicks and poults when SS was added to a basal diet.

Kidney selenium content was higher in supplemented hens than in unsupplemented hens, but the difference achieved statistical significance only for SS-supplemented animals. Even if the difference between the selenium concentration in kidney between SS and SY-supplemented hens was not statistically significant, we can suppose that when the diet is supplemented with SS, greater excretion of Se occurs via the kidney, as argued by Pan *et al.* (2007), who found a decrease in the Se concentration of kidneys when hens were fed SY compared with hens fed SS. This relationship may be explained as follows: the kidneys contain abundant capillary vessels, and these capillaries are filled with blood. When Se absorption from an inorganic Se source exceeds the nutritional or production need, excessive inorganic Se is excreted via the urinary route. On the other hand, reabsorbed Se-Met is captured in kidney capillaries and re-enters whole-body metabolism via the bloodstream, and no urinary losses of selenium in the form of selenomethionine occur.

Moreover, kidney Se content reflects the amount of Se deposited in the kidneys, as well as the Se eliminated from the body via urine (Aspila, 1991; Mahan and Parrett, 1996); selenite selenium that is not engaged in the synthesis of specialized selenoproteins does not have any metabolic route for incorporation into tissue proteins. Within a few minutes, selenite absorbed from the gut is metabolized into selenide (H_2Se), which forms nonspecific bonds with plasma albumin (Suzuki and Itoh, 1997). After multiple recycling of selenium via the selenide-to-selenite transformation pathway and its methylation, surplus inorganic selenium is rapidly excreted via the urine. However, glomerular filtration of H_2Se seems to be limited due to its albumin bond, and the rapid urinary elimination of selenium of inorganic origin is another significant disadvantage in comparison to selenoamino acids (Boldizárová *et al.*, 2001).

5.7. Conclusions

Results of our study indicate that supplementation with 0.4 ppm of selenium from SS or SY does not affect hens' zootechnical performance and blood metabolites. The only exception is the increased weight of eggs obtained from hens supplemented with Se. On the other hand, selenium—particularly in its organic form—improves eggshell quality. Specific selenium sources influence selenium distribution in hen tissues. Indeed, egg and breast muscle selenium concentrations were higher when hens were fed selenium yeast because of the greater bioavailability of organic selenium sources when compared with inorganic sources. The supply of organic selenium in the form of selenomethionine in laying hen feedstuffs benefits the human population via the food chain by helping to meet human selenium requirements that are not always satisfied by typical human diets.

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5.9. Tables and figures

Table 1. Composition of the basal diet¹

Ingredient	% as fed
Corn ground	54.00
Soybean meal ²	29.00
Calcium carbonate	9.50
Soybean oil	4.54
Monocalcium phosphate	1.40
Vitamin and mineral mix ³	1.00
Salt	0.40
DL-Methionine	0.16
Analyzed composition	% as fed
Dry Matter	88.81
ME (kcal/kg)	2835
EE	7.27
CP	17.30
NDF	8.78
Lysine	0.92
Methionine	0.43
Methionine+Cysteine	0.74
Ash	14.37
Ca	4.40
P	0.62
Nonphytate P	0.35
Se (ppm) ⁴	0.11

¹Selenium sources were added in a vitamin and mineral mix.

²44% CP

³Provided per kilogram of diet: vitamin A (retinyl acetate), 12,500 IU; vitamin D₃, 2,000 IU; vitamin E (DL- α -tocopheryl acetate), 67 IU; choline, 750 mg; niacin, 39.4 mg; pantothenic acid, 10 mg; pyroxidine, 5.1 mg; riboflavin, 5 mg; menadione, 4.4 mg; thiamin, 2 mg; folacin, 0.8 mg; biotin, 0.1 mg; vitamin B₁₂, 0.02 mg; manganese, 125 mg; zinc, 76 mg; iron, 60 mg; copper, 10 mg; iodine, 1 mg; cobalt, 0.3 mg.

⁴Analyzed selenium content of the basal diet

Table 2. Analysed content of selenium in the experimental diets.

Treatment ¹	Selenium content (ppm)
C	0.11
SS	0.46
SY	0.47

¹Treatments: C = control group; SS = sodium selenite; SY = Se-enriched yeast.

Table 3. Production performances of laying hens fed different sources of selenium¹.

Item	Treatment ²			s.e.
	C	SS	SY	
ADFI per hen (g/d)	107.52	111.25	108.08	1.69
Feed:egg mass ratio (kg:kg)	2.22	2.39	2.30	0.08
Egg production (%)	87.50	83.59	85.49	2.64
Egg mass (g/week/hen)	354.77	352.10	354.68	11.50

¹Data represent the means of 8 (2 hens per replicate) replicates. Data refer to the 56-day supplementation period for laying hens of 22 weeks of age at the beginning of the trial.

²Treatments: C = control group; SS = sodium selenite; SY = Se-enriched yeast.

Table 4. Eggshell quality of laying hens fed different sources of selenium¹.

Item	Treatment ²			s.e.
	C	SS	SY	
Egg weight (g)	57.66 ^b	59.74 ^a	59.36 ^a	0.53
Eggshell weight	5.39 ^b	5.62 ^a	5.68 ^a	0.08
Egg surface area	69.77 ^b	71.47 ^a	71.14 ^a	0.42
Eggshell (%)	9.37	9.44	9.65	0.10
Eggshell index (g/100 cm ²)	7.72 ^b	7.87 ^{ab}	8.04 ^a	0.09
Eggshell stiffness (N/mm)	148.5	156.4	159.7	5.23
Eggshell elastic modulus	14,305	14,754	14,525	461
Eggshell breaking strength	35.64 ^B	35.89 ^B	39.03 ^A	0.77
Eggshell fracture toughness	423.5	420.9	442.2	7.23
Eggshell thickness (mm)	0.359	0.361	0.369	0.003

¹Data represent the means of 128 samples per treatment. Data refer to a 56-day supplementation period.

²Treatments: C = control group; SS = sodium selenite; SY = Se-enriched yeast.

^{A, B} Means within a row lacking a common superscript are significantly different ($P < 0.01$).

^{a, b} Means within a row lacking a common superscript are significantly different ($P < 0.05$).

Table 5. Selenium content of breast muscle, liver, skin, and kidneys in laying hens fed different sources of selenium¹.

Item	Treatment ²			s.e.
	C	SS	SY	
Breast muscle (ppm)	0.42 ^B	0.39 ^B	1.22 ^A	0.19
Liver (ppm)	1.36 ^b	1.69 ^{ab}	1.84 ^a	0.12
Skin (ppm)	0.22 ^B	0.31 ^{AB}	0.40 ^A	0.04
Kidney (ppm)	0.90 ^b	1.45 ^a	1.31 ^{ab}	0.15

¹Data represent the means of 8 (1 hen per replicate) samples collected 56 days after supplementation in laying hens of 30 weeks of age. Se content values are expressed on a dry matter basis.

²Treatments: C = control group; SS = sodium selenite; SY = Se-enriched yeast.

^{A, B} Means within a row lacking a common superscript are significantly different ($P < 0.01$).

^{a, b} Means within a row lacking a common superscript are significantly different ($P < 0.05$).

Table 6. Blood metabolites of laying hens fed different sources of selenium¹.

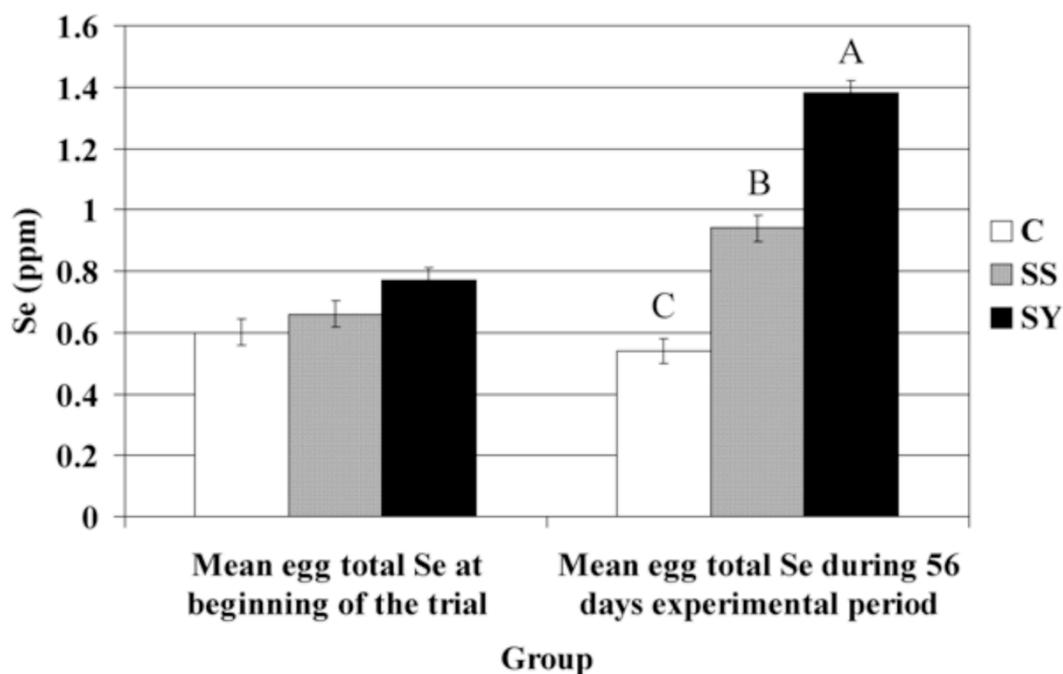
Item	Treatment ²			s.e.
	C	SS	SY	
Total protein (g/L)	47.63	46.00	51.13	1.66
Albumin (g/L)	22.13	20.75	22.38	0.81
Glucose (mmol/L)	11.78	11.95	12.05	0.51
Cholesterol (mmol/L)	2.59	3.17	3.22	0.43
Total bilirubin	3.76	5.09	5.45	0.71
ALAT (IU/L)	4.13	3.88	3.75	0.53
ASAT (IU/L)	177.88	189.75	169.88	14.21
ALP (IU/L)	1,029.50	1,224.71	935.63	354.94
GSH-Px (U/mL)	375.34 ^b	1,309.43 ^a	1,119.97 ^a	216.80

¹Data represent the means of 8 (1 hen per replicate) samples collected after 56 days of supplementation from laying hens of 30 weeks of age.

²Treatments: C = control group; SS = sodium selenite; SY = Se-enriched yeast.

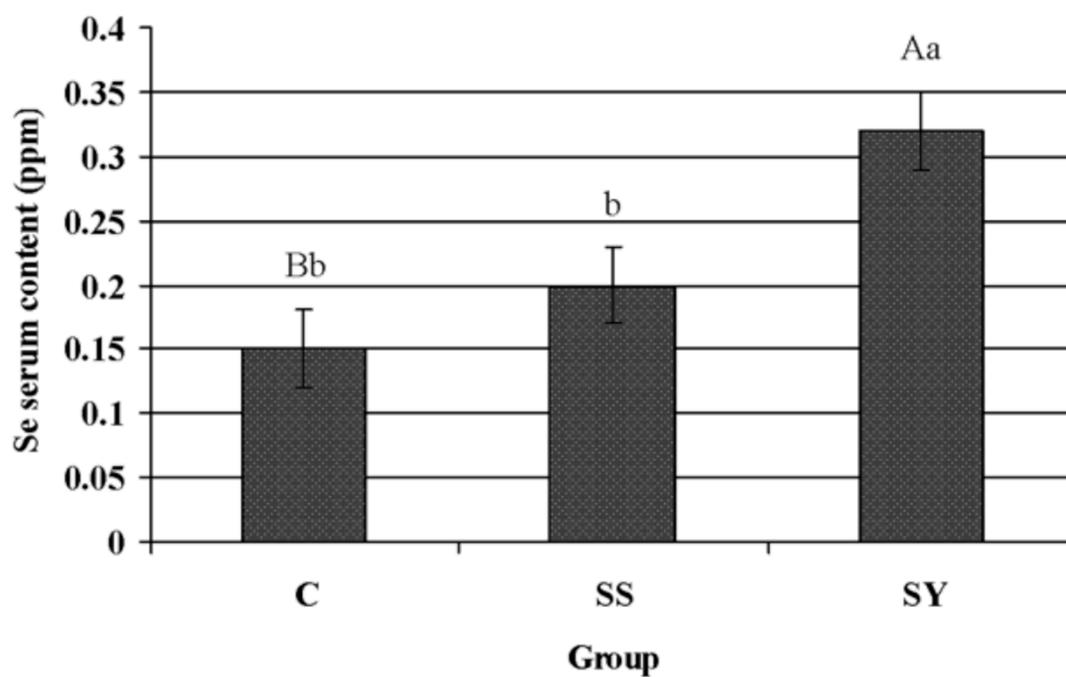
^{a, b} Means within a row lacking a common superscript are significantly different ($P < 0.05$).

Figure 1. Average selenium content of eggs produced by laying hens fed sodium selenite vs. Se-enriched yeast at the beginning of the trial and during the 56-day experimental period. Data in columns represent the means of 8 replicates (2 hens per replicate) \pm s.e. Two eggs per replicate were analyzed at days 0, 18, 36, and 56 during the experimental period. C = control group; SS = sodium selenite; SY = Se-enriched yeast.



^{A, B} Values with no common superscript are significantly different ($P < 0.01$).

Figure 2. Selenium serum concentration in laying hens fed sodium selenite vs. Se-enriched yeast at the end of a 56-day supplementation period. Data are expressed as means \pm s.e. C = control group; SS = sodium selenite; SY = Se-enriched yeast. ^{A, B} Column with no common superscript differ significantly ($P < 0.01$);



^{a,b} Values with no common superscript differ significantly ($P < 0.05$).

CHAPTER 6

General Discussion

6. General discussion

Strategies that modulate intestinal communities have experienced resurgence in interest because of the desire to reduce the use of non-therapeutic antibiotics.

The intestinal microbiota is part of a complex ecosystem that contributes substantially to the health of animals. Proposed mechanisms for the beneficial effect of Direct-Fed Microbials (DFMs) are: (I) to help in maintaining a beneficial microflora in the gastrointestinal tract by inhibiting the growth of pathogenic microorganisms (Jin *et al.* 1996) and (II) to increase nutrient utilization through improved intestinal health resulting in greater intestinal enzyme activities and nutrient availability (Nahashon *et al.* 1994).

Many strategies are currently being used to strengthen host defenses and improve weight gain by supplementing animal feed with ingredients that promote the growth of beneficial bacteria in the intestine. The common modulators of gastrointestinal tract ecology are prebiotics (oligosaccharides that promote the growth of beneficial bacteria) and probiotics (the beneficial bacteria themselves, such as lactobacilli and bifidobacteria).

The main focus this thesis was to evaluate the role of specific Direct-Fed Microbials (DFMs) on nutritional and therapeutic value of animal products and health. More specifically, in the first trial (Chapter 3) the effects of live yeast on apparent digestibility in horses were evaluated. During the last two decades, many articles have reported the effects of yeast supplementation on hindgut digestion and microbial population in horses (Morgan *et al.* 2007, Medina *et al.* 2002, Jouany *et al.* 2008, Lattimer *et al.* 2005).

Positive effects of the administration of *S. cerevisiae* were evidenced during the trial on apparent digestibility of nutrients (Chapter 3). Dry matter (DM) and organic matter (OM) digestibility were improved in horses fed live yeast by 7.3% and 8.7%, respectively. These positive findings in Live Yeast (LY) horses are in accordance with results previously reported with the use of AIA as internal markers supplying (Miraglia *et al.* 1999), and later confirmed by the study of Jouany *et al.* 2008.

Researchers speculated that improvements in digestibility are linked to the capacity of yeasts to improve microbial fermentation of fibre, and in horses such processes take place in the hindgut.

Previous research has shown that fermentation products may be stimulatory to equine hindgut digestion and can beneficially alter microbial population. However, results with yeast culture supplementation have been variable and inconsistent. Research indicates that adding yeast culture to the diet of horses can improve nutrient digestibility (Switzer *et al.* 2003, Morgan *et al.* 2007, Jouany *et al.* 2008), increase microbial populations, (Medina *et al.* 2002 and Lattimer *et al.* 2005) and maintain cecal pH (Medina *et al.* 2002 and Hall *et al.* 2005).

Increased apparent digestibility of nutrient in the present study was expected to increase dietary energy intake but, because of the lack of differences in behaviours and body weight between control and treated horses, currently it is not possible to support this hypothesis: further investigations are needed to ascertain the positive effect of live yeast administration over dietary energy availability.

Probiotics have been shown also to be involved in protection against a variety of pathogens, including *Escherichia coli* (Chateau *et al.* 1993), *Salmonella* and *Campylobacter* (Stern *et al.*, 2001), *Clostridium* and *Eimeria* (Dalloul and Lillehoj 2005). Enhancements of colonization resistance and/or direct inhibitory effects against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Probiotic strains have been shown to inhibit pathogenic bacteria both *in vitro* and *in vivo* through several different mechanisms. Several studies have been undertaken to identify various dietary supplements and probiotics to control *Eimeria* infections (Dalloul *et al.* 2003; Jang *et al.* 2007; Molan *et al.* 2009; Nweze and Obiwulu 2009, Dongjean *et al.* 2010).

In the second trial the effects of the inclusion of some probiotics (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum*) in broiler chickens infected with *Eimeria spp.* were assessed (Chapter 4).

Considering the trial on no challenge or challenge periods, in the first two weeks of the trial, the group with probiotics has shown to improve animal performance, indeed, some investigations on probiotics indicated positive responses to dietary supplementation (Mohan *et al.* 1996, Willis *et al.* 2007, Nayebpor *et al.* 2007, Mountzouris *et al.* 2007, Awad *et al.* 2009). In addition, many studies shown significant improvements in daily body weight gain and feed intake in broiler chicks receiving probiotics (Yeo *et al.* 1997, Awad *et al.* 2009, Sahin *et al.* 2009, Ashayerizadeh *et al.* 2009).

The microbial populations in the gastrointestinal tracts of poultry play a key role in normal digestive processes and in maintaining the animal's health.

Probiotics beneficially affect the host animal by improving its intestinal microbial balance. These bacteria are defined as live microorganisms that are host originated, nonpathogenic, and resistant to gastric acid and bile. They have a high affinity for attachment to the mucosal wall, produce inhibitory compounds, and adjust to immune responses (Patterson and Burkholder 2003). They produce beneficial changes in gut flora by manufacturing acids that inhibit the growth of harmful bacteria (Sun 2005). During the challenge period no differences were detected between broilers fed either probiotics and/or coccidiostat on growth performance overall the trial period. The administration of probiotics plus coccidiostat positively affected the number of intestinal fold goblet cells; these results are in agreement with Edens *et al.* (1997) who reported an increase in goblet cell numbers and mucus secretion in the intestine of chickens fed

probiotics. The presence of this mucous layer prevents bacterial translocation because to cause damage, gut pathogens must pass through this mucous layer before adhering to and invading the epithelial cells.

In the last trial of this work attention was focused to evaluate the effects of inclusion of selenium-enriched yeast in the diet of laying hens on production performances, health parameters, eggshell quality, and selenium tissue deposition (Chapter 5). Selenium (Se) is an essential trace element for animals and humans. It exists in nature in organic and inorganic forms. Some authors suggest that organic Se is an ideal additive because animals and humans absorb and retain it more than inorganic Se. It is required for growth, maintenance of health, and for physiological functions. In particular, it is essential for the proper functioning of the antioxidant enzyme glutathione peroxidase, which protects the cell by destroying free radicals. There are dual benefits from the Se supplementation of animals, namely, to improve the health and performance of animals and to influence the quality of a product (meat, milk, eggs, etc.).

Many studies have shown that the concentration and form of Se in hens' diet will influence Se content and distribution in eggs (Cantor and Scott, 1974; Cantor *et al.* 2000). Our results for egg production agree with those of Cantor *et al.* (2000), Paton *et al.* (2001), and Payne *et al.* (2005), who reported no difference in egg production when hens were fed a diet supplemented with selenium selenite or organic selenium. In contrast with several studies (Utterback *et al.*, 2005; Chantiratikul *et al.* 2008), supplementation with Se led to production of heavier eggs. However, our results are in agreement with data obtained by Rutz *et al.* (2003) showing in layers that selenium supplementation improves egg weight. Therefore the research of a rational development of new alternative strategies for good animal performance together with low or absence of pathogens in the livestock food chain has to be intensified.

The subtle manipulation of gastrointestinal microbiota in maintaining animal gut health, through diversity, stability, metabolites and crosstalk with the epithelium and the underlying immune system by probiotics and prebiotics could be a favourable route. Probiotics can find their main application in the prevention of gastrointestinal infection and disease more than a curative approach. This is because the action of probiotics is not generally aimed, as for antibiotics, to kill pathogen bacteria but they modulate the gastrointestinal environment reducing the risk of gastrointestinal disease synergistically with the immune system of the host. The trend for future could be focus on basic research to identify and characterize existing probiotics strains, determine optimal doses needed for certain strain and assess their stability through processing and digestion. For the probiotics to represent a real and effective alternative to antibiotics and chemotherapeutics it is absolutely needed to ensure their consistently high efficacy. It is important to search for ways to potentiate the efficacy of probiotic

microorganisms in all parts of the digestive tract. The efficacy of probiotics may be enhanced by selection of more efficient strains of microorganism, gene manipulations, combination of a number of strains of microorganism and combination of probiotics and synergistically acting components.

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CHAPTER 7

Summary

7. Summary

The objective of the research described in this thesis is to evaluate the effect of Direct-Fed Microbial (DFM) in horses and poultry, on digestibility and nutritional value of animal products and animal health. To achieve this objective, three different trials were designed; in the first trial was investigated the effects of live yeast on apparent digestibility in horses, in the second trial was studied the effects of the inclusion of some probiotics in broiler chickens infected with *Eimeria spp.* and in the last trial the effects of inclusion of selenium-enriched yeast in the diet of laying hens on production performances, health parameters, eggshell quality, and selenium tissue deposition were investigated.

In the first study proposed were investigated the effects of the administration of live yeast (LY) in a high-fibre diet on nutrients digestibility in mature horses. Six Italian Standardbred mares (weight: 544 ± 14 kg; age: 15.30 ± 3.9 years) in two-period crossover design were fed a basal diet (2.5% body weight [BW]) in a 70:30 forage:concentrate ratio with (LY) or without (CTR) the administration of 4.6×10^{10} colony forming unit (CFU)/d of *Saccharomyces cerevisiae* (MUCL 39885). An adaptation to the diet of 14 days, and an 18-day administration phase, with fecal collection in the last 3 days were performed for each period. Yeast was top-dressed twice a day during the concentrate meal (12:30 AM and 09:00 PM). Change in BW was measured at the beginning of each experimental phase and the diet adjusted accordingly, and individual feed intake was recorded daily. Concentrate samples were collected at the beginning of each confinement period and individual hay samples were obtained for each confinement day 38 hours before fecal collection. No influence of LY was observed on BW change ($P = .64$), feed intake ($P = .48$), hay intake ($P = .48$), or concentrate intake ($P = .47$). *S. cerevisiae* supplementation improved apparent digestibility of dry matter (64.5% vs. 60.1%, $P = .03$), organic matter (66.1% vs. 61.6%, $P = .04$), neutral detergent fiber (42.5% vs. 35.9%, $P = .04$), and acid detergent fiber (36.5% vs. 28.0%, $P = .03$) with a positive trend on crude protein ($P = .08$). In the present study, the administration of LY to horses significantly improved the digestion of the fiber fractions of the diet.

The aim of the study was to investigate the effects of the inclusion of a probiotics mix (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum*) on growth performance, health status, and intestinal morphology of broiler chickens infected with *Eimeria spp.* for a complete production cycle from hatching to slaughter. A total number of 900 Hubbard male chickens, 1d old and coming from the same hatchery, were divided in 18 pens located in 3 identical rooms (6 pens per room). At the beginning of the trial, the animals were homogeneously distributed by body weight to 3 experimental dietary treatments consisting of a basal diet plus: PC= probiotics 0.05% (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and

Cl. butyricum) + a coccidiostat; C= a coccidiostat; P= probiotics 0.1%. On the second week of life each box of the experimental groups was challenged with 400g of litter containing 2.5×10^5 oocysts of *Eimeria spp.* homogeneously distributed.

Considering the trial divided in two periods: no challenge (first two weeks) and challenge (3 to 8 weeks), in the first two weeks of the trial, birds fed the diets containing probiotics (PC and P) had significantly higher body weight (BW), average daily gain (ADG) and feed intake (FI) compared to animals fed the diet containing only a coccidiostat (C) and the feed conversion ratio (FCR) was significantly lower in the groups fed the diets containing probiotics (P and PC).

During the challenge period BW was significantly higher in birds fed the diets containing coccidiostat (C and PC), while no differences were observed for FCR, ADG and FI. The administration of probiotics plus coccidiostat (PC) positively affected the number of intestinal fold goblet cells (F) in the duodenum and ileum. In the ileum of PC group the lowest values for gland depth (G) were assessed, while F:G ratio was lower compared with C group. Fecal count of *Eimeria spp.* did not show any statistical difference between the three experimental groups at the end of the trial.

The results of this work show that the administration of probiotics (10^8 cfu/g of *Bacillus coagulans*, *B. subtilis* and *Cl. butyricum*) in normal farming condition had positively influenced the growth performance of broiler chickens.

Subsequently in challenge period no differences were detected between broilers fed either probiotics and/or coccidiostat considering; growth performance and fecal count of *Eimeria spp.*, these results suggest probiotics may have positive effects administered alone or in combination with coccidiostats in broilers chickens infected with *Eimeria spp.*

The aim of the third study is to evaluate the effect of inclusion of selenium-enriched yeast in the diet of laying hens on production performance, health parameters, eggshell quality, and selenium tissue deposition. (Chapter 5)

Forty-eight ISA Brown laying hens, each 22 weeks of age, were utilized for an 8-week trial to evaluate the bioavailability of ingested selenium yeast and its effects on egg-laying performance, eggshell quality, health parameters and tissue Se distribution. Animals were divided into 3 treatment groups: the control group (C) received a basal diet containing 0.11 mg of Se /kg DM; the inorganic Se group (SS) received the control diet plus sodium selenite at 0.4 mg/kg DM; and the Se-yeast group (SY) received the control diet plus selenium yeast at 0.4 mg/kg DM. Feed intake, egg mass ratio, and production performances were not affected by supplementation with Se, regardless of the Se source. Only egg weight was higher ($P < 0.05$) for hens with diets supplemented with Se. Eggshell weight was improved in both Se-supplemented groups, whereas breaking strength was increased by the administration of SY. Breast muscle, liver and skin

Se level was higher in the SY group than in the C group, whereas the Se level did not differ between the SS and C groups. Kidney selenium content was statistically significant only for SS animals. Eggs from SY hens had higher Se levels than those from SS hens. Results of our study indicate that supplementation with 0.4 ppm of selenium from SS or SY does not affect hens' zootechnical performance and blood metabolites. The only exception is the increased weight of eggs obtained from hens supplemented with Se. On the other hand, selenium—particularly in its organic form—improves eggshell quality. Specific selenium sources influence selenium distribution in hen tissues. Indeed, egg and breast muscle selenium concentrations were higher when hens were fed selenium yeast because of the greater bioavailability of organic selenium sources when compared with inorganic sources. The supply of organic selenium in the form of selenomethionine in laying hen feedstuffs benefits the human population via the food chain by helping to meet human selenium requirements that are not always satisfied by typical human diets.

CHAPTER 8

Acknowledgements

8. Acknowledgements

