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Use of probiotics and prebiotics: a strategy to modulate the intestinal microbiota of poultry and control *C. jejuni* colonization

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1.1 Anatomy and Physiology

The gastrointestinal tract of poultry (fig. 1.1) is relatively short and appears particularly well adapted for transforming concentrated diets into nutrients. The extremely rapid rate of passage of digesta, which is around 10 hours, implies highly efficient mechanisms of digestion and absorption. In comparison with mammals, the gastrointestinal tract of birds is distinguished by the following features:

i) replacement of the lips in mammals with beak.

ii) existence of two successive and distinct stomachs. The proventriculus or glandular stomach is the 'chemical' stomach. The gizzard, or 'mechanical' stomach ensures homogenization and a certain amount of grinding of the food.

iii) the uniqueness of the terminal region of the tract, or cloaca, which acts both as the rectum and the exit for the urino-genital system.

The development of the gastrointestinal tract is very precocious. In the embryo the primordial intestine develops from the second day of incubation. At hatching the tract represents up to 25% of the live weight. This proportion diminishes rapidly and falls to less than 5% in a 8-week old broiler.

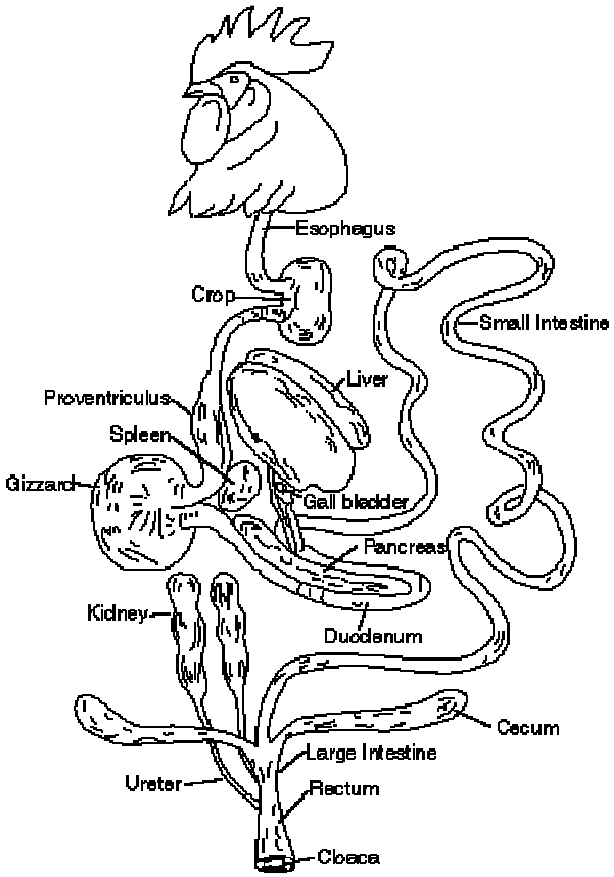


Fig. 1.1 Chicken gastrointestinal tract (from the website of the Department of Animal Science, Oklahoma State University Board of Regents).

1.1.1 Buccal cavity

The beak consists of two keratinized cases which cover the mandibles. Food particles which are grasped are transferred into the mouth without undergoing any significant transformation. Water is imbibed passively following movements of the head. Salivary glands are numerous and dispersed, in adults salivary fluid is rich in mucus which ensures both the lubrication of the food bolus to assist its passage into the oesophagus and the permanent moistening of the bucco-pharyngeal cavity. Composition of salivary fluid is analogous to that of mammals, with the presence of amylases and a large concentration of bicarbonate ions.

1.1.2 Oesophagus

This lies between the pharynx and the proventriculus and may be considered as a highly dilatable tube consisting of two parts. The first (upper) is cervical and closely linked with the arterial system and the second (lower) is intra-thoracic and found above the heart. Between this two regions is found the crop which may be considered as a simple dilatory lobe. It constitutes a reservoir regulating transit time of digesta when the bird, after a severe food restriction, is able to consume a significant amount of food within a short period of time.

The mucosa is rich in branched mucus glands and is covered with a stratified epithelium consisting of flat cells. The musculature consists of three types of muscle fiber.

Food may accumulate within the crop, be moistened and soften. The frequency of contractions within the crops varies depending upon the region under consideration. They are quicker in the cervical part and slower in the caudal area because of the difference in the degree of innervations. In this way the crop is able to receive food from the bucco-pharyngeal cavity, and the process is faster if crop contents are not passed towards the proventriculus. Emptying plays an important role in regulating the rate of passage of digesta and therefore the efficiency of the digestive process. The food bolus remains in the crop for less time when the gizzard is empty and when the food consumed is in the form of meal.

1.1.3 Proventriculus and Gizzard

On leaving the crop, chyme arrives at a small ovoid cavity surrounded by thickened wall named the glandular stomach or proventriculus (fig. 1.2). The mucosae are covered in an epithelium consisting of cylindrical cells. The numerous tubular-type glands have ducts forming rows of mammillae and the alveoli of these glands are surrounded by highly specialized cells which secrete both hydrochloric acid and proteolytic enzyme pepsinogen. The ducts open out into luminal papillae and transport gastric juice into the lumen of the proventriculus.

Under *ad libitum* feeding conditions the contents of the proventriculus as well as those of the gizzard, are predominantly acid; gastric secretion is not simply continuous but also responds to both nervous and chemical stimulation.

Secretion of hydrochloric acid, which is particularly important in the laying hen in order to solubilise between 7 and 8 grammes of calcium carbonate daily, maintains pH at levels between 1 and 2.

Chyme remains in the proventriculus for a relatively short period of time, between a few minutes and an hour, before passing into the gizzard through a narrow and short isthmus.

The gizzard is a thickened slightly biconvex organ found posterior to the sternum and which partially covers the liver lobs. External musculature is covered by a white fibrous sheath. The glandular layer synthesizes a proteinaceous substance, similar to keratin, in the form of polysaccharide-protein complex which gives rise to a thick and rugged cornified lamina covering the entire internal wall. This structure

which possesses considerable muscular strength, allows for the grinding and reduction in size of particles within chyme particularly if the bird has ingested small siliceous stones (grit) which are not attacked by hydrochloric acid. The pressure recorded within the organ when contracted is of the order of 15 cm of mercury.

The two stomach accordingly have complementary roles. The former has a secretory function, whereas that of the latter is essentially mechanical. Hydrochloric acid produced in the proventriculus continues its action within the gizzard in order to solubilise mineral salts (calcium carbonate and phosphates), to ionize electrolytes and to destroy tertiary structures of dietary proteins. In the same way pepsin (derived from pepsinogen activation), which is the sole gastric enzyme, is not effective within the lumen of the proventriculus but contributes to protein hydrolysis within the cavity of the gizzard.

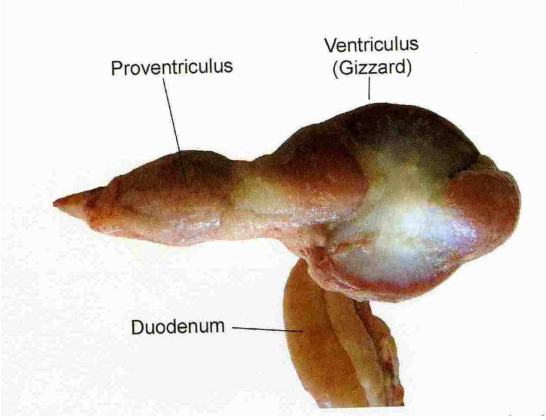


Fig. 1.2 Proventriculus and gizzard of chicken gastrointestinal tract (Grist, 2006).

1.1.4 Small intestine

In adult birds, the total length of the small intestine is approximately 120 cm, it is conventionally divided into three sections, which do not have major structural differences, being the duodenum, the jejunum and the ileum.

The duodenum is 24 cm and 'U' shaped with the two sections being bent back around the gizzard and wrapped around the pancreas. The gizzard-duodenal junction acts as a filter by only allowing small particles within the chyme to pass. The border between these two structures is covered with a thick layer of mucus having a protective role against the excessive acidity of the chyme leaving the gizzard.

The bile and pancreatic ducts enter the latter portion of the ascending branch of the duodenum at the point where, conventionally, the jejunum commences. This itself is approximately 50 cm long and is convoluted around the free side of the large mesentery. Meckel's diverticulum, regarded as the beginning of the ileum, is the vestigial omphalomesenteric duct which, in the embryo, joins the intestine in the umbilical vesicle or vitellin sac. The third portion of the small intestine is as long as the jejunum and leads to a ringed valve before branching out into the two caeca.

The internal mucosa consists of three layers. The internal one is glandular possessing enterocytes with villi as is found in mammals. Birds do not have Brunners glands but have glands or crypts of Lieberkuhn. The intermediary mucosal layer contains blood vessels and nerves. Finally the external portion consists of smooth muscle responsible for intestinal motility which is characterized by peristaltic and segmentary contractions.

Duodenal secretions or, more generally intestinal, are pale yellow. They include mucus, electrolytes and enzymes. With the exception of mucus, which is secreted throughout the intestinal tract except the gizzard, the other components are essentially of pancreatic and biliary origin.

Bile is synthesized in the liver and is carried to the duodenum through two ducts connected directly to the left lobe or indirectly to the right lobe of the liver. It is a greenish liquid, slightly acidic (pH 6) containing bile salts and lipids (cholesterol and phospholipids). Bile salts are different from those found in mammals. Lipids are emulsified under the influence of bile to facilitate the action of pancreatic lipase. Synthesis and secretion of bile develops with age of bird, with young bird being relatively unable to digest dietary lipids adequately, particularly if they contain unsaturated fatty acids. Thus the addition of bile salts to diets for young chickens improves fatty acid digestibility.

Pancreatic juice has a particularly powerful hydrolytic capability directed towards protein, carbohydrates and lipids. It has a high concentration of buffer, particularly bicarbonate, which facilitates the increase in pH of gastric chyme in order to ensure the activity of the majority of pancreatic enzymes. These enzymes are themselves secreted in the form of pro-enzymes into the intestinal lumen. Proteolytic enzymes are principally endopeptidases (e.g. trypsin, chymotrypsin, elastase).

Hydrolysis of starch, which is the principal carbohydrate of the diet, is under the influence of α-1-4-glucosidase, a glicoprotein requiring the presence of Ca^{2+} ions. Digestion of lipids, present in an emulsified form as a result of the action of bile salts, is achieved by lipase and its cofactor (colipase), a phospholipase or several esterases.

In addition to these pancreatic and bile secretions, intestinal juice contains enzymes secreted by the brush border of the small intestine. Their optimum pH of activity is approximately 6. They are enzymes specialized in the hydrolysis of oligosaccharides, for example saccharase, isomaltase and trehalase which hydrolyze saccharose, isomaltose and trehalose respectively. Saccharase and isomaltase are attached and linked to the same protein arm fixed to the wall of enterocytes in the intestinal mucosal wall. It should be also noted that, in contrast to mammals, birds do not have lactase, indicating that the very low level of lactose hydrolysis is a result solely of bacterial enzyme action.

1.1.5 Large intestine

The relatively long caeca (20 cm each in the adult) lead directly to the rectum of approximately 7cm in length, the colon being virtually absent. Each has a narrow proximal region with a smooth epithelium and a large terminal zone which is the site of significant bacterial activity. The ileo-caeco-colonic junction (fig. 1.3) controls the flux of chyme between the colon and the caeca. It relaxes to allow movement towards the colon, and contracts when the latter is distended. At this moment, the flux is directed towards the caeca or cloaca depending upon the direction of peristalsis. Caecal filling takes place at regular intervals under ad libitum feeding conditions. Evacuation of the caeca seems to result from a strong contraction which starts at the base of each of them. In contrast, frequency of emptying (5 to 8 times daily) varies with the degree of distension of the caeca, the quantity of ions H^+ present and their electrolyte content.

Digestion of food within the large intestine is minimal. There is bacterial activity which, however, does not hydrolyze cellulose or other non-starch polysaccharides.

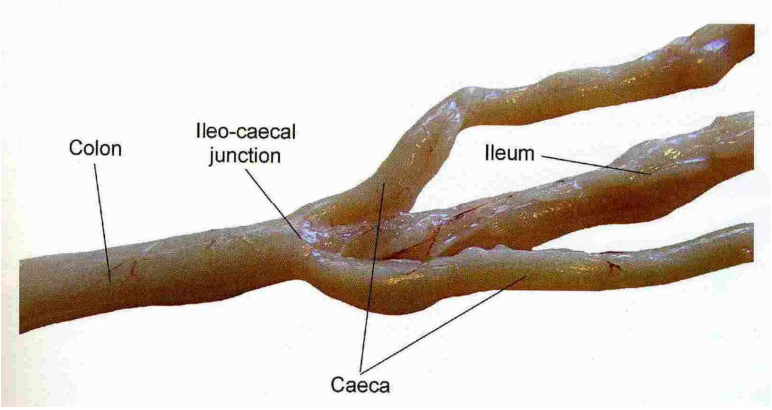


Fig. 1.3 Ileo-caecal junction and the two-caeca of chicken gastrointestinal tract (Grist, 2006).

1.1.6 Cloaca

This is divided into three portions by two transversal membranes (fig. 1.4):

i) the coprodeum: which may be regarded as a dilatation of the rectum in which faecal material accumulates

ii) the urodeum: into which the two ureters enter, and additionally, the two sperm ducts of the male and oviduct of the female.

Defecation which occurs at regular intervals is achieved through rapid contraction of the coprodeum. As a consequence of the convergence of the digestive and urinary tracts in the region of the cloaca, urine arriving from the ureters may ascend up to the caeca where water and elelctrolytes may be absorbed. Urine becomes concentrated as insoluble urates and is voided in the form of a paste covering the excrements in a white layer.

The proctodeum opens to the outside through a double sphincter (smooth internally, and rough externally). It is linked through its base to the Bursa of Fabricius which is a lymphoid organ rich in nucleoproteins which deteriorates with age. It is sometimes referred to as the cloacal thymus (Larbier and Leclercq, 1992; Scanes *et al*., 2004; Grist, 2006).

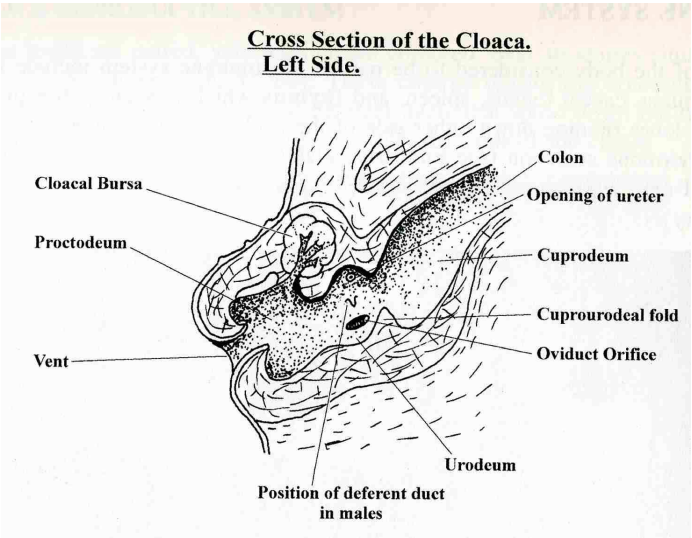


Fig. 1.4 Cross section of the chicken cloaca (Grist, 2006).

1.2 The gastrointestinal microbiota of poultry

1.2.1 Microbes of the chicken gastrointestinal tract

The gastrointestinal tract (GIT) of warm-blooded animals is densely populated by bacteria. Composition and density of the microbiota can vary a lot among individuals because it is markedly affected by the bacterial composition of the inoculum received at birth or hatch, the structure of the host intestinal epithelium and the diet (Apajalahti and Kettunen, 2006; Zhu *et al*., 2002).

At hatching the digestive tract is a sterile environment where microorganisms grow rapidly. Settlement of the microbial population depends on the egg's microbial environment at hatching which determines the order in which animals are exposed to microorganisms, their ability to colonize the intestine and their interactions (Gabriel *et al*., 2006).

Previously, the only way to characterize the microbiota was culturing on selective growth media and subsequent identification of bacteria through biochemical tests, but such methods are laborious and incomplete and, therefore, not suitable for extensive monitoring of the unknown microbiota. Recent developments in the total microbial community analysis by DNA-based methods have brought a new insight into gastrointestinal tract microbiology of chickens and many other animal species (Apajalahti *et al*., 1998; Gong *et al*., 2002; van der Wielen *et al*., 2002; Zhu *et al*., 2002; Zhu *et al*., 2003; Apajalahti *et al*., 2004). The %G+C profiling and 16S sequence analysis of chicken gastrointestinal microbiota underline that only 10% of the gastrointestinal bacteria represents previously known bacterial species. Thirty five percent represents previously unknown species with a known bacterial genus and the remaining 55% represent bacteria for which even the genus is totally unknown. Using this molecular approach 640 different species and 140 different bacterial genera have been found in the chicken gastrointestinal tract (Appajalahti *et al*., 2004).

Bacteria in the gastrointestinal tract have nutritional and spatial requirements, they derive most of their energy for reproduction and growth from dietary compounds which are either resistant to attack by digestive fluids or absorbed so slowly by the host that bacteria can successfully compete for them. Since bacterial species differ from each other in relation to their substrate preferences and growth requirements, the chemical composition and structure of the digesta largely determine the species distribution of the microbial community in the gastrointestinal tract. As a consequence, bacterial community structure is very much dependent upon the diet as the ultimate source of substrates for metabolism. Viceversa, the ability of the host digestive system to digest and absorb nutrients is, in part, dependent upon the species distribution and total population of resident microbes. Hence, changes in dietary composition or nutrient density can have dramatic effects on the intestinal microbiota populations, which in turn can influence the ability of the animal to digest and absorb dietary nutrients (Appajalahti *et al*., 2004; Gabriel *et al*., 2006).

The distribution of indigenous microbiota within the avian GIT is therefore organized qualitatively and quantitatively along vertical and horizontal regions in the GIT. The vertical distribution refers to the distribution of bacteria from the crop to the caeca. Furthermore, bacteria are distributed horizontally along the GIT and occupy the intestinal lumen, mucous lining, crypt spaces and adhere also to the epithelial cells. Each segment and horizontal layer harbors its own specific bacterial community and this depends, as already indicated on environmental factors such as nutrition, bile salts, oxygen concentration and pH of the different segments (Thomson and Applegate, 2005; van der Wielen *et al*., 2002).

The bacterial succession in chicken gastrointestinal tract starts immediately after hatching. Large numbers of anaerobic bacteria capable of decomposing uric acid comprise the cecal flora of chicks 3 to 6 h after

hatching. During the first 2 to 4 days (d) posthatch, streptococci and enterobacteria colonize the small intestine and cecum. At 4 d almost one-third of the bacteria in the chicken ceca consisted of *E. coli* and *Clostridium* species. After the first week, *Lactobacillus* predominate in the small intestine, and the cecum is colonized mainly by anaerobes (*Escherichia coli* and *Bacteroides*) with lower numbers of facultative aerobes. A typical microbiota of adult birds in the small intestine is established within 2 weeks; however, it was found that the adult cecal microbiota, which is mainly constituted of obligate anaerobes, takes up to 30 d to develop (Amit-Romach *et al*., 2004).

Molecular DGGE analyses underline that the number of bands in the profiles increase when broiler chickens grow older; this might indicate that the diversity of the dominant bacterial community in the intestinal tract also increases when broilers grow older. Moreover it seems that the dominant bacterial community in crop, duodenum and ileum within the same chicken is very similar in 4-day-old broilers. Even the similarity between the dominant bacterial community of the ceca with the other three parts of the intestinal tract is much higher in 4-day-old broilers. This suggests that the environmental conditions along the intestinal tract are rather similar and do not allow niche differentiation. When broilers age, similarity between banding patterns of crop, duodenum, and ileum decrease considerably. This indicates that environmental factors in the intestine change specifically in each compartment. These results can be important for studies related to the manipulation of the intestinal bacterial community in chickens (van der Wielen *et al*., 2002).

The maximum bacterial density is found to be reached in about one week and, after this phase of microbiota development, another one starts that can be called “maturation phase” and it is characterized by: (i) a low growth rate equal to that of the digesta passage; and (ii) gradual selection of bacteria that most efficiently adapt to the prevailing conditions.

In chicken, the main sites of bacterial activity are the crop and the caeca and, to a lesser extent, the small intestine (Gabriel et al., 2006).

The crop microbiota is mainly composed of lactobacilli attached to the epithelium and forming an almost continuous layer, and enterococci, coliforms and yeasts (Gabriel *et al*., 2006).

Bacterial density reaches at maturity 10^3-10^5 bacterial cells per gram of digesta in the proximal small intestine (duodenum) because it is characterized by rapid flow of the highly fluid digesta, while the distal small intestine (jejunum and ileum) harbors $>10^9$ bacteria cells per gram of digesta (Brisbin *et al*., 2008a, Gong *et al*., 2007). Generally the main genera of bacteria within the chicken small intestine are *Lactobacillus*, *Enterococcus* and *Clostridium*, with some bacteria from the family *Enterobacteriaceae* (Brisbin *et al*., 2008a). The most predominant *Lactobacillus* species in the upper GI tract (gizzard, duodenum, jejunum and ileum) seem to be *L. aviaries* and *L. salivarius* (Gong *et al*., 2007).

The caeca contain a more diverse community of bacteria, including species of the genera *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Escherichia*, *Fusobacterium*, *Lactobacillus*, *Streptococcus* and *Campylobacter*, and reaching $>10^{11}$ cell/g of digesta (Apajalahti and Kettunen, 2006; Brisbin *et al*., 2008a). Gong *et al*. (2007) and Bjerrum *et al*. (2006) indicated *Faecalibacterium prausnitzii* and butyrate-producing bacteria (mainly *F. prausnitzii*, *Clostridium* and *Ruminococcus*) as the largest groups in the caeca.

1.2.2 Role of the intestinal microbiota

The microbiota of the GI tract of mammals can be considered a metabolically active organ with its wide biodiversity in term of species and the high number of cells (Macfarlane and Macfarlane, 2004, Backed *et al*., 2005, Murphy *et al*., 2009).

Under normal circumstances, commensal bacteria are an essential health asset with a nutritional function and a protective influence on the intestinal structure and homeostasis. A balanced gut microbiota constitutes an efficient barrier against pathogen colonization; moreover, it produces metabolic substrates (*e.g*. vitamins and short chain fatty acids) and stimulates the immune system in a non-inflammatory manner. The intestinal microbiota in fact actively exchanges developmental and regulatory signals with the host that prime and instruct mucosal immunity (O'Hara and Shanahan, 2007; Brisbin *et al*., 2008a).

Physiological and psychological stressors, leading to dysfunction of the intestinal barrier and to the increase of intestinal permeability, have an impact on gut microbial composition and susceptibility to enteric pathogens (Gareau *et al*., 2009). In poultry production systems, birds are routinely subjected to stressors such as feed withdrawal, temperature fluctuations, and confinement during transportation augmenting disease incidence (St-Pierre *et al*., 2003; Humphrey, 2006).

1.3 Antibiotic Growth Promoters (AGPs) and animal feed regulations

1.3.1 Antibiotics

Modern food animal production depends on the use of large amounts of antibiotics for disease control (Aarestrup, 2002).

In 1949, quite by accident, while conducting nutrition studies with poultry, Jukes of Lederle Laboratory and McGinnis of Washington State University obtained startling growth responses from feeding a residue from Aureomycin production. Later experiments revealed that the supplement used by Jukes and McGinnis – the residue of Aureomycin production – supplied the antibiotic chlortetracycline. This was the birth of feeding antibiotics to livestock (Scanes *et al*., 2004b).

However, over the past few decades awareness has grown that this application creates favourable conditions for selection, spreading and persistence of antimicrobial-resistant bacteria capable of causing infections in animals and humans. It has thus become clear that antimicrobial resistance poses a threat to public and animal health and is a reason for serious concern.

In modern food animal production antimicrobial agents are normally used in one of four different ways, *i.e.* i) *therapy*: treatment of infections in clinically affected animals, preferably based on a bacteriological diagnosis, ii) *metaphylactics*: treatment of clinically healthy animals belonging to the same flock or pen as animals showing clinical signs; in this way infections may be treated before they become clinically apparent and the entire treatment period may thereby be shortened. In fact, in view of the modern production systems, this may often be the only effective approach to treat for instance large broiler flocks through water medication, iii) *prophylactics*: treatment of healthy animals in a period of stress (*e.g*. early weaning) to prevent diseases; in such cases the use of antimicrobials is indicative for general management problems, and hence in most countries is either illegal or considered imprudent; and, finally, iv) *growth promotion*: the continuous inclusion of antimicrobials in animal feed to prevent (subclinical) infections and hence promote growth; such usage is under serious debate (Aarestrup, 2002).

The primary reason for using antibiotics in poultry feeds is for their growth stimulating effect, for which they are generally used in broiler rations. The reason for the beneficial effect of antibiotics still remain obscure, but the best explanation is the disease level theory, based on the fact that antibiotics have failed

to show any measurable effect on birds maintained under germ-free conditions (Scanes *et al*., 2004b). The exact mechanisms by which these improvements occur, however, are still not fully understood. Currently, four mechanisms of growth promotion have been proposed by various scientists. Because early researches have indicated that orally dosed antibiotics do not promote growth in germ-free chicks, each of these proposed mechanisms are based on the hypothesis that the presence of bacteria in the intestine reduces animal growth, and include hypotheses that: 1) antibiotics inhibit the occurrence of sub-clinical infections, 2) antibiotics reduce production of growth-depressing microbial metabolites, 3) antibiotics reduce the use of nutrients by intestinal microbes, and 4) antibiotics allow for enhanced uptake of nutrients because they have been shown to reduce the thickness of the intestinal wall. Regardless of the fact that the exact mechanisms of antibiotic-mediated growth promotion are currently incompletely understood, most researchers support the theory that antibiotics reduce the overall numbers or diversity of gut bacteria, which may promote growth (Thompson and Applegate, 2005).

In addition to their use as growth stimulators, antibiotics are used to increase egg production, hatchability, and shell quality in poultry. They are also added to feed in substantially higher quantities to remedy pathological conditions. Antibiotics are generally fed to poultry at levels of 5 to 50 g per ton of feed, depending upon the particular antibiotic used. Higher levels of antibiotics (100 to 400 g per ton of feed) are used for disease-control purposes. The antibiotics most commonly used in poultry rations are bacitracin, virginiamycin, bambermycin, and lincomycin. High levels of calcium in a laying mash will inhibit assimilation of certain tetracycline-type antibiotics to the bloodstream and reduce their effectiveness. In all probability, antibiotics will always be used as feed additives to control and treat health problems in poultry. But the status of subtherapeutically used antibiotics as production stimulators is, at the present time, tenuous. Pressure from consumer groups and medical people may result in banning many of the antibiotics that are primarily used for medicinal purposes in humans from the list of approved production promoters. However, in the future, an increasing number of antibiotics will likely be developed specifically for the purpose of improving poultry performance. One example is that of bambermycin. This antibiotic was developed solely for use as a production promoter, serving to increase rate of gain and feed efficiency in chickens and swine. It has no medical applications, and, therefore, poses no health hazard with regards to bacteria becoming resistant to it (Scanes *et al*., 2004b).

As in human medicine, the use of antimicrobial agents in agriculture creates a selective pressure for the emergence and dissemination of antimicrobial-resistant bacteria including animal pathogens, human pathogens which have food animal reservoirs, and other bacteria that are present in food animals. These resistant bacteria may be transferred to humans either through the food supply or by direct contact with animals. The transfer of resistant bacteria from food-producing animals to humans is most evident in human bacterial pathogens which have food animal sources, such as *Campylobacter*, which has a reservoir in chickens and turkeys and *Salmonella*, which has reservoirs in cattle, chickens, pigs and turkeys. Pathogenic bacteria, such as *Campylobacter* and *Salmonella*, are not the only concern when considering antimicrobial resistance in bacteria with food animal reservoirs. Commensal bacteria, which are naturally occurring host microbiota, constitute an enormous potential reservoir of resistance genes for pathogenic bacteria. The prevalence of antibiotic resistance in the commensal bacteria of humans and animals is considered to be a good indicator of the selective pressure of antibiotic usage and reflects the potential for resistance in future infections.

Most resistant bacteria have mobile genetic elements such R-plasmids and transposons. As the reservoir of resistant commensal bacteria increases, the plasmid reservoir becomes larger and enables more frequent transfer of resistance to pathogenic bacteria including *Salmonella* and *Shigella*. *Escherichia coli*,

which is the predominant isolate of aerobic faecal microbiota in humans and most animals, has demonstrated its ability to transfer resistance genes to other species, including pathogenic bacteria (Anderson *et al*., 2005).

1.3.1 Feed additives legislation and antibiotic ban

Feed additives encompass a variety of products. According to the currently applicable legislation (EC 1831/2003, Art. 2 (2a)), "feed additives" means substances, microorganisms or preparations – other than feed materials premixtures – which are intentionally added to feed or water to perform, in particular, one or more of the functions mentioned in Article 5. Article 5 can be summarized as follows: a feed additive should favourably affect one of the following characteristics of feed or animal products; colour of ornamental fish or birds; the environment; animal production, performance or welfare through positive effects at gut level, or satisfy the nutritional needs of animals or have a coccidiostatic or histomonostatic effect (Doeschate and Raine, 2006).

At EU level, Directive 70/524 (23 November 1970) was really the first one that tried to regulate the use of feed additives across the EU Member States; but even though the directive was intended to lead to consistent legislation across the EU, it did not achieve this. Moreover, the fact that the Scandinavian countries banned antibiotic-growth promoters ahead of the rest of Europe resulted in a campaign to get these products banned in the whole of the EU. Directive 70/524 appears complicated, with many annexes and different authorization periods for different products. The industry worked with the Directive and the country-specific implementation of the Directive into law as well as possible. Regulation 1831/2003 thus sets out to review all the rules on additives, with a change of emphasis towards the protection of human health, animal health and the environment, based on precautionary principle. Regulation 1831/2003 applies directly in each Member State, and there should thus be less opportunity for country-specific rules and derogations. All additives authorized under regulation 1831/2003 will be given time-limited authorization to allow technological progress and scientific developments to be taken into account in the review of the product authorization.
The categories of additives identified in 1831/2003 are:
- Technological additives: any substances added to feed for a technological purpose.
- Sensory additives: any substance, the addition of which improves or changes the organoleptic properties of the feed, or the visual characteristics of the food derived from animals.
- Nutritional additives (such as amino acids).
- Zootechnical additives: any additive used to affect favourably the performance of animals in good health or used to affect favourably the environment.
- Coccidiostats and histomonostats.

Regulation 1831/2003 has at least stated that antibiotics, other than coccidiostats and histomonostats, might be marketed and used as feed additives only until December 31, 2005; as from January 1, 2006, those substances would be deleted from the Community Register of authorized feed additives (Castanon, 2007). The removal of these compounds in animal diet has put tremendous pressure on the livestock and poultry farms, one of the main consequences being a substantial increase in the use of therapeutic antibiotics (Casewell *et al*., 2003). It has been evidenced that AGP have long been effective in prevention of necrotic enteritis (NE) in poultry flocks and that the incidence of NE have increased in countries where AGP have been stopped (Van Immerseel *et al*., 2004).

In other ways, the ban of growth promoters demands the improvement of the hygiene from farms. It was shown that under good production conditions it is possible to reach good and competitive production results for the rearing of poultry without the continuous use of antibiotics in feeds. Moreover, safer non-antimicrobial substances have been studied as alternatives for replacing antibiotics to interact with the intestinal microbiota, including enzymes, prebiotics and probiotics or acidification of diets (Castanon, 2007).

Chapter 2. Poultry Pathogens

2.1 Zoonosis – Campylobacteriosis: the European Union survey in 2008

Zoonotic bacteria can cause clinical disease, morbidity and mortality in animals and are a major source of economic loss to the livestock and poultry industry worldwide. Moreover these enteric pathogens could be present in the animal intestinal tract asymptomatically and can be transmitted through the food chain to humans thus becoming a risk for the health as food-borne disease. Contamination of food can happen at any stage of the production chain: raw materials used in animal nutrition, feed manufacturing, farm level, slaughter plant, meat processing, retail and preparation of meat at home. To improve food safety, the industry is requested to decrease the level of contamination to zero or at least to acceptable levels depending on the pathogen (EFSA, 2007b).

In the EFSA-ECDC (2010) report, the analysis, which has been conducted in 2008, of the occurrence of infectious diseases transmitted from animals to humans shows the following figures: campylobacteriosis continued to be the most commonly reported gastrointestinal

bacterial pathogen in humans in the European Union with 190,566 (fig. 2.1) confirmed cases, even though the number of notified cases decreased by 5.0% compared with 2007. In foodstuffs, the highest proportion of *Campylobacter* positive samples was once again reported for fresh poultry meat where on average 30.1% of samples were positive. *Campylobacter* was also commonly detected from live poultry, pigs and cattle.

No clear trend in the notification rates at Community level was apparent during 2004 to 2008 (fig. 2.2) The occurrence of *Campylobacter* was high in broiler meat and broiler flocks throughout the production chain in many MSs (Member States). Broiler meat and raw milk were reported as the most important food vehicles in food-borne *Campylobacter* outbreaks in 2008 (EFSA, 2010).

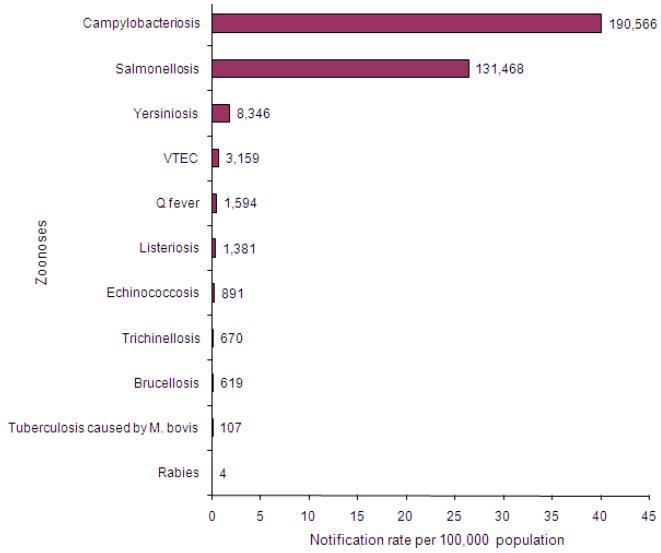


Fig. 2.1 The reported notification zoonoses rates in confirmed human cases in EU, 2008 (EFSA, 2010).

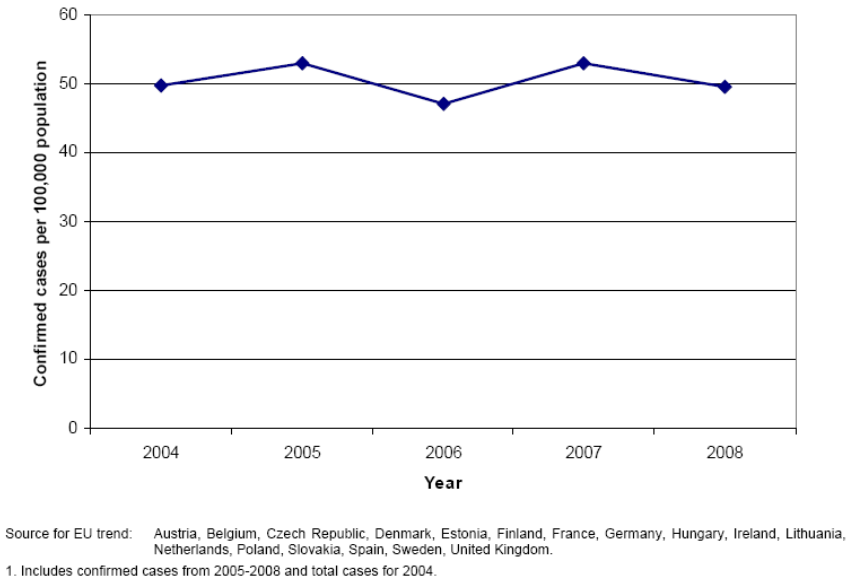
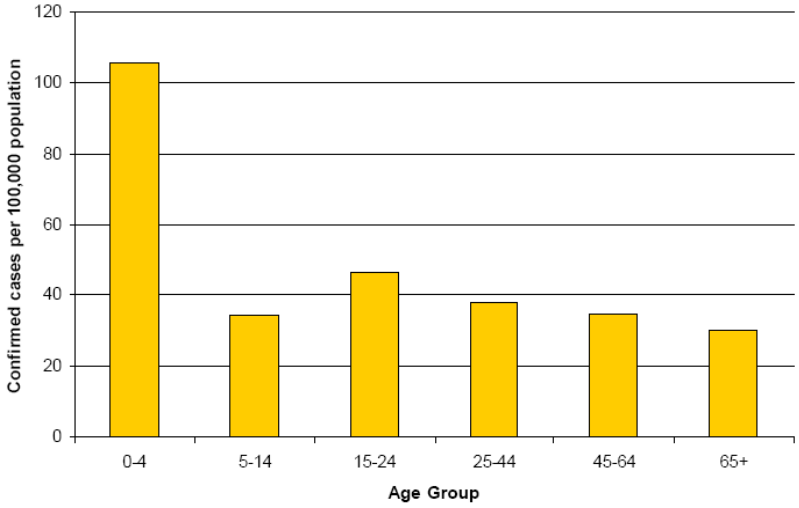


Fig. 2.2 Notification rates of reported confirmed cases of human campylobacteriosis in the EU, 2004-2008[1].

2.1.1 Human

In total, 190,566 confirmed cases of campylobacteriosis were reported by 25 MSs, which was a 5.0% decrease compared to 2007. A marked decrease in the number of cases in the Czech Republic, Germany and the United Kingdom in 2008 accounted for 65% of the reduction. Children under the age of five had the highest notification rate (105 cases per 100,000 population). Other age groups varied between circa 30 to 47 cases per 100,000 population (fig. 2.3).



Source: All MSs except Greece, Latvia and Portugal. (N= 189,293).

Fig. 2.3 Age-specific distribution of the notification rate of reported confirmed cases of human campylobacteriosis per 100,000 population, TESSy data for reporting MSs, 2008 (EFSA, 2010).

Highest numbers and notification rates of *Campylobacter* cases in humans were reported during the summer months and early autumn, from June to September (fig. 2.4). No remarkable differences were detected in the distribution of confirmed cases occurring per month between northern and southern European countries.

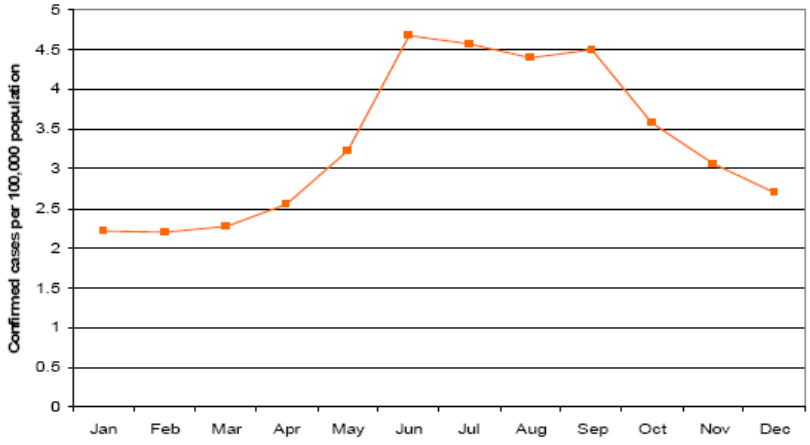


Fig. 2.4 Notification rate of reported confirmed campylobacteriosis cases in humans per 100,000 population by month, TESSy data for reporting MSs, 2008 (EFSA, 2010).

2.1.2 Foodstuffs

Broiler meat was the most frequently sampled food category in 2008, even though the number of reporting MSs was lower than in previous years, when the results from the EU-wide baseline survey are excluded. Due to fewer reporting MSs no trend analysis was performed on *Campylobacter*. The reported

occurrence of *Campylobacter* was generally at the same high level as in previous years in broiler meat, on average 30.1% of fresh broiler meat units tested positive for *Campylobacter*.
In fresh turkey meat and meat from other poultry species, the average positive findings were at similar levels as in previous years; 10.1% and 21.9%, respectively. In samples of fresh pig meat and bovine meat, *Campylobacter* was detected less frequently, at levels of 0.5% and 0.3%, respectively. In other foodstuffs *Campylobacter* was detected only occasionally.

2.1.3 Animals

In 2008, similar to previous years, the majority of data on *Campylobacter* in animals was from investigations of broilers, but data from pigs and cattle were also reported. The average prevalence of *Campylobacter* positive broiler flocks was 24.7% ranging from 6.5% to 79.0% in MSs. The lowest prevalence in broiler flocks was reported by Finland and Sweden.
For pig and cattle herds only three MSs provided data, however the prevalence in reporting MSs was generally high for pig herds (37.3-67.8%) and cattle herds (0-61.3%), which is similar to findings in previous years. The contamination of *Campylobacter* in pig and bovine meat typically decreased sharply following slaughter and remained low in meat at slaughterhouses and at retail (EFSA, 2010).

As in previous years, *Campylobacter* prevalence in live poultry and pig populations was generally at very high levels in MSs. However, lower prevalence in broiler flocks was once again reported by some Nordic countries which may imply that there are tools available to reduce *Campylobacter* colonization of broiler flocks. *Campylobacter* was also regularly detected in cattle but the prevalence was somewhat lower compared to levels in broilers and pigs. In addition, *Campylobacter* was present in other investigated animal species but not in equally high levels. Even though a high *Campylobacter* prevalence was observed in cattle and pigs, a strong decrease during the slaughter was observed in a similar manner than in previous years.
Among animal samples tested positive for *Campylobacter*, only about half of the isolates from broilers were speciated (51.7%), while speciation was more common for isolates from pigs (91.5%) and cattle (90.3%). Nevertheless, reported data indicate that *C. jejuni* was the most commonly isolated species in broilers (37.6%) and cattle (83.0%) (fig. 2.5)

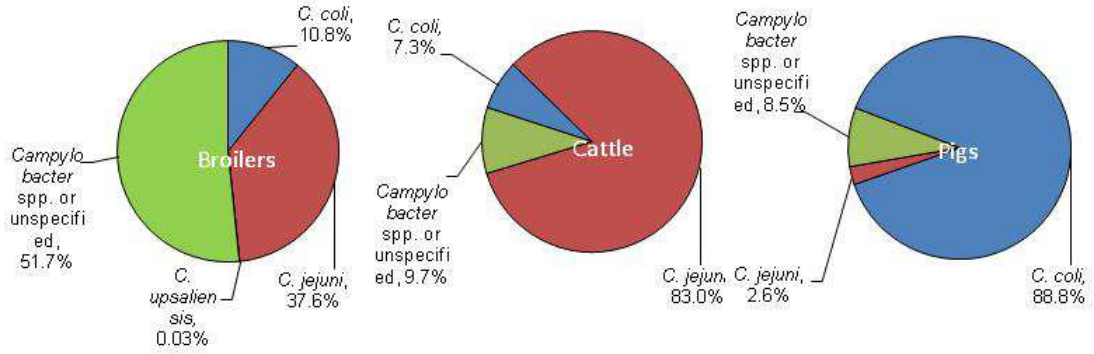


Fig. 2.5 Species distribution of positive samples isolated from broilers, cattle and pigs, 2008 (EFSA, 2010).

The numbers of *Campylobacter*-positive poultry flock are generally high, but vary by regions, seasons, and the production types (conventional, free-range and organic, etc.). It appears that the prevalence of *Campylobacter* is lower in Scandinavian countries than in other European countries, North America, and developing countries. Many prevalence studies have been conducted in Europe and the United States, which reported *Campylobacter*-positive flocks ranging from 3% to 97%. Despite the fact that the majority of on-farm surveys were conducted with broiler chickens, breeder flocks and laying hens are also commonly infected by *Campylobacter*. Seasonal variations were observed in the prevalence of *Campylobacter* flocks with a peak in worm months. The exact reason(s) for this seasonal variation is unknown, but it is proposed that the peaking prevalence of *Campylobacter* in warm months is due to increased fly population and fly mediated transmission (direct evidence is still needed to prove that flies are an important vector) (Zhang, 2008).

2.1.4 Fresh poultry meat

The occurrence of *Campylobacter* in fresh broiler meat sampled at slaughter, processing and at retail has been evaluated from 2006 to 2008 by EFSA. In 2008, as in previous years, the proportions of *Campylobacter* positive broiler meat samples varied widely between MSs (from 3.0% to 86.2%), and six out of ten MSs recorded high or very high levels (>20%) of positive samples.

The data reported in 2008 revealed a large variation in proportions of positive samples at slaughterhouse from 14.7% in Denmark to 86.2% in Spain. In Denmark, a higher proportion of positive samples was reported in 2008 compared to previous years because samples were collected during the high prevalence period. At retail, the proportion of positive poultry meat samples ranged from 8.0% in Austria to 74.6% in Slovenia. The Austrian proportion of 8.0% indicated a decrease compared to 2007 where the proportion of positive samples was 62.6%. Some decrease in the proportion of positive samples was also seen for Denmark, Germany and Spain. All other reporting countries reported an increase in the proportion of positive samples at retail.

The overall *Campylobacter* species distribution in fresh broiler meat at Community level is presented in fig. 2.6 *C. jejuni* accounted for approximately twice as many isolates as *C. coli*. Unfortunately, a high proportion of the *Campylobacter* isolates was reported only as *Campylobacter* spp.; only seven of 13 MSs reporting data on *Campylobacter* in broiler meat provided information at species level. Four MSs reported *C. jejuni* as the predominant species (more than 70% of isolates) in fresh broiler meat, while *C. coli* was reported as the predominant species (more than 54%) in three MSs (EFSA, 2010).

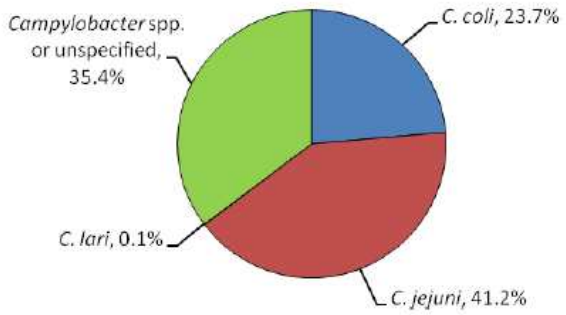


Fig. 2.6 Species distribution of *Campylobacter* isolates from fresh broiler meat, 2008 (EFSA, 2010).

2.2 Campylobacters and *C. jejuni*

2.2.1 Physiology, characteristics and human transmission

The taxonomy has changed considerably over the years and could change in the future, but to date the family *Campylobacteriaceae* includes the genera *Campylobacter*, *Arcobac ter*, and *Sulfurospirillium* and the generically misclassified *Bacteroides ureolyticus*. In regard to the genus *Campylobacter*, there are 14 species, and of these species, several are considered pathogenic to humans, causing enteric and extraintestinal illnesses. *Campylobacter* species are gram-negative, microaerophilic, non-spore-forming organisms with curved or small spiral-shaped cells that have characteristic rapid, darting, reciprocating motility and can occur in short or long chains. They range in width from 0.2 to 0.9 µm, and in length from 0.5 to 5 µm, and most species have an optimum temperature range for growth of 30 to 37°C, except for the thermophilic *Campylobacter* spp., which grow optimally at 42°C . The cells can form spherical or coccoid bodies as cultures age, and it has been postulated that certain species can have the characteristics of a viable, but not culturable state.

Campylobacter spp. have a chemoorganotrophic metabolism, and energy is derived from amino acids or tricarboxylic acid cycle intermediates due to their inability to oxidize or ferment carbohydrates. The majority of *Campylobacter* spp. reduce nitrate and nitrite. *Campylobacter* spp. have typical biochemical characteristics, which include the reduction of fumarate to succinate; a negative reaction to methyl red, acetone, and indole production; negative hippurate hydrolysis (except for most *C. jejuni* strains); and positivity for oxidase activity. *Campylobacter* spp. can be either catalase positive or negative. Broadly speaking, catalase-positive *Campylobacter* spp. are most often associated with human disease, but not in all cases (Cox *et al*., 2010).

Avian species, especially domestic poultry, are frequently infected with the members of thermophilic *Campylobacter* (*C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*), primarily *Campylobacter jejuni* and *Campylobacter coli*.

As enteric organisms, *C. jejuni* and *C. coli* are well adapted to the avian host and reside in the intestinal tract of birds. Despite extensive colonization, *Campylobacter* infections produce little or no clinical diseases in poultry.

Although thermophilic campylobacters are not significant pathogens for poultry, they are of importance to food safety and public health, with *C. jejuni* being responsible for the majority of human campylobacteriosis, followed by *C. coli* and rarely by *C. lari*. *Campylobacter* has now emerged as a leading bacterial cause of food-borne gastroenteritis in humans around the world (Zhang, 2008).

Transmission of *Campylobacter* spp. to humans generally occurs by either ingestion of contaminated food or water or by direct contact with faecal material from infected animals or persons. In humans, there are two types of illness associated with *Campylobacter* infections, and they are intestinal and extraintestinal infections. Two types of diarrhoea are usually observed with campylobacteriosis: (i) an inflammatory diarrhoea, with slimy, bloody stools containing leukocytes and fever and (ii) noninflammatory diarrhoea, with watery stools and the absence of blood and leukocytes. In some cases, intense abdominal pain, headaches, cramping, and vomiting can occur. Serious complication, such as Reiter's syndrome, Guillain-Barré syndrome (GBS), osteomyelitis, pancreatitis, nephritis, myocarditis, cystitis, septic abortion, and bacteremia in certain cases, can arise. Although campylobacteriosis does not usually lead to death, it ahs been estimated that as many as 730 people in the United States with *Campylobacter* infections die annually, often due to secondary complications. In the vast majority of cases, campylobacteriosis is mainly a self-limiting bacterial gastroenteritis, and recovery is completed in approximately 8 days, either

spontaneously or after appropriate antimicrobial therapy. However, in some instances symptoms can persist longer than 2 weeks. The population that is most susceptible to illness includes children less than 1 year of age, young adults aged 15 to 25 years, and immunosuppressed individuals.

GBS occurs in approximately 1 out of 1,000 cases. GBS cases are associated with nerve roots, causing mononuclear infiltration of peripheral nerves, and this eventually leads to primary axonal degeneration or demyelination. Molecular mimicry is believed to be the cause of GBS because a few peripheral nerves of the human neurological system share molecules similar to those of antigens on the surface of *C. jejuni* cells. Since *C. jejuni* contains a lipopolysaccharide structure (LPS) attached to the outer membrane, the core oligosaccharides of its LPS contains ganglioside-like structure, which are similar to certain human ganglioside. Upon exposure to *C. jejuni*, the immune system produces antibodies against the LPS structure as an attempt to fight the infection. Due to the similarity, antibodies attack the gangliosides on the neuromuscular junction, contributing to the appearance of the neurological symptoms. (Cox *et al*., 2010).

2.2.2 Incubation Period

Birds can be readily infected by *Campylobacter* naturally or experimentally; however, the infection usually does not cause clinical diseases, and *Campylobacter*-associated diarrhea in poultry is a rare event. Experimental studies demonstrated that colonization could occur as early as one day after inoculation. The minimal infective dose to establish colonization in day-old chicks was shown to be as low as 2 cfu, although other studies indicated higher infectious doses. Once *Campylobacter* colonization is established, it can persist in the intestinal tract for multiple weeks, but gradual decrease in the level of colonization usually occurs after a prolonged plateau period. On poultry farms, *Campylobacter* is rarely detected in birds of less than 2-3 weeks of age. The reason for this lack of infection in young birds is unclear and may be related to multiple factors including the presence of maternally-derived antibodies or differences in environmental or host-related factors. Once a flock is infected, *Campylobacter* spreads rapidly within the flock, leading to colonization of the majority of the birds within a few days. Despite the fact that *Campylobacter* infection rarely occurs in young flocks on poultry farms, newly hatched chickens can be readily infected experimentally with *Campylobacter*.

2.2.3 Clinical Signs

Campylobacter infections in poultry usually produce no clinical signs of disease under natural conditions. Some studies reported that experimental challenge of young chickens with *Campylobacter* can induce clinical diseases including watery/mucoid/bloody diarrhea, weight loss, or even mortality. Sanyal *et al*. observed watery/mucoid diarrhea in 81% of 36 to 72-hr-old birds 5 days after inoculation with *C. jejuni*, and also found that the Starbro strain of chickens was more likely to develop diarrhea than the white leghorns strain.

Campylobacter infection in commercial broilers of less than 2 weeks of age (a rare event) was found to be associated with diarrhea, decreased weight gain, and excess mortality. Despite isolated reports, many other studies did not observe any clinical diseases associated with *Campylobacter* infections in poultry.

2.2.4 Pathogenesis

Birds become infected with campylobacters via the fecal-oral route. As enteric organisms, *Campylobacter* spp. are able to survive the harsh conditions in the stomach (gizzard) as well as in small intestine and eventually reach the lower intestines, where the organisms colonize in cecal and cloacal crypts. To a lesser extent, the organism can also be recovered from the small intestines and the gizzard, and frequently

from the liver, spleen, blood and gallbladder. Several distinct features are associated with colonization of *Campylobacter* in chickens. First, it appears that *Campylobacter* does not adhere directly to intestinal epithelial cells, but mainly locates in the mucous layer of the crypts. Second, usually no gross or microscopic lesions are induced in the chickens. Third, invasion of the intestinal epithelium rarely occurs with *Campylobacter*. Even when the invasion of internal organs occurs in some cases, no clinical signs of illness are observed. Once a broiler chicken becomes infected, large numbers of the organism (up to 10^9 cfu/g faeces) can be detected in caeca and excreted in faeces for a prolonged period (Zhang, 2008).
In the chicken, as in mammals, it has been demonstrated that the mucous secretions are not only a source of nutrients for the resident microbiota, but are also a mechanism that the host microbiota may use to inhibit other bacteria. In spite of their similar function, chicken
mucins differ in structure, folding, glycosylation and charge compared to human mucins. Additionally, when compared to human mucus, chicken intestinal mucus was able to attenuate *Campylobacter jejuni* virulence which is of interest given the role of *Campylobacter* as a food-borne pathogen. Antimicrobial proteins are present at the intestinal epithelial surface and serve as another innate defense mechanism. These molecules are effective at killing a wide variety of bacteria, fungi, protozoa and viruses. One category of antimicrobial peptides named defensins are highly conserved evolutionarily and are present in mammals, birds, invertebrates and plants. Defensins are cationic proteins that function by permeabilizing the cell membrane thereby causing cell lysis. Three subfamilies of defensins exist, α-, β- and θ-defensins. To date, 13 avian β-defensins, also called gallinacins or Gal have been described. Avian macrophages, epithelial cells and heterophils have all been shown to be capable of producing gallinacins (Brisbin *et al*., 2008a).

It is likely that many genetic factors contribute to the colonization of *Campylobacter* in poultry. Published studies using genetically defined mutants revealed that flagella, DnaJ (heat shock protein), CiaB (*Campylobacter* invasion antigen B), PldA (phospholipase A), CadF (*Campylobacter* adhesion to fibronectin), CmeABC (multidrug efflux pump), MCP (a methyl-accepting chemotaxis protein), RpoN (sigma factor), the Kps locus (capsule biosynthesis proteins), the Pgl locus (protein glycosilation system), SOD (superoxide dismutase), Fur (ferric uptake regulator), and CbrR (a response regulator) all contributed to *Campylobacter* colonization in chickens. *Campylobacter jejuni* produces a cytolethal distending toxin (CDT), which is suggested to be a potential virulence factor of *Campylobacter*. Although most *Campylobacter* isolates from poultry harbour the *cdt* genes and produce toxic activity *in vitro*, the role of CDT in colonization of chickens has not been established (Zhang, 2008).

2.2.5 Immunity

Despite the commensal relationship between *Campylobacter* and the avian host, the infection indeed elicits both systemic and mucosal humoral responses. Following experimental infection of day-old chickens via oral gavage, production of *Campylobacter*-specific IgM and IgA antibodies in serum reached significant levels within 1-2 weeks of infection and peaked at weeks 4-6 postinfection, followed by gradual decreases as bird age. In contrast, detectable levels of IgG responses developed later than IgM and IgA responses, peaked at 8-9 weeks of the infection, and persisted for a longer period. Naturally occurring *Campylobacter* colonization in chickens also elicit overt immune responses, and anti-*Campylobacter* antibodies readily transfer from hens to their progenies as maternally-derived. Maternal antibody plays a partial role in protecting young chickens from infection by *Campylobacter*. A wide variety of *Campylobacter* antigens are recognized by chicken sera. There is a trend that with the development of specific anti-*Campylobacter* antibodies, the level of *Campylobacter* colonization

diminishes and some infected chickens eventually clear the infection. However the nature of protective immunity has not been elucidated, and it is unknown if humoral immunity or cellular immunity (or both) contributes to the clearance of *Campylobacter* from the host. To date there are no reports documenting cellular immune responses induced by *Campylobacter* infection in poultry (Zhang, 2008).

2.2.6 Sources and incidence in the environment and foods

Unlike many other enteric pathogens, *Campylobacter* spp. have limited spread from host to host. *Campylobacter* spp. may not be recovered by conventional cultural methods outside of the host if exposed to dry conditions or atmospheric oxygen levels for extended periods of time. *Campylobacter* enteritis can be classified as a zoonosis, because animals are the main reservoir of these organisms. *Campylobacter* spp. exist as commensals in many wild and domestic animals. This presents a risk to food safety due to the contamination of carcasses at slaughter and other foodstuffs by cross-contamination when raw or undercooked meat is handled. Contamination with this pathogen can occur at numerous stages along the food chain. This includes, but is not limited to, production, processes, distribution, handling, and preparation. *Campylobacter* spp. are fastidious organism that are capable of existing in a broad range of environments and have been sporadically recovered from rivers, costal waters, shellfish, and vegetables, but routinely recovered from sheep, cattle, swine, rodents and avian species. In poultry and cattle, *C. jejuni* is the predominant species. The majority of *Campylobacter* infections are sporadic, and outbreaks are rare but have been traced back to contaminated water, raw milk, poultry, beef, eggs, fruits, and contact with farm animals and pets. Generally speaking, the primary source of contamination of the environment and foods is believed to be from animal faeces.

Avian species, particularly poultry, are the most common host for *Campylobacter* spp.; therefore poultry is considered the main source of human illness. Studies have shown that as much as 70% of human illnesses due to *Campylobacter* spp. are caused by the consumption or handling of raw or undercooked poultry or poultry products. Increased attention has been given to reducing the level of *Campylobacter* spp. in poultry pre- and postharvest to reduce the level and incidence of raw product contamination. The ecology of *Campylobacter* spp. in poultry is not fully elucidated. Numerous studies are being conducted to determine when and how *Campylobacter* spp. gain entry into poultry flocks so that more effective intervention strategies can be employed.

Campylobacter spp. colonize the mucus layer of the intestinal tract but have been recovered from numerous tissues and organs within the bird, suggesting it is not limited to the digestive tract. In addition to the digestive tract, *Campylobacter* spp. have been isolated from the circulating blood, thymus, spleen, liver, gallbladder, unabsorbed yolk sac, ovarian follicles, and reproductive tracts of commercial poultry. In regard to the digestive tract, levels up to 10^9 CFU/g of faecal content have been shown. Two modes of transmission of *Campylobacter* into poultry flocks occur and they are horizontal and vertical transmission.

It has been shown that if a single bird in a flock is colonized, then the spread to adjacent rearing mates is rapid, and within a week *Campylobacter* prevalence in the flock can reach 100%.

The prevalence of *Campylobacter* contamination of carcasses and poultry products can vary greatly, depending on the sensitivity of the cultural procedures utilized and by the point along the process chain at which sampling is being conducted. The type of methodology employed significantly affects prevalence rates of *Campylobacter* spp. from carcasses at the final stages of processing. For example, is a less sensitive method is utilized, such as direct plating onto selective agar. Which may exclude sublethally injured cells, then the number of samples detected as positive could be greatly reduced. Including both direct plating and enrichment often allows the best probability for recovery. A question often asked is

whether the injured or stressed cells could have the ability to infect humans and cause illness. This is one reason why studies on the incidence of *Campylobacter* in poultry processing plants vary, and it is critical to consider the cultural procedures utilized and the impact those choices have on sensitivity to recover or detect the organism.

A significant high prevalence rate of *Campylobacter* spp. contamination can be found in retail poultry products and is often directly related to the prevalence rate at the farm. The reported prevalence rate varies, but on average greater than 70% of the birds are *Campylobacter* positive. In a study on supermarkets, *Campylobacter* spp. were isolated from 82%, 82% and 71% of whole chickens, breast with skin attached, and pieces respectively.

2.2.7 Survival and growth in food

Cross-contamination is a major factor that contributes to human illness. Even though *Campylobacter* spp. are sensitive to drying, high oxygen concentration, and low pH (less than or equal to 4.7), they are still one of the biggest causes of gastroenteritis.

The decimal reduction time for *Campylobacter* spp. varies, depending on the type of food product, but survival kinetics generally follow a rapid decline in numbers, which is followed by a slower rate of inactivation. This may explain the high survival rate of *Campylobacter* spp. on poultry carcasses due to the high levels of the organisms in the bird's digestive tract at the time of processing.

When environment become unfavourable for growth, *C. jejuni*, it is postulated, can enter into a viable but nonculturable (VBNC) state. The cells are metabolically active and show signs of respiratory activity but are unable to be cultured through conventional methodology procedures. The VBNC state was first described by Rollins and Colwell (1986), who postulated that it could play a role in human infection and illness. The VBNC state arises from exposure to sublethal adverse environmental conditions, and recovery occurs by passage of the organism to a susceptible host. The significance of the VBNC state remains unclear and controversial, but as the understanding of these phenomenon unfolds, this could shed light on how *Campylobacter* spp. survive and persist in certain food commodities and go undetected in dry environments.

Poultry meat, which is frequently contaminated with the organism, may be responsible for as much as 70% of sporadic campylobacteriosis. Contamination is thought to originate from the intestinal tract of primarily avian species (mainly poultry) and then spread to the meat during transport and processing, thought it has also been demonstrated that broiler crops, particularly after a feed withdrawal prior to transport to the processing facility, may harbour large numbers of *Campylobacter* bacteria. Crops burst more often than cecal pouches or other parts of the gut and can contaminate previously *Campylobacter*-free carcasses. As birds enter the plant, levels of *Campylobacter* in the intestinal tract can be as high as 10^7 CFU/g cecal contents, and when whole carcasses with feathers are rinsed, 10^6 CFU/ml of rinse can be recovered. External contamination often increase during transport from grow-out houses to the processing plant. Generally, *Campylobacter* counts decrease in the scalding tank, increase during removal of feathers (picking), and are at their highest immediately following evisceration.

Many continue to say that there is no evidence to suggest egg transmission and hatchery contamination when the evidence is not only present, but in recent years overwhelming. It has never been said that vertical transmission is the only source of contamination of a flock, but it is definitely a source, and additional evidence continues to mount. If the research community continues to ignore published facts, then this source will always be present and the level of contamination of commercial poultry will never be eliminated in reference to *Campylobacter* (Cox et al., 2010)

Chapter 3. Alternatives to antibiotics: Probiotics Prebiotics and Synbiotics

3.1 Probiotics

Many definitions have been proposed for the term probiotic. The most recent one is "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). In this definition it is implicit that a health effect must be demonstrated for the probiotics.

The beneficial modes of action include: regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function (Salminen *et al*., 1996), expression of bacteriocins (Mazmanian *et al*., 2008), enzymatic activity inducing absorption and nutrition (Hooper, 2002; Timmerman *et al*., 2005), immunomodulatory effects (Salzman *et al*., 2003), inhibition of procarcinogenic enzymes and interference with the ability of pathogens to colonize and infect the mucosa (Gill, 2003). The expected health-promoting characteristics and safety criteria of probiotics are shown in Table 3.1.

Table 3.1. Expected characteristics and safety criteria of probiotics.

Non toxic and non pathogenic
Accurate taxonomic identification
Normal inhabitant of the targeted species
Survival, colonization and being metabolically active in the targeted site, which implies:
resistance to gastric juice and bile
persistence in the GIT
adhesion to epithelium or mucus
competition with the resident microbiota
Production of antimicrobial substances
Antagonism towards pathogenic bacteria
Modulation of immune responses
Ability to exert at least one scientifically-supported health promoting properties
Genetic stability
Amenability of the strain and stability of the desired characteristics during processing, storage and delivery
Viability at high populations
Desirable organoleptic and technological properties when included in industrial processes

3.1.1 Regulatory considerations

Significant progress in legislation for the safety evaluation of probiotics, has been made in USA, Canada, and Europe (EFSA, 2005a; HC, 2006; FAO/WHO, 2002); however, no unique standard is available. In the USA, microorganisms considered safe for human consumption are awarded GRAS status (Generally Regarded As Safe) by the Food and Drug Administration. In Europe, the European Food Safety Authority (EFSA) has introduced the concept of Qualified Presumption of Safety (QPS) similar in purpose to the GRAS approach. The QPS concept provides a generic assessment system for use within EFSA that in principle can be applied to all requests received for the safety assessments of microorganisms deliberately introduced into the food chain (EFSA, 2005b). EFSA has published a list of microorganism, which possess a known historical safety, proposed for QPS status (EFSA, 2007a).This list does not include *Enterococcus* species, even if *E. faecium* shows a long history of apparent safe use in food or feed. The main reason is due to the possibility of carrying transmissible resistance to antibiotics by *Enterococcus* spp. (EFSA, 2007a).

A list of the probiotic species for studies or application in animal feed is showed in table 3.2; these data derived from extensive literature and internet search of commercial products.
Lactobacillus, *Enterococcus*, *Bacillus* and *Saccharomyces* are actually the most used probiotics in livestock and poultry.
Many studies indicate that the organisms cited on the labels of certain probiotic products are not actually contained within the product (Huff, 2004; Mattarelli *et al*., 2002; Wannaprasat *et al*., 2009). It is necessary to indicate clearly on the label of the products the name of the exact taxonomic species of probiotics utilized in order to avoid confusion and misidentification.
Regulatory bodies should carefully monitor and control these indications. Another important point is the viability and consequently derived concentrations of viable bacteria of probiotic preparations at the moment of the administration to the animals. It is fundamental to study proper formulations which will allow the maximum viability of the bacteria species utilized. The shelf life of these products also needs to be advertised to the final user.

3.1.2 Efficacy of probiotics

The use of probiotics in animal feeding could be enhanced by a preliminary in vitro screening: anti-microbial activity, survival in the GIT, adhesive studies and antibiotic susceptibility are among the main probiotic properties that should be analysed to assess functionality and safety. The advanced molecular methods, *e.g*. microarrays, will improve the detection of these multiple characteristics allowing also the analysis, at genomic level, of phenotypic and genetic properties useful for industrial production.
Probiotic activity could be related to genera, species, or strains. An approach in probiotic application could be the use of mixtures of strains belonging to different genera or species (Timmerman *et al*., 2004).
Timing and duration of the administration of probiotics may be a factor affecting efficacy. Another determinant may be the age of the animals; during early life, colonization patterns are instable and newborn animals are then susceptible to environmental pathogens. Initial colonization is of great importance to the host because the bacteria can modulate expression of genes in epithelial cells thus creating a favourable habitat for themselves (Siggers *et al*., 2007).

Table 3.2 List of probiotics applied or studied for application in animal feed.

Genus	**Species[(a)]**
Bacteria	
Bifidobacterium	*B. animalis (B animalis* subsp. *animalis)* *B. lactis (B. lactis* subsp. *lactis)* *B. longum (B. longum* subsp. *longum)* *B. pseudolongum (B. pseudolongum* subsp. *pseudolongum)* *B. thermophilum*
Enterococcus	*E. faecium* *E. faecalis*
Lactobacillus	*L. acidophilus* *L. amylovorus* *L. brevis* *L. casei(L. casei* subsp. *casei)* *L. crispatus* *L. farmicinis* *L. fermentum* *L. murinus* *L. plantarum (L. plantarum subsp. plantarum)*

	L. reuteri *L. rhamnosus* *L. salivarius* *L. sobrius (L. amylovorus)*
Lactococcus	*L. lactis* subsp. *cremoris* *L. lactis* subsp. *lactis*
Leuconostoc	*L. citreum* *L. lactis* *L. mesenteroides*
Pediococcus	*P. acidilactici* *P. pentosaceus* subsp. *pentosaceous*
Propionibacterium	*P. freudenreichii*
Streptococcus	*S. cremoris* *S. faecalis* *S. faecium* *S. infantarius* *S. salivarius subsp. salivarius* *S. salivarius subsp. thermophilum*
Bacillus	*B. cereus var. toyoi* *B. licheniformis* *B. subtilis*
Yeasts	
Saccharomyces	*S. cerevisiae* *S. boulardii (S. cerevisiae)* *S. pastorianus* (synonym of *Saccharomyces carlsbergensis)*
Kluyveromyces	*K. fragilis*
Fungi	
Aspergyllus	*A. orizae* *A. niger*

[(a)]In bracket valid taxonomic name.

3.1.3 Genus *Bifidobacterium*

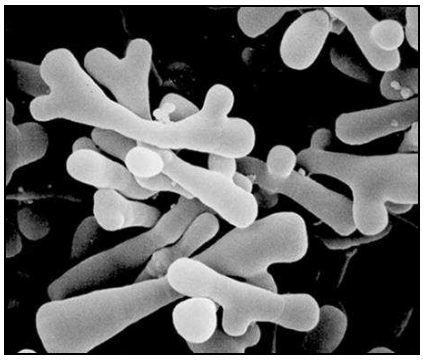


Fig. 3.1 Scanning electron micrographs of *Bifidobacterium* spp.

In 1900, Tissier observed and isolated in the feces of breast-fed infants a bacterium with a strange and characteristic Y shape (fig. 3.1) and called it "*Bacillus bifidus*" (Tissier, 1899). This bacterium was anaerobic, Gram-positive and did not produce gas during its growth (Tissier, 1899). He proposed its inclusion in the family *Lactobacillaceae*. For a long time, bifidobacteria were included in the genus *Lactobacillus*. In the 8th edition of *Bergey's Manual of Determinative Bacteriology* bifidobacteria were classified for the first time in the genus *Bifidobacterium* and comprised eight species.
Nowadays, according to Taxonomic Outline of the Prokaryotes, the genus *Bifidobacterium* belongs to the phylum *Actinobacteria*, class *Actinobacteria*, sub-class *Actinobacteridae*, order *Bifidobacteriales*, family *Bifidobacteriaceae*. The other genera belonging to this family are: *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia* and *Scardovia*.

Bifidobacterium longum is the most common species in the human gut and has been isolated both in infants and adults (Biavati *et al*., 2000). A strong genetic link has been outlined between *B. longum* and *B. infantis* with DNA-DNA homologies values in the 65%-80% range (Scardovi *et al*., 1986). Moreover, a group of strains isolated in calves with a 75%-80% homology with both *B. longum* and *B. infantis*, has been described. Researchers have concluded that *B. infantis* and *B. longum* can form a single species, a "continuum", the middle position of which is taken by the strains isolated in calves. Recently (Mattarelli *et al*., 2008), with the aid of different genotypic techniques, Mattarelli and co-workers proposed a new classification of 3 biotypes of *B. longum* in 3 subspecies: *B. longum* subsp. *longum* subsp. nov., *B. longum* subsp. *infantis* comb. nov. and *B. longum* subsp. *suis* comb. nov.

Physiology and metabolism

Bifidobacteria are Gram-positive polymorphic branched rods that occur singly, in chains or in clumps. They are non-spore forming, non-motile and non-filamentous. They are anaerobic : their sensitivity to oxygen changes in relation to the species and the different strains of each species. Bifidobacteria are chemoorganotrophs, having a fermentative type of metabolism. They produce acid but not gas from a variety of carbohydrates. They are catalase negative (with some exceptions). Their genome GC content varies from 42 mol% to 62 mol% (Biavati and Mattarelli, 2001).
The optimum temperature for growth is 37-41 °C, while no growth occurs below 20 °C and above 46 °C. Growth at 45 °C seems to discriminate between animal and human strains. Bifidobacteria are acid-tolerant microorganisms.

The optimum pH is between 6.5 and 7.0 and no growth is recorded below pH 4.5. Bifidobacteria are in fact acid tolerant but they are not acidophilic microorganisms.
Bifidobacterium produces lactic and acetic acid from glucose.
The global equation is:

2 glucose + 5 ADP + 5 P → 3 acetate + 2 lactate + 5 ATP

This peculiar metabolic pathway is called "fructose-6-phosphate shunt" or "bifidus shunt". The key enzyme of this pathway is fructose-6-phosphate-phosphoketolase, which is considered a taxonomic character for the identification on the genus level (Biavati and Mattarelli, 2001). Different species produce variable amounts of acetate, lactate ethanol and formate under the same conditions. The bifidobacteria utilize a great variety of mono- and disaccharides as carbon sources and are able to metabolize also complex carbohydrates that are normally not digested in the small intestine. This feature should give an ecological advantage to colonizers of the intestinal environment where complex carbohydrates, such as mucin, are present either because they are produced by the epithelium of the host or because they are introduced through diet.

Bifidobacteria in animals and probiotic application

Studies on the intestinal microbiota, carried out mostly on domestic animals, have revealed a complex microbiota: *Bacteroides*, eubacteria, anaerobic lactobacilli, anaerobic Gram-positive cocci, spirillaceae and often bifidobacteria. Almost all chickens, dogs, pigs, rats and hamsters presented bifidobacteria, although in a smaller quantity than lactobacilli. Mice, rabbits and horses rarely displayed bifidobacteria, and cats and minks never had them. Many factors influence the composition of bifidobacteria microbiota in animals: the age, the species and the diet of the host.
Some species apparently are host-specific: *B. magnum* and *B. cuniculi* have only been found in rabbit faecal samples, *B. pullorum* and *B. gallinarum* only in the intestine of chickens and *B. suis* only in piglet faeces (Matteuzzi *et al*., 1971; fig. 3.2).

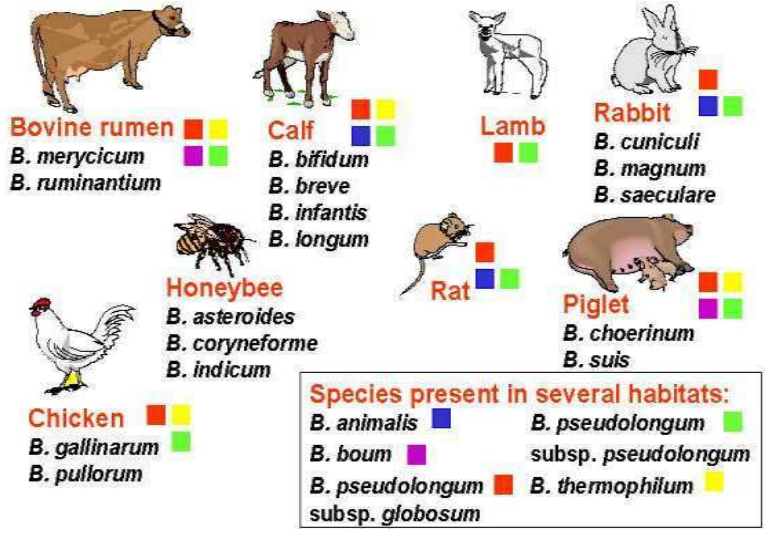


Fig. 3.2. *Bifidobacterium* species found in animals.

In the intestinal tracts of animals and humans bifidobacteria are considered one of the key genus. Their presence in high number is associated to good health status of the host. There is a general believe that *Bifidobacterium* are helpful in maintaining appropriate balance of the microbiota in the GIT reducing the risk of pathogen infection.
Several species are host specific (Biavati and Mattarelli, 2006). Bifidobacteria possess very promising probiotics properties; they are frequently used in food and pharmaceutical preparations and their application in animal feeding is increasing. Due to the long history of safe use of bifidobacteria, many species are proposed for QPS status.

3.1.4 Genus *Lactobacillus*

The genus *Lactobacillus* (fig. 3.3) belongs to the lactic acid bacteria (LAB), a group of Gram-positive, catalase-negative bacterial species which are able to produce lactic acid as the main end-product of the carbohydrate fermentation.

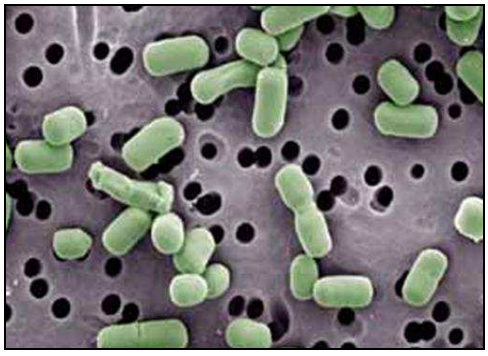


Fig. 3.3 *Lactobacillus brevis*, image from SEM

Lactobacillus is a well-characterized genus belonging to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae*.
They are gram-positive non-sporeforming rods that are catalase negative, usually nonmotile, or motile by peritrichous flagella.
Their growth temperature ranges from 20 to 53°C, the optimum being between 30 and 40°C and their pH ranges from 5.5 to 6.2. They are aerotolerant anaerobes, and the microaerophilic atmosphere with 5 to 10% CO2. enhance their growth. Glucose is used either homofermentatively or heterofermentatively, and they have complex nutritional requirements for amino acids, carbohydrates, peptides, nucleic acid derivatives, vitamins, salts, fatty acids, or fatty acid esters.
Lactobacillus includes 113 recognized species and 16 subspecies and the type species is *Lactobacillus delbrueckii* Leichmann 1896 (Beijerinck, 1901). The genus *Lactobacillus* is very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties. This heterogeneity can be seen in the range of moles percentage G+C of the genomic DNA of species included in the genus, which ranges range being 32 to 54%.

Metabolism and nutritional requirements

Lactobacilli possess efficient carbohydrate fermentation pathways coupled to substrate level phosphorylation. A second substrate level phosphorylation site is the conversion of carbamyl phosphate to CO_2 and NH_3.

Two main sugar fermentation pathways can be identified among lactobacilli: the Embden-Meyerhof pathway which results almost exclusively in lactic acid as an end product (homolactic fermentation) and the 6-phosphogluconate pathway producing significant amounts of other end products such as ethanol, CO_2, acetate, formate, or succinate, in addition to lactic acid (heterolactic fermentation). Each species has its own exact nutritional requirements which are often strain specific. In general, they require carbohydrates as energy and carbon sources as well as nucleotides, amino acids, and vitamins. Thiamine is necessary only for the growth of the heterofermentative lactobacilli, while pantothenic acid and nicotinic acid are required by all species. The requirements for riboflavin, pyridoxal phosphate, folic acid and p-aminobenzoic acid vary widely among the various species, riboflavin being the most frequently required, whereas biotin and vitamin B_{12} are requested by only a few strains.

Probiotic action and application

Lactobacillus spp. are among the most frequent and better characterized microorganisms used as a probiotic. Important considerations in the choice of a probiotic include safety, functional aspects and technological aspects (Donohue *et al*. 1998).
Many of the species are significant constituents of the normal gut microbiota of humans and animals, and their occurrence and number are host dependent. Several species of the genus are intentionally introduced in the food chains, being involved in a range of food and feed fermentations, and applied as probiotics in humans and animals (Hammes and Hertel, 2007). However, an increasing number of reports stated that these microorganisms might occasionally be involved in human diseases, where *L. casei* and *L. rhamnosus* are the most common (Vesterlund *et al*., 2007). No report can be found on safety concerns related to lactobacilli in animals. Due to the long history of safe use, a list of species has been proposed for QPS status (EFSA, 2007a).

3.1.5 Competitive exclusion

Competitive exclusion (CE), also indicated as the Nurmi concept, has its origin on the finding that the newly hatched chicken could be protected against Salmonella colonization of the gut by dosing it with a suspension of gut content prepared from healthy adult chickens (Nurmi and Rantala, 1973). The introduction of CE bacteria from the gut content should occur early in life, such that the CE bacteria are preferentially established in the gastrointestinal system to become competitive or antagonistic to opportunistic pathogens. Because of the use of undefined preparations from the cecal or fecal material could result in the transmission of pathogens, regulatory restrictions for probiotic microorganisms (SCAN, 2000) made this kind of products difficult to be authorized. However, CE products with defined and identified microorganism have been developed and applied in animal breeding (Schneitz, 2005).

3.1.6 Microencapsulation

Microencapsulation is defined as a technology of packaging solids, liquids or gaseous materials in miniature, sealed capsules that can release their contents at controlled rates under the influences of specific conditions. A microcapsule consists of a semipermeable, spherical, thin, and strong membrane surrounding a solid/liquid core, with a diameter varying from a few microns to 1 mm. A brief description of microencapsulation techniques for encapsulation probiotic microorganisms is given in table 3.3. In a broad sense, encapsulation can be used for many applications in the food industry, including stabilizing the core material, controlling the oxidative reaction, providing sustained or controlled release (both temporal and time-controlled release), masking flavours, colours or odours, extending the shelf life and

protecting components against nutritional loss. Food-grade polymers such as alginate, chitosan, carboxymethyl cellulose (CMC), carrageenan, gelatin and pectin are mainly applied, using various microencapsulation technologies (Anal and Singh, 2007).

The maintenance of the viability and functionality of the probiotics until they reach their destination in the gut is one of the key requirements for their beneficial action. It has clearly been shown that the extreme acidic environmental conditions in the human and animal stomach can seriously decrease the number of living cells reaching the intestine. In this regard microencapsulation has been attempted to enhance the survival of probiotic bacteria during processing, storage and particularly gastric transit (Heidebach *et al*., 2009).

Tab. 3.3 Techniques and processes used for encapsulating probiotic microorganisms (Anal and Singh, 2007).

Microencapsulation techniques	**Type of materials for coating**	**Major steps in processes**
Spray-drying	Water- soluble polymers	(i) preparation of the solutions including microorganisms (ii) atomization of the feed into spray (iii) drying of spray (iv) separation of dried product form
Spray-congealing	Waxes, fatty acids, water-soluble/ insoluble polymers, monomers	(i) preparation of the solutions containing core (ii) solidification of coat by congealing the molten coating materials into non-solvent (iii) removal of non-solvent materials by sorption, extraction or evaporation techniques
Fluidized-bed coating/	Water-insoluble/soluble polymers, lipids, waxes	(i) preparation of coating solutions (ii) fluidization of core particles (iii) coating of core particles with coating solutions
Extrusion	Water- soluble/ insoluble polymers	(i) preparation of coating solution materials (ii) dispersion of core materials (iii) cooling or passing of core-coat mixtures through dehydrating liquid
Coarcevation/phase separation tech.	Water- soluble polymers	(i) core material is dispersed in a solution of coating polymer, the solvent for the polymer being the liquid manufacturing vehicle phase (ii) deposition of the coating, accomplished by controlled, physical mixing of the coating and core materials in the vehicle phase (iii) rigidifying the coating by thermal, cross-linking or desolvation techniques, to form self-sustaining microcapsules
Electrostatic method	Oppositely charged polymers/compounds	(i) mixing of core and coating materials (ii) extrusion of mixtures of core-coatingmaterials in oppositely charged solutions (iii) freeze-dry or oven-dry of microcapsules/microspheres/beads

3.2 Prebiotics

Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). For a dietary substrate to be classed as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolyzed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial commensal bacteria in the large intestine such as the bifidobacteria, (3) fermentation of the

substrate should induce beneficial luminal/systemic effects within the host (Scantlebury-Manning and Gibson, 2004). The effects of dietary fiber on upper and lower gastrointestinal tract are shown in Table 3.4. Most identified prebiotics are carbohydrates and oligosaccharides normally occurring in human and animal diet, with different molecular structures; dietary carbohydrates such as fibers, are candidate prebiotics, but most promising are non-digestible oligosaccharides (NDOs). NDOs which meet the critical point of the definition are fructo-oligosaccharides (FOS, oligofructose, inulin), galacto-oligosaccharides (GOS) or transgalacto-oligosaccharides (TOS), and lactulose; however a large number of other NDOs, to which less rigorous studies have been so far applied are glucooligosaccharides, glycooligosaccharides, lactitol, isomaltooligosaccharides, maltooligosaccharides xylo-oligosaccharides, stachyose, raffinose, and sucrose thermal oligosaccharides have also been investigated (Patterson and Burkholder, 2003). Although mannanoligosaccharides (MOS) have been used in the same manner as the prebiotics listed above, they do not selectively enrich for beneficial bacterial populations.

Investigation on the mode of action of mannanoligosaccharide pointed out that these compounds are able to bind to mannose-specific lectin of Gram-negative pathogens that express Type- 1 fimbrieae such as *Salmonella* and *E. coli* resulting in their excretion from the intestine (Baurhoo *et al*., 2007; Thomas *et al*., 2004).

Dietary modulation of the human gut flora has been carried out for many years. In humans, prebiotic addition to the diet has brought positive aspects to the gut microbial balance. The use of prebiotics in animal production, as possible alternative to antimicrobial growth promoters, has given contradictory results, while their use in the modulation of the gut microbial equilibrium is worthwhile. They contribute to the establishment of a healthier microbiota where bifidobacteria and/or lactobacilli become predominant and exert possible health-promoting effects at the expense of more harmful species.

Tab. 3.4 Intestinal functions assigned to prebiotics.

Dietary fibers and gastrointestinal functions	
Effect on upper GI tract	Resistance to digestion
	Retarded gastric emptying
	Increased oro-caecal transit time
	Reduced glucose absorption and low glycaemic index
	Hyperplasia of the small intestinal epithelium
	Stimulation of secretion of intestinal hormonal peptides
	Acting as food for colonic microbiota
	Acting as substrates for colonic fermentation
	Production of fermentation end products (mainlt SCFAs)
	Stimulation of saccharolytic fermentation
Effect on upper GI tract	Acidification of the colonic content
	Hyperplasia of the colonic epithelium
	Stimulation of secretion of colonic hormonal peptides
	Bulking effect on stool production
	Regularization of stool production (frequency and consistence)
	Acceleration of caeco-anal transit

3.2.1 FOS, fructooligosaccharides

Fructooligosaccharides are natural food ingredients commonly found in varying percentages in dietary foods. They are present in > 36.000 plant species. They are present as storage carbohydrate, together with inulin, in a number of vegetables and plants including wheat, onion, bananas, garlic and chicory. These oligosaccharides are manufactured by two different general methods, which result in slightly different end products. In the first method they are produced from the disaccharide sucrose using the transfructosylation activity of the enzyme β-fructofuranosidase (or fructosyltransferase). The second method is instead the controlled enzymatic hydrolysis of the polysaccharide inulin.

For that concern the synthesis of FOS from sucrose, the enzyme source can be divided into two classes: one is plant such as asparagus, sugar beet, onion, Jerusalem artichoke etc.; the other consists of bacterial and fungal origins such as *Aspergillus* sp., *Aureobasidium* sp., *Arthrobacter* sp., *Fusarium* sp., etc. The dual name of the enzyme implied in the synthesis, that is fructosyltransferase and fructofuranosidase, derives on the hydrolytic nature of this enzyme. The production yield of FOS using enzymes originated from plants is low and mass production of enzyme is limited by seasonal condition, therefore industrial production depends chiefly on fungal enzymes from either *Aureobasidium* sp. or *A. niger*. Moreover these enzymes are bigger and more stable than those of plants.

As for the production of TOS a high concentration of the substrate (sucrose) is required for efficient reaction. The FOS formed in this process contain between two and four β(1→2)-linked fructosyl units linked to a terminal α-D-glucose residue. These are named: 1-kestose (GF2, glucose-fructose2), 1-nystose (GF3) and 1F-fructosylnystose (GF4) (fig. 3.4).

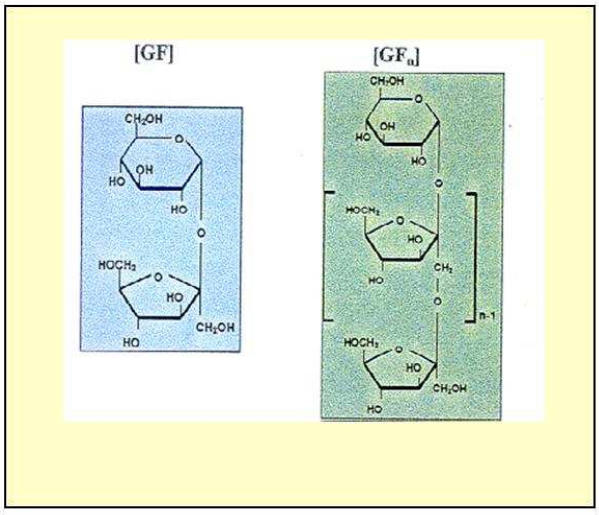


Fig. 3.4 General structure of sucrose derived FOSs.

Recent developments in industrial enzymology have made the large-scale production of FOSs by enzymatic means possible. The industrial processes for the production of FOSs can be divided into two classes: the first is the batch system using soluble enzymes and the second is the continuous one using immobilized enzymes or whole cells. The immobilized enzyme column is essentially superior to the immobilized cell column from the practical point of view, but the operational stability of the immobilized cells is higher. The sterilization procedure can be done through heat or ultraviolet, but the ultraviolet method is more favorable because colorization of the reaction products may occur from heat sterilization.

Glucose and small amounts of fructose, as well as unreacted sucrose, are removed from the oligosaccharide mixture using chromatographic procedure to produce FOS products of higher purity. However pure products are not easily available, the maximum FOS content is known to be 55-60% on a dry substance basis. The structures of FOSs synthesized in cell-free enzyme systems are essentially identical to those produced by whole cell systems.

The relative sweetness of 1-kestose, nystose and 1F-fructosylnystose to 10% sucrose solution are 31, 22 and 16%, respectively. FOSs are highly hygroscopic; it is difficult to keep the lyophilized products stable under atmospheric conditions for prolonged periods. There is a strong indication that FOSs resembles sucrose in many properties such as solubility, freezing and boiling points, crystal data, etc. (Jong, 1996).

As regards the production of fructooligosaccharides through inulin hydrolysis, the mixture formed by this process closely resembles the mixture produced by the transfructosylation process. However, not all the β(1→2)-linked fructosyl chains end with a terminal glucose. Additionally, the oligosaccharide mixture produced from inulin hydrolysis contains longer fructo-oligomer chains than that produced by the sucrose transfructosylation process.

Some manufacturers have also tried to extract the so called inulin-type fructans (or FOS) directly from plants, and the only plant that has so far been used for this purpose belongs to the *Compositae* family, *i.e.* chicory (*Cichorium intybus*). In fact native chicory inulin is a non-fractionated inulin extract from fresh roots that always contain glucose, fructose, sucrose and small oligosaccharides. However the classical industrial process involves the extraction of inulin and its hydrolysis. The roots of chicory look like small oblong-shaped sugar beets. Their inulin content is high (more than 70% of dry matter) and fairly constant from year to year. The extraction step is by diffusion in hot water, in a manner very similar to the extraction of sucrose from sugar beets. The raw extract is the refined by using technologies from the sugar and starch industries, such as ion exchangers. Then the material is evaporated and spray-dried. The subsequent step is the hydrolysis of inulin, followed eventually by spray drying. Hydrolysis is catalyzed either by exo-inulinase, by the combined action of exo- and endo-inulinases, or solely by endo-inulinase. Although the best source of these enzymes is *Kluyveromyces fragilis* that produces only an exo-inulinase, most inulin-hydrolyzing enzymes of yeast origin have both exo- and endo-inulinase activity. The partial enzymatic hydrolysis of inulin using an endo-inulinase produces, as we have already said, oligofructose that is a mixture of both GpyFn (glucose-fructose) and FpyFn (fructose-fructose) molecules in which the DP varies from 2 to 7 with DPav = 4 (average DP). It is composed primarily of lower DP oligosaccharides, namely 1-kestotriose, 1,1-kestotetraose and 1,1,1-kestopentaose, as well as inulobiose, inulotriose and inulotetraose. Even though the inulin hydrolysate and the synthetic compound have a slightly different DPav (4 and 3.6 respectively), the terms oligofructose or fructooligosaccharides shall be used to identify both. Oligofructose has quality similar to sucrose or glucose syrup. It is more soluble than sucrose and provides ~ 30-50% of the sweetness of sucrose. Oligofructose in food industry contributes body to dairy products and humectanty to soft backed goods, depresses the freezing point in frozen dessert, provides crispness to low fat cookies and act as a binder in nutritional bars in the same way as sugar but with added benefits and low calories, fiber enrichment and other nutritional properties (Inulin-type Fructans, 2005).

Oligofructose, together with inulin, is the most studied and well-established prebiotic.

In vitro data with mixed cultures, which mimic the real situation in the large bowel, have shown that the growth of bifidobacteria is selectively stimulated in such a way that these bacteria become largely predominant over the other populations.

In all the nutritional trials so far reported that have tested for the effect of FOSs on human microbiota, the increase in the number of bifidobacteria has been reported and it has been observed that:

- The number of bifidobacteria becomes significant and reaches its maximum probably in less than a week;
- Remains as long as the intake of the probiotic continues;
- Progressively (within 1-2 weeks) disappears when the intake stops.

It has been demonstrated also that the intake of FOSs reduces significantly the count of *Bacteroides*, fusobacteria and clostridia.

The increase in bifidobacterial flora is accompanied with other beneficial effects such as: modulation of intestinal functions, increase of stool weight, decrease of faecal pH (probably linked to the suppression of the production of putrefactive substances in the colon), modulation of cholesterol levels and modulation of mineral metabolism (Roberfroid, 2005).

3.2.2 GOS, galactooligosaccharides

Galactooligosaccharides are manufactured from lactose using the transgalactosylase activity of β-galactosidase. They are therefore often referred as transgalactosylated oligosaccharides (TOS). This enzyme is a hydrolase enzyme and works by transferring galactose from lactose to water. Under condition of high lactose concentration, the enzyme utilises lactose as an alternative acceptor to water resulting in the formation of galactooligosaccharides. A variety of enzyme reactor configurations based upon free or immobilised β-galactosidases have been used to produce these NDOs (Rastall and Gibson, 2002).

The transgalactosylation reaction leads to the formation of a mixture of oligosaccharides varying from DP 3 to DP 6 (DP, degree of polymerisation), with the average containing 3-4 sugar moieties. The general structure of TOSs is: β-D-gal-(1→6)-[β-D-gal]n-(1→4)-α-D-glu (fig. 3.5).

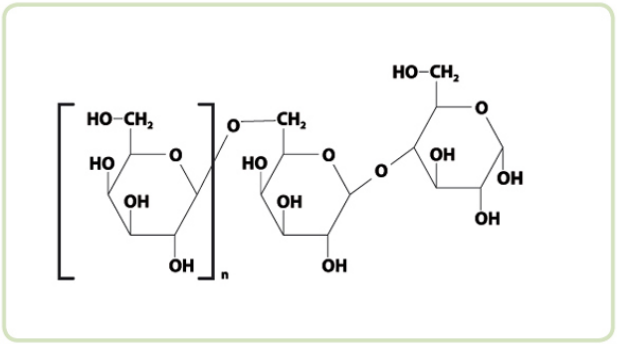


Fig. 3.5 Chemical structure of Galactooligosaccharides

The linkages between the galactose units, the efficiency of transgalactosylation, and the components in the final products depend on the enzymes and the conditions of the reaction. Using β-galactosidases derived from *Bacillus circulans* or *Cryptococcus laurentii*, the glycoside bonds between two galactose units are mainly β1-4 bonds (4'-TOS). While using enzymes from *Aspergillus oryzae* or *Streptococcus thermophilus* glycoside bonds are mainly β1-6 (6'-TOS). In standardized large scale production using the enzyme from *B. circulans*, more than 55% of the lactose is converted to TOS.

The lactose used as substrate for TOS production is usually purified from cow's milk whey. The main products are trisaccharides, namely 4'- or 6'-galactosyllactose and also longer oligo (≥ 4 units).

TOS are available in liquid and powder form. The relative sweetness of the product is about 35% that of sucrose. It is stable and is resistant to high temperature, to low pH and to long-term storage.

The indigestibility of TOS in vivo has been demonstrated, TOS resists digestion and absorption in the small intestine and reaches the caecum and colon, where it is fermented by the colonic bacteria. 4'-Galactosyllactose is selectively utilized by all the *Bifidobacterium* strains tested compared with lactulose and raffinose whose specificity is less remarkable. But also strains of other genera are able to use TOS, such as strains of *Lactobacillus* and *Bacteroides*. However, the utilisation of NDOs by bifidobacteria is usually mediated by the hydrolyzing enzymes they produce, and many strains produce glycolytic enzymes which hydrolyze a wide variety of monosaccharide units and different glycoside bonds. Other enteric bacteria, on the contrary, have enzymatic activities that are less varied and with a weaker activity (Sako *et al*., 1999). In vitro fermentations with human faecal or rat caecal microbiota indicate that TOS increases the production of acetate and propionate. Follow on studies have addressed the galactooligosaccharides in respect to pure batch cultures. They have demonstrated that these carbohydrates are readily fermentable by bifidobacteria, some but not all strains of *Bacteroides*, lactobacilli and *Enterobacteriaceae* but not by eubacteria, fusobacteria, clostridia, and most strains of streptococci.

TOS have demonstrated positive effects on calcium absorption and have prevented bone loss in some animal research. In preliminary studies, TOS have shown some ability to lower triglyceride levels. TOS are now used as sweeteners by themselves, especially in fermented milk products, breads, jams, etc. For example TOS in bread are not broken down by yeasts and render the bread excellent in taste and texture. Fermented milk products containing probiotic bacteria with added TOS are commercially available in Japan and in Europe. Baby foods and specialized foods for elderly are promising fields of application of TOS.

3.3 Synbiotics

Synbiotics may be defined as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract (Gibson and Roberfroid, 1995). The acquisition of data on the efficacy of synbiotic products as feed additives in livestock and poultry needs further investigation. However, results on *in vivo* trials are promising, either in young animals or adults: the coupling of a probiotic and prebiotic could also yield a synergistic effect in the reduction of food-borne pathogenic bacterial populations in food animals prior to slaughter (Bomba *et al*., 2002).

Chapter 4. Application of Probiotics

The adaptation to the post hatching period and the increased stressors, deriving from practices used in modern broiler production, *e.g*. feed changes or imbalances, transportation, processing at the hatchery and high stocking densities (Pinchasov and Noy, 1993), may weaken immune functions and thus predispose broilers to colonization of the gastrointestinal tract by bacterial pathogens, posing a threat to birds health and food safety. Among pathogens, Salmonella spp. has been the most studied because of its ability to infect chickens and hens increasing the risk of contamination through the food chain (Humphrey, 2006). In the last years, application studies have been extended to other bacteria such as *Campylobacter jejuni* and *Clostridium perfringens*, which could be both considered an emerging and increasing threat for poultry and human health (Humphrey *et al*., 2007; Van Immerseel *et al*., 2004). Probiotics could be a possible strategy to control pathogens shedding and thus maintain a healthy indigenous gut microbiota.

The application of probiotics in poultry is strictly associated with the concept of competitive exclusion (CE). Since the first applications on new hatched chicks, several experiments with undefined and defined probiotic cultures have been developed and successfully applied to control and reduce *Salmonella* colonization. Moreover, it has been shown experimentally that the CE treatment also protect chicks against *C. jejuni*, *Listeria monocytogenes*, pathogenic *E. coli*, *Yersinia enterocolitica* and *C. perfringens* (Nisbet, 2002; Schneitz, 2005).

A variety of well-characterized probiotic strains have been selected to evaluate the modulation of the avian gut microbiota and the protection against a variety of pathogens; however there has been a recent increase in the investigation of the effect of feeding *Lactobacillus* spp. to broilers, focusing on strains previously selected *in vitro* for adhesion properties and antimicrobial activity (Patterson and Burkholder, 2003).

Higgins *et al*. (2008) showed that *Lactobacillus*-based probiotic cultures reduced significantly *Salmonella enteritidis* recovery in challenged neonatal broiler chicks. Furthermore, the administration by vent application, compared to traditional application by drinking water, resulted in significant reduction of *S. enteritidis* one hour following oral challenge. In a previous trial, the same probiotic cultures affected the concentration of *S. enteritidis*, both in cecal tonsils and in caeca content, whereas no relevant results were obtained towards *S. thipymurium* (Higgins *et al*., 2007).

No differences in cecal and colonic counts were observed testing the efficacy of *L. johnsonii* F19185 in reducing the colonization and shedding of *S. enteriditis* in newly hatched chicks; nevertheless, the colonization of *E. coli* O78K80 and *Clostridium perfringens* were compromised significantly (La Ragione *et al*., 2004). Lactobacilli were also successful in decreasing mortality due to necrotic enteritis from 60% to 30% in a challenge trial, when they were given orally to day-old chicks (Hofacre *et al*., 2003).

To date, few studies evidenced a possible role of probiotics in preventing the shedding of *Campylobacter jejuni* at the level of primary production, although in vitro studies reported a strong antimicrobial activity of several species of *Lactobacillus* towards this pathogen (Chaveerach *et al*., 2004; Fooks and Gibson, 2002). Willis and Reid (2008) showed that *C. jejuni* presence was lower in broiler chickens fed with a standard diet supplemented with a minimum presence of 10^8 cfu/gr of *L. acidophilus*, *L. casei*, *Bifidobacterium thermophilus*, and *E. faecium*.

With regard to probiotic microorganisms, other than *Lactobacillus* spp., Vila *et al*. (2009) reported a reduction of *S. enteritidis* colonization and invasion by feeding continuously spores of the probiotic strain *B. cereus* var. *toyoi*, both in broiler chickens and white leghorn chickens.

In a study conducted by La Ragione and Woodward (2003), 1-day-old and 20-day-old specific pathogen free chicks were dosed with a suspension of *B. subtilis* spores prior to challenge with *S. enteritidis* and *C. perfringens*; the treatment suppressed completely the persistence and colonisation of both pathogens.

Studies testing the use and efficacy of *Bifidobacterium* spp., following pathogen challenge, have not yet been described. Mainly, authors have been focused on the beneficial impact on the gut microbiota and growth performance (Estrada *et al*., 2001; Jung *et al*., 2008).

The use of bifidobacteria in poultry feeding is, to our knowledge, less common with respect to lactobacilli administration.

Along with the control of foodborne pathogens in the avian gut, selected probiotic cultures, mainly *Lactobacillus* spp., may also potentially increase performance parameters; among poultry farmers, objectives such as increasing growth rate, improving feed conversion and meat quality are undoubtedly of primary importance.

Kalavathy *et al*. (2003) found that a supplementation of twelve *Lactobacillus* strains in broiler diets improved the body weight gain, feed conversion rate and was effective in reducing abdominal fat deposition.
Mountzouris *et al*. (2007) investigated the efficacy of selected probiotic bacteria, isolated from the gut of healthy chickens (*Lactobacillus reuteri, L. salivarius, Enterococcus faecium, Bifidobacterium animalis* and *Pediococcus acidilactici*) and on body weight, feed intake and feed conversion ratio of broiler chickens; overall the probiotic formula added to water and feed displayed a growth-promoting effect that was comparable to avilamycin treatment. In addition, the probiotic cultures modulated the composition and the enzymatic activities of the cecal microbiota, resulting in a significant probiotic effect.
The available body of literature offers a variety of conflicting results concerning the efficacy of probiotics for increasing growth performance in broilers; inconsistent results have been also reported from other authors (Estrada *et al*., 2001; O'Dea *et al*., 2006) showing a confusing state of the art; Timmerman *et al*., (2006) underlined the importance of way and timing in the administration as main factors affecting the efficacy of the probiotic preparation.
Eggs production has been also investigated in relation to probiotic application; Davis and Anderson (2002) reported that a mixed cultures of *Lactobacillus acidophilus, L. casei, Bifidobacterium thermophilus* and *Enterococcus faecium*, improved egg size and lowered feed cost in laying hens. Moreover, probiotics increase egg production and quality (Kurtoglu *et al*., 2004; Panda *et al*., 2008).

The prebiotic approach has not a long history of use in broiler chickens (Yang *et al*., 2009). However, application studies have been increasing in the last years to assess their effect on gut health, performance, and reduction of pathogen shedding. Xu *et al*., (2003) found a dose-dependent effect of fructooligosaccharides (FOS) on average daily gain; whereas Juskiewicz *et al*. (2006) reported no impact on the performance or productivity of turkeys after feeding for eight weeks with different amounts of FOS.
By feeding chycory fructans to broilers, Yusrizal and Chen (2003a) showed an improvement in weight gain, feed conversion, carcass weight and serum cholesterol decrease; additionally, the supplementation of fructans resulted in increase of lactobacilli counts in the gastrointestinal tract and *Campylobacter* and *Salmonella* decrease (Yusrizal and Chen, 2003b). Klessen *et al*. (2003) described decreased *C. perfringens* number and a reduction in bacterial endotoxin levels by adding 0,5% of fructan-rich Jerusalem artichokes syrup in broilers drinking water.
No weight gain was observed in turkeys fed with two different concentration of inulin and mannanoligosaccharides (MOS) (Stanczuk *et al*., 2005), whereas Sims *et al*. (2004) reported an improvement on live weight after feeding turkeys a standard diet supplemented with MOS. Yeast cell wall containing MOS reduced intestinal *Salmonella* concentrations by 26% in broiler chicks compared with chicks fed with an unsupplemented diet (Spring *et al*., 2000). Thitaram *et al*. (2005), with different amounts of isomaltooligosaccharide (IMO), showed a significant 2-log reduction in the level of inoculated *S. enterica* serovar Typhimurium present in the caeca of young broiler chickens. Feed consumption, feed conversion and feed efficiency were not significant compared to the control; however, the IMO containing diets significantly increased the number of the intestinal bifidobacteria. Feeding young chicks with five different oligosaccharides (inulin, oligofructose, mannanoligosaccharide, short-chain fructooligosaccharide, and trans-galactooligosaccharide) did not registered any significant responses in weight gain for any of the oligosaccharides; moreover the study outlined that high dosage of prebiotics can have negative effects on the gut system and retard the growth rate of birds (Biggs *et al*., 2007).

Likewise, a recent study reported no effects in body weight, feed intake and feed conversion ratio in broiler chickens fed with a standard diet and GOS at two different concentrations; however the study clearly showed a significant increase in the intestinal bifidobacteria population (Jung *et al*., 2008). Mainly, prebiotics seem to enhance selectively lactobacilli and bifidobacteria populations and reduce colonization by pathogenic bacteria (Baurhoo *et al*., 2009; Biggs and Parsons, 2008).

Results on animal performance, either with a probiotic or a prebiotic treatment, are often contradictory and mostly affected by the microorganisms or compound chosen, the dietary supplementation level, and duration of use. In many cases, the environmental and the stress status of the animals are not reported or considered as the experimental setting are often too far from the farm conditions.

Recent development and applications of synbiotic products have been focused on the assessment of beneficial effects in poultry health and production; however, there is still scarce information available to date. Mohnl *et al*. (2007) found that a synbiotic product had a comparable potential to improve broiler performance as avilamycin treatment.

A *Lactobacillus* spp.-based probiotic product, in combination with dietary lactose, was successfully assessed, improving body weight and feed conversion in *Salmonella*-challenged turkeys (Vicente *et al*., 2007). Li *et al*. (2008), adding FOS and *B. subtilis* to the diet, observed that average daily gain and feed conversion ratio were improved; diarrhoea and mortality rate were reduced compared to aureomycin treatment.

A considerable increase in the bifidobacteria, lactobacilli and total anaerobes populations has been shown by using a diet containing a combination of a galacto-oligosaccharide and *Bifidobacterium lactis*; in contrast, no effect on body weight, feed intake and feed conversion has been observed (Jung *et al*., 2008).

Awad *et al*. (2009) investigated the effect of a dietary treatment with a synbiotic product (a combination of *E. faecium*, a prebiotic derived from chicory, and an immune modulating substances derived from sea algae) on broiler chickens. The body weight, average daily weight gain, carcass yield percentage, and feed conversion rate were significantly increased compared with the control, whereas no increase in organs weight was found with exception for the small intestine; a significant increase in the villus height in both duodenum and ileum was also observed.

Overall, all the authors agreed that a synbiotic product displayed a greater effect than individual preparations (Awad *et al*., 2009; Jung *et al*., 2008; Revolledo *et al*., 2009; Vandeplas *et al*., 2009). This coupling could represent an important and synergic strategy to improve gut health of chickens from the first days of life and control pathogen release in the environment decreasing the risk of foodborne infections in humans. Thus, future research and applications in field trials are necessary to look for new combination with the aim to produce standard safe composition at a high functional level (Gaggia, F. *et al*., 2010).

Chapter 5. Molecular approaches to study the microbiota: Real-Time PCR

5.1 Molecular methods to study intestinal microbiota

It is now generally accepted in microbial ecology that cultivation-based approaches provide an incomplete picture of microbial diversity in the gastrointestinal tract because only a minority of microbes can be obtained in culture. Therefore the application of molecular approaches, especially those focused on 16S ribosomal RNA sequence diversity, have become popular as they enable researchers to bypass the cultivation step. These approaches have provided considerable information about microbial ecosystems, including the GI tract (Zoetendal and Mackie, 2005).

Studies on chicken caecal microbiota, by both culture based (Barnes, 1972, 1979; Barnes *et al*., 1972; Mead and Adams, 1975) and culture-independent (Gong *et al*., 2002; Zhu *et al*., 2002; Lu *et al*., 2003) methods, have indicated that this environment is dominated by obligate anaerobes, but a diverse range of species have been detected. The traditional culture-based methods of assessing mammalian gastrointestinal tract community structure are extremely laborious, and it has been estimated that only 10–60% of total bacteria from this environment are able to be cultured (Barnes, 1972, 1979; Barnes *et al*., 1972; Salanitro

et al., 1974; Mead, 1989). Non-culture methods for assessing gut microbial ecology (reviewed in Zoetendal et al. 2004), such as the construction and analysis of 16S rDNA clone libraries (Gong *et al*., 2002; Zhu *et al*., 2002; Lu *et al*., 2003), for example, have been instrumental in the discovery of new intestinal bacterial groups. Molecular indices of diversity, such as the community fingerprinting tools DGGE (Knarreborg *et al*., 2002; van der Wielen *et al*., 2002), T-RFLP (Gong *et al*., 2002) and %G + C profiling (Apajalahti *et al*., 2001), have also provided insight into chicken gut microbial ecology. Although these procedures have proved useful for detecting community structure shifts, with the exception of fluorescent in situ hybridization- based studies (Zhu and Joerger, 2003), they have the drawback that they are typically not quantitative. Real-time PCR, on the contrary, can be quantitative as the number of target gene copies in DNA directly extracted from an environmental sample can be determined. Using group-specific primer sets, the abundance of a particular gene marker for a defined group in the community can be estimated by comparison to a standard curve (Wise and Siragusa, 2007).

Molecular methods have also permitted to develop more accurate protocols for pathogen detection along the food chain. Traditional diagnostic methods are commonly based on selective enrichment of the target pathogens. Even though these methods are standardized and efficient, they suffer from serious disadvantages, such as that they are time-consuming and expensive, correct analysis can be difficult due to lack of expression of phenotypic properties, and detection of viable but-non-culturable cells (VBNCs) is almost impossible at present. In an effort to overcome these limitations, DNA-based detection methods have been developed. One of the most promising methods is real-time PCR (rtPCR) due to its speed, cost effectiveness and sensitivity, specificity, selectivity, high degree of automation and the possibility of target quantification. A number of conventional PCR assays have been described for the identification and characterization of *Campylobacter* species from a spectrum of sample types, including stools (Piddock *et al*., 2000; Houng *et al*., 2001; Maher *et al*., 2003), food products (Bohaychuk *et al*., 2005; Englen *et al*., 2003), water (Reiter *et al*., 2005) and cultures (Sails *et al*., 2003), using a variety of gene targets such as, *hipO*, *glyA*, *23S rRNA*, *16S rRNA*, *ceuE* and *mapA* (Debretsion *et al*., 2007).

Diagnosis of this important pathogen is difficult due to special growth requirements and low infectious doses. Water and poultry are major sources of infections. The main reservoir of *C. jejuni* in poultry is the caecum, with an estimated content of 10^6–10^8 cells/g. If a flock is infected with *C. jejuni*, the majority of the birds in that flock will harbor the bacteria. Diagnosis at the flock level could thus be an important control point (Rudi *et al*., 2002).

For this pourpose, different rtPCR method that identifies species-specific detection of *Campylobacter* species, and in particular *C. jejuni* in naturally infected chicken fecal samples (Rudi *et al*., 2004; Rudi *et al*., 2002; Lund *et al*., 2004), chicken caeca (Rudi *et al*., 2004; Skånseng *et al*., 2006) and meat products (Yang *et al*., 2003; Yang *et al*., 2004; Sailis *et al*., 2003) had been developed.

5.2 Real-time PCR

The possibility of following the increase in the number of targets during the amplification by monitoring the increase in the fluorescent signal is the key to performing real-time PCR. By observing the point where the fluorescence crosses a threshold level, or crossing point value or Cp value (depending on the equipment, also known as a Ct value), a cycle number can be acquired for samples with different initial DNA concentrations. If the initial concentration is high, the threshold level will be crossed earlier than when the initial concentration is low (fig. 5.1). By measuring the Ct value for samples with known concentrations, standard curves can be made that can then be used for absolute quantification. The standard curve that is created prior to quantification of unknown samples gives important information about two parameters. First, it shows the detection window, or the range over which data points can be acquired. It is, however, important to notice that a linear relationship is used for quantification, and that sometimes not all points (especially at the window borders) fit a linear relationship (figure 6.2). That is why a distinction can be made between the detection window (i.e., the window over which detection is obtained) and the linear range of amplification (i.e., the window over which a linear relationship of the standard curve can be obtained). The second parameter that can be derived from the standard curve is the amplification efficiency (AE) through the following equation: $AE = (10^{(-1/slope)}) - 1$. When the theoretical optimum of a target doubling in each cycle is reached, the slope of the standard curve will be –3.32 and the value of AE will be 1.00. The AE can be used in several ways. First of all, deviations from the optimal value of 1.00 indicate that the PCR is not performing optimally, either because of inhibition or because of a suboptimal PCR setup. Therefore, the AE is an excellent tool with which to perform PCR optimization. Unfortunately, there seems to be no consensus yet in the scientific community about the correct way to analyze quantitative data and to create standard curves for real-time PCR. Most published data show standard curves constructed of one data set whereas others analyze and use multiple data sets to calculate the AE (Wolffs and Rådström, 2006).

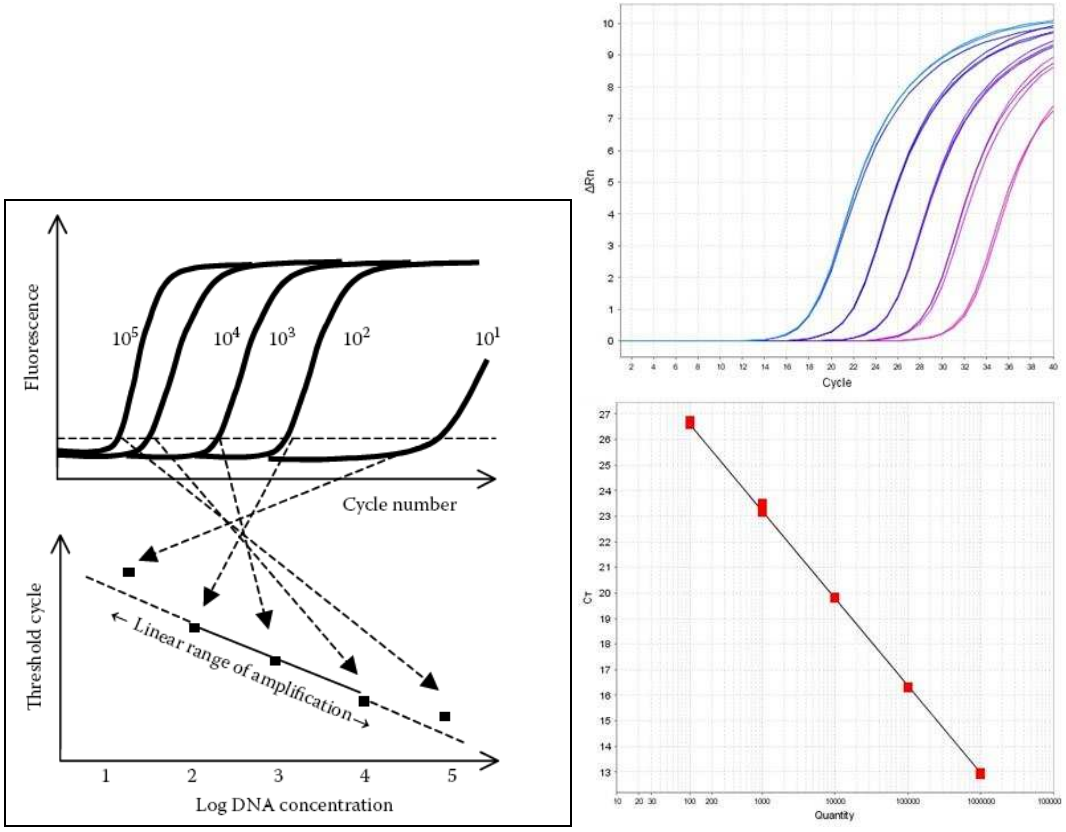


Fig. 5.1 Schematic overview of the generation of a standard curve used for real-time quantitative PCR (Walffs and Rådström, 2006).

5.2.1 Non-specific Real-Time chemistry

The standard method for nonspecific real-time detection of PCR amplicons is use of fluorescent double-stranded (ds)DNA intercalating dyes such as SYBR Green™ I or SYBR Gold™. Both of these commercial dyes are DNA minor groove binding dyes that fluoresce after interacting with dsDNA (fig. 5.2).

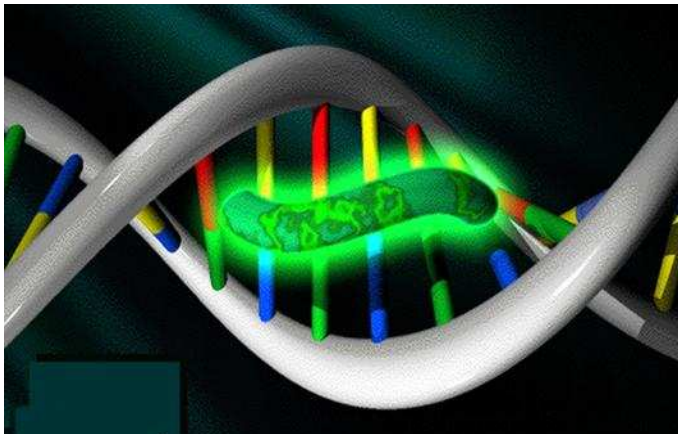


Fig. 5.2 Interaction of SybrGreen intercalating dye with double-stranded DNA and subsequent fluorescence under appropriate wavelength. The interaction is not sequence-specific.

Most real-time PCR instruments are programmed to read near the emission and excitation wavelength spectrum of SYBR Green™ (495 and 537 nm, respectively). This dye is very light sensitive, degrading quickly following dilution to working concentrations, but when fully active, allow the user to obtain real time fluorescence emission data (relative fluorescence units on the y-axis of a plot) as a function of cycle number on the x-axis. Since relative fluorescence units for each sample are plotted during the exponential phase of amplification, results are quantitative and thus useful for determining copy number and genome equivalents from template DNA purified from food. SYBR Green™ I has been used as an alternative to ethidium bromide for staining DNA in agarose gels, but it is also useful for real-time PCR detection assays in food systems, such as quantification of pathogen in humans, animal and food products. Due to the logistical difficulty in optimizing QC-PCR assays, the approach has limited potential for large-scale applications, particularly in light of many of the real-time chemistries. In addition to simply quantitative detection of target pathogenic or spoilage bacteria in foods, intercalating dyes such as SYBR Green™ I allow one to discriminate among amplicons in a multiplex PCR reaction by using melt curve analysis. The melt curve analysis allows also to detect non-specific amplification, such as primer-dimers. This approach subjects the PCR reactions to slow and continual heating to 95°C while monitoring fluorescence over time. Since each amplicon of a varying length and/or GC content will melt at a slightly different temperature, fluorescence will decrease incrementally according to the population of products in the reaction tube. Once conditions are optimized, the negative derivative of the fluorescence vs. temperature line will allow for small sequence differences, and certainly differences in length of products to become apparent (fig. 5.3). Melt curve analysis has been applied primarily for mutation screening in specific clinical pathogens, but also may be useful for food pathogen detection.

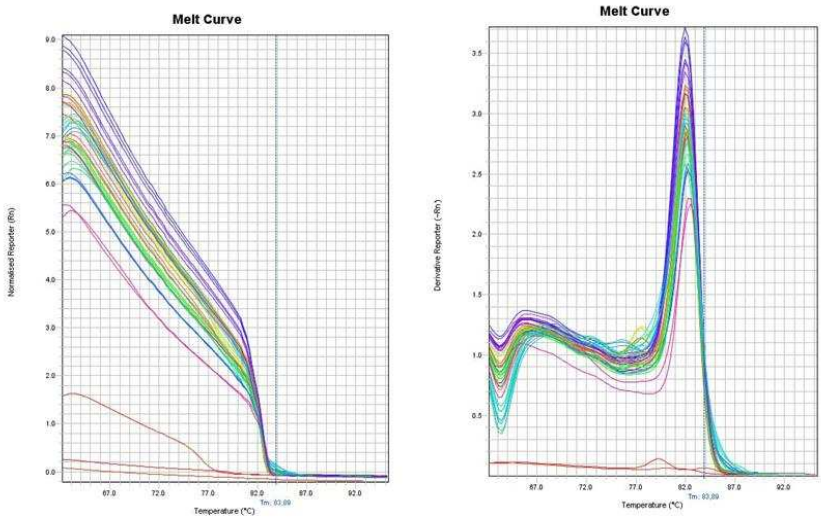


Fig. 5.3 Example of a melting curve and its derivative.

5.2.2 Specific Real-Time chemistry

A diverse array of fluorescently labeled probes are in use clinically and industrially for sequence-specific detection of target DNA or RNA, and many of these have been applied in food analysis. The primary category of these involves fluorescence resonance energy transfer (FRET) between a specific fluorophore and a quencher group. Perhaps the most widely used FRET conjugate pair for real-time PCR assays includes the fluorophore FAM (fluorescein) and the quencher TAMRA. The resonance energy from the fluorophore is passed to the appropriate quenching moiety, and if in close proximity (as described below

for specific primer and probe regimes), generates low levels, if any, detectable fluorescence as measured by a PCR cycler with fluorimeter capabilities. If separated or alone in solution, the fluorophore will not be quenched and the resonance energy will be emitted as a detectable fluorescent signal at the appropriate wavelength. Depending on the format of the PCR assay, the signal generated will be directly correlated with the amount of target DNA present or amplicon concentration. Regardless of the specific means in which the fluorophore/ quenching pair is applied, the basis remains the same, and includes the added advantage of sequence specificity that dsDNA intercalating dyes do not offer. One of the earliest uses for the FRET-based probe approach was the 5'-nuclease (TaqMan) assay, first described as a radioisotopic system, but soon modified to be based on fluorogenics. The 5'-nuclease activity incorporates a target gene-specific primer set and a dual-labeled probe that will hybridize to a region on one of the template strands within the primer annealing sites. During the extension phase of a PCR cycle, the 5'-3' exonuclease activity of Taq-polymerase will cleave the 5' fluorophore from the terminal end of the hybridized probe, separating it from the quenching moiety, eliciting fluorescence at a specific wavelength (fig. 5.4). Depending on the instrument being used for real-time detection, the investigator may choose to use multiple TaqMan primer and probe combinations in the same reaction tube for multiplexing, with each being detected in a unique optical channel at the respective wavelength. Regardless, TaqMan is a specific and sensitive assay for detection of pathogenic and/or spoilage bacteria in food. In recent years, the TaqMan approach has been reported for different pathogens, such as for example *E. coli* O157:H7 in raw milk and other foods (Bohra *et al*., 2001; Buerk *et al*., 2002), *Salmonella* spp. in meat and seafood (Chen *et al*., 1997; Kimura *et al*., 1999), *Campylobacter jejuni* from poultry, shellfish, and other commodities (Padungtod *et al*., 2002; Sails *et al*., 2003), and *L. monocytogenes* in dairy foods (Cox *et al*., 1998; Lunge *et al*., 2002). These representative studies illustrate the versatility of the TaqMan assay for a very diverse array of foods to detect pathogens to levels as low as 10^1 cfu per ml, although frequently following several hours of pre-enrichment (McKillip and Drake, 2006).

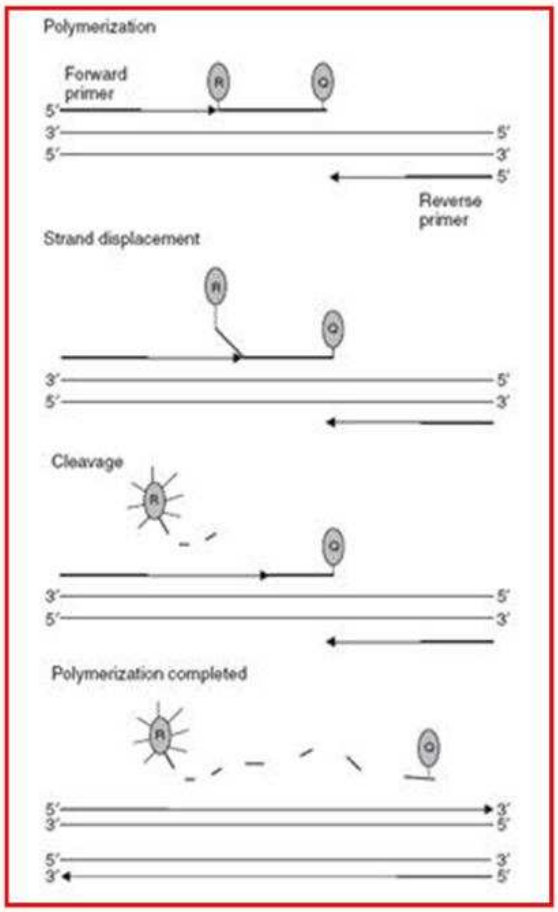


Fig. 5.4 Mechanism of TaqMan 5’ nuclease assay for real-time detection of PCR products using FRET-labeled probe internal to the sequence-specific primers. R denotes the reporter dye while Q represents the quenching moiety.

PART 2: AIM OF DISSERTATION

The avian gut microbiota has received increased attention in the past decade. Researches on poultry microbiota mainly focus on minimizing food-borne illness in humans, improving animal nutrition and reducing the use of antibiotics as growth-promoters.

Antibiotics have often been often used in animal breeding as growth promoters to improve feed efficiency and to control the so called "production related" bacterial infections *e.g.* infections associated with early weaning, high animal densities, poor sanitary conditions and frequent transportations. Two of the most important zoonoses that constitute a threat for human health are campylobacteriosis and salmonellosis (EFSA, 2010). However, concerns about development of antimicrobial resistance and transfer of antibiotic resistance genes from animal to human microbiota, led to withdrawal approval for antibiotics as growth promoters (AGPs) in the European Union since January 1, 2006.

New food additives, *e.g.* probiotics and prebiotics, could represent a strategy to improve intestinal health and growth performance.

The term probiotic was defined recently by the FAO/WHO as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". These beneficial effects include: regulation of intestinal microbial homeostasis, stabilization of the gastrointestinal barrier function, expression of bacteriocins, enzymatic activity inducing absorption and nutrition, immunomodulatory effects, inhibition of procarcinogenic enzymes and interference with the ability of pathogens to colonize and infect the mucosa.

A prebiotic compound was defined by Gibson and Robertfroid as "a non digestible feed ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves gut health". Certain oligosaccharides are considered to be prebiotics compounds because they are not hydrolyzed in the upper gastrointestinal tract and are able to favorably alter the colonic microbiota. Many oligosaccharides, when fed to animals, can reach the colon undegraded and provide a carbohydrate substrate for the growth of beneficial microorganisms, such as bifidobacteria and some lactic acid bacteria, which are thought to create conditions unfavorable to pathogens growth.

Probiotics and prebiotics could be, therefore, considered a powerful tool for intestinal microbiota modulation to improve the health status of the animal.

The aim of this research was the formulation of a new synbiotic formula for chicken feed, in order to improve chicken intestinal health and control pathogen spread. Two separate *in vivo* trials were first planned to select an appropriate probiotic strain and an effective prebiotic compound to create the synbiotic formula. The final synbiotic product was then tested *in vivo* with a peculiar formula that comprised the microencapsulation of the probiotic. The shelf-life of the microorganism is a crucial point for the application of probiotic bacteria in animal feed. The microencapsulation technique improves bacteria survival at environmental conditions protecting bacteria from oxygen, low temperature, humidity, osmotic stress etc. For this reason microencapsulation could be considered an effective strategy for the supplementation of probiotic additives in intensive farming, where problems for feed rationing could be encountered.. The shelf-life of the microencapsulated product was evaluated before the *in vivo* trial. Moreover, considering the pressure of the European Union about the substitution of *in vivo* trial with appropriate *in vitro* experiment, the two administered probiotic strains were also tested on chicken intestinal cell-line in order to further characterize these strains.

PART 3: MATERIALS AND METHODS

Chapter 6. In vivo trials

The procedure to allow the *in vivo* study has been approved by the ethic committee of the University of Bologna according to the Italian Legislation.
The animal management agreed with the European directive 86/609/CEE regarding the protection of animals for experimental use.

6.1 Study Design

6.1.1 Probiotic trial

The broiler chickens for this trial were provided by a farming business (Petitoni Dante); before acceptance animals were visited by a veterinarian and immediately settled in collective boxes (fig. 6.1). No artificial light was used. Animals were fed with common poultry feed without active pharmaceutical ingredients *ad libitum* and free access to water.
A period of 5 days before starting the experiment has been necessary for animal naturalization. Every single broiler was identified with a number in a label, applied on its leg. Animal clinical conditions were observed daily and noted down; individual weighting was recovered before starting treatment, after 15 days of supplementation and at the end of the experiment. Temperature and relative humidity of the room were monitored along the entire period.
24 males and 24 females were subdivided into 3 groups depending on weight (homogeneity criteria). Every group were formed by 16 animals and then identified as follows:

- Group 1 (Control)
- Group 2 (Treated – *Lactobacillus plantarum* PCS 20, isolated from Slovenian cheese) (PCS group)
- Group 3 (Treated – *Bifidobacterium longum* PCB 133, isolated from new born infant faeces) (PCB group)

The two formula were administered as follows:

- 16 broiler chickens, 8 female and 8 male (15-20 days old) after a period of naturalization were treated for 14 days with *L. plantarum* PCS 20 (10^8 CFU/day). The probiotic suspension was administered orally by gavage (fig. 6.1).
- 16 broiler chickens, 8 female and 8 male (15-20 days old) after a period of naturalization were treated for 14 days with *B. longum* subsp. *longum* PCB 133 (10^8 CFU/day). The probiotic suspension was administered orally by gavage(fig. 6.1).
- 16 broiler chickens were held as control. – I order to manage also control broilers in the same way, these chickens were administered with 1 ml of skim milk solution through oral gavage.

Ten animals were selected from each group for faecal sampling; faecal samples were collected at time zero (T0), after 15 days of probiotic administration (T1) and after 21 days (T2), *i.e.* after a wash out period.

Fig. 6.1 Collective boxes for broiler chickens and gavage administration of probiotic strains.

6.1.2. Prebiotic trial

The broiler chickens were provided by "Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna"; before acceptance, animals have been visited from a veterinarian and immediately settled in collective boxes. Artificial light has been used to guarantee 12 light-hours and 12 dark-hours. Animals were fed with common poultry feed without active pharmaceutical ingredients *ad libitum* and free access to water.

A period of 6 days before starting the experiment has been necessary for naturalization. Every single broiler was identified with a number in a label, applied on their leg.

Clinical conditions were observed daily and noted down; individual weighting were carried out at their arrival and at the end of the experimental treatment. Room temperature was monitored along the entire period.

20 males and 22 females were subdivided into 3 groups depending on weight (homogeneity criteria). Every group were formed by 14 animals and then identified as follows:

- Group 1 (Control) (CTR group)
- Group 2 (Treated - FOS Actilight®) (FOS group)
- Group 3 (Treated - GOS CUP Oligo P) (GOS group)

The two formula were administered as follows:

- 14 broiler chickens, 7 female and 7 male (40 days old) after a period of naturalization were treated for 14 days with Actilight®. The concentration of 0.5% of the prebiotic Actilight® was mixed with the feed and administered daily to chickens for 14 days.
- 14 broiler chickens, 7 female and 7 male (40 days old) after a period of naturalization were treated for 14 days with CUP Oligo P. The concentration of 3% of the prebiotic CUP Oligo P was mixed with the feed and administered daily to chickens for 14 days.

- 14 broiler chickens were held as control.

Ten animals were selected from each group for faecal sampling; faecal samples were collected at time zero (T0), after 15 days of probiotic administration (T1) and after 21 days (T2), *i.e.* after a wash out period.

6.1.3 Synbiotic trial

The broiler chickens for this trial were provided by a farming business (Petitoni Dante); before acceptance animals were visited by a veterinarian and immediately settled in collective boxes. No artificial light was used. Animals were fed with common poultry feed without active pharmaceutical ingredients *ad libitum* and free access to water.
A period of 7 days before starting the experiment has been necessary for animal naturalization. Every single broiler was identified with a number in a label, applied on their leg. Animal clinical conditions were observed daily and noted down; individual weighting was recovered before starting treatment, after 15 days of supplementation and at the end of the experiment. Room temperature and relative humidity were monitored along the entire period.
14 males and 14 females were subdivided into 2 groups depending on weight (homogeneity criteria). Every group were formed by 14 animals and then identified as follows:
Group 1 (Control) (CTR group)
Group 2 (Treated with Synbiotic product) (SYN group)

The synbiotic formula was composed as follow:

- 1 g microencapsulated *B. longum* subsp. *longum* PCB 133 /100 g of feed (> 10^9 cfu/g)
- g of GOS CUP Oligo P/100 g of feed (3%)

The synbiotic formula was mixed to normal powder feed three times during the trial through an automatic feed blender.

The synbiotic formula was administered as follows:

- 14 broiler chickens, 7 female and 7 male (40 days old) after a period of naturalization were treated for 14 days with the synbiotic formula. The synbiotic formula was mixed to normal feed at a concentration indicated above.
- 14 broiler chickens were held as control.

Ten animals were selected from each group for faecal sampling; faecal samples were collected at time zero (T0), after 14 days of synbiotic administration (T1) and after 21 days (T2), *i.e.* after a wash out period.

6.2. Microbial growth condition

6.2.1 Pure cultures

Strains of *Lactobacillus* spp. (*L. rhamnosus* ATCC 7469^T, *L. delbrueckii subsp. lactis* ATCC 12315^T, *L. acidophilus* J 14 [provided by Dr Sozzi], *L. plantarum* PCS 20 [fig. 6.1]), *Bifidobacterium* spp. (*B. animalis* subsp. *lactis* DSM 10140^T, *B. animalis* subsp. *lactis* Ra18 [from rabbit faeces], *B. animalis* subsp. *lactis* P32 [from chicken faeces], *B. gallinarum* ATCC 33777^T, *B. longum* subsp. *longum* PCB

133, *B. longum* subsp. *infantis* ATCC 15697^T, *B. longum* subsp. *suis* ATCC 27533^T, *B. longum* subsp. *longum* ATCC 15707^T, *B. pseudolongum* subsp. *pseudolongum* DSM 20094 from chicken, *B. pseudolongum* subsp. *pseudolongum* ATCC 25526^T from pig, *B. pullorum* ATCC 27685^T), *C. jejuni* subsp. *jejuni* CIP 70.2^T (Collection Institute Pasteur, fig. 6.4) and *C. jejuni* subsp. *jejuni* ATCC 29428 have been cultivated to extract DNA from pure cultures to prepare standard curves.
B. animalis subsp. *lactis* Ra18, *B. animalis* subsp. *lactis* P32 and *B. longum* subsp. *longum* PCB 133 (fig. 6.3) derived from the Bologna University Scardovi culture Collection of Bifidobacteria (BUSCOB).
Lactobacilli and bifidobacteria strains were grown respectively in "de Man Rogosa Sharpe" broth (MRS) (Merck, code: 1.10660) and "Trypticase-Phytone-Yeast Extract" (TPY) broth (Biavati and Mattarelli, 2001), incubated in anaerobiosis, at 37 °C for 48 h. *Campylobacter jejuni* was grown on Müller-Hinton Broth (Oxoid, code CM0405) and Müller Hinton Broth supplemented with 1.5% Agar Bacteriological (Agar N.1, Oxoid, code LP0011) supplemented with *Campylobacter* Growth Supplement liquid (Oxoid, code SR0232) in microaerophilic conditions at 42°C.

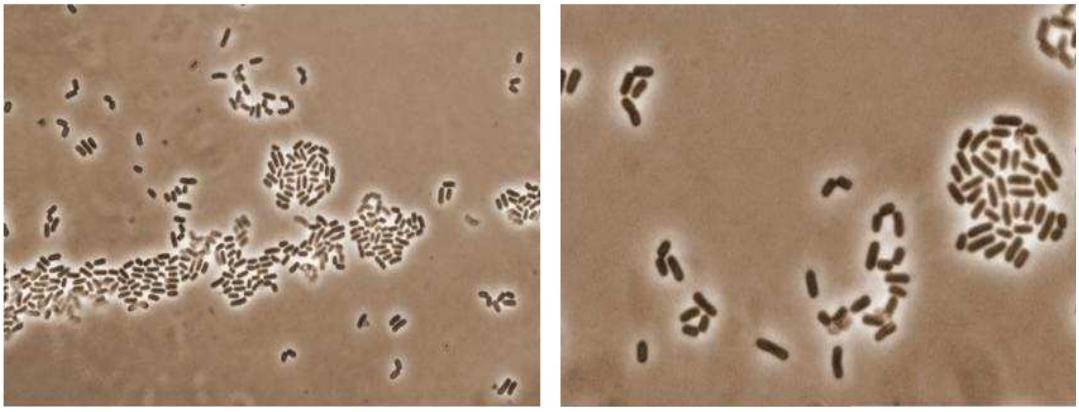


Fig. 6.2 *Lactobacillus plantarum* PCS 20, photomicrograph magnification 800x and 1200x.

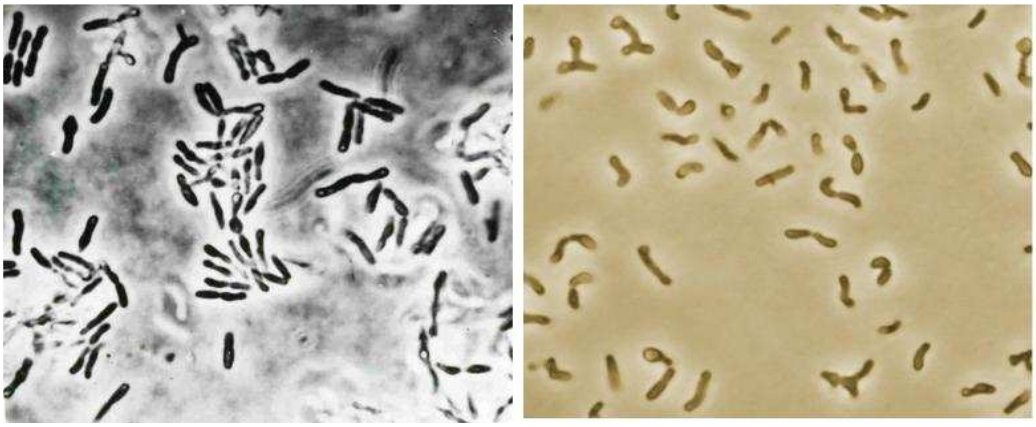


Fig. 6.3 *Bifidobacterium longum* subsp. *longum* PCB 133, photomicrograph magnification 1500x and 1200x.

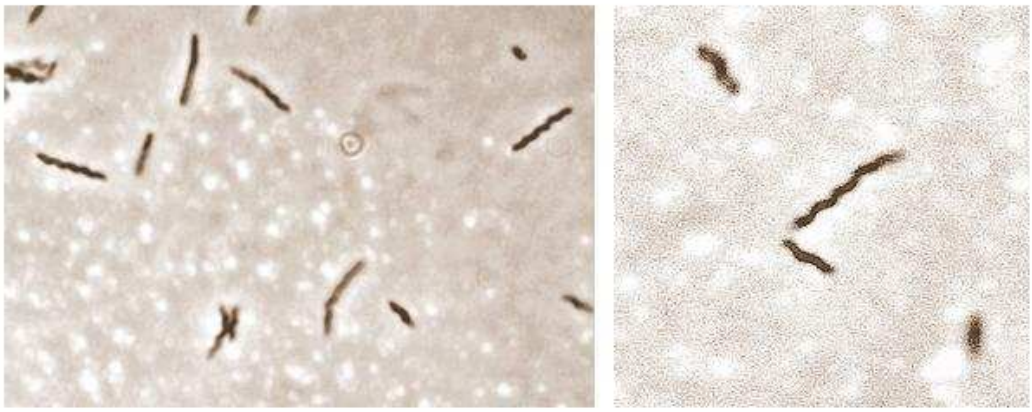


Fig. 6.4 *Campylobacter jejuni* subsp. *jejuni* CIP 70.2, photomicrograph magnification 1800x and 2500x.

6.2.2 Dose preparations

250 doses of each strain selected for the in vivo trial (*B. longum* subsp. *longum* PCB 133 and *L. plantarum* PCS 20) were prepared at the concentration of approximately 10^8 CFU. *B. longum* subsp. *longum* PCB 133 and *L. plantarum* PCS 20 were cultured for 18-24 hours at 37°C in anaerobic conditions in TPY medium and MRS medium, respectively. The two cultures were washed twice in PBS buffer, centrifuged and suspended at the defined concentration in 1 ml of skim milk. Doses containing only skim milk were also prepared for the control tests. After preparation doses were immediately stored at -80°C. Bacterial counts on the frozen doses were performed after storage (T0), after 15 (T1) and 21 days (T2) to check viability.

6.3. Sampling

To collect the samples each chicken was transferred in a single cage with the low part made of wire netting and an aluminium paper was settled under the cage; no stimulus was applied.
Faecal samples were collected in sterile vials and kept at -120°C; faeces for the molecular analysis were immediately processed for DNA extraction.

6.4 DNA-extraction

6.4.1 DNA extraction from pure cultures

10 ml of culture were harvested and washed twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, [pH 7.6]), resuspended in 1 ml TE containing 15 mg lysozime and incubated at 37°C overnight. Cells were lysed with 3 ml of lysis buffer (100 mM Tris-HCl, 400 mM NaCl , 2 mM EDTA, pH [8.2]), 220 µl SDS (10% w/v) and 150 µl proteinase K (>600 mAU/ml, solution) and incubated for 2 hours in water bath at 60°C. One ml of saturated NaCl solution was added and the suspension was gently inverted twice. Pellets were harvested through centrifugation (5000 X g) at room temperature for 15 minutes. After the transfer of clean supernatants in new tubes, DNA was precipitated with 2.5 volumes of cold ethanol (95%) and resuspended in 300 µl of TE buffer (Rossi *et al*.,2000).

6.4.2 DNA extraction from faecal samples

DNA extraction optimization

Initially DNA extraction from faecal samples was performed with three different methods in order to select the best protocol evaluating DNA-quality and extraction-time ratio. The three compared methods include: two extraction kits (QIAamp DNA Stool Mini Kit [Qiagen, Cat. No. 51504] and Ultra Clean Faecal DNA kit [MO BIO, Cat. No. 12811-S] and the benzyl chloride extraction (Zhu *et al*., 1993; Matsuki *et al*., 1999).
DNA purity and concentration was evaluated with a spectrophotometer (Beckman coulter, DU®730). Extracted DNA was stored at -20°C.

DNA extraction

Genomic DNA was isolated from 200 mg of each faecal sample using the QIAamp DNA Stool Mini Kit (Qiagen West Sussex, UK) following manufacturer's instructions. The recommended lysis temperature was increased to 95 °C to improve bacterial cell rupture. The DNA was stored at −20 °C until analysis.

6.5. Protocols Optimization for qPCR

6.5.1 Primer selection

Different sets of primers (tab. 6.1) have been evaluated for target bacteria analyzed in this work: *B. longum*, *B. plantarum*, *Bifidobacterium* spp., *Lactobacillus* spp., *Campylobacter* spp. and *C. jejuni*. Primers have been previously tested in qualitative PCR .

TABLE 6.1 Primer sets evaluated for qPCR

	Primer Sequence	Amplicon lenght (bp)	Refernces
Lactobacillus plantarum			
planF	5'-CCG TTT ATG CGG AAC ACC TA -3'	318	Torriani *et al*., 2001
pREV	5'-TCG GGA TTA CCA AAC ATC AC-3'		
Bifidobacterium longum			
IDB51F	5'-CGG TCG TAG AGA TAC GGC TT-3'	301	Youn *et al*., 2008
IDBC1R	5'-ATC CGA ACT GAG ACC GGT T-3'		
BIL-1	5'-GTT CCC GAC GGT CGT AGA G-3'	153	Wang *et al*., 1996
BIL-2	5'-GTG AGT TCC CGG CAT AAT CC-3'		
BiLONg-1	5'-TTC CAG TTG ATC GCA TGG TC-3'	277	Matsuki *et al*., 1998
BiLONg-2	5'-TCS CGC TTG CTC CCC GAT-3'		
Bifidobacterium spp.			
BifTOT-F	5'-TCG CGT CYG GTG TGA AAG-3'	243	Rinttilä, 2004
BifTOT-R	5'-CCA CAT CCA GCR TCC AC-3'		
Lactobacillus spp.			
F-Lac	5'-GCA GCA GTA GGG AAT CTT CCA-3'	349	Castillo, 2006
R-Lac	5'-GCA TTY CAC CGC TAC ACA TG-3'		
Campylobacter spp.			
UC-Fw	5'-CCG CAA CGA GCG CAA CCC ACG-3'	172	Keramas, 2003
UC-Rev	5'-CAT TGT AGC ACG TGT GTC-3'		
CampTOT-F	5'-GGA TGA CAC TTT TCG GAG-3'	246	Wise and Siragusa, 2007
CampTOT-R	5'-AAT TCC ATC TGC CTC TCC-3'		
Campylobacter jejuni			
cj hip	5'-GGAGAGGGTTTGGGTGGTG-3'	735	Lawson,1998
	5'-AGCTAGCTTCGCATAATAACTTG-3'		
HIPj-Fw	5'-GTA CTG CAA AAT TAG TGG CG-3'	149	Keramas, 2003
HIPj-Rev	5'-GCA AAG GCA AAG CAT CCA TA-3'		

6.5.2 Qualitative PCR

To test the specificity of the different sets of primers qualitative PCR analysis was performed first of all on DNA extracted from pure cultures and also on DNA extracted from faecal samples, to test the presence of inhibition substances (TGradient, Biometra®; PTC-100 Peltier Thermal Cycler, Bio-Rad).
Reagent concentrations and thermal cycle conditions (tab. 6.2), have been optimized for the different primers; amplified products were subjected to gel electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining.

Master mix and PCR cycle for primer cj hip as been performed as previously described (Lawson, 1998).
20 µl master mix for primers BiLONg-1/BiLONg-2, BIL-1/BIL-2 PlanF/pREV, CampTOT-F/CampTOT-R and IDB51F/IDBC1R was composed by 10 µl of HotStartTaq® *Plus* Master Mix (Qiagen, West Sussex, UK, 2x concentrated master mix, containing 3 mM $MgCl_2$ and 400 µM of each dNTP), BSA 0.1 mg/ml, forward and reverse primers at a concentration of 0.25 µM (BiLONg-1/BiLONg-2, BIL-1/BIL-2 PlanF/pREV) 0.3 µM (IDB51F/IDBC1R) and 0.4 µM (CampTOT-F/CampTOT-R). Primers UC-Fw/UC-Rev and HIPj-Fw/HIPj-Rev have been used at a concentration of 0.4 µM adding 0.75 mM $MgCl_2$ to the previously described master mix . BifTOT-F/BifTOT-R and F-Lac/R-Lac have been used in qualitative PCR adding BSA 0.12 mg/ml and 0.7 mM $MgCl_2$ (F-Lac/R-Lac). The concentration of these primers was respectively 0.5 µM and 0.25 µM.
DNA (2 µl) from a pure culture was added to PCR reactions at a concentration of 5 ng/ µl while DNA (2 µl) extracted from faecal samples was usually used not diluted, depending on sample DNA concentration.

TABLE 6.2 Annealing temperatures for qualitative PCR

Taget Bacteria	Initial denaturation	Denaturation	Annealing temperature (°C)	Extension	N. cycles	Final extension
Lactobacillus plantarum planF/pREV	94°C - 3min	94°C - 30 sec	56°C - 10 sec	72°C - 30 sec	30	72°C - 5 min
Bifidobacterium longum IDB51F/IDBC1R	95°C - 5 min	94°C - 30 sec	65°C - 30 sec	72°C - 1min	35	72°C - 10 min
BIL-1/BIL-2	95°C - 5 min	94°C - 30 sec	50°C - 30 sec	72°C - 1min	35	72°C - 10 min
BiLONg-1/BiLONg-2	95°C - 5 min	94°C - 30 sec	56°C - 30 sec	72°C - 1min	35	72°C - 10 min
Bifidobacterium spp. BifTOT-F/BifTOT-R	95°C - 5 min	94°C - 30 sec	59°C - 30 sec	72°C - 1min	35	72°C - 10 min
Lactobacillus spp. F-Lac/R-Lac	95°C - 5 min	94°C - 30 sec	61°C - 30 sec	72°C - 1min	35	72°C - 10 min
Campylobacter spp. UC-Fw/UC-Rev	95°C - 5 min	94°C - 30 sec	62°C - 30 sec	72°C - 1min	35	72°C - 10 min
CampTOT-F/CampTOT-R	95°C - 5 min	94°C - 30 sec	61°C - 30 sec	72°C - 1min	35	72°C - 10 min
Campylobacter jejuni cj hip	94°C - 2 min	66°C-1 min	66°C - 1min	72°C - 1min	30	72°C - 3min
HIPj-Fw/HIPj-Rev	95°C - 5 min	94°C - 30 sec	62°C - 30 sec	72°C - 1min	35	72°C - 10 min

6.5.3 Standard curve preparation

Standard curves were prepared amplifying DNA extracted from pure cultures with species- or genus-specific primers (tab. 6.1) (Verity Thermal Cycler, Applied Biosystems). The amplified products were purified with the NucleoSpin® Extract II Kit (Macherey Nagel, Cat. No. 740.609.250) and than spectrophotometrically quantified.(Biophotometer, Eppendorf-Italia, Milan, Italy). The results were converted into gene copy number per microlitre of the obtained standards. The PCR amplifications were also checked by 1.5% agarose gel electrophoresis. Then the standard curves were prepared making 10-fold dilutions of target amplicons. Qualitative PCR reactions were performed as described in paragraph 1.5.2.

6.5.4 qPCR

The assays were performed with a 20 µl PCR amplification mixture containing 10 µl of Fast SYBR® Green Master Mix (Applied Biosystems), optimized concentrations of primers (tab. 6.3), H_2O molecular grade and 2 µl DNA extracted from faecal samples at a concentration of 5ng/µl for all the assay except C. jejuni quantification. For pathogen quantification DNA extracted from faecal samples was not diluted. The primer concentrations were optimized through primer optimization matrices in a 48-well plate and evaluating the best Ct/ΔRn ratio. The data obtained are then transformed to obtain the number of bacterial cells/g faeces according with the rRNA copy number available at the rRNA copy number database (tab. 6.4)(Klappenbach *et al.*, 2001; Lee *et al.*, 2009). Equations and coefficients of determination for the different assays are reported in tab. 6.5.

TABLE 6.3. qPCR cycles and primers concentration for qPCR using SybrGreen chemistry

Taget Bacteria	Initial denaturation	Denaturation	Annealing temperature (°C)	N. cycles	Fw	Rev
Lactobacillus plantarum planF/pREV	95°C - 1min	95°C - 3 sec	60°C - 30 sec	40	250 nM	300 nM
Bifidobacterium longum IDB51F/IDBC1R	95°C - 1min	95°C - 3 sec	64°C - 30 sec	40	200 nM	300 nM
Bifidobacterium spp. BifTOT-F/BifTOT-R	95°C - 1min	95°C - 3 sec	62.5°C - 35sec	40	200 nM	300 nM
Lactobacillus spp. F-Lac/R-Lac	95°C - 1min	95°C - 3 sec	62°C - 30 sec	40	200 nM	200 nM
Campylobacter spp. UC-Fw/UC-Rev	95°C - 1min	95°C - 3 sec	60°C - 30 sec	40	200 nM	300 nM
Campylobacter jejuni HIPj-Fw/HIPj-Rev	95°C - 1min	95°C - 3 sec	62°C - 30 sec	45	200 nM	100 nM

TABLE 6.4 16S rDNA copy number of different genera and species

Genus- Species Targets	Primer Targets	Gene copy number mean*
Bifidobacterium spp.	16S rDNA	3,57
Bifidobacterium longum	16S rDNA	4
Lactobacillus spp.	16S rDNA	5,71
Campylobacter spp.	16S rDNA	2,92
Campylobacter jejuni	Hippuricase gene	1

* (Klappenbach *et al.*, 2001; Lee *et al.*, 2009)

TABLE 6.5 qPCR equations and R^2 for the different assay

Target	Equation	R^2
Lactobacillus spp.	Ct= -3.432x + 36.26	1
Bifidobacterium spp.	Ct= -3.462x + 39.915	0.998
Campylobacter spp.	Ct= -3.408x + 35.71	0.999
Lactobacillus plantarum	Ct= -3.752x + 37.594	0.997
Bifidobacterium longum	Ct= -3.38x + 38.175	0.98
Campylobacter jejuni	Ct= -3.674x + 40.732	0.994

6.6 Statistical analysis

Data were analysed by analysis of variance with the GLM procedure of SAS considering the control group, the two different treatments and three times of sampling (for the synbiotic trail only control group and synbiotic group were present). The statistical analysis was based on a Repeated Measures Analysis of Variance. The "polynomial" option used in the algorithm indicates that the transformation used to implement the repeated measures analysis is an orthogonal polynomial transformation. A contrast statement was applied to compare the different sampling times.
Moreover statistical significances were confirmed by comparing the different sampling times (T0-T1-T2) with *t*-test for each group, with SAS software using the MEANS procedure.

Chapter 7. In vitro analysis of microencapsulated bacteria survival in feed

The two microencapsulated microorganisms, *Bifidobacterium longum* subsp. *longum* (PCB 133) and *Lactobacillus plantarum* (PCS 20), provided by Probiotical s.r.l., were tested for their survival during a period of time. The analyses were performed in presence and in absence of poultry feed.

Aliquots of microencapsulated microorganisms were divided into different bags, with (50:50 wt/wt) and without feed, and the bags were kept open at room temperature.
At definite time (T0, T+7days, T+15days, T+30days and T+60days) these samples were processed as follows:
aliquots of microencapsulated products, mixed with feed or not, were suspended in Buffer Borate (pH 8.4) and shaken in a stomacher for some minutes; serial dilutions were prepared and then plated in TPY (*B. longum* subsp. *longum*) and MRS (*L. plantarum*).
Plates were incubated anaerobically at 37°C and plate count was performed after 48 h.

Chapter 8. In vitro screening of Bifidobacterium and Lactobacillus strains on B1OXI cell-line

8.1 Bacterial strains and growth condition

Three strains (tab. 8.1) of *Lactobacillus plantarum* (PCS 20, PCS 22 and PCS 25) isolated from cheese, (Department of Microbiology, Biochemistry and Biotechnology, Faculty of Agriculture, University of Maribor) were grown anaerobically in MRS broth (Merck, Darmstadt, Germany) at 37°C. *Lactobacillus rhamnosus* LGG was grown anaerobically in MRS broth (Merck, Darmstadt, Germany) at 37°C. Strains of bifidobacteria (Bologna University Scardovi Collection of Bifidobacteria, University of Bologna, Italy) were grown anaerobically in TPY broth 37°C and are listed in Table 1. All strains were maintained at -20°C in 15% (v/v) glycerol (Merck).

Tab. 8.1 Bacterial strains used in this study

Strain	**Species**
B632	*Bifidobacterium breve*
B1412	*Bifidobacterium longum* subsp. *infantis*
B1975	*Bifidobacterium longum* subsp. *longum*
B2021	*Bifidobacterium breve*
B2055	*Bifidobacterium longum* subsp. *longum*
B2091	*Bifidobacterium bifidum*
B2101	*Bifidobacterium longum*
B2150	*Bifidobacterium breve*
B2192	*Bifidobacterium longum* subsp. *longum*
B2274	*Bifidobacterium breve*
B7003	*Bifidobacterium pseudocatenulatum*
B7740	*Bifidobacterium longum* subsp. *infantis*
B7947	*Bifidobacterium pseudocatenulatum*
B7958	*Bifidobacterium longum* subsp. *longum*
B8452	*Bifidobacterium longum* subsp. *longum*
PCB 133	*Bifidobacterium longum* subsp. *longum*

8.2 Growth and maintenance of the cell culture

The chicken intestinal cell line (B1OXI) was provided by Department of Microbiology, Biochemistry and Biotechnology, Faculty of Agriculture, University of Maribor, Slovenia (fig. 8.1). The cell line was routinely grown in advanced Dulbecco-modified Eagle's Medium (DMEM) (Sigma-Aldrich, Grand Island, USA), supplemented with 10% or 5% foetal calf serum (Lonza, Basel, Switzerland), L-glutamine (2 mmol/L, Sigma), penicillin (100 units/ml, Sigma) and streptomycin (1mg/ml, Fluka, Buchs, Switzerland) in 25 cm^2 culture flasks (Corning, New York, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air until a confluent monolayer was obtained.

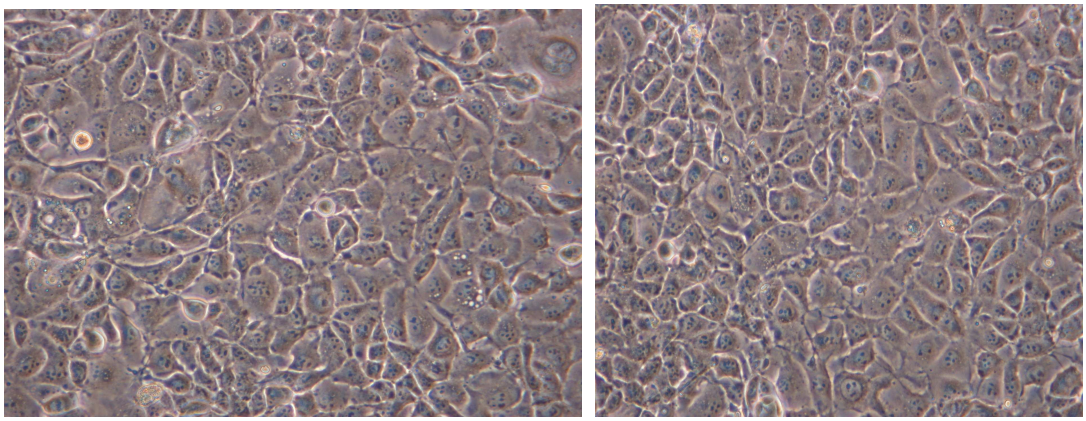


Fig. 8.1 B1OXI cell line (image kindly provided by Department of Microbiology, Biochemistry and Biotechnology, Faculty of Agriculture, University of Maribor, Slovenia)

8.3 Cytotoxicity of putative probiotic bacteria

To test cytotoxic activity exerted by bacteria on cell monolayer cells were seeded at $1x10^6$ cell/ml using 96 well/plates. When confluent monolayers were attained cells were washed twice with PBS to remove remnant traces of antibiotics and exposed to serial dilutions of bacterial strains from $1x10^8$ to $1x10^4$ cfu/ml and then incubated at 37°C for 90 minutes in a humidified atmosphere of 5% CO_2 and 95% air. After incubation the supernatants of the strains were collected, serially diluted and plated on agar plates to determine the amount of bacteria present in the suspensions.

The monolayers were washed with PBS to remove the excess of bacteria and the plates were fixed and stained with crystal violet (0.01%) in ethanol, rinsed with water, dried at 55°C and dissolved with 10% (v/v) acetic acid. Photometric quantitative detection of crystal violet previously retained in living cells was measured spectrophotometrically at 595 nm with a microplate reader (Multiskan, Thermo Electron Oy, Vaanta, Finland).

8.4 Determination of metabolic activity (mitochondrial activity) of cell culture exposed to probiotics

The metabolic activity of B1OXI was measured by the MTT assay. The protocol is based on tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction by metabolically active cells that result in the intracellular purple formazan (N'-amino-N-iminomethanimidamide), which can be solubilized and quantified by a spectrophotometer. Bacterial pellet was resuspended in cell growth media without phenol red and supplements in indicated concentrations (from 1x108 to 1x104 cfu/ml) and seeded onto washed (2X) confluent monolayers of B1OXI.

Cell monolayers with the added probiotic suspension were incubated for 90 min at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After incubation the monolayer was washed carefully with PBS and 200 µl of DMEM without phenol red and supplements were added in each well together with 20 µl of MTT solutions, and then incubated 75 min. Solubilization of formazan was achieved after addition of 0.04% HCl in isopropanol. The optical density was measured at 570 nm using a microplate reader (Multiskan, Thermo Electron Oy, Vaanta, Finland).

8.5 Determination of H_2O_2

The bacterial pellet was resuspended in cell growth media without phenol red and supplements in indicated concentrations and added to washed (2X) confluent monolayers of B1OXI in 96-well plates as described above. Monolayers treated with the bacterial suspension were incubated for 90 min at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

The release of H_2O_2 was determined transferring 50 µl of supernatant in a new 96-well plate and by the addition of 50 µl 0.01% peroxidase and 100 µl TMB solution (diluted with distillated water (1:1)). After 15 min incubation at room temperature optical density was measured at 450 nm by use of microplate reader (Multiskan, Thermo Electron Oy, Vaanta, Finland).

8.6 Adhesion ability of putative probiotic bacteria

Adhesion assay was performed on B1OXI cell line monolayer in a 96-well plate. Bacteria were resuspended in DMEM as described above at a concentration of 10^8 cfu/ml, seeded on cell monolayer and incubated 90 min at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Supernatants were then collected, serially-diluted and plated on agar plates.
Monolayer was washed with PBS and trypsinised. Trypsinisation was stopped after 5 min by adding 80 µl DMEM without phenol red and with 5% of FCS. The percentage of adherent bacteria was determined by agar plate counts of serially-diluted suspensions plated on MRS agar plates for lactobacilli and on MRS agar supplemented with 0.5% cystein for bifidobacteria strains. Plates were incubated 24 h at 37°C in anaerobiosis.

8.7 Statistical analysis

Analysis of numeric value was done by unpaired Student's t test. A *p*-value < 0.05 was considered significant for all assays. All results are expressed by the mean ratios (%, ± SD) of absorbance in treated wells as compared to those in negative control wells.

PART 3: RESULTS

Chapter 9. In vivo trials

9.1 Protocol Optimization

9.1.1 DNA extraction

Three different DNA extraction methods were used to evaluate the best protocol to process faecal samples. Two examples of DNA spectrophotometric values are reported in table 9.1, comparing the three different methods. Considerations on extraction tests could be summarized as follow:

1) kits (QIAamp DNA Stool Mini Kit [Qiagen] and Ultra Clean Faecal DNA kit [MoBio] were clearly faster methods than benzyl chloride extraction protocol, the DNA A_{260}/A_{280} ratio was satisfactory in the main, but they provided less concentrated DNA samples, compared to benzyl chloride, in a final volume of 200 μl TE using 200 mg of faeces;

2) benzyl chloride protocol gave a higher DNA concentration than kits in a final elution volume of 100 μl TE using fewer faecal material (30 mg) but this method was time consuming. Moreover benzyl chloride is a toxic substance (R40-R45-R48) and the protocol implied the use of liquid N_2.

For these reasons the use of a DNA extraction kit was considered the most convenient method. QIAamp DNA Stool Mini Kit (Qiagen West Sussex, UK) particularly provided higher DNA concentration and purity compared to Ultra Clean Faecal DNA kit (MO BIO, Cat. No. 12811-S).

Tab. 9.1 Comparison of three extraction protocols

	QIAamp DNA Stool Mini Kit		Ultra Clean Faecal DNA kit		Benzyl Chloride extraction	
	A_{260}/A_{280}	[DNA] μg/ml	A_{260}/A_{280}	[DNA] μg/ml	A_{260}/A_{280}	[DNA] μg/ml
Sample 1	2,094	165,03	0,593	77,808	2,021	134,58
Sample 2	1,9	92,238	1,789	14,22	2,08	155,23

9.1.2 Qualitative PCR

Bifidobacterium spp. and Lactobacillus spp.

Primers BifTOT-F/BifTOT-R (Rinttilä, 2004) and F-Lac/R-Lac (Castillo, 2006) used to amplify respectively *Bifidobacterium* spp. and *Lactobacillus* spp. gave good amplification reactions in qualitative PCR as shown in fig. 9.1 and 9.2.

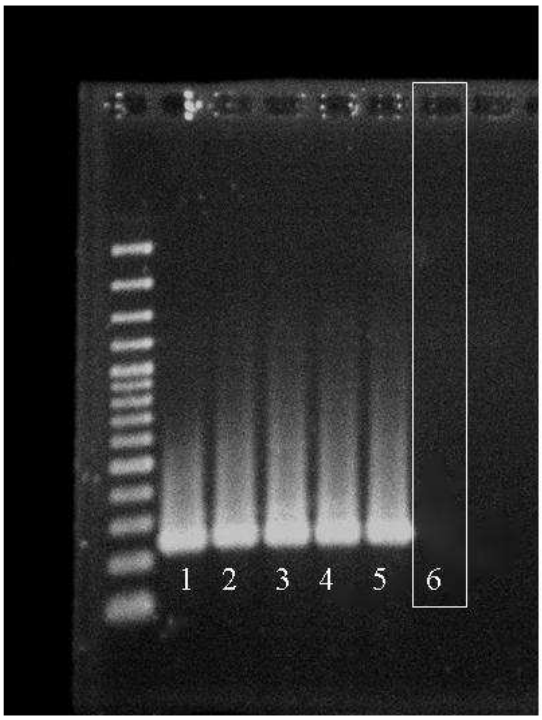


Fig. 9.1 Primers BifTOT-F/BifTOT-R. 1) *B. longum* subsp. *suis* ATCC 27533^{T}, 2) *B. longum* subsp. *infantis* ATCC 15697^{T}, 3) *B. longum* susp. *longum* ATCC 15707^{T}, 4) *B. longum* subsp. *longum* PCB 133, 5) *B. animalis* subsp. *lactis* Ra18, 6) Master Mix negative control.

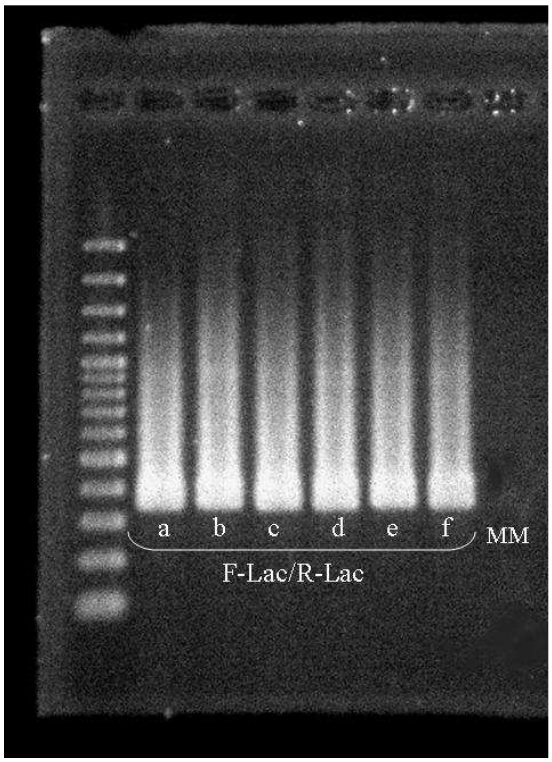


Fig. 9.2 Primers F-Lac/R-Lac. a-e)Amplification of DNA extracted from random faecal samples, f) amplification of DNA extracted from *L. plantarum* PCS 20 pure culture. MM: master mix negative control.

Lactobacillus plantarum

The DNA extracted from *Lactobacillus plantarum* PCS 20 was amplified with planF/pREV primers (Torriani *et al*., 2001). The amplification reaction was visualized with gel electrophoresis (fig. 9.3). The specificity of this primer set was also tested on *L- pentosus* (*L. plantarum* closely related species) and primers do not cross-react with this species (data not shown). In fig.9.3 (b) it is also possible to see the purified amplicon of this primers set, used in real time PCR to construct the standard curve.

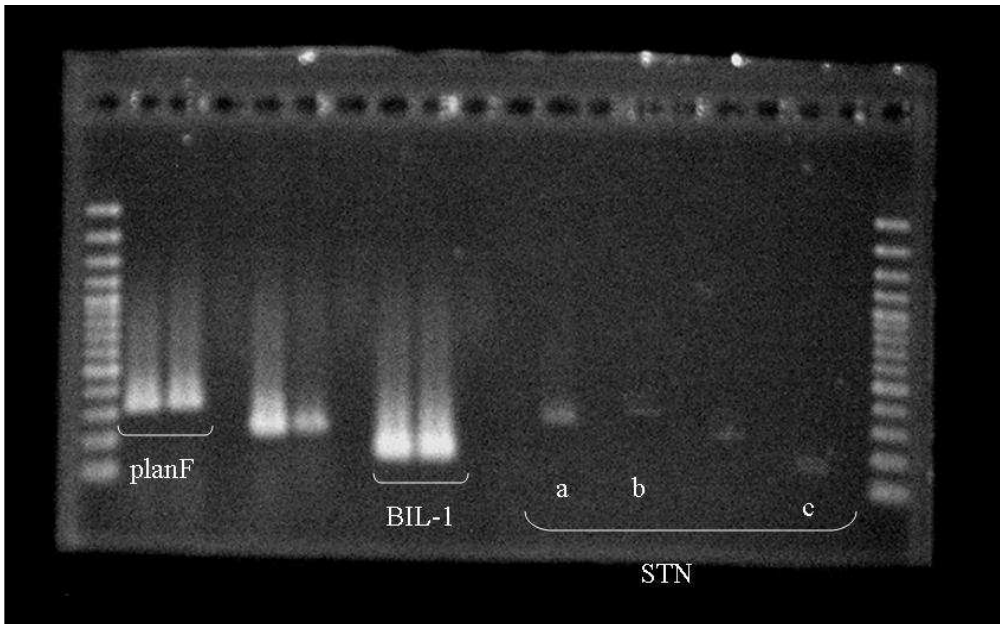


Fig. 9.3 PlanF: amplification of *L. plantarum* PCS 20 with planF/pREV primers. BIL-1: amplification of *B. longum* subsp. *longum* PCB 133 with BIL-1/BIL-2 primers. a) BiLONg-1/BiLONg-2 standard, b) planF/pREV standard and c) BIL-1/BIL-2 standard - purification of the amplicons obtained with the listed primers and visualization on agarose-gel.

Bifidobacterium longum

Three different sets of primers have been used in qualitative PCR to test the specificity of *B. longum* available primers towards different bifidobacteria species.

Primer set BiLONg-1/BiLONg-2 shows a lack of specificity for some *Bifidobacterium longum* related species (fig. 9.4)

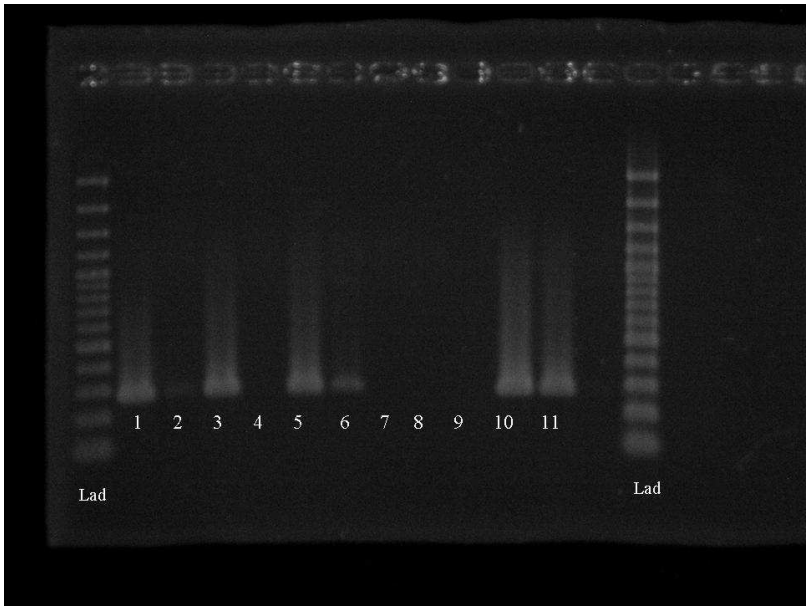


Fig.9.4 Primer BiLONg-1/BiLONg-2. Lad) ladder, 1) *B. longum* subsp. *longum* PCB 133, 2) *B. animalis* susp. *lactis* Ra18, 3) *B. longum* subsp. *longum* ATCC 15707^{T}, 4) *B. pseudolongum* DSM 20094 (isolated from chicken), 5) *B. longum* subsp. *infantis* ATCC 15697^{T}, 6) *B. pseudolongum* ATCC 25526^{T} (isolated from pig), 7-8-9) Master mix (negative control), 10-11) *B. longum* subsp. *longum* PCB 133.

BIL-1/BIL-2 primers also showed cross-reactions with some bifidobacteria strains that do not belong to the species *B. longum* as shown in fig. 9.5.

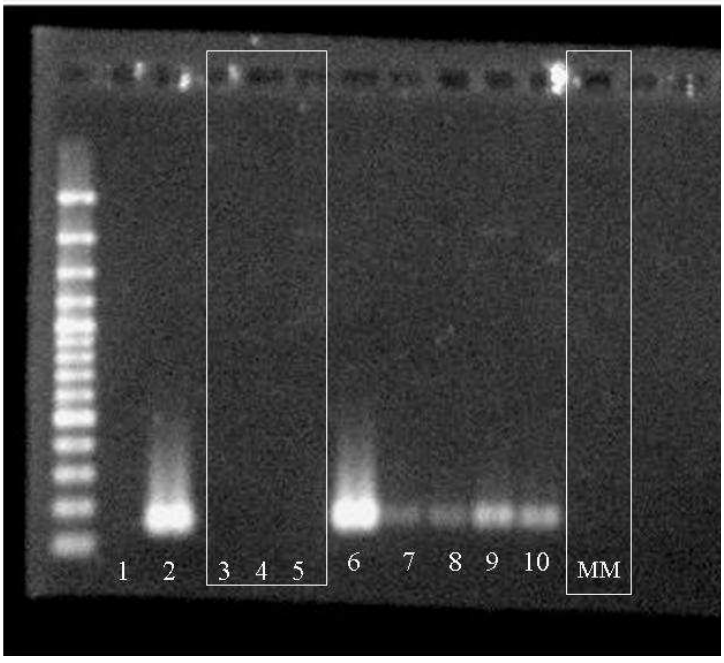


Fig. 9.5 Primer BIL-1/BIL-2. 1) *B. pullorum* ATCC 27685^{T}, 2) *B. longum* subsp. *longum* PCB 133, 3) *B. gallinarum* ATCC 33777^{T}, 4) *Bifidobacterium animalis* subsp. *lactis* P32, 5) *B. pseudolongum* subsp. *pseudolongum* DSM 20094, 6) *B. longum* susp. *longum* ATCC 15707^{T}, 7) *B. animalis* subsp. *lactis* Ra18, 8) *B. longum* subsp. *suis* ATCC 27533^{T}, 9) *B. pseudolongum* subsp. *pseudolongum* ATCC 25526^{T}, 10) *B. animalis* subsp. *lactis* DSM 10140^{T}, MM) Master Mix negative control.

Primer IDB51F/IDBC1R seemed to be the best candidate primers for real-time PCR reactions because do not cross react with other species in qualitative PCR (fig. 9.6 and 9.7). The annealing temperature is high (65°C) and probably this increase the specificity of the assay.

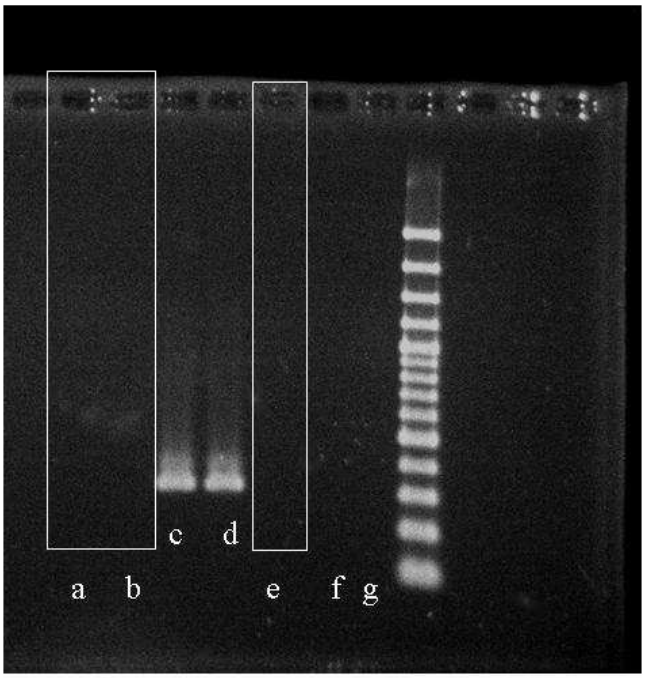


Fig. 9.6 Primer IDB51F/IDBC1R. a) *B. longum* subsp. *suis* ATCC 27533^{T}, b) *B. longum* subsp. *infantis* ATCC 15697^{T}, c) *B. longum* subsp. *longum* ATCC 15707^{T}, d) *B. longum* subsp. *longum* PCB 133, e) *B. animalis* subsp. *lactis* Ra18, f-g) Master Mix negative control.

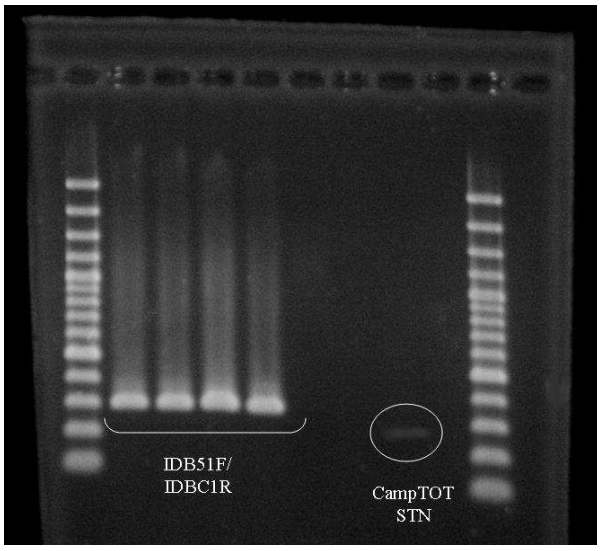


Fig. 9.7 Primers IDB51F/IDBC1R DNA amplification of *B. longum* subsp. *longum* PCB 133 pure culture. CampTOT stn: purification of the amplicon obtained with CampTOT primers and visualization on agarose-gel.

Campylobacter spp and Campylobacter jejuni

Two different primer sets were used to amplify *Campylobacter* spp. Both UC primers and CampTOT gave clear amplification reactions as visualized on agarose gel (fig. 9.8 and 9.9). The purified standards (fig. 9.7 and 9.9) also gave an evident single band on agarose gel. Both primers have been used in real time PCR to design an appropriate assay to quantify *Campylobacter* spp. (as described in section 9.2).
Campylobacter jejuni DNA was amplified using primers targeted to hippuricase gene. The hippuricase (or hippurate hydrolase or benzoyl-glycine aminohydrolase) is an enzyme that hydrolyzes hippurate to form benzoate and glycine. Among thermophilic *Campylobacter* only *C. jejuni* possesses the hippuricase gene.

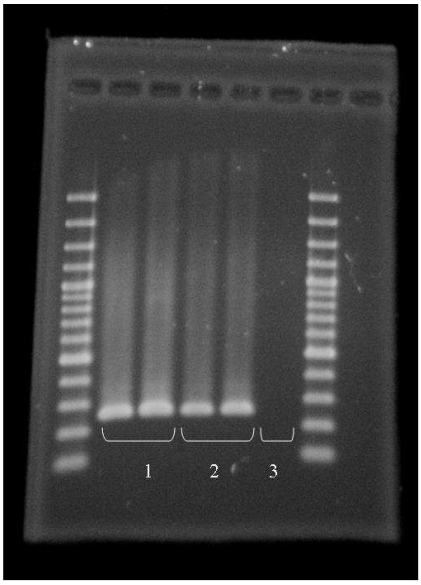


Fig. 9.8 CampTOT-F/CampTOT-R. 1) *C. jejuni* subsp. *jejuni* CIP 70.2^{T}, 2) *C. jejuni* subsp. *jejuni* ATCC 29428, 3) Master mix, negative control.

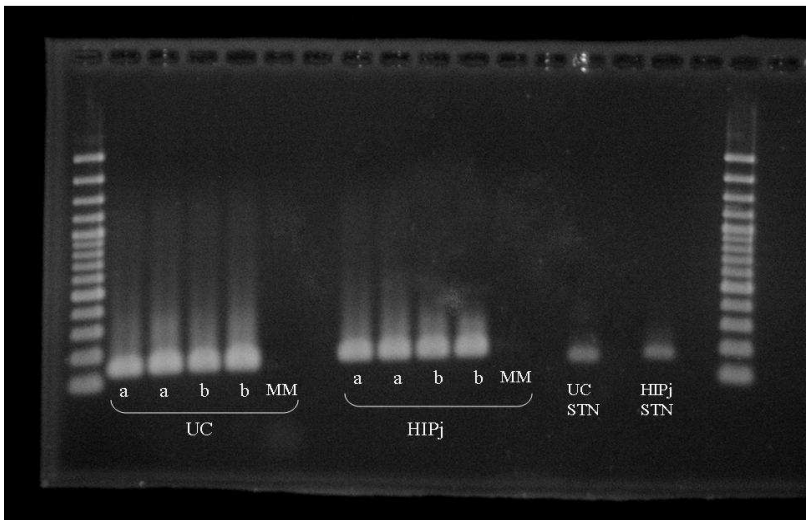


Fig. 9.9 Primers UC-Fw/UC-Rev (UC) and HIPj-Fw/HIPj-Rev. (HIPj) a) C. *jejuni* subsp. *jejuni* CIP 70.2^{T}, b) *C. jejuni* ATCC 29428. UC STN and HIPj STN: purification of the amplicons obtained with UC and HIPj primers and visualization on agarose-gel.

9.2 qPCR

9.2.1 Probiotic trial

Two different probiotic bacteria (*B. longum* subsp. *longum* PCB 133 and *L. plantarum* PCS 20) were chosen for their antimicrobial activity against *C. jejuni* for an *in vivo* experiment on broiler chickens (Santini et al., submitted).. Bacteria (~ 10^8 cfu) were administered orally from frozen culture in skim milk solution through oral gavage for 15 days. Control group (CTR) was also administered with 1 ml of skim milk solution through oral gavage, in order to .have comparable conditions for all animals. Faecal samples were collected from ten animals in each group before starting supplementation, after 15 days of probiotic administration and also after 21 days, *i.e.* one week after stopping probiotic intake (washout period). Faecal samples were processed to quantify specific bacterial group or species, first of all to quantify the probiotic bacteria administered.
Temperature and relative humidity of the room were monitored along the entire period and reported in table 9.2.

TABLE 9.2 Environmental conditions during probiotic trial

Temperature	**Relative Humidity**
N. observations: 509	N. observations: 509
Observation interval: 1 h	Observation interval: 1 h
Higher °T: 36.77 °C	Higher relative humidity: 64.55%
Lowest °T: 21.18 °C	Lowest relative humidity: 20.62%
Mean °T: 29.00 °C	Mean relative humidity: 41.04%
SD: 3.58 °C	SD: 9.21%

Throughout the feeding trials all the chickens were healthy. There were no signs of diarrhoea, weight loss or loss of appetite.
Animal weight was recorded for the 16 animals in each treated group and in control group before starting treatment, after 15 days of supplementation and at the end of the experiment as shown in tables 9.3 to 9.5 and figures 9.10 to 9.12.

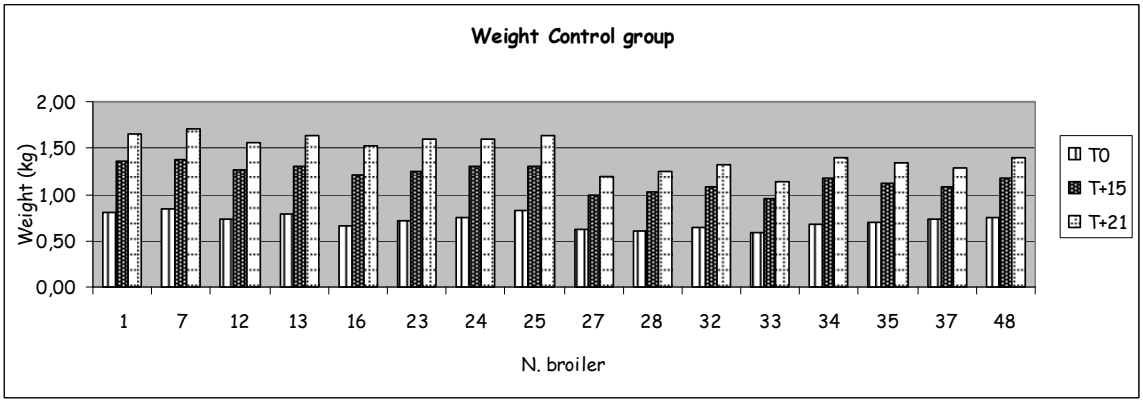


Fig. 9.10 Weight of 16 broilers of the control group at time zero (T0), after 15 days of probiotic administration (T+15) and after 21 days (T+21). Chickens N.1 to N.25 are male; chickens N.27 to N.48 are female.

TABLE 9.3 Weight of 16 broilers of the control group at time zero (T0), after 15 days (T+15) and after 21 days (T+21)

	N. animal	Sex	T0	T+15	T+21
Group 1 - Control group	1	M	0,81	1,35	1,66
	7	M	0,85	1,38	1,70
	12	M	0,74	1,27	1,56
	13	M	0,78	1,31	1,64
	16	M	0,66	1,21	1,52
	23	M	0,72	1,25	1,59
	24	M	0,76	1,31	1,60
	25	M	0,83	1,30	1,64
	27	F	0,63	0,99	1,20
	28	F	0,61	1,03	1,24
	32	F	0,65	1,09	1,32
	33	F	0,59	0,95	1,13
	34	F	0,68	1,18	1,39
	35	F	0,70	1,12	1,34
	37	F	0,73	1,08	1,28
	48	F	0,76	1,17	1,40
Sum			**11,50**	**18,99**	**23,21**
Mean			**0,719**	**1,187**	**1,451**
SD			**0,078**	**0,133**	**0,185**

TABLE 9.4 Weight of 16 broilers administered with *L. plantarum* PCS 20 at time zero (T0), after 15 days of probiotic administration (T+15) and after 21 days (T+21)

	N. animal	Sex	T0	T+15	T+21
Group 2- PCS 20	2	M	0,82	1,33	1,59
	3	M	0,84	1,41	1,72
	5	M	0,74	1,27	1,55
	9	M	0,75	1,30	1,61
	10	M	0,78	1,34	1,57
	14	M	0,86	1,39	1,59
	19	M	0,78	1,35	1,61
	20	M	0,66	1,10	1,34
	31	F	0,64	1,11	1,34
	36	F	0,79	1,24	1,45
	38	F	0,68	1,13	1,34
	39	F	0,61	1,02	1,19
	40	F	0,59	0,99	1,16
	42	F	0,72	1,24	1,41
	46	F	0,73	1,20	1,39
	49	F	0,65	1,06	1,26
Sum			**11,64**	**19,48**	**23,12**
Mean			**0,728**	**1,218**	**1,445**
SD			**0,083**	**0,135**	**0,167**

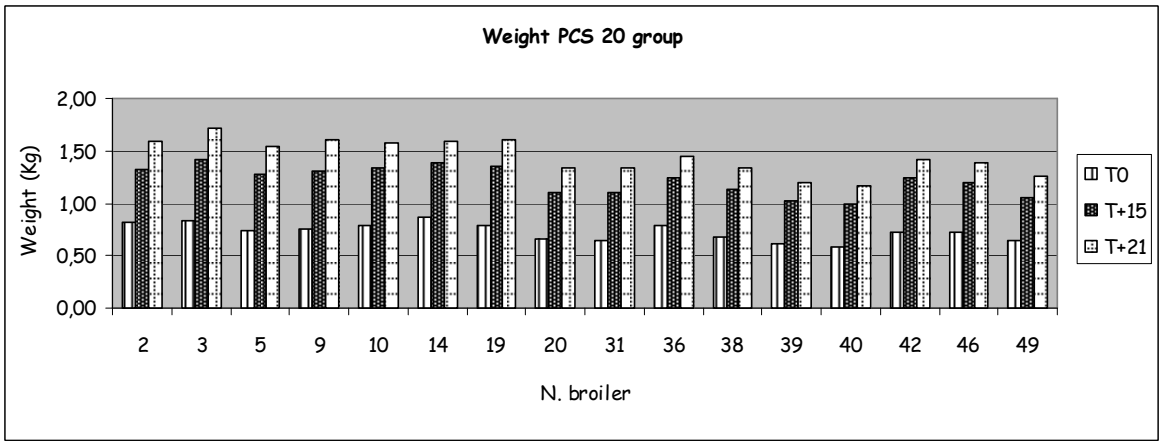


Fig. 9.11 Weight of 16 broilers administered with *L. plantarum* PCS 20 at time zero (T0), after 15 days of probiotic administration (T+15) and after 21 days (T+21). Chickens N.2 to N.20 are male; chickens N.31 to N.49 are female.

TABLE 9.5 Weight of 16 broilers administered with *B. longum* subsp. *longum* PCB 133 at time zero (T0), after 15 days of probiotic administration (T+15) and after 21 days (T+21)

	N. animal	Sex	T0	T+15	T+21
Group 3- PCB 133	4	M	0,83	1,40	1,70
	6	M	0,74	1,21	1,50
	11	M	0,78	1,34	1,62
	17	M	0,68	1,15	1,40
	18	M	0,80	1,33	1,53
	21	M	0,86	1,40	1,67
	22	M	0,76	1,33	1,59
	26	F	0,69	1,03	1,21
	30	F	0,68	1,15	1,34
	41	F	0,81	1,47	1,47
	43	F	0,72	1,14	1,33
	44	F	0,60	0,95	1,12
	45	F	0,64	1,08	1,30
	47	F	0,73	1,17	1,35
	50	F	0,62	1,06	1,25
	51	M	0,84	1,51	1,77
Sum			**11,78**	**19,72**	**23,15**
Mean			**0,736**	**1,233**	**1,447**
SD			**0,080**	**0,167**	**0,190**

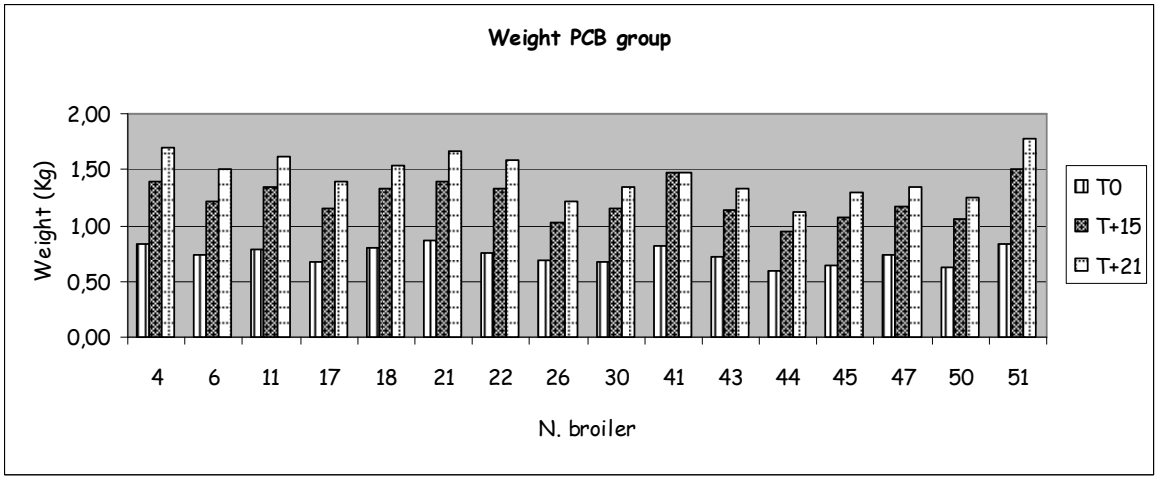


Fig. 9.12 Weight of 16 broilers administered with *B. longum* subsp. *longum* PCB 133 at time zero (T0), after 15 days of probiotic administration (T+15) and after 21 days (T+21). Chickens N.4 to N.22 and chicken N.51 are male; chickens N.26 to N.50 are female.

The statistical ANOVA analysis performed with GLM procedure with SAS software detected no significant weight differences in broiler groups. As can be clearly seen by bar-diagrams, there was a significant difference ($p<0.05$) in weight between male and female in every group, and the difference increased with time ($p<0.01$ at T+21)

The results of qPCR on *Lactobacillus plantarum* PCS 20 showed the absence of this microorganism in faeces at the detectable level of the assay.
Bifidobacterium longum, on the other hand, was present in all the broilers administered with PCB 133 (fig. 9.13), even if not in high number (tab. 9.6). The variation between T0 and T1 was significant ($p<0.01$) while the decrease between T1 and T2 was not. *Bifidobacterium longum* was absent in control group faecal samples (fig. 9.14) because this microorganism is a commensal species of the human intestinal microbiota.

Tab. 9.6 Mean values (log cfu/g faeces) of *Bifidobacterium longum* ± SEM

	T0	**T1**	**T2**
CTR	0	0	0
PCB	0	4.27 ± 0.07	3.88 ± 0.19

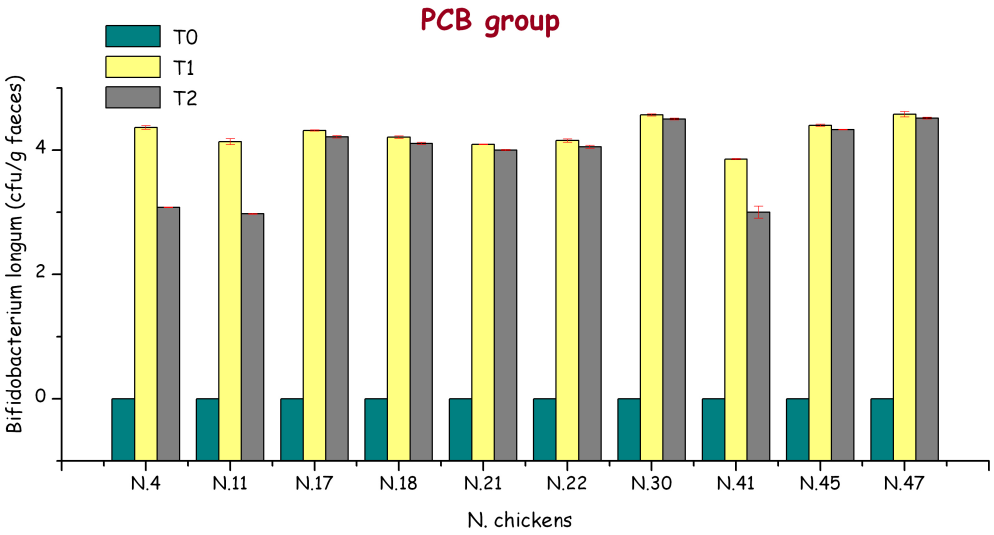


Fig. 9.13 *Bifidobacterium longum* quantification in faecal samples of the PCB group.

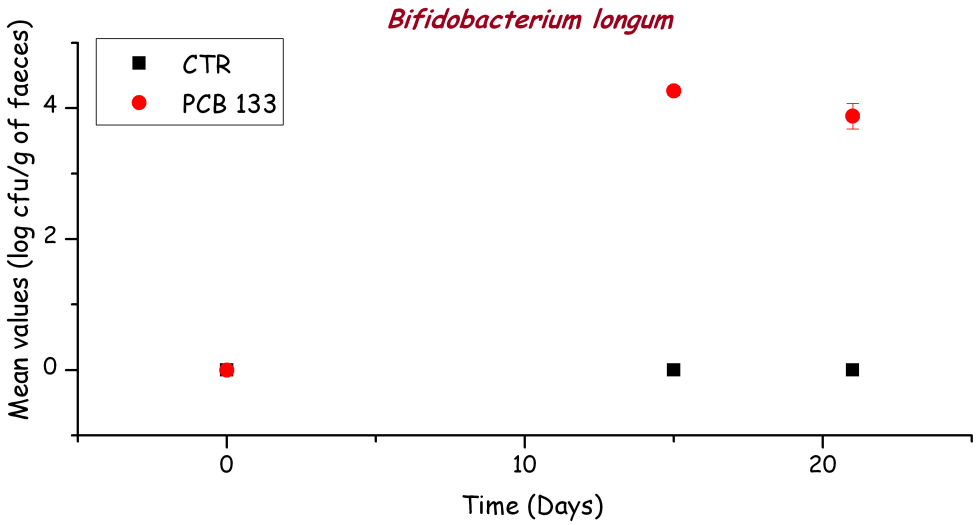


Fig. 9.14 Mean values comparison for *Bifidobacterium longum* in control group and PCB 133 group.

Bifidobacterium spp. were quantified in control group and PCB 133 administered group. The ANOVA analysis showed no significant variations of *Bifidobacterium* spp. in both groups (fig. 9.15 to 9.17 and tab. 9.7).

Tab. 9.7 Mean values (log cfu/g faeces) of *Bifidobacterium* spp. ± SEM

	T0	**T1**	**T2**
CTR	7.18 ± 0.11	6.83 ± 0.22	7.21 ± 0.16
PCB	7.19 ± 0.10	7.12 ± 0.17	7.19 ± 0.13

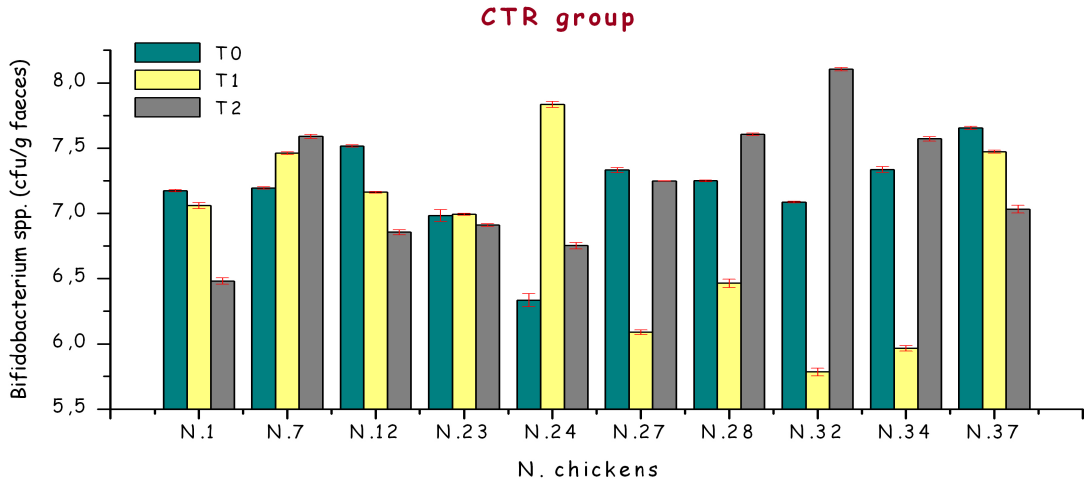


Fig. 9.15 *Bifidobacterium* spp. quantification in faecal samples of the control group.

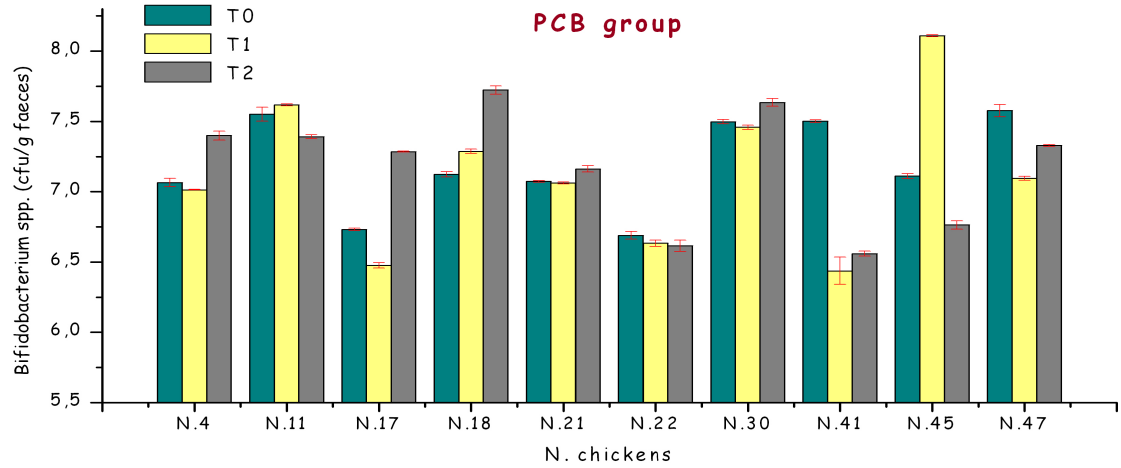


Fig. 9.16 *Bifidobacterium* spp. quantification in faecal samples of the PCB 133 group.

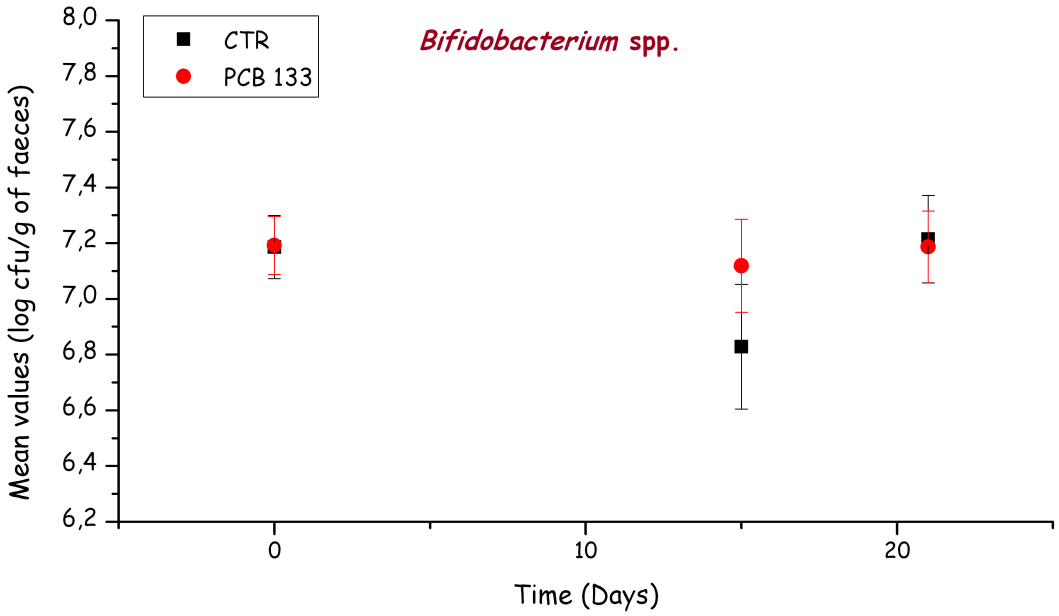


Fig. 9.17 Mean values comparison for *Bifidobacterium* spp. in control group and PCB 133 group.

Campylobacter spp. in the PCB group remained stable during the experiment as shown in tab. 9.8. and fig. 9.18 and 9.19. In the control group *Campylobacter* slightly decreased between T0 and T1 and then increased significantly at T2 (compared with T0, $p<0.05$) (fig. 9.20).

Tab. 9.8 Mean values (log cfu/g faeces) of *Campylobacter* spp. ± SEM

	T0	**T1**	**T2**
CTR	7.27 ± 0.15	6.87 ± 0.25	7.51 ± 0.05
PCB	7.33 ± 0.18	7.10 ± 0.31	7.32 ± 0.07

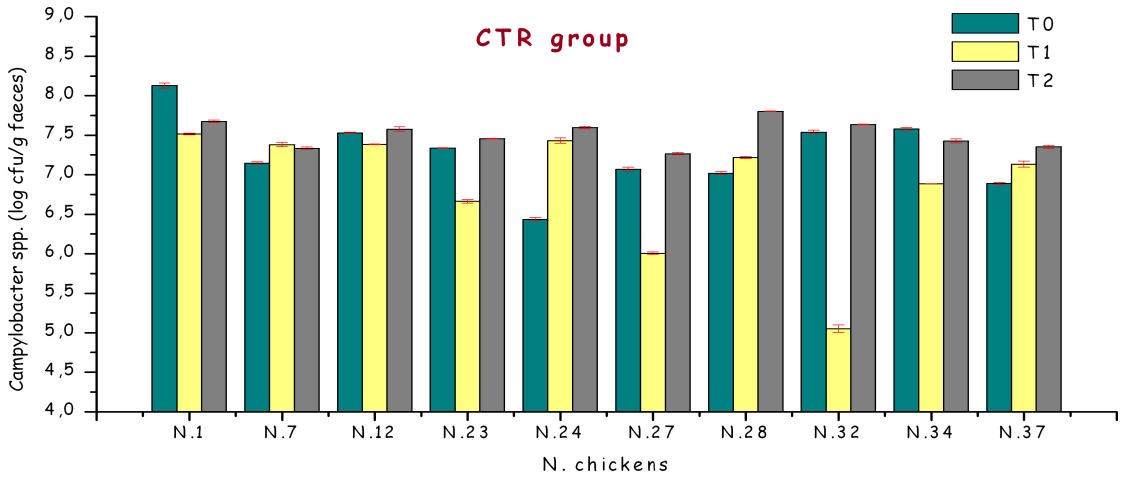


Fig. 9.18 *Campylobacter* spp. quantification in faecal samples of the control group.

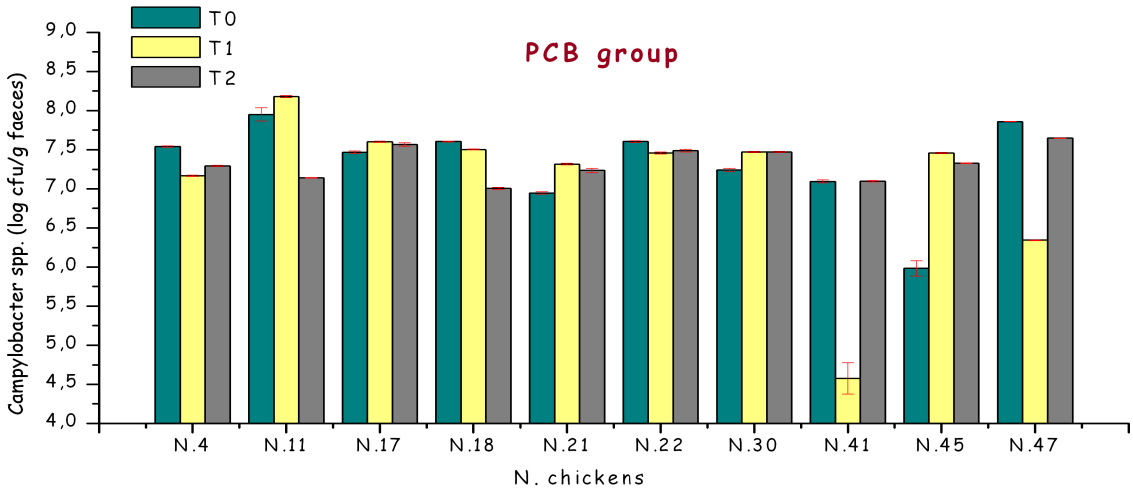


Fig. 9.19 *Campylobacter* spp. quantification in faecal samples of the PCB 133 group.

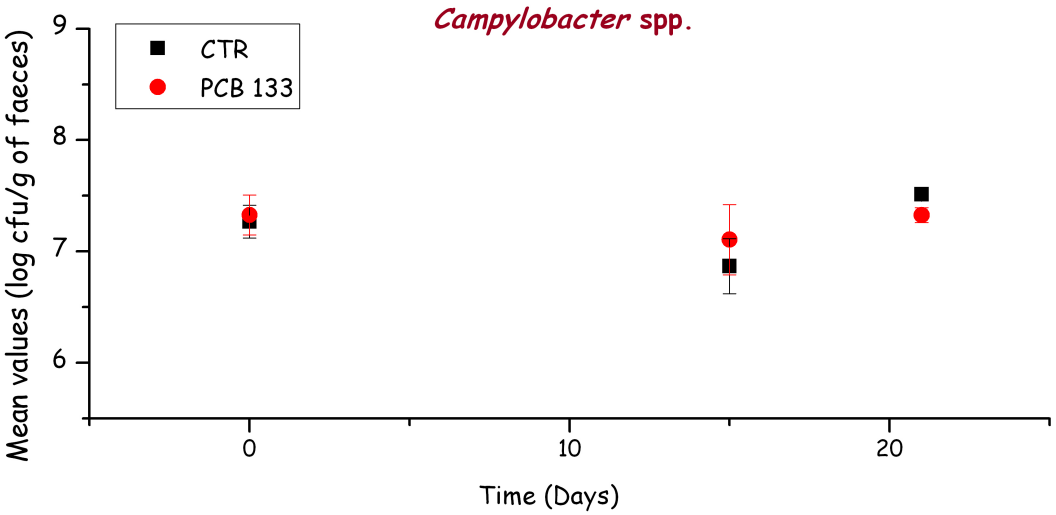


Fig. 9.20 Mean values comparison for Campylobacter spp. in control group and PCB 133 group.

ANOVA analysis on *Campylobacter jejuni* quantification showed a significant time*treatment interaction (p<0.05). *C. jejuni* decreased in treated group after 15 days of probiotic supplementation and continued to decrease after stopping the administration (not significantly) (fig.9.22 and 9.23). The pathogenic microorganism had not significant variations in control group (tab. 9.9 and fig. 9.21).

Tab. 9.9 Mean values (log cfu/g faeces) of *Campylobacter jejuni* spp. ± SEM

	T0	**T1**	**T2**
CTR	4.42 ± 0.15	4.28 ± 0.13	4.30 ± 0.12
PCB	4.87 ± 0.19	4.16 ± 0.26	4.04 ± 0.18

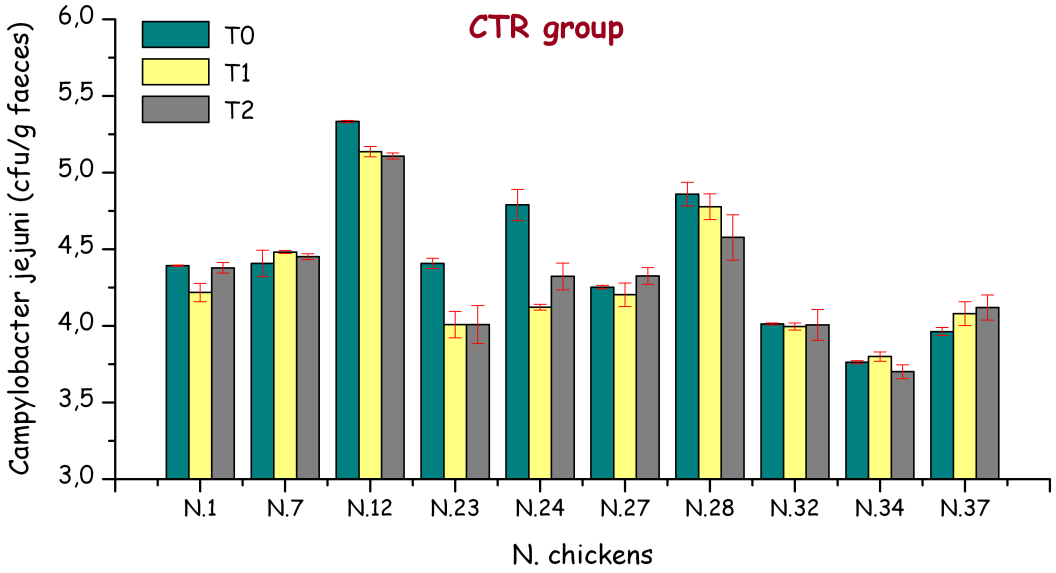


Fig. 9.21 *Campylobacter jejuni* quantification in faecal samples of the control group.

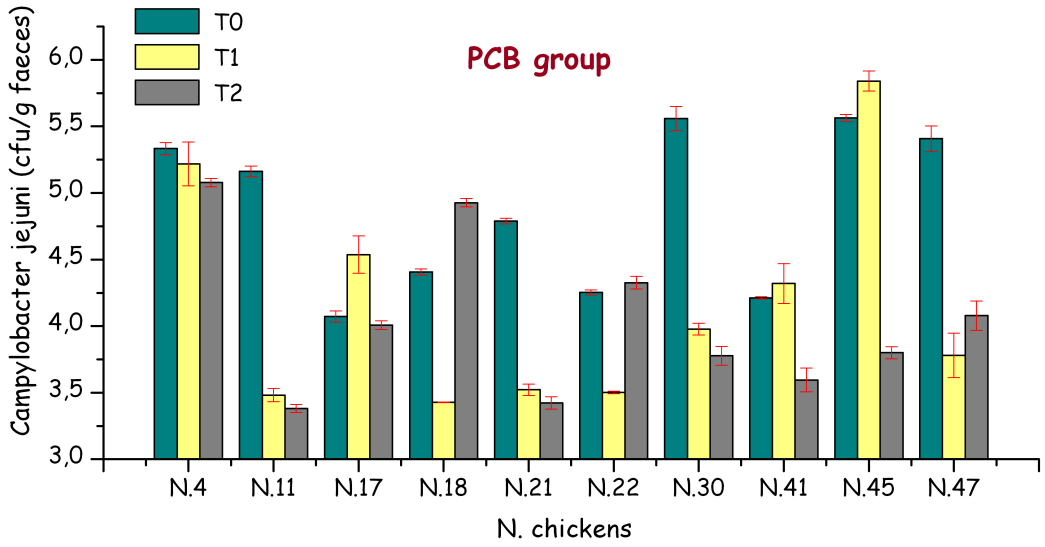


Fig. 9.22 *Campylobacter jejuni* quantification in faecal samples of the PCB 133 group.

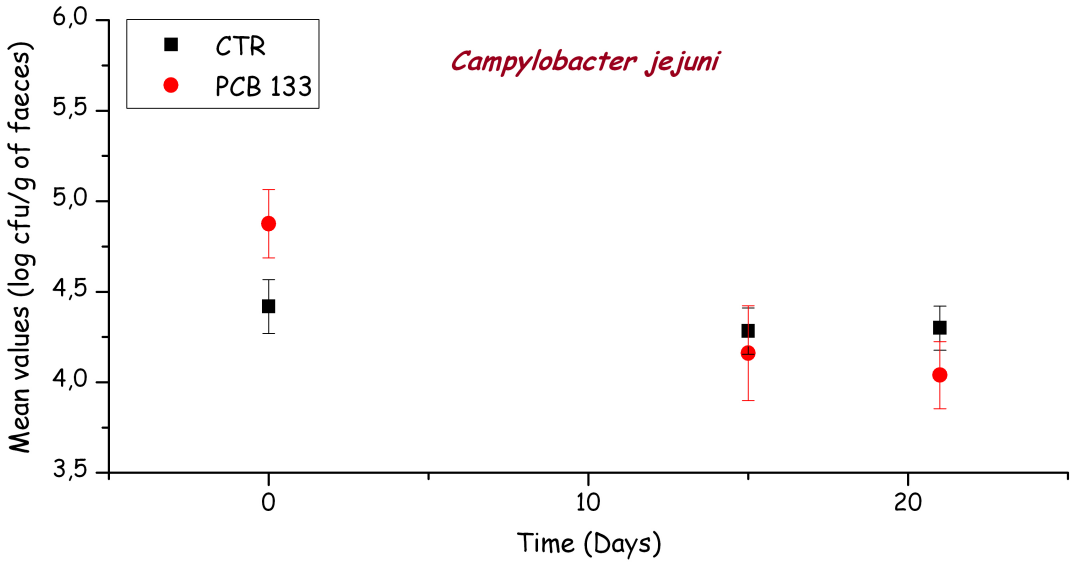


Fig. 9.23 Mean values comparison for *Campylobacter jejuni* in control group and PCB 133 group.

9.2.2 Prebiotic trial

Two different prebiotic compounds, a fructololigosaccharide (FOS) and a galactooligosaccahride (GOS) were administered at a dose respectively of 0.5% and 3% to broiler chickens for 15 days, mixed with poultry normal feed. Faecal samples were collected from 10 animals in each group before starting supplementation, after 15 days of prebiotic administration and also after 21 days, *i.e.* one week after stopping prebiotic intake. Faecal samples were processed to quantify specific bacterial groups or species.

Room temperature was monitored along the entire period and values are reported in table 9.10.

TABLE 9.10 Environmental conditions during probiotic trial

Temperature
N. observations: 8116
Observation interval: 5 min
Higher °T: 19.46 °C
Lowest °T: 8.02 °C
Mean °T: 13.695 °C
SD: 2,095 °C

Throughout the feeding trials all the chickens were healthy. There were no signs of diarrhoea, weight loss or loss of appetite. Animal weight was recorded for all 42 animals before starting treatment and at the end of the trial (T+21) (table 9.11 to 9.13 and fig.9.24 to 9.26).

TABLE 9.11 Weight of 14 broilers of the control group at time zero (T0) and after 21 days (T+21)

	N. animal	Sex	T0	T+21
Group 1- Control Group	75	M	1,21	1,71
	77	M	1,17	1,86
	69	M	0,94	1,49
	63	M	0,94	1,53
	76	M	0,93	1,49
	80	M	0,91	1,28
	42	F	0,91	1,30
	56	F	0,87	1,26
	53	F	0,87	1,28
	57	F	0,85	1,22
	41	F	0,85	1,24
	45	F	0,84	1,26
	62	F	0,64	0,94
	47	F	0,49	0,40
Sum			**12,42**	**18,26**
Mean			**0,887**	**1,304**
SD			**0,179**	**0,346**

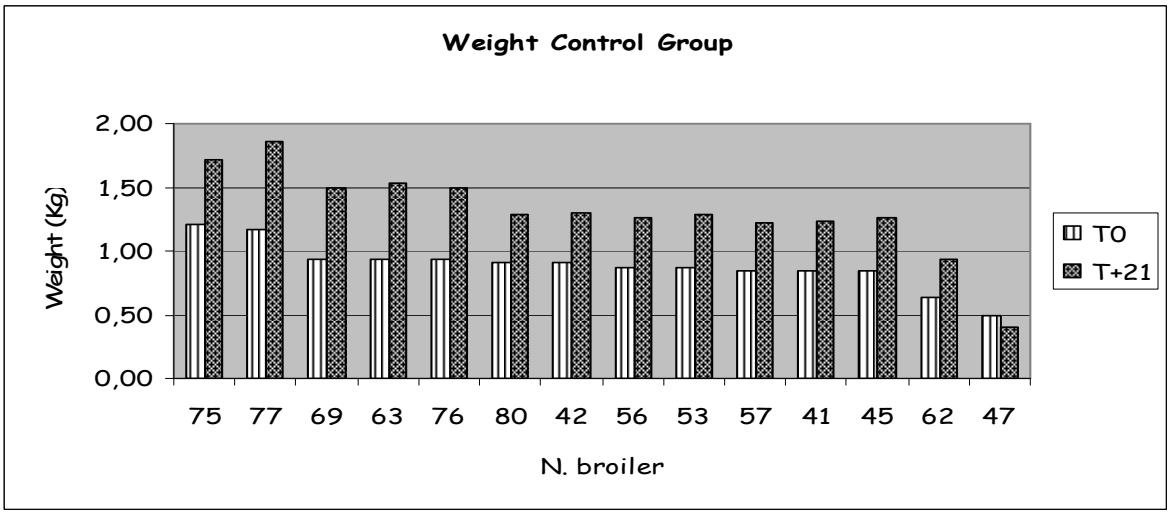


Fig. 9.24 Weight of 14 broilers of control group at time zero (T0) and after 21 days (T+21). Chickens N.75 to N.80 are male; chickens N.42 to N.47 are female.

TABLE 9.12 Weight of 14 broilers administered with FOS at time zero (T0) and after 21 days (T+21).

	N. animal	Sex	T0	T+21
Group 2- FOS Group	43	F	0,81	1,17
	44	F	0,80	1,15
	46	F	0,75	1,13
	49	F	0,81	1,16
	55	F	0,83	1,24
	58	F	0,77	1,08
	61	F	0,74	1,10
	65	M	1,04	1,55
	66	M	1,03	1,56
	67	M	1,06	1,71
	68	M	0,97	1,54
	70	M	0,97	1,55
	71	M	1,05	1,62
	73	M	0,98	1,49
Sum			**12,61**	**19,05**
Mean			**0,901**	**1,361**
SD			**0,123**	**0,230**

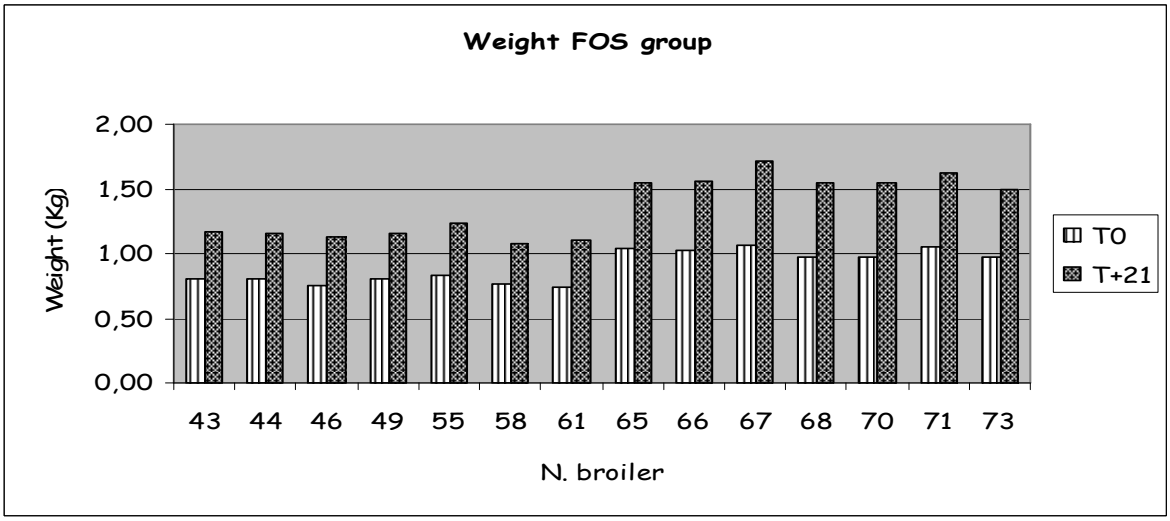


Fig. 9.25 Weight of 14 broilers administered with FOS at time zero (T0) and after 21 days (T+21). Chickens N.43 to N.61 are female; chickens N.65 to N.73 are male.

TABLE 9.13 Weight of 14 broilers administered with GOS at time zero (T0) and after 21 days (T+21).)

	N. animal	Sex	T0	T+21
Group 3- GOS Group	48	F	0,74	1,00
	50	F	0,83	1,12
	51	F	0,81	1,12
	52	F	0,83	1,19
	54	F	0,79	1,14
	59	F	0,82	1,19
	60	F	0,71	1,08
	64	M	1,00	1,58
	72	M	1,01	1,65
	74	M	1,01	1,50
	78	M	0,99	1,54
	79	M	1,08	1,58
	81	M	1,01	1,59
	82	M	1,00	1,60
Sum			**12,63**	**18,88**
Mean			**0,902**	**1,349**
SD			**0,122**	**0,244**

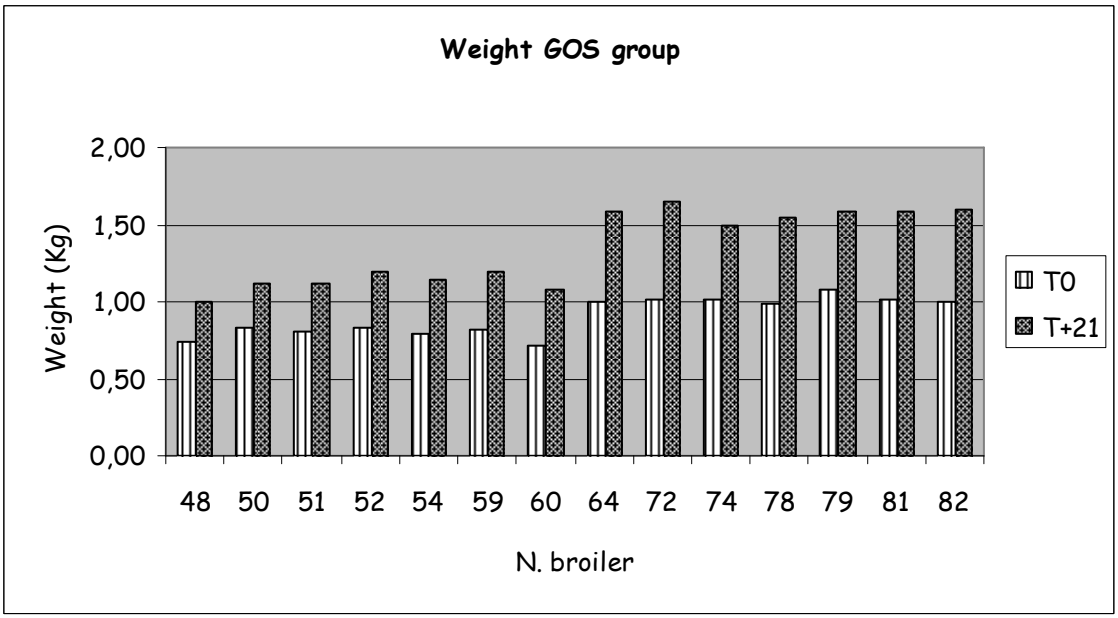


Fig. 9.26 Weight of 14 broilers administered with GOS at time zero (T0) and after 21 days (T+21). Chickens N.48 to N.60 are female; chickens N.64 to N.82 are male.

The statistical ANOVA analysis performed with GLM procedure with SAS software detected no significant weight differences in broiler groups. As can be clearly seen by bar-diagrams also in this trial there was a significant difference ($p<0.05$) in weight between male and female in every group, and differences increased with time ($p<0.01$ at T+21).

Lactobacillus spp.: the ANOVA analysis between groups showed that there was a significant time effect ($p<0.01$), *Lactobacillus* population varies with time in each treated group (FOS-GOS), but the interaction between time and treatment was not significant. However mean values (tab. 9.14) showed a slight decrease of *Lactobacillus* population after 21 days (T2) in all groups. The paired *t*-test evidences that this decrement is significant in GOS group ($p<0.05$) comparing T0-T2 (fig. 9.29 and 9.30), on the other hand in FOS treatment after 15 days an increase can be recorded in 7 out of 10 animals (fig. 9.28 and 9.30) with a paired *t*-test ($p<0.05$) but subsequently, after stopping supplementation, *Lactobacillus* population decreases reaching starting values. Variations in control group (CTR group) were not significant (fig. 9.27 and 9.30).

Tab. 9.14 Mean values (log cfu/g faeces) of *Lactobacillus* spp. ± SEM

	T0	**T1**	**T2**
CTR	7.85 ± 0.15	8.02 ± 0.17	7.73 ± 0.12
FOS	7.62 ± 0.1	7.97 ± 0.1	7.57 ± 0.15
GOS	7.94 ± 0.17	7.88 ± 0.1	7.54 ± 0.18

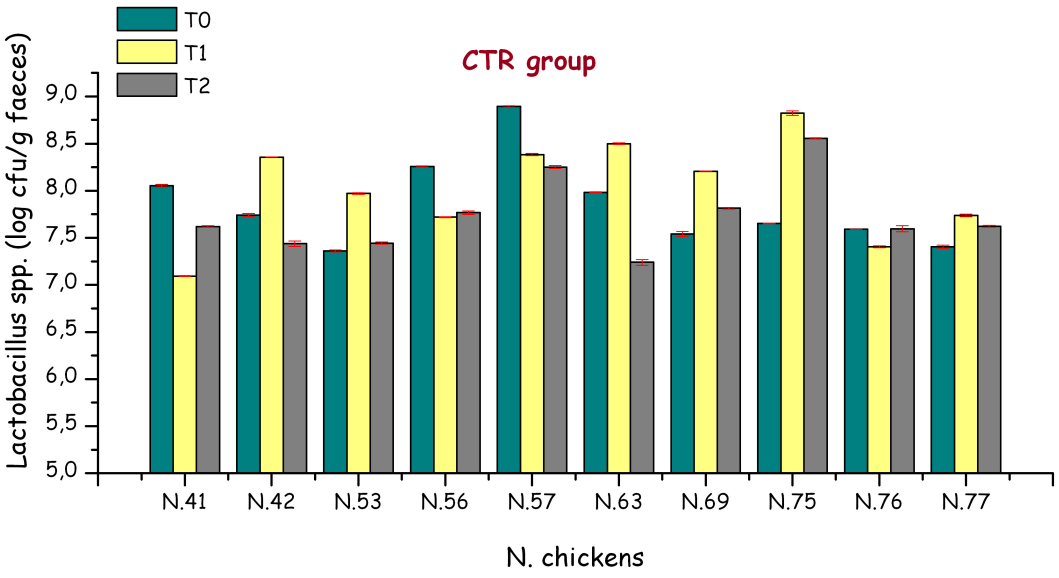


Fig. 9.27 *Lactobacillus* spp. quantification in faecal samples of the control group.

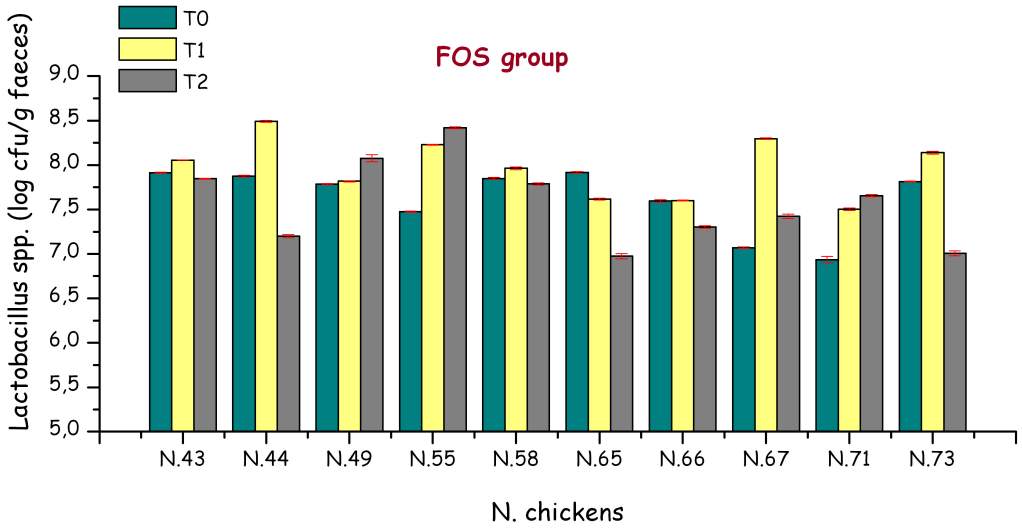


Fig. 9.28 *Lactobacillus* spp. quantification in faecal samples of the FOS group.

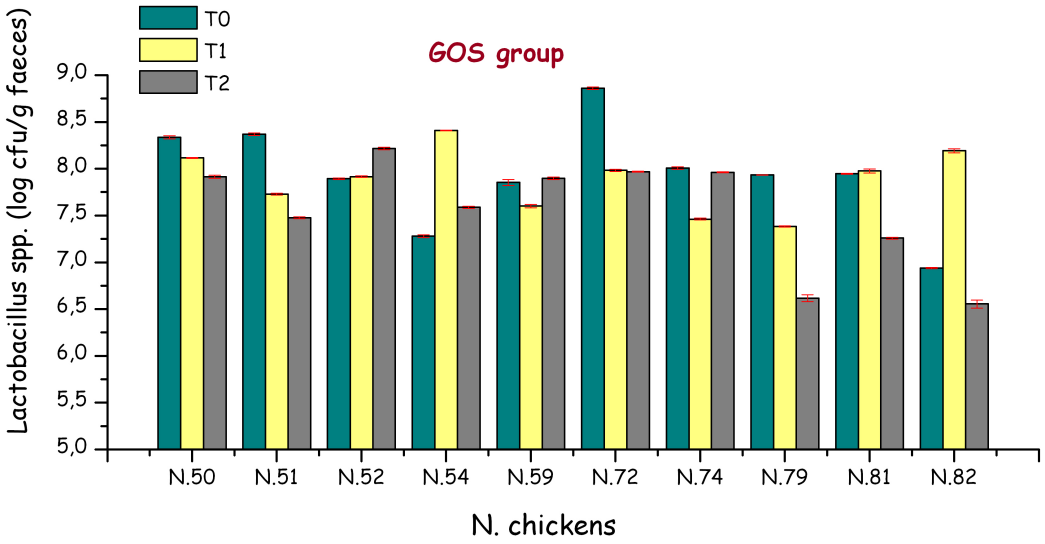


Fig. 9.29 *Lactobacillus* spp. quantification in faecal samples of the GOS group.

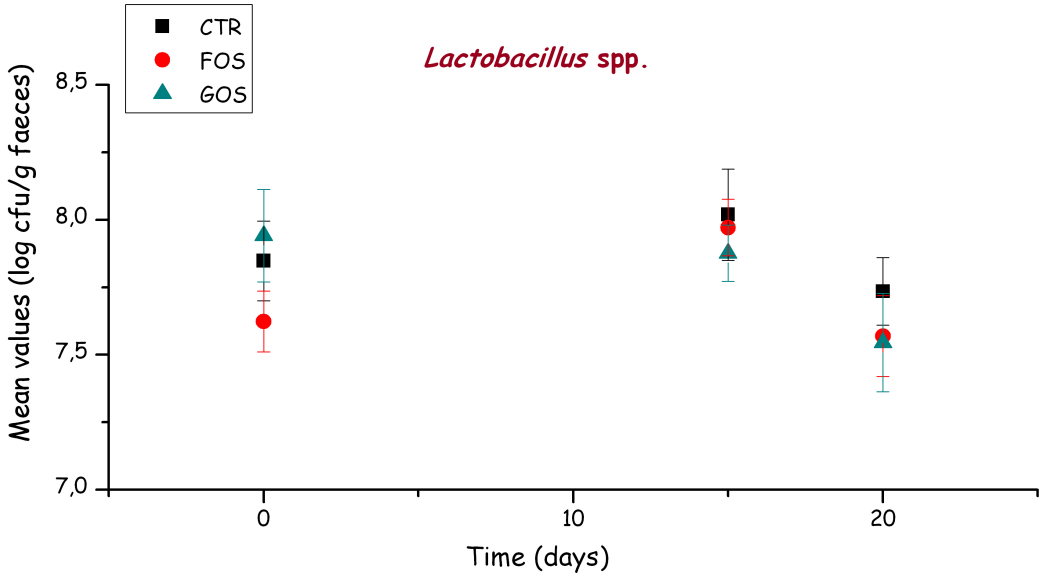


Fig. 9.30 Mean values comparison for *Lactobacillus* spp. in the three groups

Bifidobacterium spp. increased with time in all three groups (fig. 9.31 to 9.33 and tab. 9.15). It was interesting to underline the significant increase in control group ($p<0.05$), however the ANOVA analysis showed the significant effect of time*treatment interaction comparing FOS and GOS administration ($p<0.05$). The paired *t*-test between sampling times confirmed the significant increase in GOS group after 15 days of supplementation ($p<0.05$) as can be seen also in fig. 9.33 and 9.34. In broilers supplemented with GOS, the slight decrease of *Bifidobacterium* spp. observed during the last week was not significant. In FOS group, on the contrary, *Bifidobacterium* population had a not significant increase at T1 but had a significant decrease at T2 ($p<0.05$) (fig. 9.34).

Tab. 9.15 Mean values (log cfu/g faeces) of *Bifidobacterium* spp. ± SEM

	T0	**T1**	**T2**
CTR	6.46 ± 0.15	7.17 ± 0.15	7.37 ± 0.15
FOS	6.48 ± 0.16	6.73 ± 0.14	6.18 ± 0.28
GOS	5.99 ± 0.17	6.91 ± 0.23	6.80 ± 0.15

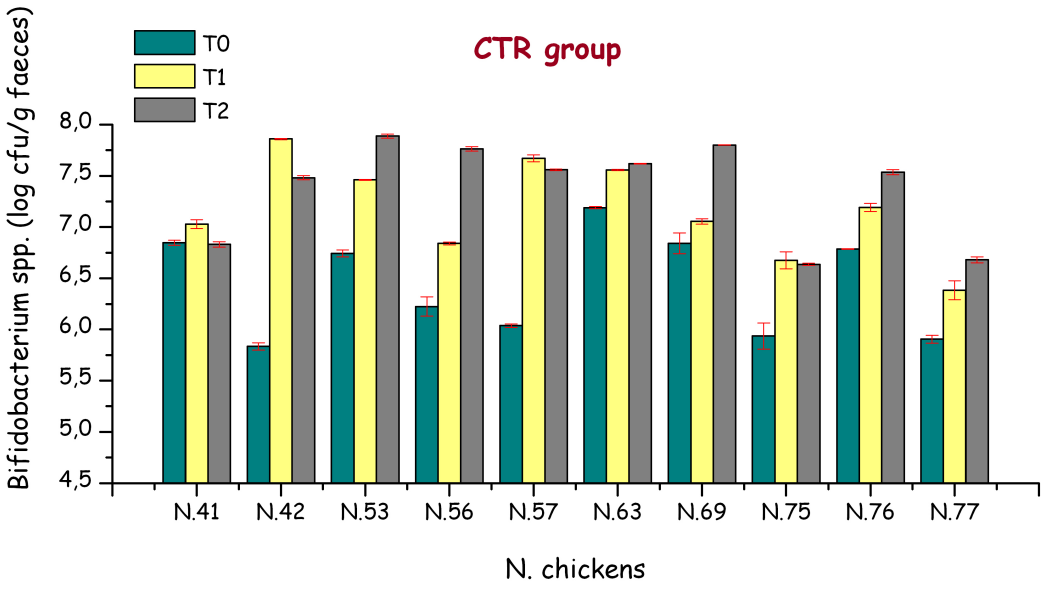


Fig. 9.31 *Bifidobacterium* spp. quantification in faecal samples of the control group

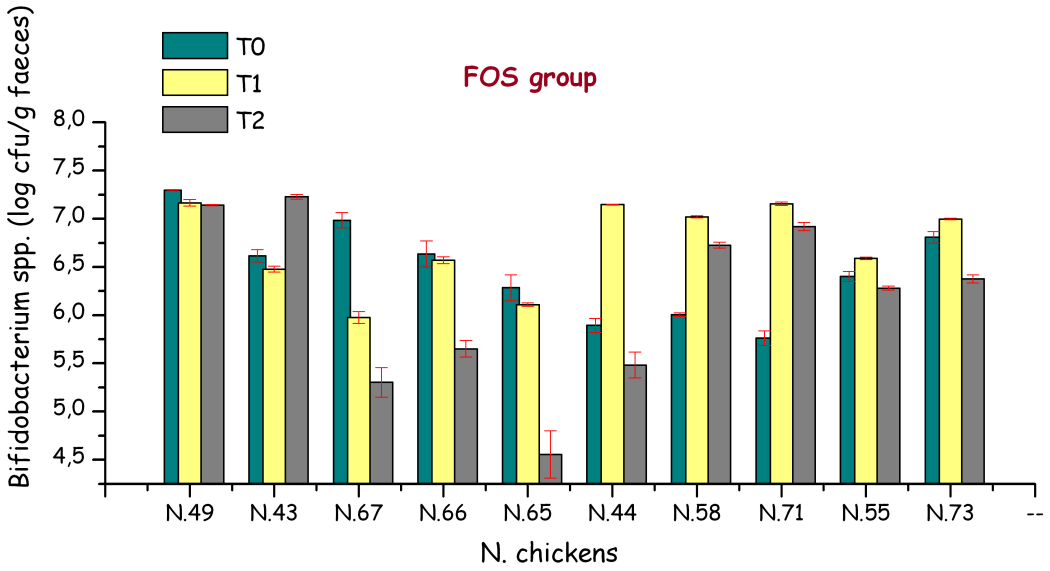


Fig. 9.32 *Bifidobacterium* spp. quantification in faecal samples of the FOS group

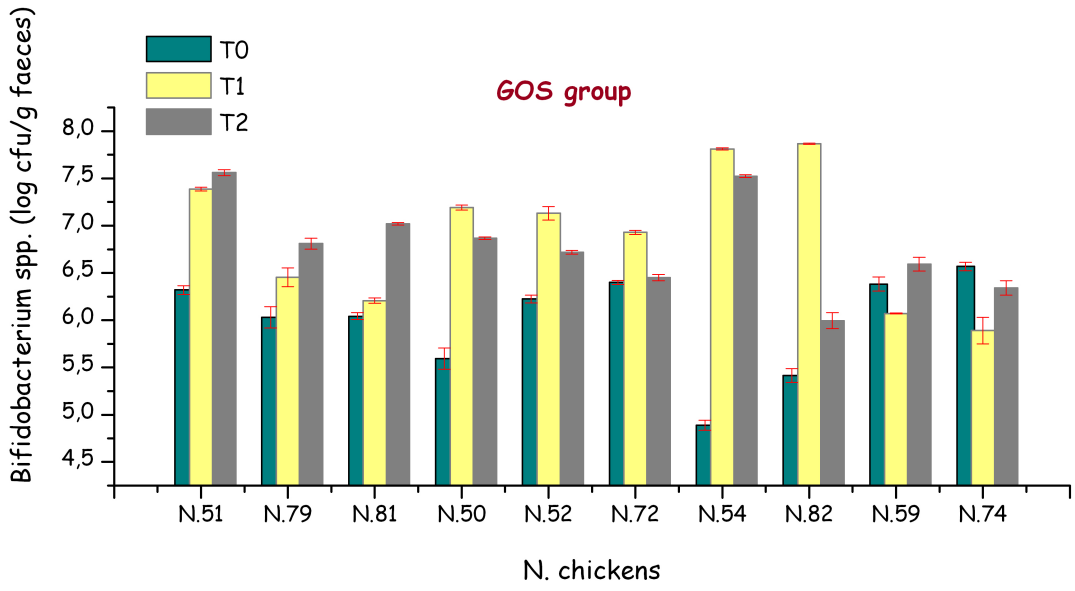


Fig. 9.33 *Bifidobacterium* spp. quantification in faecal samples of the GOS group

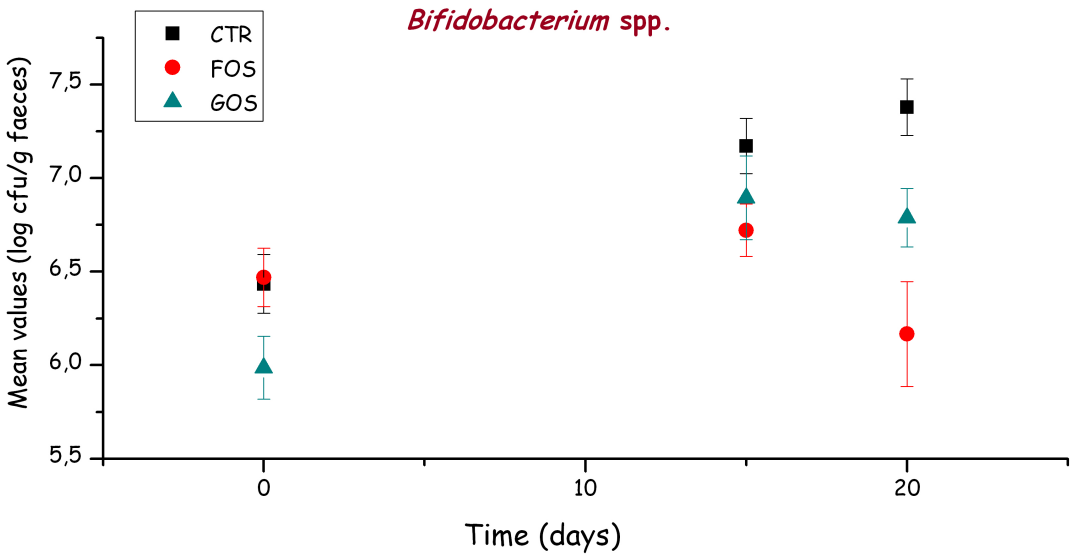


Fig. 9.34 Mean values comparison for *Bifidobacterium* spp. in the three groups.

Results showed that the starting mean values for *Campylobacter* spp. were similar in all groups (tab. 9.16 and fig. 9.38). Analysis of variance with SAS software adding contrast statement to compare the different sampling time between groups showed a significant difference between T0 and T2 (p<0.01). *Campylobacter* population decreased in groups supplemented with prebiotic compounds and the effect is more evident in GOS treated broilers that after 15 days of supplementation had a significant decrease with a p<0.01 (fig. 9.36 to 9.38).

Tab. 9.16 Mean values (log cfu/g faeces) of *Campylobacter* spp. ± SEM

	T0	T1	T2
CTR	8.41 ± 0.17	8.28 ± 0.19	8.50 ± 0.12
FOS	8.48 ± 0.05	8.11 ± 0.21	7.79 ± 0.21
GOS	8.29 ± 0.08	7.98 ± 0.05	7.84 ± 0.16

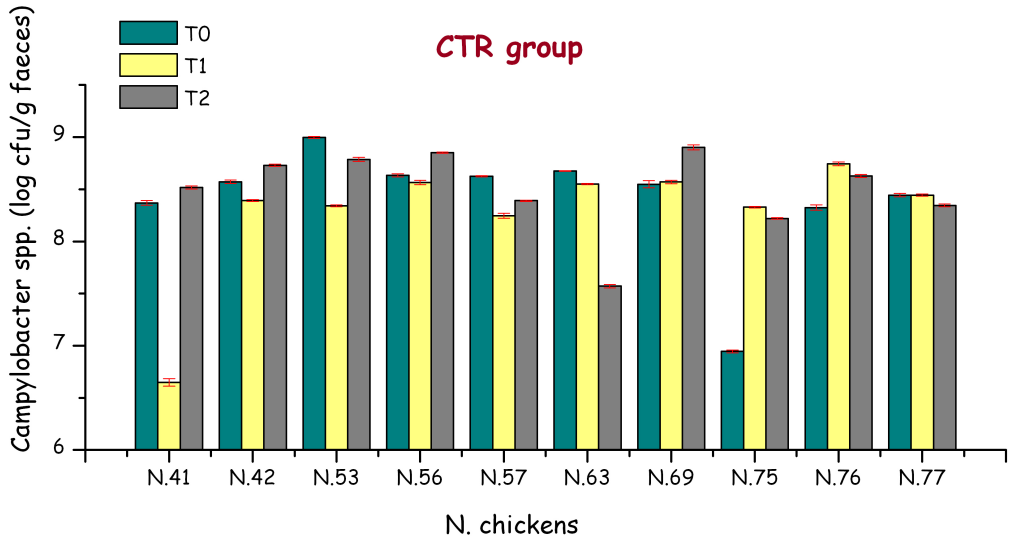


Fig. 9.35 *Campylobacter* spp. quantification in faecal samples of the control group

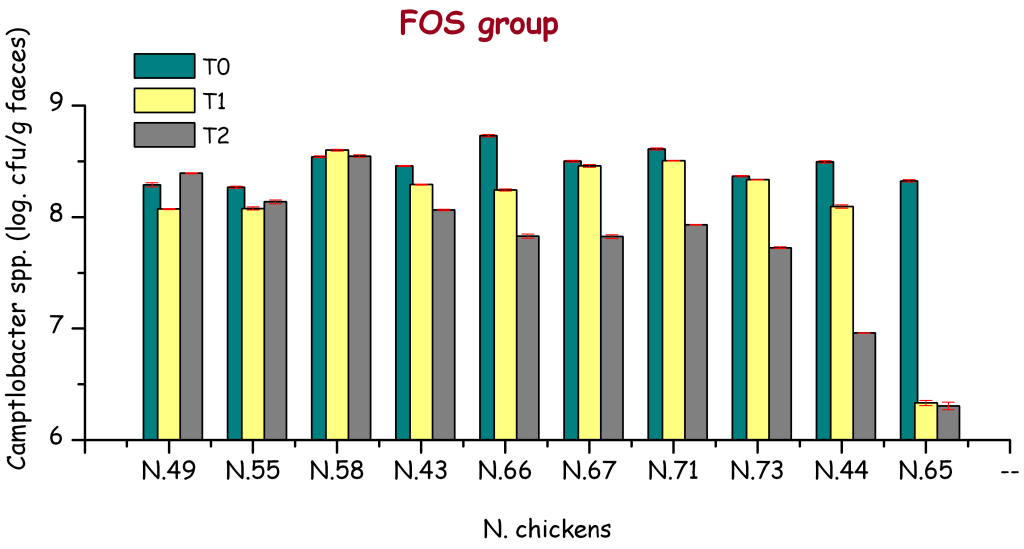


Fig. 9.36 *Campylobacter* spp. quantification in faecal samples of the FOS group

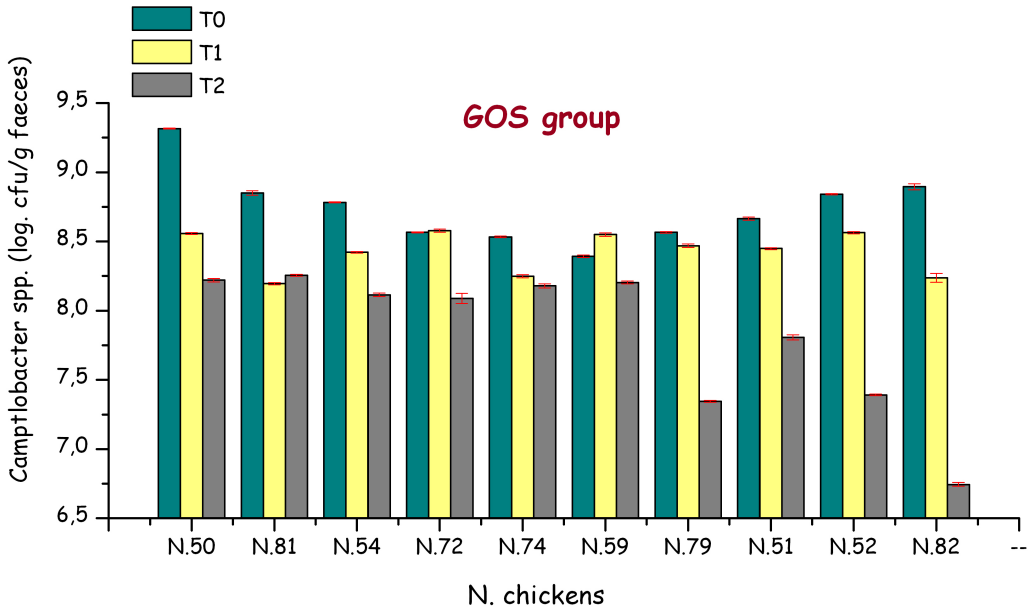


Fig. 9.37 *Campylobacter* spp. quantification in faecal samples of the GOS group

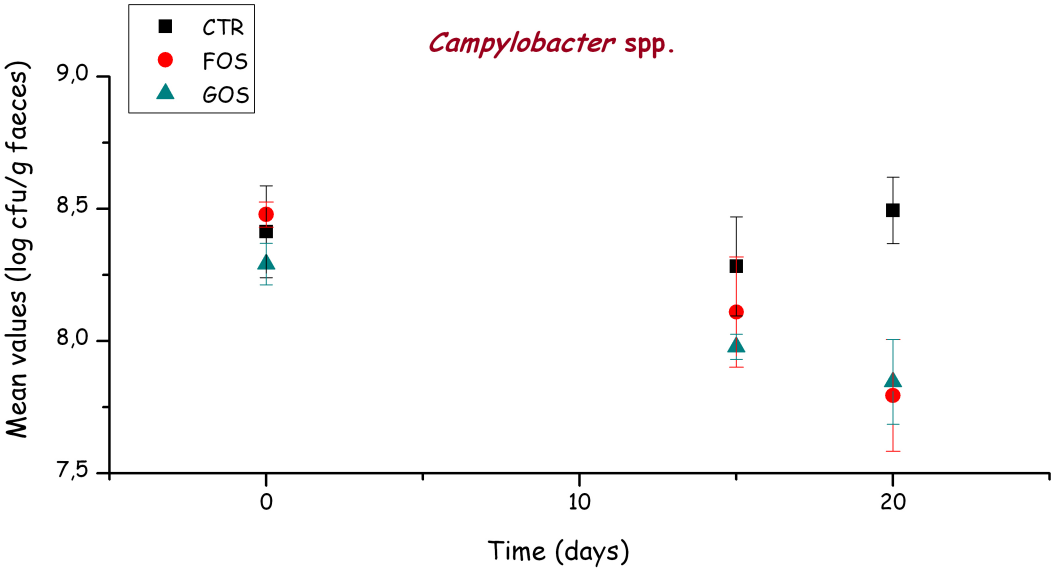


Fig. 9.38 Mean values comparison for *Campylobacter* spp. in the three groups.

9.2.3 Synbiotic trial

The synbiotic formula, composed by a galactooligosaccharide (GOS, 3%) and the microencapsulated *Bifidobacterium longum* subsp. *longum* PCB 133, was administered to broiler chickens (SYN group) for 15 days, mixed with poultry normal feed. Faecal samples were collected from ten animals in each group before starting supplementation, after 15 days of prebiotic administration and also after 21 days, *i.e.* one week after stopping synbiotic intake. Faecal samples were processed to quantify specific bacterial groups or species.

Room temperature and relative humidity were monitored along the entire period and reported in table 9.17.

TABLE 9.17 Environmental conditions during synbiotic trial

Temperature	**Relative Humidity**
N. observations: 326	N. observations: 326
Observation interval: 2 h	Observation interval: 2 h
Higher °T: 24.412 °C	Higher relative humidity: 88.512%
Lowest °T: 13.882 °C	Lowest relative humidity: 41.936%
Mean °T: 18.039 °C	Mean relative humidity: 66.607%
SD: 2.310 °C	SD: 11.010%

Throughout the feeding trial all the animals were healthy. There were no signs of diarrhoea, weight loss or loss of appetite.

Animal weight was recorded for 14 animals in both groups before starting treatment, after 15 days of supplementation and at the end of the experiment as shown in tables 9.18 and 9.19 and figures 9.39 and 9.40.

TABLE 9.18 Weight of 14 broilers of the control group at time zero (T0), after 15 days (T+15) and after 21 days (T+21)

	N. animal	**Sex**	**T0**	**T+15**	**T+21**
Group 1- Control Group	2	F	1,85	2,47	2,57
	3	F	2,14	2,64	2,77
	5	F	1,99	2,60	2,73
	10	F	2,12	2,62	2,72
	11	F	2,05	2,68	2,76
	13	F	1,94	2,44	2,62
	14	F	1,68	2,22	2,32
	15	M	2,54	3,35	3,49
	16	M	2,10	3,04	3,25
	18	M	2,86	3,86	4,08
	19	M	2,34	3,27	3,52
	20	M	2,57	3,53	3,69
	25	M	2,76	3,62	3,66
	26	M	2,77	3,71	3,89
Sum			**31,71**	**42,05**	**44,07**
Mean			**2,265**	**3,004**	**3,148**
SD			**0,376**	**0,544**	**0,568**

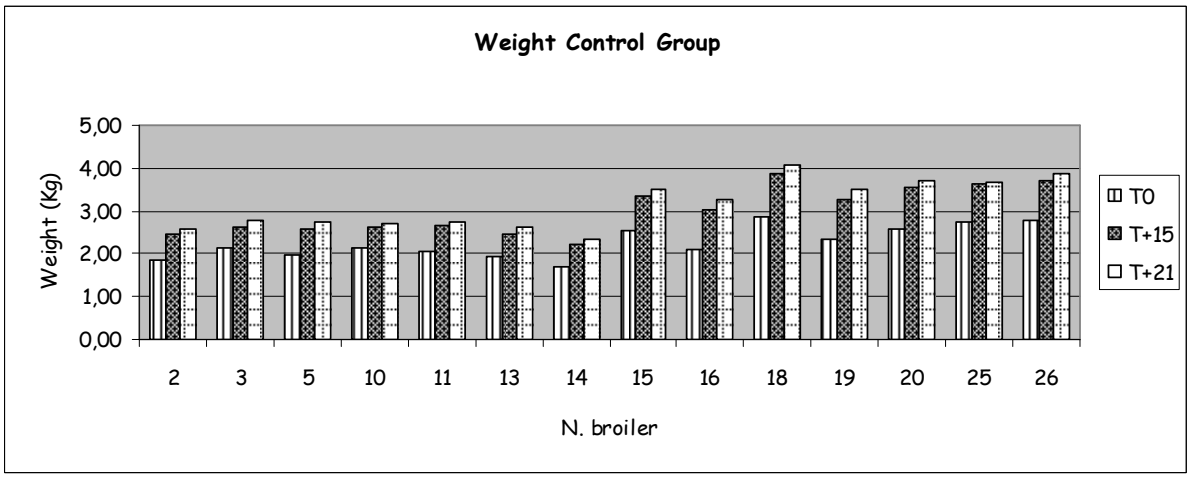


Fig. 9.39 Weight of 14 broilers of the control group at time zero (T0), after 15 days (T+15) and after 21 days (T+21). Chickens N.2 to N.14 are female; chickens N.15 to N.26 are male.

TABLE 9.19 Weight of 14 broilers administered with the synbiotic formula at time zero (T0), after 15 days of supplementation (T+15) and after 21 days (T+21)

	N. animal	Sex	T0	T+15	T+21
Group 2- Synbiotic Group	1	F	1,81	2,37	2,45
	4	F	2,16	2,63	2,84
	6	F	1,95	2,40	2,56
	7	F	2,07	2,45	2,59
	8	F	1,94	2,50	2,69
	9	F	1,91	2,42	2,52
	12	F	1,65	2,20	2,37
	17	M	2,44	3,27	3,49
	21	M	2,66	3,48	3,68
	22	M	2,57	3,33	3,6
	23	M	2,83	3,79	4,06
	24	M	2,94	3,92	4,12
	27	M	2,57	3,25	3,45
	28	M	2,23	2,90	2,98
Sum			**31,73**	**40,91**	**43,40**
Mean			**2,266**	**2,922**	**3,100**
SD			**0,403**	**0,575**	**0,615**

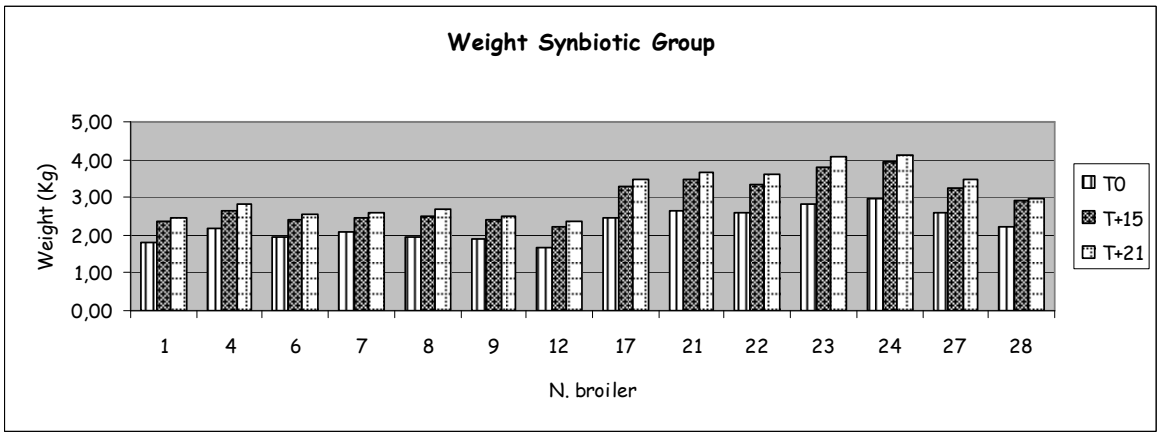


Fig. 9.40 Weight of 14 broilers administered with the synbiotic formula at time zero (T0), after 15 days of supplementation (T+15) and after 21 days (T+21). Chickens N.1 to N.12 are female; chickens N.17 to N.28 are male.

The statistical ANOVA analysis performed with GLM procedure with SAS software detected no significant weight differences between broiler groups. As can be clearly seen by bar-diagrams also in this trial there was a significant difference ($p<0.01$) in weight between male and female in both groups, at any time.

The ANOVA analysis between groups showed that there was no effect on *Lactobacillus* spp. population that remained stable during the experiment as shown by mean values in tab. 9.20 and fig. 9.43. However, considering bar diagrams of single chickens, it is quite evident that there was a high variability. An increase of *Lactobacillus* spp. was observed for example in five out of ten chickens in control group, a decrease was on the contrary reported for the five remaining broilers (fig. 9.41). The same trend can be stressed in the synbiotic group (fig. 9.42).

Tab. 9.20 Mean values (log cfu/g faeces) of *Lactobacillus* spp. ± SEM

	T0	**T1**	**T2**
CTR	7.87 ± 0.17	8.03 ± 0.2	7.85 ± 0.24
SYN	7.77 ± 0.17	7.81 ± 0.15	7.79 ± 0.12

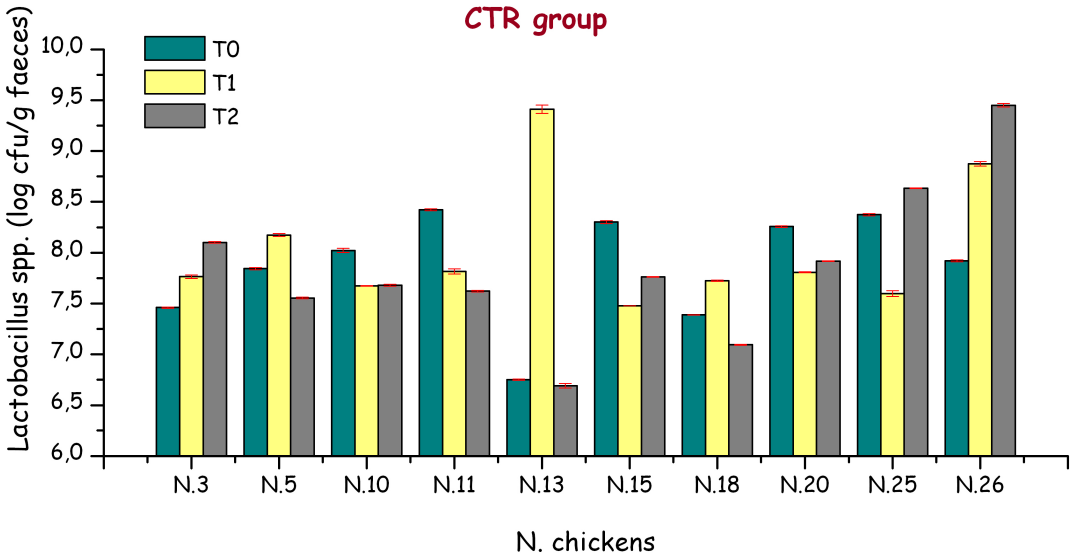


Fig. 9.41 *Lactobacillus* spp. quantification in faecal samples of the control group.

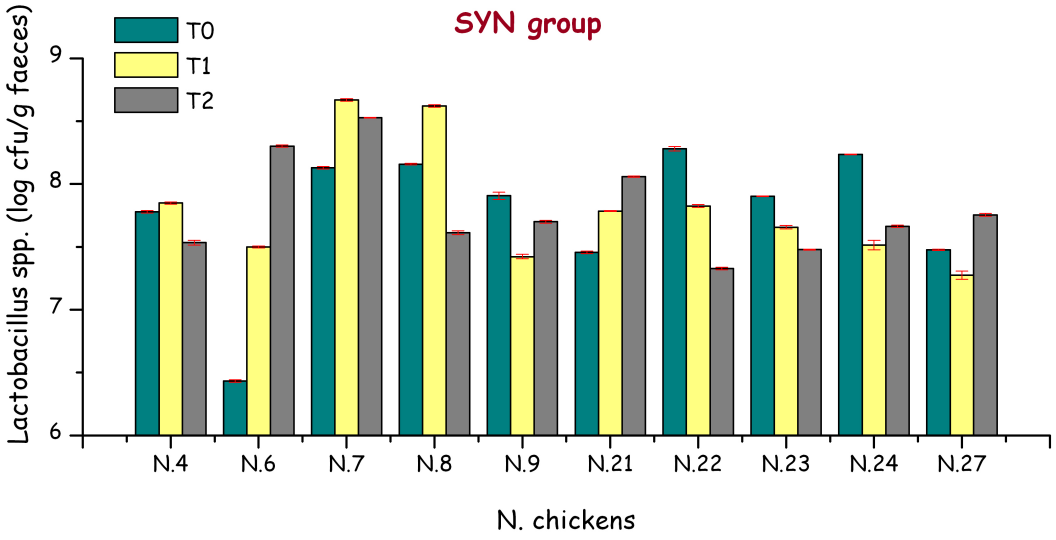


Fig. 9.42 *Lactobacillus* spp. quantification in faecal samples of the synbiotic treated group

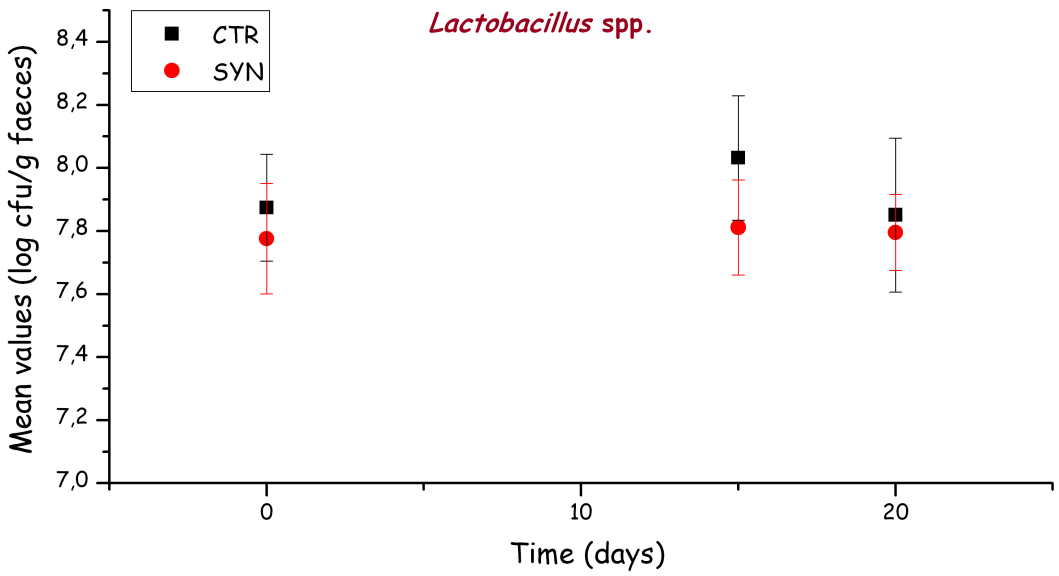


Fig. 9.43 Mean values comparison for *Lactobacillus* spp.

Quantification results of *Bifidobacterium* spp. showed a high variability at T0 both in the control and in the synbiotic group (fig. 9.44 and 9.45). However, while in control group the variability remained constant during the 21-day trial, in broilers supplemented with the synbiotic formula a clear reduction of intra-group variability can be observed (fig. 9.45 and 9.46). Even if the difference between starting mean values in control and treated group is about 1 log (tab. 9.21) the ANOVA analysis showed a significant difference ($p<<0.01$) between groups after 15 days of synbiotic intake, confirmed also by the significant ($p<<0.01$) time*treatment interaction.

Tab. 9.21 Mean values (log cfu/g faeces) of *Bifidobacterium* spp. ± SEM

	T0	**T1**	**T2**
CTR	6.56 ± 0.43	6.17 ± 0.29	5.54 ± 0.22
SYN	5.54 ± 0.22	7.96 ± 0.05	6.85 ± 0.10

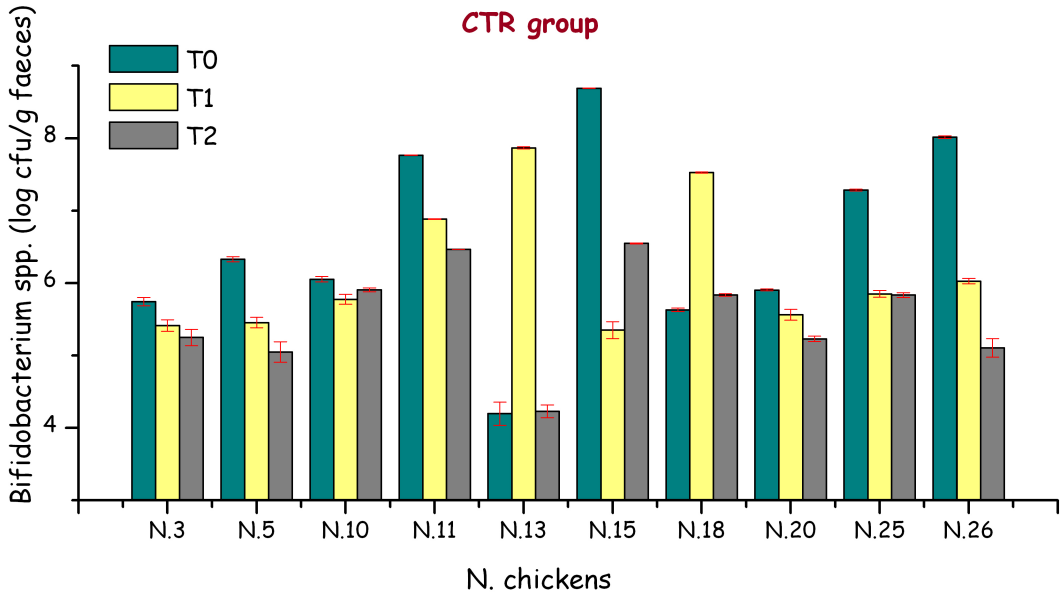


Fig. 9.44 *Bifidobacterium* spp. quantification in faecal samples of the control group.

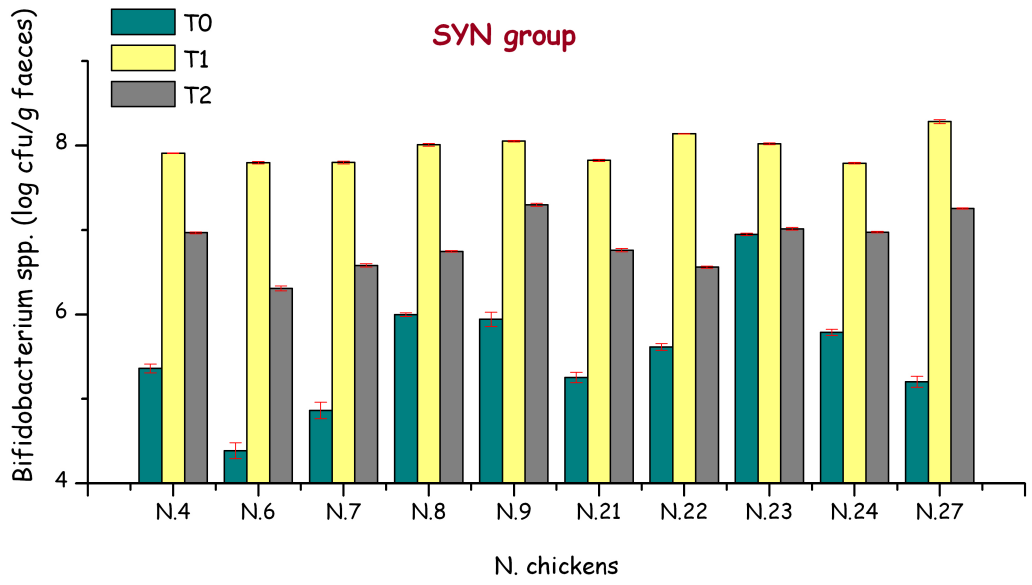


Fig. 9.45 *Bifidobacterium* spp. quantification in faecal samples of the synbiotic treated group.

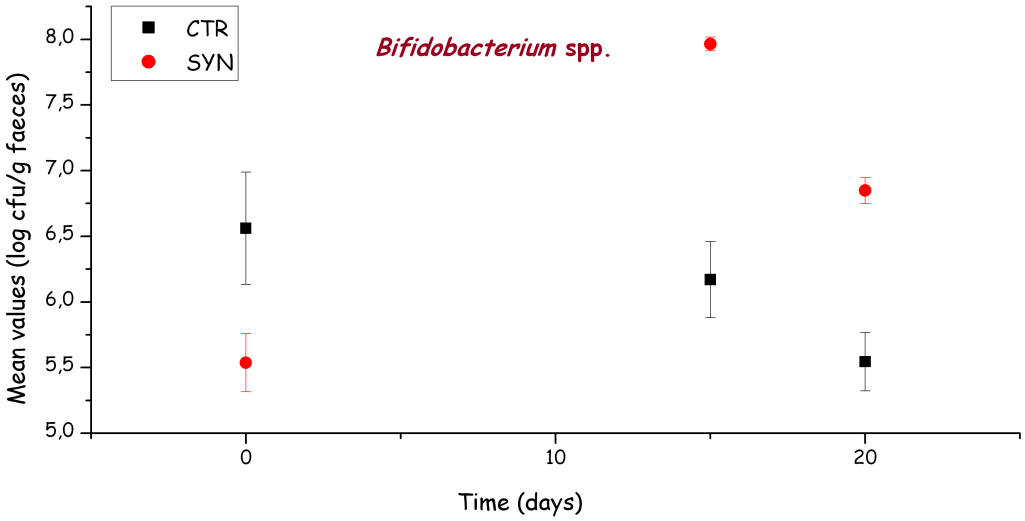


Fig. 9.46 Mean values comparison for *Bifidobacterium* spp.

Quantification of *Bifidobacterium longum* was performed only on DNA samples extracted from SYN group, taking into consideration that *B. longum* is a bifidobacterial species of the human gastrointestinal tract. It was therefore absent in chicken faeces. The results showed that *B. longum* significantly ($p<<0.01$) colonized the chicken gastrointestinal tract after 15 days of synbiotic formula administration and after stopping supplementation *B. longum* population decreased ~ 1.2 log ($p<0.01$) (tab. 9.22 and fig. 9.47 and 9.48).

Tab. 9.22 Mean values (log cfu/g faeces) of *Bifidobacterium longum* spp. ± SEM

	T0	T1	T2
CTR	0	8.17 ± 0.04	6.94 ± 0.1
SYN	0	0	0

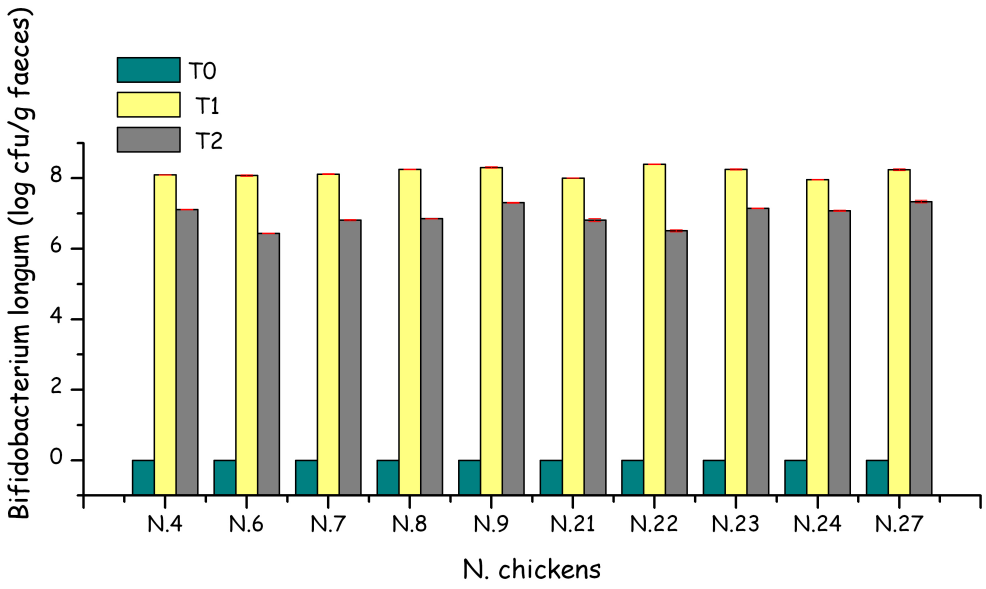


Fig. 9.47 *Bifidobacterium longum* subsp. *longum* PCB 133 quantification in faecal samples of the synbiotic treated group.

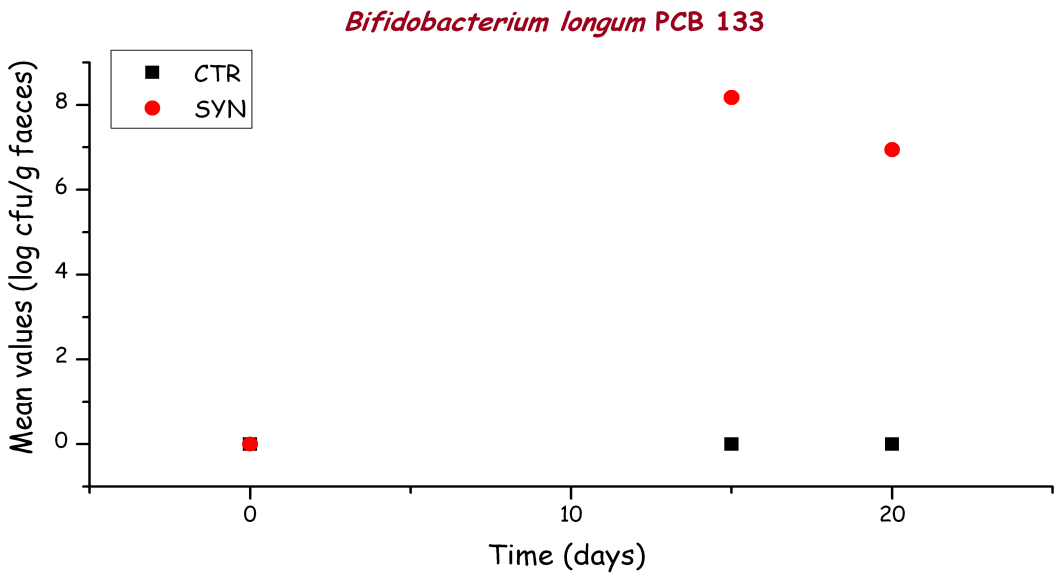


Fig. 9.48 Mean values comparison for *Bifidobacterium longum*

The ANOVA analysis between groups showed that there was no effect on *Campylobacter* spp. population that remained stable during the experiment as show by means in tab. 9.23 and fig. 9.51. *Campylobacter* population is also fairly constant between animals as shown by bar diagrams (fig. 9.49 and 9.50).

Tab. 9.23 Mean values (log cfu/g faeces) of *Campylobacter* spp. ± SEM.

	T0	**T1**	**T2**
CTR	7.52 ± 0.31	7.63 ± 0.05	7.07 ± 0.31
SYN	7.24 ± 0.23	7.44 ± 0.12	7.41 ± 0.19

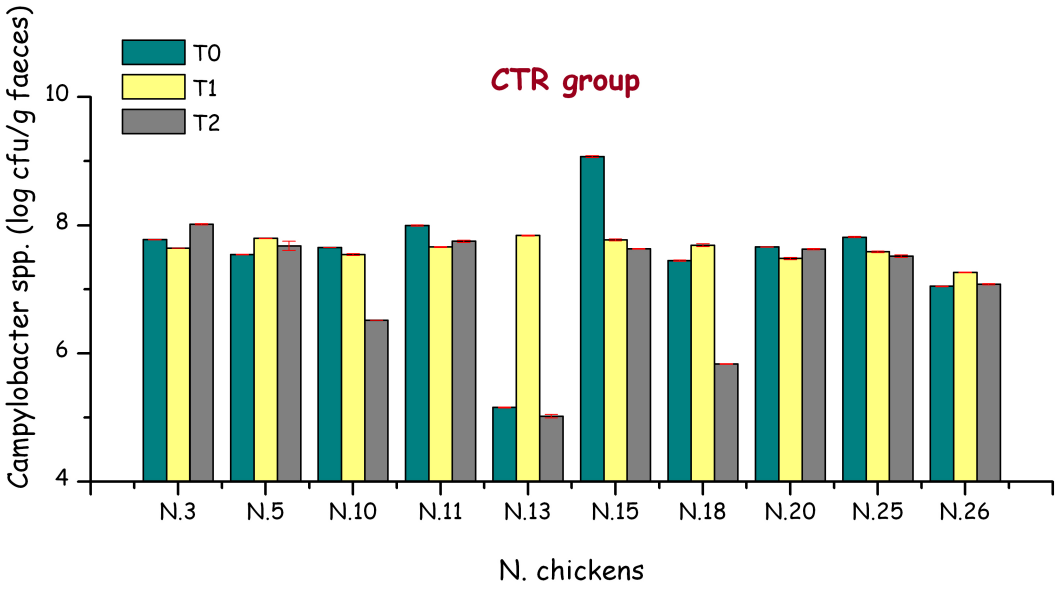


Fig. 9.49 *Campylobacter* spp. quantification in faecal samples of the control group.

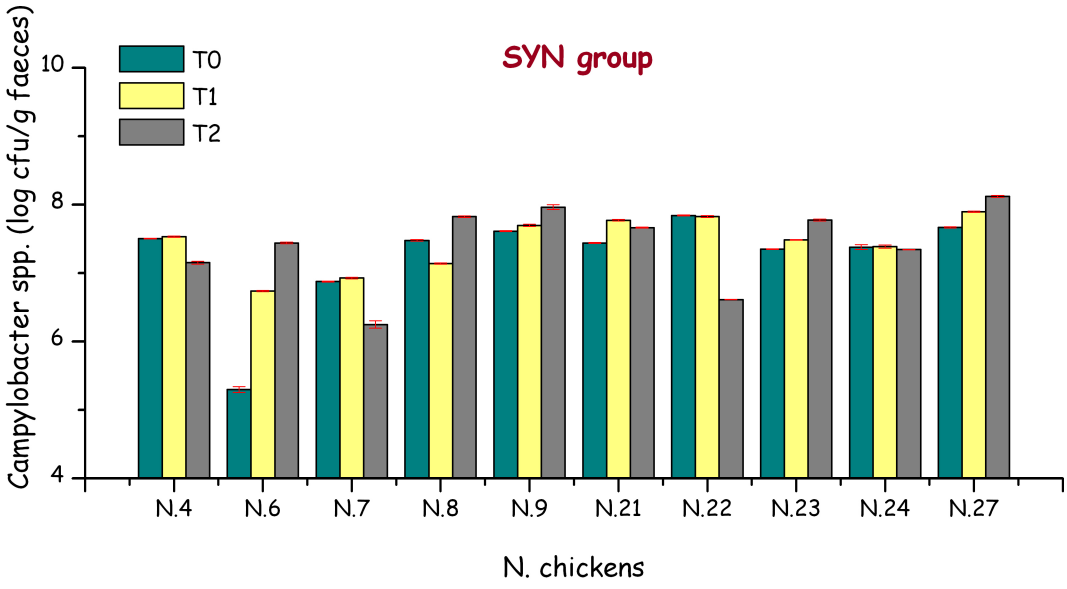


Fig. 9.50 *Campylobacter* spp. quantification in faecal samples of the synbiotic treated group.

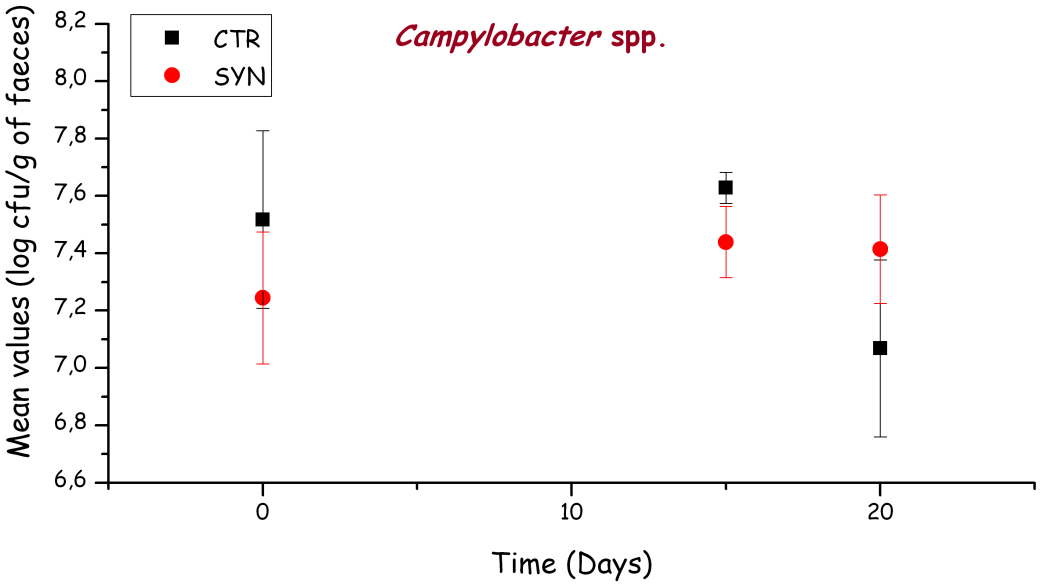


Fig. 9.51 Mean values comparison for *Campylobacter* spp.

ANOVA analysis on *Campylobacter jejuni* quantification showed that there was not a significant difference between CTR group and SYN group at T0 while after 15 days of treatment on SYN group the *C. jejuni* population significantly (p<0.05) decreased in the latter group (tab. 9.24 and fig. 9.54). The same decrement was reached also by CTR group but one week later, with the same significance (p<0.05). However it was important to observe the intra group variability of pathogen quantification (fig. 9.52 and 9.53)..

Tab. 9.24 Mean values (log cfu/g faeces) of C*ampylobacter jejuni* spp. ± SEM.

	T0	T1	T2
CTR	5.99 ± 0.38	5.53 ± 0.27	4.85 ± 0.46
SYN	5.66 ± 0.23	4.61 ± 0.35	5.43 ± 0.32

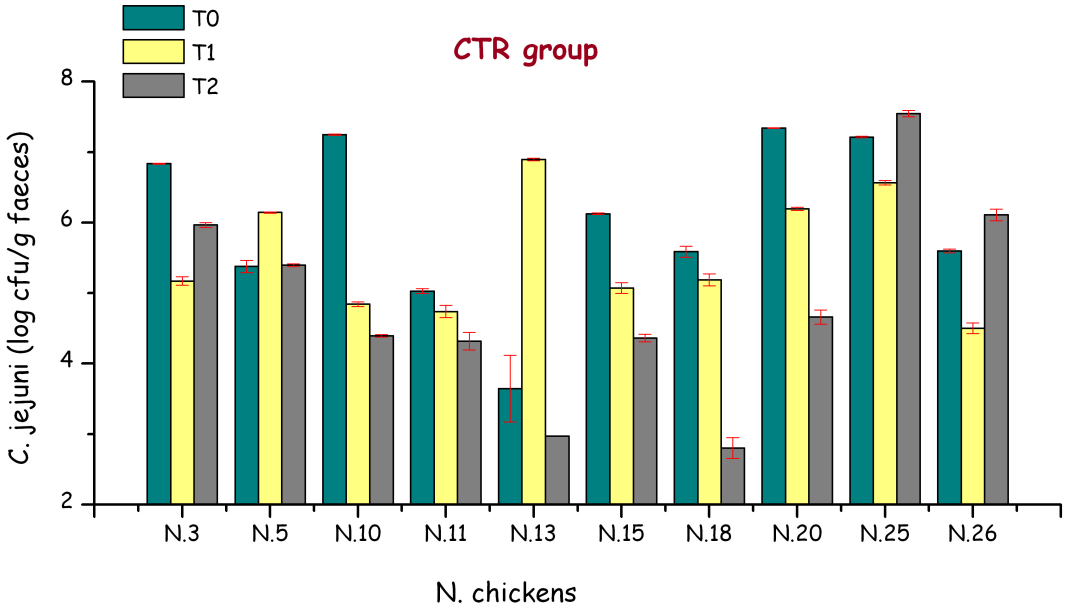


Fig. 9.52 *Campylobacter jejuni* quantification in faecal samples of the control group.

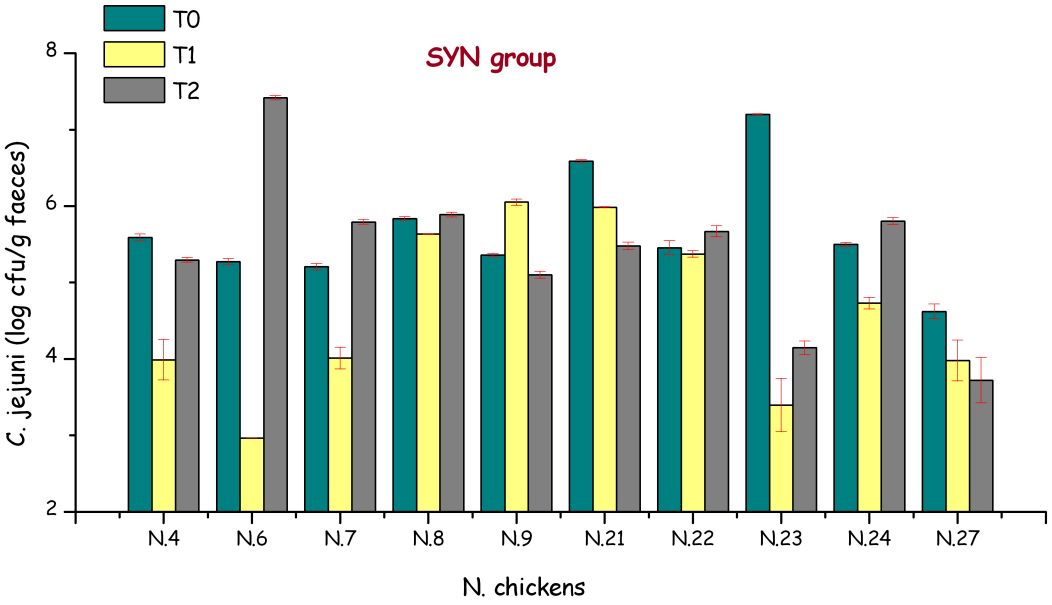


Fig. 9.53 *Campylobacter jejuni* quantification in faecal samples of the synbiotic treated group.

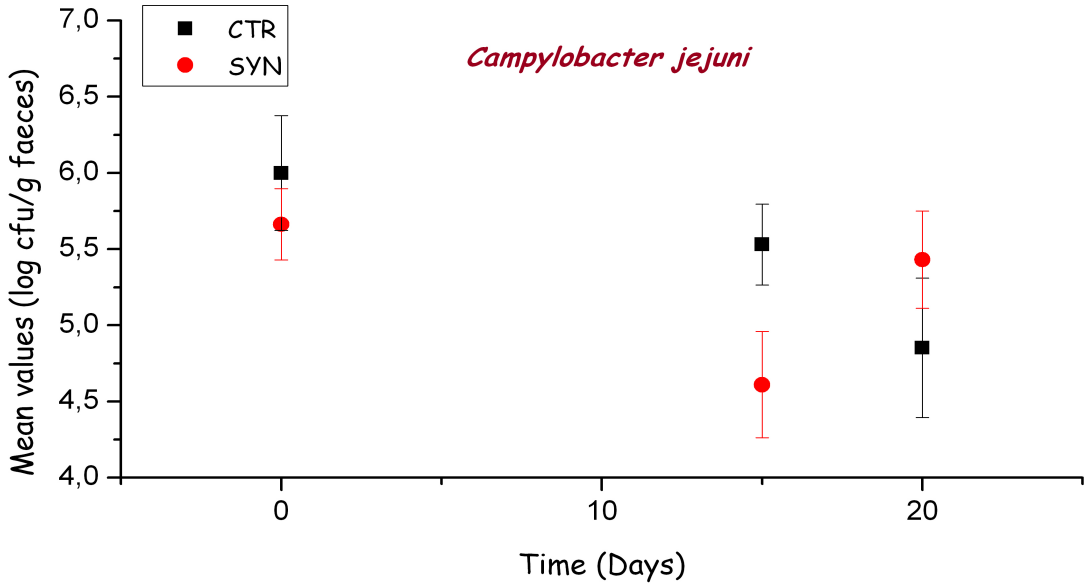


Fig. 9.54 Mean values comparison for *Campylobacter jejuni*.

Chapter 10. In vitro analysis of microencapsulated bacteria survival in feed

The stability of the microencapsulated probiotic strains was analyzed to assess their shelf life, in particular when mixed with feed.

Aliquots of microencapsulated microorganisms were divided into different bags, with (50:50 wt/wt) and without feed, and the bags were kept open at room temperature.

The viable cell concentration obtained by Probiotical S.p.A. on PCB 133 and PCS 20 batches was $\geq 1x10^9$ CFU/g. The results obtained showed that the vitality of the microorganisms remained quite constant (tab. 10.1 and 10.2, fig. 10.1 and 10.2. Values are expressed in log CFU/g).

Tab. 10.1 Variation of concentration of pure microencapsulated products during time (days).

	T_0	T+7	T+14	T+30	T+60
PCS 20 log CFU/g	11,3	11,5	10,7	10,5	9,6
PCB 133 log CFU/g	10,4	9,5	9,5	9,5	8,9

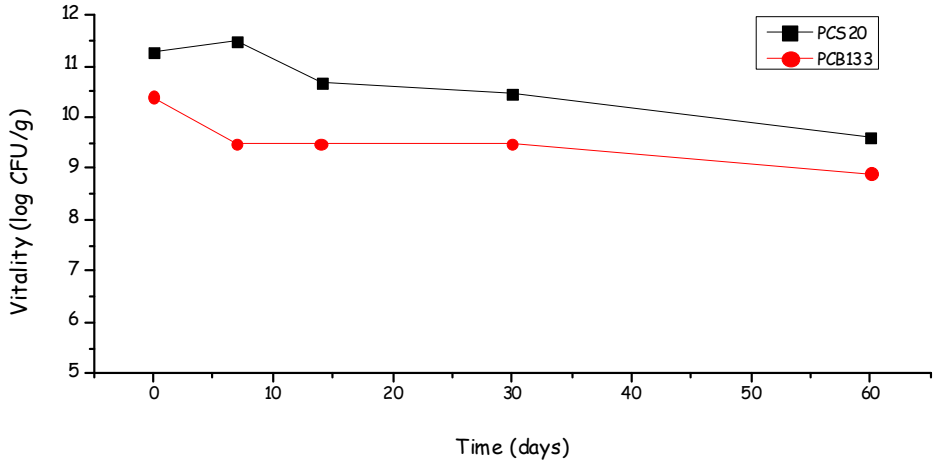


Fig. 10.1 Trend of pure microencapsulated microorganism survival.

Tab. 10.2 Variation of the concentration of microencapsulated probiotics mixed with poultry feed (50:50 wt/wt) during time (days).

	T_0	T+7	T+14	T+30	T+60
Mixed PCS 20 log CFU/g	11,6	11,6	10,3	10,1	8,6
Mixed PCB 133 log CFU/g	8,9	8,8	8,6	8,2	7,6

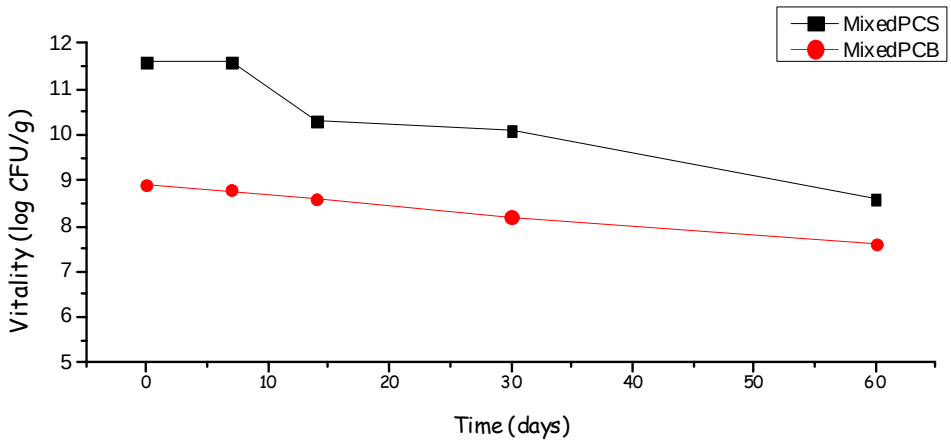


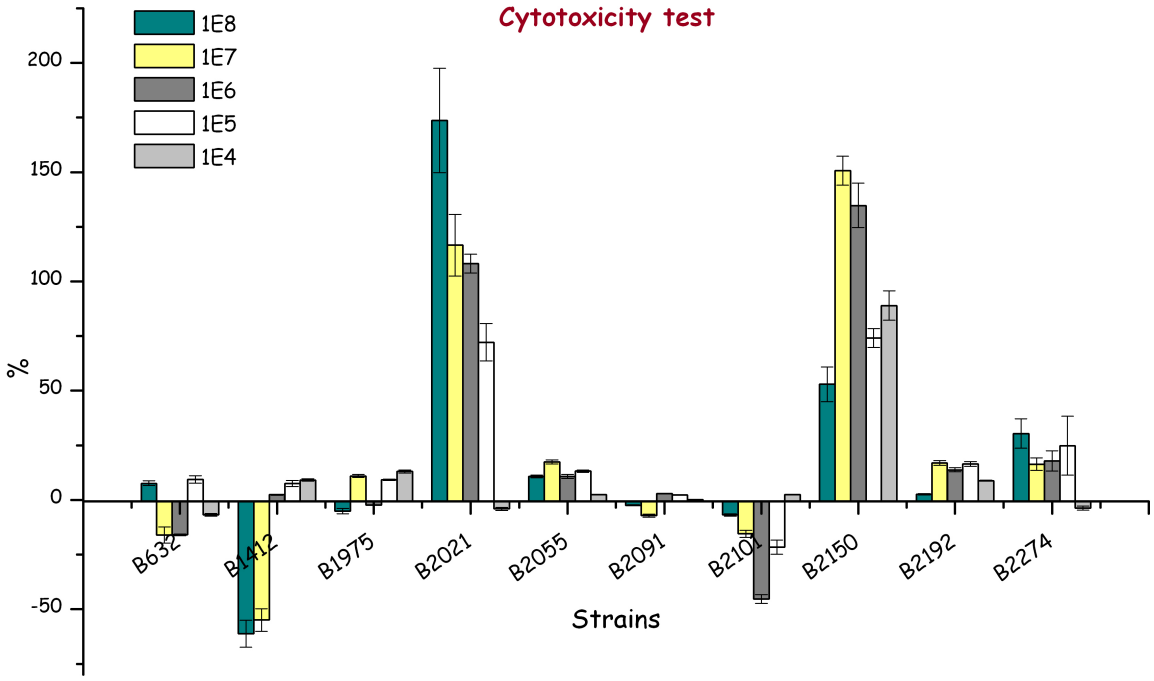
Fig. 10.2 Trend of microencapsulated bacteria survival when mixed with poultry feed.

Chapter 11. In vitro screening of bifidobacteria strains on B1OXI cell-line

B. longum subsp. *longum* PCB 133 and *Lactobacillus plantarum* PCS 20 were further characterized on non-transformed chicken intestinal cell-line (B1OXI, provided by Department of Microbiology, Biochemistry and Biotechnology, Faculty of Agriculture, University of Maribor, Slovenia). Together with the two probiotic strains other bifidobacteria and *Lactobacillus* strains were characterized in order to find out other potential probiotic strains.

11.1 Cytotoxicity test

Half of the assayed strains showed to reduce the B1OXI cell viability when applied at the highest concentration (10^8 cells/ml) (fig. 11.1), that is to say that very high concentration could result toxic for epithelial cells (it should be born in mind that 10^8 bacteria are applied on 0.33 cm^2, the area of each well). However the *t*-test showed a not significant reduction of the cell viability ($p>0.05$) for six strains in respect to negative control (*i.e.* the epithelial cells incubated in the same conditions without adding bacteria); whereas it was significant for four strains: *B. longum* subsp. *infantis* B1412, *B. longum* B2101, *L. plantarum* PCS 22 and *Lactobacillus rhamnosus* LGG (fig. 11.1). Six strains on the contrary gave a significant ($p<0.05$) high value of A_{595} after co-incubation with epithelial cells at a bacterial concentration of 10^7 to 10^5 cell/ml. Especially *B. breve* B2021, *B. breve* B2150, *B. pseudocatenulatum* B7003, *B. longum* subsp. *longum* PCB 133 and *L. plantarum* PCS 25 gave interesting results (fig. 11.1). Also *L. plantarum* PCS 20 at concentration of 10^5 and 10^4 cell/ml had a significant *p*-value.



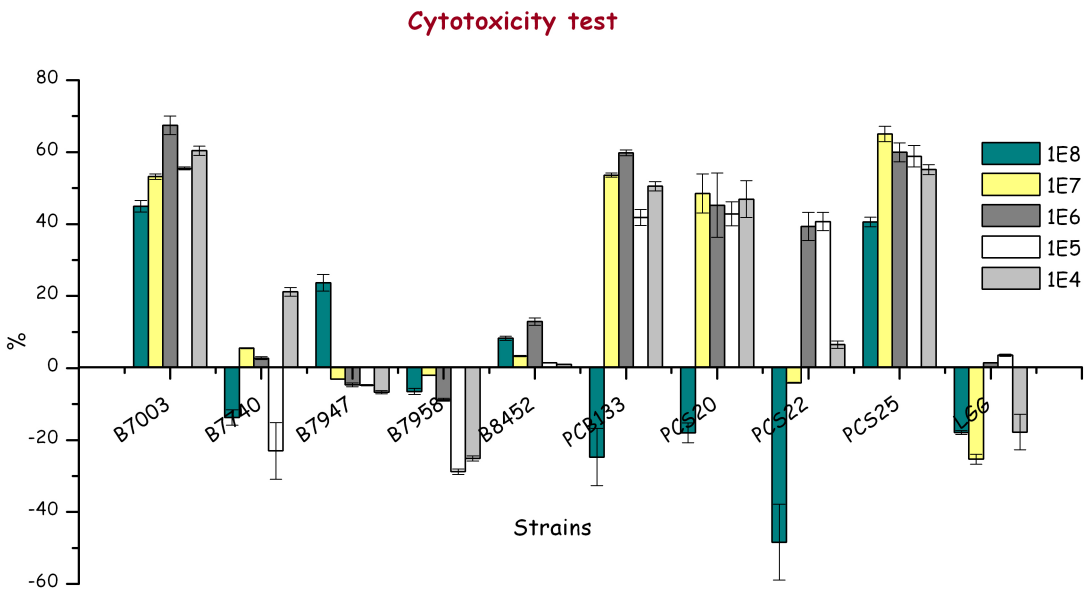


Fig. 11.1 Results of cytotoxicity test of *Bifidobacterium* and *Lactobacillus* strains on B1OXI cells. Bacteria were inoculated at 10^8 to 10^4 cell/ml (results are expressed by the mean ratios (%, ± SD) of absorbance in treated wells as compared to those in negative control wells).

11.2 Metabolic activity enhancement

The metabolic activity of intestinal epithelial cells was measured with the MTT test (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide). A mitochondrial dehydrogenase reduced the yellow MTT compound into a blue compound (formazan). Colour intensity was proportional to metabolically active cells.

Results showed that almost all strains were able to enhance the metabolic activity of cells. Some bifidobacteria strains gave very significant results in comparison with non treated cells, as for example *B. breve* B2021, *B. longum* subsp. *longum* B2055, *B. bifidum* B2091, *B. longum* B2101, *B. breve* B2150, *B. breve* B2274, *B. pseudocatenulatum* B7003, *B. longum* subsp. *infantis* B7740, *B. longum* subsp. *longum* PCB 133, *L. plantarum* PCS 20 and PCS 25 (fig. 11.2). However other strains seemed to decrease the metabolic activity of cells: *B breve* B632, *B. longum* subsp. *infantis* B1412, *B. longum* subsp. *longum* B1975.

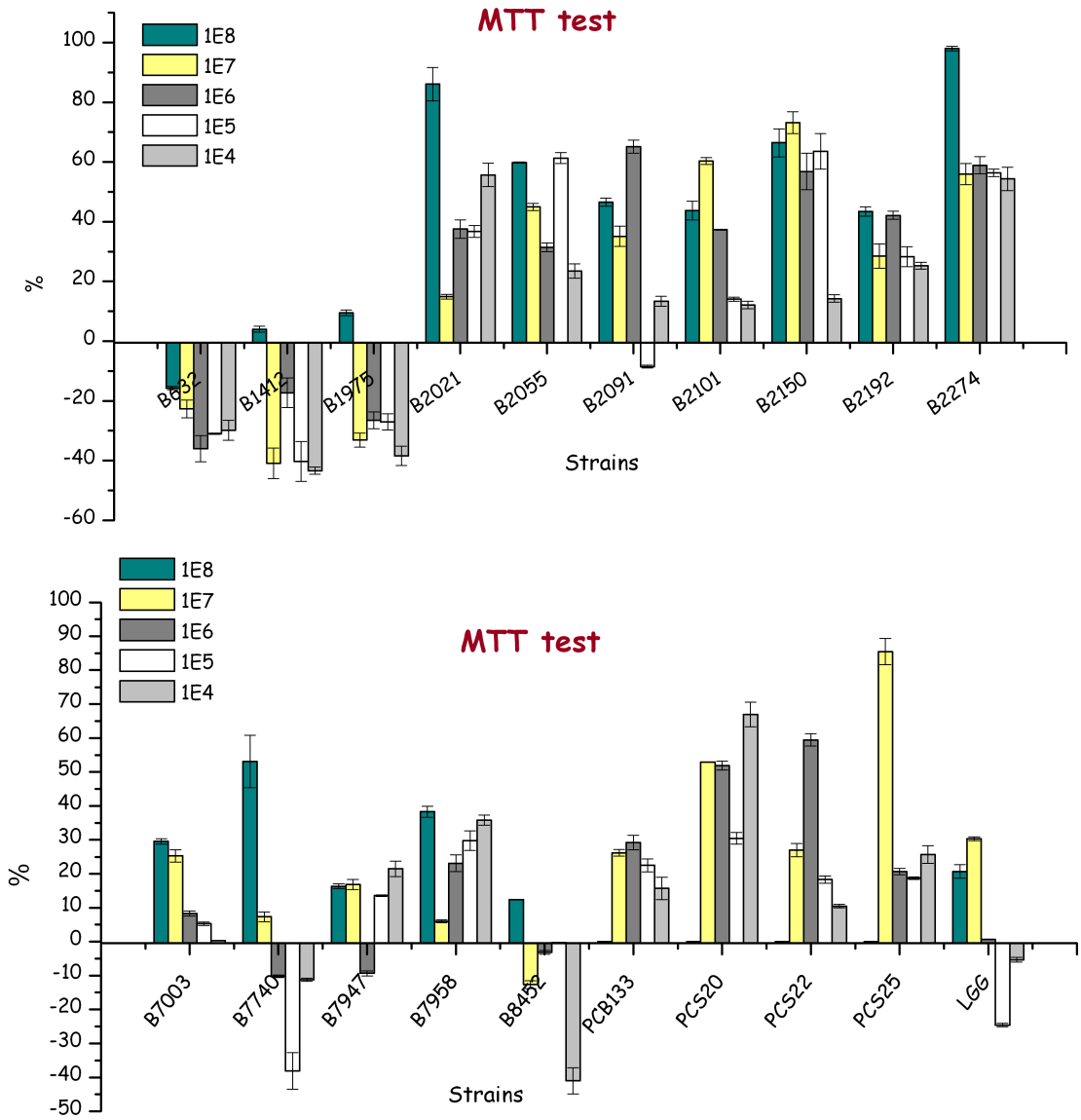


Fig. 11.2 MTT test results for *Bifidobacterium* and *Lactobacillus* strains incubated on B1OXI cells at a concentration of 10^8 to 10^4 cell/ml (results are expressed by the mean ratios (%, ± SD) of absorbance in treated wells as compared to those in negative control wells).

11.3 Total H_2O_2 production

The H_2O_2 test revealed that some strains were able to stimulate the H_2O_2 production in B1OXI cells when the monolayer was exposed to high bacterial concentration (10^8-10^7 cell/ml). Strains *B. breve* B632, *B. breve* B2021, *B. breve* B2274, *B. longum* subsp. *infantis* B7740, *B. longum* subsp. *longum* B8452, *B. longum* subsp. *longum* PCB 133 and LGG showed to stimulate a significant production of hydrogen peroxide when incubated on cell monolayer at 10^8 cell/ml. *L. plantarum* PCS 25 and *B. longum* subsp. *longum* B8452 stimulated H_2O_2 secretion at a concentration of 10^7 cell/ml (p= 0,042 and 0,039 respectively) (fig. 11.3).

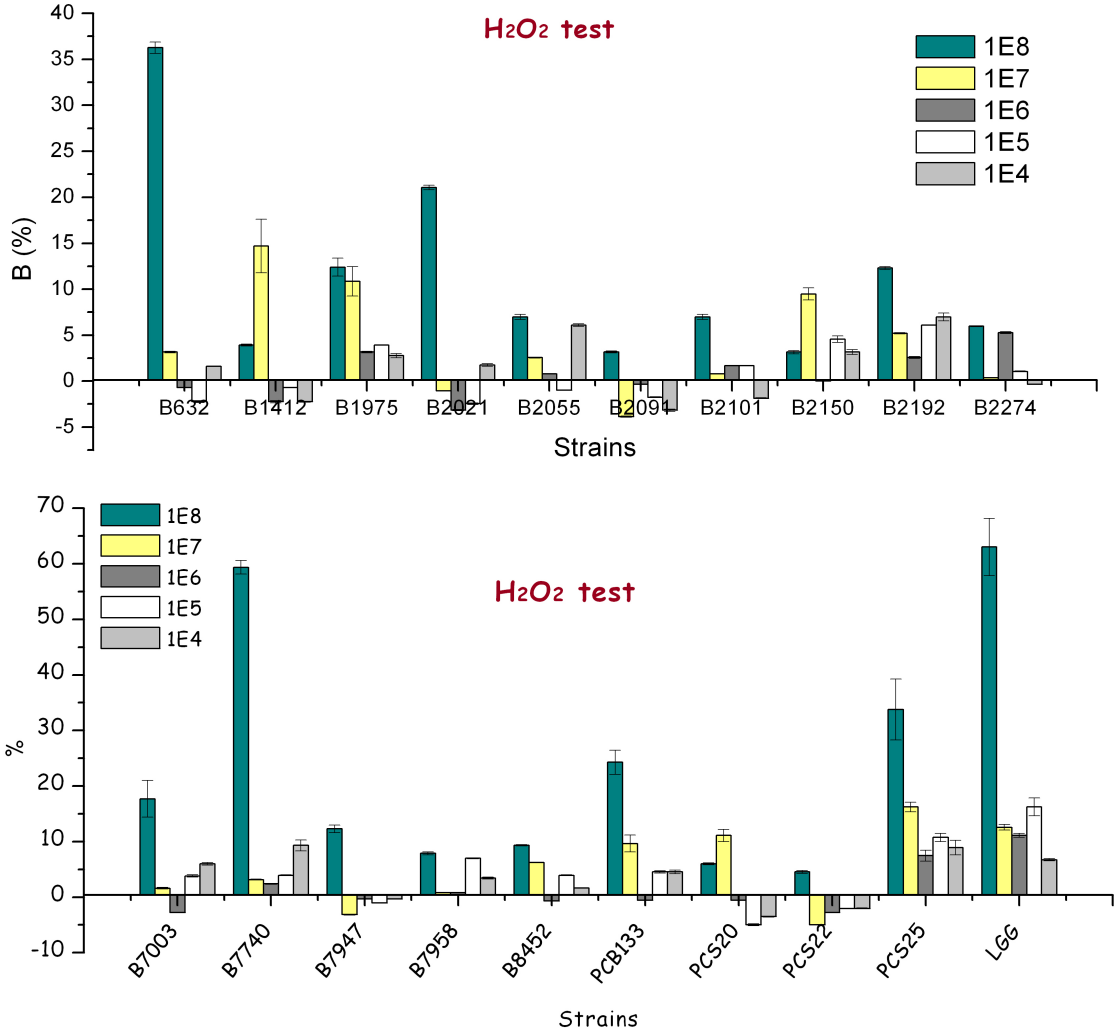


Fig. 11.3 Production of H_2O_2 in B1OXI cells incubated with potential probiotic strains (results are expressed by the mean ratios (%, ± SD) of absorbance in treated wells as compared to those in negative control wells).

11.4 Adhesion ability

All bacteria strains tested showed to be able to adhere to the B1OXI cell line. The *L. plantarum* strains (PCS 20, PCS 22, PCS 25) and one strain of *Bifidobacterium breve* (B2150) had a strong adhesion ability, the supernatants and adherent cells counts were more than 10^8 cell/ml after 90 min incubation (tab. 11.1, fig. 11.4). 7 strains of *Bifidobacterium* had an adhesion greater than 80% (tab. 2). Only three strains showed low adhesion to epithelial cells (< 50%): B1412 (*Bifidobacterium longum* subsp. *infantis*), B2055 (*B. longum* subsp. *longum*) and B2101 (*B. longum*). The two strains of *B. pseudocatenulatum* B7003 and B7947 had an adhesion around 70-80%. The adhesion data were not available for *B. breve* B2021, *B. longum* subsp. *longum* B2192 and *B. longum* subsp. *longum* B7958. However the supernatant of B7958 after incubation showed a high cell count per ml (> 10^8), it is possible that this strain had a weak adhesion capability so after the washing steps bacterial cells were washed away. Concerning the LGG strain used as positive control it showed good adhesion ability (about 70%).

	ADH	SUPER
B632	81,08%	80,29%
B1412	37,50%	42,47%
B1975	83,57%	86,29%
B2021	n.a.	n.a.
B2055	45,67%	25%
B2091	83,74%	unc
B2101	28,76%	n.a.
B2150	unc	unc
B2192	n.a.	n.a.
B2274	81,26%	n.a.
B7003	68,98%	n.a.
B7740	82,66%	unc
B7947	75%	unc
B7958	n.a.	unc
B8452	81,23%	unc
PCB 133	86,01%	unc
PCS 20	unc	unc
PCS 22	unc	unc
PCS 25	unc	unc
LGG	71,75%	unc

Tab. 11.1 Adhesion assay results. ADH: percentage of adherent cells in respect of initial inoculum; SUP: percentage of bacterial cells recovered on supernatants. n.a.: not available data, unc: uncountable plates.

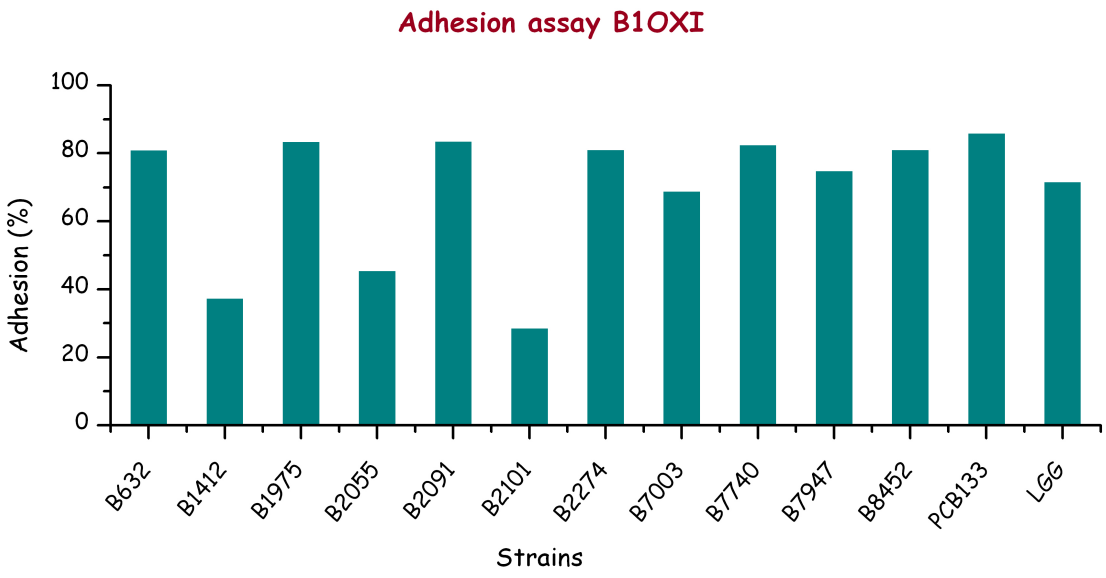


Fig. 11.4 Adhesion assay, the graph shows the percentage of adherent cells. Countless strains are not shown (*L. plantarum* PCS 20, PCS 22, PCS 25 and *B. breve* B2150).

PART 4: DISCUSSION

Chapter 12. In vivo trials

The gastrointestinal (GI) microbiota of mammals and birds is characterized by its high population density, wide diversity and complexity of interactions. While all major groups of microbes are represented, bacteria predominate. Importantly, bacterial cells outnumber animal (host) cells by a factor of ten and have a profound influence on nutritional, physiological and immunological processes in the host animal (Zoetendal *et al*., 2004).

The relationship between the host animal and its gut microbiota can therefore be viewed as a balance between mutualism and pathogenicity. In farm animals, the routine inclusion of antibiotic growth promoters (AGPs) in diets had a beneficial effect on the growth and efficiency of feed conversion, probably by beneficially modulating the gastrointestinal microbiota and suppressing the growth of pathogens. Concerns, however, over the possible selection for genes conferring resistance to therapeutic antibiotics had led to question the practice of using AGPs in commercial settings (Dumonceaux *et al*., 2006). Subsequently, several reports very clearly and definitively concluded that the links between sub-therapeutic usage of antibiotics and antimicrobial resistance among zoonotic bacteria really existed (Bager, 1998; Caprioli *et al*., 2000; van den Bogaard and Stobberingh, 2000). In the year 2006, the EU officially banned the usage of all antibiotics for the sole purpose of growth promotion in poultry and livestock (Halfhide, 2003). Therapeutic use of appropriate antibiotics is now allowed via prescription only through a veterinarian. The impact of this political decision has had a dramatic influence on the methods used to produce broilers, turkeys and table eggs, and one of the main consequence of this tremendous pressure on livestock and poultry farms was a substantial increase in the use of therapeutic antibiotics (Casewell *et al*., 2003).

It was evidenced that AGPs were effective in prevention of necrotic enteritis (NE) in poultry flocks and that the incidence of NE increased in countries where AGPs were stopped (Van Immerseel *et al*., 2004). There is the need to look for viable alternatives that should enhance the natural defence mechanisms of animals and reduce the massive use of antibiotics (Verstegen and Williams, 2002).

One way is to use specific feed additives or dietary raw materials to favourably affect animal performance and welfare, particularly through the modulation of the gut microbiota which play a critical role in maintaining host health (Tuohy *et al*., 2005). A balanced gut microbiota constitutes an efficient barrier against pathogen colonization, produces metabolic substrates (*e.g.* vitamins and short chain fatty acids) and stimulates the immune system in a non inflammatory manner (Brisbin *et al*., 2008a; Brisbin *et al*., 2008b; Haghighi *et al*., 2006; Haghighi *et al*., 2005). In this context probiotics, prebiotics and synbiotics could be a possible solution. The main effects of these feed additives are the improved resistance to pathogenic bacteria colonization and enhanced host mucosa immunity; thus, as a consequence of a reduced pathogen load, an improved health status of the animals (Choct, 2009; Williams *et al*. 2001) and a reduced risk of foodborne pathogens in foods can be achieved.

Therefore, during the past decades several studies addressed the concept of probiotics, prebiotics and synbiotics for use in the poultry industry as an alternative to antibiotic growth promoters.

The synbiotic approach has not a long history of use in broiler chickens. Application studies have been increasing in the last years to assess its effect; however, information available to date is scarce.

Recent researches confirmed the efficacy of specific synbiotic formula on growth performance (*e.g*. body weight and feed conversion rate) and intestinal morphology (*e.g.* increase in the villus height) of broiler

chickens (Awad *et al*., 2009; Awad *et al*., 2008). Moreover, a considerable increase in the bifidobacteria, lactobacilli and total anaerobes populations has been shown, for example, when feeding a diet containing a combination of a galacto-oligosaccharide and *Bifidobacterium lactis* (Jung *et al*., 2008).
Overall, all the authors agreed that a synbiotic product displayed a greater effect than individual preparations (Awad *et al*., 2009; Jung *et al*., 2008; Revolledo *et al*., 2009; Vandeplas *et al*., 2009). This coupling could represent an important and synergistic strategy to improve gut health of chickens from the first days of life and control pathogen release in the environment, decreasing the risk of food-borne infections in humans. Thus, future research and applications in field trials are necessary to look for new combination with the aim to produce standard safe compositions at a high functional level.

The aim of this research was just the formulation of a new synbiotic formula for chicken feed, a formula that could enhance chicken health status, increasing bifidobacteria population and also decreasing pathogen load. Concerning pathogen we focused on *C. jejuni* analysis. This pathogen is, in fact, an increasing threat (EFSA, 2010) at European level and chickens are asymptomatic reservoir of this microorganism at intestinal level (Zhang, 2008).
At first, two separate *in vivo* trials were planned to select an appropriate probiotic microorganism and an effective prebiotic compound to create the synbiotic formula.
Lactobacillus plantarum PCS 20 and *Bifidobacterium longum* subsp. *longum* PCB 133 were chosen for this *in vivo* study for their *in vitro* antimicrobial activity against *Campylobacter jejuni* and for their good adhesion ability on intestinal cell-line. Analyses clearly showed the ability of *B. longum* subsp. *longum* PCB 133 to survive during the gastric transit and be recovered in all chicken faecal samples even if in lower amount compared with the inoculum dose. *B. longum* subsp. *longum* PCB 133 is an intestinal human-derived strain and bifidobacteria population percentage in chicken intestine is lower in comparison to the percentage present in human colon. However, considering administration method (gavage) and the fact that, prior to administration, probiotics were thawed and subsequently injected in chicken gastrointestinal tract, it is relevant that the microorganism was recovered from 100% of administered broilers. Moreover, its persistence was also established one week after stopping supplementation. Regarding the absence of *Lactobacillus plantarum* in DNA samples extracted from faeces of broilers administered with PCS 20 strain, some hypothesis could be formulated. Even if *Lactobacillus plantarum* is a commensal species in human intestine, this strain was isolated from cheese. Moreover *Lactobacillus* spp. constitutes a large part of chicken crop and intestinal microbiota; therefore, competition for a *Lactobacillus* strain is greater compared with the competition encountered by a bifidobacteria strain. It should bed kept in mind also that the detection limit of the real-time PCR instrument used in this study is 10 gene copy number per well, that is to say that if a single-copy number gene is used for quantitative assay it is not possible to detect bacteria with a concentration lower than 3.5 log cfu/g faeces (of course if a multiple copy number gene is used to quantify a specific microorganism, the detection limit is lower). It could be stated also that if a probiotic microorganism does not reach an appropriate concentration in the intestinal environment, probably it could not exert a significant beneficial action on animal health (Fuller, 1995). For this reason, subsequent analysis on chicken microbiota were performed only on control group and on chickens administered with *B. longum* subsp. *longum* PCB 133.
Bifidobacterium spp. quantification was not affected by probiotic supplementation because the starting population was high (~7 log cfu/g faeces) and 4 log cfu/g of faeces of *B. longum* subsp. *longum* cannot affect the mean of total bifidobacteria. The *Campylobacter* spp. quantification revealed a significant increase of total *Campylobacter* in control group after 21 days; this could also be due to a stressing situation caused by frequent manipulation. Concerning *Campylobacter jejuni* quantification, even if the

variability among chickens is grater for this microorganism, a significant reduction was reported in PCB 133 treated group while in control group the pathogen quantification did not change with time.

The use of prebiotics in animal production, as a possible alternative to antimicrobial growth promoters, has given contradictory results, while their use in the modulation of the gut microbial equilibrium is worthwhile (Geier *et al*., 2009; Yusrizal and Chen, 2003a; Yusrizal and Chen, 2003b; Thitaram *et al.*, 2005). They contribute to the establishment of a "healthier" microbiota where bifidobacteria and/or lactobacilli become predominant and exert possible health-promoting effects at the expense of more harmful species. Application studies have been increasing in the last years to assess their effect on gut health, performance, and reduction of pathogen shedding. Xu *et al*. (2003) found a dose dependent effect of fructooligosaccharides (FOS) on average daily gain; whereas Juskiewicz *et al*. (2006) reported no impact on the performance or productivity of turkeys after feeding for eight weeks with different amounts of FOS. A recent study reported no effects on body weight, feed intake and feed conversion ratio in broiler chickens fed with a standard diet and GOS at two different concentrations; however the study clearly showed a significant increase in the intestinal bifidobacteria population (Jung *et al*., 2008).
Mainly, prebiotics seems to enhance selectively lactobacilli and bifidobacteria populations and reduce colonization by pathogenic bacteria (Baurhoo *et al*., 2009; Biggs and Parsons, 2008). Results on animal performance, either with a probiotic or a prebiotic treatment, are often contradictory and mostly affected by the microorganisms or compounds chosen, the dietary supplementation level, and duration of use. In many cases, the environmental and the stress status of the animals are not reported or considered as the experimental setting are often too far from the farm conditions (Gaggia *et al*., 2010).
In our study, after an *in vitro* evaluation on probiotic strains ability to ferment galactooligosaccharides (GOS) and fructooligosaccharides (FOS) at different concentrations (data not shown), broiler chickens were fed with a 0.5% FOS and 3% GOS for 15 days. Animal conditions and environment were the same of the previous trial, but chicken supplier was different. Broilers used in probiotic trial were provided by a farming business while chickens used for the prebiotic experiment were provided by "Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna". This constitutes a big variable for the basic microbiota composition of birds. Information provided by suppliers demonstrated the big differences in animal management, fist of all with respect to egg hatching. In the farming business no special procedures for egg hatching were applied, they used extensive farming methods and after hatching chicks were just put in a clean room. Concerning broilers provided by the Zooprophylactic institute birds were reared with sophisticated techniques. Eggs were sterilized, incubated in sterile incubators and at hatching chicks were put in sterile rooms to avoid cross contamination. This is, of course, one possible explanation for the absence of *Campylobacter jejuni* in all the chickens used in this experiment.
The evaluation of the different performance of the two prebiotics on broiler chickens was carried out comparing the results obtained on bifidobacteria and *Campylobacter* populations. *Bifidobacterium* spp. quantification after supplementation clearly showed a beneficial effect of GOS supplementation compared to FOS and also the decrease of *Campylobacter* spp. was significant. It is important to remember that the intestinal epithelial cells (enterocytes) of the chicken gastrointestinal tract do not produce lactase, for this reason it could be supposed that GOS supplement is entirely used by intestinal beneficial bacteria. The results of this trial showed GOS as the best probiotic supplement for the synbiotic formula.

The final *in vivo* experiment was arranged to test the efficacy of the new formula composed in the end by the *Bifidobacterium longum* and the galactooligosaccharides (GOS). For this experiment the probiotic

microorganism was microencapsulated and provided by Probiotical s.r.l. The microencapsulation technique guaranteed a better survival through gastric transit of the probiotic bacterium being the lipidic coating resistant to acidic pH. The calculated daily intake was higher compared to probiotic trial ($<10^9$ cfu/day) and the quantified *B. longum* microorganisms in faeces confirmed the advantage of this way of administration. PCB 133 was recovered in 100% of supplemented animals. The combined application of probiotics and prebiotics has different effects from those of the individual supplements, but it does not simply result in additive or synergistic effects (Roller *et al*., 2004). In synbiotic supplemented broilers, the significant increase of *Bifidobacterium* spp. confirmed the efficacy of the microencapsulated synbiotic formula. Moreover pathogen quantification showed a significant decrease in broilers supplemented with the synbiotic formula. Results on control group also indicated a significant decrease of *Campylobacter jejuni* after 21 days (but not after 15 days). We have to consider, however, that animals are not stressed as in a real farming business environment, and the result is in any case interesting in an industrial perspective when it is necessary to diminish the pathogen load before slaughter in a short time.

Mathematical models that simulate different strategies to reduce zoonosis infections showed that if the flock prevalence was reduced for example two times then the number of cases associated with consumption of chicken meat would also be reduced approximately two times. This is because there is a one-to-one relationship between the two parameters (Rosenquist *et al*., 2003). Several countries have implemented or are at the point of implementing strategies to reduce the number of *Campylobacter* contaminated broiler flocks and among these strategies the study on the efficacy of probiotics and prebiotic is evaluated.

Chapter 13. In vitro screening of bifidobacteria strains on B1OXI cell-line

In this study the probiotic properties of some *Bifidobacterium* spp. and *Lactobacillus plantarum* strains were tested on chicken epithelial cell line (B1OXI). The indigenous intestinal microbiota is a fundamental part of human and animal body, sometimes scientists refer to it as a "forgotten organ" (O'Hara and Shanahan, 2006) to stress its importance for the host. The complexity of the gut microbiota has been extensively studied and the disruption of its balance (dysbiosis) following antibiotic administration, stress, infections, dietary changes leads to a series of modifications of intestinal permeability, mucosal immune system and intestinal physiology in general. Dysbiosis leads to a greater susceptibility to pathogen colonization of the gastrointestinal tract, the indigenous microbiota has in fact a fundamental role in protecting host intestine against pathogen colonization (La Ragione *et al*., 2004). For these reasons, the screening of potential probiotic strains that are able to stimulate the gut immune system takes on an important meaning especially in animal husbandry, for food safety control. Intestinal immune cells are therefore able to trigger a faster immune response against invading pathogens; both through the innative (ROS production) and adaptive immunitary response (macrophage and lymphocyte activation and cytokines production). However if the stimulation is too high it can lead to tissue inflammation (Brisbin *et al*., 2008a) (Haghighi *et al*., 2005).

Analyzing the results obtained in this study the cytotoxicity test evidenced that almost all strains were not cytotoxic while the MTT test showed that only some strains were able to increase the metabolic activity of cells. Regarding the adhesion test it is necessary to underline that adhesion is one of the most important properties for a probiotic bacteria because it allows probiotic microorganisms to persist in the host and therefore to exert their beneficial properties. *Lactobacillus* strains confirmed their good adhesion ability

that in this study is higher in respect to *Bifidobacterium* strains. Probable their strong adhesion also facilitates the enhancement of the metabolic activity of B1OXI cells, as shown in the MTT test (especially for PCS 20 strain).

Some bifidobacteria strains had a very low adhesion even if they succeeded in stimulating the mitochondrial activity; probably they can secrete stimulating compounds for epithelial cells (Park *et al*., 1999) (Lin and Chang, 2000).

In this work some of the strains tested could be chosen for subsequent tests because they showed almost a good response for all tests, for example *B. longum* subsp. *longum* B2055 and *B. breve* B2101 resulted not cytotoxic, their stimulation of the metabolic activity of epithelial cells was significant and they were also able to induce a low release of H_2O_2 ($p<0.1$), however these strains had also a low adhesion ability. *B. bifidum* B2091 was not cytotoxic, seemed to strongly enhance the mitochondrial activity and showed a good adhesion but it didn't stimulate H_2O_2 production.

Regarding *B. breve* B2150 it could be considered the best strain because of the significant value obtained with cytotoxicity and MTT tests and also for the high adhesion ability. Also *B. pseudocatenulatum* B7003 and *B. longum* subsp. *longum* PCB 133 had good properties; PCB 133 was able to stimulate H_2O_2 production unlike B7003 but *B. pseudocatenulatum* B7003 significantly enhanced the epithelial cells mitochondrial activity.

Among *L. plantarum* strains the best results had been obtained with PCS 20 and PCS 25. PCS 20 showed good results with the cytotoxicity test at a concentration of 10^5-10^4 cells/ml and gave a significant mitochondrial stimulation of B1OXI cells for all the concentrations used, however PCS 25 was also not cytotoxic and was able to stimulate mitochondrial activity and H_2O_2 production in epithelial cells when incubated at high concentration (10^7 cells/ml).

A lot of research works have investigated the possible stimulation of cytokine release after cell exposure to probiotic bacteria. This is recommended as the next step for a subsequent selection of these probiotic strains.

Appendix: the Pathogen Combat Project: "A pan-European alliance to fight food-borne pathogens"

The research described in this thesis is framed within EU 6th Framework Programme (www.pathogencombat.com) PathogenCombat. It is an integrated project that began on 1st of April 2005 and run until March 2010. PathogenCombat consists of 44 partners in Europe and Australia: 24 are research institutions and organizations, 17 are SMEs and 3 are industrial partners. Food safety is of fundamental importance to the European consumer, the food industry and the economy. The impact on trade and competitiveness is substantial. Despite the significant investment in the field, the incidence of food derived diseases is increasing in the EU. PathogenCombat aims at dealing with this pan-European problem through a holistic, multidisciplinary approach towards threats from new/emerging pathogens in the entire food chain. A number of advanced platforms will be developed to investigate the survival and virulence expression of pathogens in feed and food and on contact surfaces in the food chain including the intestinal tract of farm animals. The platforms, several of which are used for the first time in food safety studies, include bioimaging, laser tweezers, phage display/convergent evolution, functional mammalian cell models, functional genomics and microarrays. New/emerging foodborne bacteria, yeast, filamentous fungi and viruses are targeted for milk and dairy products, ruminants, poultry and pigs and their meat products. The overall and specific objectives of PathogenCombat can briefly be described as follows:

- The production of safe food with no or acceptably low levels of pathogens.
- The determination of factors in the food chain, which enable the viability, persistence and virulence of pathogens.
- The detection and prediction of the occurrence and virulence of pathogens in the food chain with molecular biology based culture independent techniques and microarrays.
- The determination of host-pathogen interaction with functional cell model replacing the use of experimental animals.
- The prevention of pathogen transmission along the food chain through new processing technologies and systems, protective cultures and new information on host-pathogen interaction.
- The application of PathogenCombat deliverables in the food chain/SMEs.
- The control of pathogens throughout the food chain with new mathematical models.
- The development of a Food Safety Management System, which incorporates the deliverables of PathogenCombat.
- The creation of a Small and medium Enterprises (SME) Network including dissemination of knowledge, dissemination of results and training of SMEs and consumer awareness on food safety.

The aim of the Work Package 13 “Application in the Food Chain” is related to the application of the knowledge and tools produced within the project and to the development of support measures to food industries.
SMEs and Industrial partners involved in the project, along the last three years of project, aim to apply and improve:
New detection methods and prediction of the occurrence and virulence of pathogens in the food chain and at time of consumption with molecular biology based on culture independent techniques and microarrays.
New processing technologies and systems, new hygienic design, protective and probiotic cultures and new information on host-pathogen interaction to prevent pathogen transmission along the food chain.
New mathematical models for pathogen control throughout the food chain and at time of consumption.
Their Food Safety Management System preventing microbial food borne diseases.

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