Scuola di Dottorato in Scienze Veterinarie
per la Salute Animale e la Sicurezza Alimentare

Università degli Studi di Milano

GRADUATE SCHOOL OF VETERINARY SCIENCES
FOR ANIMAL HEALTH AND FOOD SAFETY
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Doctoral Program in Animal Nutrition and Food Safety

Academic Year: 2011-2012

Inclusion of a species-specific probiotic or calcium diformate in young calves diets: effects on gut microbial balance, health status and growth performance

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CHAPTER 1

General Introduction

1 General Introduction

1.1 Calf breeding in Italy

1.1.1 The veal calves breeding

The veal calves breeding usually include males and females of dairy breeds exceeding the replacement heifers. These animals arrive in the fattening facilities in the first weeks of life and here they remain for a period of 180 days about, until to achieve a body weight of 200-220kg.
The distinguishing peculiarities of the veal calves' meat are achieved through a diet based on milk replacerer, being completed with a minimum amount of concentrate.
The industrialization of this type of farming began in 1960 with the ability to take advantage of the high surplus milk powder produced in northern Europe, leading to a rapid growth of this sector in the early 90s. The veal calves, subsequent to *colostrum* administration are transferred in the fattening centres of fattening and fed with milk replacer powder for a period of 180 - 200 days until the achievement of a live weight of 200-250 kg.
Italy is the third largest country at communitarian level in the breeding of veal calves (18% of total production) after France (31%) and the Netherlands 27% (Suarez et al., 2006); in this regard Lombardy region alone is able to cope the half of the national veal calves request.
In the EU, public opinion has always been influencing the agricultural policies and with regard to veal production, in the last decade the increased public concern on animal welfare has resulted in stricter regulations on veal production (Directive 97/2/EC; Directive by the Council of Europe, 1997). In the past, the provision of solid feeds (either concentrate or roughage) to veal calves was deliberately avoided by believing that this practice would have a negative effect on milk intake, feed conversion and mainly meat color and quality. But as a result of the new EU policies, since 1997 feeding of solid feeds has become mandatory for the veal industry. The reason for the new EU legislation was the potential improvement in health and well being of the calves when fed diets stimulating rumen function (Directive 97/2/EC; Directive by the Council of Europe, 1997).

1.1.2 Replacement heifers breeding

Italy represents one of the leading countries in the herd of dairy cows. Particularly, in the Lombardy region, the breeding of dairy cattle has a portfolio consisting of more than 580.000 sibjects, covering a central role in the production of almost 40% of Italian milk and participating in a major share of the total assets of census cows (1.880.000 heads about, ISMEA, 2011).
In each single farm on the territory, the birth of female calves goes to belong to the park of future heifers needed to restock inside the herd, as the result of the substitution of retirement or not-producing cows.
The replacement, which presents inside the breeding an average rate of 35% (data referred to Lombardy region; Campiotti, 2009) is a direct opportunity to improve the genetic value of the herd, but the range of time after partum and after weaning represents an important cost item, which in most cases does not allow to obtain an adequate number of heifers or an adequate productive potentiality of the same.
A proper approach to the newborn dairy calves, externalized through proper nutrition and management, it's the first and fundamental step for a correct examine of the herd's future.

1.2 Elements of physiology of nutrition in calf

The stomach of ruminants is divided into four compartments: the rumen, the reticulum, the omasum and the abomasums. At birth the calf is considered a functional monogatric because these structures are rudimentary and undeveloped; particularly papillary growth, rumen wall muscularization and vascularisation are minimal. The phase of transition from a pre-ruminant to a ruminant is critical and can be influenced by feeding management, consequently, it is important to know and understand the factors responsible of initiating the cellular growth and maturation of the rumen tissues and how they can be influenced by the composition of the diet (concentrates and roughages, Fig 1). Moreover, specifically for veal calves, being raised at high intake levels of milk replacer, it is also important to identify areas of potential interactions between the milk replacer and rumen development.

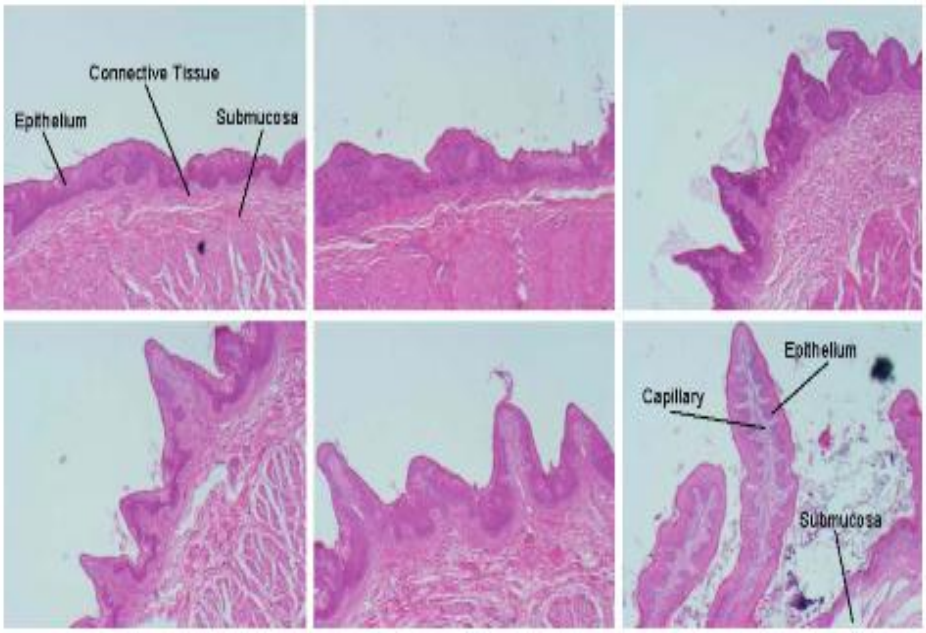

Figure 1: The progression of cellular differentiation and growth considering 3 to 35 days of life, in a grain fed dairy calves

Prior to transitioning from a pre-ruminant to a ruminant, growth and development of the ruminal absorptive surface area (papillae), is necessary to enable absorption and utilization of microbial digestion end products, specifically rumen volatile fatty acids (VFA) (Heinrichs, 2005). These VFA (the major are acetic, propionic, butyric, and valeric acids) are produced by naturally occurring microbes in the rumen and are absorbed and metabolized by the rumen epithelium (Davis and Drackley, 1998).

Presence and absorption of volatile fatty acids is indicated to stimulate rumen epithelial metabolism and may be key in initiating rumen epithelial development, maintaining rumen papillae growth, size and function (Baldwin and McLeod, 2000). The stimulatory effects of different volatile fatty acids are not equal, with butyrate being most stimulatory, followed by propionate. ß-hydroxybutyrate (BHBA) is converted from butyrate as it is oxidized by the rumen epithelial cells and passes through the rumen wall and therefore is a measurement of rumen epithelial metabolism (Lesmeister and Heinrichs, 2004).

Quigley et al. (1991) reported that blood BHBA concentrations increased when calf starter was offered from four days of age and continued to increase proportionately with calf starter intake. Butyrate metabolism by the epithelium appears to increase concomitantly with decreasing rumen pH and increasing butyrate concentrations (Baldwin and McLeod, 2000).

A pH in the optimum range must occur for the establishment and survival of a diverse and stable population of microorganisms. During the first few weeks of life, rumen fermentation activity is low and pH is high. Ruminal pH is controlled by multiple factors including relative concentration of bases, acids, and buffers (Owens et al., 1998), the primary base in the rumen is ammonia (NH_3), with lactate being the primary acid and bicarbonate and phosphate acting as major buffers. A decrease in NH_3 concentration is attributed to ruminal microbial proliferation, due to the increase of microbial use of available NH_3 (Crocker et al, 1998). Rumen epithelial metabolic activity may however occur independently of volatile fatty acid production but numerous researchers have indicated that ingestion of dry feeds and the resultant microbial end products sufficiently stimulate rumen epithelial development (Heinrichs, 2005): it is likely that diets composed of milk, concentrates, or forages affect the rate and extent of rumen epithelial growth differently. Dietary and age differences have been found to alter papillae density of the developing rumen; however, significant differences due to dietary treatment are seldom reported for papillae density in calves, commonly reported as the number of papillae in a fixed area (Lesmeister et al., 2004).

1.2.1 Liquids Feeds and Rumen Development

Milk or milk replacer is initially the primary diet of neonatal dairy calves; its chemical composition and the shunting effect of the esophageal groove limit its ability to stimulate rumen development. Calves receiving only milk/milk replacer exhibit minimal rumen epithelial metabolic activity and volatile fatty acid absorption, which once again does not increase with age. However, ruminal size of the milk-fed calf, regardless of rumen development, has been shown to increase proportionately with body size. Therefore, while a milk/milk replacer diet can result in rapid and efficient growth, it does little to prepare the pre-ruminant calf for weaning or utilization of grain and forage based diets.

Solid feeds, unlike liquid feeds, are preferentially directed to the reticulo-rumen for digestion (Church, 1988). The intake of first stimulates rumen microbial proliferation and production of microbial end products; however, solid feeds have a different efficacy to stimulate rumen development based on multiple chemical characteristics, on chemical composition (casein, starch, cellulose, and minerals) so the resultant microbial digestion end products have the greatest influence on epithelial development (Nocek et al., 1984). In 4 week old calves papillae length and rumen wall thickness were significantly greater when they were fed calf starters containing steam-flaked corn over those fed dry-rolled and whole corn when these corn supplements made up 33% of the calf starter (Fig.2) (Lesmeister and Heinrichs, 2004). This study showed that the type of grain processing too can influence rumen development in young calves.

However concentrate diets with small particle size and low abrasive value (Greenwood et al., 1997) increased volatile fatty acid production, decreased rumen buffering capacity, and subsequently decreased rumen pH are factors commonly associated with occurrences of parakeratosis. Abrasive value has been defined as a feed's efficacy in physically removing keratin and/or dead epithelial cells from the rumen epithelium (Greenwood et al., 1997).

Therefore, increased feed particle size, especially with forages or coarsely-ground concentrates, maintains epithelial and papillae integrity and absorptive ability via physical removal of the keratin layer, increased rumination and rumen motility, increased salivary flow and buffering capacity, and development of mature rumen function and environment.

In contrast to concentrate's advantages for epithelial development, forages appear to be the primary stimulators of rumen muscularization development and increased rumen volume (Zitnan et al., 1998). Large particle size, high effective fiber content, and increased bulk of forages or high fiber sources physically increase rumen wall stimulation, subsequently increasing rumen motility, muscularization, and volume (Vazquez-Anon et al., 1993; Zitnan et al., 1998). As discussed earlier, increases in rumen muscularization and volume have occurred independently of epithelial development.

Figure 2: External and internal appearance of a 4 week-old calf rumen

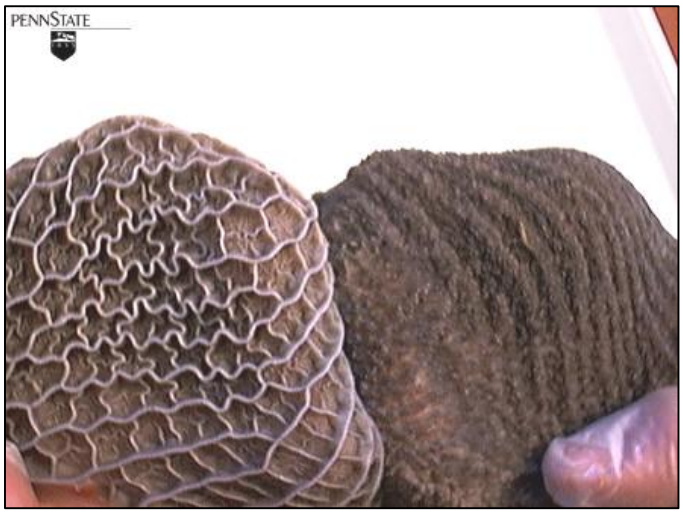

Figure 3: Age 4 weeks - Diet: milk and grain.

Figure 4: Age 12 weeks - Diet: milk and hay.

The rumen of the calf at 4 weeks of age is more developed than the 12-week-old calf that received no grain (Pictures from: PennState University On-Line Web Site)

Figure 5: Age 6 weeks - Diet: milk and grain.

Figure 6: Age 8 weeks - Diet: milk and grain.

In the figures (**Figures 2-6**) the rumen of the calf at four weeks of age is more developed than the 12-week-old calf that received no grain . Continued grain feeding through 6 or 8 weeks leads to further papillae growth and development. Since grain intake is strongly related to rumen development and since we normally cannot see the inside of calves' rumens, grain intake can be used as an indirect estimator of rumen development and a criterion for weaning. (Pictures from PennState Univesity).

1.3 Critical points of veal and replacement heifers breeding

The veal calves are particularly susceptible to stress syndromes, caused by environmental conditions and management. Moreover, the coverage of colostral antibodies is extremely variable. The physiologic immunodeficiency affect both the innate immune system an the adaptive system into its components humoral and cell-mediated immunity. These negative phenomena are added to mismanagement and lack of oversight of the animal health status that cause increased sensitivity to the disease in these so delicate stages and can be formed as the farm of origin, as the final step into the breeding of livestock. The farms of origin are often represented by herds of dairy cows, in which calves are not included in business plans (especially males or females in excess) and are almost considered as a by-product, which results in an uncorrected management of their first days of life. The move by the breeding to rise to one in which the animals will complete the production cycle, is another critical point in their management and the calves often suffer a transport too early, on the timing of umbilical wound healing: in this regard, the Regulation (EC) n. 1/2005 laying down rules for the transport of live animals, states that can not be handled calves under 10 days of age. The situation may be exacerbated by unfavourable environmental conditions on the fattening farms, where they are inevitably conveyed animals of various origin and therefore with a highly differentiated profile of *colostrum* immunity, determining a risk of multiple cross-contamination.
In the fattening companies the health status is a particularly critical factor and results in an economic loss, not only for the eventual death of the animal but also as regards the additional costs antibiotic treatments and the reduction of the growth of the animals.
The onset of health problems can't be often attributed to specific etiologic agents but to multiple factors such as the type of farming and facilities, the competence of the operators,. Therefore, many of the diseases found in the modern manufacturing units in intensive nature are classified as diseases influenced by environmental factors and/or managerial act as stressors. The action of these stressors may be limited in time or, in the worst case and in the absence of corrective measures, last for the entire life of the animal. Maier and Watkins (1998) proposed a close relationship between stress and immune function: the immunological changes due to stress are similar to those induced by infection. In this context, the immune response, stress and the inflammatory process are a set of responses activated by the body to neutralize the stimuli that alter homeostasis (Ottaviani, 1998).

In dairy farm context, for the proper management of the future heifers is essential a correct management of the newborn from the first moments of life which presupposes a proper process management, food and health, as well as an adequate structural support.
Nutrition and management of young calves is in-fact a key parameter for their health and profitability of dairy, but together with the management issues that frequently needed, infectious agents and environmental stressors are able to interact with the animal causing greater susceptibility to pathogens of the same, with particularly evident during the delicate period of weaning.
Recent results from studies on 80 farms in the Lombardy region show that the average mortality in the first month of life of replacement heifers is around 15%, with a mortality rate of 8.9% at delivery (ideally <5%) and 6.4% after the first month of life (Aral, 2003).
Studies carried out by Huzzey and Overton (2010) have also revealed the need to monitor on dairy cows the trend over time (in particular during the transition phase) of stress indicators as predictors of metabolic diseases and immune deficiencies (in particular the endogenous cortisol): it is therefore of primary importance to correlate the status of maternal health with that of the calf in the immediate post-partum period. It has been shown how the environmental stressors in cattle induce an increase in acute phase proteins such as haptoglobin and serum amyloid A protein (Arthington et al., 2003; Lomborg et al., 2008): in addition to cortisol is therefore important to monitor the status of these indicators. The study of strategies that impose a nutritional and managerial stricness becomes crucial.

1.4 Diseases of the newborn calf

Rate of calf illness and mortality vary considerably among individual farms, depending on the calf management program. Calfhood diseases have a significant financial impact on dairies. The costs associated with calfhood disease include treatment costs, replacement costs, genetic loss, and impaired future performance.

1.4.1 Intestinal disease

Neonatal diarrhoea, or scours, accounts for between 50 and 75 % of deaths in dairy calves under three weeks of age. The cause is often multi-factorial and includes exposure to one or more infectious agents (Tab.1) as well as management and environmental factors including colostral management, sanitation, housing, grouping strategies, ventilation, stress, and nutrition. Infectious agents live for long periods in the environment, the major route of infection for most of these agents is from contact with infective faeces in the environment, or ingestion of faecally-contaminated feed or water.
Infectious agents or their toxins cause damage and inflammation in the lining of the small and/or large intestine. This results in an increase in the secretion of fluids and electrolytes into the intestine, an impaired ability of the intestine to reabsorb fluids and electrolytes, and an impaired ability to digest and absorb nutrients. The calf can lose blood, water, electrolytes, and nutrients such as proteins and sugars in the scours. Severe diarrhoea leads to rapid dehydration and often death since the most common cause of death in scouring calves is dehydration.
Many of the infectious agents that cause diarrhoea in calves can also cause disease in humans (e.g. *E. coli*, Salmonellosis, Cryptosporidiosis).

1.4.2 Septicemia

Septicemia often occurs during the first week of life and is defined as the presence of bacteria and their toxins in the bloodstream. While often caused by *E. coli* (septicemic colibacillosis), many other bacteria including *Salmonella* sp. or *Mycoplasma bovis* may also be involved. These bacteria may enter the bloodstream by crossing a damaged intestinal wall or through the open navel of the newborn calf. These bacteria may travel through the blood to infect other organs including the brain (meningitis), heart, lungs, liver, kidneys, eyes, or joints.

1.4.3 Cryptosporidiosis

Cryptospotidium is a protozoan parasite. After ingestion it adheres to the lining of the intestine wall and causes damage to the microvilli, the finger-like projections that are important for absorption of water and nutrients. There is no effective treatment that works directly against cryptosporidiosis. Both *coccidia* and *cryptosporidia* are resistant to antimicrobials. Additionally, *cryptosporidium* is highly resistant to most disinfectants.

Infectious Agent	Age of affected calves	Transmission
Bacteria		
Escherichia coli	Usually less than 3 to 5 days	F/O
Salmonella typhimurium	Usually 2 to 6 weeks	F/O + C/M+ S/N
Clostridium perfringens C	Usually 5 to10 days	F/O
Viruses		
Rotavirus	Usually 7 to 14 days	F/O
Coronavirus	Few days to several weeks	F/O
Bovine virus diarrhea	Any age	F/O + C/M +S/N
Protozoal Parasites		
Coccidiosis	From 17 days to 6 months	F/O
Cryptosporidium	Usually 5 to 35 days	F/O

Table 1: Main gastrointestinal infectious agents and their methods of transmission (F/O= faecal/oral; C/M=colostrum/milk; S/N= Salival/Nasal)

1.4.4 Pneumonia

Pneumonia is an infection that causes inflammation and damage to the calf's lungs, and is the second most common health problem for young calves. While this commonly occurs around 4 or 5 weeks of age, it can also occur in younger and older calves. Although the infection is caused by bacteria and viruses, the calf's environment and management are also very important factors in causing the disease. Humid, moist air, drafts, exposure to wet or chilling cold conditions, and sudden temperature swings are risk factors for pneumonia. Build-up of ammonia and other gases in confinement housing irritate the calf's respiratory tract, damaging its defense mechanisms against infection. Other stressors that increase the calf's risk for pneumonia include crowding, transportation, weaning, and exposure to infectious agents through the co-mingling of calves in group pens or by housing of calves with older animals. Thus, pneumonia is caused as much by poor environment and management as it is by exposure to infectious agents.

Bacterial respiratory pathogens include *Pasteurella multocida, Pasteurella hemolytica, Corynebacterium pyogenes, Mycoplasma dispar,* and *Hemophilus somnus*. Viral respiratory pathogens include *Infectious Bovine Rhinotracheitis (IBR), Parainfluenza-3 virus (PI-3), Bovine Respiratory Syncitial Virus (BRSV),* and *Bovine Virus Diarrhoea* (BVD).

1.4.5 Preventing the introduction and spread of disease

Biosecurity is essential in calf health management. This involves increasing calf's level of immunity against disease while decreasing the calf's contact with infectious agents. Improving a calf's disease resistance requires proper colostrum management, a quality nutrition and vaccination program, and minimizing environmental and other stressors. Minimizing the risk of exposure to pathogens considers environmental management, housing, grouping of animals, sanitation, and control of potential carriers of disease such as people, animals or equipment.

1.5 Feed Additives

The first goal of the livestock production is the delivery of safe foods for human consumption taking into account to welfare of the animal and respect of the environment. In the past, antibiotics have been included in animal feed at sub-therapeutic levels, acting as growth promoters (Dibner and Richards, 2005).
The transference of antibiotic resistance genes from animal to human microbiota lead to banning the use of antibiotics as growth promoters in the European Union since 1st January 2006.
There is need to look for viable alternatives that could enhance the natural defence mechanism of animals and reduce the massive use of antibiotics (Gaggia et al., 2010) and into this contest the use of feed additives has been an important part.
The additives are mainly used in animal nutrition to improve the quality of feed and the animals' performance and health, and consequently to guarantee the safety of animal origin food.
According to Regulation (EC) No 1831/2003 and No 767/2009, feed additives may not be put on the market unless authorization, following a scientific evaluation demonstrating that the additive has no harmful effects, on human and animal health and on the environment. The European Food Safety Authority (EFSA) is responsible for conducting the evaluation of the data submitted requesting authorisations that are granted for specific animal species, specific conditions of use and for ten years periods.
The additive can be a *technological additive***,** which influences the technological aspects of the feed but not directly influences the nutritional value of the feed (e.g. preservatives, antioxidants, emulsifiers, stabilizing agents, acidity regulators, silage additives); a *sensory additive***,** which improve the palatability of a diet by stimulating appetite (e.g. flavours, colorants); a *nutritional additive* which supply specific nutrient required by the animals (e.g. vitamins, minerals, amino acids, trace elements); a *zootechnical additive* which improve the nutrient status of the animal enabling more efficient use of the nutrients present in the diet (e.g. digestibility enhancers, gut flora stabilizers) or it can be a *coccidiostats or histomonostats***,** used to control intestinal health of poultry through direct effects on the parasitic organism concerned.

1.6 Probiotics

Probiotics are feed additives containing microbial species that are considered to be non-pathogenic normal flora. They are also referred to as "direct-fed microbials" and many definitions have been proposed for the term "probiotic". The more widely accepted one is "live microorganism which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). The expected characteristics and safety criteria of probiotics are:

- 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 gastro intestinal tract
- 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
- Genetically 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

1.6.1 History and legal basis in the EU on probiotics in feed

All additives used in animal feed, including yeasts and bacteria, are strictly regulated within the EU legislative framework.
The microbial feed additives were covered by the Council Directive 70/524/EEC concerning additives in feeding stuffs. The Directive was amended a lot of times, the last one was by the Council Directive 96/51/EC. In 2003, these Directives were repealed by the new Regulation (EC) No. 1831/ 2003 of the European Parliament and of the Council on additives for use in animal nutrition which sets out the rules for its authorisation, use, monitoring, labelling and packaging. In the Regulation (EC) No. 1831/2003, the micro-organisms re included in the category 'zootechnical additives' and as functional group within the 'gut microflora stabilisers' defined as micro-organisms or other chemically defined substances, which, when fed to animals, have a positive effect on the gut microflora. Under this Regulation, specific labelling requirements are needed for micro-organisms such as the expire data of the guarantee or storage life from the data of manufacture, the directions for use, the strain identification number, and the number of CFU per gram. In the EU, there is a positive list of the micro-organisms to be included in market products; this list contains some columns which describe the following specifications:

- EU number
- additive
- chemical formula, description
- species or category of animal
- maximum age
- minimum content (colony forming units per kilogram, cfu/kg) in the complete feeding stuffs
- maximum content (cfu/kg) in the complete feeding stuffs
- other provisions
- period of authorisation.

With respect to the guidelines for the assessment of micro-organisms, the Council Directive 87/153/EEC established the composition of the submission dossier for all feed additives. The Commission has updated these guidelines in 1994 introducing specific requirements for enzymes and microorganisms (Commission Directive 94/40/EC).

In 2001, the Directive 87/153/EEC was amended by the Commission Directive 2001/79/EC. Until may 2003, the risk assessment of animal feed additives in Europe was the responsibility of the Scientific Committee of Animal Nutrition (SCAN) (Anadòn et al., 2006). After this date, the European Food Safety Authority (EFSA) took over the functions of SCAN. While EFSA provide expert scientific advice to the European Commission the approval and risk management of a probiotic product is responsibility of the EC and its constituent member's states.

The requirements for a novel probiotic product required by EU regulations on animal feed additives are the identification and characterization to species level, and the efficacy data must be provided in support of any claims made for the product. Some characteristics are requested to product such as no adverse effects on the health of performance, the product must be safe for the operator, have no adverse effects upon exposure and also the product must not pose a risk to the safety of the end-consumer (SCAN, 2001).

Significant progress in legislation for the safety evaluation of probiotics has been made. The European Food Safety Authority (EFSA) has introduced the concept of Qualified Presumption of Safety (QPS) that 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, 2005). According to recent evaluation (Wassenaar and Klein, 2008), QPS system appears more flexible because it takes into account additional criteria to evaluate the safety of bacterial additives such as a history of safe use the food industry and the acquisition of antibiotic resistance or virulence determinants. EFSA has published a list of microorganism, which possess a known historical safety, proposed for QPS status (EFSA, 2007). Micro-organisms used in animal feed in the European Union (EU) are mainly bacterial strains of Gram-positive bacteria belonging to the types *Bacillus* (*B. cereus* var. *toyoi, B. licheniformis*, *B. subtilis*), *Enterococcus* (*E. faecium*), *Lactobacillus* (*L. acidophilus*, *L. casei*, *L. farciminis*, *L. plantarum*, *L. rhamnosus*), *Pediococcus* (*P. acidilactici)*, *Streptococcus* (*S. infantarius*); some others probiotics are microscopic fungi such as strains of yeast belonging to the *Saccharomyces cerevisiae* species and *kluyveromyces*. The 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, 2007). Often not valid taxonomic designations are used in scientific publications or in commercial preparations. *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 and often the products contain other species than those claimed on the label (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 administration to the animals.

The claim made by the producer about the preparation should reflect the actual composition of the food until the "best-before" date of the product at the recommended storage conditions with a decrease of one or two logarithmic units at maximum (Czinn and Blanchard, 2009); often the number of probiotic bacteria found in the products were below the one declared or they were absent (Wannaprasat et al., 2009). It is fundamental to study proper formulations which will allow the maximum viability of the utilized bacteria species.

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

Table 2: List of probiotics studied for application in animal feed

1.6.2 Gut microbiota

In last 15 years the use of probiotics strains in animal production has been increased. These probiotics strains can modulate the balance and activities of the gastrointestinal microbiota in which are responsible to gut homeostasis. The intake of probiotics supplemented in ration and provided to the animals, can strongly affect the structure and activities of the gut microbial communities leading to promoting health and improving the performance in livestock, when it is impaired by numerous factors, such as dietary and management constraints. The understanding of the digestive ecosystems in terms of microbial composition and functional diversity is fundamental to modulate the gastrointestinal tract (GIT) of domestic animals providing to them the possibility to maintain the homeostasis of these complex microbial communities, which can be composed of bacteria, protozoa, fungi, archaea, and viruses, thus promoting a reduction of the incidence of diseases. Therefore considerable researches during 30 years are characterizing the domestic animals´GIT. The welfare, health and feed efficiency of the animals can be affected by different factors, many of them, environmental factors. Among these factors, feeding practices, composition of animal diets, farms management and productivity constraints can influence the microbial balance in GIT, whose role is fundamental to gut homeostasis and its reduction consequently can affect efficiency digestive When occurs the reduction of microbial in GIT, some reactions as digestion and fermentation of plant polymers are impaired, since the action of the microbiota on gut is strongly related with the realization these reactions, and the animals also are impaired by the fact these polymers to be of particular importance to the herbivorous (Chaucheyras-Durand and Durand, 2010).

Under normal circumstances, commensal bacteria are an essential health asset with a nutritional function and a protective influence on the intestinal structure and homeostasis. The intestinal microbiota protects against infections, and actively exchanges developmental and regulatory signals with the host that primes and instructs mucosal immunity. Although the intestinal microbiota is complex and the role of most of the bacteria in providing benefit to the host is not clear, bacterial species of the genera *Lactobacillus* and *Bifidobacterium* have been shown to supply protection against enteric infections. By enhancing the beneficial components of the gut microbiotas it is possible to treat various intestinal disorders and maintain host well-being (O'Hara and Shanahan, 2007). Moreover, access to beneficial microorganisms has been suggested to be one of the selective advantages of social behaviour in animals (Ley et al., 2008). In particular, the close proximity of individuals in livestock or poultry farms could facilitate the host–host transmission of microbiota.

Therefore, in high population density, it is important to maintain a "healthy" microbiota as a barrier against pathogen infection. A key issue is to identify and know the species present in the gut microbiota of the different animals. Studies on the survey of the gut bacterial communities in 60 species of mammals based on 16S rRNAanalysis showed that diet, host phylogeny and gut morphology influence the microbial ecology of the gastrointestinal tract (Ley et al., 2008). If mammals are classified as monogastric and polygastric, their microbiota clusters into groups that correspond to these categories. However, the composition of the faecal microbiota is also a strong predictor of the host physiology status. The major microbial groups in monogastric animals (such as pig, chicken, rabbit and man) are *Bacteroides*, *Clostridium, Bifidobacterium, Eubacterium, Lactobacillus*, *Enterobacteriaceae*, *Streptococcus, Fusobacterium, Peptostreptococcus* and *Propionibacterium*. In polygastric animals, (such as cow, sheep and lamb), the rumen is the most important microbial ecosystem with the predominance of fiber-degrading groups belonging to Fibrobacter, *Ruminococcus*, *Butyrivibrio* and *Bacteroides* together with major groups such as *Prevotella*, *Selenomonas, Streptococcus, Lactobacillus* and *Megasphaera*. Some anaerobic fungi and ciliate protozoa and a large number of methanogens are also present in the rumen (Mackie et al., 2000). In mammals, the percentage of the different microbial groups varies between individuals, depending on age (Mueller et al., 2006) and on the health/pathological status (Abt and Artis, 2009). Diet is an additional factor influencing the gut microbiota; herbivores contain a higher number of bacterial phyla, while carnivores the fewest number, and omnivores are at an intermediate level (Ley et al., 2008).

1.6.3 Efficacy of probiotics

The use of probiotics in animal feeding could be enhanced by a preliminary in vitro screening: antimicrobial activity, survival in the GIT, adhesion studies and antibiotic susceptibility are among the main probiotic properties that should be analysed to assess functionality and safety. The advanced molecular methods, such as microarrays, will improve the detection of these multiple characteristics, also allowing the analysis 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).

Dose, timing and duration of the administration of probiotics may be a factor affecting efficacy: in acute infectious diarrhoea, higher dose of probiotic given for short period of time seems to be more effective than lower doses (Sazawal et al., 2006).
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 favorable habitat for themselves (Siggers et al., 2007)

1.6.4 Probiotics and immune modulation

The maturation of the humoral immune mechanisms can be conducted by microbial colonization, this events can promote the circulation of the IgA and IgM secreting cells.
The other important factor that can be affected by microbial colonization on the gut of different animals particularly the ruminants are the balance of the different T helper subsets. The memory B and T cells migrate to effectors sites in consequence these events. Other mechanisms to immune modulation are followed by active proliferation local induction of certain cytokines and production of secretion antibodies as IgA (Perdigon et al., 1999; Al-Saiady et al., 2010). When the host is exposure to the antigen, immune cells respond releasing cytokines from host direct the subsequent immune responses. The low-dose tolerance immunity TGF-B associated in via local cytokine is the man mechanisms which the gut associated lymphoid tissue maintains homeostasis. Some lactic acid bacteria can induces the production of proinflammatory cytokines, tumor necrosis factors alpha and interleukin-6 from human peripheral blood mononuclear cells. A strain of *Lactobacillus casei* can inhibit the growth of pathogenic strains as *Pseudomonas aeruginosa* and *Listeria monocytogenes* leading to an increase in the level of macrophages (Fleige et al., 2008; Frizzo et al., 2010; Buddington et al., 2011). Others strains as *Lactobacillus acidophilus* and *Bifidobacterium bifidum* could inhance non-specific immunity and concluded that specific lactic acid bacteria could play a role in specific age groups, specific neonates or the elderly (Fleige et al., 2008).

1.6.5 Probiotics in dairy cows and cattle

Studies on the application of probiotics in ruminants have been performed considering both the health status of the animals and the economics parameters. The most significant effects of probiotics have been demonstrated when they have been included in the diet of animals during particularly stressful: at weaning, at the beginning of lactation period and after a dietary shift.
In adult ruminants, probiotics have mostly been selected to target the rumen compartment, which ecosystem consists of wide diversity strictly anaerobic bacteria, ciliate protozoa, anaerobic fungi and archea. Regarding bacterial probiotics, lactate-producing bacteria (*Enterococcus, Lactobacillus*) which would sustain a constant level of lactic acid, thus allowing the lactate-utilising species to flourish (Nocek et al., 2002; Nocek and Kautz, 2006) may represent a possible means to limit acidosis in high-concentrate fed animals. *Megasphaera elsdenii* or *Propionibacterium* spp., which utilise lactate as an energy source, could be administere as direct-fed microbials to avoid ruminal lactate engorgement (Klieve et al., 2003; Stein et al., 2006). A growing interest for using probiotics is to reduce digestive carriage by adult ruminants of human pathogens, such as *Escherichia coli* O157 or *Salmonella.* Among zoonotic pathogens, *E.coli* O157:H17 is the main health threat for ruminants, and cattle are considered the major reservoir (Lejeune and Wetzel, 2007), playin an important role in epidemiology of human infections (Griffin and Tauxe, 1991). To reduce carriage shedding of this coliform strain have been successfully applied to adult cattle (Schamberger and Diez-Gonzalez, 2002). Certain strain of *Lactobacillus acidophilus* have shown to decrease numbers of *E.coli* O157 in feedlot cattle faeces (Tabe et al., 2008) and also appear to reduce shedding of *Salmonella enterica* (Stephens et al., 2007). In particular, steers fed a diet containing *Lactobacillus acidophilus* NP51 showed a reduction of *E.coli* O157 faecal shedding by 57% (Younts-Dahl et al., 2004) and by 35% in beef cattle (Peterson et al., 2007). Other autors have found greater reductions (Brachears et al., 2003; Tabe et al., 2008).
In dairy cow, the yeast strain supplementation have been shown to improve performance, the most consistent effects being an increase in dry matter intake and milk production (Jouany, 2006; Stella et al., 2007; Desnoyers at al., 2009). Other benefits have been related to greater total culturable ruminal bacterial population densities and cellulolytic microorganism (Chaucheyras-Durand et al., 2008) and increase fiber digestibility (Guedes et al., 2008; Marden et al., 2008). The study of Krehbiel et al. (2003) too suggest that probiotics fed alone or in combination with fungal cultures might be efficacious for increasing milk production by lactating dairy cows.

In beef cattle, undergo a variety of stresses, such as recent weaning, transport, fasting, assembly, vaccination, castration, and dehorning the probiotics potentially increase some growth parameters when fed to finishing cattle (Krehbiel et al., 2003), not consistent in other studies (Younts-Dahl et al., 2005; Peterson et al., 2007; Vasconcelos et al., 2008).
The greatest performance response to the bacterial probiotic generally occurred within the first 14 d of the receiving period (Krehbiel et al., 2003).

1.6.6 Probiotic in calves

- *Bacterial microflora population in newborn calves*

The colonization of newborn's gastrointestinal tracts starts during the delivery and it is initially strongly influenced by the manner and environment of birth, by the diet, use of antibiotics, genetic background and environment of the individual. Bifidobacterial population have been detected as part of this complex ecosystem where they may provide health-promoting benefits to the host (Vlkovà et al., 2006). Generally, bacteria colonization starts with *E.coli* detectable in all areas of the digestive tract of calves eight hours after birth, lactobacilli and streptococci being detectable after one day of life. In healthy animals, lactobacilli quickly colonize the gut (Smith, 1965; Karney et al., 1986). Microbial colonization of the rumen occurs rapidly after birth, with a large population of strictly anaerobic bacteria presents after two days (Fonty et al., 1987). Although bifidobacteria were often isolated from calf feces (Scardovi, 1986), only a scarce information exists on their occurrence and quantity in calf gut and no data were available about bifidobacterial counts in other parts of the gastrointestinal tract of young animals.

- *Use of probiotics in newborn calves*

In the neonate and in stressed calves, the microbial population is in transition and extremely sensitive; abrupt changes in diet or the environment can cause alterations in microbial populations in the gastrointestinal tract. Neonatal-calf diarrhoea, most often caused by enterotoxigenic *E.coli*, is an important cause of morbidity and mortality in young ruminants. Moreover, faecal counts of lactobacilli normally are higher than coliforms in healthy animals and reversed in those suffering from diarrhea.

Feeding probiotics to suckling calves has shown to improve digestion with beneficial actions on gut health: the rate, the severity and length of diarrhoeal episodes are reduced (Lejeune and Wetzel., 2007; Morrison et al., 2010; Riddel et al., 2010). The decreased incidence of diarrhea might be associated with a consistently increased shedding of *Lactobacillus* (Abu-Tarboush et al., 1996) and an inconsistent decreased shedding of coliforms (Krehbiel et al., 2003) in feces in response to supplements of *Lactobacillus*.

Consequently to the positive response on gut and general health (Fleige et al., 2008; Al-Saiady et al., 2010; Frizzo et al., 2010), the effect of probiotic on growth performance is investigated (Timmerman et al., 2005; Frizzo et al., 2010; Morrison et al., 2010; Riddel et al., 2010). Improved weight gain and rumen development have been reported in young calves with several bacterial and yeast strain supplementation (Mokhber et al., 2007 Adams et al., 2008; Frizzo et al., 2008; Kawakami et al., 2010; Nagashima et al., 2010; Frizzo et al., 2011).

To select the most effective and specific potential probiotic for veal calves, the identification at the clonal level of the calves' microbiota is crucial, since different strains within the same species may have different probiotic properties (Soto et al., 2010, Xiao-Hua et al., 2010; Ripamonti et al., 2011). The strains naturally residing in the digestive system of veal calves can provide protection against gastrointestinal infections. In addition, it has been recognised that functionality of multistrain and multispecies probiotics could be more effective than that of monostrain probiotics (Sanders et al., 2003; Hong et al., 2005; Timmerman et al., 2005; Gavini et al., 2006; Corcionivoschi et al., 2010). The advantages of administering multistrain and multispecies probiotics include the enhanced capability of colonizing the gastrointestinal tract and to combine the different mechanisms of action of each strain in a synergistic way.

1.7 Acidifiers in animal production

According to Regulation (EC) No 1831/2003 and No 767/2009, only additives that have been through an authorization procedure may be placed on the market. In European Union organic acids such as formic, propionic and several others (lactic, citric, fumaric and sorbic acids) as well as their salts (e.g., calcium formate, calcium propionate) are considered *technological additives* which influences the technological aspects of the feed. This does not directly influence the nutritional value of the feed but may do indirectly by improving its handling or hygiene characteristics.

Organic acids are weak acids with at least one carboxylic group (-COOH) and a carbon chain having not more than 10 C–atoms, normally C1 to C7. This distinguishes them from the fatty acids with longer carbon chains (Theron and Lues 2007).

Acidifiers have been used for decades in commercial compound feeds, mostly for feed preservation (Ricke, 2003), for which formic and propionic acids are particularly effective. Moreover acidification of milk replacers for use in rearing young calves has been widely practiced and tested in Europe (Jaster et al., 1990).

Actually the market for organic acids in Europe is expected to continue to grow, in Northern Europe was already beginning to adopt these products before the EU ban of antibiotics like growth promoter, based on the expectation that acids would emerge as a 'cost-effective, performance enhancing option for feed industry' (Kochannek, 2011).

The salts of organic acids have the advantage of being less corrosive, less odorous and easier to handle than free acids. Another rather new form used in animal nutrition is coated acids: the coating allows a stepwise release of the acid so that it can pass through the small intestine and also act directly in the large intestine (Piva et al., 1997; Piva et al., 2002). Factors influencing the decision of which acid to use in animal nutrition, its form and its dosage depends on the efficacy but also on the costs which can vary considerably among the different commercially available organic acids.

The main mode of action of organic acids is through their antimicrobial effects enhancing the growth performance, the magnitude of which is dependent on the chemical properties of the individual acid or acid salt.

1.7.1 Use of acidifying in livestock

Benefits from the use of dietary acidifiers include positive effects on growth performance and health status (Tab.3).

	Effective Form	Effects
Feed	H^+	-pH reduction
		-reduction of acid binding capacity
		-reduction of microbial growth
	H^+ and anion	-antibacterial effects
Intestinal Tract	H^+	-pH reduction in stomach and duodenum
		-improved pepsin activity
	Anion	-complexing agents for cations
	H^+ and anion	-antibacterial effects
		-change in microbial concentrations
Metabolism		-energy supply

Table 3: The different uses of acidificants in livestock, and their main effects (Lückstäd, 2006)

1.7.2 Acidified milk/milk replacers

Acidifying of milk has some main reasons: conservation of the milk, killing pathogens inside it and destruction of some antibiotics, but also as a way of reducing the pH in the calves digestive tract and thereby prevent diarrhoea caused by bacteria like *E. coli* (Jaster et al., 1990). The acidification can be caused by adding to cold milk (10°C) organic acids like formic acid, propionic acid or citric acid (Davis and Drackley, 1998), added carefully and mixed properly in order to achieve a homogenous liquid. Davis and Drackley (1998), reported an improvement in the performance of calves fed acidified milk, but other studies show no advantage to calf growth (Jaster et al., 1990; Raeth-Knight et al., 2009). The acidified milk should ideally have a pH of >5.0 in order to impair the growth of *E.coli* (Cherrington et al., 2008; Constable, 2009) and for stable preservation (Tomkins and Jaster, 1991).

1.7.3 Growth promoting effects

Organic acids may influence mucosal morphologhy or induce alterations in gut microflora thought bacteriostatic or bactericidal actions, as well as enhance endogenous enzyme activity, stimulate pancreatic secretions and they also serve as substrates in intermediary metabolism (Partanen and Mroz, 1999). It is also hypothesized that acidifiers could be related to the reduction of gastric emptying rate, the energy source in intestine, the chelation of minerals, the stimulation of digestive enzymes and the provision of an energy source in the distal gastointestinal tract. Organic acid supplementation can reduce dietary buffering capacity, which is expected to slow down the proliferation and/or colonization of undesirable microbes, e.g. *Escherichia coli*, in the gastro-ileal region, resulting in reduction of scouring (Partanen and Mroz, 1999; Partanen, 2001).

In general it is believed that acidifiers can enhance the growth performance by:

a. Improving gut health by promoting the beneficial bacterial growth, while inhibiting growth of pathogenic microbes (through reduction of pH and buffering capacity of diets). A reduced buffering capacity of diets containing organic acids is also expected to slow down the proliferation and/or colonization of undesirable microbes, e.g. *E. coli*, *clostridia* in the gastro-ileal region (jejunum, cecum) (Partanen and Mroz, 1999; Biagi et al*.,* 2003). In addition organic acids or their salts could not improve the animal growth performance, but they could indirectly increase cecal pH and cecal ammonia concentrations (Biagi et al., 2007).

b. Stimulating - improving pancreatic secretions (Harada et al., 1986), which increase the digestibility, absorption and retention of protein and amino acids (Blank et al., 1999, Kemme et al., 1999) and minerals (such as Ca, P, Mg and Zn - particularly Ca and P) (Jongbloed et al., 2000; Valencia, 2002; Omogbenigun et al., 2003) in the diet. Although opposite results have also been reported (Radecki et al., 1988), it is generally considered that dietary organic acids or their salts lower gastric pH, resulting in increased activity of proteolytic enzymes and gastric retention time.

c. Influencing of gut morphology by promoting changes in the digestive function and microbial ecology and fermentation (Piva et al., 2002; Manzanilla et al., 2004). Some organic acids act positively on microbial growth and ammonia production by pig cecal microflora. Kirchgessner and Roth (1998), have proposed that organic acids may stimulate intermediary metabolism resulting in improved energy or protein/amino acid utilization. The use of some organic acids has been found to reduce the formation of biogenic amines (such as cadaverine and putrescin) that have unfavourable effects on growth and feed conversion. The growth stimulation effects of formic, acetic and propionic acids are partly caused by their inhibitory effect on biogenic amines (Eckel et al., 1992).

1.7.4 Mechanism of action

- *The acid-buffering capacity*

Addition of organic acids reduces dietary pH curvili nearly depending on the acid pKa value and buffering capacity (Bolduan et al. 1988) of the diet. The pH-lowering effect of different organic acids is reduced in the following order: tartaric acid>citricacid>malic acid> fumaric acid>lactic and formic acids>acetic acid> propionic acid. Salts of organic acids have only a small influence on dietary pH, but the addition of protein and mineral sources to the diet weakens the pH-lowering effect of the acid (Roth and Kirchgessner, 1998). It seems reasonable to assume that the buffering capacity of feed can be considerably influenced by the selection of feed ingredients, and it may in part reflect the differences in the effectiveness of acidifiers.
In general, organic acids lower dietary buffering capacity, whereas certain salts of organic acids can increase it. The greatest acidification benefits have been observed in diets formulated from cereals and plant proteins, while the growth-promoting effect in diets containing milk products is small (Giesting et al., 1991). The latter presumably holds true when lactose in milk products is converted to lactic acid by lactobacilli in the stomach, creating the desired reduction in pH and thus reducing the need for diet acidification (Easter, 1988).

- *The antimicrobial activity*

The reduction of pH due to inclusion of organic acids conducts to an antimicrobial effect can prevent the growth of bacteria (especially Gram negative bacterial species, like *Salmonella spp.* and *E. coli*), yeasts and moulds. In the stomach, the pH is decreased, reducing the concentration of all the types of bacteria. In the small intestine, only the organic acids with antibacterial activity are able to inhibit bacteria growth. This is the main reason that the use of these acids has been proposed as a way of preventing or reducing the incidence of diarrhoea in young pigs (Jensen et al., 2001; Tsiloyiannis et al., 2001; Piva et al., 2002; Papatsiros et al., 2011). Thus, the organic acids are divided into two large groups. In the first group are included those with indirect effect on the decrease of the bacterial population by pH reduction and acting mainly on the stomach because the animal organism has the capability of preventing the decline in the acidity in the small intestine by buffering the medium with bicarbonate (fumaric, citric, malic and lactic acids).

In the other group, are involved those organic acids (formic, acetic, propionic and sorbic acid), that have the ability to reduce the pH and affect directly Gram-bacteria by interfering in the bacterial cell with complex enzymes. The antimicrobial effects of the organic acid ions, which act by controlling bacterial populations in the upper gastrointestinal tract, are responsible for the beneficial effects of these acids (Roth and Kirchgessner, 1998). Moreover, organic acids can also enhance the effects of antibiotics by improving their absorption (Radecki et al., 1988; Eidelsburger et al., 1992). In addition, acidifiers can have an initial eradicating effect on bacteria in the feed (Lueck, 1980) and remain there as a first barrier, preventing re-contamination.

- *Mechanism of antimicrobial activity of organic acids*

As undissociated organic acids are lipophilic, they can cross the cell membrane of Gram negative bacteria, such as *Salmonella*. Once inside the cell, the higher cytosolic pH causes the acid to dissociate, releasing hydrogen ions, which consequently reduces the intracellular pH. Microbial metabolism is dependent on enzyme activity, which is depressed at lower pH. To redress the balance, the cell is forced to use energy to expel protons out across the membrane via the H-ATPase pump to restore the cytoplasmic pH to normal. Over a period of exposure expelling protons also leads to an accumulation of acid anions in the cell (Lambert and Stratford, 1998), which inhibits intracellular metabolic reactions, including the synthesis of macromolecules, and disrupts internal membranes. Lactic acid bacteria are less sensitive to the pH differential across the cell membrane, and thus remain unaffected. Inhibition of microbial growth by this mode of action has been exploited for thousands of years in food preservation; organic acids are natural by-products of microbial metabolism.

- *The anion model of organic acid toxicity*

Fermentative bacteria produce organic acids when oxygen is not available as terminal electron acceptor, but they differ greatly in the types of acids that they produce. Because the oxidation of one molecule must be coupled to the reduction of another, anaerobic bacteria often produce several acids. Fermentation acids are inhibitory when the pH is low but some bacteria are much more resistant than others.
Traditionally, microbial growth inhibition by organic acids was explained by the ability of these acids to pass across the cell membrane, dissociate in the more alkaline interior and acidify the cell cytoplasm (Kashket, 1987).

For many years it was assumed that bacteria maintained a slightly alkaline intracellular pH, but this assumption was largely based on work with laboratory cultures of *E. coli* (Padan et al., 1981).

It is now clear that many fermentative bacteria have the ability to let their intracellular pH decline when the extracellular pH becomes highly acidic. This decline in intracellular pH necessitates a metabolism that can tolerate a lower pH, but the strategy appears to be highly adaptive. When intracellular pH remains high, the pH gradient across the cell membrane can become very large. The protons can be pumped back out of the cell, but the pH gradient causes a logarithmic accumulation of the fermentation acid anions. By letting intracellular pH decrease, the bacterium has a much smaller pH gradient across the cell membrane and it is protected from anion accumulation.

Continuous culture studies with *E. coli* K-12 and O157: H7 indicated that the two strains diferred greatly in their sensitivity to acetate at pH 5.9, and the ability of O157: H7 to tolerate more acid than K-12 could be correlated with ability to decrease intracellular pH (Diez-Gonzalez and Russell 1997). These experiments also revealed another important observation about fermentation acid toxicity. When the intracellular acetate concentration of K-12 increased there was a nearly equal molar increase in intracellular potassium. These results indicated that fermentation acid anion accumulation was at least in part an osmotic stress. Recent work with *Clostridium sporogenes,* a silage and food contaminant, indicated that it accumulated lactate anion at acidic pH values in accordance with the pH gradient across the cell membrane, but lactate anion accumulation caused a secondary effect (Flythe and Russell, 2007). When lactate anion increased, the cells lost intracellular glutamate, and its fermentation scheme of amino acid deamination is dependent on glutamate transaminase. The final result was a virtually complete inhibition of ammonia production. The antimicrobial activity of organic acids on other bacterial species has not been correlated with intracellular pH regulation, but bacteria that could be classified as neutrophiles seem to be more sensitive than those that are acid tolerant. For example, the minimal inhibitory concentration (MIC) of acetic acid is 250 times lower for *Bacillus subtilis* than lactobacilli (Hsiao and Siebert, 2002). The anion model of organic acid toxicity explains why bacteria differ in their sensitivity to organic acids, but it does not provide information on the antibacterial effect of one acid versus another. The MIC for acetic, butyric, lactic and caprylic acid in *E. coli* are less than 4 g/l, but this same bacterium is approximately 10-times more resistant to malic, tartaric and citric acid (Hsiao and Siebert, 2002). This observation indicates that factors such as chain length, side chain composition, p*K*a values, and hydrophobicity could affect the antimicrobial activity.

1.7.5 Formic acid

In animal nutrition the use of formic acid and its salts have been the subject of investigation due to an antimicrobial action. Formic acid (also called methanoic acid) is the simplest carboxylic acid and its mainly used as formate (the salt is less corrosive and less toxic than the free acid). It can be found in plants and insects where it mainly serves as defending agent. On the other hand, formate is an integral part of the metabolism, especially in the transfer of 1-C intermediates and in the synthesis of purines (Partanen and Mroz 1999).
Its chemical formula is HCOOH or HCOH. It is an important intermediate in chemical synthesis and occurs naturally, most notably in the venom of bee and ant stings. In fact, its name comes from the Latin word for ant, *formica*, referring to its early isolation by the distillation of ant bodies. Esters, salts, and the anion derived from formic acid are referred to as formates.

- *Physical properties*

Formic acid is a colorless liquid which freezes at 8.4° and boils at 101°. The dielectric constant is 58. It has a sharp pungent odor and is miscible in all proportions with water.
Formic acid tends to form azeotropes and a variety of these are known. Among the more interesting are those containing tertiary ammonium formats which, as shall be seen, have some interesting properties. Formic acid exhibits severe toxicity as both an acute (short duration) local and acute systemic hazard. It shows slight chronic (long duration) systemic toxicity and moderate chronic local toxicity. Thus, exposure to skin, ingestion, and inhalation should be avoided (Gibson, 1969).

- *Chemical properties*

The aldehyde-like reducing properties of formic acid encouraged some early chemists to postulate that formic acid is abnormal in structure More modern techniques have shown this not to be the case, except that the ether C-O bond is abnormally short, indicating much double bond character

This undoubtedly contributes to its relatively high acidity. Formic acid is the strongest of the unsubstituted alkanoic monoacids. It has a p*K*a of 3.77 as compared with 4.77 for acetic acid. Its relatively high acidity is due to the lack of alkyl groups and their attendant electron release by an inductive effect.
This electron release causes destabilization of the carboxylate anions resulting from ionization of the higher monocarboxylic acids (Gibson, 1969).

- *Calcium formate and calcium diformate*

Calcium formate (CaFO), Ca(HCOO), is the calcium salt of formic acid, HCOOH. It is also known as food additive E238 in food industry. The mineral form is very rare and called formicate. It is known from a few boron deposits. It may be produced synthetically by reacting calcium oxide or calcium hydroxide with formic acid (Lide et al., 1998).
Physically, calcium formate is an odourless, free-flowing white crystalline solid and it is a convenient source of calcium and formate ions for aqueous solutions.
Calcium formate actually has different uses in the industry: **leather tanning** (as a masking agent in the chrome-tanning process), **cement additive** (CaFO imparts a number of properties desirable in the final product, e.g. increased hardness and decreased setting time in comparison to ordinary cements), **gas generation** (a mixture of calcium formate with ammonium nitrate or with chlorate, perchlorate, peroxide, superoxide or permanganate of an alkali metal evolves rapidally large volumes of gas when ignited), **flue-gas desulfurization, safety explosives** (**c**alcium formate can be used as the fuel component in explosives designed to have improved deflagration behaviour), **de- icing agent** (mixtures of calcium formate with urea are excellent agents for the melting of ice), **silage treatment** (calcium formate is commonly used as a component of an ensiling agent for silage treatment).
The calcium formate in animal feed acts as a feedstock preservative and an acidifying effect on the gastro-intestinal tract (Kil et al., 2011), and as a source of calcium in cows and calves (Xu et al., 1998). The main difference between calcium formate and calcium diformate, both used in animal feed supplementation, is the percentage of inclusion of calcium and formic acid. The calcium formate presents the 50 percent of calcium and the same percentage of formic acid while the calcium diformate presents the 75 percent of calcium and the 25 percent of formic acid.

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CHAPTER 2

Objectives

2 Objectives

The ban on the use of antibiotics as feed additives in animal nutrition has led to a worldwide search and implementation of alternative strategies for preventing the growth of pathogenic bacteria in farm animals, to maintain and improve the health status and performance.

The objective of this thesis was to evaluate the effect both a species-specific probiotic (*Bacillus coagulans*, *Lactobacillus animalis* and *Lactobacillus paracasei* spp. *paracasei*) than an acidifier (calcium diformate) in calves. To achieve this objective, three different trials were designed. In the first trial the effects of a species-specific probiotic inclusion in 96 veal calves diet was investigate (Chapter 3), the second one studied the effects of the same administered ptrobiotic on 22 dairy calves (Chapter 4) and the last examined the effects of inclusion of calcium diformate in the diet of 36 veal calves (Chapter 5). The studies evaluated the effects of the administrations on health status, gut microbial balance and growth performance. On veal calves the effects of probiotic and acidifier on slaughter performance and histological survey were further evaluated.

CHAPTER 3

Specie-specific probiotic supplement in veal calves diet: effects on zootechnical and microbial parameters in standard rearing conditions.

3. Specie-specific probiotic supplement in veal calves diet: effects on zootechnical and microbial parameters in standard rearing conditions.

3.1 Abstract

The aim of the study was to evaluate the effects of the administration of a species-specific probiotic supplement to veal calves on performance and microbial parameters in standard rearing conditions. Ninety six male Friesian veal calves (49.31 ± 1.38kg of body weight and 20±5 days of life) were divided *at random* in two homogeneous groups of 48 animals each from the arrival in the farm and fed either a basal diet (C) or a basal diet plus $1.8x10^9$ CFU/head/day of a probiotic supplement containing *Bacillus coagulans*, *Lactobacillus animalis* and *Lactobacillus paracasei* spp. *paracasei* in a 35:30:35 ratio (T) for a total of 180 days. Starting from 10th day from arrival and monthly until the end of the trial, individual body weight (BW) was recorded and average daily gain (ADG) was computed. At the same time, on the half of animals per group, faecal samples were collected for faecal score (FS) evaluation, Lactobacilli count, *Escherichia coli* count and Lactobacilli/*E.coli* ratio, while blood samples were collected for haematological, haematochemical and immunological parameters evaluation. During the whole experimental period daily health status and therapeutic treatments were recorded for General Health Score (GHS) determination. At slaughter 10 animals per group were analysed for macroscopically injuries detection, and gut samples were collected for histological analyses. On each subject carcass weight, dressing percentage, fattening condition, carcass grade (SEUROP classification) and meat pH were recorded. During the trial were considered six times (corresponding to the each month of fattening): from time 0, referred to the 10th day from arrival, to the last considered month (time 6). BW resulted significantly higher ($P \leq 0.05$) in C calves than T at the 5th (C=226.76kg *vs* T=223.27kg) and 6th (C=267.14kg *vs* T=263.56kg) considered times while ADG was not different between groups. Faecal consistency was significantly higher in T group at the 4th sampling ($P \leq 0.01$) as faecal Lactobacilli content (C=8.37 Log_{10} *vs* T=8.49 Log_{10}; $P \leq 0.05$). This trend could explain the better GHS observed in supplemented calves during the trial.

At different times, some haematological parameters such as basophils, aspartate amino transferase (AST), bilirubin, glucose, urea, bactericidal and complement were higher ($P<0.05$) in C group, while non-esterificated fatty acids (NEFA) and lactate dehydrogenase (LDH) showed lower values than T ($P<0.05$). At slaughter no differences were observed for carcass weight, dressing percentage, carcass grade and meat pH. The *cecum* histological examination revealed a less evident de-epithelialisation and a greater integrity of the epithelium surface in T subjects than C, while in both the *ileum* than in *cecum* a numerical increase of the intestinal crypts' depth was detected. The administration of species-specific probiotic was able to improve the gut microbiological balance and health status, with no evident effects on growth performance.

3.2 Introduction

Italy represents one of the largest Europe market for the veal calves trading. The veal calf, among the livestock, is predisposed to stress syndromes caused by breeding conditions and management: these subjects are immunologically immature and the antibody levels are often extremely variable. This aspect tends to be amplified by the breeding procedures in the farms: the calves are often transported too early (not healed umbilical wound), and do not receive an adequate *colostrum* administration in the early days of life, showing consequently a marked sensitivity to conditioned diseases in the first weeks of life.
The different animals' origin corresponding to a highly differentiated *colostrum* immunitary profile, exacerbates the unfavourable environment conditions in fattening farms.
The gastrointestinal and respiratory diseases most affect the veal calves: diarrhoea is a major cause of mortality while respiratory problems cause economic losses related to the use of treatments, and lower growth performance (Timmerman et al., 2005).
In this context is necessary to reduce the use of antibiotics, given that the microorganism's resistance is currently considered one of the major public health problems. Has been demonstrated that the resistant strains selection is able to colonize the gut and consequently the environment. In particular, resistant strains of *Escherichia coli* and Enterococci has been isolated from the veal calves' intestinal content, in relation to the massive use of antimicrobial substances as growth promoters in the past (Dir.97/72/CE; Busani et al., 2003).
To search new nutritional strategies allows to combine the economic needs of farmer with the protection of consumers, the use of probiotics to improve the performance of livestock is well know for a long time by now. These products are used in the zootechnical practice to maintain the balance of the intestinal microflora in order to ensure a better growth performance (Krehbiel et al., 2003) and they are defined by Fuller (1989) as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance".
The probiotic action is expressed at various levels on intestinal microflora, intestinal epithelial cells (Bontempo et al., 2006; Di Giancamillo, 2007) and the immune-system (Sheridan et al., 1994). Especially for ruminants, the probiotic dietary supplementation can improve microbial conditions in both the rumen and the lower digestive tract (Elam et al., 2003) resulting in a better ability to absorb nutrients (Giardini and Villa, 2007). The aim to be achieved with the probiosis in calves is the maintenance of good digestive process of the calf, which is reflected on a positive general health status.

Currently probiotics in mixed culture are often used, but with low interest to the species-specifity of the strains contained, with consequent loss of part of the positive effect. The term "species-specificity" means the award of the probiotic microorganism quality to a single clone and not to the species. In fact the selection of lactic strains naturally resident in the digestive tract of veal calves, allows enhancing the barrier effect against pathogenic bacteria thereby protecting the intestinal mucosa from infection better than other non-species-specific probiotics

The literature highlights the importance of the specificity strain for its probiotic characteristics. To optimize the use of probiotic compounds in the farms the identification of probiotic bacteria level clone species-specific isolated directly from veal calves is crucial. It is also noted that is recognized a feature to a greater formulated multi species/multi strain compared to administration of a single species or a single strain (Timmerman et al., 2004).

The objective of this study was to evaluate within a made feedstuff, the effectiveness of the probiotic species-specific selected in order to improve the health conditions of calves by maintaining the bowel.

3.3 Materials and Methods

The experimental trial was divided into two phases. The first was performed *in vitro* and it allowed us to produce the probiotic formulation and its subsequent technological, microbiological and stability checks.
During the second work phase an *in vivo* trial was carried out in order to evaluate the efficacy of the probiotic on the health status and productive performances of the veal calves in standard farming conditions.

3.3.1 Probiotic biomass production process and in vitro check of the product

The design and formulation of the probiotic compound was carried out at the Department of Veterinary Sciences for Health, Animal Production and Food Safety.
The three selected strains (*Lactobacillus animalis* DUP 5009, *Lactobacillus paracasei* ssp.*paracasei* DUP 13077 and *Bacillus coagulans* RiboGroup 189-444) were produced through fermentation in twin jet laboratory mixers.
Initially the strains were grown on nutrient agar, then inoculated into nutrient broth, thus optimising the formulation of the growing means based on fundamental growth and fisical parameters. The process of production of the *Bacillus coagulans* was optimised for the formation of thermoresistant endospores.
The biomass development was measured by the means of the optical density and of the dry weight of known volumes of centrifuged cultures followed by lyophilisation.
The vital charge was measured by the means of life count on powder samples. The obtained powders were kept at 4°C until the formulation of the probiotic compound.
At the same time, microbiological analyses were carried out (method IDF STD 50B:1985) using serial dilutions 1:10 and triple analyses to evaluate the stability of the produced strains (Marossi et al., 1999), in order to test the product stability at room temperature, at 42°C and at refrigeration temperature. The stability of the formulation was also tested in seasonal stress conditions (i.e., freezing/defrosting and 37°C-refrigeration).
The control of the quantity of every microorganism in the mixture was obtained thanks to a morphological evaluation of the the strain colonies. To evaluate the cloning conservation of the strains, ribotyping was performed on liophylised elements.

The evaluation of the contaminant microorganisms was carried out both on the liophylised elements in pool, and on each individual liophylised strain by the means of liquid culture, to the exception of those used for the count of coagulase-positive staphylococci analysed through streaking.
Determination Coliform bacteria ISO 4832:1991 with growth medium: Violet Red Bile Agar; determination of *E.coli* Method as per Afnor- Bio 12/5-01/99 with growth medium: Coli ID; determination coagulase-positive staphylococci ISO 6888-1:1999 growth medium used: Baird Parker Agar. Finally, for the coagulase test a commercial test was used. The method used for yeasts and molds was ISO 7954:87 and the used growth medium was Sabouraud Dextrose Agar w/cloramphenicol. In order to evaluate the overall Mesophile bacteria count the method ISO 4833:91 was applied on Gelisato Agar growth medium; whilst for the determination of *Salmonella* spp the method ISO 6579:2002 was applied. The composition of the formulation used for this experimental test was the following: 30% of *Lactobacillus animalis* DUP 5009; 35% *Lactobacillus paracasei* spp. *paracasei* DUP 13077; 35% *Bacillus coagulans* RiboGroup 189-444.
In order to better understand the microbical activity of the strains, the inhibition test on dish was carried out. The strains used for the test were the following: *Escherichia coli* ATCC 25988, *Listeria monocytogenes* ATCC 19115, *Salmonella tiphymurium* ATCC 14028.

3.3.2 Animals and Experimental Design

Ninety-six veal calves of Friesian Breed, 94 males and 2 females (20±5 days of life, 49.31±1.38 kg of body weight) were randomly divided into 2 experimental groups composed of 48 animals each.
Control group (C) received a basal diet (consisting in milk replacer *plus* concentrate), while treated group (T) received the same basal diet as C but supplemtented with the species-specific probiotic compound (10^9 UFC/animal/day). Both experimental diets were isoproteic and isoenegetic following Nutrient Research Councill (NRC, 2000) reccommendations for veal calves for each phase of the rearing system, the probiotic dosage was doubled in case of a individual antibiotic treatment.
The calves were located in the same building at the same environmental conditions, not artificially conditioned and in collective boxes of 6 calves each equipped with a concrete slatted floor. The experimental planning of the *in vivo* phase was divided into two periods: the first lasted for 10 days and it allowed the animals to get used to the management conditions and procedures (adaptation period): during this period the same C basal diet used was administered both to control and trated animals. The second, period lasted for 180 days and consisted of dietary supplementation of the species-specific probiotic compound to T animals. The milk replacer (Tab.1) was administered to all the experimental animals twice a day, at 7:00AM and 5:00PM; while the concentrated (Tab.2) was given at 12:00AM starting from their 31st day of life.
From the beginning of the trial until the end of the experimental period, calves were feed increasing amounts of milk ranging from 2 litres/meal to 9.5litres/meal. During the trial three different milk powders were used from a startet type, during the first 80 days, to a growing-finishing type until slaughter.
The passage from starter milk replacer to growing-finishing milk replacer was obtained mixing the two milk powder in increasing concentration for the last one.
The inclusion of milk powder was 100g/L during the first 100 days of the trial, while a mean concentration of 135 g/L was adopted during the last three months. Reconsitution temperature of milk was 72°C, while service temperature was 37°C. The amount of administered concentrated feed* composed by 50% of corn grain and 50% of corn silage, (starting from the 31st day of test) was initially equal to 200gr/calf/day and it was increased up to 700gr/day at the end of the trial.

***** Milk powder and concentrate were provided by Zoogamma s.p.a. (VanDrie Group. Borgo Satollo, Ghedi, Brescia)

Chemical Analyses (% a.f.)	Start FE (% a.f.)	Elite 20 (% a.f.)	Elite 100 (% a.f.)
Humidity	5.00	5.00	5.00
Raw protein	22.00	19.00	20.00
Raw fibre	0.50	0.30	---
Fat	18.00	17.00	18.50
Ash	8.00	7.80	7.80
Vit.A (IU)	25,000.00	25,000.00	25,000.00
Vit. D_3	3,000.00	3,000.00	3,000.00
Vit. E (mg)	150.00	50.00	50.00
Vit. C (mg)	150.00	40.00	40.00
Fe (mg)	50.00	---	---
Cu (mg)	10.00	5.00	5.00

Table 1: Administered milk replacer: chemical anlyses of the three different administered type

Chemical Analyse	Dry Matter (%)	As Fed (%)
Humidity	42.31	---
Raw protein	19.96	7.62
Raw fibre	4.25	2.50
Fat	4.41	2.59
Ash	4.25	2.50
Starch	76.32	44.87

Table 2: Administered concentrate: chemical analyse

3.3.3 Growth performance

The individual BW of each calf was recorded montly during the trial, starting from the 10th day from the arrival in the farm by an electronic precision scale (100 grams sensitivity). According to recorded BW, the ADG was subsequently calculated.

3.3.4 Faecal parameters and microbiological status

In order to highlight calves with enteritis problems (diarrhoea) and evaluate the livestock nutritional management, extimated FS was recorded monthly for the evaluation of the macroscopic status of the faeces of each calf. Each animal was given a mark from 1 to 5 (1: very hard faeces; 5: very liquid faeces). Moreover individual faecal samples were taken monthly on a half of the calves by rectal stimulation. Faecal sample were subsequently pooled (three animals of the same box per pool), stored in vials with transport medium (Faecal TM enteric Plus, Oxoid, Basingstoke, UK) at 4°C, and immediately delivered to laboratories. Pooled faecal samples were than analysed within 24 hours using the official methods IDF STD 50B:1985 on growth medium MRS (DeMan Rogosa Sharp Agar) for the identification of Lactobacilli; whilst in order to identify *E.coli* the AFNOR-Bio 12/5-01/99 method on growth medium Coli ID was used. The serial dilutions applied to both determinations were equal to 1:10.

3.3.5 Health Status

During all the trial period the health status of the calves was constantly monitored and all antibiotic treatments performed were recorded, on both individual and collective basis.
Incidence of diarrhoea and therapeutic tratments lead to the calculation of the General Health Score (GHS) index for each calf as reported by Timmerman et al. (2005) with the following formula:

15 – (1 **x** n. total days with diarrhoea) – (2 **x** n. of individual therapeutic treatments for digestive system pathologies) – (3 **x** n. of individual therapeutic treatments for breathing system pathologies) – (2 **x** n. of mass therapeutic treatments).

Animals affected by pathologies episodes were isolated from the other animals and lodged in a specific dedicated area. Incidence of pathology and mortality rate were recoded during all the experimental trial.

3.3.6 Haematological parameters

Starting from the 10th day from the arrival and monthly, blood samples were collected on half of the tested animals for each experimental group. Samples were taken from the jugular vein by the means of a 10ml vacuum test tube (Terumo Venosafe 10 ml VF-109SHL, Terumo Europe L.V., Leuven, Belgium). The refrigerated samples were subsequently delivered at the laboratory determinations.
The considered haematological, hematochemical and ossidative stress parameters were as follow: red blood cell count (RBC), haemoglobin (HB), hematocrit (HT), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), platelets (PLT), white blood cells (WBC) and leukocyte formula, total proteins, albumin, alfa-, beta-, gamma-globulins, albumins/globulins ratio, not-esterified fatty acids (NEFA), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), total bilirubin, creatin kinase (CK), glucose, creatinine, urea, anti-oxidant power (OXID) and reactive oxygen metabolites (ROM's).

3.3.7 Slaughter Parameters

Upon slaughtering (180th test day), 10 calves per group were necroscopically analysed to determine the presence of macroscopic lesions. Immediately after slaughtering, the whole gastrointestinal tract was isolated and a $1cm^3$ sample of its surface was taken from the small intestine and the *cecum*. All the samples taken were stored in paraformaldehyde in PBS at 4%. They were subsequently tested for: histological profile of the small and *cecum* intestine, structural aspect of the intestinal mucous membrane, evaluation of the height of the intestinal villi and crypts and their relationship (V:C ratio), immunohistochemical parameters (small and *cecum* intestine: anti-PCNA and TUNEL method), immunohistochemical/histometric (small and *cecum* intestine: count of the epithelial cells with PCNA-positive nucleus and TUNEL-reactive) and immunohistochemical parameters (small and *cecum* intestine: anti IgA).
Moreover for each analysed calf any eventual macroscopic lung lesions were evaluated (Redaelli et al., 2006). Also any inflammations of the lung parenchyma, of the bronchial tube and of the pleural membrane were taken into consideration. A sample of the lung parenchyma was taken from each of the selected subjects in order to perofrm an histological evaluation of lesions of the lung tissue, giving a mark from 0 to 5 according to the growing seriousness of the lesions.
The carcass dressing percentage was evaluated for each of the slaughtered calves. The same carcasses were classified according to the SEUROP category assignment as per the EC regulations 1208/81 and 1026/91 (S=superior; E= excellent; U=very good; R=good; O=rather good; P=mediocre).
At the same time the meat pH was evaluated 45 minutes and 24 hours after death.

3.4 Statistical Analysis

Live BW, ADG, FS, GHS, blood parameters, and meat pH were analysed by a MANOVA procedure for repeated measures of SAS/STAT, (Version V8, 1999, SAS Inst, Inc., NC, U.S.A.) including the fixed effect of the treatment, day, and the interaction treatment x day and considering the box or the animal as the experimental unit.

Lactobacilli count, *Escherichia coli* count, Lactobacilli/*E.coli* ratio and slaughtered data were analysed by a General Linear Model (GLM) procedure of (SAS/STAT, Version V8, 1999, SAS Inst, Inc., NC, U.S.A.) including the fixed effect of the treatment and considering the group as the experimental unit. The significance was declared at $P \leq 0.05$.

Histometric data (villus height, crypt depth, V:C ratio) were analysed by ANOVA using the mixed procedure of the SAS package (Version V8, 1999, SAS Inst, Inc., NC, U.S.A.). The model included treatment as fixed effect and the calf as random effect. The data were presented as least squared means ± SE. Differences between least squared means were analysed by orthogonal contrast and considered significant at $P \leq 0.05$.

3.5 Results

3.5.1 Growth performance

In the present trial, dicreased BW was found in T animals than C on months 5 and 6 (Tab.3), (respectively -3,49kg and -3,58kg), while no differences in ADG between the groups were foundd (table 4).

	TIME	GROUP			P		
	(Month)	C	T	SEM	GROUP	TIME	G*T
BW	Initial	50.02	48.59	1.38	≤0.01	≤0.01	0.96
(kg/h)	1	71.11	68.89				
	2	99.51	98.11				
	3	145.80	143.29				
	4	181.22	179.68				
	5	226.76[a]	223.27[b]				
	6	267.14[a]	263.56[b]				

Table 3: Body weight (BW) registered during the trial in in veal calves ([a,b]=P≤0.05)

	TIME	GROUP			P		
	(Month)	C	T	SEM	GROUP	TIME	G*T
ADG	0-1	0.73	0.70	0.07	0.30	≤0.01	0.65
(kg/h/d)	1-2	0.98	1.00				
	2-3	1.36	1.36				
	3-4	1.42	1.58				
	4-5	1.34	1.30				
	5-6	1.44	1.39				

Table 4: Average daily gain (ADG) evaluated during the trial in in veal calves

3.5.2 Microbiological and faecal

Faecal score was significantly improved in T group on the 4th month of the trial (Tab.5).

ITEM	TIME (Month)	GROUP C	GROUP T	SEM	P GROUP	P TIME	P G*T
Faecal Score	Initial	1.25	1.60	0.16	0.09	0.61	0.08
	1	1.56	1.27				
	2	1.56	1.58				
	3	1.51	1.27				
	4	1.86^{A}	1.27^{B}				
	5	1.53	1.44				
	6	1.45	1.31				

Table 5: Macroscopical evaluation of the feces (Faecal score) during the trial in veal calves (^{A,B}=P≤0.01)

No differences were detected between the two experimental groups for Lactobacilli faecal content except for increased values in T animals in the 4th month of the trial (P<0.05).

	TIME	GROUP		
ITEM	**(Month)**	**C**	**T**	**SEM**
Lactobacilli (Log_{10})	1	8.12	7.81	0.17
	2	8.45	8.43	0.17
	3	8.39	8.62	0.19
	4	8.37[b]	8.49[a]	0.17
	5	8.35	8.88	0.17
	6	8.49	8.63	0.17
	Average	**8.36**	**8.48**	**0.07**

Table 6: Lactobacilli count in feces of veal calves during the trial ([a,b]=P≤0.05)

In the same way both *E.coli* (tab.7) and Lactobacilli/*E.coli* ratio (tab. 8) were not influenced by species-spoecific probiotic compound.

	TIME	GROUP		
ITEM	**(Month)**	**C**	**T**	**SEM**
E.coli (Log_{10})	1	4.16	3.91	0.27
	2	6.66	6.60	0.26
	3	6.86	6.81	0.26
	4	6.86	6.95	0.25
	5	6.86	6.88	0.25
	6	6.79	7.01	0.25
	Average	**6.36**	**6.36**	**0.25**

Table 7: *Escherichia coli (E.coli)* count in feces of veal calves during the trial

ITEM	TIME (Month)	GROUP C	T	SEM
Lactob./*E.coli* ratio (Log_{10})	1	4.03	3.95	0.31
	2	1.74	1.83	0.31
	3	1.52	1.80	0.33
	4	1.51	1.55	0.30
	5	1.49	2.00	0.30
	6	1.70	1.68	0.31
	Average	**2.00**	**2.13**	**0.12**

Table 8: Lactobacilli/*E.coli* ratio in feces of veal calves during the trial

3.5.3 Health status

The GHS did not show any significant difference between the two experimental groups (Tab.9); however, it must be outlined a tendence to increased values in T group than C starting from time 2 that couple with the higher isolation rate for severe pathologies (4,17% T *vs* 16,67% C) and number of deaths in C calves (mortality equal to 8.33% *vs* 2.08% of group T).

ITEM	TIME (Month)	GROUP C	T	SEM	P GROUP	TIME	G*T
GHS	1	9.35	8.89				
	2	11.58	11.87				
	3	10.67	11.48				
	4	12.48	13.91				
	5	11.81	12.83				
	6	8.96	10.64				

Table 9: General health status evaluation (GHS) in veal calves during the trial

3.5.4 Haematological parameters

The haematological, hematochemical, immunological paramenters were similar in the two groups for most of the sampling times (Tab.10 a,b; Tab 11a,b).
Significant initial (time 0) lower average corpuscular volume (MCV) values were detected in group T (C = 35 fl *vs* T = 33 fl; $P \leq 0.01$), while lower content of basophiles was detected in T calves at time 2 (C = 1.3% *vs* T = 0.8%; $p < 0.05$). Despite the little dietary Fe intake, the haemoglobin values were within the superior limit fixed by the law (7.3 g/dl) for all test period in both experimental groups.

ITEM	TIME (Month)	GROUP C	GROUP T	SEM	P GROUP	P TIME	P G*T
RBC	Initial	8.46	8.64	0.20	0.29	<0.01	0.85
(M/µl)	1	9.11	9.22				
	2	9.80	9.55				
	3	9.40	9.16				
	4	8.75	8.55				
	5	8.68	8.43				
	6	8.10	7.81				
HCT	Initial	29.77	28.54	0.05	<0.01	<0.01	<0.01
(%)	1	27.66	26.77				
	2	29.08[a]	26.04[b]				
	3	25.06	23.73				
	4	24.84	23.54				
	5	23.36	21.78				
	6	23.36	21.78				
HGB	Initial	9.35	8.86	0.25	0.02	<0.01	0.86
(g/dl)	1	8.97	8.74				
	2	9.45	9.52				
	3	9.33	8.99				
	4	8.48	7.91				
	5	8.46	8.04				
	6	7.97	7.42				
MCV	Initial	35.13[A]	33.14[B]	0.50	<0.01	<0.01	<0.01
(fl)	1	30.36	29.14				
	2	30.00	29.04				
	3	29.68	28.78				
	4	28.58	27.98				
	5	28.89	28.02				
	6	28.89	27.98				
MCH	Initial	11.05[b]	14.59[a]	0.09	0.91	0.04	0.51
(pg)	1	9.82	9.34				
	2	10.20	9.79				
	3	10.02	9.62				
	4	9.58	9.04				
	5	9.82	9.27				
	6	9.83	1.27				

Table 10a: Haematological parameters in veal calves during the trial ([A,B]=P≤0.01; [a,b]=P≤0.05)

ITEM	TIME (Month)	GROUP C	GROUP T	SEM	P GROUP	P TIME	P G*T
MCHC	Initial	31.46	31.01	0.22	0.91	<0.01	0.39
(g/dl)	1	32.38	32.63				
	2	33.91	34.38				
	3	34.26	34.21				
	4	33.64	33.32				
	5	34.00	34.20				
	6	34.05	34.06				
RDW	Initial	29.53	28.73	4.21	0.98	0.69	0.32
(%)	1	35.50	26.25				
	2	27.76	27.51				
	3	27.27	25.58				
	4	26.71	26.21				
	5	25.37[b]	37.41[a]				
	6	24.97	25.89				
PLT	Initial	1191.87	1268.47	60.28	0.01	<0.01	0.97
(K/µl)	1	697.97	838.62				
	2	790.15	888.29				
	3	700.54	748.78				
	4	817.02	851.51				
	5	703.66	811.43				
	6	627.96	752.68				

Table 10b: Haematological parameters in veal calves during the trial ([a,b]=P≤0.05)

ITEM	TIME (Month)	GROUP		SEM	P		
		C	T		GROUP	TIME	G*T
WBC	Initial	7.48[a]	6.32[b]	0.35	0.35	<0.01	0.58
(K/µl)	1	6.95	6.63				
	2	7.01	6.82				
	3	7.75	7.67				
	4	7.76	7.93				
	5	7.36	7.37				
	6	7.03	7.16				
Neutrophils	Initial	30.97	29.35	2.30	0.40	<0.01	0.36
(%)	1	40.54	44.99				
	2	32.43	34.72				
	3	29.46	27.51				
	4	32.03	28.68				
	5	38.05	33.03				
	6	31.46	28.24				
Limphocytes	Initial	58.24	58.07	2.40	0.56	<0.01	0.32
(%)	1	42.76	37.5				
	2	52.32	50.27				
	3	57.60	58.33				
	4	57.34	60.62				
	5	48.74	53.55				
	6	55.47	60.32				
Monocytes	Initial	10.32	11.86	0.65	0.49	<0.01	0.45
(%)	1	15.56	15.9				
	2	13.96	13.6				
	3	11.43	12.8				
	4	9.62	9.86				
	5	12.13	12.02				
	6	11.06	10.06				

Table 11a: Leukocyte formula in veal calves during the trial ([a,b]=P≤0.05)

ITEM	TIME (Month)	GROUP		SEM	P		
		C	T		G	T	G*T
Eosinophils	Initial	0.14	0.14	0.23	0.89	<0.01	0.75
(%)	1	0.22	0.47				
	2	0.37	0.57				
	3	0.71	0.56				
	4	0.18	0.04				
	5	0.48	0.65				
	6	1.44	0.97				
Basophils	Initial	0.29	0.55	0.13	0.89	<0.01	0.05
(%)	1	0.92	1.14				
	2	1.33^{A}	0.77^{B}				
	3	0.77	0.74				
	4	0.81	0.81				
	5	0.57	0.76				
	6	0.53	0.39				

Table 11b: Leukocyte formula in veal calves during the trial (^{A,B}=P≤0.01)

Hepatic, renal and muscle-skeletal functionalities related parameters did not show differences between the test groups (Tab.12a-d) in most of the cases.
Some few differences were detected at different times for AST (T6: C=3.4 UI/L *vs* T=3.0 UI/L; P<0.05), bilirubin (T2: C=3.6µmol/L *vs* T=2.83.6µmol/L; P<0.05), glucose (T6, C=61g/L *vs* T= 58,5 g/L; P<0.05), NEFA (T3: C=0,3 mmol/L *vs* T=0.4mmol/L; P<0.05), urea (T0: C=3.9mmol/L *vs* T=3.4mmol/L; P<0.05; T1: C=1.6mmol/L *vs* T=1.1mmol/L; P<0,05), and LDH (T3: C=127UI/L *vs* T=236UI/L; P<0,05). Immunological parameters were not influenced by the dietary administration of the species-specific probiotic compound except for lower values for the bactericidal activity (T1: C = 77,5 % *vs* T = 47,1 %; P<0.05), for the complement (T1: C = 27,3 UEC H50/150µl *vs* T = 18,8 UEC H50/150µl; P<0.05), and the lysozyme (at time T0, T1, T3 and T6) in T than C.

ITEM	TIME (Month)	GROUP C	GROUP T	SEM	P GROUP	P TIME	P G*T
AST	Initial	60.96	58.67	3.16	0.06	<0.01	0.11
(IU/L)	1	56.13	52.82				
	2	66.35	60.24				
	3	70.38	64.33				
	4	75.59	83.81				
	5	65.24	61.2				
	6	113.55	102.34				
Bilirubin	Initial	4.18	4.19	0.22	0.07	<0.01	0.28
(µMol/L)	1	3.63	2.83				
	2	4.32^{A}	4.18^{B}				
	3	4.47	4.11				
	4	4.83	4.53				
	5	3.19	2.98				
	6	4.19	4.40				
Glucose	Initial	6.00	5.72	0.20	0.07	<0.01	0.04
(Mmol/L)	1	6.52	6.58				
	2	5.35	6.02				
	3	6.52	6.93				
	4	6.50	6.44				
	5	5.30	5.22				
	6	6.17	7.11				
Tot.Protein	Initial	61.08	58.53	0.80	0.08	<0.01	0.60
(g/L)	1	59.51	58.58				
	2	58.25	58.04				
	3	61.56	62.14				
	4	66.68	66.15				
	5	58.81	57.55				
	6	68.09	66.88				
NEFA	Initial	0.20	0.21	0.24	0.10	<0.01	0.49
(Mmol/L)	1	0.23	0.24				
	2	0.27	0.27				
	3	0.34	0.42				
	4	0.28	0.30				
	5	0.38	0.39				
	6	0.24	0.25				

Tab 12a: Biochemical and immunological parameters in veal calves during the trial (^{A,B}=P≤0.01)

ITEM	TIME (Month)	GROUP		SEM	P		
		C	T		GROUP	TIME	G*T
Creatinine	Initial	110.44	107.09	2.41	0.91	<0.01	0.71
(µMol/L)	1	66.16	62.54				
	2	52.29	52.08				
	3	63.36	63.97				
	4	71.49	74.14				
	5	59.39	60.76				
	6	63.45	65.01				
Urea	Initial	3.85	3.44	0.12	<0.01	<0.01	0.79
(Mmol/L)	1	1.57	1.09				
	2	1.89	1.58				
	3	1.73	1.59				
	4	2.67	2.44				
	5	2.33	2.06				
	6	2.86	2.54				
GGT	Initial	48.50[a]	38.21[b]	1.30	0.65	<0.01	0.72
(IU/L)	1	25.00	23.17				
	2	22.04	19.50				
	3	22.14	19.57				
	4	24.67	22.87				
	5	22.00	20.59				
	6	29.60	27.70				
CK	Initial	218.79	189.33	60.32	0.21	<0.01	0.61
(IU/L)	1	113.35	99.38				
	2	153.78	131.88				
	3	126.77	119.00				
	4	174.00	199.17				
	5	85.76	82.18				
	6	252.30	270.39				
LDH	Initial	2069.29	1906.63	9.81	<0.01	<0.01	0.40
(IU/L)	1	2101.26	2051.04				
	2	2479.48	2311.08				
	3	2663.77	2367.09				
	4	2688.22	2806.00				
	5	2507.57	2431.50				
	6	3538.40	3311.00				

Tab 12b: Biochemical and immunological parameters in veal calves during the trial ([a,b]=P≤0.05)

ITEM	TIME (Month)	GROUP			P		
		C	T	SEM	GROUP	TIME	G*T
Lisozyme (µg/ml)	Initial	1.03	1.41	0.08	<0.01	0.04	0.82
	1	0.96	1.39				
	3	1.07	1.39				
	6	1.16	1.63				
Battericidal (%)	Initial	93.57	87.72	3.55	<0.01	<0.01	<0.01
	1	77.55	47.14				
	3	91.48	89.96				
	6	83.14	83.68				
Haptoglobin (Mg/ml)	Initial	0.03	0.01	0.02	0.88	0.16	0.32
	1	0.01	0.007				
	3	0.03	0.048				
	6	0.048	0.05				
Complement (UEC H50/150 µMol)	Initial	13.94	15.28	1.20	0.10	<0.01	<0.01
	1	27.3	18.82				
	3	18.92	19.28				
	6	17.2	18.26				
Albumin G (g/L)	Initial	27.18	27.48	5.19	0.91	0.12	1.00
	1	24.52	25.79				
	3	36.35	36.61				
	6	27.42	28.11				

Tab 12c: Biochemical and immunological parameters in veal calves during the trial

ITEM	TIME (Month)	GROUP C	GROUP T	SEM	P GROUP	P TIME	P G*T
Alpha Globuline	Initial	9.92	9.20	2.20	0.91	0.31	1.00
(g/L)	1	8.40	8.67				
	3	12.30	12.02				
	6	12.58	10.60				
Beta Globuline	Initial	7.12	7.17	0.25	0.58	<0.01	0.47
(g/L)	1	6.59	6.90				
	3	7.67	8.14				
	6	10.93	10.7				
Gamma Globuline	Initial	6.57	6.82	1.60	0.65	<0.01	0.51
(g/L)	1	7.52	8.72				
	3	12.52	9.41				
	6	13.53	13.28				
Alfa/Gamma	Initial	1.18	1.21	0.03	0.92	<0.01	0.45
Globuline	1	1.11	1.07				
	3	1.07	1.07				
	6	0.79	0.83				

Tab 12d: Biochemical and immunological parameters in veal calves during the trial

The oxidative stress parameters did not show any significsnt differences between the experimental groups, highliting however the presence of an adaptative stress in the calves with high levels of OXID at time 0 (C=513 μmol HClO/ml *vs* T=475 μmol HClO/ml; P<0.05) (Tab.13).

ITEM	TIME (Month)	GROUP C	GROUP T	SEM	P GROUP	P TIME	P G*T
OXID	Initial	513.57	475.03	7.44	0.03	<0.01	<0.01
(μMol HClO/m)	1	261.73	258.28				
	6	189.42	191.11				
ROMS	Initial	4.41	4.26	0.24	0.60	<0.01	0.82
(Mmol H_2O_2)	1	1.19	1.25				
	6	2.03	1.83				

Tab 13: Evaluated oxidising stress parameters in veal calves during the trial

3.5.5 Slaugher parameters

Slaughtering performance did not show any significant differences for carcass weight and dressing percentagfe between the groups (Tab.14).

	GROUP			
ITEM	**C**	**T**	**SEM**	**P**
Carcass Weight (Kg)	148.77	146.61	2.17	0.50
Dressing Percentage (Kg)	55.75	55.69	0.51	0.94

Table 14: Carcass weight and dressing percentage of veal calves fed a species-specific probiotic

Both pH at 45 minutes and 24 hours after slaughter, did not show any differences between the groups (Tab.15).

		GROUP			P		
ITEM	**TIME**	**C**	**T**	**SEM**	**GROUP**	**TIME**	**G*T**
pH *(mixed)*	Initial	6.22	6.24	0.04	0.59	≤0.01	0.387
	45' *	6.70	6.68				
	24h*	5.73	5.80				

Table 15: Carcass pH at zero, 45 minutes and 24 hours after slaughter in veal calves fed a species-specific probiotic compound

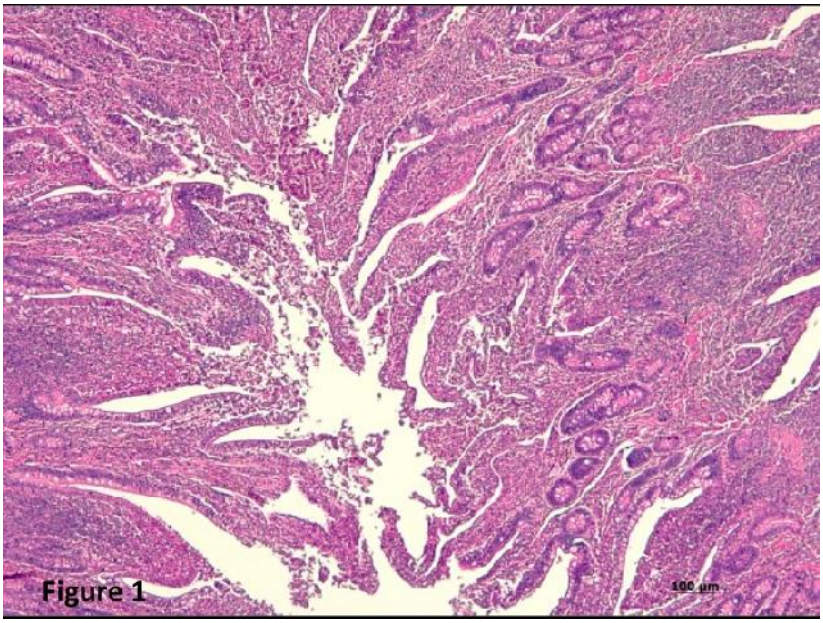
The SEUROP classification of carcasses revealed both increased type R and Type P frequence of carcasses in T group, while C animals had type O carcasses in most of the cases. (Tab.17).

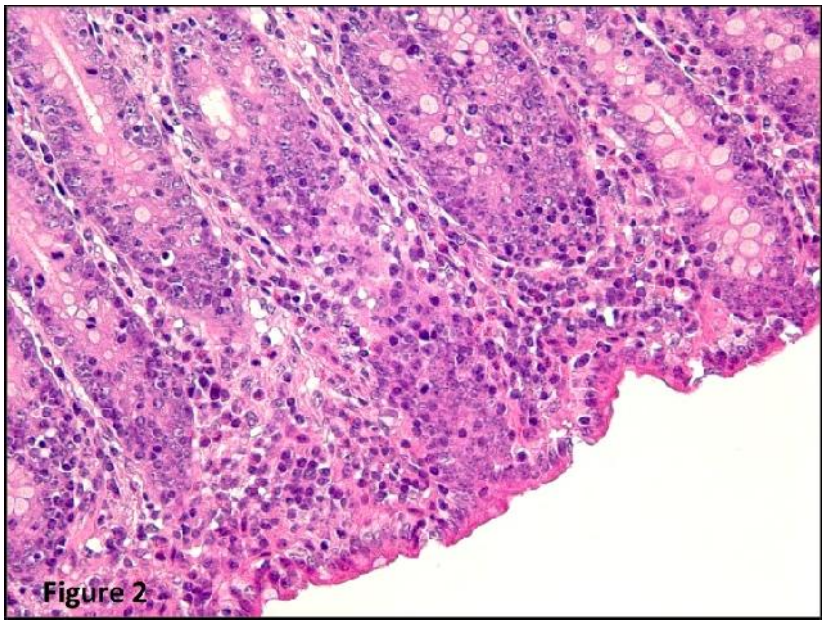
EUROPE CLASSIFICATION	CONTROL	TREATED
S	0	0
E	0	0
U	0	0
R	0	4.26% (2 subjects)
O	97.5% (39 subjects)	91.49% (43 subjects)
P	2.5% (1 subject)	4.26% (2 subjects)

Table 17: SEUROP classification of veal calves carcass

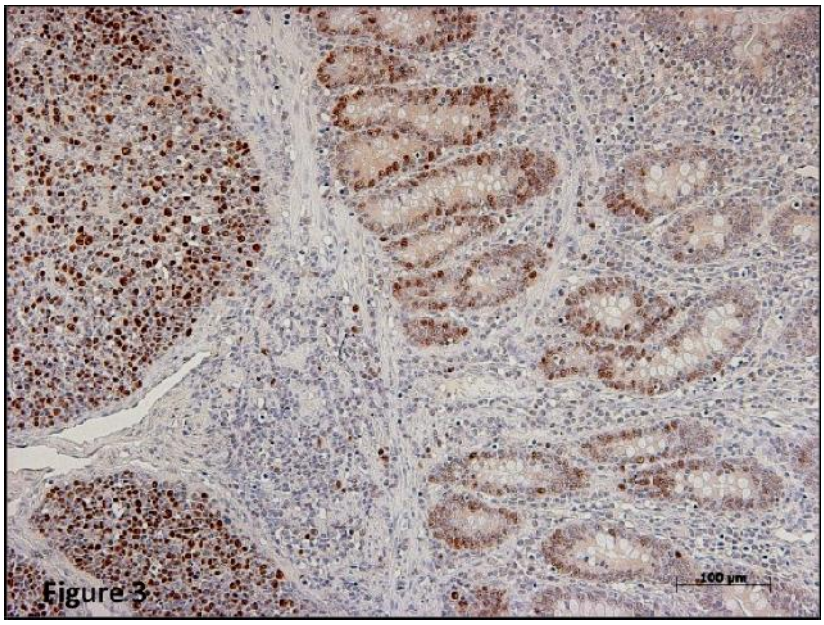
3.5.6 Histological and histometrical parameters

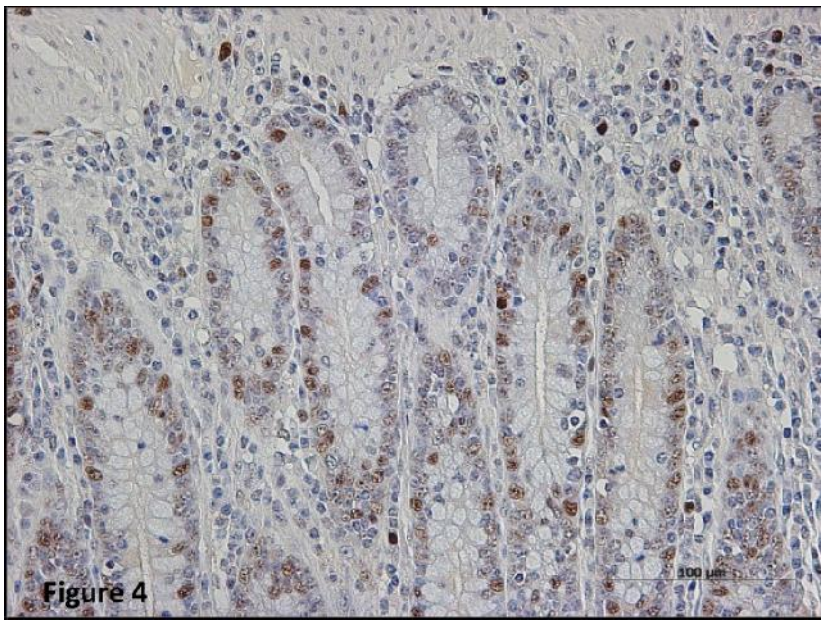
Considering the histological and histometrical evaluation of intestinal samples of calves upon slaughtering, it did not show any difference between the groups; however, the possible protective effect of the probiotic compound tested was confirmed by the presence of less serious histopathologies in *cecum* for T group, with a greater integrity of the superficial epithelium.
The administration of the species-specific probiotic compound in the calves diet was able to determine a numerical increase of the depth of the crypts in the ileum and the caecum intestine, while dicreasing the number of cells in replication at the level of *ileum* and *cecum* crypts (Fig.1-2). Obtained results on histological examination also showed a tendency to a reduction in the number of cells in replication, apoptotic activity in the *ileum* lymphatic tissue, and Ig-A positive immunocompetent cells in the GALT (gut-associated lymphoid tissue) of treated calves (Fig. 3-6).



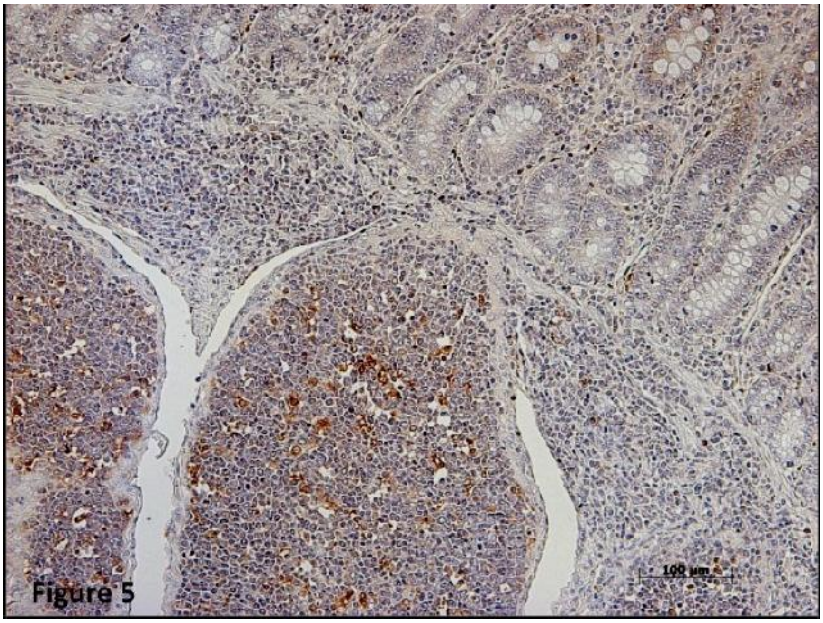


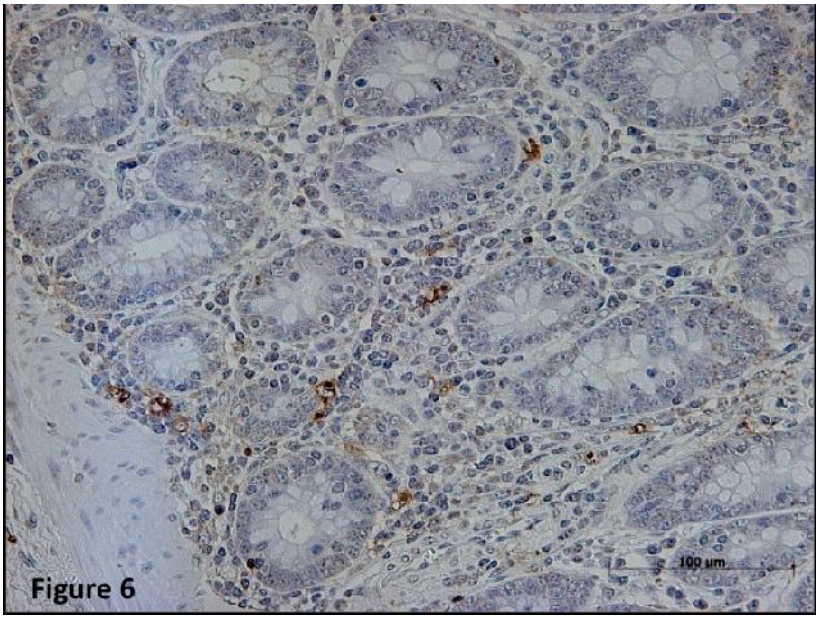
Figures 1 and 2: Microanatomical aspects of *ileum* (Fig.1, C group, 40X) and *cecum* (Fig. 2, T group, EE 200X). A chronic catarrhal enteritis with a severe de-epithelialisation and a loss of superficial epithelium can be observed in the *ileum*. C*ecum* microanatomical aspects do not evidence epithelial injuries in T subjects.





Figures 3 and 4: Microanatomical aspects of *ileum* (Fig.3, group C, anti-PCNA 100X; Fig.4, group T, anti-PCNA 200X). Immunoreactive to anti-PCNA are observed both in epithelial cells of the intestinal crypts and in immunocompetent cells of the TLD and the GALT (mainly lymphocytes).

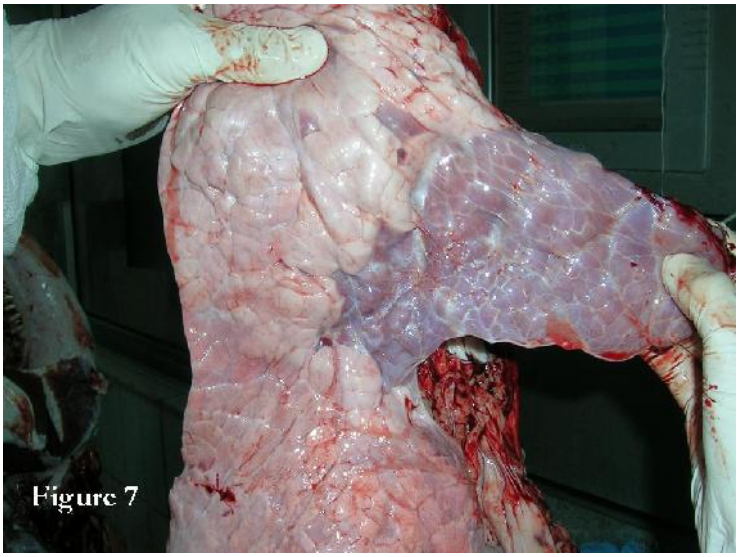


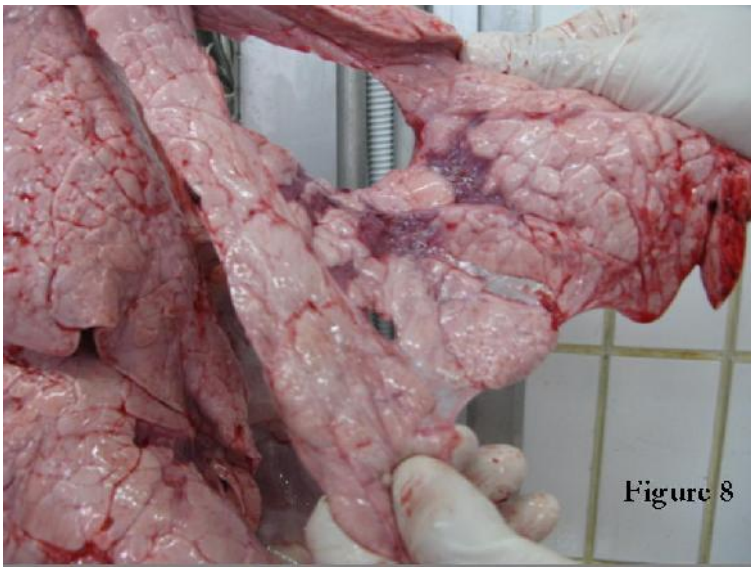


Figures 5 and Figures 6: Microanatomical aspects of *ileum* (Fig.5, T group, TUNEL 100X; Fig.6, C group, TUNEL 200X). Apoptosis in the TLD and the GALT (mainly macrophages) in both groups of animals are evident.

3.5.7 Lungs evaluation at the slaughter

The evaluation of lungs upon slaughtering showed chronic pleural lesions in most of the calves (adherences) and lesions of the pulmonary parenchyma. The observed lesions could be attributed to subacute-chronical inflammatory forms, generally quite common in veals calves. Histological samples analysis confirmed the presence of subacute-chronical bronchial pneumonia (Fig. 7 and 8) and interstitial pneumonia.





Figures 8 and 9: Different extensions of bronchial pneumonia in lungs of veal calves

3.6 Discussion

The concern on massive use of antibiotics in animal production impose the use of alternative substances that may be effective on both animals' health and consumer's health. Among different compounds, probiotics seem to be able to influence performance goes throught the henhancing of the positive intestinal microflora (Lalles et al., 2007; Corcionivoschi et al., 2010; Frizzo et al., 2011), the reduncing of diarrhoea incidence (Timmerman et al., 2005; Mokhber et al., 2007; Nagashima et al., 2010; Frizzo et al., 2010; Kawakami et al., 2010), and the improving both the innate and acquired immunity (Fleige et al., 2008; Frizzo et al. 2010; Al-Saiady et al., 2010).

Anyway a number of studies have been carried out in order to determine the effects of probiotic dietary supllementation on growth performance, but the obtained results have so far been discordant. In some cases the probiotics given to veal calves not always allowed to highlight increased body weight or dressing percentage at slaughtering (Bakhshi et al., 2006; Riddell et al., 2010) altought some authors found positive results (Frizzo et al., 2010; Kawakami et al., 2010; Nagashima et al., 2010; Al. Saiady et al., 2010; Hossaini et al., 2010).

Such inconsistencies are not easily interpreted: the sensibility to the probiotic is dependent to different factors (Timemrman et al., 2005) as the type, the strain and species-specificity of the administered compound (Ohashi et al., 2006; Ripamonti et al., 2011) where the most species-specific the probiotic is, the most effective should be (Vinderola et al., 2004).

Whether it is true that the action of probiotics is obtained by the improvment of the intestinal dismicrobism and, as a consequence, of the overall health status, there are however many factors (intrinsic and extrinsic) that can influence the calf's status. In addition to this, in not particularly stressful managerial conditions, the effects of the administration of such compunds could be hidden.

In the present study ADG did not show any difference between the two experimental groups confirming previous studies (Bakhshi et al., 2006; Riddell et al., 2010). The lack of increased preformance can be partially explained also by the use of antibiotics during the trial that can have influenced the mechanism of action of the probiotics compound.

To confirm this hypothesis, starting from the last mass antibiotic administration (3rd test time), a better faecal consistency was observed (significant at 4th time) in T group: this could indicate a better stability of the intestinal microbial conditions. Such tendency was confirmed by the faecal count of lactobacilli and, even if not significant, by an increased Lattobacilli/*E.coli* ratio in the group

supplemented with the species-specific probiotic compound in agreement with Signorini et al. (2010).
Moreover a better intestinal microbiological balance in calves fed probiotic can be observed by the improvement of an indirect health indicator such as GHS or by the lower incidence of mortality rate observed
The blood determinations did not show relevant differences between the two experimental groups, confirming what stated by some authors previously (Riddel et al., 2010; Adams et al., 2010; Al-Saiady et al., 2010). In the present study there was no haemoglobin and hematocrit increase in agreement with Kim et al. (2011), and the protidemy trend was characterised by an initial reduction of the antibody quantity of colostral origin, followed by an antibody title increase as reported by Kim et al. (2011).
Differently from Fleige et al. (2007), in the present study no positive immunemodulatory effect in collected blood samples was observed in calves fed the species-specific probiotic compound. Considering that such parameters may be influenced by stressogen factors, it is possible to hypothesize that both the use of antibiotics during the trial, and the lack of high environmental stress factors can have masked the expected positive effects.
The intestine not colonised by pathogenic germs is capable of absorbing some substances and amminoacids more efficiently (Elam et al., 2003). If a thinner lamina propria results in more efficient nutrient absorption, then ADG and feed efficiency should be improved.
In the present study the obtained results of the immunohistochemical and istometrical considered parameters agree with the hypothesis that species-specific probiotics dietary administration may be indirectly linked to a protecive action of the intestinal mucous membrane, with the preservation of the barrier integrity, expecially in the *cecum* (Connolly et al., 2008).
The lower incidence of serious *cecum* histopathological lesions evidenced during the trial in probiotic-fed calves with a less evident disepithelization confirm findings by previous authors (Waters et al., 1999; Casas et al., 2000; Blatter et al., 2001) who found positive results over the intestinal ephitelium integrity in animals fed probiotics.

3.7 Conclusions

The present study showed how species-specific probiotic compound dietary administration to veal calves can positively affects the intestinal mucosal integrity and the microbial population of the gastro intestinal tract with some positive results over health status of treated animals, although a strong negative effect of antibiotic treatments can be outlined over effectiveness of treatment.

3.8 References

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CHAPTER 4

Species-specific probiotic in newborn female calves: microbiological and general health status, growth performance, haematological parameters and cell mediate immune response.

4 Species-specific probiotic in newborn female calves: microbiological and general health status, growth performance, haematological parameters and cell mediate immune response.

4.1 Abstract

This study evaluated the effects of the administration of a species-specific multistrain probiotic (*Lactobacillus animalis-Lactobacillus paracasei* subsp. *paracasei-Bacillus coagulans*; 30:35:35) on health and performance parameters of newborn Friesian female calves during the first month of life.
Twenty-two calves were divided in two groups: control (C) fed with milk replacer and concentrate as a basal diet, and treatment (T), fed C diet plus 1g/head/d of probiotic from the second day after birth to the end of the first month of life. Faecal samples were collected weekly for the count of Lactobacilli and *Escherichia coli*; blood samples were collected and analyzed weekly. Individual faecal score (FS) was recorded daily, General Health Score (GHS) was calculated at the end of the trial, and cell mediate immune response was evaluated by skin test at 7 and 28 days of life. Body weight (BW), biometrical parameters and Average Daily Gain (ADG) were recorded weekly while Feed Intake (FI) and Feed Conversion Rate (FCR) were recorded for the overall period. Higher faecal Lactobacilli/*E. coli* ratio on day 28 of life (3.73 Log UFC/g *vs*. 2.02 Log UFC/g; $P<0.05$) and improved faecal consistency at 6, 25 and 27d of life were found in T group. Final body weight (48.92 Kg *vs* 46.92 Kg; $P<0.05$) and hearth girth (81.16 cm *vs* 78.49 cm; $P<0.05$) were significantly higher in T group. Concentrate FI (%DM) was significantly higher in T group overall the trial period, while ADG, FCR and milk replacer FI (%DM) were not influenced by probiotic administration. GHS showed increased mean value, but non significantly, in T group (T=20.45 *vs* C=18.91). Increased haemoglobin (10.04g/dl *vs* 8.60g/dl) and hematocrit (26.68% *vs* 22.17%) plasma content at 8d in T group with lower eosinophils percentage (0.05% *vs* 0.22%) were found at 8d in T group, while basophiles content was increased at 28d in species-specific probiotc-fed animals than C (0.21% *vs* 0.16%, respectively; $P<0.05$). The administration of a species-specific probiotic compound during the first month of life of newborn calves improved gut microflora, increased performance and some biometric parameters.

4.2 Introduction

Calf health is a critical factor linked to the economics of intensive breeding: not only health in the first weeks of life is strictly linked to the mortality rate, but also to the impact on morbidity with increased antibiotic and feed costs, couples with reduced animal growth performance. In the first month of life, calves possess an extremely unstable intestinal microflora (Postema et al., 1987; Lucàs et al., 2007) and are easily susceptible to the proliferation of pathogenic microorganisms: as a consequence they are frequently subjected to gastrointestinal diseases, in particular to episodes of diarrhoea. Many of the problems that affect the performance of young calves are indeed related to low digestion and reduced absorption of nutrients due to gut colonization of pathogens (Signorini et al., 2011). Healthy animals have a equilibrate and balanced intestinal microflora, in particular the intestinal tract is colonized by microbiota that develop in a beneficial symbiosis with the host (Kurzak et al., 1998), allowing an optimal growth rate. The treatments, frequently used to contrast calf diarrhoea and intestinal diseases, are often based on antibiotics or other antimicrobial agents. The use of mass antibiotic treatments in livestock determined serious and alarming consequences such as the development of antimicrobial resistances among the microbial population. Lactic acid bacteria, usually found in the intestinal microflora of humans and animals (Schneider et al., 2004) are widely recognized for their beneficial properties such as the balance of the gut microorganisms, the inhibition of pathogenic bacteria colonization and the improvement of Gut-Associated Lymphoid Tissue (GALT) (Corcionivoschi et al., 2010; Morrison et al., 2010; Riddel et al., 2010). It is well known that feeding probiotic to calves determine an improvement of gut health. The subsequent positive effect leads to an increase in digestion efficiency and consequently to better growth performance (Timmerman et al., 2005; Frizzo et al., 2010; Kawakami et al., 2010). In addition, the higher effectiveness of the use of multispecies-multistrain probiotics was demonstrated than that of monostrain products (Timmerman et al., 2005), thanks to the synergism of different mechanisms of action (Timmerman et al., 2004). The aim of this study was the evaluation of the effects of the administration of a multispecies-multistrain species-specific probiotic, previously developed (Ripamonti et al. 2011), to newborn Friesian female calves in the first month of life, with particular interest on gut microbial health, general health status, growth performance, haematological parameters and immune mediate cell response.

4.3 Materials and Methods

4.3.1 Experimental design and administration of probiotic

A total number of 22 newborn Friesian female calves, were divided into 2 different groups: Control group (C), fed with a standard milk replacers/concentrates diet, and Treated group (T), fed with the same diet supplemented with 1g/die of a species-specific multistrain probiotic powder (*Lactobacillus animalis-Lactobacillus paracasei-Bacillus coagulans*, 30:35:35% with final concentration of $1{,}8x10^{10}$ CFU/g). Calves were randomly assigned to treatments and were started on probiotic intake by 2nd day of age. Each calf was placed into individual outdoor box sized 2x1.25 m in order to prevent cross contamination. The trial lasted for the first 28 days of life and occurred in the months between January and June 2011. Calves were fed individually twice a day (7.00am-17.00pm) starting from the second day of life, with non-medicated milk replacer (MR) containing 22.5% of crude protein, 18% of fat, 9.0% of ashes, 1.75% of lysine, 0.55% of methionine and 0.50% of cystine. The milk powder (130g/L) was reconstituted in hot water (65°C) and fed at a temperature of 39°C in the bucket. The initial powder administered amount was on average 393 g/head/d, while the final was 514 g/head/d. Quantitative of milk replacer refused by a single calf were measured at each feeding. At the same time a commercial pelleted starter mixture (SM) composed by wheat bran, soybean meal, corn flake, corn flour, corn gluten feed, sunflower meal, dried beet pulp, cane molasses and mineral-vitamin supplementation, was offered ad-libitum from a bucked once daily. The starter chemical composition on DM basis was: 20.28% crude protein, 4.38% crude fat, 8.46% crude fibre, 30.01% neutral detergent fibre (NDF) and 8.37% ashes. The total refusal of SM was quantified at the end of the trial. Water was ad libitum available since the second day of life.

4.3.2 Microbiological and gut health markers

Faecal samples on day 2, 8, 14, 21 and 28 of life were collected from each female calf by rectal stimulation, stored in vials with transport medium (Faecal ™ enteric Plus, Oxoid, Basingstoke, UK) kept refrigerated (4°C) until delivered to the laboratory and analyzed the same day. Ten grams of each sample were diluted with 90 mL of Buffered Peptone Water (Oxoid) and homogenized in a Stomacher for 1 min (Seward Stomacher 400 blender Mixed Homogenizer, International PBI, Milano, Italy). Serial 10-fold dilutions were plated onto MRS agar medium (Oxoid) and TBX agar (ISO 16649-2) (Oxoid) for the enumeration of lactobacilli and *Escherichia coli*, respectively. MRS agar plates were incubated in anaerobic jars (Anaerojar, Oxoid) with Anaerogen kit (Oxoid) at 37°C for 48 h, while TBX agar plates were incubated aerobically at 44°C for 24 h. After incubation, the agar plates were assessed for growth and typical colonies were counted. Individual faecal scores were measured daily on a scale of 1 to 5 (1 = watery, 5 = firm; Zaaijer and Noordhuizen, 2003), from 2 to 27 days. General health score (GHS) was calculated using the formula performed by Timmerman et al. (2005) modified as following: 28-(1×total number of diarrheic days)− (2×number of individual therapeutic treatments for digestive diseases) − (3×number of individual therapeutic treatments for respiratory diseases) − (2×number of individual therapeutic treatments for infections other than digestive or respiratory) − (2×number of antibiotic treatments on a herd basis).

4.3.3 Growth performance

Milk replacer intake was registered daily throughout the experimental period, while the starter consumption was recorded daily by the difference between offered amount and total daily refusals. Starting from daily intake of milk replacer, the intake of air-dry milk replacer was calculated as (milk offered- milk refused) x inclusion rate of dry-air milk replacer per litre of water. Body weight was recorded weekly by an electronic weighing system (model BF/E 1425E) and ADG and FCR were subsequently calculated. At 2, 8, 14, 21 and 28 days after birth biometric parameters, such as hearth girth (HG), body length (BL), withers height (WH) and hip width (HW) were measured by a calibrated meter according to Hoffman (1997).

4.3.4 Haematological parameters

Haematological parameters were determined at 2, 8, 14, 21, and 28 days of life: two tubes of blood were drawn from the jugular vein of each animal into 10ml lithium heparin vacuum tubes (Terumo Venosafe 10 ml VF-109SHL, Terumo Europe L.V., Leuven, Belgium). All tubes were immediately placed on ice and transferred to the laboratory. Blood samples were processed using an auto-mated veterinary haematology analyzer (Nihon Kohden, Cell Tac a, MEK 6108, Tokyo, Japan) for the determination of hematocrit (HCT), red blood cells count (RBC), mean cell volume (MCV), haemoglobin (HB), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), platelets (PLT), platelet distribution width (PDW), mean platelets volume (MPV), percentage of large platelets (PLCR), plateletcrit (PCT), neutrophils, red cells distribution width (RDW), lymphocytes and monocytes. Moreover white blood cell count (WBC), basophiles, neutrophils and eosinophils content was assessed in whole blood samples with a Hemat 8 (SEAC, Calenzano, Florence, Italy).

4.3.5 Skin test

Cell-mediate immune response was evaluated determining double skin thickness in response to phytohaemoagglutinin injection (PHA, Sigma Chemicals, St. Louis, MO, USA) using the test procedure described by Lacetera et al. (1999). The test was performed at 8 and 28 days after birth, in either instance succeeding intradermal injection of 250 μg PHA diluted in 0.1 ml of sterile PBS (Sigma) to a top-part shaved area of right shoulder using an automatic syringe for intradermal injection (Veterinaria Strumenti, Padova, Italy). Sterile PBS (0.1 ml) was injected to the corresponding area of left shoulder in order to check any skin response to PBS alone. Double skin thickness to both areas was measured with a constant tension caliper (Mitutoyo Italiana, Lainate -MI-, Italy) before (time 0) and 24 hours after PHA injection. Values obtained at 24 h were considered the maximum PHA response (Lacetera et al., 1999).

4.4 Statistical analysis

Data relative to growing performance, microbiological markers, blood parameters, faecal score, skin test at 8 and 28 days of life were processed by a mixed procedure of SAS 9.2 (SAS INSTITUTE SAS Inc, NC, USA). The statistical model included the following main effects and interactions: treatment, day of treatment and treatment by day of treatment. Probability values $P>0.05$ were considered non-significant. All data in the text and tables are presented as least square means ± s.e.

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

Where μ= general mean; T_i= effect of *ith* treatment (i=1-2); D_j= effect of day of treatment; $(TXD)_{ij}$ = effect of the interaction between treatment and day of treatment; e_{ij} = casual effect of each observation.Data relative to milk replacer and concentrate feed conversion rate, general health score and total skin thickness were analysed by a General Linear Model procedure of SAS 9.2 (SAS INSTITUTE SAS Inc, NC, USA).

4.5 Results

4.5.1 Microbiological parametersin feces, faecal score, and general health status

Faecal E. coli and Lactobacilli counts were not significantly different in the two experimental groups even if a trend to lower content of E. coli was found in T group at 28 days of life (C=5.01; T=3.76 Log CFU/g) (Tab1). As a sum of effects Lactobacilli/*E. coli* ratio showed higher values in T group at the end of the trial, (T=3.73 *vs.* C=2.02; P<0.05) (Fig.1).

	TIME	GROUP			P		
ITEM	**(Day)**	**C**	**T**	**SEM**	**GROUP**	**TIME**	**G*T**
E. Coli							
(LogUFC/g)	2	6.28	5.72	0.48	0.16	<0.01	0.53
	8	5.34	5.05				
	14	4.12	4.55				
	21	4.49	3.96				
	28	5.01	3.76				
Lactobacilli							
(LogUFC/g)	2	7.91	7.71	0.39	1.00	<0.01	0.62
	8	8.46	8.08				
	14	7.75	7.71				
	21	7.53	7.64				
	28	7.03	7.49				

Tab 1: Lactobacilli and *E. coli* counts in control (C) and treated (T) groups

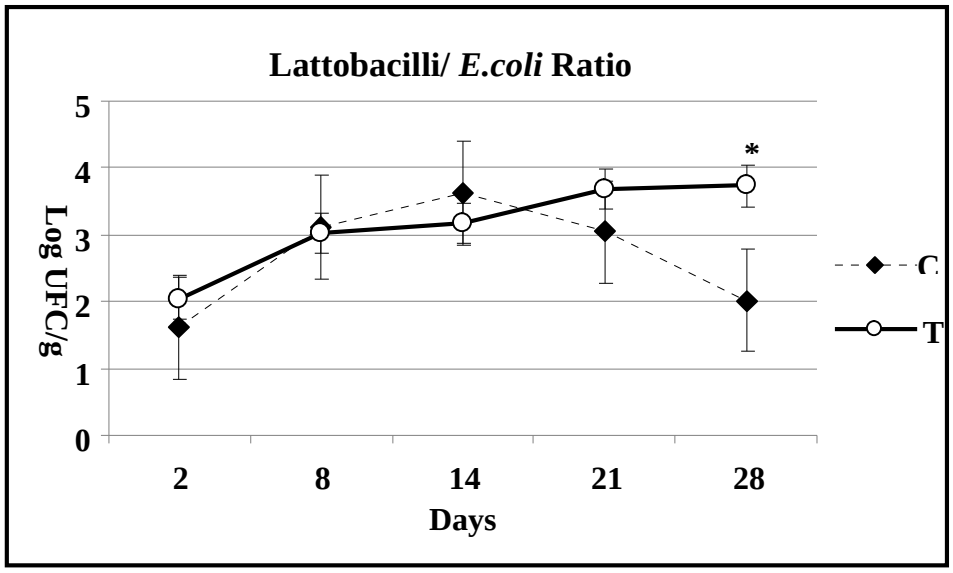

Figure 1: Lactobacilli/*E. coli* ratio in control (C) and treated (T) groups (*P< 0,05)

Faecal score showed significantly higher values in T group at 6 (C=1.82 *vs* T=2.64; P<0,05), 25 (C=2.00 *vs* T=2.91; P<0.01), and 27 days of life (C=2.36 *vs* T=3.09; P<0.05) (Fig. 2), while GHS was not influenced by the treatments althought numerically a higher mean value was detected in T (T=20.45 *vs* C=18.91)

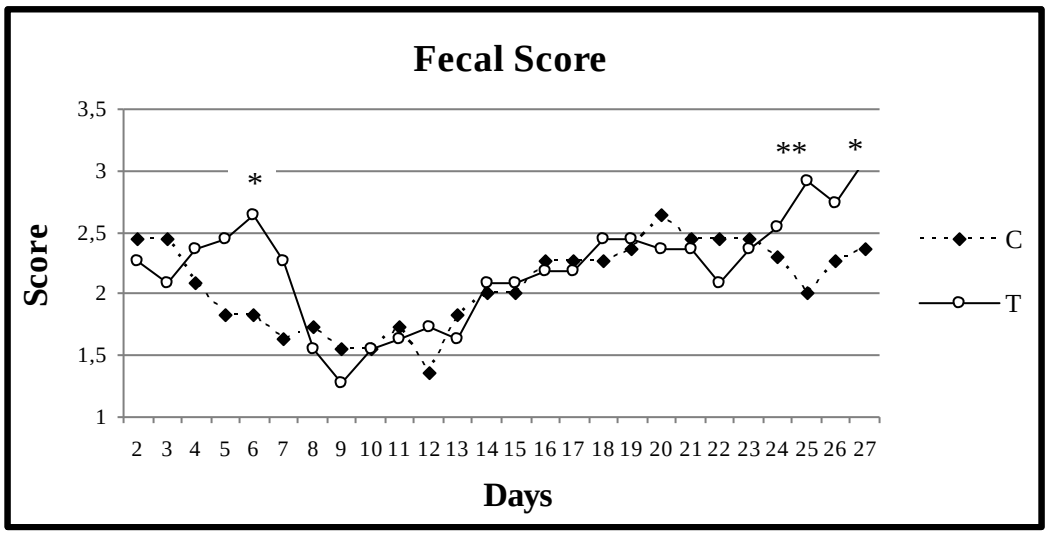

Figure 2: Trend of faecal score (FS) in treated (T) and control (C) groups (**P<0.01;*P < 0.05)

4.5.2 Growth performance

All calves were healthy during the trial and no animal deaths were registered. Increased BW (Tab. 2) was found in T animals than C at the end of the trial (48.92 kg *vs* 46.92 kg; P≤0.04), while no differences were detected between groups on biometrical parameters, with the exception increased mean HG in T group (81.16 cm *vs.* 78.49 cm; P≤0.05). The total milk replacer intake was not different between the two experimental groups, while total concentrate intake was higher in treated group (14.77kg *vs* 12.56 kg; P<0.05) than C. FCR was not found to be different between C and T.

ITEM	TIME (Day)	GROUP C	GROUP T	SEM	P G	P T	P G*T
Body weight							
(kg/head)	2	39.40	39.92	0.56	0.12	<0.01	0.31
	8	41.49	40.86				
	14	41.84	43.06				
	21	43.80	44.72				
	28	46.92[b]	48.92[a]				
Biometric Measurements							
Withers height (cm)	2-28	79.65	79.85	1.35	0.80	<0.01	0.98
Hearth girth (cm)	2-28	78.49[b]	81.16[a]	1.26	0.01	<0.01	0.99
Hips width (cm)	2-28	22.49	23.22	0.58	0.05	<0.01	0.87
Body length (cm)	2-28	63.22	62.94	1.51	0.78	<0.01	0.92
Average Daily Gain							
(kg/head/day)	2-28	0.42	0.29	0.06	0.54	<0.01	0.16
	9-14	0.19	0.34				
	15-21	0.28	0.29				
	22-28	0.44	0.52				
Total ADG	2-28	0.29	0.37	0.04	0.22		
Feed Intake							
Milk replacer (g/h/d)	2-28	451.02	449.08		0.83	<0.01	0.99
Calf starter (tot kg/dm)	2-28	12.56[a]	14.77[b]	0.68	0.03		
Feed Convers. Rate							
Milk replacer+starter	2-28	3.80	3.00	0.37	0.14		

Table 2: Growth performance in treated (T) and control (C) groups ([A,B] P<0.01; [a,b] P<0.05)

4.5.3 Hematological parameters

Haematological results are reported in Tables 3(a,b) and 4. No differences were detected during the trial for RBC, MCV, MCH, MCHC, PLT, PDW, MPV, PLCR, PCT, and RDW. Hematocrit and haemoglobin serum content was statistically different at 8 days of life (respectively C=22.17% *vs* T=26.68%, P<0.05 and C=8.60g/dl *vs* T=10.04P, <0.05). During the trial HCT content was slightly below the phisyological limits in both groups in some sampling times, but these values can be considerate acceptable in very young calves (Riddel et al., 2010). Lymphopenia, neutrophilia and monocytosis (were detectable in in C and T animals without any significant diffeence. The values referred to white blood cell count were within the physiological limits, and no statistical differences were observed between the groups, except for basophiles at 28 days (C=0.16% *vs* T=0.21%;P<0.05) and eosinophils at 8 days (C=0.22% *vs* T=0.05%; P<0.01).

ITEM	TIME (Day)	Group C	Group T	SEM	P GROUP	P TIME	P G*T
Hct	2	23.79	27.08	1.46	<0.01	<0.01	0.97
(%)	8	22.17[b]	26.68[a]				
	14	22.04	24.61				
	21	20.46	23.52				
	28	28.32	21.69				
Rbc	2	6.33	7.00	0.41	<0.01	<0.01	0.973
(M/μl)	8	6.12	7.07				
	14	6.09	6.54				
	21	5.47	6.25				
	28	4.81	5.76				
Mcv	2	37.67	38.78	0.59	0.05	0.07	0.36
(fl)	8	36.23	37.84				
	14	36.34	37.61				
	21	37.62	37.89				
	28	38.50	37.97				
Hbg	2	8.80	9.93	4.65	<0.01	0.74	0.93
(g/dl)	8	8.60[b]	10.04[a]				
	14	8.67	9.54				
	21	8.59	9.27				
	28	8.44	9.29				
Mch	2	14.14	14.41	0.74	0.71	<0.01	0.73
(pg)	8	14.10	14.49				
	14	14.27	14.80				
	21	16.04	15.38				
	28	18.06	16.84				
Mchc	2	37.49	37.24	1.58	0.25	<0.01	0.93
(g/dl)	8	38.99	38.26				
	14	39.60	39.26				
	21	42.47	40.51				
	28	46.62	44.13				

Table 3a: Haematologica parameters in control (C) and treated (T) groups ([A,B] P<0.01; [a,b] P<0.05)

ITEM	TIME (Day)	GROUP C	GROUP T	SEM	P GROUP	P TIME	P G*T
Plt	2	606.64	543.18	59.58	0.49	<0.01	0.80
(k/μl)	8	758.33	823.45				
	14	702.09	743.70				
	21	647.18	677.20				
	28	519.73	576.50				
Pdw	2	72.40	75.60	2.74	0.18	<0.01	0.94
(fl)	8	69.85	74.36				
	14	66.82	67.12				
	21	65.90	67.55				
	28	66.00	67.83				
Mpv	2	64.50	64.90	1.13	0.61	<0.01	0.99
(fl)	8	62.86	63.36				
	14	59.63	60.37				
	21	59.20	59.89				
	28	60.17	59.67				
Plcr	2	43.80	49.70	4.92	0.28	<0.01	0.91
(fl)	8	36.00	43.82				
	14	23.86	25.75				
	21	23.60	26.33				
	28	23.50	21.83				
Pct	2	39.10	35.50	4.63	0.56	<0.01	0.85
(%)	8	49.57	52.18				
	14	42.27	47.50				
	21	36.50	39.33				
	28	34.33	37.16				
Rdw	2	274.64	271.45	6.50	0.92	0.08	0.81
(fl)	8	284.11	285.55				
	14	294.00	284.90				
	21	285.64	290.20				
	28	277.27	281.60				

Table 3b: Haematological parameters in control (C) and treated (T) groups (^{A,B} P<0.01; ^{a,b} P<0.05)

ITEM	TIME (Day)	GROUP C	GROUP T	SEM	P GROUP	P TIME	P G*T
Wbc	2	8.66	9.95	0.89	0.96	0.94	0.67
(k/μl)	8	8.98	9.73				
	14	9.37	8.99				
	21	9.02	8.39				
	28	9.42	8.53				
Monocytes	2	12.14	10.61	1.55	0.59	<0.01	0.63
(%)	8	9.35	8.74				
	14	15.65	13.58				
	21	14.90	15.96				
	28	16.96	17.44				
Limphocytes	2	19.01	22.45	4.12	0.15	<0.01	0.99
(%)	8	22.28	26.90				
	14	31.10	33.00				
	21	34.87	37.12				
	28	38.28	40.88				
Basophils	2	0.13	0.16	0.02	0.37	0.07	0.29
(%)	8	0.13	0.14				
	14	0.16	0.14				
	21	0.16	0.16				
	28	0.16^{b}	0.21^{a}				
Neutrophils	2	66.64	62.94	4.14	0.28	<0.01	0.99
(%)	8	65.03	62.34				
	14	51.27	51.01				
	21	48.34	44.58				
	28	42.69	38.79				
Eosinophils	2	0.10	0.13	0.03	0.70	0.76	0.02
(%)	8	0.22^{A}	0.05^{B}				
	14	0.04	0.10				
	21	0.03	0.07				
	28	0.06	0.07				

Table 4: Leukocyte formula in control (C) and treated (T) groups (^{A,B} P<0.01; ^{a,b} P<0.05)

4.5.4 Skin test

There were no differences between the two groups in terms of skin thickness at PBS injection sites and hence they were not used to correct PHA-induced thickness. The skin thickness reaction to PHA injection did not increased with time up to 24 hours post injection in both groups at 8 and 28 days of life (Table 5).

	TIME	GROUP			P		
ITEM	**(Day)**	**C**	**T**	**SEM**	**GROUP**	**TIME**	**G*T**
Skin Thickness (mm)	7 (h24-h0)	0.40	0.65	0.13	0.89	0.12	0.11
	28 (h24-h0)	0.48	0.19				

Table 5: Skin test in control (C) and treated (T) groups (^{A,B} P<0.01; ^{a,b} P<0.05)

4.6 Discussion

In our study we evaluated the effects of the administration of a species-specific probiotic compound on gut microbial health, general health, growth performance, haematological parameters and cell mediate immune response of newborn female calves in the first month of life. Gut microbial balance is one of the most important factors which concur to obtain animal health status; its importance has been underlined in particular for young calves, which immune system is immature and prone to debilitating diarrhoea and respiratory diseases (Tsuruta et al., 2009). Oral administration of Lactic Acid Bacteria (LAB) probiotics has been already recognized as a promoter of intestinal microbial balance and growth performance (LeJeune and Wetzel, 2007; Al-Saiady, 2010; Hossaini et al., 2010, Kawakami et al., 2010, Nagashima et al., 2010). The beneficial effects of probiotics are possibly due to the transient proliferation in the digestive tract, developing a microbial defense against the proliferation of pathogenic bacteria; in particular lactic acid bacteria are recognized to be effective against *Escherichia coli* and *Salmonella* spp. In our trial we considered Lactobacilli and *E. coli* counts as microbiological indicators of gut microbial health: their role has been already underlined by other authors, who detected clearly higher Lactobacilli/coliforms ratio in healthy calves than in calves suffering from diarrhoea (Abu-Tarboush et al., 1996; Frizzo et al., 2010). Results from the present trial evidenced a better Lactobacilli/*E. coli* ratio, indicating a favourable equilibrium in the microflora of these animals, especially during the last phase of the trial in agreement with Timmerman et al. (2005), and Stella et al. (2007) but in goats, and Frizzo et al. (2011) in calves who underlined a higher Lactobacilli/*coliforms* when supplementing LAB inoculums. The improved gut health status observed in our study was confirmed by increased faecal score in T group with a lower frequency of episodes of diarrhoea in agreement with Kawakami et al. (2010). Although GHS was not different between the experimental groups, numerically higher values could suggest a healthier status of T calves. as reported by Timmerman et al. (2005). The improvement of Lactobacilli/*E. coli* ratio and faecal consistency as indices of health status of the calves could explain the higher final BW detected in probiotic-fed group as reported by several authors (Frizzo et al., 2008; Bakr et al., 2009; Al-Saiady et al., 2010; Hasunuma et al., 2011). In fact it is well known as many troubles affecting the growth rates are caused by the failure in nutrients digestion and absorption (Davis and Drackley, 2001). In the present trial weekly ADG tended to be higher in treated group from the second week of life, confirming data by Timmerman et al. (2005), Frizzo et al. (2008) and Kawakami et al. (2010) who registered a significant difference from the second till the fifth week. Calf Starter total feed

intake was higher in treated group in agreement with some other authors (Timmerman et al., 2005; Frizzo et al., 2008; Kawakami et al., 2010): this effect is particularly important, as the dry matter intake in early stage of life is necessary to assure the proper growth of pre-stomachs, although the literature reports inconstant results in different studies (Abe et al., 1995; Bakshi et al., 2006; Frizzo et al., 2008; Kawakami et al., 2010), probably due to the microorganisms used and experimental conditions. Haematological profile of veal calves used for the trial followed generally the physiological trend for the first phases of life. Considering HCT and HGB, a gradual decrease during the trial was detected. Such trend could be considered physiological because normally observed from birth until the first weeks of life of young calves due to the diet composition (Mohri et al., 2009). The type of delivery is associated to different haematological profile, having an influence on oxygenation levels and on a-specific immunitary profile in neonatal calves (Probo et al., 2011). Young calves can also be subjected to recruitment problems or malabsorption in the first weeks, leading to a condition of anaemia, defined as a decrease below normal range of RBC, and HGB (Sarker et al., 2010). In our trial RBC resulted slightly below the normal range at the third week for C group and fourth weeks for T group, but this trend should be considered physiological in very young animals. A slight lymphopenia, associated with monocytosis and neutrophilia was revealed for the whole experimental period in both groups, but similar values can be usually detected in very young animals, and do not represent a pathological condition (Mohri et al., 2011). In the present study HGB levels were significantly higher in T group at 8 days of life; this effect has been observed also in other studies, but literature data are inconstant and this item should be further investigated (Fleige et al., 2008; Matsumoto et al., 2009; Al-Saiady et al., 2010; Riddell et al., 2010; Kim et al., 2011). The reaction to PHA inoculums has been already evaluated in several species, but till now no trials were conducted on dairy calves supplemented with probiotic. In our trial no differences in dermal reaction to skin test between the groups were observed at 8 or 28 days of life. The absence of statistical evidences in skin test during the first weeks of life have been observed also by Masucci et al. (2010) in young buffalo calves, evidencing the presence of a positive response only during later age (11 weeks).

4.7 Conclusions

The administration of a species-specific multistrain probiotic compound improved intestinal balance after one month of treatment, consequently increasing some growth parameters of veal calves na d reducing the incidence of enteric probems, although a direct effect on cell-mediated immune response was not observed. Further studies must be conducted to investigate the immune parameters in neonatal calves, also in different stress conditions

4.8 Acknowledgements

The present experimental trial was carried as part of the research project n. 1340 "Study and evaluation of a species-specific probiotic for veal calves" (BIOVAPRO) funded through Plan for Research and Development of the Lombardy Region (2007-2009), DG Agriculture, d.g.r. 8/3620, 28/11/2006.

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CHAPTER 5

Calcium diformate in calf diet: effects of two different dosages on growing performance, metabolism, health status, and Ca and formate organ content

5 Calcium diformate in calf diet: effects of two different dosages on growing performance, metabolism, health status, and Ca and formate organ content

5.1 Abstract

The trial involving 36 male Friesian calves was conducted to determine the effect of calcium diformate administration and its inclusion level on growth performance, intestinal balance, metabolism, health status, and calcium and formate organ content. The calves (44.55kg±5.51kg of body weight, 25±10 days old), were divided in three homogeneous groups of twelve subjects each: C fed with a basal diet, T1 fed with a basal diet plus 1%/milk replacer of calcium diformate, and T3 fed a basal diet plus 3%/milk replacer of calcium diformate for a total of 84 days of supplementation. During the experimental period daily Feed Intake (FI), Average Daily Gain (ADG) and Feed Conversion Rate (FCR) were determined. At 0, 42 and 84 days of trial individual blood samples were collected in order to detect haematological and biochemical parameters, Ca and FO content. At the same time faecal samples were collected for Lactobacilli, total coliforms and *E.coli* count along with faecal pH. Weekly body weight (BW) and faecal score (FS) were recorded, while the health status was constantly monitored and the cause of deaths analyzed by necropsy. At the 91st day of trial the calves were slaughtered: any presence of ulcers were detected, individual carcass weight and dressing percentage were determined, and kidney and liver weight recorded. On seven animals per group liver, kidney, muscle and fat samples were collected and total Ca and formate were evaluated. The administration of calcium diformate decreased dry matter intake from either milk powder, concentrate and both milk powder and concentrate together with increasing level of calcium diformate in the diet (C=1419,05g/h/d; T1=1217,78g/h/d; T3=1190,73g/h/d; P≤0.01). As a result, final body weight in treated animals was lower than control calves (C=121,00kg; T1=112,17kg; T3=108,74kg; P≤0.01). Mean ADG in 3%/milk replacer CaFO calves was decreased if compared to C subjects (C=0.87kg/h/d *vs* T3=0.73kg/h/d; P<0.01), while a similar feed conversion rate was detected between the experimental groups. Faecal score was decreased in T3 animals (P≤0.05) than control while no differences were found for faecal pH, *E.coli* or Lactobacilli. Higher total *coliforms* count was evidenced in T1 animals than C (7.45 Log_{10} *vs* T1=2.75Log_{10} respectively; P≤0.05).

At slaughter no difference was observed for dressing percentage, while carcass weight resulted higher in C compared to T3 (C=67.49kg *vs* T3=58.87; P≤0.05). Organ weight and organ formate and calcium content were similar between the experimental groups.
Red blood cells count (RBC), haemoglobin (HB) and hematocrit (HT) resulted higher (P≤0.05) in C and T3 than T1, while control calves showed higher platelets (P≤0.05) value than T1, and greater (P≤0.05) triglycerides than T3. Mean cell haemoglobin (MCH) value resulted higher (P≤0.05) in T3 subjects than the other groups. Increased formate blood content in 3% of calcium diformate/milk replacer animals did not show any differences for organ formate content thus giving no accumulation. Immune response was not affected by the treatment and necropsies on dead animals suggest no interaction among treatment and death itself. The histological examination revealed altered structural aspects of the intestinal mucosa in all the experimental corresponding with no detectable differences between control T1 and T3 groups. Epithelial detachment in the intestinal villi associated with mucous secretion excess and some diffuse mixed cellular infiltration sub mucosa and *lamina propria* were highlighted. The sub mucosal Brunner glands were in normal physiological conditions in C, but showed empty and enlarged lumen in treated animals. While not showing toxic effects in veal calves, the administration of calcium diformate is not useful in promoting the growth performance and gut balance. In conclusion the calcium diformate supplementation, even if not showing toxic effects in veal calves, isn't helpful to increase the intestinal microbial balance and the growth performance.

5.2 Introduction

Feeding practices, composition of animal diets and farm management can influence the microbial balance in the gastrointestinal tract and, subsequently, affect feed efficiency and the animal health.
Under current husbandry conditions, veal calves are often affected by diarrhoea, which is the main cause of morbidity and mortality in the early life of veal calves (Timmerman et al., 2005).
Immunity against pathogen and viruses begins with the dam. Calves are born with an immune system that is naive, and must be "taught" before it is able to respond fully to pathogenic challenges on its own. Various factors can cause the high incidence of intestinal and respiratory diseases in veal calves: after birth calves are separated from their mothers, preventing the calf from picking up the protective gut microflora from (Fuller, 1989). The second and the third week of life have been found to be the most critical in terms of calf mortality and they could be viewed as a "weak" period, where the effectiveness of passive immunity is lessening and adaptive immunity is not fully functional (Wilde, 2008). Furthermore, at young age, the calves are faced with major stress events like transportation, dietary changes and exposure to a variety of infectious agents. Subsequently, animals are predisposed to the loss of the barrier function on the gut (Nabuurs et al., 2001; Soderholm and Perdue, 2001), and may suffer from impaired immune function (Timmerman et al., 2005). Moreover, the protective potential of the microbial gut flora tends to decrease (Cray et al., 1998).
Thus, a preventive intervention is the recommended strategy. As a preventive measure alternative to use of antibiotics, acidifiers are recommended (Palenzuela et al., 2003; Ribeiro et al., 2009) due to their bacteriostatic and bactericidal action (Young and Foegeding, 1993).
Organic acids have been used for decades in commercial compound feeds, mostly for feed preservation, where formic and propionic acids are particularly effective. In the European Union, several organic acids and their salts (e.g., calcium formate, calcium propionate) are used under the classification "feed preservatives". Acidifiers are the most reliable product group of the non-antibiotic growth promoters available in Europe, and can also be used safely and effectively with other additives (Lückstädt et al., 2011).
Acids added to diets may provide benefits through maintaining a low gastric pH that may increase nutrient digestion and reduce pathogen survival (Mroz, 2003; Kim et al., 2005). The main mechnaism of action of organic acids is through their antimicrobial effects, the magnitude of which is dependent on the chemical properties of the individual acid or acid salt.

Briefly, the undissociated organic acids are lyophilised, so they can cross the cell membrane of Gram negative bacteria such as *Salmonella*. Once inside the cell, the higher cytosolic pH causes the acid to dissociate, releasing hydrogen ions, which subsequently reduces the intracellular pH. Microbial metabolism depends on enzyme activity, which is depressed at lower pH. To redress the balance, the cell is forced to use energy to expel protons out across the membrane. Over a period of exposure to organic acids, this can be sufficient to kill the cell. Lactic acid bacteria are less sensitive to the pH differential across the cell membrane, and, thus, they remain unaffected (Lückstädt et al., 2011). Therefore, the improvement in growth performance resulting from dietary inclusion of organic acid salts is due to an antimicrobial effect (Kirchgessner et al., 1992). Formic acid is added to the diets in the form of its salt (K e Ca), which results more manageable than pure form and more efficient to promote the growth performance (Paulicks et al., 1996; Kirchgessner et al., 1997). The administration of formic acid salts leads to a higher digestibility of the diet (Roth et al., 1998) especially when milk replacers contain lower levels of milk by-products (Lawlor et al., 1992): the acid lactic production induced from lactose can decrease the stomach pH masking the acidificant effects of the formate.
A high calcium formate inclusion in the diet can give rise to toxicity phenomenon in dairy cows (Scott e Van Wijk, 2000), while further trials should be conducted with relation to calves.
The aim of the trial was to evaluate the effects of two different dietary dosages of calcium diformate on growing performance, metabolism, health status, Ca and formate organ content in veal calves.

5.3 Materials and methods

5.3.1 Animals and Experimental Design

A total number of 36 Friesian male calves coming from different farms (25±10 days old, body weight of 44.55±5.51kg) were divided into three homogeneous groups on the basis of initial BW on the 7th day from arrival. The subjects were allocated to six different boxes (two boxes for each treatment) and received the correspondent experimental dietary treatment consisting of a basal diet (C group) plus 1%/milk replacer of calcium diformate (T1 group) or 3%/milk replacer of calcium diformate (T3 group). The test had two main phases named F1 and F2 respectively. F1 lasted for 7 days (adaptation period, no experimental treatment was performed) and F2 lasted for 84 days (days 8-91), during which the groups received the correspondent dietary treatment, for a total of 91 days of the experimental study. During the first seven days after the calves' arrival, the basal diet was administered and no measurements were performed in order to reduce the animal stressors, except for feed records. The calves were allotted indoor with environmental contrled conditions and reared on concrete slatted floor with straw. each experimental box had the same area of approximately 17.50 m^2 , with a density of 2.9 animals/m^2 each box was equipped with individual buckets and feeders in order to determine milk and feed consumption. Experimental animals were fed twice a day at 07:00am am and 6.00pm, In the first 47 days from the arrival a *prestarter* milk powder type was administered, while a *starter* type was provided from 48th day up to the end of the trial (Tab.1 and 4). The milk powder was mixed with hot water at a temperature of 70°C and a concentration of 110g/L at the beginning of the trial, 125g/L until 60 days of treatment and 140g/L subsequently. Increasing milk replacer amounts were administered according with the standard procedures related to the age of the animals. The meal was administered approximately at 38°C, controlled with an electronic thermometer. The calves initially received 2L/head/meal, increased of 0.5L/head/meal weekly for a total of 8 litres per meal at the end of the trial.
The provided commercial concentrate, composed by 50% of corn grain and 50% of corn silage (Tab.1) was administered once a day (12.00am) starting from the 14th day from arrival at the initial rate of 50g/head/day until 250g/head/day at the end of the experimental period. The orts were removed, weighed and recorded at each meal.

During the supplementation period the administered diets were adjusted for an equal Ca content (Tab.2 and 3).The experimental premix was prepared weekly in order to include the proper amount of CaCO3 and calcium diformate in the respective diets (Tab.2 and 3).
Calves had free access to drinking water at all times and the experimental diets were able to meet the requirements stated by the NRC for the different stages of growth.

****** Milk powder and concentrate provided by Zoogamma s.p.a. (VanDrie Group. Borgo Satollo, Ghedi, Brescia)

Chemical analysis (%DM)	Milk Powder Prestarter	Milk Powder Starter	Concentrate
Moisture	7.00	8.70	42.31
Crude protein	22.04	21.14	12.96
Crude fat	18.17	19.06	4.41
Ash	7.05	7.97	4.25
Crude Fibre	---	---	7.55
NDF	---	---	20.50
Starch	---	---	76.32
Ca	0.43	0.41	---
Ptot	0.70	1.27	---
Formic acid	0.04	≤0.01	---
DE(kcal/kg)	4,800.00	4,783.00	---
ME(kcal/kg)	4,670.00	4,665.00	---

Table 1: Composition and chemical analysis of the calves concentrate, prestarter and starter milk powder diet without any addition of CaCO3 and of calcium diformate.

				Prestarter		Starter	
Group	**Premix[a]**	**Milk Powder[a]**	**Ca from premix[a]**	**Ca from milk[a]**	**Total Ca Content[b]**	**Ca from milk[a]**	**Total Ca content[c]**
C	2.53	97.47	0.936	0.360	**1.30**	0.361	**1.30**
T1	2.69	97.31	0.936	0.360	**1.30**	0.360	**1.30**
T3	3.00	97.00	0.936	0.359	**1.30**	0.359	**1.30**

(Prestarter Calcium Content, %a.f.=0.61; Starter Calcium Content, %a.f=0.37)

Table 2: Inclusion of experimental premixes in milk powder and target Ca content (calculated) in the diet of veal calves (a=kg a.f; b= kg/100kg a.f.; c=g/100kg a.f.)

Group	Calcium diformate (g)	Ca From Ca Diformate(g)	CaCO3* (g)	Ca from CaCO3 (g)	Total amount of premix (g)	Total Ca amount (g)
C	---	0.00	2,529.73	936.00	2,529.73	936.00
T1	1,000.00	312.00	1,686.48	624.00	2,686.48	936.00
T3	3,000.00	936.00	---	0.00	3,000.00	936.00

Table 3: Composition of experimental premixes and calculated Ca target content

	C		T1		T3	
Chemical analysis (%DM)	**Prestarter**	**Starter**	**Prestarter**	**Starter**	**Prestarter**	**Starter**
Moisture	7.10	7.50	7.80	8.00	7.30	6.40
Crude protein	21.85	19.78	22.56	19.57	21.79	19.55
Ether extract	18.41	18.16	18.44	18.37	18.34	17.95
Ash	9.10	10.30	9.24	10.08	9.06	9.44
Ca	1.26	1.45	1.19	1.26	1.24	1.38
P tot	0.86	0.89	0.78	1.00	0.78	0.92
Formic acid	0.04	≤0.01	0.74	0.74	2.46	2.44
DE(kcal/kg)	4,560.00	4,408.00	4,594.00	4,431.00	4,570.00	4,366.00
ME(kcal/kg)	4,439.00	4,308.00	4,467.00	4,332.00	4,449.00	4,267.00

Table 4: Analyzed chemical composition of the experimental milk powders (%DM).

5.3.2 Growth performance and feed analyses

Initial individual body weight (BW) was evaluated upon arrival. Subsequently the individual BW was recorded weekly with an electronic weighing scale (sensitivity 100 g).
Starting from day seven, ADG was calculated and FCR estimated based on a daily individual total dry matter intake (milk replacer and concentrate consumption) (DMI).
On the seventh day from test beginning, the *prestarted* milk powder type was collected and subsequently analyzed for dry matter content (DM), raw protein content (CP), fat content (EE), ash content, calcium (Ca) and phosphorus (P) and for the starter type on 48th day of trial.
On the 14th day from the start, the concentrate was sampled for dry matter content, protein content, fat content, ash content, NDF value calcium and phosphorus. A sample of the concentrate given to the animals on 14th day from arrival was analyzed for DM, CP, EE, ash, NDF, Ca and P content.

5.3.3 Haematological parameters

On days 0 (7th day from arrival), 42 and 84 of the treatment, individual blood samples taken from the jugular vein were collected into three 10 ml vacuum tubes (Terumo Venosafe 10 ml VF-109SHL, Terumo Europe L.V., Leuven, Belgium). The tubes were immediately placed in ice, transferred to the laboratory and automatically analyzed by Sysmex 2000i (Sysmex corporation) for red blood cells count (RBC), haemoglobin (HB), hematocrit (HT), reticulocytes, reticulocytes production index (RPI), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red cells distribution width (RDW), platelets (PLT), mean platelets volume (MPV), plateletcrit (PCT), platelet distribution width (PDW), white blood cell (WBC). The leucocytes formula (neutrophils, limphocytes, monocytes, eosinophils and basophils) was assessed in whole blood samples with a Hemat 8.
The samples were further analyzed for some blood chemical parameters: albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALAT), gamma-glutamyl transferase (GGT), creatinine, glucose, total cholesterol, triglycerides, total protein, bilirubin, urea, uric-acid, iron (Fe), magnesium (Mg), phosphorus (P) determined by Cobas ® Mira Classic (Roche Diagnostics).
Moreover haptoglobin, pepsinogen calcium (ISTISAN 1996/34) and formic acid (MI 397 rev 0 2007) were determined in collected blood samples.

5.3.4 Faecal parameters and gut microbial balance

On days 0 (7th day from arrival), 42 and 84 of the treatment, faecal samples from six animals per group were collected by rectal stimulation, stored in vials with transport medium (Faecal TM enteric Plus, Oxoid, Basingstoke, UK) kept refrigerated (4°C) and analyzed in order to determine the Lactobacilli count (ISO 15214:1998), *E.coli* count (ISO 16649-2:2001), total coliforms (ISO 4832:2006) and faecal pH.
The individual faecal score was recorded weekly on a scale of 1 to 5 (1 = watery, 4 = firm; Larson et al., 1978), and diarrhoea incidence was calculated.

5.3.5 Health Status

During the whole trial, the health status was checked and recorded daily; signs of toxicity and illness were recorded, unexpected deaths were necropsied.
The occurrence of diarrhoea was evaluated daily.

5.3.6 Slaughter Parameters and Intestinal morphology

At the end of the trial (91 days after arrival, 84 days of treatment) the individual carcass weight and dressing percentage were evaluated at the slaughterhouse; moreover, the individual liver and kidney weight were determined. The presence of gastrointestinal ulcers was individually detected at necropsy.
On 8 animals per group liver, kidney, muscle and fat samples were collected and total Ca were evaluated by atomic absorption with spectrophotometer (ISTISAN 1996/34) and formate content were determined by Boehringer Mannheim/R-Biopharm (R-Biopharm AG Landwehrstr.54 D-64293, Germany) modified kit for tissues as suggested from the producer.
Eight calves for each group were selected at slaughtering for histomorphological analysis. The entire intestine was removed and fragments of proximal duodenum were sampled from each animal and promptly fixed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS) pH 7.4 for 24 h at 4°C. The specimens were then dehydrated in graded alcohols, cleared with xylene and embedded in paraffin. After dewaxing and rehydration, serial microtome sections (4-μm thick) were stained with Haematoxylin-Eosin (HE) sequential staining to ascertain structural detail.
Histometric evaluation was used to determine 10 *villous* height (V), 10 crypts depth (C) and the relative *villous* height to crypt depth ratio (V:C) per section.

5.3 Statistical analysis

Body weight, ADG, FI, haematological, microbiological analyses and faecal parameters were analysed by a MANOVA procedure for repeated measures of SAS/STAT (Version V8, 1999, SAS Inst, Inc., NC, U.S.A.), including the fixed effect of treatment, the day, and the interaction treatment per day considering the animal as the experimental unit.
Carcass weight, dressing percentage, organs weight and their Ca and Fo content were analysed by a General Linear Mode (GLM) procedure of SAS/STAT (Version V8, 1999, SAS Inst, Inc., NC, U.S.A.) including the fixed effect of treatment and considering the animal as the experimental unit. The significance was declared at $P \leq 0.05$.
Histometric data (*villous* height, crypt depth, V:C ratio) were analysed through ANOVA using the mixed procedure of the SAS package. The model included treatment as fixed effect and the calves as random effect. The data were presented as least squared means ± SE. Differences between least squared means were analysed by orthogonal contrast and considered significant at $P < 0.05$.

5.4 Results

5.5.1 Growth performance

The growth performances during the trial in veal calves are reported in Table 6 (a,b and c). No differences in BW were detected among experimental groups at the beginning of the trial; while at the end, T1 animals evidenced a lower BW compared to C animals (C=121.00kg; T1=112.17; T3=108.74; $P \leq 0.01$). During the experimental period, starting from the first week of treatment, both T1 and T3 groups significantly decreased BW in comparison with C animals; whilst no differences were observed among the treated groups.
As early as the 5th week of supplementation and until the end of the trial, milk powder DMI was statistically lower in both T1 and T2 groups than in control. The same trend was observed for concentrate DMI from the 8th considered week; while no difference was detected among the treated animals. As a result, total DMI significantly decreased from the fifth week of administration (C=1,419.05g/h/d, T1=1,217.78 g/h/d, T3=1.190,73 g/h/d; $P \leq 0.01$).

The supplementation significantly reduced the total ADG in T3 compared to C (0.73kg/d *vs.* 0.87kg/d respectively; P≤0.01); the same trend was observed in both T1 and T3 groups during the 1st and the 6th week of supplementation and also at 8th week in T3 subjects.
Considering all the trials, no influence of calcium diformate administration was determined on FCR calculated on milk powder, concentrate or both pooled togheter. Differently, at 2nd,6th and 9th week from the beginning of the supplementation of calcium, the feed efficiency calculated on the milk powder given to C subject improved significantly in comparison with what recorded for the animals in T3. An opposite trend could be seen during the third week of trial compared to the third observed time.
Both total FCR and FCR calculated over milk supllementation were similar in groups T1 and T3, except for a lower value in T3 detected at week 9 from the beginning of treatment.

ITEM	TIME (week)	GROUP C	GROUP T1	GROUP T3	SEM	P GROUP	P TIME	P G*T
DMI	2	672.86	651.33	653.14	40.43	0.01	0.01	0.01
Milk P.	3	793.63	771.19	769.15				
(g/h/d)	4	907.77	849.56	866.08				
	5	1,042.14^{a}	984.77	920.68^{b}				
	6	1,261.08^{Aa}	1,133.90^{Ab}	934.13^{B}				
	7	1,443.00^{A}	1,217.47^{Ba}	1,078.27^{Bb}				
	8	1,538.58^{A}	1,188.33^{B}	1,213.99^{B}				
	9	1,634.94^{A}	1,313.30^{B}	1,399.32^{B}				
	10	1,763.67^{A}	1,347.80^{B}	1,304.78^{B}				
	11	1,752.88^{A}	1,477.37^{B}	1,589.33^{B}				
	12	1,959.46^{A}	1,669.80^{B}	1,601.50^{B}				
	2-12	1,342.73^{A}	1,145.89^{B}	1,120.94^{B}	12.07	<0.01		
DMI	2	22.90	20.71	21.44	2.56	0.01	0.01	0.01
Concen.	3	31.53	30.53	31.85				
(g/h/d)	4	39.80	41.09	37.74				
	5	58.26	56.28	55.51				
	6	70.33	67.00	67.39				
	7	81.19	82.21	79.05				
	8	88.23^{A}	78.79^{B}	83.66				
	9	99.73^{Aa}	89.27^{B}	92.43^{b}				
	10	106.01^{A}	95.39^{B}	96.35^{B}				
	11	118.21^{A}	106.78^{B}	95.93^{C}				
	12	123.34^{A}	122.68^{A}	106.30^{B}				
	2-12	76.32^{A}	71.88^{B}	69.79^{B}	0.76	<0.01		
Total	2	695.77	672.04	674.58	42.29	0.01	0.01	0.01
DMI	3	825.16	801.71	800.99				
(g/h/d)	4	947.57	890.64	903.82				
	5	1,100.41^{a}	1,041.05	976.19^{b}				
	6	1,331.42^{Aa}	1,200.90^{Ab}	1,001.51^{B}				
	7	1,524.18^{A}	1,299.67^{Ba}	1,157.32^{Bb}				
	8	1,626.81^{A}	1,267.12^{B}	1,297.65^{B}				
	9	1,734.67^{A}	1,40257^{B}	1,491.75^{B}				
	10	1,869.67^{A}	1,443.19^{B}	1,401.14^{B}				
	11	1,871.08^{A}	1,584.14^{B}	1,685.26^{B}				
	12	2,082.80^{A}	1,792.48^{B}	1,707.80^{B}				
	2-12	1,419.05^{A}	1,217.78^{B}	1,190.73^{B}	12.56	<0.01		

Table 6a: Growth performance in veal calves during the weeks of the trial
(^{A,B,C}= P≤0.01; ^{a,b}=P≤0.05)

	TIME	GROUP				P		
ITEM	**(week)**	**C**	**T1**	**T3**	**SEM**	**GROUP**	**TIME**	**G*T**
BW	0	48.13	45.25	46.95	1.25	≤0.01	≤0.01	≤0.01
(kg)	1	52.92^{a}	49.75	49.10^{b}				
	2	56.75^{a}	52.38^{b}	52.80^{b}				
	3	60.08	56.71	57.30				
	4	65.79^{a}	62.12^{b}	61.70^{b}				
	5	71.29^{Aa}	65.38^{B}	66.65^{b}				
	6	78.63^{A}	71.38^{B}	70.35^{B}				
	7	86.79^{A}	77.71^{B}	77.35^{B}				
	8	91.96^{A}	81.92^{B}	79.85^{B}				
	9	98.96^{A}	88.17^{B}	86.46^{B}				
	10	106.29^{A}	95.88^{B}	94.07^{B}				
	11	113.71^{A}	104.25^{B}	101.74^{B}				
	12	121.00^{A}	112.17^{B}	108.74^{B}				
ADG	1	0.68^{A}	0.64^{a}	0.31^{Bb}	0.09	≤0.01	≤0.01	≤0.01
(kg/d)	2	0.55	0.38	0.53				
	3	0.48	0.62	0.64				
	4	0.82	0.77	0.63				
	5	0.79^{a}	0.46^{b}	0.71				
	6	1.05^{A}	0.86^{a}	0.53^{Bb}				
	7	1.17^{a}	0.91^{b}	1.00				
	8	0.74^{A}	0.60	0.36^{B}				
	9	1.00	0.89	0.89				
	10	1.05	1.10	1.09				
	11	1.06	1.20	1.10				
	12	1.04	1.13	1.00				
	1-12	0.87^{A}	0.80	0.73^{B}	0.03	<0.01		

Table 6b: Growth performance in veal calves during the weeks of the trial
(^{A,B,C}= P≤0.01; ^{a,b}=P≤0.05)

	TIME	GROUP				P		
ITEM	**(week)**	**C**	**T1**	**T3**	**SEM**	**GROUP**	**TIME**	**G*T**
FCR Milk powder	2	1.18^{b}	1.41	1.76^{a}	0.21	0.20	<0.01	0.018
	3	2.08^{A}	1.33^{B}	1.32^{B}				
	4	1.30	1.14	1.23				
	5	1.41^{b}	2.10^{a}	1.67				
	6	1.27^{B}	1.54	2.01^{A}				
	7	1.32	1.32	1.07				
	8	2.39	2.35	2.22				
	9	1.79	1.48^{b}	2.08^{a}				
	10	1.80	1.31	1.33				
	11	1.73	1.26	1.74				
	12	1.95	1.49	1.91				
	2-12	1.66	1.52	1.66	0.06	0.20		
Total FCR	2	1.22	1.45	1.81	0.21	0.22	0.01	0.02
	3	2.16^{A}	1.38^{B}	1.37^{B}				
	4	1.35	1.19	1.28				
	5	1.48^{b}	2.22^{a}	1.76				
	6	1.34^{B}	1.64	2.18^{A}				
	7	1.39	1.41	1.15				
	8	2.53	2.53	2.37				
	9	1.90	1.58^{b}	2.21^{a}				
	10	1.91	1.41	1.43				
	11	1.85	1.35	1.85				
	12	2.07	1.60	2.04				
	2-12	1.75	1.61	1.77	0.06	0.22		

Table 6c: Growth performance in veal calves during the weeks of the trial
(^{A,B,C}= P≤0.01; ^{a,b}=P≤0.05)

5.5.2 Haematological evaluations

The Haematological result on blood collected samples are reported in Table 7. and 8. Most of serum parameters were not influenced by the treatments and were in the suggested ranges for calves (Hege et al., 2006; Mohri et al., 2007). Red Blood Cells, Hb, and Ht decreased in T1 compared to T3 (RBC and Ht=$P<0.01$; Hb=$P<0.05$) and C (RBC=$P<0.01$; Hb and Ht=$P<0.05$) calves, on the other hand, PLT resulted higher in T1 than in C group ($P<0.05$). The white blood cell count in animals fed 3.0%/milk replacer of calcium diformate was higher in comparison with T1 animals ($P<0.05$), while MCHC was lower than in C subjects ($P<0.05$). Triglycerides content resulted lower in T3 calves than in Control group ($P<0.01$), but the mean values was anyway included in the suggested ranges. ALAT and total protein serum content were found to be lower than the suggested range in the three experimental groups, while GGT and haptoglobin were higher. Pepsinogen content resulted lower in T1 group than C and T3 groups ($P<0.05$) while P level was higher in C in comparison both T3 ($P<0.01$) and T1 ($P<0.05$)calves. Uric acid showed a difference between the treated groups: T1 showed a higher value compared to T3 ($P<0.05$). The group being fed 3.0%/milk replacer calcium diformate highlighted the greatest value in Ca and formate content in respect to T1 and C ($P<0.05$).
No statistical differences were detected in the leukocyte formula among the three experimental groups.

ITEM		GROUP C	T1	T3	SEM	P Ca
Haematological parameters						
RBC	(10^6/μl)	5.51^{a}	4.95^{Bb}	5.62^{A}	0.18	0.02
Hb	(g/dl)	9.06^{A}	8.13^{Bb}	8.71^{a}	0.02	≤0.01
Ht	(%)	19.78^{A}	17.78^{B}	20.20^{A}	0.55	≤0.01
Reticulocytes	(%)	---	---	---	---	---
RPI		---	---	---	---	---
MCV	(fl)	36.05	35.91	36.04	0.43	0.97
MCH	(%)	17.95	17.98	16.46	0.61	0.17
MCHC	(%)	49.56^{b}	50.01^{b}	45.52^{a}	1.29	0.04
RDW	(%)	27.62	26.94	27.54	0.33	0.28
PLT	(10^3/μl)	542.97^{b}	606.89^{a}	580.65	22.72	0.13
MPV	(fl)	5.91	6.00	5.93	0.06	0.55
PCT	(%)	0.36	0.37	0.37	0.03	0.97
PDW	(%)	6.47	6.73	6.76	0.12	0.15
WBC	(10^3/μl)	8.41	7.81^{b}	9.06^{a}	0.41	0.12
Serum parameters						
Albumin	(g/L)	30.21	30.93	28.73	0.29	0.22
ALP	(UI/L)	384.48	305.83	326.53	35.25	0.26
ALAT	(UI/L)	66.77	66.44	60.05	3.93	0.42
GGT	(UI/L)	37.75	34.78	28.81	4.67	0.41
Creatinine	(μmol/L)	97.20	98.38	93.63	1.86	0.19
Glucose	(mmol/L)	5.52	5.30	5.11	0.21	0.39
Cholesterol	(mg/dL)	67.79	65.85	58.72	6.81	0.62
Triglicerydes	(mg/dl)	38.32^{A}	33.08	29.63^{B}	2.29	0.04
Total Protein	(g/L)	57.83	55.78	55.80	0.78	0.09
Bilirubin	(μmol/Ll)	3.83	3.88	3.86	0.16	0.97
Haptoglobin	(g/L)	0.49	0.55	0.69	0.06	0.61
Pepsinogen	(mμTyr/)	1.31^{a}	0.96^{b}	1.36^{a}	0.01	0.03
Urea	(mol/L)	2.64	3.13	2.96	0.21	0.25
Uric-Acid	(mg/dl)	0.97	0.94^{a}	1.02^{b}	0.05	0.14
Fe	(μg/dl)	77.73	87.25	85.51	9.93	0.76
Mg	(mmol/L)	0.89	0.88	0.88	0.01	0.91
P	(mmol/L)	3.20^{Aa}	2.87^{b}	2.59^{B}	0.05	≤0.01
Ca	(mmol/L)	2.68^{b}	2.73	2.83^{a}	0.04	0.06
Formate	(g/100g)	≤0.01^{B}	≤0.01^{B}	0.02^{A}	≤0.01	≤0.01

Table 7: Haematological parameters, biochemical and metabolic values in veal calves during the trial (^{A,B}= P≤0.01; ^{a,b}=P≤0.05).

ITEM		GROUP C	T1	T3	P SEM	Ca.Diform.
Leukocyte formula						
Neutrophils	(%)	27.85	25.32	28.16	2.00	0.59
Limphocytes	(%)	53.68	56.42	54.08	1.89	0.55
Monocytes	(%)	13.46	14.48	12.88	0.89	0.48
Eosinophils	(%)	4.22	3.33	3.72	0.71	0.66
Basophils	(%)	1.21	1.14	1.07	0.05	0.29

Table 8: Leukocyte formula in veal calves during the trial.

Considering the single sampling (Tab.9a,b) at time 0, RBC ($P<0.05$), Hb ($P<0.01$), Ht ($P<0.05$), RDW ($P<0.05$) basophiles ($P<0.05$) and total protein ($P<0.05$) resulted higher in C than T1; while Hb ($P<0.05$), pepsinogen ($P<0.01$), and uric-acid ($P<0.05$) resulted lower in T1 calves compared to T3 groups; PDW, instead, was greater in T3 compared to C ($P<0.05$). On 42nd day of administration, Ht was higher in C ($P<0.05$) and T3 ($P<0.01$) compared to T1, while MCH ($P<0.05$) and MCHC ($P<0.05$) resulted greater in T1 than T3. The same trend was observed for PLT: higher in T1 group than in C calves ($P<0.05$). In the third considered time, WBC was greater in T3 than T1 ($P<0.05$). On the contrary, basophiles were lower in T3 than T1 ($P<0.05$) (Tab.10).
During the trial the metabolic status of the animals in the three experimental groups was found to be very similar with minor variations in serum parameters (Tab.11a,b).
Initial GGT was higher in C animals than in T3 ($P<0.05$). Most of the differences were evidenced on 42nd day of the trial, when albumin was greater in T1 than in C ($P<0.05$); on the contrary, the triglycerides highlighted a higher value in C than T1 ($P<0.05$) and T3 ($P<0.01$), and in T1 resulted higher than T3 ($P<0.05$). At the end of the trial, the creatininine resulted lower in T3 than T1 ($P<0.05$).
The P content, upon the second sampling, was higher in not supplemented animals than T1 ($P<0.01$) and the latter was higher than T3 ($P<0.01$) (Tab.11c), while the Ca serum presence was greater in C and T1 groups than T3 ($P<0.01$). The groups T3 and T1 highlighted a higher formate content ($P<0.01$) than C subjects (Tab.11c).

ITEM	TIME (Day)	GROUP C	GROUP T1	GROUP T3	SEM	P GROUP	P TIME	P G*T
RBC	0	7.54^{a}	6.61^{b}	7.26	3.03	0.02	≤0.01	0.39
(10^6/µl)	42	5.10	4.35^{b}	5.45^{a}				
	84	3.90	3.91	4.15				
Hb	0	10.75^{A}	9.48^{Bb}	10.45^{a}	0.35	≤0.01	≤0.01	0.21
(g/dl)	42	8.68^{A}	7.28^{B}	8.15				
	84	7.76	7.63	7.52				
Ht	0	23.38^{a}	24.56^{b}	26.59	0.95	≤0.01	≤0.01	0.37
(%)	42	18.31^{a}	15.17^{Bb}	19.04^{A}				
	84	13.71	13.64	14.99				
MCV	0	36.26	37.41	36.86	0.75	0.97	0.05	0.41
(fl)	42	36.53	35.69	35.34				
	84	35.35	34.62	35.92				
MCH	0	14.48	14.77	14.80	1.08	0.17	≤0.01	0.56
(%)	42	18.68	18.94^{a}	15.87^{b}				
	84	20.67	20.23	18.70				

Table 9a: Haematological parameters in veal calves at 0, 42 and 84 days of the trial (^{A,B}= P≤0.01; ^{a,b}=P≤0.05)

ITEM	TIME (Day)	GROUP C	GROUP T1	GROUP T3	SEM	P GROUP	P TIME	P G*T
MCHC	0	39.80	39.35	40.02	2.23	0.04	≤0.01	0.33
(%)	42	50.47	52.20[a]	44.47[b]				
	84	58.42	58.49	52.08				
RDW	0	30.34[a]	28.48[b]	29.76	0.57	0.28	≤0.01	0.20
(%)	42	26.14	25.53	26.76				
	84	26.37	26.81	26.09				
PLT	0	641.58	721.83	678.64	39.32	0.13	≤0.01	0.50
(10^3/µl)	42	450.83[b]	566.67[a]	550.55				
	84	536.48	532.17	512.76				
MPV	0	5.92	6.04	6.02	0.09	0.55	0.37	0.90
(fl)	42	5.89	5.94	5.93				
	84	5.91	6.04	5.85				
PCT	0	0.38	0.45	0.41	0.04	0.97	0.04	0.73
(%)	42	0.36	0.35	0.34				
	84	0.35	0.31	0.37				
PDW	0	6.70[b]	6.78	7.01[a]	1.15	0.15	0.08	0.77
(%)	42	6.35	6.66	6.78				
	84	6.35	6.77	6.49				
WBC	0	7.34	7.15	7.63	0.72	0.12	≤0.01	0.75
(10^3/µl)	42	8.78	8.07	8.95				
	84	9.11	8.23[b]	10.59[a]				

Table 9b: Haematological parameters in veal calves at 0, 42 and 84 days of the trial ([A,B]= P≤0.01; [a,b]=P≤0.05)

ITEM	TIME (Day)	GROUP C	GROUP T1	GROUP T3	SEM	P GROUP	P TIME	P G*T
Leukocyte	**formula**							
Neutrophils	0	22.07	8.83	11.53	3.33	0.59	≤0.01	0.11
(%)	42	32.81	38.21	38.58				
	84	28.68	28.86	34.36				
Lymphocytes	0	71.57	83.47	81.75	3.03	0.55	≤0.01	0.13
(%)	42	46.83	43.33	43.25				
	84	42.63	42.48	37.26				
Monocytes	0	6.90	9.13	5.40	1.52	0.48	≤0.01	0.29
(%)	42	15.48	15.65	13.18				
	84	18.01	18.67	20.06				
Eosinophils	0	---	0.02	0.05	1.24	0.66	≤0.01	0.71
(%)	42	3.43	1.38	3.62				
	84	9.23	8.58	7.50				
Basophils	0	0.88[a]	0.56[b]	0.76	0.09	0.29	≤0.01	0.09
(%)	42	1.45	1.43	1.37				
	84	1.30	1.42[a]	1.07[b]				

Table 10: Leukocyte formula in veal calves at 0, 42 and 84 days of the trial
([A,B]= P≤0.01; [a,b]=P≤0.05)

ITEM	TIME (Day)	GROUP C	GROUP T1	GROUP T3	SEM	P GROUP	P TIME	P G*T
Albumin	0	28.21	28.85	29.12	0.50	0.22	0.01	0.17
(g/L)	42	30.35^{b}	31.85^{a}	31.49				
	84	32.08	32.09	30.96				
ALP	0	122.21	129.37	135.47	55.75	0.26	≤0.01	0.71
(UI/L)	42	539.68	408.73	481.47				
	84	491.55	379.40	362.19				
ALAT	0	65.65	63.14	53.05	6.22	0.42	0.50	0.60
(UI/L)	42	69.66	70.50	56.80				
	84	65.00	65.68	70.28				
GGT	0	69.09^{a}	54.98	38.59^{b}	7.39	0.41	≤0.01	0.28
(UI/L)	42	22.99	22.66	23.50				
	84	21.16	26.68	24.33				
Creatinine	0	108.64	105.23	105.13	2.95	0.19	≤0.01	0.58
(μmol/L)	42	92.81	95.53	91.46				
	84	90.16	94.38^{a}	84.29^{b}				
Glucose	0	5.55	5.03	4.70	0.34	0.39	0.34	0.34
(mmol/L)	42	5.24	5.09	5.59				
	84	5.78	5.77	5.03				
Cholesterol	0	55.36	45.46	42.58	10.63	0.62	≤0.01	0.87
(mg/dL)	42	59.75	70.96	53.22				
	84	88.25	81.13	80.34				
Triglicerydes	0	22.95	25.42	23.25	3.71	0.04	≤0.01	0.02
(mg/dL)	42	47.09^{Aa}	33.92^{b}	22.98^{Bc}				
	84	44.92	39.92	42.67				

Table 11a: Biochemical and metabolic parameters at 0, 42 and 84 days of the trial (^{A,B}= P≤0.01; ^{a,b,c}=P≤0.05)

ITEM	TIME (Days)	GROUP C	GROUP T1	GROUP T3	SEM	P GROUP	P TIME	P G*T
Total Protein	0	53.28[a]	50.40	48.50[b]	1.25	0.09	≤0.01	0.61
(g/L)	42	58.74	57.48	57.95				
	84	61.46	59.45	60.30				
Bilirubin	0	3.34	3.97	3.70	0.26	0.97	0.34	0.48
(µmol/L)	42	4.11	3.97	3.88				
	84	4.04	3.72	4.01				
Haptoglobin	0	0.67	0.75	0.67	0.08	0.61	≤0.01	0.95
(g/L)	42	0.51	0.55	0.61				
	84	0.28	0.36	0.39				
Pepsinogen	0	1.40	0.97[B]	1.67[A]	0.02	0.03	0.36	0.74
(mµTyr/L)	42	1.30	1.00	1.22				
	84	1.26	0.92	1.18				
Urea	0	4.78	5.00	4.14	0.36	0.25	≤0.01	0.11
(mmol/L)	42	1.71[b]	2.57	3.08[a]				
	84	1.43	1.82	1.66				
Uric-Acid	0	1.22	1.12[b]	1.26[a]	0.05	0.14	≤0.01	0.38
(mg/dL)	42	0.86	0.91	0.99				
	84	0.82	0.79	0.81				

Table 11b: Biochemical and metabolic parameters at 0, 42 and 84 days of the trial ([A,B,C]= P≤0.01; [a,b]=P≤0.05)

ITEM	TIME (Days)	GROUP C	GROUP T1	GROUP T3	SEM	P GROUP	P TIME	P G*T
Fe	0	184.18	204.62	172.25	16.78	0.76	≤0.01	0.61
(µg/dL)	42	23.08	25.62	32.43				
	84	25.92	31.53	51.84				
Mg	0	0.88	0.89	0.92	0.02	0.91	≤0.01	0.25
(mmol/L)	42	0.89	0.84	0.84				
	84	0.92	0.91	0.91				
P	0	2.77	2.70	2.68	0.04	≤0.01	≤0.01	≤0.01
(mmol/L)	42	3.29^{A}	2.49^{B}	1.76^{C}				
	84	3.55	3.42	3.32				
Ca	0	2.43	2.57	2.55	0.07	0.06	≤0.01	0.27
(mmol/L)	42	2.82^{A}	2.85^{A}	2.19^{B}				
	84	2.80	2.76	2.75				
Formate	0	≤0.01	≤0.01	0.01	≤0.01	≤0.01	≤0.01	≤0.01
(g/100g)	42	≤0.01^{B}	≤0.01^{B}	0.04^{A}				
	84	0.01	0.01	0.01				

Table 11c: Calcium and Formate content at 0, 42 and 84 days of the trial (^{A,B}= P≤0.01; ^{a,b}=P≤0.05)

5.5.3 Faecal score and Microbiological status

The data regarding faecal score avaluation, pH determination and microbiological status are reported in table 12.
During the trial, the faecal score was significantly lower in T3 animals compared to C group ($P<0.05$), while no differences were detected during each considered time. Faecal pH did not show differences between groups, and the same trend was observed on 0, 42nd and 84th day of supplementation. Considering all the trial, C group highlighted an higher presence of coliforms than T1 ($P<0.01$). The coliforms and *E.coli* were statistically higher ($P<0.01$) on day 0 in T1 group than in T3 and C and on the 84th day, instead, C groups showed an higher *E.coli* values than T3 ($P<0.01$).

	TIME	GROUP				P		
ITEM	**(Week)**	**C**	**T1**	**T3**	**SEM**	**GROUP**	**TIME**	**G*T**
Faecal Score	0	2.31	2.19	2.20	0.11	0.04	≤0.01	1.00
(Scale)	1	2.50	2.44	2.53				
	2	2.54	2.61	2.58				
	3	2.71	2.73	2.65				
	4	2.71	2.65	2.60				
	5	2.67	2.77	2.63				
	6	2.81	2.71	2.65				
	7	2.85	2.79	2.70				
	8	2.98	2.98	2.88				
	9	3.04	2.96	2.83				
	10	2.85	2.90	2.75				
	11	2.88	2.83	2.65				
	12	2.90	2.77	2.68				
	0-12	2.75[a]	2.72	2.64[b]	0.04	0.04		
	Days							
Faecal pH	0	7.28	7.84	7.63	0.34	077	0.02	0.57
	42	8.09	7.28	7.85				
	84	6.90	6.80	6.92				
	0-84	7.42	7.31	7.46	0.16	0.46		
Coliforms	0	8.20[B]	9.52[A]	8.04[B]	0.25	0.03	≤0.01	0.02
(Log_{10})	42	6.79	6.57	6.74				
	84	6.03[B]	6.27	6.91[A]				
	0-84	7.00[B]	7.45[A]	7.23	0.17	0.03		
E. coli	0	8.19[B]	9.45[A]	7.89[B]	0.27	0.17	≤0.01	≤0.01
(Log_{10})	42	6.94	6.21	6.59				
	84	5.95[b]	6.45	6.90[a]				
	0-84	7.03	7.37	7.13	0.12	0.07		
Lactobacilli	0	9.58	9.70	9.76	0.39	0.28	≤0.01	0.52
(Log_{10})	42	8.74	8.84	7.78				
	84	9.50	9.30	9.14				
	0-84	9.27	9.28	8.89	0.18	0.17		

Table 12: Faecal parameters and microbiological status in veal calves at 0, 42 and 84 days of the trial ([A,B]= P≤0.01; [a,b]=P≤0.05)

5.5.4 Health status during the trial

During the first phase of the trial, the calves received an antibiotic treatment (amoxicillin *plus* colistin) and a *Cryptosporidium parvum* prevention treatment; at the end of the adaptation period, all animals involved in the trial were found in good general health conditions. Diarrhoea was detected during the three weeks following beginning of the treatment (Tab. 13). No diarrhoeic phenomena were evidenced for the rest of the trial period in the three experimental groups. T1 and T3 animal had diarrhoea during the first week of the trial and with less days of presence than the C animals that, on the contrary, evidenced the problem in one subject until the third week of the study.

Group	Week 1		Week 2		Week 3	
	n. of calves	Diarrhoea*	n. of calves	Diarrhoea*	n. of calves	Diarrhoea*
C	3	3.67	1	2.00	1	3.00
T1	4	3.00	0	0.00	0	0.00
T3	4	3.25	0	0.00	0	0.00

* Days mean

Table 13: Number of calves and mean days in diarrhoea during the first three weeks of the trial

At the beginning of the second phase a total number of 1 animal in C, 3 animals in T1 and 4 animals in the T3 group were sent to the Veterinary Faculty Clinics. Moreover, 1 subject of T3 group was treated two times during the trial by the Veterinary Clinics.

Most of the therapeutic treatments were performed due to respiratory problems and hyperthermia with a concomitant general health depression.

During the trial, 3 animals from T3 group died. The performed necropsy evidenced an increase in gastrointestinal tract lynphonodes dimension, while no clinic signs were detected on esophagus mucosae.

In detail, all dead subjects showed moderate to severe ruminal constipation due to the presence of large amounts of dry ingesta and multifocal mucosal hemorrhages.

The first calf showed multifocal to coalescing necrotizing hemorrhagic foci and, at histological examination, micotic ife (*mucor micosis*) was found.

The second animal evidenced moderate fibrinous peritonitis, moderate catarrhal abomasitis and mild but diffuse catarrhal enteritis.

The third subject had moderate *fibrinous pleuritis* and severe, diffuse, gangrenous bronchial pneumonia with huge and diffuse pulmonary edema.

5.5.5 Slaughtering evaluations

Slaughtering weight resulted lower in treated gropus compared to C (C=121.00kg *vs* T1=112.17kg *vs* T3=108.74kg; P<0.01).
Carcass weight was lower (P<0.01) in T3 (58.87kg) than C (67.49kg) (Tab.14), while the administration of calcium diformate did not influenced organ Ca and formiate content, organ weight, and carcass dressing percentage.

5.5.6 Histological and histometrical analyses at the slaughter

At slaughtering no differences were detected from a clinical examination of lungs, liver, kidney and total gastrointestinal tract of the animals. In particular no ulcers were found in the three experimental groups and no differences were detected in villous height, cripth dept and their ratio (Tab.15).
The microscopic examination of the intestinal sections revealed altered structural aspects of the intestinal mucosa corresponding to catarrhal enteritis with no detectable differences between C calves and T calves. Epithelial detachment in the intestinal *villous* associated with mucous secretion excess was detected (Fig.1,3 and 5). Some diffuse mixed cellular infiltration (Fig. 4 and 6) in the *sub mucosa* and *lamina propria* was also detected. The most evident difference between C and T1/T3 calves was concerning the sub mucosal Brunner glands, which were fully normal in either aspect in C calves (Figs 1, 2) but showed empty and enlarged lumen in treated animals (Figures. 3,4,5 and 6). In addition, the histological signs of mucous secretions appeared scarce.

	GROUPS				P
ITEM	**C**	**T1**	**T3**	**SEM**	**CaFO**
Slaughtering weight (kg)	121.00^{A}	112.17^{B}	108.74^{B}	0.76	0.01
Carcass weight (kg)	67.49^{A}	63.25	58.87^{B}	2.55	0.02
Dressing percentage (%)	55.81	56.54	55.75	1.19	0.78
Organ weight (g)					
Liver	2,628.92	2,376.67	2,208.00	293.68	0.43
Kidney	543.83	496.50	545.75	42.73	0.46
Organ Formiate content (g/100gDM)					
Liver	≤0.01	≤0.01	≤0.01	---	---
Kidney	≤0.01	≤0.01	≤0.01		
Muscle	≤0.01	≤0.01	≤0.01		
Fat	≤0.01	≤0.01	≤0.01		
Organ Ca content (mg/100gDM)					
Liver	272.83	212.33	273.23	42.46	0.52
Kidney	225.11	328.36	244.95	48.83	0.25
Muscle	455.86	379.23	515.27	99.59	0.63
Fat	99.40	138.50	123.49	20.70	0.42

Table 14: Slaughter performance of veal calves and calcium and formiate organ content (A,B=P≤0.01;a,b=P P≤0.05)

	GROUP		
MEASURE*	**C**	**T1**	**T3**
Villi height (μm)	344.43±9.37	331.67±8.84	348.47±10.02
Crypts depth (μm)	355.44±7.70	352.76±7.26	373.28±8.24
V:C ratio	0.98±0.02	0.95±0.02	0.94±0.02

Table 15: Effect of the calcium diformate on villous height, crypt depth and their ratio in veal calves (*values are means±SEM)

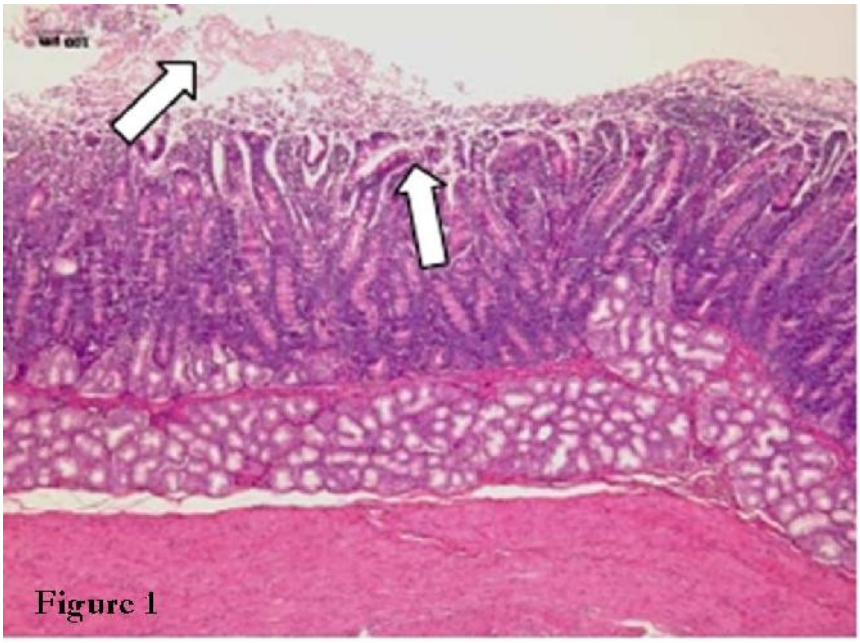


Figure 1

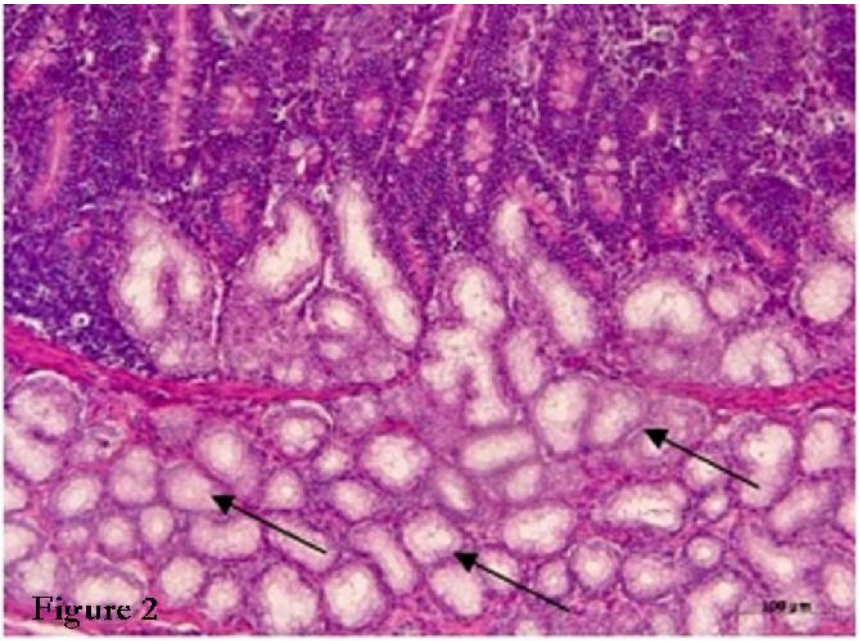


Figure 2

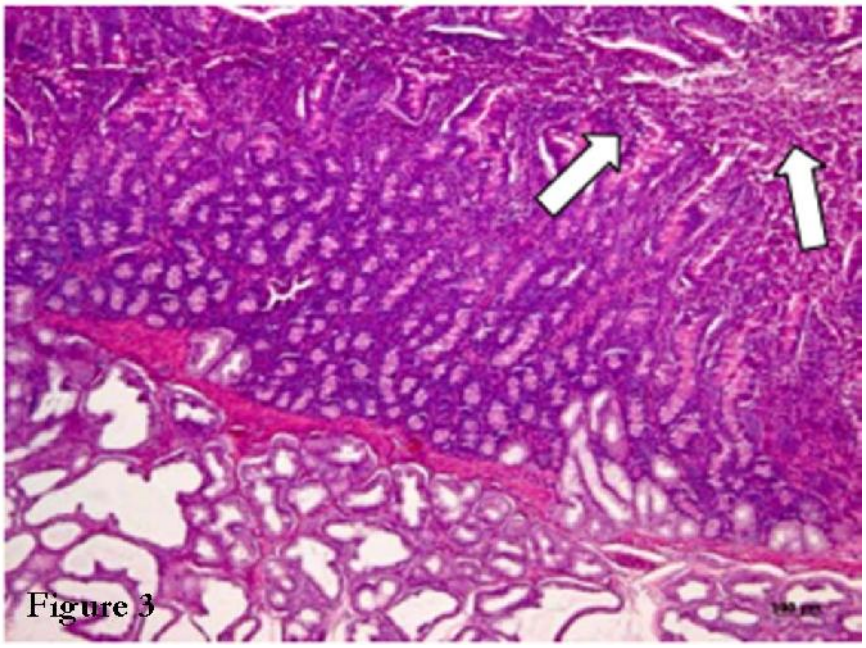


Figure 3

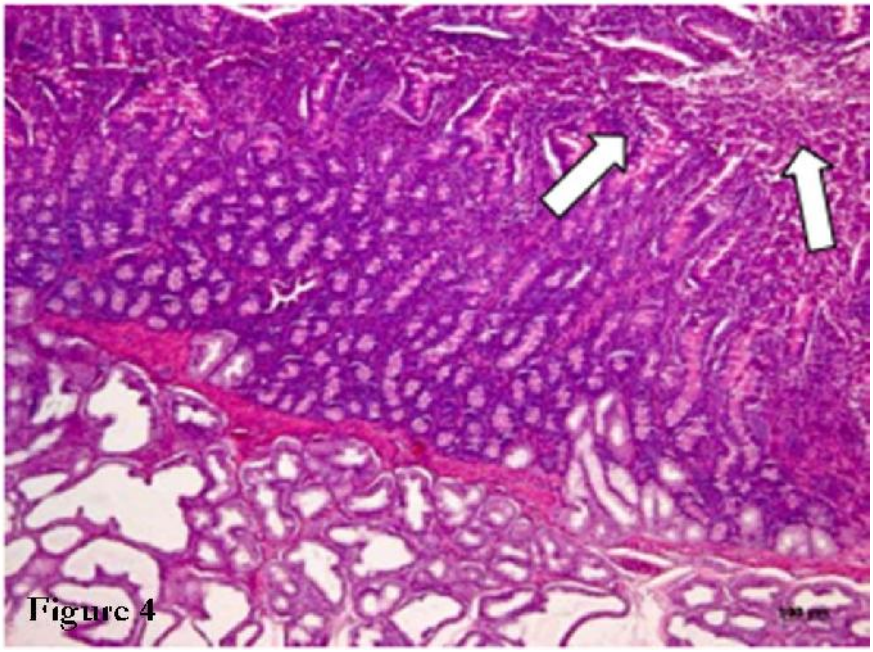
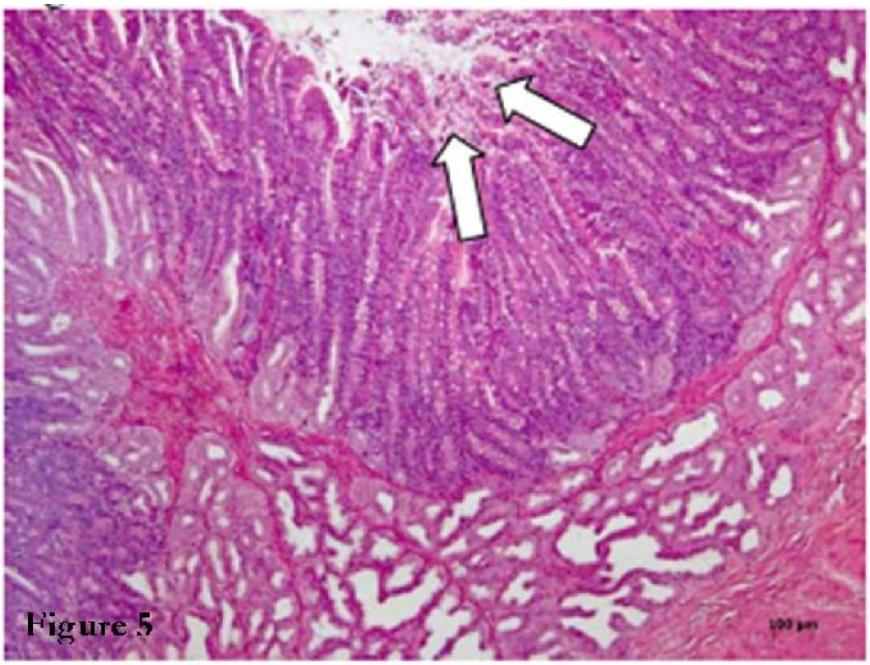
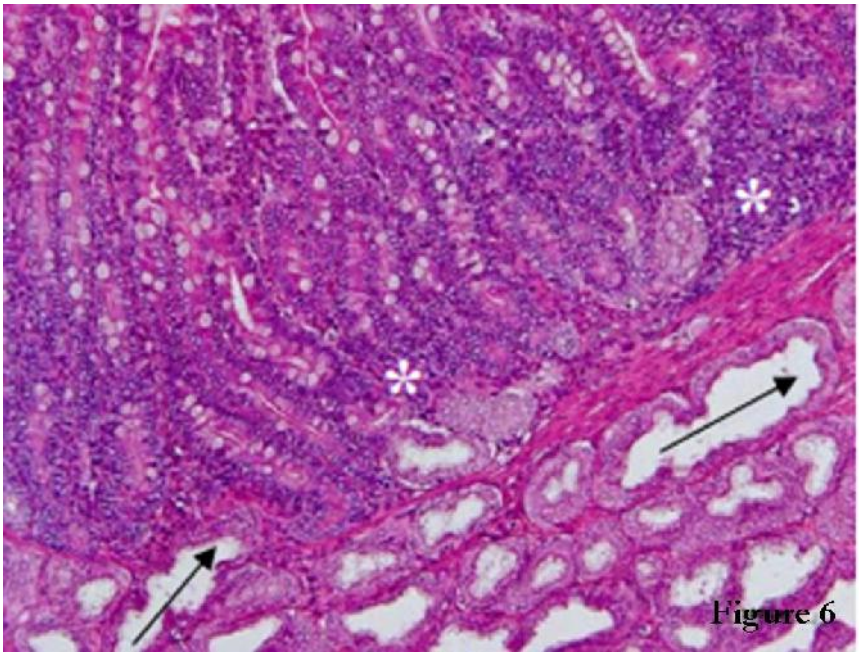


Figure 4





Figures 1-6: In the intestinal *villous* it can be observed an epithelial detachment associated with mucous secretion excess (Figure 1,3 and 5). In the *sub mucosa* and *lamina propria* it can be highlighted a diffuse mixed cellular infiltration (Figure 4 and 6 granulocytes, lymphocytes and plasma cells; white asterisks) The sub mucosal Brunner glands are fully normal in either aspect in C calves (Fig. 1, 2; in Fig 2: black thin arrows) but show empty and enlarged lumen in treated animals (Figures. 3,4,5 and 6; in 4 and 6: black thin arrows).

5.6 Discussion

The veal calves are very often subject to enteric and respiratory diseases due to multifactorial stress and the administration of substances alternative to antibiotics is critical for the consumer's safety.
In this context, the major outcomes using organic acids include bactericidal and bacteriostatic actions (Luckstadt et al., 2011).
The formic acid salts administered in form of calcium formate present not-recent and not extensive literature, especially concerning veal calves. Only a few studies were conducted to verify the effects of administration of this compound in piglets and broilers, as well as to determine the toxicity at different doses in dairy cows but, in some cases, the reported results in literature are conflicting.
In piglets, a reduction of dry matter intake was observed (Bindas et al., 1998), on the contrary, Bosi et al. (2005) found an increase of DMI that lead to similar BW but increased ADG between treated and no treated animal confirming results obtained Bindas et al. (1998) and Kirchgessner et al. (1997) who also found increased feed effiency.
The improvement of growth performance using acidifiers in the drinking water was also evidenced in poultry by Cornelison et al. (2005), but higher inclusion levels of organic acids could reduce weight gain and feed efficiency (Patten et al., 1997). More recently, however, as a result of correct microflora balancing, Philipsen et al (2006) observed an increase in broiler food efficiency, promoting the growth performance. Positive effects on ADG and FCR were also found in chicken with a combination of formic acid and propionic (Desai et al., 2007), raising gastric proteolysis and improving the proteins and amino acids digestibility (Samanta et al., 2010).
Differently from some authors, the results obtained in the present trial on growth performance of veal calves showed a reduced dry matter intake.
The general observed intake reduction effect of milk replacer could be attributed to a lower product palatability that consequently leads to negative findings over growth and slaughtering performance in the treated groups as reported by Partanen and Mroz (1999) who found higher level of organic acids responsible for a reduction of BW as a result of decreased feed intake
Moreover it appears that the response of growth paramenters to the acid supplementation is associated with several factors such as the acid type, the inclusion level, the acid buffering capacity of the basal diet, the stage of growth and the health status of treated animals (Che et al., 2012). Among these factor,, growth promoting effects of dietary organic acids appear to be dependent on the chemical composition of the diet. The greatest benefits have been observed when diets were formulated with cereals, while the growth promoting effect was

smaller in diets containing milk products (Easter, 1988; Partanen and Mroz, 1999). In addition to this, a high intake of calcium is known to enhance the formation of calcium fatty acid soaps in the digest, which also reduces fat digestibility (Xu et al, 1999). Moreover Ravindran and Kornegay (1993) suggested that a better performance could be expected in suboptimal rearing conditions.

Several haematological values in young calves differ from adult reference intervals. These age-related factors should be kept in mind when assessing hematologic values in calves (Hege et al., 2006). In the present trial most of haematological variations observed in calcium-diformiate supplemented animals respect to basal-diet fed calves are negligible considering the dietary treatment not influencing the haematological balance.

Evidenced RBC count variations during the present trial, expecially in the first 5 to 8 weeks of life than later, could be attributed to iron content or iron availability of diet as reported by Hege et al. (2006).

Serum pepsinogen has been used to detect abomasal necrosis (Zadnik and Mesaric, 1999) and is used with haptoglobin as a biochemical marker of abomasal inflammation in dairy cows supplemented with calcium formiate (McIntyre et al., 2002). The supplementation of organ acid could reduce stomach pH, stimulating the pepsinogen to pepsin conversion (Luckstadt et al., 2011): in the present trial serum pepsinogen content resulted higher in C and T3 calves than T1, so it canot be attributable to the treatment in accordance with McIntyre et al. (2002).

Moreover the decreased levels of Haptoglobin in all the considered groups during the trial should confirm the absence of an inflammatory status.

In the entire considered period, faecal score was decreased in treated animals than control contrarily to Jaster et al. (1990) and Ribeiro et al. (2009) who did not observe any variations of faecal score in calves, but results can be often variable in calves fed acidifiers (Zanetti et al., 1999; Heinrichs et al., 2003; Timmerman et al., 2005).

Higher faecal coliforms and, in particular, *E. coli* during the trial in T groups are not in agreement with findings by Luckstadt et al. (2011) and the supposed to bactericidal mechanism of action of salts of organic acids that should result in a Gram- reduction (i.e. Salmonelle and *E.coli*), as observed in piglets (Bindas et al.,1998) and broilers (Izat et al., 1990; Al-Tarazi and Alshawackeh, 2003). Pathogenic bacteria reduction by lower intestinal pH valueds, hould esitate in a better nutrient absortion due to the lower competion for nutrients, with less digestive problems such as diarrhoea (Ribeiro et al., 2009): in the present trial no alteration of intestinal pH was observed.

However this mechanism may not be effective, because organic acids with pH value of 5.0 or higher would probably not have bactericidal or bacteriostatic

effects and calves fed organic acids reduce the production of hydrochloric acid through the gastric juice to compensate the hight feed acidity (Jaster et al., 1990). Moreovoer, according to Jaster et al. (1990) and Merchen et al. (1996), animals react to the intestinal acidity producing greater amounts of bile and pancreatic juice to make the intestinal pH stable and to provide the appropriate environment for the action of digestive and absorptive enzymes.

The incidence of mortality and the causes of sudden deaths observed during the trial can not be ascribed to dietary treatments excluding a toxic effect by the absence of ulcers and changes in the duodenal mucosa at the time of death although reported data concerning calcium diformiate toxicity are conflicting. McIntyre et al. (2002) do not show signs of toxicity at the slaughterhouse in adult cows supplemented with Ca formate, even if abomasal inflammation was highlighted, while Scott et al. (2000) observed necrotic abomasal areas in cows.

No differences were shown regarding the duodenal morphology differently from Bosi et al. (2005), who observed a higher *villous* height, but in piglets, attributable to a good intestinal function and absence of inflammatory phenomena or diarrhoea that may affect the morphological appearance of the intestine.

5.7 Conclusions

The administration of both 1% and 3%/milk powder of calcium diformiate in veal calves decreased the growing performance of the treated animals, but not carcass dressing percentage, while no major differences were detected between the experimental groups in haematological, serum, and immune response investigated parameters.

5.8 References

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CHAPTER 6

General Discussion

6 General Discussion

In order to obtain the maximum economic yield in veal calf's and replacement heifer's breeding, it is essential to optimize the growth performance and the health status. Various factors such as a good health status, a proper management and correct applicated nutritional strategies are essential to avoid economic losses. The health status of the young calves is mined by enteric and respiratory diseases: specifically the neonatal diarrhoea represents the main cause of mortality and morbidity and it is often due to an enteric pathogen: the *Escherichia coli.* The enterohaemorrhagic *E.coli* (EHEC) is recognized as an infectious of food products (Lee et al., 2007), whose contamination can occur by the ingestion of uncooked food, polluted with the faeces of infected animals. The European Community ban of antibiotics like growth promoters (2006) induced to test more additives, for instance probiotics and acidifiers, able to promove the growth performance through the improvement of health status and intestinal and immunitary status.
In our experimental trials, the administration of a species-specific probiotic (*Bacillus coagulans*, *Lactobacillus animalis* and *Lactobacillus paracasei* spp. *paracasei*) both in veal calves and replacement heifers, and an acidifier (calcium diformate) in veal calves, did not allow to obtain univocal effects in relation to microbial status, health status and growth performance.
It has been possible observed how, in respect of a different management and a different breeding production target, the general health status is overall improved, iven if not significantly, in both the experimental trials wich have provided the probiotic as a supplement in the diet.
In fact, despite the veal calves often do not receive an adequate *colostrum* administration and the veal rearing involves a stressing situation, the probiotic allows to highlight an improvement of animals' health compared to the control animals, not only through a better GHS score, but also reducing the mortality and morbidity rate as reported by Timmerman et al. (2005). This trend is also confirmed by some authors who stated as the response to the treatment is clearly visible mainly in the animals with compromised health status (Spanhaak et al., 1998; Timmerman et al., 2004).

Probiotic administration is reported to henhance health status through the improvement of immune condition (Fleige et al., 2008; Frizzo et al., 2010; Buddington et al., 2011) and the reduction of the respiratory (Hatakka et al., 2001; Tsuruta et al., 2009) and enteric (Mokhber et al., 2007; Nagashima et al., 2010; Frizzo et al., 2010; Kawakami et al., 2010) pathologies: in our trials the lower severity of enteric diseases is demonstrated by an higher faecal consistency. Otherwise the administration of calcium diformate, in spite of the supplement's antimicrobial action could improved the general health status, does not allow to observe neither a reduction of mortality and morbidity rate nor an improvement of faecal consistency. The better faecal consistency in animals represents an index of a better gut microbial status: this balance is undermined in the calves subject to enteric pathologies in the few weeks of life (Gaggia et al., 2010). The *Lactobacillus* genus population is a natural component of intestinal microflora in several species (Schneider et al., 2004) and also in calves (Rada et al., 2004). The population of Lactobacilli is able to influence the process of homeostasis (Novik et al., 2006) which allows the control of certain gut epithelium function (Heyman and Menard., 2002), as well as the control of some pathogens' species that colonized the gut such as *Salmonella* (Gill et al., 2001) and *E.coli* (Shu and Gill, 2002). In good healthy condition, the Lactobacilli count is higher than the coliforms count, but the situation is reversed in case of intestinal pathologies and stress (Abu-Tarbush et al., 1996; Signorini et al., 2007). In the latter case it can be observed an unbalanced ratio between Lattobacilli and *E.coli*.

Our two trials confirm the positive effects reported in literature in calves supplemented with probiotic, emphasizing a statistical higher value referred to Lactobacilli count (Lalles et al., 2007; Corcionivoschi et al., 2010; Frizzo et al., 2011; Kawakami et al., 2010) at the fourth considered sampled in veal calves, and a better significant trend in the Lactobacilli/*E.coli* ratio in dairy calves. In veal calves this tendency is concurrently with the suspension of mass antibiotic treatments corresponding to the third considered month. The organic acid bactericidal action, in which the lactic acid bacteria are extremely less sensitive (Luckstadt et al., 2011) is not confirmed in our trial in veal calves, in contrast with a reduction of gram negative bacteria observed in broilers (Izat et al., 1990; Mroz et al., 2005; Mikkelsen et al., 2009) and in piglets (Dennis et al., 2004; Lyinch et al., 2007; Correge et al., 2010) and an increment in the population of Lactobacilli and Bifidobacteria in broilers (Luckstadt and Theobald, 2009).

Thus the observed enhancement of the intestinal microbial status in calves supplemented with probiotic can be related with the improvment of health status and faecal consistency, thas should lead to higher growth performance. In our trials, the administration of probiotic in veal calves showed no increase in body weight, unlike what is observed in dairy calves, the last one in agreement with Al-Saiady et al. (2010) and Hossaini et al. (2010). In the latter, the concentrate intake also significantly improved in the whole considered period, according with Timmerman et al. (2005) and LeJeune and Wetzel (2007), while weight gain and feed efficiency did not change in both reported trials. Anyway still at the moment literatures are conflicting: some authors reported no improvement in body weight (Frizzo et al., 2010; Morrison et al., 2010) or in relation to feed efficiency (Bakhshi et al., 2006; Riddel et al., 2010). These discrepances would be attributed to different factors, indivuals and managerials. It also can be assumed that the administration of antibiotic treatments of mass may not allow a total completion action of the probiotic. The absence of any enhancing in growth performance in calves supplemented with calcium diformate, contrary to what observed in calves by Woodford et al. (1987) but in agreement with Jaster et al. (1990) and Xu et al. (1999) is instead due to a significantly reduced feed intake wich results in a statistically lower body weigth in treated animals and a deterioration of the slaughter performance. From the presented trials, it can be concluded that the administration of a species-specific probiotic compound can determine several positive effects on growth performance and health status as a function of an improvement of the microbiology in the gut, although it should be noted that an high incidence of antibiotic treatments can affect the efficacy of the product, as found in our test in veal calves. This ipotesis is supported by the data reported *in vitro* that show an higher inhibition halos in plates treated with eight different classes of antimicrobial agents (Ripamonti et al., 2012 *in press*), while the administration of calcium diformate in calves should be further explored, pointing out the correct dose which can improve the intestinal microbial status.

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CHAPTER 7

Summary

7 Summary

The purpose of the present trials was the evaluation of the effects both a species-specific probiotic administration in veal (1st trial) and dairy calves (2nd trial) than an acidifier supplementation in veal calves (3rd trial) on health status, gut microbial balance and growth performance. On veal calves the effects of probiotic and acidifier on slaughter performance and histological survey were further evaluated.

The aim of first the study was to evaluate the effects of the administration of a species-specific probiotic supplement to veal calves on performance and microbial parameters in standard rearing conditions. Ninety six male Friesian veal calves (49.31 ± 1.38kg of body weight and 20±5 days of life) were divided *at random* in two homogeneous groups of 48 animals each from the arrival in the farm and fed either a basal diet (C) or a basal diet plus $1.8x10^9$ CFU/head/day of a probiotic supplement containing *Bacillus coagulans*, *Lactobacillus animalis* and *Lactobacillus paracasei* spp. *paracasei* in a 35:30:35 ratio (T) for a total of 180 days. Starting from 10th day from arrival and monthly until the end of the trial, individual body weight (BW) was recorded and average daily gain (ADG) was computed. At the same time, on the half of animals per group, faecal samples were collected for faecal score evaluation (FS), Lactobacilli count, *Escherichia coli* count and Lactobacilli/*E.coli* ratio, while blood samples were collected for haematological, haematochemical and immunological parameters evaluation. During the whole experimental period daily health status and therapeutic treatments were recorded for General Health Score (GHS) determination. At slaughter 10 animals per group were analysed for macroscopically injuries detection, and gut samples were collected for histological analyses. On each subject carcass weight, dressing percentage, fattening condition, carcass grade (SEUROP classification) and meat pH were recorded. During the trial were considered six times (corresponding to the each month of fattening): from time 0, referred to the 10th day from arrival, to the last considered month (time 6). BW resulted significantly higher ($P \leq 0.05$) in C calves than T at the 5th (C=226.76kg *vs* T=223.27kg) and 6th (C=267.14kg *vs* T=263.56kg) considered times while ADG was not different between groups. Faecal consistency was, significantly higher in T group at the 4th sampling ($P \leq 0.01$) as faecal Lactobacilli content (C=8.37 Log_{10} *vs* T=8.49 Log_{10}; $P \leq 0.05$). This trend could explain the better GHS observed in supplemented calves during the trial.

At different times, some haematological parameters such as basophils, aspartate amino transferase (AST), bilirubin, glucose, urea, bactericidal and complement were higher ($P<0.05$) in C group, while non-esterificated fatty acids (NEFA) and lactate dehydrogenase (LDH) showed lower values than T ($P<0.05$).

At slaughter no differences were observed for carcass weight, dressing percentage, carcass grade and meat pH. The *cecum* histological examination revealed a less evident de-epithelialisation and a greater integrity of the epithelium surface in T subjects than C, while in both the *ileum* than in *cecum* a numerical increase of the intestinal crypts' depth was detected. The administration of species-specific probiotic was able to improve the gut microbiological balance and health status with not no evident effects on growth performance.

The second study evaluated the effects of the administration of a species-specific multistrain probiotic (*Lactobacillus animalis-Lactobacillus paracasei* subsp. *paracasei-Bacillus coagulans*; 30:35:35) on health and performance parameters of newborn Friesian female calves during the first month of life.

Twenty-two calves were divided in two groups: control (C) fed with milk replacer and concentrate as a basal diet, and treatment (T), fed C diet plus 1g/head/d of probiotic from the second day after birth to the end of the first month of life. Faecal samples were collected weekly for the count of Lactobacilli and *Escherichia coli*; blood samples were collected and analyzed weekly. Individual FS was recorded daily, GHS was calculated at the end of the trial, and cell mediate immune response was evaluated by skin test at 7 and 28 days of life. Body weight, biometrical parameters and ADG were recorded weekly while Feed Intake (FI) and Feed Conversion Rate (FCR) were recorded for the overall period. Higher faecal Lactobacilli/*E. coli* ratio on day 28 of life (3.73 Log UFC/g *vs*. 2.02 Log UFC/g; $P<0.05$) and improved faecal consistency at 6, 25 and 27d of life were found in T group. Final body weight (48.92 Kg *vs* 46.92 Kg; $P<0.05$) and hearth girth (81.16 cm *vs* 78.49 cm; $P<0.05$) were significantly higher in T group. Concentrate FI (%DM) was significantly higher in T group overall the trial period, while ADG, FCR and milk replacer FI (%DM) were not influenced by probiotic administration. GHS showed increased mean value, but non significantly, in T group (T=20.45 *vs* C=18.91). Increased haemoglobin (10.04g/dl *vs* 8.60g/dl) and hematocrit (26.68% *vs* 22.17%) plasma content at 8d in T group with lower eosinophils percentage (0.05% *vs* 0.22%) were found at 8d in T group, while basophiles content was increased at 28d in species-specific probiotc-fed animals than C (0.21% *vs* 0.16%, respectively; $P<0.05$). The administration of a species-specific probiotic compound during the first month of life of newborn calves improved gut microflora, increased performance and some biometric parameters.

The last trial involving 36 male Friesian calves was conducted to determine the effect of calcium diformate administration and its inclusion level on growth performance, intestinal balance, metabolism, health status, and calcium and formate organ content.

The calves (44.55kg±5.51kg BW, 25±10 days old), were divided in three homogeneous groups of twelve subjects each: C fed with a basal diet, T1 fed with a basal diet plus 1%/milk replacer of calcium diformate, and T3 fed a basal diet plus 3%/milk replacer of calcium diformate for a total of 84 days of supplementation. During the experimental period daily FI, AD and FCR were determined. At 0, 42 and 84 days of trial individual blood samples were collected in order to detect haematological and biochemical parameters, Ca and FO content. At the same time faecal samples were collected for Lactobacilli, total coliforms and *E.coli* count along with faecal pH. Weekly BW and FS were recorded, while the health status was constantly monitored and the cause of deaths analyzed by necropsy. At the 91st day of trial the calves were slaughtered: any presence of ulcers were detected, individual carcass weight and dressing percentage were determined, and kidney and liver weight recorded. On seven animals per group liver, kidney, muscle and fat samples were collected and total Ca and formate were evaluated. The administration of calcium diformate decreased dry matter intake from either milk powder, concentrate and both milk powder and concentrate together with increasing level of calcium diformate in the diet (C=1419,05g/h/d; T1=1217,78g/h/d; T3=1190,73g/h/d; P≤0.01). As a result, final body weight in treated animals was lower than control calves (C=121,00kg; T1=112,17kg; T3=108,74kg; P≤0.01). Mean ADG in 3%/milk replacer CaFO calves was decreased if compared to C subjects (C=0.87kg/h/d *vs* T3=0.73kg/h/d; P<0.01), while a similar feed conversion rate was detected between the experimental groups. Faecal score was decreased in T3 animals (P≤0.05) than control while no differences were found for faecal pH, *E.coli* or Lactobacilli. Higher total coliforms count was evidenced in T1 animals than C (7.45 Log_{10} *vs* T1=2.75Log_{10} respectively; P≤0.05). At slaughter no difference was observed for dressing percentage, while carcass weight resulted higher in C compared to T3 (C=67.49kg *vs* T3=58.87; P≤0.05). Organ weight and organ formate and calcium content were similar between the experimental groups.
Red blood cells count (RBC), haemoglobin (HB) and hematocrit (HT) resulted higher (P≤0.05) in C and T3 than T1, while control calves showed higher platelets (P≤0.05) value than T1, and greater (P≤0.05) triglycerides than T3. Mean cell haemoglobin (MCH) value resulted higher (P≤0.05) in T3 subjects than the other groups. Increased formate blood content in 3% of calcium difromate/milk replacer animals did not show any differences for organ formate content thus giving no accumulation. Immune response was not affected by the treatment and necropsies on dead animals suggest no interaction among treatment and death itself. The histological examination revealed altered structural aspects of the intestinal mucosa in all the experimental corresponding with no detectable differences between control T1 and T3 groups.

Epithelial detachment in the intestinal villi associated with mucous secretion excess and some diffuse mixed cellular infiltration sub mucosa and *lamina propria* were highlighted. The sub mucosal Brunner glands were in normal physiological conditions in C, but showed empty and enlarged lumen in treated animals. While not showing toxic effects in veal calves, the administration of calcium diformate is not useful in promoting the growth performance and gut balance. In conclusion, the species-specific probiotic administration in veal and dairy calves can improve the gut microbial balance and consequently the general health status, although no effect on growth performance, while the calcium diformate supplementation, even if not showing toxic effects in veal calves, isn't helpful to increase the intestinal microbial balance and the growth performance.