Alma Mater Studiorum – Università di Bologna

Dottorato di ricerca in:

SCIENZE DELLA NUTRIZIONE E DEGLI ALIMENTI

FEED AND FOOD SCIENCE

XXII Ciclo

Settore scientifico disciplinare di afferenza: AGR 18

Natural compounds to control clostridial and salmonella infections in food animals

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ESAME FINALE ANNO 2010

A me stessa…
“chi fa da se, fa per tre”

L'UOMO DI SCIENZA

La scienza è rigore imprescindibile.
La razionalità sovraintende guardinga,
la conoscenza accresce nel tempo,
la saggezza è la meta lontana.

Non ci sono vie di fuga,
i confini sono marcati.

La scienza è inevitabilmente scienza.

L'uomo è fantasia inaudita.
La debolezza schierata lo attende,
la paura paralizza l'azione,
la felicità sprigiona energia.

Non esiste logica nella vita,
la passione elude ogni limite.

L'uomo è inevitabilmente uomo.

E poi c'è… l'uomo di scienza.

V.P.

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1. LITERATURE REVIEW

In Europe, the 1990s was the decade in which the "mad cow disease" (BSE) scandal hit the European food sector, the implementation of the Single European Market began, and thus a time where new issues about the integration and harmonisation of procedures in food production, processing and regulation emerged. The BSE case resulted in a collapse of public confidence in the organisations handling food safety. The crisis helped put food safety at the top of the political agenda in Europe. As a result food regulation underwent serious reorganisation in EU (Binnis, 2009; Holm and Halkier, 2009).

European food safety regulation before the 1990s has been described as fragmented, reflecting a food market made up of national markets in which national governments have used food safety regulation as a competitive tool for domestic markets (Berhauer and Carduff, 2006). National regulations mirror different national food cultures and the aspects of food they prioritise and focus on. In response to the BSE scandal the EU Commission prepared a White Paper on Food Safety which stated: "The European Union needs to re-establish public confidence in its food supply, its food science, its food law and its food controls" (EU Commission, 2000). The changes on the agenda were not only within the food producing sector, but also in the legal and regulatory framework, in the division of responsibility between social actors, in the public authorities in charge of food control, and in the conduct of the science lying behind the regulation of food safety (Giorgi and Lindner, 2009; Holm and Halkier, 2009).

Knowledge derived through scientific research has resulted in the development and use of new technologies that have had a profound impact on food animal agriculture. These advances have increased the efficiency of food production and providing significant quantities of high-quality food. The bottom line is how enhance food production as well as animal health and ensure public health and sustainable agriculture (Oliver *et al.*, 2009).

More than 200 known disease are transmitted through food by a variety of agents, including fungi, viruses, parasites, and bacteria. Foodborne illness is a major factor contributing to morbility and mortality in worldwide. Many zoonotic and nonzoonotic source of microbial pathogens could breach the food safety barrier. Food safety begins

with soil, plant, or animal, and continues within the plant or animal through various stages of production and processing (Oliver *et al.*, 2009).

There has been a surge of research activity into pathogens reduction strategies that were mainly inspired by the HACCP initiative. All food-processing plants were required to have an HACCP system in place that include the ability to trace foodborne pathogens back to the production unit. The food production unit needs a system to detect the origin of the contamination as well as effective measures to reduce microbial contamination (Oliver *et al.*, 2009).

Food can become contaminated by a variety of factors: zoonotic or nonzoonotic, direct or indirect. Farm-associated pathogenic bacteria are directly or indirectly associated as risk factors in the entire commercial food chain. Animal activity on the farm, manure management, and effluent discharge influence bacterial populations in farm soil as well as associated pathogenic flora. New technologies to reduce bacterial contamination based on prebiotics or probiotics for competitive exclusion seem promising. New tools for pathogen detection and pathogen modelling hold considerable promise for influencing research and measurable out comes in food safety (Wiedmann, 2003).

1.1. FOOD SAFETY LEGISLATION

The EU Commission's White Paper on Food Safety (2000) states that the essential role of the Internal Market "is to offer to consumers a wide range of safe and high quality products coming from all Member States" (EU Commission, 2000). As the food production chain is becoming increasingly complex, the health of consumers can only be adequately protected if every link in this chain is "as strong as the others". Restoring public confidence thus involved all parts of the food sector. That is, a farm to table policy was required. Five key principles guided the Commission's suggestions: clear definitions of the roles and responsibilities of stakeholders in the food chain; traceability of feed and food and their ingredients; transparency of food policy; risk analysis as the foundation on which food safety policy is based; and the application of the precautionary principle in risk management decisions. Last but not least, the need to involve individual consumers actively in food safety policy, and in the handling of food safety, was underlined (Holm and Halkier, 2009). An attempt was made, then, to encourage and direct societal actors to take part in the strategies for handling the problem of food safety. In this respect, the new EU food policy is an example of the alleged shift in political management, from the "dirigiste" approach towards a more regulatory approach (Majone, 1994).

The EU system has been described as the "Regulatory State par excellence" (Knill and Lenschow, 2004). It adopts a mode of governance which builds on regulatory capacities already existing within regulated sectors, such as the in-house control systems in modern food industry, thus blurring borders between public and private authority. It changes top-down authoritative control by decentralising regulatory processes, allowing access to and spreading responsibility across economic and social actors such as food manufacturers, food retailers and consumers. This new form of governance allows governmental centres of command and control to be replaced by semi-autonomous agencies such as the new risk assessment agencies in Europe, which often operate outside the hierarchical control of the central administrations (Majone, 1994). In this manner power is thought to be delegated from politicians to experts. This offers a solution to the problem of lack of expertise among policy-makers (Holm and Halkier, 2009).

The new EU initiatives in relation to food safety policy were deeply inspired by the changes in the United Kingdom.

1.1.1. The New European Food Regulation

The White Paper on Food Safety lays down the key principles for governing food law in the EU – these principles were translated into law through the General Food Law, Regulation EC 178/2002.

In January 2002 the new European Food Regulation was adopted (Regulation (EC) 178/2002). Thereby a framework was provided to ensure a coherent approach in the development of food legislation in order to ensure the free movement of food and feed in the EU. The regulation aims at ensuring a high level of protection of human life and health. It covers all stages of food/feed production and distribution and was to be implemented by all member states no later than 2007 (Marvin *et al.*, 2009). The primary responsibility for ensuring the safety of food rests with the food business. The regulation establishes in EU law that risk assessment, risk management and risk communication provide the basis for food law. Transparency of legislation and effective public consultation are seen as essential elements of building greater consumer confidence. In all countries it is now official policy that principles for food safety inspection have changed. The adoption of HACCP principles which are suggested in the regulation implies that post-factum product control is substituted by process control. Part of the EU regulation was the establishment of new organisations and new institutional division of responsibilities. (Holm and Halkier, 2009).

1.1.2. Institutional Reorganization

The new risk policy was therefore to cover the whole food production chain from plough to plate and to separate responsibility for risk analysis from that of risk management. An independent authority - the European Food Safety Authority (EFSA) - was set up in 2002 with main responsibility for risk assessment of all categories of potential food hazard (Holm and Halkier, 2009; Kleter *et al.*, 2009).

The EFSA was established in 2002 as an autonomous agency following the decision in the framework of the European food safety law to separate the two tasks of risk management and scientific risk assessment. EFSA took over the task of carrying out and coordinating risk assessment and communication at European level. The scientific work of EFSA is carried out by 10 panels. Each of the panels may initiate a study on their own or upon request by one of the risk managers at Member State level or the European Commission. The European Commission will almost always ask the EFSA to carry out

a scientific assessment when confronted with a request for an authorization of a product or company (Giorgi and Lindner, 2009).

Risk management was placed in the General Directorate for Health and Consumer Protection (DG Sanco) which became responsible for ensuring food safety through farm-to-table measures. A Standing committee on the Food Chain and Animal Health and a Rapid Alert System for Food and Feed (RASFF) were set up. Under the RASFF system, members, such as national food control authorities of the European Union (EU) member states, are obliged to notify any measures regarding to food safety, such as recalls of food and feed products and arrestment of imported consignments not complying with food legislations. The introduction of RASFF has further formalized as procedure of centralized reporting that existed before RASFF (Giorgi and Lindner, 2009; Kleter *et al.,* 2009).

All countries reduced the number of organisations responsible for food safety and to clarify the division of responsibilities. In most countries new organisations were built with an overall responsibility for food safety for the whole food chain. A clear separation between consumer and producer interests was a priority. The new EU risk policy was therefore based on the separation of three steps: assessment, management and communication. This was attempted by placing risk assessment at the new independent European Food Safety Authority and by placing risk management and communication at the General Directorate for Health and Consumer Protection (DG Sanco) in collaboration with food control systems of member states. EFSA was set up independently from government in an attempt to restore confidence in scientific expertise, to re-establish neutrality of 'science' and thus to create an independence lacking to the "government scientist" (Wales *et al.,* 2006). Integral to the new EU risk policy was the intention to separate responsibility for legislation from that of inspection. In the EU, legislation is the responsibility of the Council of Ministers, whereas responsibility for enforcement lies with DG Sanco (Holm and Halkier, 2009).

The European Commission publishes weekly overviews of RASFF alert and information notification on its website. In addition, it publishes annual reports of the notifications. These annual reports provide an overview of the numbers of notifications and categories of food products and hazards that they pertained to. Some members of the RASFF network, including the Italian, German, and Swedish authorities, also publish updates and reviews of the data reported through RASFF, sometimes with a particular focus on their national situation.

Numerous projects were sponsored by the European Commission, such as SAFE FOOD. This is the acronym of an integrated project sponsored mainly by the European Commission as part of it's the Sixth Framework Program for Research and Technology Development. This project aims to develop and ameliorate methods for risk assessment and risk analysis of food safety. SAFE FOOD consist of various work packages addressing specific topics within the field, including the use of advanced analytical methods for detecting changes in crops caused by agricultural practices; the use of advanced statistical methods to estimate cumulative human exposure to multiple food contaminants and natural toxicants; consumer perception of food safety management; institutional arrangements for implementing food safety policies; and the development of a new risk analysis model for food safety (Kleter *et al.*, 2009).

1.1.3. WTO framework: SPS and TBT agreements

The EU scope is to provide the highest level of protection of human health and to facilitate both national and international food trade. At international level, a risk analysis approach is fundamental for the implementation of the World Trade Organisation (WTO) Sanitary and Phytosanitary Agreement (SPS Agreement) (WTO, 1995). At European level, Regulation (EC) No.178/2002 (OJEU, 2002) sets the general principles and requirements of food safety law including the need of a risk analysis approach, and it also establishes the European Food Safety Authority (EFSA) (Binnis, 2009; Hugas and Tsigarida, 2008).

The international food policy regime as it relates to trade is defined by two WTO-agreements: the Agreement on the application of sanitary and phytosanitary measures (hereinafter referred to as the SPS agreement) and the agreement on technical barriers to trade (hereinafter referred to as the TBT agreement) (Giorgi and Lindner, 2009).

When adopting sanitary and phytosanitary (SPS) measures, each country is entitled to establish an own appropriate level of protection, under the condition that this is 'applied only to the extent necessary to protect human, animal or plant life or health', is based on scientific principles (SPS, Article 2.2), and does not discriminate between members (SPS, Article 2.3), that is, it is not used for protecting domestic markets from international importers. The obligation to base SPS measures on scientific principles obliges members to either base their measures on international standards (SPS, Article 3), or on scientific risk assessment (Articles 5.1, 5.2, and 5.3).

If a member chooses to base its SPS measure on international standards, guidelines and recommendations, the SPS agreement recommends three standard-setting reference organizations, also called the 'three sister organisations': in the case of human health this is the Codex Alimentarius Commission (CAC); in the case of animal health it is the World Organization for Animal Health (OIE); and in the case of plant health it is the International Plant Protection Convention (IPPC). Adopting the standards of the WTO reference organizations is not obligatory (Giorgi and Lindner, 2009).

TBT measures cover technical regulations, standard, and conformity assessment producers. The TBT agreement covers all technical regulations, standards and conformity assessment procedures except when these are sanitary or phytosanitary measures.

Whereas the TBT agreement attends to labels established for reasons other than those intended to protect human, animal or plant health, the SPS Agreement attends to labels intended to protect human, animal or plant health (Giorgi and Lindner, 2009).

1.1.4. Standardization agencies

Standards institutes exist in most countries and are in charge of elaborating technical standards and guidelines in various sectors. Today most national standards institutes operate in a coordinated way within the international framework established by ISO and CEN. The European Committee for Standardization (CEN) develops technical standards (EN standards) for analytical methods used for establishing levels of contamination, or methods applied in sampling and analysis.

At the international level, the International Standardization Organization (ISO) brings together standards institutes from 157 countries from either government or industry. It is set up as a non-governmental organization and claims to occupy "a special position between the public and private sectors"(Giorgi and Lindner, 2009).

1.1.5. The new role of consumer

The outcome of the BSE crisis was thus increased priority to consumer interests and protection of consumer health. Thus 'the consumer' became a central food policy actor (Marvin *et al.*, 2009). Traceability and labelling was seen as a tool for consumer control over all steps of the food chain, that is, as a means of direct control by consumers on market actors.

Consumers were not to be protected from market excess but seen as an omnipresent and unpredictable figure that needed to be taken into account. Further, focus was now on the consumer as an individual market actor and not as a member of a political organisation (Halkier and Holm, 2008). Even though the interests and concerns of consumers are framed in different manners by various actors in the countries (Halkier *et al.*, 2007) the shift towards a clear priority of consumer protection and consumer interests can be found in several countries. Securing the credibility of the food production chain is the first proposed objective of the new agency for Food Quality and Safety proposed in 2000. In some countries this new prioritising of consumer interests also show in new policies regarding the workings of the administrative system, incorporating consumer organisations as 'concerned parties' in legislative processes and public administration. In the new regulation consumers were no longer seen as weak individuals in need of protection, but were called upon as competent and independent actors able to and responsible for making relevant choices in the food market. In order for consumers to be able to live up to this, full information about food products was necessary. This was the aim of legislation about labelling and traceability. In most countries the development towards the Single European Market and the need for national economies to adapt to it is an important driver behind regulatory and institutional change. Thus references to the new demands of traceability are frequently made in all countries, when discussions are raised about the need to adjust national food control policies to the EU system and adopt the principles of HACCP. It is thus clear that the regulatory changes following the new EU food policies in some countries are carried through on the initiative of private market actors, while in others, the state and the public authorities have acted as vanguard of the necessary reforms.

1.1.6. Microbiological hazards

Following the recommendations of the EFSA Committee on Veterinary Measures (SCVPH), and using the Codex Principles for the establishment and application of microbiological criteria (1997), a new legislation was enacted in 2006. This is Regulation 2073/2005/EC on microbiological criteria for foodstuffs. Regulation 2073/2005/EC distinguishes between "food safety criteria" and "process hygiene criteria". The food safety criterion is mandatory and defines "the acceptability of a product or a batch of foodstuffs applicable to products ready to be placed on the market". The process hygiene criterion applies to the production process and is not

mandatory. The expectation, of course, is that given the mandatory nature of food safety criteria and assuming an adequate number of controls, process hygiene criteria will be established by default. The Annex to Regulation 2073/2005/EC specifies for several food categories a set of relevant criteria such as the microbiological limits, the analytical reference method, the sampling design and the frequency of sampling. In the majority of cases, the analytical reference method relies on ISO or EN standards (Giorgi and Lindner, 2009).

Various types of microbiological hazards have been reported through the RASFF system, including bacteria, moulds, and viruses. Bacteria constitute the largest group of microbiological hazards, with more than a hundred specific microbe species, subspecies, and strains. The most important product categories are seafood, meat, poultry, spice and condiment, animal feed, and dairy products. As regards bacteria, Salmonella and its subspecies are the most numerous. *Salmonella typhimurium*, including the phage types DT104, DT108, and U302, mainly occur in meat and poultry; while *Salmonella enteritidis* mainly is reported as microbiological contaminations of egg products (Kleter *et al.*, 2009).

1.2. FOODBORNE PATHOGENS OF INTEREST

Many foodborne pathogens can have habitats in food-producing animals and in the farm environment. These pathogens can enter meat and milk products during slaughter or at milking, or can contaminate raw vegetables when soil is fertilized with improperly composted animal manure (McEwen and Fedorka-Cray, 2002). There is evidence to support the concept that significant increase in the incidence of foodborne illness is related to changes in animal husbandry practices and to the handling and processing of food of animal origin (Committee on the Review of the Use of Scientific Criteria and Performance Standard for Safe Food, National Research Council, 2003)

Specific groups of disease-causing microorganism are consistently associated with the food-producing animal environmental.

The results on the occurrence of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks that were reported from the Member States and analysed by EFSA show that in 2005 the two most commonly reported zoonotic diseases in the European Union were campylobacteriosis and salmonellosis (Hugas and Tsigarida, 2008).

From the standpoint of preharvest food safety in general and human health in particular, *Salmonella* spp., *E. coli*, *Campylobacter jejuni*, and *Listeria monocytogenes* are important foodborne pathogens affecting public health. These pathogens are the leading causes of foodborne morbidity and mortality, and they are carried by cattle, poultry, and swine and are found in their associated farm environments. Epidemiological data suggest that other pathogens, including *Staphylococcus aureus*, *Clostridium perfringens*, and *Bacillus cereus* are important pathogens that have origins on farms (Oliver *et al.*, 2009).

All these bacteria are the major pathogens of animal origin transmitted through food, and they are found in animal feces; therefore, contamination of carcasses and food products by animal feces is likely to be a principal mode by which foodborne pathogens reach the consumer. Cattle, sheep, swine, chicken, and turkeys are principal reservoirs, but wild birds and various mammals that are common in farm environments can be also a source of these pathogens (D'Aoust *et al.*, 2008; Meng *et al.*, 2008; Nachamkin, 2008; Swaminathan *et al.*, 2008). The contamination cycle in food-producing animals is through ingestion of feed and water that can be contaminated by feces. The use of nontreated manure as fertilizer, the spread of slurry, and the use of recycled wastewater disseminate these pathogens even more. Stresses on animals caused by poor

management, types and quantities of feeds increase susceptibility to infection and shedding of foodborne pathogens (Cray *et al.*, 1998).

1.2.1. Foodborne pathogens: common characteristics and control strategies on farm

Several epidemiological characteristics are common to foodborne pathogens of interest:

- Foodborne pathogens are shed in feces and gastrointestinal secretions or excretions of healthy animals. Shedding is sporadic and is caused by reinfection from sources in the environment.
- Cattle, swine, and poultry are believed to be the primary reservoirs, but birds and various mammals that are common in farm environments were also identified as reservoirs.
- The contamination cycle is as follows: infection occurs initially by ingestion of contaminated feeds and water, followed by shedding of food pathogens in feces that, in turn, contaminate feeds and animal drinking water, causing new infections and reinfection of convalescent animals.
- Stress caused by poor management and by the types and quantities of animal feedstuffs increases their susceptibility to infection and the shedding of foodborne pathogens.
- Feeds and water contaminated with feces and secretions or excretions from animals are the vehicles for additional contamination in the environment, including other mammals, birds, and insects. The use of nontreated manure as fertilizer and the spread of slurry and recycled wastewater further disseminate contamination.

The model in which the presence of pathogens depends on ingestion of contaminated feed, followed by amplification in animal hosts and fecal dissemination in the farm environment. Colonization of the gastrointestinal tract and amplification of *E. coli* O157:H7, *Salmonella*, *C. jejuni*, and *L. monocytogenes* appear to be required stages in the cell cycles. Shedding of foodborne pathogens in feces and distribution in the environment where food-producing animals live lead to animal reinfection and persistence of the pathogen on the farm (Olivier *et al.*, 2009).

Production units are major reservoirs for foodborne pathogens, that can reach the human population by direct contact, ingestion of raw contaminated food, or contamination during the processing of milk.

By breaking the infection-reinfection cycle, it is possible to reduce foodborne pathogen shedding and therefore the spread of foodborne pathogens among food-producing animals and in the farm environment.

From a management point of view, it is practicable to focus on selected groups of pathogens. However, many of the pathogens are asymptomatic for the animal harbouring or shedding them. Previous experience in pathogen reduction strategies, pathogen eradication strategies, or both amply testifies that postharvest packing or processing in itself is not adequate to reduce the risk of food safety consistently. Many experts now believe that pathogen reduction and HACCP strategies have resulted in noticeable changes in food safety risk reduction. It is imperative that even if human pathogens cannot be completely eliminated preharvest, their intended reduction is a logical end point that could reduce morbidity and mortality (Olivier *et al.*, 2009).

1.3. CLOSTRIDIUM SPP. IN FOODSTUFFS

Anaerobic spore-forming bacteria spoil a wide range of foods including dairy products, meat and poultry products, fresh and canned fruits and vegetables, typically producing gas and/or putrid odours. A few of those species can cause illness.

The species of the genus *Clostridium* most commonly involved in food-borne illness are *Clostridium perfringens* and *C. botulinum*. Intoxication due to *C. perfringens* is usually brief, self-limiting, and is rarely fatal. However, the neurotoxins of *C. botulinum* are among the most toxic naturally-occurring substances and cause severe food-borne illness, sometimes fatal, with symptoms continuing for several months.

Clostridia occur commonly in soil, dust, the aquatic environment and in the intestines of animals. Consequently, *C. perfringens* and *C. botulinum* can be present in a wide range of foods. Good Agricultural Practices and Good Hygienic Practices contribute to reducing numbers of clostridia by minimising contamination with soil and animal faeces (Songer, 1996; García and Heredia, 2009).

C. perfringens is commonly present in foods and ingredients, occasionally at hundreds per gram. *C. botulinum* is present less frequently, normally at a few spores per kg. Spores of both *C. perfringens* and *C. botulinum* can be eliminated from foods by heating.

1.3.1. Clostridium perfringens as microbiological hazard

Clostridium perfringens may be the most widely occurring pathogenic bacterium and is certain the most important cause of clostridial enteric disease in animal and potentially lethal foodborne diseases in humans, including food poisoning and necrotic enteritis in the 1940's, since when outbreaks have been reported (Songer, 1996; EFSA, 2005; García and Heredia, 2009). Some types of C. perfringens (mainly type A) are consistently recovered both from the intestinal tracts of animals and from the environment, while others (types B, C, D, and E) are less common in the intestinal tracts of animals and can occasionally be found in the environment in areas where disease produced by these organism is enzootic (Songer, 1996)

C. perfringens is a gram-positive, spore forming, anaerobic bacterium and natural inhabitant of soil and the intestinal tracts of several animals, including humans. The ubiquitous nature of this bacterium and its spores makes it a frequent problem for the

food industry and establishments where large amounts of food are prepared because it grows well on meat and poultry products (García and Heredia, 2009; Golden *et al.*, 2009; EFSA, 2005).

This bacterium could be controlled properly following safety rules such as adequate heating and cooling of food during processing.

The genus *Clostridium* consists of a diverse group of bacteria that are unable to grow in the presence of oxygen and have the ability to form heat-resistant endospores. This bacterium is the most prolific toxin-producing species within the clostridial group (Songer, 1996; García and Heredia, 2009).

Food poisoning from *C. perfringens* gives rise to abdominal pain, nausea and acute diarrhoea 8-24 h after the ingestion of large numbers of the organism, a proportion of which survive the acid conditions of the stomach. The illness is usually brief and full recovery within 24-48 h is normal. However, death occasionally occurs in the elderly or otherwise debilitated patients (Songer, 1996; EFSA, 2005; García and Heredia, 2009).

C. perfringens food poisoning is not a reportable disease; however, in the United States, the Center for Disease Control and Prevention (CDC) estimates that 250,000 cases of *C. perfringens* type A food poisoning occur annually (García and Heredia, 2009; Golden *et al.*, 2009).

The symptoms of the disease are caused by an enterotoxin. *C. perfringens* is grouped into 5 types A-E according to the exotoxins (soluble antigens) produced. Types A, C and D are pathogens for humans, types B, C, D and E, and possibly A also, affect animals. The enterotoxin produced by types A and C is distinct from the exotoxins and is responsible for the acute diarrhoea that is the predominant symptom of *C. perfringens* food poisoning (Songer, 1996; EFSA, 2005; García and Heredia, 2009).

The beta-toxin of type C appears to be the necrotic factor in the disease enteritis necroticans jejunitis ("pig-bel"). Type A strains are responsible for gas gangrene (myonecrosis), necrotizing colitis, peripheral pyrexia, septicaemia as well as food poisoning. The enterotoxin involved in food-poisoning have been reviewed (Songer, 1996; EFSA, 2005; García and Heredia, 2009).

The disease-causing enterotoxin of *C. perfringens* (CPE) is produced during sporulation. To cause disease vegetative cells of *C. perfringenes* have to be ingested with the food. The vegetative cells that survive the acidity of the stomach sporulate in the intestinal lumen. During lysis of the mother-cells to release the spores, CPE is also released. Subsequently, the CPE is converted to a more active toxin by trypsin and

chymotrypsin, after which it binds to receptors present on the brush-border membrane of the intestinal epithelial cells (Songer, 1996; EFSA, 2005). The bound CPE inserts into the cell membrane and pores are produced that makes the cells permeable for ions and small molecules. As a consequence a reversal of ileal transport of water is induced (Songer, 1996; EFSA, 2005).

It is important to recognise that not all *C. perfringens* are able to produce the enterotoxin that causes foodborne disease. Foodborne illness is caused when food becomes contaminated with large numbers of vegetative bacterial cells ($>10^6$ CFU/g) of *C. perfringens* type A isolated that carry the *cpe* gene (EFSA, 2005; García and Heredia, 2009; Golden *et al.*, 2009).

C. perfringens is ubiquitous and widely distributed in soil, dust, vegetation and raw foods. It is part of the normal flora of the intestinal tract of man and animals. Although the clostridia are anaerobes, *C. perfringens* is one of the less fastidious species and is able to grow under conditions that are not strictly anaerobic (Songer, 1996). The spores exhibit a range of resistance to heating, and spores of some strains readily survive cooking.

C. perfringens type A food poisoning usually results from either improper cooling or temperature maintenance of food, preparation of food a day or more in advance, or inadequate reheating of food. Although *C. perfringens* spores are the main source of concern in food products, vegetative cells may occasionally cause health problems in nonheat-treated foods or by recontamination of heat-treated foods (García and Heredia, 2009; Golden *et al.*, 2009). To limit the growth of *C. perfringens* on meat and poultry products, the Food Safety Inspection Service (FSIS) published a final rule that establishment a performance standard for *C. perfringens* during production of RTE/PC food (Golden *et al.*, 2009).

C. perfringens can be detected in a wide range of foods as a result of contamination by soil or with fecal matter e.g. meat, poultry, fish, vegetables, dairy products, dehydrated foods such as soups and gravies, spices, milk, gelatin, pasta, flour, soy protein and animal feeds.

Animal carcasses and cuts of meat can become contaminated with *C. perfringens* from contact with soil or animal feces, or during slaughtering and processing. Many organisms that compete with *C. perfringens* are killed when meat and poultry are cooked, but *C. perfringens* spores are difficult to eliminate (García and Heredia, 2009; Golden *et al.*, 2009).

Illness occurs after ingestion of large numbers of enterotoxin-producing vegetative cells of *C. perfringens*, some of which survive the acid conditions of the stomach and subsequently form spores in the large intestine, at the same time producing enterotoxin. From reported and investigated outbreaks, approximately 10^8 vegetative cells per serving are necessary to cause diarrhoea. This occurs a few hours after consuming food, usually cooked meat or gravy, in which multiplication *of C. perfringens* has occurred and vegetative cells are present. The illness commonly lasts only ca 12-24 h, and the symptoms are usually not serious enough to consult a physician – hence the recorded number of outbreaks may be an underestimate.

C. perfringens has the capacity to grow in protein-rich foods held at temperatures above 12°C. Not all strains of *C. perfringens* have the capacity to produce enterotoxin. Cooked meat and poultry are the foods most commonly involved in *C. perfringens* food poisoning outbreaks. Fish and fish products are rarely implicated.

C. perfringens requires more than a dozen amino acids and several vitamins for its growth, both of which are typically present in meat. The leading food vehicles for this bacterium in the United States are meats, notably beef and poultry, and meat-containing products, such as gravies, stews, and Mexican food. Ready-to-eat and partially cooked (RTE/PC) meat and poultry products were the focus of the risk assessment, too (García and Heredia, 2009; Golden *et al.*, 2009).

C. perfringens type A food poisoning usually results from either improper cooling or temperature maintenance of food, preparation of food a day or more in advance, or inadequate reheating of food (García and Heredia, 2009; Golden *et al.*, 2009). Almost all outbreaks are the result of cooling slowly, or holding without refrigeration, allowing multiplication of *C. perfringens*, numbers reaching 10^6-10^7 cells/g, implying an infective dose of the order of 10^8 vegetative cells of enterotoxin-producing *C. perfringens* (EFSA, 2005).

Occasionally illness has been caused by pea soup prepared on a catering (food service) scale. Even after slow cooling and multiplication of *C. perfringens* that survives cooking; thorough re-heating will inactivate the vegetative cells and prevent illness. In the Netherlands, pea soup is traditionally composed of cut vegetables (peas, celery, leek, and carrots) and pork meat. It has a neutral pH. It is usually produced in winter and often in large quantities e.g. at sporting events (EFSA, 2005).

Although *C. perfringens* spores are the main source of concern in food products, vegetative cells may occasionally cause health problems in nonheat-treated foods or by

recontamination of heat-treated foods (García and Heredia, 2009; Golden *et al.*, 2009). To limit the growth of *C. perfringens* on meat and poultry products, the Food Safety Inspection Service (FSIS) published a final rule that establishment a performance standard for *C. perfringens* during production of RTE/PC food (Golden *et al.*, 2009)

Risk factors

C. perfringens posses several attributes that have contributed significantly to its ability to cause foodborne illness. First of all, It has an ubiquitous distribution in the natural environment and it is present in most raw food product materials, including vegetables and meat products. Therefore, its presence in many food products must be accepted., giving it ample opportunity to contaminate food. Overall, it has the ability to form heat-resistant spores. In second time, *C. perfringens* has the ability to grow quickly in foods, allowing the bacteria to reach the high levels that are necessary for food poisoning. Finally, *C. perfringens* is capable of producing an intestinally active enterotoxin (CPE) that is responsible for the characteristic gastrointestinal symptoms of *C. perfringens* food poisoning (Songer, 1996; García and Heredia, 2009).

The extent of multiplication of *C. perfringens* in food is determined mainly by the storage temperature. In addition, *C. perfringens* requires a protein-rich substrate for growth, explaining why foods associated with *C. perfringens* outbreaks are limited to meat and poultry products, gravy, casseroles and pea soup, usually after temperature-abuse after cooking. Low numbers of *C. perfringens* spores often survive cooking and multiply to food- poisoning levels during slow cooling and unrefrigerated storage of prepared foods. Larger quantities of food, large joints of meat, and large containers of pea soup are difficult to cool quickly. Hence cooling rate and temperature of storage are risk factors.

Herbs, spices and seasonings may contain *C. perfringens* in numbers varying from <100 - 500/g (Songer, 1996; EFSA, 2005; García and Heredia, 2009). Use of herbs and spices is considered by some to be a risk factor. However, there is no evidence that adding even substantial amounts of herbs and spices to cooked foods is a real risk factor for C. perfringens-induced disease. Herbs and spices containing *C. perfringens* will only cause disease if the cooked food in question is not cooled adequately or left-overs are not reheated sufficiently (70-72°C throughout) and is therefore only a temperature-related risk factor (EFSA, 2005).

Initial contamination of food

The initial contamination of foods with *C. perfringens* is difficult to control. While Good Agricultural Practices (GAP) can help to reduce numbers of infectious pathogens such as salmonellae, the only means of reducing the initial load of bacterial spores is to minimise contamination of raw foods by soil and by animal faeces. Good Hygienic Practices (GHP) in the manufacturing environment minimise chances of recontamination. There is some evidence that *C. perfringens* can reside in pipelines and contaminate product, resulting in spoilage of the product (EFSA, 2005).

Because *C. perfringens* occurs so commonly in the environment, many foods will be contaminated. Experience has demonstrated that if foods are handled properly during all stages of distribution, retailing and consumer use, the likelihood of multiplication of clostridia is very low. Heating food to 68-70°C kills vegetative cells but not spores (Songer, 1996; EFSA, 2005; García and Heredia, 2009; Golden *et al.*, 2009).

Growth limitation of *C. perfringens* in the food chain

There are numerous publications illustrating the importance of rate of chilling and holding temperatures on growth of *C. perfringens* (Songer, 1996; EFSA, 2005; García and Heredia, 2009; Golden *et al.*, 2009). The optimum temperature for growth of *C. perfringens* is 43-47°C. Growth does not occur below 10-12°C. Most meat and poultry products receive a cook much lower than the sterilizing process for low-acid canned foods, and spores of *C. perfringens* survive. If the product then cools slowly, surviving spores germinate and multiply rapidly. After heating, meat and poultry products should be cooled from 55°C to below 15°C as quickly as is reasonably possible. US regulations serve as a guide, requiring the product's internal temperature not to remain between 54.4°C and 26.7°C for more than 1.5 h or between 26.7°C and 4.4°C for more than 5 h (FSIS, 1999).

However, study of *C. perfringens* in commercially cooked products during chilling and refrigerated storage concluded that the above chilling regimes are more severe than necessary to maintain safe product (EFSA, 2005; Golden *et al.*, 2009).

Inactivation of clostridia in the food chain

Heating is the most reliable method of inactivating bacterial spores. The rate of inactivation of spores is dependent on the temperature of heating, spores dying more rapidly as temperature rises. The heat resistance of spores is also affected by properties

of food (pH, water activity, fat content). Spores are more heat resistant at low a_w and neutral pH values. Spores are more heat sensitive at low pH values (below ca pH 4.5); hence canned fruits are made safe and shelf-stable without refrigeration by much lower heat processes than those applied to low acid canned foods. (EFSA, 2005).

Until 20-25 years ago inactivation of microbes in food processing used heating. Today many novel processes are being explored e.g. high hydrostatic pressure, pulsed electrical fields, irradiation used alone, or in combination with heat (EFSA, 2005).

Several chemical and physical stresses can inactivate vegetative cells of *Clostridium* spp. and a proportion of their spores. Conditions in the food (pH, a_w, fat content) may influence their effectiveness.

For many centuries the curing of meat and fish has relied upon curing salts to control the growth of *C. perfringens*. Concerns that sodium nitrite in meat products might result in the formation of carcinogenic N-nitrosamines and other nitrosated products led to extensive research, mainly in North America and Europe, to try to find alternatives to sodium nitrite and sodium nitrate. Some anti-clostridial activity was identified in potassium sorbate and certain polyphosphates, parabens, antioxidants, nisin, and sodium lactate when used in combination with other conditions (EFSA, 2005).

Cured meat producers have minimised the risk of formation of nitrosamines by reducing the amount of nitrite used, and eliminating nitrate from most cured products.

If contamination is on farm?

Epidemiological data suggest that *C. perfringens* is an important pathogen that has origins on farms. So, contamination of carcasses is another important point to control (Oliver *et al.*, 2009). Reduce presence of *C. perfringens* on farm means to prevent and reduce contamination of carcasses.

There are many reports about different ways of controlling the number of *C. perfringens*. In most cases antibiotics or ionophores anticoccidials were used. Due to the development of antibiotic resistance, concern about the effect of growth promoting antibiotics in animal feed on public health, and the approaching ban of nutritional antibiotics from feed in the EU, we need alternative methods to control the proliferation of C. perfringens in the digestive tract of animals, in particular of poultry (Mitsch *et al.*, 2004).

C. perfringens proliferation is associated to an enteric disease in poultry known as necrotic enteritis, that can be costly to both the economy and animal welfare. The

disease is also of significance in preharvest food safety as the use of dietary antibiotics has been considered a potential link to the emergence of antibiotic-resistant strains of zoonotic microorganism in food animals (Si *et al.*, 2009).

1.4. SALMONELLA SPP. AND FOOD SAFETY

Foodborne salmonellosis is responsible for over 600 deaths and 1,4 million illnesses in US annually (Dunkley *et al.,* 2009). In 1999, 22% of all culture-confirmed Salmonella infected individuals were hospitalized. Salmonella have also been commonly associated with foods such as raw meat poultry, eggs, and dairy products and cause a large fraction of the food-related deaths in the US annually. In Europe, the number of human cases was reported to be greater than 100,000 in 1997. In the past few years, the incidence of salmonellosis has shown a significant decrease across Europe and in the US since 1996. Approximately 60% of human cases reported to the CDC in 2001 were caused by four serotypes including *S. Typhimurium*, *S. Enteritidis*, *S. Newport*, and *S. Heidelberg* (EFSA, 2007; Callaway *et al.*, 2008; Dunkley *et al.*, 2009).

Salmonellosis remained the second most frequent zoonosis with 176,395 reported human cases despite a fall of 9.5 % to an incidence rate of 38.2 compared to 2004. *Salmonella* was most often reported from Member States for fresh broiler and pig meat where proportions of positive samples were detected up to 18%. The reported proportions of positive findings in bovine meat were generally lower than 2%. In table eggs, findings of positive *Salmonella* samples ranged from 0% to 6%, but over the past 5 years an overall decreasing trend was observed. In animal populations, *Salmonella* was most frequently detected in poultry flocks. Particularly, the results of the mandatory control programme for *Salmonella* in breeding flocks (*Gallus gallus*) at European level indicated that 6% of the parent-breeding flocks for laying hens and 5% of parent-breeding flocks for broiler were infected with *Salmonella* (EFSA, 2007; Hugas and Tsigarida, 2008).

Salmonella spp. have been linked with illness among many animal species and humans, and are one of the most commonly reported causes of human foodborne disease. *Salmonella* live in the intestinal tract of various animal species and therefore represent a major reservoir for human foodborne disease.

Studies have shown that *Salmonella* infection may be present on farms in the absence of clinical disease. Healthy animals can become carriers and shed *Salmonella* for long periods. Humans become infected primarily through fecal contamination of food products or water; however, direct contact with infected animals is another source of contamination, especially for farm families. Many of the >2,500 *Salmonella enterica*

serotypes are isolated frequently from clinically infected animals. *Salmonella enterica* serovars *Typhimurium, Enteritidis, Javiana, Hadar, Kentucky,* and *Anatum* are among these serotypes, and *Salmonella Typhimurium* DT 104 is of particular concern to public health agencies because of its multiple antibiotic resistance genes.

Because fecal shedding of *Salmonella* is one of the principal modes of on-farm contamination, the question of how fecal shedding can be reduced is very relevant to human health. Research has demonstrated that reduction of *Salmonella* fecal shedding in poultry and swine production units is possible through the modification of management practices. Several control points that could be important for on-farm reduction of *Salmonella* include the presence of carrier animals, the exposure of neonates to feces from sick animals, environmental hygiene, the use of recycled water, contaminated feeds, the use of contaminated water to irrigate forage crops, the spreading of nontreated manure, and infected birds and rodents (EFSA, 2007; Oliver *et al.*, 2009).

In 1980, WHO formulated three lines of defence for the control of *Salmonella* which are still valid and may be used for other zoonotic agents. The first line focuses on the control of *Salmonella* in the food producing animal; the second line of WHO recommendations refers to the prevention or reduction of contamination of the carcasses; the third line of defence concentrates on the prevention of contamination during the final preparation of the food by industry and consumer (Hugas and Tsigarida, 2008).

Salmonella are pathogens but can frequently live in animals as a transient member of the intestinal microbial population without causing disease. Thus, reliance on animal looking sick is not an effective indicator of *Salmonella* colonization. Food animals are the primary vector for transmitting salmonella to humans. Chickens, turkeys, and eggs can all be infected with *Salmonella*. The intestinal tracts of finishing and breeding swine as well as that of beef and dairy cattle can contain *Salmonella*. Further outbreaks of salmonellosis have been linked to improper pasteurization of dairy products or improperly cooked ground beef. Other routes of exposure of humans to *Salmonella* include water runoff from farms or swine effluent lagoons, and direct animal or fecal contact (Callaway *et al.*, 2008).

Thus, *Salmonella* are relatively widespread in the environment and within food animals, and attempts to understand and control this pathogen must be equally broad based. Because *Salmonella* can live undetected in food animals but still pose a risk to human

consumers, control strategies must be tailored to specific animal species yet be applicable to large numbers of animals (Callaway *et al.*, 2008; Martín-Peláez *et al.*, 2008).

1.4.1. What is *Salmonella*?

Salmonella are gram-negative bacteria comprising 2 species and 6 subspecies; the most important of which is *Salmonella enterica* infection in humans causes severe illness and can be an intracellular pathogen. *Salmonella enterica* causes illness in humans by passing from the intestinal tract into the epithelium, where it causes inflammation and systemically releases an enterotoxin and a potent endotoxin. *Salmonella* exists in a typical fecal-oral life cycle, although it can be spread through the nasal cavity to the gut. *Salmonella enterica* comprises over 2,500 known serovars that are pathogenic to humans or animals. A *Salmonella* serotype would commonly be known simply as *Salmonella Typhimurium*, rather than as *S. enterica enterica Typhimurium*. Some serotypes, such as *Typhimurium*, can be utilitarian and infect many species of animals, including man (Callaway *et al.*, 2008).

Adaptation allows *Salmonella* to exist as a pathogen in a suitable host environment, or as a transient member of the gastrointestinal population in a less-than-ideal host environment. Some serotypes can live in food animals without causing illness; however, when host animals and their carried serotypes are consumed by humans, then foodborne illness can result.

Although *Salmonella* serotype influences the extent and outcome of human illness, elimination or treatment strategies are not different between serotypes.

Although the relative importance of serotype has been overstated in regard to the development of pathogen reduction strategies, serotype is still critical information to understand the spread of *Salmonella* through the food chain. In USA, over the past 5 years, the number of *Salmonella*-positive samples in ground beef has decreased and the percentage of positive samples from broilers has increased. This increase in broilers has led to the 2006 implementation of a "*Salmonella* attack plan" by USDA Food Safety and Inspection Service that focuses on an increased sampling frequency in "dirty" plants (Callaway *et al.*, 2008).

Seasonality of fecal shedding is critical to understanding the flow of *Salmonella* through the food chain. There is a correlation between shedding in animals and human outbreaks. Shedding by food animals can approach zero during the winter months and

reaches its peak in summer and early fall especially in cattle and swine, and human outbreaks also peak during this period (EFSA, 2007; Callaway *et al.*, 2008).

Although a physical correlation to temperature exists, it must be noted that the internal temperature is not the sole source of the observed seasonality. Other potential factors for seasonality of pathogen shedding include thyroid hormones and melatonin level as reported by Edrington *et al.* (2006, 2007).

Salmonella in farm environments

Salmonella spp. can be found widely on farms of many types, including those for beef and dairy cattle, swine farrowing and finishing facilities, and poultry farms. The *Salmonella* isolates came from all materials examined on the farms.

Illness from salmonellosis in the bovine is seen predominantly in young calves, although occasionally it seen in adult cattle as well. *Salmonella* have been isolated from the feces of healthy dairy cattle, where the pathogen may exist as a normal member of the gastrointestinal population or as a transient member of gastrointestinal microbial population. Cattle can carry many different serotypes of *Salmonella*.

Swine can be asymptomatic reservoirs of foodborne pathogenic bacteria that are transmissible to humans via consumption of contaminated pork products or through the environment (Callaway *et al.*, 2008).

Salmonella infections in porcines damage both health and productivity. Intestinal infection results in the destruction or turnover of the intestinal mucosa, inducing inflammatory diarrhoea. Furthermore, antibiotic resistance in *Salmonella* species found in farm animals can lead to increased morbility and mortality, due to reduced efficacy of therapeutic antibiotics. Economic losses associated with *Salmonella* are not only due to infection in farm animals but also through entry into the human food chain, where they can cause zoonotic infections in humans (Martín-Peláez *et al.*, 2008)

Foodborne pathogenic bacteria such as *Salmonella* can persist in the environment or within a herd at subclinical levels for years. The most common *Salmonella* serotypes isolated from swine include *Derby*, *Typhimurium*, and *Infantis* (Si *et al.*, 2006; Callaway *et al.*, 2008).

Salmonella infection of farm animals is from multiple source and also has the capacity to cause asymptomatic infections, thereby increasing dissemination. Feeding management strategies are capable to exerting some control on *Salmonella* infection (Martín-Peláez *et al.*, 2008)

Pigs may become colonized with *Salmonella* by ingesting contaminated feces. Placing swine in *Salmonella*-contaminated pens for a large period before slaughter can also result in the colonization of pigs immediately before entry into the food chain (Callaway *et al.*, 2008).

Salmonella is found commonly in chickens and turkeys, and it spreads easily from bird to bird through a fecal-oral route within poultry houses. *Salmonella* also can be spread via other reservoirs thus, the need for stringent biosecurity and pest control plans on most poultry farms.

Salmonella Typhimurium an Enteritidis are the human illness-causing serovars most commonly associated with poultry meat and eggs. Both can cause illness in poultry and are isolated from clinically ill birds, but are frequently present as an asymptomatic infection, allowing them to enter the food chain without triggering a simple detection tripwire (Van Immerseel *et al.*, 2006; EFSA, 2007; Callaway *et al.*, 2008; Dunkley *et al.*, 2009).

Salmonella is a serious threat to broiler and egg production, both as a direct food safety threat in poultry meat and eggs and via vertical transmission to a new generation of infected broilers or layers. Because *Salmonella* can survive in the gut of birds or invade host tissues, it can be transmitted to consumers through various routes. For example, *S.* Enteritidis can invade the ovaries and be directly encapsulated in eggs, or it can live in the intestinal tract and enter eggs through cracks in the shell as the egg intersects the intestinal tract in addition to being transmitted through poultry meat. Fertilized eggs can be infected with *Salmonella* via semen. Thus, when an infected egg is hatched, the chick can already contain Salmonella, which can then be spread quickly to “clean” birds through contact, as well as through the common fecal-oral routes (Callaway *et al.*, 2008; Dunkley *et al.*, 2009).

There has been a great deal of research aimed at understanding what effect stresses have on populations of *Salmonella*, especially dietary and transportation stresses (Callaway *et al.*, 2008). Colonization of the gastrointestinal tract by pathogenic microorganisms is linked to environmental factors of the digesta and therefore, the composition and amount of ingested feed can influence colonization (Martín-Peláez *et al.*, 2008).

1.5. FOOD PATHOGENS INTERVENTION AND REDUCTION STRATEGIES

The ability of foodborne pathogens from food animals to be widely disseminated through the food chain, further emphasizing the need to reduce foodborne pathogenic bacteria in the live animal before they contact human consumers (Greathead, 2003; Burt, 2004; Diez-Gonzales, 2007; Oliver *et al.*, 2009).

With the growing industrialization of the production and transport of food, human illnesses from indirect contact have become increasingly noted.

Some of the most promising improvements aimed at enhancing food safety have focused on the development of interventions that work at the live-animal level. Live-animal, or on-farm, intervention strategies can be loosely grouped into 2 categories: procommensal strategies or directly antipathogen strategies (Diez-Gonzales, 2007; Oliver *et al.*, 2009).

Procommensal strategies use a native (or introduced) microbial ecosystem against pathogens by capitalizing on competition for nutrients and environmental niches. Directly antipathogenic strategies, on the other hand, specifically kill (or inhibit) pathogens via a variety of mechanisms.

A procommensal strategy is defined as the establishment of a nonpathogenic microbial intestinal population that reduces, excludes, or kills pathogenic bacteria, including foodborne pathogens. Simply put, procommensal strategies promote the growth of groups of bacteria that are competitive with, or even antagonistic to, the pathogens of interest. Procommensal strategies used in food animals include probiotics, which are microbial cultures that are fed to animals to maintain a constant flow of commensal organisms through the gut environment; competitive exclusion, defined as the establishment of a microbial population in a naive food animal gut; and prebiotics.

Antipathogenic strategies are the most straightforward of the intervention strategies because they directly attack the pathogen of interest. However, because foodborne pathogenic bacteria typically do not have any unusual properties within the gut of food animals, they are difficult to target directly without significant “collateral damage” on the rest of the microbial population. However, a variety of antipathogen strategies can be used to address pathogen populations in food animals, including antibiotics and bacteriocins, organic acids, and essential oil (Oliver *et al.*, 2009).

Numerous nutritional additives are either already in use or have been proposed as means to reduce or eliminate pathogens or as a means to improve growth and feed conversion (Joerger, 2003; Diez-Gonzales, 2007).

The primary mode of action of growth promoting feed additives arises from stabilizing feed hygiene (e.g., through organic acids), and even more from beneficially affecting the ecosystem of gastrointestinal microbiota through controlling potential pathogens. This applies especially to critical phases of an animals' production cycle characterized by high susceptibility to digestive disorders, such as the weaning phase of piglets or early in life of poultry. Due to a more stabilized intestinal health, animals are less exposed to microbial toxins and other undesired microbial metabolites, such as ammonia and biogenic amines. Consequently, growth-promoting feed additives relieve the host animals from immune defence stress during critical situations and increases the intestinal availability of essential nutrients for absorption, thereby helping animals to grow better within the framework of their genetic potential (Windisch *et al.*, 2008).

1.5.1. Bacteriocins

Bacteria have many complex mechanisms to resist antibiotics, and the widespread use of antibiotics in both human medicine and animal agriculture has led to the widespread dissemination of antimicrobial resistance genes. Because of concerns about the dissemination of antimicrobial resistance, it is likely that prophylactic use of medically important antibiotics as growth promotants in food-producing animals will become completely prohibited (Joerger, 2003; Diez-Gonzales, 2007; Oliver *et al.*, 2009).

Some bacteria produce proteinaceous compounds, known as bacteriocins, lethal to bacteria other than the producing strain, that can inhibit the growth of foodborne pathogenic bacteria, including *C. botulinum*, *Bacillus* spp., *E. faecalis*, *E. coli*, *Salmonella*, and *Listeria* (Carolissen-Mackay *et al.*, 1997; Joerger, 2003; Diez-Gonzales, 2007; Oliver *et al.*, 2009).

As with any antimicrobial compound, the issue of resistance also has to be considered for bacteriocins. Although the mechanism of action is not known for all bacteriocins, most of the low molecular weight bacteriocins appear to interact with the bacterial membrane. Resistance is therefore usually the result of changes in the membrane of bacteria targeted by a bacteriocin, but inactivation by degradation has been observed for nisin. Until recently, development of resistance to bacteriocins was not considered as affecting resistance to currently used antibiotics (Joerger, 2003; Oliver *et al.*, 2009).

Compared to antibiotics, most bacteriocins are relatively specific and can only affect a limited number of bacterial species. Bacteriocins of lactic acid bacteria can be inhibitory to many Gram-positive organisms, but they have little effect on Gram-negative species due to the protective effect of the outer membrane. Among the different types of bacteriocins, colicins probably have the greatest specificity because many of them only affect strains within the same species. The specificity of bacteriocins can be particularly advantageous for applications in which a single bacterial strain or species is targeted without disrupting other microbial populations. In the case of pathogens as target organisms that colonize the gastrointestinal tract of poultry, cattle and swine, the use of bacteriocin-producing strains would have little effect on most beneficial intestinal bacteria (Joerger, 2003; Diez-Gonzales, 2007; Oliver *et al.*, 2009).

In contrast to the currently used antibiotics, bacteriocins are often considered more natural because they are thought to have been present in many of the foods eaten since ancient times. The bacteriocin nisin actually has GRAS (generally recognized as safe) status (Joerger, 2003; Oliver *et al.*, 2009). Nisin and other bacteriocins produced by lactic acid bacteria have received a great deal of attention because they are produced by bacteria largely considered beneficial to human health and to food production (Joerger, 2003).

One of the potential benefits of using bacterocins in livestock is the stimulation of animal productivity. However, due to the specificity of bacteriocins it is very unlikely that their growth enhancement would be similar to the effect of antibiotics. In recent years, several reports have indicated that ruminal microorganisms are capable of producing a variety of bacteriocins and some of these organisms have been isolated for an eventual application to manipulate the rumen (Diez-Gonzales, 2007).

The application of bacteriocin-producing bacteria for improvements in productivity has not been limited to cattle, as several researchers have explored the use of probiotic strains capable of producing bacteriocins to increase the growth rate of swine. In poultry, the use of bacteriocin has been mainly targeted for the control *Salmonella*. The potential improvement of productivity in animals mediated by the utilization of bacteriocin could be based on the inhibition of specific groups of organisms (Joerger, 2003; Diez-Gonzales, 2007).

The utilization of bacteriocin-producing bacteria as a pre-harvest food safety strategy is considered as one of the most viable interventions for reducing the gastrointestinal colonization of livestock by foodborne pathogens. These bacteria can easily be

administered to animals by mixing dried or wet cultures with feed or drinking water, and depending on the ability of the particular probiotics strain to colonize the gastrointestinal tract they could be fed sporadically or continuously. The feeding of bacteriocin-producing bacteria can have a direct effect on reducing the existing populations of foodborne pathogens such as *Salmonella* and *Escherichia coli* O157:H7, and long-term colonization with bacteriocin-producing bacteria would prevent further re-introduction of the pathogenic bacteria (Diez-Gonzales, 2007).

Despite the enormous potential of bacteriocin-producing bacteria to increase animal productivity and to reduce the likelihood of foodborne disease, there are relatively few studies that have investigated the factors influencing their applicability (Diez-Gonzales, 2007).

The administration of bacteriocin-producing bacteria rather than the bacteriocins themselves might be a more cost-effective approach, but significant progress in developing suitable producer strains will have to be made before such an approach will be feasible. Few studies have addressed the fate of bacteriocins in the intestinal tract, but some data suggest that some of the low molecular weight bacteriocins can survive at least some of the intestinal environments and possibly could be administered with feed (Joerger, 2003; Diez-Gonzales, 2007).

For uses involving purified bacteriocins, cost of the compounds can become a significant barrier. Production of all but the smallest bacteriocins is currently only imaginable by culture of natural or genetically engineered producer organisms (Joerger, 2003).

1.5.2. Organic acids

Man has used fermentations as a method of food preservation for more than 6000 years, but now it appears that fermentation acids also have value as feed or drinking water additives. Commercial preparations appear to enhance digestibility and diet palatability, thus improving feed conversion and growth of animals, including pigs and poultry (Partanen and Mroz, 1999; Canibe *et al.*, 2001; Hismiogullari *et al.*, 2008; Van Immerseel *et al.*, 2009). Some acid mixtures prevent mould growth on feed, and claims of increased egg production have been made. Pathogen control has also been reported, but the peer-reviewed scientific literature has few definitive studies (Canibe *et al.*, 2001; Van Immerseel *et al.*, 2009). Until recently, the use of short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA) and other organic acids was largely based on their

antimicrobial activity outside the intestinal tract (Partanen and Mroz, 1999; Van Immerseel *et al.*, 2009).

Finally, organic acids (OA) acts as protectors of the gut, through very different ways, including mucosal growth, intestinal barrier function stregthening, anti-oxidant and anti-infiammatory capacities, and anti-bacterial properties (Lallès *et al.*, 2009).

Bacterial metabolism of OA

Bacteria can use organic acids as both carbon and energy sources. In *E. coli,* the hydrophobic long-chain fatty acids (LCFA) (≥C12) are transported across the cell membrane by carrier mechanisms, in which the fadL (outer membrane proteins) and the fadD proteins (inner membrane) are involved. FadL carries LCFA to the periplasmatic space and fadD is an acyl- CoA synthetase. Once the acyl-CoA molecules are formed inside the cell, degradation occurs through the b-oxidation pathway, yielding multiple acetyl-CoA molecules. Degradation of LCFA having an odd number of carbon atoms also yields propionyl-CoA as an end product. Whether MCFA (C6 to C10) can be transported by carrier proteins or are able to diffuse freely across the cell membrane in undissociated form is less clear, but also the fadD protein and the b-oxidation pathway are used for metabolization. The LCFA and MCFA can also be used for incorporation in the membrane as phospholipids. SCFA (≤ C4) presumably cross the outer membrane mainly through diffusion in the undissociated form. Once inside the cell, they can be converted to their CoA thioester forms. Butyric acid is converted to butyryl-CoA by the acetoacetyl-CoA transferase system (AtoAD system), converted to acetoacetyl- CoA by the fadB/E system, and then further breakdown to acetyl-CoA is performed by the atoB gene product (Van Immerseel *et al.*, 2009). Thus as an example, butyric acid is converted to two molecules of acetyl-CoA. Propionic acid, either taken up from the environment or generated as an endproduct of degradation of LCFA with an odd number of carbon atoms is metabolized in *Salmonella* and *E. coli* in the methylcitrate cycle. Propionyl-CoA reacts with oxaloacetate to form 2-methylcitrate, which is converted through a series of reactions, to succinate and pyruvate. These products can be used in the citric acid cycle. Although it is thought that acetate can diffuse across the cell membrane, an acetate permease (ActP) was detected in *E. coli*. In *E. coli* and *Salmonella*, acetate is converted to acetyl-CoA by either acetyl-CoA synthetase or the sequential action of acetate kinase and phosphotransacetylase. Acetyl-CoA, generated by either the b-oxidation pathway, by butyric acid breakdown or by acetate conversion,

can be used for oxidation in the citric acid cycle and for replenishing intermediates of the citric acid cycle via the glyoxylate shunt (Van Immerseel *et al.*, 2009).

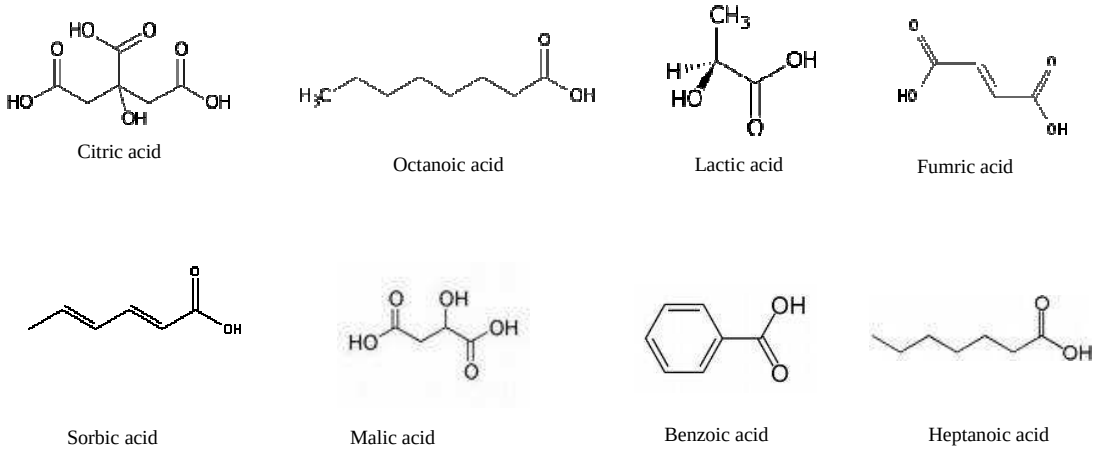

Figure 1.1. *Chemical structures of main organic acids with antimicrobial activity.*

Meccanism of antimicrobial activity of OA

Fermentative bacteria produce organic acids when oxygen is not available as a 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 (Partanen and Mroz, 1999; Van Immerseel *et al.*, 2009). The simplest fermentation is conversion of sugar to lactate, and many lactobacilli, streptococci, lactococci and enterococci have a scheme that is virtually homolactic when sugar is plentiful.

However, when sugars are scarce, all of these bacteria are capable of switching to a fermentation that produces acetate, formate and ethanol, so ATP production can be enhanced.

Bacteria capable of utilizing fatty acids are found in stagnant anaerobic environments, but these bacteria grow very slowly, and fermentative environments are typically acidic. 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 (Canibe *et al.*, 2001; Van Immerseel *et al.*, 2009).

Organic acids were compared with synthetic uncouplers that could remain membrane associated, and shuttle protons in a cyclic manner to dissipate the proton-motive force. The problem with this analogy is the fact that organic acid anions are charged and not lipid permeable (Hismiogullari *et al.*, 2008; Van Immerseel *et al.*, 2009).

Why are some bacteria so much more sensitive than others?

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*. 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 is protected from anion accumulation (Canibe *et al.*, 2001; Mroz, 2003; Van Immerseel *et al.*, 2009).

Fermentation acid anion accumulation was at least in part an osmotic stress. Recent work with *C. 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. When lactate anion increased, the cells lost intracellular glutamate, and the fermentation scheme of amino acid deamination is dependent on glutamate transaminase.

The lower the external pH, the more undissociated weak acid will be available (based upon pK_a values) to cross the membrane and affect internal pH. To overcome the lowering of internal pH, several amino acid decarboxylases can be induced; these elevate the internal pH by consuming a proton during decarboxylation and they then exchange the decarboxulation end-product for a new substrate via a membrane-bound antiporter (Bearson *et al.*, 1997). One example is lysine decarboxylase (CadA) coupled with the lysine-cadaverine antiporter (CadB) of *S. Typhimurium*. The CadA decarboxylates intracellular lysine to cadaverine and consumes a proton in the process. Cadaverine is then exchanged for fresh lysine from the surrounding environment via the CadB antiporter (Park *et al.*, 1996).

The final result was a virtually complete inhibition of ammonia production (Van Immerseel *et al.*, 2009). 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 neutrophils seem to be more sensitive than those that are acid tolerant.
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.
Factors such as chain length, side chain composition, pKa values and hydrophobicity could affect the antimicrobial activity (Van Immerseel *et al.*, 2009).

1.5.3. Essential oil

An essential oil is a mixture of fragrant, volatile compounds, named after the aromatic characteristics of plant materials from which they can be isolated. The term "essential" was adapted from the theory of "quinta essential" proposed by Paracelsus who believed that this quintessence was the effective element in a medical preparation (Greathead, 2003; Lee *et al.*, 2004).
Essential oils are very complex mixtures of compounds and their chemical compositions and concentrations of individual compounds are variable. Because of the large variation in composition, the biological effects, if any, of essential oil may differ (Greathead, 2003; Burt, 2004; Lee *et al.*, 2004).
Essential oil basically consist of two classes of compounds, the terpenes and phenylpropenes. Depending on the number of 5-carbon building blocks, terpenes can be sub-divided into mono-, sesqui-, and di-terpenes. Further derivates of terpenes are typified by the presence or absence of a ring structure, double bond, addition of oxygen or stereochemistry. Terpenes and phenylpropenes are synthesized by the mevalonic and shikimic pathway, respectively (Greathead, 2003; Lee *et al.*, 2004).
Essential oil, however, are a relatively new class of feed additives and we are still rather limited in knowledge regarding modes of their action and aspects of their application. Essential oils can be used as phytogenic feed additives incorporated into diets to improve productivity of livestock through amelioration of feed properties, promotion of the animals' production performance, as well as improving quality of food derived from those animals (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008).

Use of feed additives is usually subject to restrictive regulations. In general, they are considered as products applied by the farmer to healthy animals for a nutritional purpose on a permanent basis in contrast to veterinary drugs.

In the European Union, for example, feed additives need to demonstrate identity and traceability of the entire commercial product, efficacy of the claimed nutritional effects including absence of possible interactions with other feed additives, as well as safety to the animal, to the user, to the consumer of animal-derived products, and to the environment. Problems with feed additive legacy may, therefore, arise especially with phytogenic feed additives addressed to explicit health claims or in case of plant derived substances suspected to modulate metabolism (Windisch *et al.*, 2008).

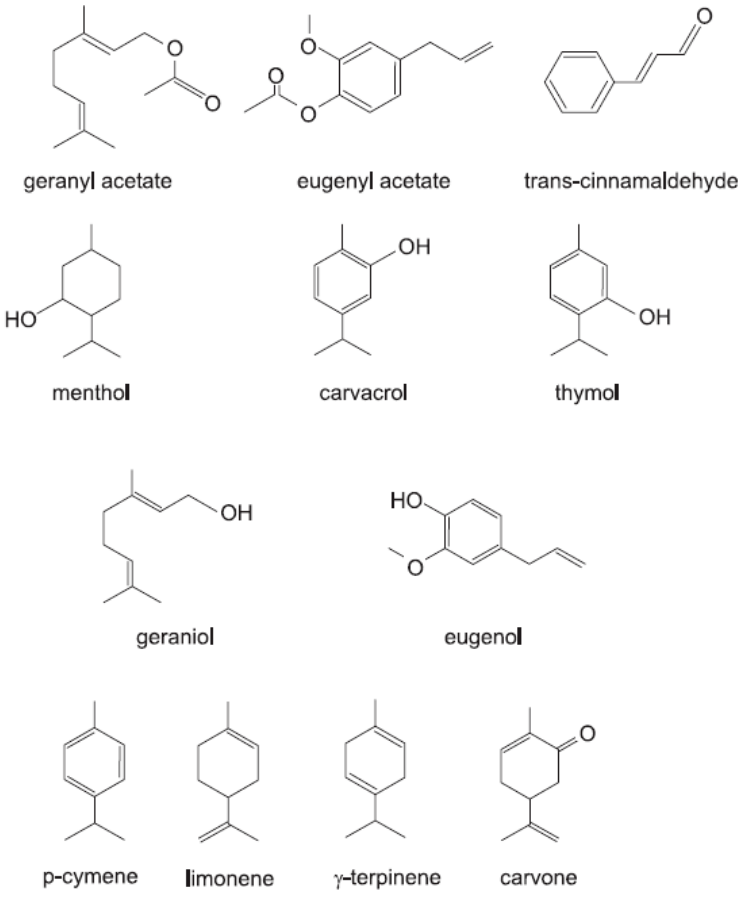

Figure 1.1. *Chemical structures of main substances of essential oil.*

Antioxidative properties are well described for essential oil (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008). Among a variety of plants bearing antioxidative constituents, the volatile oils from the mint plants have been attracting the greatest interest, especially products from rosemary. Its antioxidative activity arises from phenolic terpenes. Other *Labiatae* species with significant antioxidative properties are thyme and oregano, which contain large amounts of the monoterpenes thymol and carvacrol (Windisch *et al.*, 2008).

The antioxidant property of many phytogenic compounds may be assumed to contribute to protection of feed lipids from oxidative damage like antioxidants usually added to diets (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008). The principal potential of feed additives containing herbal phenolic compounds from *Labiatae* plant family to improve oxidative stability of animal derived products has been demonstrated for poultry meat, pork, rabbit meal, and eggs (Windisch *et al.*, 2008).

Phytogenic feed additives are often claimed to improve flavor and palatability of feed, thus, enhancing production performance (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008). However, the number of studies having tested the specific effect of phytogenic products on palatability by applying a choice feeding design is quite limited. They show dose-related depressions of palatability in pigs fed essential oils from fennel and caraway, as well as from thyme and oregano herbs. On the other hand, there are numerous reports on an improved feed intake through phytogenic feed additives in swine (Windisch *et al.*, 2008).

A wide range of spices, herbs, and their extracts are known from medicine to exert beneficial actions within the digestive tract: stimulation of digestive secretions, bile, and mucus, and enhanced enzyme activity are proposed to be core mode of nutritional action (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008). Phytogenic feed additives were also reported to stimulate intestinal secretion of mucus in broilers, an effect which was assumed to impair adhesion of pathogens and thus to contribute to stabilizing the microbial eubiosis in the gut of the animals.

Herbs and spices are well known to exert antimicrobial actions *in vitro* against important pathogens (Windisch *et al.*, 2008). The active substances are largely the same as mentioned previously for antioxidative properties, with phenolic compounds being the principle active components (Greathead, 2003; Burt, 2004; Lee *et al.*, 2004; Windisch *et al.*, 2008). The antimicrobial mode of action is considered to arise mainly from the potential of the hydrophobic essential oils to intrude into the bacterial cell

membrane, disintegrate membrane structures, and cause ion leakage (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008). Microbiological analysis of minimum inhibitory concentrations (MIC) of plant extracts from spices and herbs, as well as of pure active substances, revealed levels that considerably exceeded the dietary doses when used as phytogenic feed additives (Burt *et al.*, 2004; Windisch *et al.*, 2008). Another implication of antimicrobial action of phytogenic feed additives may in be improving the microbial hygiene of carcasses. Indeed, there are isolated reports on the beneficial effects of essential oils from oregano on microbial load of total viable bacteria, as well as of specific pathogens (e.g., salmonella) on broiler carcasses (Windisch *et al.*, 2008).

Several are the effects of essential oil as growth promoters. Data on swine vary widely from depressions in production performance to improvements similar to those observed with common growth promoters, such as antibiotics, organic acids, and probiotics. For poultry, the majority of experimental results indicate reduced feed intake at largely unchanged weight gain or final body weight, leading to an improved feed conversion when feeding phytogenic compounds (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008). Of course, the wide variation in biological effects induced by phytogenics reflects the experimental approaches to test suitability of these substances for use as growth promoting feed additives to swine and poultry and includes also failures in selecting proper plants, active components, and efficacious dietary doses. Recent studies with swine and poultry indicated stabilizing effects of phytogenic feed additives on the ecosystem of gastrointestinal microbiota. These effects are also typical for organic acids, which are known to exert a major part of their biological efficacy mainly through stabilizing the microbial eubiosis in the gastrointestinal tract. Morphological changes in gastrointestinal tissues due to phytogenic feed additives may provide further information on possible benefits on the digestive tract (Greathead, 2003; Burt, 2004; Lee *et al.*, 2004; Windisch *et al.*, 2008); however, the literature available does not provide a consistent picture. Available reports show increased, unchanged, and reduced villi length and crypt depth in the jejunum and colon for broilers and pigs treated with phytogenic feed additives.

Improved digestive capacity in the small intestine may be considered an indirect side effect of feed additives stabilizing the microbial eubiosis in the gut. An improved prececal digestive capacity reduces the flux of fermentable matter into the hind gut and, thus, lessens the post-ileal microbial growth and the excretion of bacterial matter in

feces, respectively. Because bacterial protein is the dominant fraction of total fecal protein, an improved prececal digestive capacity may result indirectly in an increased apparent digestibility of dietary protein (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008).

Besides efficacy, application of phytogenic feed additives to livestock also has to be safe to the animal, the user, the consumer of the animal product, and the environment (Greathead, 2003; Lee *et al.*, 2004; Windisch *et al.*, 2008). Regarding exposed animals, adverse health effects cannot generally be excluded in case of an accidental overdose. For the user, the handling of pure formulations of such feed additives usually needs protective measures because they are potentially irritating and can cause allergic contact dermatitis (Burt, 2004; Windisch *et al.*, 2008). With respect to consumer safety, the phytogenic feed additives cannot be relieved from determination of possible undesired residues in products derived from animals fed those products (Windisch *et al.*, 2008).

Phytogenic feed additives are claimed to exert antioxidative, antimicrobial, and growth promoting effects in livestock, actions which are partially associated with an enhanced feed consumption supposedly due to an improved palatability of the diet (Greathead, 2003; Burt, 2004; Lee *et al.*, 2004; Windisch *et al.*, 2008). Whereas available results do not support a specific amelioration of palatability, the antioxidative efficacy of some phytogenic compounds to protect quality of feed, as well as that of food derived from animals fed those substances cannot be ruled out. With respect to antimicrobial action, some observations *in vivo* support the assumption for the general potential of phytogenic feed additives to contribute to a final reduction of intestinal pathogen pressure. When compared with antimicrobial feed additives and organic acids, the phytogenic substances currently used in practice seem to similarly modulate relevant gastrointestinal variables, such as microbial colony counts, fermentation products, digestibility of nutrients, gut tissue morphology, and reactions of the gut associated lymphatic system. In addition, phytogenic products may stimulate intestinal mucus production, which may further contribute to relief from pathogen pressure through inhibition of adherence to the mucosa (Windisch *et al.*, 2008).

2. OBJECTIVES

In the contest of improving food safety, as required by the European Authority and consumer, the attention was focused on microbiological risk in food animal. *Clostridium perfringens* and *Salmonella* spp. were the major causes of human infection. In particular, to prevent microbiological risk is necessary decrease slaughterhouse cross-contamination. To obtain this result, the studies can be focused on the control of pathogens presence in farm and reduction of intestinal tract colonization of swine and poultry.

In this scenario the objectives of this work were:

- to screen the antimicrobial power of different organic acids and flavours against foodborne pathogens, which represent a threat to both animal productivity and human health, to find possible synergisms among those compounds;
- to evaluate the role of such compounds and their possible synergistic effect against pathogens in an *in vitro* system which simulate intestinal environment;
- to evaluate natural compounds *in vivo* as feed additives to control pathogens infections.

To reach these objectives 3 groups of experiments were planned:

- determination of the Minimal Inhibitory Concentration (MIC). Study was planned to assess the MIC of organic acids and flavours against *Clostridium perfringens* and *Salmonella Typhimurium*, and find the presence of synergistic effects.
- Intestinal fermentation. The study was to evaluate the role of association of organic acids and flavours to control *Salmonella Typhimurium* infection and modulate fermentation parameters in an *in vitro* system (batch culture techniques) that simulate swine intestinal environment.
- *In vivo* studies. In the first study it was evaluate the role of microencapsulated blend of organic acids and flavours to control *Clostridium perfringens* infection in broilers; the second study was to evaluate the effect of a bacteriocin and its producer strain *Pediococcus pentosaceus* to reduce *C. perfringens* infection in broilers.

3. EVALUATION OF ANTIMICROBIAL ACTIVITY OF SEVERAL ORGANIC ACIDS AND FLAVORS AGAINST CLOSTRIDIUM PERFRINGENS AND SALMONELLA TYPHIMURIUM

3.1. Aim of the study

The antibacterial strength of such substances can be determined by broth dilution method and optical density measurement (Smith-Palmer *et al.*, 1998), and the minimal inhibitory concentration (MIC) of a substance can be defined as the minimal concentration of such substance inhibiting visible growth of test organism used at a given infectious dose after a given amount of time (Karapinar and Aktug, 1987; Onawunmi, 1989; Hammer *et al.*, 1999; Delaquis *et al.*, 2002).

Aim of the study was to evaluate of the capacity of some organic acids and flavors to control the growth of *C. perfringens* and *S. Typhimurium*, and the possible combination effect of a given organic acid and a given flavor to improve their individual antibacterial activities when combined at different concentrations during 24 h of incubation, in order to study their possible use in animal husbandry to reduce microbial infections and microbial carcass contamination at slaughter.

3.2. Materials and method

Bacterial strains and growth conditions

The bacterial strains used to test the substances in this study were *Clostridium perfringens* ATCC 13124 and *Salmonella enterica subs. enterica, serov. Typhimurium* H2665 DT104.

C. perfringens was stored in Cooked Meat (CM broth, Difco Laboratories, Division of Becton Dickinson and Company, Sparks, MD, USA) at room temperature. Clostridium working seeds were obtained by inoculating 100µL of stock culture in 10 mL of Reinforced Clostridial Medium (RCM) broth (Oxoid Spa, Bansingstoke, UK) and incubated at 37°C in anaerobic conditions through two subsequent incubations of 24h.

S. Typhimurium was stored at –20°C in Brain Hart Infusion (BHI) broth with glycerol 20% (v/v). The strain was revitalized by inoculation in BHI and incubation at 37 °C in aerobic conditions through two subsequent incubations of 24h.

Chemical substances

Organic acids and flavours objects of the study were: thymol (Fluka), carvacrol (Fluka), vanillin (Fluka), limonene (Fluka), and lactic acid (Fluka), all purchased by Sigma-Aldrich Chemie Gmbh, Steinheim, Germany, cinnamaldehyde, benzoic acid, citric acid, fumaric acid, and DL-malic acid (Sigma-Aldrich Corporation, St. Louis, MO, USA). The molecules were diluited in RCM broth for *C. perfringens* tests and in BHI broth for S. *Typhimurium* tests as described below (EP 1391155B1, Vetagro S.p.A, Italy)

Broth dilution method

Stock solutions of each acid were prepared by dissolving it in deionized water, correction to pH 6.5 followed by sterilization using a filter with a membrane pore size of 0.22 μm (Millipore Corporation, Bedford, MA, USA).

Organic acid working solutions were prepared by serial two-fold dilutions of each stock solution with RCM or BHI broth (pH 6.5) in order to obtain the lowest concentrations to be tested depending on the type of acid.

Flavor stock solutions were obtained by dissolving them in ethanol (≤ 5%, v/v) (Merck, Darmstadt, Germany) in order to reach 7.28 mM. All the solutions were adjusted to pH 6.5 and sterilized by filtration (pore diameter 0.22 μm). Working broth solutions of each flavor were prepared by diluting them with RCM or BHI broth at pH 6.5 (flavor stock solution: RCM or BHI broth = 3: 1). Further concentrations were obtained in the same way until 1.96 mM was reached. Final concentrations tested in 96-wells microtiter plates were:

- flavors (carvacrol, thymol, vanillin, cinnamaldehyde, and limonene) at: 1.82, 1.46, 0.98, 0.73, 0.49 mM;
- malic acid and lactic acid at: 500, 250, 125, 62.5, 31.25, 15.63 mM;
- citric acid and benzoic acid at: 250, 125, 62.5, 31.25, 15.63, 7.82 mM;
- fumaric acid at: 125, 62.5, 31.25, 15.63, 7.82, 3.91 mM

Each flavor was tested alone and in combination with each organic acid at concentrations previously indicated.

MIC determination in microtiter plates.

Clostridium and *Salmonella* inhibition study was performed with 10^4 CFU/well: each well (96-wells microtiter plates) was filled with 100 μl of bacterial inoculum (10^5 CFU/ml) and with 100 μl of each solution. The negative control wells were inoculated in the same way with 100 μl of a 10^5 CFU/ml culture and 100 μl of RCM or BHI broth

(pH 6.5) without the addition of any substance. Each substance was tested two times per plate.

A blank control with ethanol 5% (v/v) was assessed in order to verify that the highest concentration of ethanol used to dissolve flavors was not inhibiting the growth of bacteria.

C. perfringens plates were incubated at 37 °C in anaerobic conditions, while *S. Typhimurium* plates were incubated at 37°C in aerobic conditions. Optical density (OD) as bacteria growth index was measured after 24h of incubation using a spectrophotometer (Tecan Spectra Classic, Tecan Group Ltd, Switzerland) at lambda (λ) = 630 nm.

Broth turbidity at 10^4 CFU/mL could not be observed; increasing of turbidity was considered as a positive indicator of bacterial growth after 24h of incubation. For each bacterial strains the growth response was plotted against each concentration of the compounds. The minimal inhibitory concentrations (MIC) were determined as the lowest concentration of the substance tested which completely inhibited the increase of turbidity of bacterial cultures after 24h of incubation.

To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC index) was calculated for each substance in each combination (Ohran *et al.*, 2005). The following formulas were used to calculate the FIC index:

$$\sum FIC = FIC\ A + FIC\ B,$$

were FIC A was the ratio between the MIC of substance A in combination and MIC of substance A alone, and FIC B was the ratio between the MIC of the substance B in combination and MIC of substance B alone. Synergy was defined as a $\sum FIC \leq 0.5$; an additive effect was defined when $0.5 < \sum FIC < 2$. Indifference was defined as $2 < \sum FIC < 4$, and antagonism was defined as a $\sum FIC \geq 4$.

Statistical analyses

The experiment was made in duplicate. For each bacterial strain, OD data were analyzed for each tested compound by One-way ANOVA; the differences among means of groups were obtained using Newman-Keuls t-test based on the variances derived from ANOVA (GraphPad Prism 4.0; GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $P<0.05$.

3.3. Results

Clostridium perfringens

As described in table 3.1, MIC of carvacrol was 1.46 mM, even if at 0.98 mM carvacrol reduced *C. perfringens* growth of 84%. For organic acids were determined MIC value only for citric, benzoic and malic acid, 62.5 mM, 125 and 250 mM, respectively. For all the other acids MIC was not determined because the highest concentrations tested were not effective in inhibiting *Clostridium* growth. As showed citric acid was more effective against *C. perfringens*. Lactic acid showed to have inhibitory effect at high concentration during the 24h duration of the study (fig. 3.1).

The MIC of combination showed that, even if any acid alone did not inhibit *C. perfringens*, the same acid in combination with carvacrol had an antibacterial activity, reducing bacteria growth.. Carvacrol at 0.98 mM instead of 1.46 mM, in association with fumaric acid at 125 mM. completely inhibited *C. perfringens*.

As described by FIC index, there was an additive effect between carvacrol and citric, malic or benzoic acid.

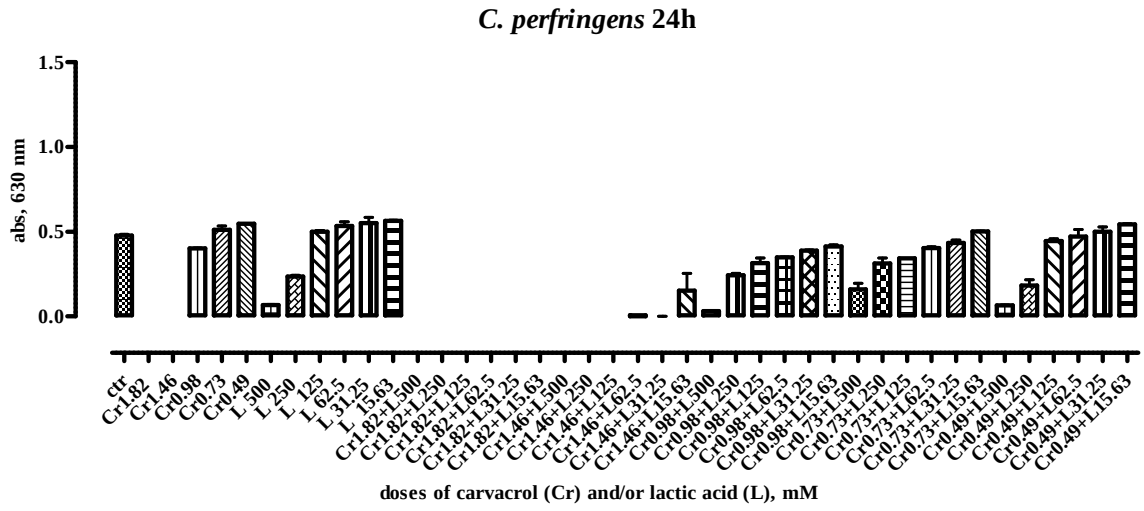

Figure 3.1. *Absorbance (λ=630 nm) values of C. perfringens growth after 24h of incubation. Column bars indicate mean ± SD. Each column is the mean of two values. Cr= carvacrol; L= lactic acid.*

***Table 3.1**. Antibacterial effect of carvacrol and organic acids alone or in combination, against 10^4 CFU/ml of C. perfringens after 24h of incubation. MIC values are expressed as mM. (n=2). NI= Not Inhibitory.*

MIC Carvacrol	MIC Citric	MIC Carvacrol + Citric	FIC
1.46	62.5	0.98 + 7.82	0.8
		0.73 + 31.25	1.0
		0.49 + 31.25	0.8
MIC Carvacrol	**MIC Malic**	**MIC Carvacrol + Malic**	**FIC**
1.46	250	0.98 + 31.25	0.8
		0.73 + 125	1.0
MIC Carvacrol	**MIC Benzoic**	**MIC Carvacrol + Benzoic**	**FIC**
1.46	125	0.98 + 62.5	1.2
		0.73 + 62.5	1.0
MIC Carvacrol	**MIC Fumaric**	**MIC Carvacrol + Fumaric**	**FIC**
1.46	NI	0.98 + 125	-
MIC Carvacrol	**MIC Lactic**	**MIC Carvacrol + Lactic**	**FIC**
1.46	NI	NI	-

MIC of thymol (tab. 3.2) was not determined because the highest concentrations tested were not effective in inhibiting *Clostridium* growth, but at 1.82 and 1.46 mM reduced clostridia growth of 80%. For organic acid MIC values were determined for malic (500mM) and benzoic (125 mM). When acids were associated with thymol MIC values were reduced. *C. perfringens* growth was inhibited by malic acid and thymol in combination at 125 and 0.98 mM, or at 250 and 0.73 mM, or at 250 and 0.49 mM, respectively, whereas thymol at 0.98 mM, 0.73 and 0.49 mM did not decrease optical density values. MIC of benzoic acid and thymol in combination was 62.5 and 0.98 mM, respectively. For citric acid MIC was not determined because the highest concentration tested was not effective in inhibiting *Clostridium* growth, but when used in association with thymol clostridia growth was inhibited at 125 and 0.98 mM, or 250 and 0.73 mM, for citric acid and thymol, respectively. MIC of fumaric or lactic acid with thymol was

not determined, even if figure 3.2 showed a reduction of growth when thymol was used in association at 1.82 mM, 1.46 or 0.98 mM.

***Table 3.2**. Antibacterial effect of thymol and organic acids alone or in combination, against 10^4 CFU/ml of C. perfringens after 24h of incubation. MIC values are expressed as mM. (n=2). NI= Not Inhibitory.*

MIC Thymol	**MIC Citric**	**MIC Thymol + Citric**	**FIC**
NI	NI	0.98 + 125 0.73 + 250 0.73 + 50	-
MIC Thymol	**MIC Malic**	**MIC Thymol + Malic**	**FIC**
NI	500	0.98 + 125 0.73 + 250 0.49 + 250	-
MIC Thymol	**MIC Benzoic**	**MIC Thymol + Benzoic**	**FIC**
NI	125	0.98 + 62.5	-
MIC Thymol	**MIC Fumaric**	**MIC Thymol + Fumaric**	**FIC**
NI	NI	NI	-
MIC Thymol	**MIC Lactic**	**MIC Thymol + Lactic**	**FIC**
NI	NI	NI	-

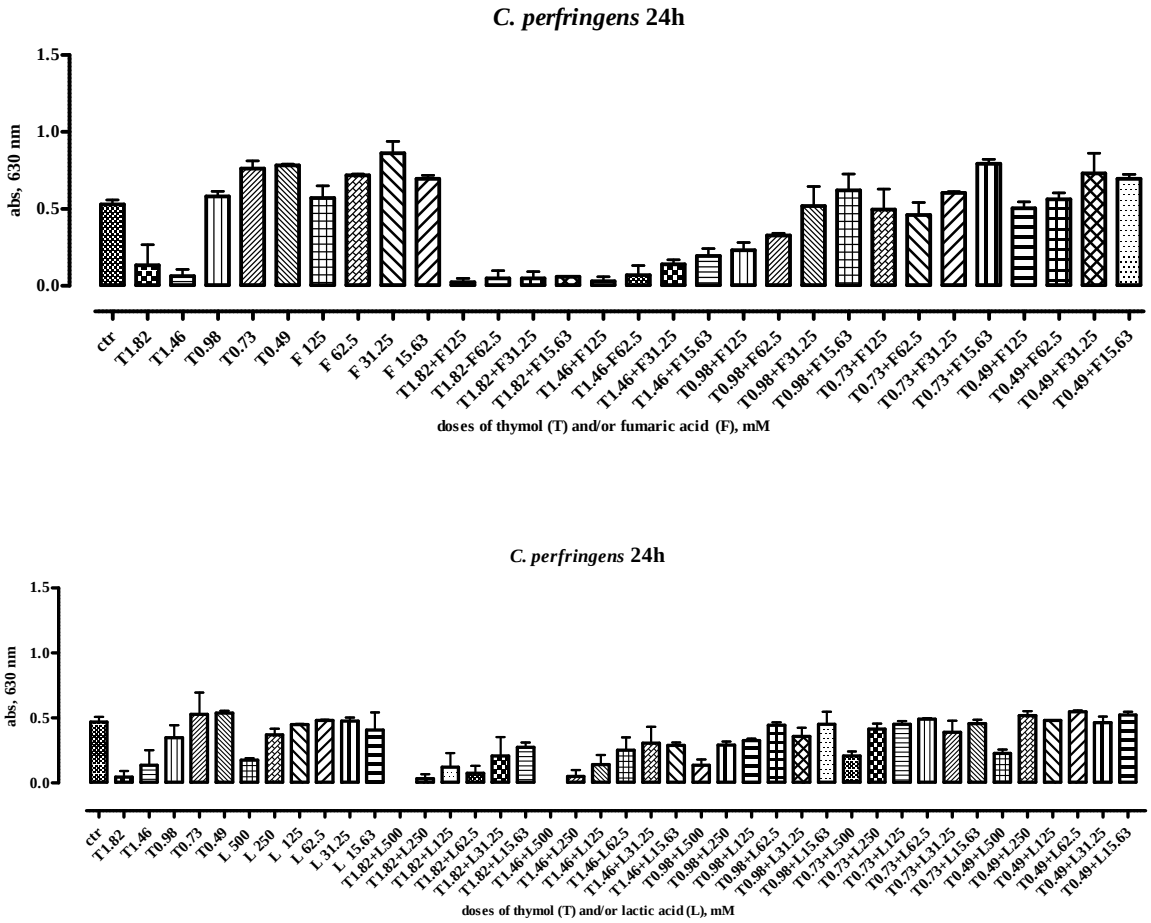

Figure 3.2. *Absorbance (λ=630 nm) values of C. perfringens growth after 24h of incubation. Column bars indicate mean ± SD. Each column is the mean of two values. T= thymol, F= fumaric acid; L= lactic acid.*

MIC of cinnamaldehyde (tab. 3.3) was 1.46 mM. For organic acid MIC values were determined for citric (31.25 mM), malic (250 mM) and benzoic (125 mM). When acids were associated with cinnamaldehyde MIC values were reduced. *C. perfringens* growth was inhibited by benzoic acid and cinnamaldehyde in combination at 31.25 and 0.73 mM, respectively, whereas cinnamaldehyde at 0.73 mM did not decrease optical density values. MIC of lactic acid and cinnamaldehyde in combination was 250 and 1.82 mM, or 500 and 1.46 mM, respectively. When citric acid was used in association with cinnamaldehyde clostridia growth was reduced by 65% at 7.82 and 0.73 mM, or by 69% 15.63 and 0.49 mM, for citric acid and cinnamaldehyde, respectively. MIC of fumaric acid with cinnamaldehyde was not determined, but bacteria growth was reduced at 62.5

and 0.98 mM, or 15.63 and 0.98 mM for fumaric acid and cinnamaldehyde, respectively. Even if for lactic acid MIC was not determined because the highest concentration tested was not effective in inhibiting *Clostridium* growth, figure 3.3 showed a reduction of growth when cinnamaldehyde was used in association at 1.82 or 1.46 mM, and for all combination with lactic acid at 500 mM.

***Table 3.3**. Antibacterial effect of thymol and organic acids alone or in combination, against 10^4 CFU/ml of C. perfringens after 24h of incubation. MIC values are expressed as mM. (n=2). NI= Not Inhibitory.*

MIC Cinnamaldehyde	MIC Citric	MIC Cinnamaldehyde + Citric	FIC
1.46	31.25	NI	-
MIC Cinnamaldehyde	**MIC Malic**	**MIC Cinnamaldehyde + Malic**	**FIC**
1.46	250	NI	-
MIC Cinnamaldehyde	**MIC Benzoic**	**MIC Cinnamaldehyde + Benzoic**	**FIC**
NI	125	0.73 + 31.25	-
MIC Cinnamaldehyde	**MIC Fumaric**	**MIC Cinnamaldehyde + Fumaric**	**FIC**
1.46	NI	NI	-
MIC Cinnamaldehyde	**MIC Lactic**	**MIC Cinnamaldehyde + Lactic**	**FIC**
NI	NI	1.82 + 250 1.46 + 500	-

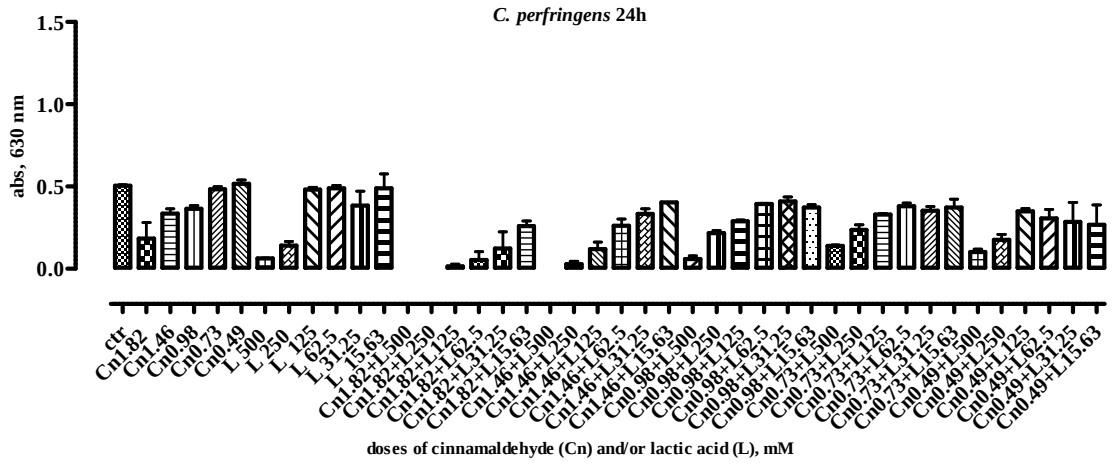

***Figure 3.3.** Absorbance (λ=630 nm) values of C. perfringens growth after 24h of incubation. Column bars indicate mean ± SD. Each column is the mean of two values. Cn= cinnamaldehyde; L= lactic acid.*

MIC of vanillin (tab. 3.4) was not determined because the highest concentration tested did not inhibit clostridia growth. For organic acid MIC values were determined for citric (62.50 mM), malic (250 mM) and benzoic (125 mM). When acids were associated with vanillin the effects on inhibition of clostridia were not significant. Only lactic acid in combination with vanillin at 250 or 500 mM (fig. 3.4) showed an inhibition of bacteria growth of 80%.

***Table 3.4**. Antibacterial effect of thymol and organic acids alone or in combination, against 10^4 CFU/ml of C. perfringens after 24h of incubation. MIC values are expressed as mM. (n=2).* NI = *Not Inhibitory.*

MIC Vanillin	**MIC Citric**	**MIC Vanillin + Citric**	**FIC**
NI	62.50	NI	-
MIC Vanillin	**MIC Malic**	**MIC Vanillin + Malic**	**FIC**
NI	250	NI	-
MIC Vanillin	**MIC Benzoic**	**MIC Vanillin + Benzoic**	**FIC**
NI	125	0.73 + 31.25	-
MIC Vanillin	**MIC Fumaric**	**MIC Vanillin + Fumaric**	**FIC**
NI	NI	NI	-
MIC Vanillin	**MIC Lactic**	**MIC Vanillin + Lactic**	**FIC**
NI	NI	NI	-

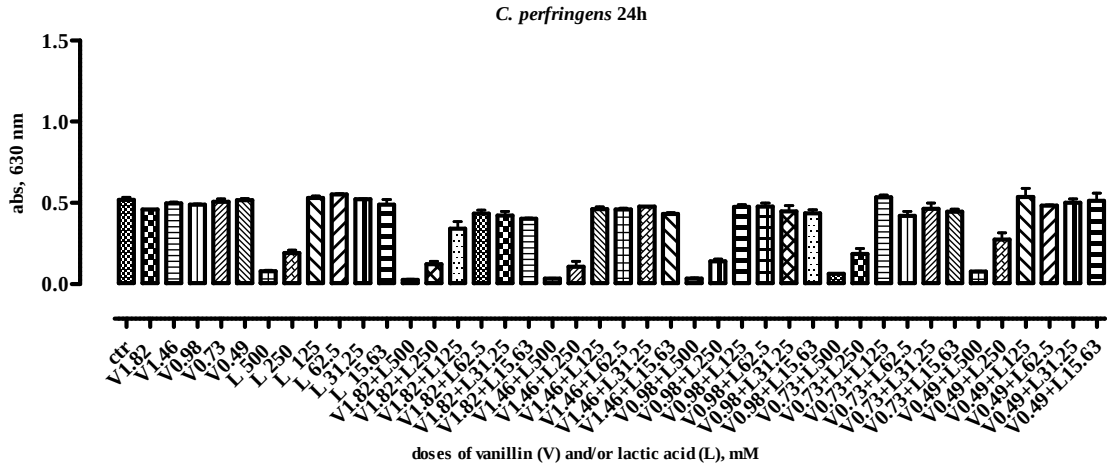

***Figure 3.4.** Absorbance (λ=630 nm) values of C. perfringens growth after 24h of incubation. Column bars indicate mean ± SD. Each column is the mean of two values. V= vanillin; L= lactic acid.*

MIC of limonene and organic acids were not determined because the highest concentration tested not inhibited *C. perfringens* growth. There were no differences in bacteria growth between the several concentrations of limonene tested. Only benzoic acid in association with limonene showed a dose-dependent reduction of bacteria growth probably because of benzoic acid inhibitory effect (fig. 3.5).

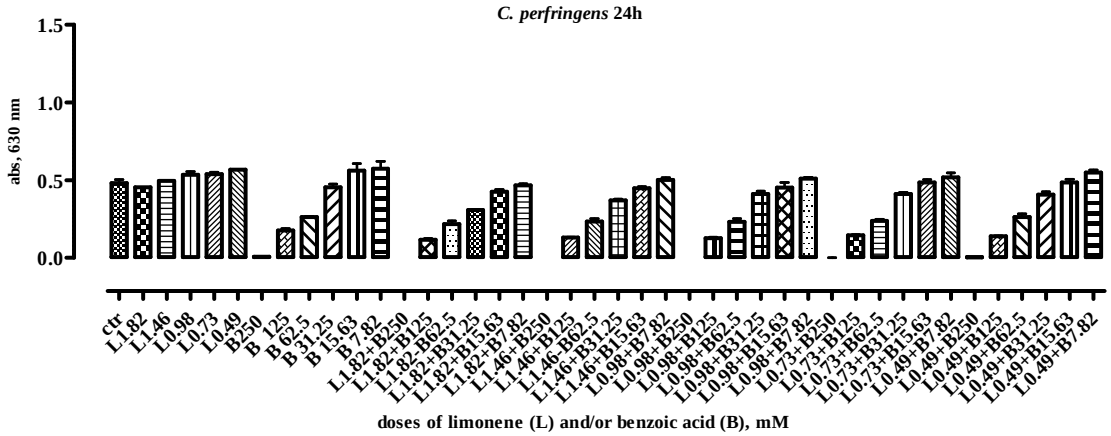

Figure 3.5. *Absorbance (λ=630 nm) values of C. perfringens growth after 24h of incubation. Column bars indicate mean ± SD. Each column is the mean of two values. L= limonene; B= benzoic acid.*

S. Typhmurium

MIC of carvacrol (tab. 3.5) was not determined because the highest concentrations tested were not effective in inhibiting *Salmonella* growth, but alone was responsible for a reduction of 60% in bacteria growth compared to control. For organic acid MIC value was determined only for benzoic at 62.5 mM. When carvacrol was associated with organic acid, *Salmonella* growth was inhibited as showed in table 3. In these cases *Salmonella* was inhibited at low acids concentration, while carvacrol concentration in association was high: two MIC of citric acid and carvacrol in combination was 7.82 and 1.82 mM, or 7.82 and 1.46 mM, respectively; one MIC of malic acid and carvacrol in combination was 15.63 and 1.82 mM, respectively; MIC of fumaric acid and carvacrol in combination was 3.91 and 1.82 mM, respectively. Citric and malic acids showed a *Salmonella* growth inhibition dose- dependent (fig. 3.6).

Table 3.5. *Antibacterial effect of carvacrol and organic acids alone or in combination, against 10^4 CFU/ml of S. Typhimurium after 24h of incubation. MIC values are expressed as mM. (n=2). ND= Not Inhibitory.*

MIC Carvacrol	**MIC Citric**	**MIC Carvacrol + Citric**	**FIC**
NI	NI	1.82 + 7.82 1.46 + 7.82 0.98 + 125 1.46 + 25 0.98 + 50	-
MIC Carvacrol	**MIC Malic**	**MIC Carvacrol + Malic**	**FIC**
NI	NI	1.82 + 15.63 1.46 + 62.5 0.98 + 250 0.73 + 500 0.49 + 500	-
MIC Carvacrol	**MIC Benzoic**	**MIC Carvacrol + Benzoic**	**FIC**
NI	62.5	1.46 + 31.25 0.98 + 31.25	
MIC Carvacrol	**MIC Fumaric**	**MIC Carvacrol + Fumaric**	**FIC**
NI	NI	1.82 + 3.91	-
MIC Carvacrol	**MIC Lactic**	**MIC Carvacrol + Lactic**	**FIC**
NI	NI	1.46 + 500	-

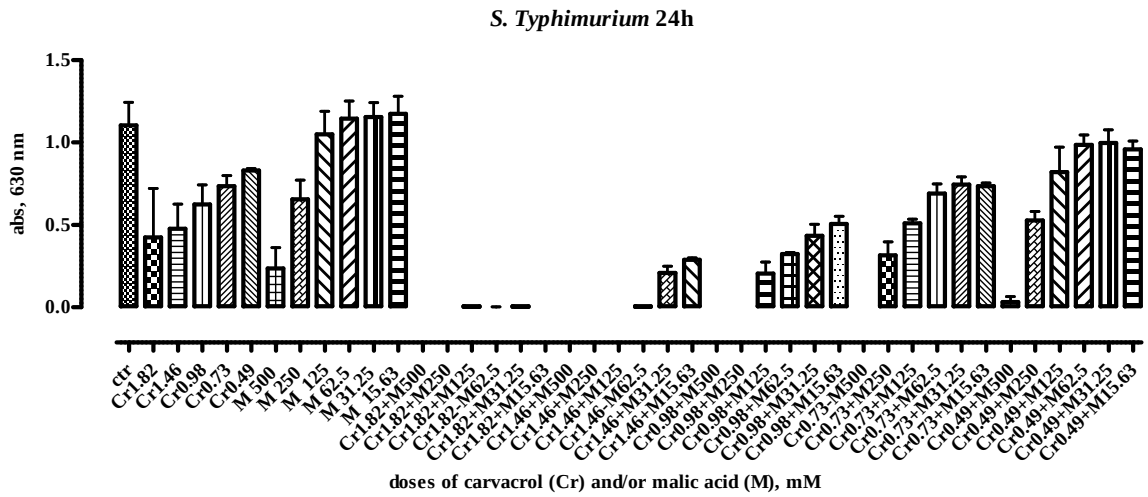

Figure 3.6. *Absorbance (λ=630 nm) values of C. perfringens growth after 24h of incubation. Column bars indicate mean ± SD. Each column is the mean of two values. Cr= carvacrol; C= citric acid; M= malic acid.*

As showed in table 3.6, MIC of thymol was 1.46 mM. For organic acids, only MIC of benzoic acid was determined at 62.5 mM. MIC of benzoic acid and thymol in combination was 31.25 and 0.98 mM, respectively. In this case FIC index was calculated and showed an additive effect between substances. *Salmonella* growth was inhibited by combination of high acids concentration and low thymol concentration: one MIC of malic acid and thymol in combination was 500 and 0.49 mM, respectively; MIC of lactic acid and thymol in combination was 500 and 0.98 mM, respectively.

Even if citric acid alone did not determined an inhibition of bacteria, when used in association with thymol bacteria growth was inhibited at low concentration: MIC of citric acid and thymol in combination was 31.25 and 0.98 mM, or 62.5 and 0.73 mM, respectively. Citric and malic acids showed a *Salmonella* growth inhibition dose-dependent (fig. 3.7).

Table 3.6. *Antibacterial effect of thymol and organic acids alone or in combination, against 10^4 CFU/ml of S. Typhimurium after 24h of incubation. MIC values are expressed as mM. (n=2). ND= Not Inhibitory.*

MIC Thymol	**MIC Citric**	**MIC Thymol + Citric**	**FIC**
1.46	NI	0.98 + 31.25 0.73 + 62.5 0.73 + 50 0.49 + 50	-
MIC Thymol	**MIC Malic**	**MIC Thymol + Malic**	**FIC**
1.46	NI	0.98 + 125 0.73 + 250 0.49 + 500	-
MIC Thymol	**MIC Benzoic**	**MIC Thymol + Benzoic**	**FIC**
1.46	62.5	0.98 + 31.25	1.2
MIC Thymol	**MIC Fumaric**	**MIC Thymol + Fumaric**	**FIC**
1.46	NI	NI	-
MIC Thymol	**MIC Lactic**	**MIC Thymol + Lactic**	**FIC**
1.46	NI	0.98 + 500	-

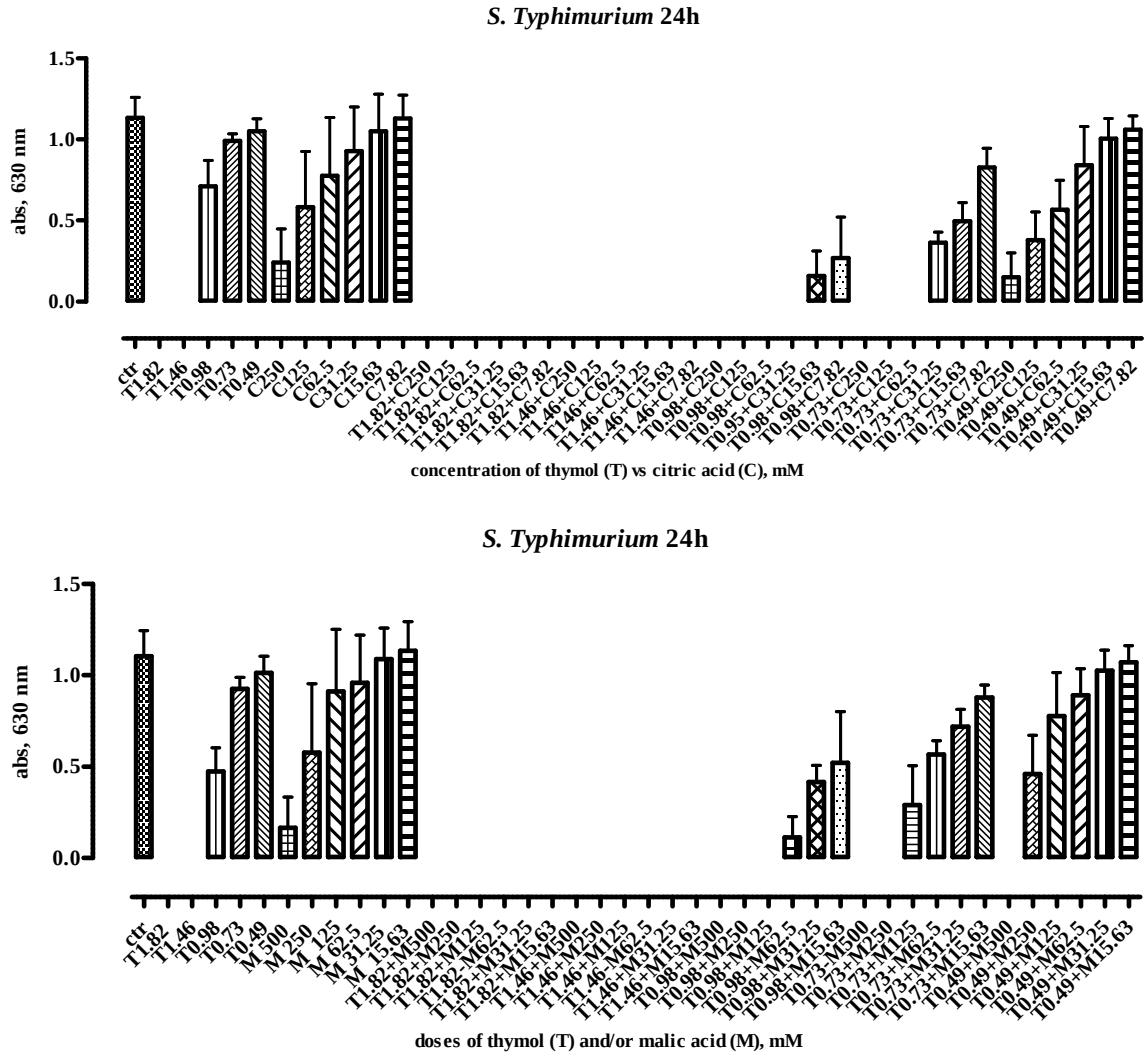

Figure 3.7. *Absorbance (λ=630 nm) values of C. perfringens growth after 24h of incubation. Column bars indicate mean ± SD. Each column is the mean of two values T= thymol; C= citric acid; M= malic acid..*

MIC of cinnamaldehyde was not determined because the highest concentration tested not inhibited *Salmonella* growth. For organic acids, MIC was determined for malic (500 mM) and benzoic acids (125 mM). When cinnamaldehyde was associated with organic acids, *Salmonella* growth was inhibited as showed in table 3.7.

Table 3.7. *Antibacterial effect of thymol and organic acids alone or in combination, against 10^4 CFU/ml of S. Typhimurium after 24h of incubation. MIC values are expressed as mM. (n=2). ND= Not Determined.*

MIC Cinnamaldehyde	MIC Citric	MIC Cinnamaldehyde + Citric	FIC
NI	NI	1.82 + 31.25 1.46 + 7.82 0.98 + 31.25 0.73 + 125	-
MIC Cinnamaldehyde	**MIC Malic**	**MIC Cinnamaldehyde + Malic**	**FIC**
NI	500	1.82 + 15.63 1.46 + 125 0.98 + 250 0.73 + 250	-
MIC Cinnamaldehyde	**MIC Benzoic**	**MIC Cinnamaldehyde + Benzoic**	**FIC**
NI	125	1.82 + 62.50 0.73 + 62.50	-
MIC Cinnamaldehyde	**MIC Fumaric**	**MIC Cinnamaldehyde + Fumaric**	**FIC**
NI	NI	1.82 + 7.82 1.46 + 125	-
MIC Cinnamaldehyde	**MIC Lactic**	**MIC Cinnamaldehyde + Lactic**	**FIC**
NI	NI	1.82 + 31.25 1.46 + 250	-

MIC of vanillin was not determined because the highest concentration tested not inhibited *Salmonella* growth. For organic acids, MIC was determined for malic (500 mM) and benzoic acids (125 mM). When vanillin was associated with organic acids, *Salmonella* growth not showed a significant reduction.

The same results were founded for limonene alone or in combination with organic acids.

3.4. Discussion

MIC at 24h were determined in experiments where OA and flavours were tested in combination against *Clostridium perfringens* and *Salmonella Typhimurium*. In particular, it was also possible to determine the MIC of substances that, when tested singularly, induced only a partial reduction in growth but not complete inhibition.

Substances that showed a MIC value when tested singularly, showed a reduction of the MIC value when tested in combination.

At considered concentrations of OA or flavours, showed a MIC value higher than the MIC value detected when the same substance was tested in combination with organic acids or flavours, respectively.

Carvacrol showed a higher inhibition of *C. perfringens* than thymol. Carvacrol showed a lower inhibition of *Salmonella* than thymol.

When carvacrol was tested in combination with OAs the result was a higher inhibition of *C. perfringens* and *Salmonella* than the inhibition induced by thymol in combination with the same organic acid.

Several studies reported bactericidal activity of plant extracts and organic acids on spoiling bacteria, moulds, and on pathogens such as *S. aureus*, *Salmonella* spp., *E. coli*, *Listeria monocytogenes* (Burt and Reinders, 2003; Lee *et al.*, 2004; Burt *et al.*, 2005; Peñalver *et al.*, 2005; Gutierrez *et al.*, 2008), but few authors studied antimicrobial activity of natural substances against *C. perfringens*.

Several studies disagree with the effects of flavours against gram-positive or gram-negative bacteria. Same authors demonstrated that different structural and chemical composition of the cells wall of gram-positive bacteria results more sensitive to antimicrobials action than gram-negative bacteria (Nikaido and Varra, 1985; Lis-Balchin, 2003). From data of Si *et al.* (2006) appears that flavours have a gram-negative bacteria specifically as target.

In these MIC experiment carvacrol had a strong antimicrobial activity against both *C. perfringens* and *Salmonella*. Data were confirmed by authors that obtained the same carvacrol antimicrobial activity against both gram-positive and gram-negative bacterial pathogens (Dorman and Deans, 2000; Lambert *et al.*, 2001; Peñalver *et al.*, 2005).

The hydrophobic constituents of essential oils are capable of accessing to the periplasm of gram-negative bacteria through the porin proteins of outer membrane (Helander *et al.*, 1998). Carvacrol and thymol can disrupt the outer membrane of bacteria, causing the release of membrane-associated material from the cell to the external medium and an increased permeability of the nucleus. It is thought that membrane perforation or binding is the main mode of action of such compounds (Shapiro and Guggenheim, 1995; Strauss and Hayler, 2001). Structural features such as the aromatic ring, or the presence of hydroxylic group (e.g. thymol and carvacrol) alter polarity and topography

of a molecule, therefore changing the affinity to different binding sites in the bacteria (Si *et al.*, 2006).

Even if carvacrol and thymol differ in the chemical structure only by the position of hydroxyl group, in the present study they showed a different antimicrobial action.

Ultee *et al.* (2002) hypothesized that the hydroxyl group and the presence of a system of delocalized electrons are important for the antimicrobial activity of phenolic compounds, such as carvacrol and thymol. Such a particular structure would allow compounds to act as proton exchanger, thereby reducing the gradient across the cytoplasmic membrane. The resulting collapse of the proton motrice force and depletion of the ATP pool lead eventually to cell death. The presence of free hydroxylic group play an important role on antimicrobial activity of carvacrol and thymol (Ben Arfa *et al.*, 2006). Other authors focused on the importance of position of the hydroxyl group. López *et al.* (2007) demonstrated the difference in activity between carvacrol and thymol against *S. choleraesuis*, and the position of the hydroxyl group seems to influence their activity against gram-negative bacteria.

Cinnamaldehyde showed an antimicrobial activity when associated with organic acids. Although cinnamaldehyde is known to be inhibitive to growth of *E. coli* O157:H7 and *S. typhimurium* at similar concentrations to carvacrol and thymol, it did not disintegrate the outer membrane or deplete the intracellular ATP pool. The carbonyl group is thought to bind to proteins, preventing the action of amino acid decarboxylases in *E. aerogenes* (Burt, 2004)

Vanillin and limonene did not showed a significant antibacterial effect alone or in combination with organic acids.

Organic acids resulted less effective than flavours if tested at the same molar concentration. The mode of action of organic acids is primarly associated to the fact that undissociated organic acid can penetrate the bacteria cell wall and disrupt the normal physiology of bacteria pH sensitive (Gauthier, 2002). After the undissociated acid passed through the bacterial cell wall, it dissociates, because of the inner cell pH, releasing H^+ and anion COO^-. The internal pH decreases and because pH sensitive bacteria do not tolerate large variation of cytoplasmic pH values, a specific mechanism (H^+-ATPase pump) acts to bring the pH inside the bacteria to a physiological level. This phenomenon requires energy and it can stop the growth of the bacteria or even kills them. A lower internal pH involves others mechanisms: inhibition of glycolysis, prevention of active transport, interference with signal transduction (Lambert and

Stratford, 1999; Gauthier, 2002). The anionic part of the acid is trapped inside the bacteria because it can not diffuse freely through the cell wall, and its accumulation becomes toxic (Roe *et al.*, 1998). Different bacteria showed different levels of sensitivity to different organic acids under specific circumstances.

The present study let us to conclude that the *in vitro* results of inhibition effect of a flavour, in particular carvacrol or thymol, and an organic acid in combination could be a promising strategy to reduce *C. perfringens* and *Salmonella* contamination.

4. ROLE OF SEVERAL ASSOCIATION OF ORGANIC ACIDS AND NATURE-IDENTICAL COMPOUNDS IN SWINE BATH-CULTURE TECNIQUES

4.1. Aim of the study

Purpose of this part of the study was to screen the role of citric and sorbic acids, thymol and carvacrol in modulating the intestinal microflora of pigs in an *in vitro* fermentation system (bath-culture techniques), which simulate the intestinal environment and microflora. In particular, the study focalized on the antimicrobial effects of these substances against *S. Typhimurium*.

A two step procedure was applied:

1. digestion of the feed through enzymatic reactions;
2. fermentation of digested diet with intestinal content, substances under investigations, and *S. Typhimurium*.

4.2. Materials and method

Digestion

A commercial standard diet for pigs was digested *in vitro* to simulate gastric and pancreatic digestion as described by Verveake *et al.* (1989).this was a stepwise procedure with an incubation of feed (25 g; particle size<1 mm) in 500 mL of pepsin solution (0.2% pepsin w/v, HCl 0.075 N; P7000 from porcine gastric mucosa; Sigma Chemical, St. Louis, MO, USA) in a shaking bath at 37°C for 4 h. at the end of the 4h incubation, the solution was adjusted to pH 7.5 with NaOH 0.1 N. in the second step, 500 mL of a pancreatin-$NaHCO_3$ mixture solution (10g/L w/v pancreatin of 1M $NaHCO_3$; P1500 from porcine pancreas; Sigma Chemical, St. Louis, MO, USA) was added and the mixture was reincubated for 4 h at 37°C to simulate pancreatic digestion. Composition of the phosphate buffer solution was as follows: 26.2 mM Na_2HPO4, 46.7 mM $NaHCO_3$, 3.3 mM NaCl, 3.1 mM KCl, 1.3 mM $MgCl_2$, 0.7 mM $CaCl_2$ (Martillotti *et al.*, 1987). After enzymatic digestion, the preparation was centrifuged (3,000 × g, 10 min, 4°C), washed twice with distilled water, recentrifuged (3,000 × g, 5 min, 4°C), and dried at 60°C overnight. The digested diet was used as the substrate in the *in vitro* fermentation studies.

Batch Culture Fermentations

Within 20 min after slaughter of pigs (six animals, 10 months old, live weight approximately 160 kg) cecal content were collected and kept in a sealed nylon bag at 39°C during transfer to the laboratory. Cecal content was diluited with buffer (1:3) and filtered through six layers of cheese cloth. The filtered liquid was used as inoculum. The buffer composition (McDougall, 1948) was as follows:

9.8 g $NaHCO_3$ + 0.57 g KCl + 0.079 g $CaCl_2 \cdot 6H_2O$ + 9.3 g $Na_2HPO_4 \cdot 12H_2O$ + 0.67 g NaCl + 0.12 $MgSO_4 \cdot 7 H_2O$ in 1 L of distilled water. Buffer pH was then adjusted to pH 6.7 by adding 3N HCl. The buffer solution kept at 39°C and flushed with CO_2 for 20 minutes before use. The inoculum was dispensed into five 10 mL glass syringes (5 mL of inoculum in each syringe) and five 50 mL vessels (previously flushed with CO_2, 25 mL of inoculum in each vessel) per treatment, containing 20 and 100 mg of pre-digested diet, respectively. Syringes and vessels were sealed and incubated at 39°C for 24 h.

Two experiments were performed to evaluate the antimicrobial activity against *Salmonella Typhimurium* of natural compounds. In the first, batch culture fermentations were performed in order to evaluate several combined doses of citric acid and thymol, or citric acid and carvacrol. The second was performed to evaluate several combined doses of sorbic acid and thymol, or sorbic acid and carvacrol.

In each experiment, eight dietary treatment were investigated in vessels. Of these, in both experiment, one was a control diet (CTR), and the other was a control diet inoculated with *Salmonella Typhimurium* H2662 DT104 (10^6 CFU/ml) (S).

In the first experiment, dietary treatments investigated were *Salmonella Typhimurium* infected control diet added with:

- citric acid and thymol (pH 6.7) at 62.50 and 2.72 mM, respectively (CT1);
- citric acid and thymol at 31.25 and 1.36 mM, respectively (CT2);
- citric acid and thymol at 15.63 and 0.68 mM, respectively (CT3);
- citric acid and carvacrol (pH 6.7) at 62.50 and 2.72 mM, respectively (CC1);
- citric acid and carvacrol at 31.25 and 1.36 mM, respectively (CC2);
- citric acid and carvacrol at 15.63 and 0.68 mM, respectively (CC3).

In the second experiment, dietary treatments investigated were *Salmonella Typhimurium* infected control diet added with:

- sorbic acid and thymol (pH 6.7) at 25 and 2.72 mM, respectively (ST1);
- sorbic acid and thymol at 12.50 and 1.36 mM, respectively (ST2);

- sorbic acid and thymol at 6.25 and 0.68 mM, respectively (ST3);
- sorbic acid and carvacrol (pH 6.7) at 25 and 2.72 mM, respectively (SC1);
- sorbic acid and carvacrol at 12.50 and 1.36 mM, respectively (SC2);
- sorbic acid and carvacrol at 6.25 and 0.68 mM, respectively (SC3).

Citric acid, sorbic acid, thymol, and carvacrol were purchased from Sigma-Aldrich (Chemie Gmbh, Steinheim, Germany).

Gas production was measured as described by Mencke *et al.* (1979), using 10 mL glass syringes and recording the cumulative volume of gas produced every 30 min. In syringes treatments were the same of vessels, excepted for treatment S and all treatments were not infected with *Salmonella*.

Samples of fermentation fluid were collected from each vessel at time 0, 4, 8, and 24 h after incubation in shaking bath for ammonia analysis and microbial counts of *Salmonella*; pH was determined at the end of the fermentation.

Chemical analyses of fermentation fluid and bacterial counts.

Ammonia in fermentation fluid and intestinal chymus was measured as described by Searcy *et al.* (1967).

Viable counts of *Salmonella* in fermentation samples were measured by plating serial 10-fold dilutions onto Brilliant Green Agar (OXOID, Bansingstoke, UK) according to he manufacturer conditions. There were five plate-replicates per treatment. Brilliant Green Agar were incubated for 24 h at 37°C under aerobic conditions.

Statistical analyses.

A modified Gompertz bacterial growth model (Zwietering *et al.*, 1992) was used to fit gas production data. This model assumes that substrate levels limit growth in a logarithmic relationship (Schofield *et al.*, 1994) as follows:

$$V = V_F \exp \{ - \exp [1 + (\mu_m e/V_F)(\lambda - t)]\},$$

where symbols have the meanings assigned by Zwietering *et al.* (1990): V= volume of gas produced at time t, t= fermentation time, V_f= maximum volume of gas produced, μ_m= maximum rate of gas production, which occurs at the point of inflection of the gas curve and λ= the lag time; as the time-axis intercept of a tangent line at the point of inflection.

The duration of the exponential phase was calculated from the parameters of the Gompertz equation, as suggested by Zwietering *et al.* (1992) with the following:

exponential phase (h) = V_F /(μ_m e){ 1 – ln[(3 -√5)/2]}.

Curve fitting was performed using the program GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Total gas production, maximum rate of gas production, duration of the exponential phase, ammonia, pH, bacterial counts, and SCFA data were analyzed by ANOVA using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA) in a completely randomized design. Each syringe and vessel formed the experimental unit. The differences among means of groups were analyzed using the Newmann-Keuls test. Differences were considered statistically significant at P<0.05.

4.3. Results

First trial: citric acid and thymol or carvacrol

Gas production curves were accurately described by the modified Gompertz model (r^2=0.98). Gompertz growth model showed that compared to control, all the blends of citric acid and carvacrol (CC1, CC2, CC3), and the blend of citric and thymol at 62.5 and 2.92 mM (CT1) and citric acid and thymol at 31.25 and 1.46 mM (CT2), significantly increased gas production by 60% (P<0.05) (tab. 4.1).

Maximum rate of gas production was significant high (P<0.05) for all treatments with additives.

The duration of the exponential phase did not exhibit any statistical difference.

Results suggested that citric acid stimulates intestinal fermentation, probably acting as substrate of microflora.

Ammonia concentration was measured in the fermentation fluid after 4 and 8h from the beginning of the experiment. After 4 and 8h of fermentation, no changes occurred in ammonia production (tab. 4.2).

Measurements of fermentation fluid pH from each treatment were assessed at the end of the experiment. The blend of citric acid and carvacrol at 15.63 and 0.68 mM (CC3) showed a pH value lower compared to control (-2.28%, P<0.05). While the blend with citric acid and thymol at 62.50 and 2.72 mM (CT1) and citric acid and carvacrol at

62.50 and 2.72 mM (CC1) showed a pH value significantly higher compared to CTR (+2.28% and 1.88%, respectively, P<0.05).

Microbial counts (tab 4.3) showed a significant lower number of *Salmonella* cells at 4 and 8h only in CTR group , in which diet was not infected with *Salmonella*, compared to the other treatments (-30%, P<0.05). At 24h, *Salmonella* growth was significant lower for treatments with citric acid and carvacrol at 62.50 and 2.72 mM (CC1, -26%, P<0.05), and citric acid and carvacrol at 31.25 and 1.36 mM (CC2, - 46%, P<0.05), compared to CTR. *Salmonella* counts for S group was numerically lower at 24h compared to the same group at the other time-points.

These results showed that carvacrol was more effective as antimicrobial additive, compared to thymol.

Table 4.1. *First trial. Modified Gompertz equation fitted to gas production data from the 24h in vitro incubation of swine intestinal inoculum. Values are indicated as means ± SD. Different letters within the same column are significantly different (P<0.05). CTR= basal diet; CT1= basal diet with citric acid at 62.50 mM and thymol at 2.72 mM; CT2= basal diet with citric acid at 31.25 mM and thymol at 1.36 mM; CT3= basal diet with citric acid at 15.63 mM and thymol at 0.68 mM; CC1= basal diet with citric acid at 62.50 mM and carvacrol at 2.72 mM; CC2= basal diet with citric acid at 31.25 mM and carvacrol at 1.36 mM, respectively; CC3= basal diet with citric acid at 15.63 mM and carvacrol at 0.68 mM;* V_f*= maximum volume of gas produced (mL);* μ_m*= maximum rate of gas production (mL* h^{-1}*); log phase= exponential phase (h).*

Treatment	V_f	μ_m	Log phase	n
CTR	2.94 ± 1.57 **a**	0.23 ± 0.11 **a**	11.80 ± 3.04	5
CT1	7.74 ± 2.08 **b**	0.57 ± 0.15 **b**	9.83 ± 5.73	4
CT2	6.21 ± 2.06 **b**	0.64 ± 0.18 **b**	8.76 ± 2.09	5
CT3	5.23 ± 1.72 **ab**	0.51 ± 0.17 **b**	10.13 ± 4.87	5
CC1	8.71 ± 0.79 **b**	0.67 ± 0.09 **b**	9.50 ± 5.58	4
CC2	6.76 ± 2.04 **b**	0.69 ± 0.09 **b**	8.90 ± 2.51	5
CC3	6.69 ± 2.45 **b**	0.76 ± 0.19 **b**	8.66 ± 3.79	5

Table 4.2. *First trial. Ammonia values (mmol L^{-1}) at 4 and 8h, and pH values at 24h of an in vitro incubation of swine intestine inoculum. Values are indicated as means ± SD. Different letters within the same column are significantly different (P<0.05). CTR= basal diet; S= basal diet infected with Salmonella Typhimurium (10^6 CFU/mL); CT1= infected basal diet with citric acid at 62.50 mM and thymol at 2.72 mM; CT2= infected basal diet with citric acid at 31.25 mM and thymol at 1.36 mM; CT3= infected basal diet with citric acid at 15.63 mM and thymol at 0.68 mM; CC1= infected basal diet with citric acid at 62.50 mM and carvacrol at 2.72 mM; CC2= infected basal diet with citric acid at 31.25 mM and carvacrol at 1.36 mM; CC3= infected basal diet with citric acid at 15.63 mM and carvacrol at 0.68 mM.*

Treatment	4h	8h	pH	n
CTR	20.30 ± 2.67	22.50 ± 2.60	6.67 ± 0.09 **b**	5
S	19.95 ± 4.29	23.24 ± 2.71	6.63 ± 0.07 **b**	5
CT1	24.14 ± 4.14	26.86 ± 6.36	6.82 ± 0.02 **c**	5
CT2	24.70 ± 2.22	29.35 ± 1.88	6.71 ± 0.01 **b**	5
CT3	22.48 ± 2.00	24.55 ± 3.16	6.71 ± 0.04 **b**	5
CC1	25.67 ± 4.47	29.12 ± 8.02	6.79 ± 0.06 **c**	5
CC2	22.25 ± 4.35	24.29 ± 5.85	6.63 ± 0.03 **b**	5
CC3	21.60 ± 2.29	29.22 ± 1.77	6.51 ± 0.03 **a**	4

Table 4.3. *First trial. Counts of Salmonella Typhimurium (log10 CFU mL^{-1}) at 4, 8 and 24h of an in vitro incubation of swine intestinal inoculum. Values are indicated as means ± SD. Different letters within the same column are significantly different (P<0.05). N.D.= under the detection limit. CTR= basal diet; S= basal diet infected with Salmonella Typhimurium (10^6 CFU mL^{-1}); CT1= infected basal diet with citric acid at 62.50 mM and thymol at 2.72 mM; CT2= infected basal diet with citric acid at 31.25 mM and thymol at 1.36 mM; CT3= infected basal diet with citric acid at 15.63 mM and thymol at 0.68 mM; CC1= infected basal diet with citric acid at 62.50 mM and carvacrol at 2.72 mM; CC2= infected basal diet with citric acid at 31.25 mM and carvacrol at 1.36 mM; CC3= infected basal diet with citric acid at 15.63 mM and carvacrol at 0.68 mM.*

Treatment	4h	n	8h	n	24h	n
CTR	4.84 ± 0.25 **a**	5	4.08 ± 0.92 **a**	5	3.17 ± 1.09 **b**	5
S	6.72 ± 0.19 **b**	3	7.06 ± 0.08 **b**	3	4.00 ± 0.28 **b**	5
CT1	7.53 ± 0.11 **c**	4	7.00 ± 0.14 **b**	3	4.77 ± 0.30 **c**	5
CT2	7.10 ± 0.15 **b**	3	6.82 ± 0.20 **b**	5	4.21 ± 0.23 **b**	5
CT3	7.06 ± 0.18 **b**	4	6.77 ± 0.21 **b**	4	3.55 ± 1.07 **b**	4
CC1	6.98 ± 0.26 **b**	4	N. D.	5	2.94 ± 0.75 **ab**	4
CC2	7.39 ± 0.32 **c**	5	6.84 ± 0.16 **b**	4	2.16 ± 0.25 **a**	5
CC3	7.32 ± 0.32 **c**	3	6.66 ± 0.15 **b**	3	3.95 ± 0.20 **b**	3

Second trial: sorbic acid and thymol or carvacrol

Gompertz growth model showed no statistically difference in gas production for the blends with sorbic acid and thymol (ST1, ST2, ST3) (tab. 4.4).

The treatment with sorbic acid and carvacrol at 25 and 2.72 mM (SC1) showed a reduction in gas production compared to CTR and blend with the same concentration of sorbic acid and thymol (ST1) (-32% and -40%, respectively).

Analysis of maximum rate of gas production reflected the same tendency of results obtained from analysis of gas volume. Treatment with sorbic acid and carvacrol at 25 and 2.72 mM (SC1) exhibited a maximum rate of gas production significant lower respect to ST1 and CTR (-57%, $P<0.05$).

The duration of exponential phase of the blend with sorbic acid and carvacrol at 25 and 2.72 mM was significant higher then CTR (+74%, $P<0.05$).

However results suggest that blend of sorbic acid and carvacrol at 25 and 2.72 mM (SC1) slowed down metabolic activity of bacteria, enhancing the duration of exponential phase, and decreasing gas production.

Ammonia (tab. 4.5) concentration was significantly increased after 4h by SC1 compared to CTR (+47%, $P<0.05$); after 8h ammonia concentration was significant enhanced by SC1 and ST1 when compared to CTR (+29% and +42%, respectively, $P<0.05$).

The treatments with sorbic acid and thymol at 25 and 2.72 mM and at 12.50 and 1.36 mM significantly lowed pH values when compared to control (6.6 versus 6.9, for ST1 and ST2 versus CTR, respectively, $P<0.05$).

Microbial counts after 4, 8, and 24h showed no significant difference among treatments (tab 4.6). After 4h, there were no difference between treatments with lower concentration of both essential oil (ST3 and SC3) and treatment with *Salmonella* infected control diet (S); while blends with thymol at 2.72 and 1.36 mM (ST1 and ST2, respectively) reduced *Salmonella* growth by 11%.

Microbial counts showed that only the blend with sorbic acid and carvacrol at 25 and 2.72 mM (SC1) inhibited Salmonella growth during 24h of fermentation.

Table 4.4. *Second trial. Modified Gompertz equation fitted to gas production data from the 24h in vitro incubation of swine intestinal inoculum. Values are indicated as means ± SD. Different letters within the same column are significantly different (P<0.05). CTR= basal diet; ST1= basal diet with sorbic acid at 25 mM and thymol at 2.72 mM; ST2= basal diet with sorbic acid at 12.50 mM and thymol at 1.36 mM; ST3= basal diet with sorbic acid at 6.25 mM and thymol at 0.68 mM; SC1= basal diet with sorbic acid at 25 mM and carvacrol at 2.72 mM; SC2= basal diet with sorbic acid at 12.50 mM and carvacrol at 1.36 mM, respectively; SC3= basal diet with sorbic acid at 6.25 mM and carvacrol at 0.68 mM;* V_f*= maximum volume of gas produced (mL);* μ_m*= maximum rate of gas production (mL* h^{-1}*); log phase= exponential phase (h).*

Treatment	V_f	μ_m	Log phase	n
CTR	5.09 ± 0.47 **ab**	0.49 ± 0.05 **b**	9.43 ± 1.17 **a**	5
ST1	5.84 ± 0.41 **b**	0.49 ± 0.10 **b**	8.84 ± 5.24 **a**	4
ST2	5.19 ± 0.51 a**b**	0.52 ± 0.02 **b**	9.03 ± 0.93 **a**	5
ST3	4.87 ± 0.52 **ab**	0.46 ± 0.03 **b**	9.63 ± 1.14 **a**	5
SC1	3.46 ± 0.82 **a**	0.21 ± 0.12 **a**	16.43 ± 4.68 **b**	5
SC2	5.46 ± 0.44 **b**	0.45 ± 0.04 **b**	10.83 ± 0.39 **a**	5
SC3	4.17 ± 2.07 **ab**	0.48 ± 0.22 **b**	8.33 ± 3.31 **a**	5

Table 4.5. *Second trial. Ammonia values (mmol* L^{-1}*) at 4 and 8h, and pH values at 24h of an in vitro incubation of swine intestine inoculum. Values are indicated as means ± SD. Different letters within the same column are significantly different (P<0.05). CTR= basal diet; S= basal diet infected with Salmonella Typhimurium (*10^6 *CFU/mL); ST1= infected basal diet with sorbic acid at 25 mM and thymol at 2.72 mM; ST2= infected basal diet with sorbic acid at 12.50 mM and thymol at 1.36 mM; ST3= infected basal diet with sorbic acid at 6.25 mM and thymol at 0.68 mM; SC1= infected basal diet with sorbic acid at 25 mM and carvacrol at 2.72 mM; SC2= infected basal diet with sorbic acid at 12.50 mM and carvacrol at 1.36 mM; SC3= infected basal diet with sorbic acid at 6.25 mM and carvacrol at 0.68 mM.*

Treatment	4h	n	8h	n	pH	n
CTR	8.68 ± 0.93 **a**	5	9.72 ± 1.70 **ab**	5	6.88 ± 0.15 **b**	5
S	11.23 ± 0.48 **ab**	5	8.06 ± 1.66 **a**	5	6.79 ± 0.04 **b**	5
ST1	11.74 ± 2.95 **ab**	5	13.83 ± 0.97 **c**	5	6.59 ± 0.04 **a**	5
ST2	8.52 ± 2.93 **a**	5	11.03 ± 1.18 **ab**	5	6.59 ± 0.10 **a**	5
ST3	10.29 ± 1.05 **ab**	5	9.69 ± 0.78 **b**	5	6.75 ± 0.13 **b**	5
SC1	12.77 ± 0.78 **b**	5	12.52 ± 1.43 **c**	5	6.83 ± 0.03 **b**	5
SC2	8.05 ± 2.98 **a**	5	10.14 ± 2.07 **ab**	4	6.88 ± 0.07 **b**	5
SC3	11.28 ± 1.77 **ab**	5	10.55 ± 1.12 **ab**	5	6.85 ± 0.02 **b**	5

Table 4.6. *Second trial. Counts of Salmonella Typhimurium (log10 CFU mL^{-1}) at 4, 8 and 24h of an in vitro incubation of swine intestinal inoculum. Values are indicated as means ± SD. Different letters within the same column are significantly different (P<0.05). N.D.= under the detection limit. CTR= basal diet; S= basal diet infected with Salmonella Typhimurium (10^6 CFU/mL); ST1= infected basal diet with sorbic acid at 25 mM and thymol at 2.72 mM; ST2= infected basal diet with sorbic acid at 12.50 mM and thymol at 1.36 mM; ST3= infected basal diet with sorbic acid at 6.25 mM and thymol at 0.68 mM; SC1= infected basal diet with sorbic acid at 25 mM and carvacrol at 2.72 mM; SC2= infected basal diet with sorbic acid at 12.50 mM and carvacrol at 1.36 mM; SC3= infected basal diet with sorbic acid at 6.25 mM and carvacrol at 0.68 mM.*

Treatment	4h	n	8h	n	24h	n
CTR	4.52 ± 0.82	4	4.19 ± 0.58	3	3.67 ± 0.21	2
S	5.20 ± 0.76	5	4.49 ± 0.59	5	3.57 ± 0.79	2
ST1	4.61 ± 0.81	5	4.82 ± 0.86	5	3.61 ± 0.63	5
ST2	4.59 ± 0.30	5	4.99 ± 0.50	5	3.73 ± 0.61	4
ST3	5.33 ± 0.50	5	4.95 ± 0.44	5	3.41 ± 0.24	3
SC1	N. D.	5	N. D.	5	N. D.	5
SC2	5.43 ± 0.60	5	4.93 ± 0.46	5	3.32 ± 0.71	2
SC3	5.19 ± 0.25	5	4.92 ± 0.31	3	3.98 ± 0.34	5

4.4. Discussion

Comparison between *in vitro* fermentation with two organic acids showed that sorbic acid inhibited fermentation activity, as indicated by maximum gas production, and *Salmonella* growth at the highest concentration tested.

Carvacrol and thymol have the same chemical structure. The only difference is OH^- position on the phenolic ring, and probably for this reason this compounds showed different antimicrobial activity.

In particular, carvacrol in combination with an organic acid is more active against *Salmonella* compared to thymol.

Syringes showed the ability of citric acid at different concentrations to stimulate fermentation activity of cecal microflora, resulting in a significant increase in the gas production in all treatments in which this acid was present.

In caecum, citric acid represented a substrate for microflora. It is known that several bacterial strains can use citric acid as an energy source.

Modulate the activity of microflora can improve available energy and nutrients, and reduce production of toxic substances.

Sorbic acid was more effective to contain the gas production and, combined with carvacrol, has allowed a general slowdown in fermentation.

Antibacterial activity of substances against *Salmonella Typhimurium* was analyzed within vessels: carvacrol was more active in combination with citric acid than with sorbic acid.

Combination of citric acid 31.25 mM and carvacrol 1.36 mM showed a reduction of *Salmonella* growth of 46%.

Total inhibition of *Salmonella* growth was achieved in treatments with 25 mM sorbic acid plus 2.72 mM carvacrol.

These results, particularly the second one, suggest that the use of carvacrol in combination with an OA could represent an effective approach to contain *Salmonella* gastrointestinal infections in pigs.

5. ANTICLOSTRIDIAL MICROENCAPSULATED BLEND OF ORGANIC ACIDS AND NATURAL IDENTICAL FLAVOURS IN A BROILERS CHALLENGE STUDY

5.1. Aim of the study

The aim of this study was to investigate the efficacy of a microencapsulated blend of organic acid and natural identical flavours as a tool to contain negative effects, in terms of health and loss of performance, associated to *Clostridium* proliferation in the intestine of broilers in the absence of antibiotic growth promoters.

5.2. Materials and method

Bacterial cultures and growth conditions

Throughout the study, *C. perfringens* type A (ATCC 13124) was revitalized and subcultured in reinforced clostridial broth (Oxoid Ltd.) in anaerobic conditions at 37°C for 24 h.

***Table 5.1**. Basal diet composition and chemical analysis (% as fed basis).*

	Floor pen study	
	I phase (0-14 d)	II phase (15-42 d)
Ingredients		
Soft wheat meal	39.0	40.0
Soybean meal	40.0	35.0
Corn meal	13.9	16.5
Soybean Oil	3.2	5.0
Dicalcium phosphate	1.9	1.9
Calcium carbonate	0.6	0.5
Vitamins and minerals[1]	0.5	0.5
Salt (NaCl)	0.2	0.2
Lysine HCL	0.16	-
Methionine DL	0.3	0.2
Sodium bicarbonate	0.15	0.15
Analyzed Nutrients		
Dry matter	87.91	89.55
Crude protein	25.08	21.71
Ether extract	5.30	6.75
Crude fibre	2.85	2.65
Ash	5.99	5.76
Metabolizable Energy Kcal/kg	2838	2920

[1] providing per kg: vit. A: 2.500.000 UI; cholecalciferol: 15 mg; vit. E: 15.000 UI; vit. K: 1.200 mg; vit. B1 : 400 mg ; vit. B2 : 1.600 mg ; Pantothenic acid: 2.500 mg; vit. B6: 1.200 mg; Biotin: 30 mg; Folic acid: 250 mg; vit. C: 20.000 mg; vit. PP : 8.000 mg ; vit. B12 : 6 mg ; Cu : 1.000 mg ; Fe : 10.000 mg ; Mn : 30.000 mg ; Se : 40 mg ; Zn : 15.000 mg ; I : 200 mg ; Co : 40 mg.

Animals and Diets.

Two-hundred and sixteen male broilers Ross 508 1-day-old (34.7 ± 2.2 g) were randomly allocated in 18 pens divided in 3 experimental groups (d0): a negative control group (CTR) fed the basal diet (tab. 5.1); a group inoculated with *C. perfringens* (CP); a group (AVI) inoculated with *C. perfringens*, and fed a diet supplemented with 300 ppm of a microencapsulated blend of organic acid and nature-identical compounds (EP 1391155B1; Vetagro S.p.A., Italy).

All diets were formulated as isoproteic and isoenergetic, without antibiotic growth promoters and coccidiostatic drugs. Birds were fed *ad libitum* for 42 days. On day 0, 14, and 42 since the beginning of the study, BW and ADFI were recorded. ADG and FCR during the periods 0-14, 15-42 and 0-42 days, were calculated.

The study was conducted in the research facilities of CERZOO (Piacenza, Italy), which is Good Laboratory Practices-certified. The ethical committee of the University of Bologna reviewed and approved the experimental protocol.

Evaluation of Growth Performance.

On d 0, 14, and 42 since the beginning of the study, BW and ADFI were recorded. Average daily gain and FCR during the periods of 0 to 14, 15 to 42, and 0 to 42 d were calculated.

Fecal and Intestinal Sampling.

Excreta from 5 birds/pen were sampled on d 11, 12, and 13, and on each day, the 5 birds/pen samples were pooled to have 1 sample per pen. Samples were then plated on to violet red bile, Rogosa, and tryptose-sulfite-cycloserine agar for the enumeration of coliforms, LAB, and *C. perfringens*, respectively. On d 14, twelve birds per treatment (2 birds/pen) were killed and analyzed for intestinal lesions and *C. perfringens* colonic counts. On d 42, twenty-four birds per treatment (4 birds/pen) were slaughtered, and ileum and cecum were sampled for the enumeration of coliforms, LAB, and *C. perfringens* as described previously.

Statistical Analysis.

The pen was the experimental unit for growth performance and fecal counts, whereas each animal was the experimental unit for intestinal counts. Data were analyzed with one-way ANOVA, followed by Fisher post-test to compare the means of groups (SAS Software, release 2002-2003, SAS Institute, Milan, Italy). Differences were stated as significant at $P \leq 0.05$.

5.3. Results

Health Status of Animals, Intestinal Lesions, and Bacterial Counts.

Mortality was 4.2% and 6.9% for CP and AVI groups, respectively, whereas it was null for the CTR unchallenged animals. Although in dead animals there were no clinical signs and typical lesions of NE, it could be presumed that the bacterial challenge may have had a negative influence on broiler health status. The necroscopic examination of animals slaughtered at 14d and 42d did not show typical lesions of NE. No statistical differences were found among treatments for coliforms, LAB and *C. perfringens* in excreta, colon, and ileum counts. Statistical differences were found among feeding treatments for lactic acid bacteria counts in caecum content at 42d: the values are lower in CP group vs. CTR and AVI groups (-4.56% and -4.65%, respectively; P=0.0455).

***Table 5.2**. BW, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion rate (FCR) of broilers.*

	Treatment[1]			Statistics[2]	
Item	CTR	CP	AVI	SE	P-value
Initial BW, g	34.7	35.2	34.7	0.22	0.19
I phase (0-14d)					
ADFI, g	39.4	37.5	38.8	2.36	0.84
ADG, g	16.7	16.5	16.0	0.45	0.55
FCR	2.38	2.30	2.43	0.19	0.90
14 d BW, g	269	266	273	6.43	0.72
II phase (15-42d)					
ADFI, g	139.4[b]	133.3[ab]	129.3[a]	2.56	0.04
ADG, g	71.1	68.0	70.6	1.04	0.11
FCR	1.96	1.96	1.83	0.05	0.11
Final BW, g	2266	2244	2254	29.16	0.86
Overall (0-42d)					
ADFI, g	101.9[b]	96.8[ab]	93.9[a]	2.00	0.04
ADG, g	50.7	48.4	49.2	0.85	0.18
FCR	2.01	2.01	1.91	0.05	0.30
EPEF[*]	268.42	254.76	260.12	-	-

[a,b] Means with different superscript within the same row differ significantly (P<0.05).
[1] CTR = negative control group; CP = *C. perfringens* challenged control group; AVI= *C. perfringens* challenged group fed diet supplemented with a microencapsulated blend of organic acids and nature-identical compounds (produced by Vetagro S.p.A., Italy).
[2] n = 6; SE= Standard error.
[*] EPEF (European Production Efficency Factor)= [BW(kg) * Liveability(%)/Age at depletion (days) * F:G]*100

Growth performance.

Data are shown in table 5.2. No statistical differences were found among treatments for ADFI, ADG, and FCR ratio. In the 2nd phase, ADFI was significantly lower for AVI vs CTR (-7.25%; P=0.04). During the same period, ADG was numerically lower in CP group *vs.* CTR and AVI groups (68.0g vs. 71.1g and 70.6g, respectively; P=0.11); FCR ratio was numerically lower in AVI group *vs.* CTR and CP groups (1.83 *vs.* 1.96 and 1.96, respectively; P=0.11). Overall, ADFI was significantly lower for AVI group compared to CTR group (-7.85%; P=0.04), with no significant changes in overall ADG, FCR, and final BW, even if FCR was numerically lower for AVI group compared to CTR and CP (1.91 *vs.* 2.01, respectively). EPEF showed a value numerically higher for AVI compared to CP (260.12 *vs.* 254.76, respectively)

5.4. Discussion

Subacute enteritis is very frequent, with great economic implication associated to lower digestion and absorbtion, feed efficiency and impairment of growth performance (Kaldhusdal *et al.*, 2001; Loveland and Kaldhusdal, 2001; Hofacre *et al.*, 2003; Van Immerseel *et al.*, 2004).

This study aimed to induce subacute necrotic enteritis in broiler chickens, in order to understand if the supplementation of a microencapsulated blend of organic acid and nature-identical compounds could restore growth performance to regular levels.

No effect was found in the number of viable *C. perfringens* cells in the intestines of slaughtered chickens, but the number of lactic acid bacteria in CTR and AVI was higher than CP (data not showed). This might be explained by considering the competitive effect of *in vivo* intestinal microbial content. Probably microbial competition can be restore by AVI, that could have other bacterial target and could stimulate proliferation of lactic acid bacteria, instead to directly reduce *C. perfringens* proliferation.

During the second phase, and in overall study, animals feeding AVI showed lower ADFI compared to CTR (7%). These results can be caused by challenge with *C. perfringens*, because CP group showed lower values compared to CTR (5%), even if these data were no statistically significant.

ADFI differences had no effect on final body weight of birds, that showed no statistical difference among treatment. FCR among groups showed no statistical differences, even

if FCR was numerically lower for AVI (-5%) compared to CP and CTR. These results evidenced that the use of a microencapsulated blend of organic acids and flavours helped birds to restore weight to levels comparable to unchallenged animals. The blend used probably acts on intestinal microflora, stimulating positive bacteria such as lactic acid bacteria and increasing the competitiveness for the environment. Restore normal performance conditions results in decreasing of economic lost for the farmer as indicated by higher EPEF of AVI group compared to CP (+2%) .

6. PEDIOCIN A IMPROVES GROWTH PERFORMANCE OF BROILERS CHALLENGED WITH CLOSTRIDIUM PERFRINGENS

6.1. Aim of the study

The aim of this study was to investigate the efficacy of the anticlostridial pediocin A from *P. pentosaceus* FBB61 (WO/2004/087189; Piva and Casadei, 2004) as a tool to contain negative effects, in terms of health and loss of performance, associated to *Clostridium* proliferation in the intestine of broilers in the absence of antibiotic growth promoters, through 2 subsequent investigations (Grilli *et al.*, 2009).

6.2. Materials and method

Bacterial cultures and growth conditions

Throughout the studies, *P. pentosaceus* FBB61 (ATCC 43200) and its isogenic mutant were revitalized and subcultured in M17 broth (Oxoid Ltd., Basingstoke, UK) supplemented with 1% (wt/vol) glucose at 34°C for 18 h, whereas *C. perfringens* type A (ATCC 13124) was revitalized and subcultured in reinforced clostridial broth (Oxoid Ltd.) in anaerobic conditions at 37°C for 24 h.

Expression and purification of Pediocin A

Semipurified pediocin A for antimicrobial assay was obtained as described previously by Casadei *et al.* (2009). For the *in vivo* studies, pediocin A preparation was obtained as follows. A *P. pentosaceus* FBB61 cultural broth was collected to define the sample titer of pediocin A and bacteria concentration. The so-obtained broth was used in the appropriate amount for the floor pen study, whereas for the pilot study, the cultural broth underwent a purification step through centrifugation at 16,500 × *g* at 4°C for 10 min. Supernatant was collected and filtered with Stericup (Millipore Corporation, Bedford, MA) through a membrane with pores of 0.45-μm diameter and then titered for pediocin A. Titration of activity of pediocin A was performed through an agar spot test technique. Briefly, from each supernatant filtered produced, 20-μL aliquots were delivered into wells of a M17 agar plate previously seeded with 20 μL of *P. pentosaceus* FBB61-2 (Daeschel and Klaenhammer, 1985) fresh overnight culture and incubated at 39°C overnight. The sample titer [activity units (AU)/mL] was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn.

Antimicrobial Assay

An antimicrobial assay was conducted to assess the most effective dose of pediocin A against *C. perfringens* to be used in *in vivo* challenge studies. The minimal inhibitory concentration (MIC) of pediocin A against *C. perfringens* type A (ATCC13124) was determined using a broth dilution method. The test was performed in disposable tubes containing two-fold dilutions (320 to 0.6 activity units AU/ml) of semi purified pediocin A in a reinforced clostridial broth (Oxoid Ltd, Basingstoke, UK). An overnight culture of *C. perfringens* was prepared and adjusted so that the final concentration in each tube was approximately 10^4 CFU/ml. The tubes were incubated anaerobically at 37°C for 24 and 48 h. Bacterial growth was indicated by the presence of turbidity in the tube and measured by optical density (OD) at 600 nm (UltroSpec 3000 Pharmacia Biotech, Biochrom, Ltd, Cambridge, UK). The MIC was determined as the first tube, in ascending order, were the OD was 0.00. To confirm MIC, 100 µl of broth from each tube after 48 h of incubation was plated onto RCM agar. After 24 h of incubation the growth of viable cells was observed. The MIC was the lowest concentration which resulted in significant decrease in OD values where >99.9% or more of the initial inoculum was killed (Cosentino *et al.*, 1999).

Pilot Study

Birds and Diets.

Thirty-six female broilers Ross 508 (44.8 ± 1.8 g) were obtained 1-day hatch, and 12 chickens were placed in each of 3 150x60cm isolation units equipped with bedding straw, drinkers, heating lamps, and a filtered air supply. Each isolation unit was assigned to one of the 3 experimental groups: a negative control (CTR), fed basal diet (tab. 6.1); a positive control, fed the same diet added with supernatant filtrate of a cultural broth of the isogenic mutant *P. pentosaceus* FBB61-2 devoid of pediocin A expression (Bac-); a treated group (Bac+), fed the control diet supplemented with supernatant filtrate of a cultural broth of *P. pentosaceus* FBB61 (Bac+). In Bac+ group, pediocin A was provided at 80 AU/g of feed. All birds were challenged with C. perfringens.

Birds were fed *ad libitum* for 21 days. On day 0, 9, 14, and 21 from the beginning of the study, BW and average daily feed intake (ADFI) were recorded. Average daily gain (ADG), and feed conversion rate (FCR) during the periods 0-9, 0-14, 15-21, 0-21 days,

were calculated. The study was conducted in the facilities of the University of Bologna, whose ethical committee reviewed and approved the experimental protocol.

Microbial challenge

On day 9, animals were challenged by intracrop administration of vaccinal oocysts at a dose 50 times higher than the recommended one to produce a mild intestinal coccidiosis, and favour C. perfringens infection (Shane *et al.*, 1985).

A fresh overnight culture of *C. perfringens* type A was administered *per os* on day 14, 15 and 16, twice daily (10^6 CFU/bird).

Evaluation of Growth Performance.

On d 0, 9, 14, and 21 from the beginning of the study, BW and ADFI were recorded. Average daily gain and feed conversion rate (FCR) during the periods of 0 to 9, 0 to 14, 15 to 21, and 0 to 21 d were calculated.

Fecal and Intestinal Sampling.

One pool of feces was collected from each isolator at 11, 12, 13, and 14 d, to perform oocyst counts with an optical microscope. At 21 d, 5 birds per group were killed, analyzed for intestinal lesions, and ileal contents were sampled and plated on to violet red bile, Rogosa, and tryptosesulfite- cycloserine agar (Oxoid Ltd.) for enumeration of coliforms, lactic acid bacteria (LAB), and *C. perfringens*, respectively.

Statistical Analysis.

The isolator was the experimental unit for ADFI and FCR calculations, and for oocysts shedding. These data did not undergo statistical analysis, whereas each animal was the experimental unit for BW, ADG calculations, and microbiological counts, and were analyzed by 1-way ANOVA followed by Tuckey post-test (Graphpad Software 4.1, San Diego, CA). Differences were stated as significant at $P < 0.05$.

***Table 6.1**: Basal diet composition and chemical analysis (% as fed basis).*

	Pilot study	Floor pen study	
		I phase (0-14 d)	II phase (15-42 d)
Ingredients			
Soft wheat meal	35.0	39.0	40.0
Soybean meal	30.0	40.0	35.0
Corn meal	27.2	13.9	16.5
Soybean Oil	3.0	3.2	5.0
Dicalcium phosphate	2.0	1.9	1.9
Calcium carbonate	1.3	0.6	0.5
Vitamins and minerals[1]	0.5	0.5	0.5
Salt (NaCl)	0.3	0.2	0.2
Lysine HCL	0.3	0.16	-
Methionine DL	0.3	0.3	0.2
Sodium bicarbonate	0.1	0.15	0.15
Analyzed Nutrients			
Dry matter	88.69	87.91	89.55
Crude protein	22.22	25.08	21.71
Ether extract	5.43	5.30	6.75
Crude fibre	3.08	2.85	2.65
Ash	6.19	5.99	5.76
Metabolizable Energy Kcal/kg	2990	2838	2920

[1] providing per kg: vit. A: 2.500.000 UI; cholecalciferol: 15 mg; vit. E: 15.000 UI; vit. K: 1.200 mg; vit. B1 : 400 mg ; vit. B2 : 1.600 mg ; Pantothenic acid: 2.500 mg; vit. B6: 1.200 mg; Biotin: 30 mg; Folic acid: 250 mg; vit. C: 20.000 mg; vit. PP : 8.000 mg ; vit. B12 : 6 mg ; Cu : 1.000 mg ; Fe : 10.000 mg ; Mn : 30.000 mg ; Se : 40 mg ; Zn : 15.000 mg ; I : 200 mg ; Co : 40 mg.

Floor Pen Study

Animals and Diets.

Two-hundred and sixteen male broilers Ross 508 1-day-old (34.7 ± 2.2 g) were randomly allocated in 18 pens divided in 3 experimental groups (d0): a negative control group (CTR) fed the basal diet (tab. 6.1); a group inoculated with *C. perfringens* (CP); a group (PA) inoculated with *C. perfringens*, and fed a diet supplemented with *P. pentosaceus* FBB61 (10^7 CFU/g), and pediocin A, providing 60 and 40 AU/g fed in the 1st and 2nd phase diets (0-14d; 15-42d), respectively. All diets were formulated as isoproteic and isoenergetic, without antibiotic growth promoters and coccidiostatic drugs. Birds were fed *ad libitum* for 42 days. On day 0, 14, and 42 since the beginning of the study, BW and ADFI were recorded. ADG and FCR during the periods 0-14, 15-42 and 0-42 days, were calculated.

The study was conducted in the research facilities of CERZOO, which is Good Laboratory Practices-certified. The ethical committee of the University of Bologna reviewed and approved the experimental protocol.

Evaluation of Growth Performance.

On d 0, 14, and 42 since the beginning of the study, BW and ADFI were recorded. Average daily gain and FCR during the periods of 0 to 14, 15 to 42, and 0 to 42 d were calculated.

Fecal and Intestinal Sampling.

Excreta from 5 birds/pen were sampled on d 11, 12, and 13, and on each day, the 5 birds/pen samples were pooled to have 1 sample per pen. Samples were then plated on to violet red bile, Rogosa, and tryptose-sulfite-cycloserine agar for the enumeration of coliforms, LAB, and *C. perfringens*, respectively. On d 14, twelve birds per treatment (2 birds/pen) were killed and analyzed for intestinal lesions and *C. perfringens* colonic counts. On d 42, twenty-four birds per treatment (4 birds/pen) were slaughtered, and ileum and cecum were sampled for the enumeration of coliforms, LAB, and *C. perfringens* as described previously.

Statistical Analysis.

The pen was the experimental unit for growth performance and fecal counts, whereas each animal was the experimental unit for intestinal counts. Data were analyzed with 1-way ANOVA, followed by Fisher post-test to compare the means of groups (SAS Software, release 2002-2003, SAS Institute, Milan, Italy). Differences were stated as significant at $P \leq 0.05$.

6.3. Results

The MIC of pediocin A against 10^4 CFU/ml of *C. perfringens* was 20 AU/ml and 40 AU/ml after 24 and 48 h of incubation, respectively.

Pilot Study

The MIC of pediocin A against 10^4 CFU/ml of *C. perfringens* was 20 AU/ml and 40 AU/ml after 24 and 48 h of incubation, respectively.

Health Status of Animals, Intestinal Lesions, Oocyst Shedding, and Bacterial Counts.

Only one animal died in the CTR group, but the cause of death was not associated to NE, but with colibacillosis. Macroscopic evaluation of intestinal mucosa showed specks of blood with focal distribution throughout the intestine, as well as hemorrhage areas. Generally, there was a lack of marked differences between the Bac+ and Bac- groups. No brownish or diphteric pseudo-membranes were observed. Gram stained smears of intestinal mucosa demonstrated rod-shaped bacteria with typical *C. perfringens*

morphology. Oocyst shedding was less pronounced for Bac+ and Bac- groups than for CTR throughout the collection days. There were no statistical differences among treatments for coliforms, LAB and *C. perfringens* in ileal samples.

Growth Performance.

Data are showed in table 2. During the 1st period (0-9d), ADG was significantly higher for Bac+ vs. CTR and Bac- (+31% and +21% vs. CTR and Bac-, respectively; P < 0.01), and consequently, BW was higher for Bac+ vs. CTR and Bac- (+24% and +17% vs. CTR and Bac-, respectively; P < 0.01). On d14, ADG was still higher for Bac+ compared to CTR (+23%, P = 0.02), and BW was higher for Bac+ and Bac- compared to CTR (+23% and +14% respectively; P = 0.02). No statistical differences were found among treatments for growth performance on d21. EPEF value was numerically higher for Bac+ compared to CTR (+3%).

***Table 6.2**: BW, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion rate (FCR) of broilers in the pilot study*

	Treatment[1]			Statistics[2]	
Item	CTR	Bac-	Bac+	SEM	P-value
Initial BW, g	44.8	43.2	45.4	1.60	0.24
0-9d					
ADFI, g	27.41	23.70	27.41	-	-
ADG, g	15.45^{b}	16.79^{b}	20.31^{a}	1.85	<0.01
FCR	1.34	1.10	1.08	-	-
9d BW, g	183.8^{b}	194.3^{b}	228.3^{a}	14.24	<0.01
0-14d					
ADFI, g	34.29	33.45	33.69	-	-
ADG, g d	21.20^{b}	24.20^{ab}	26.07^{a}	2.28	0.02
FCR	1.62	1.38	1.29	-	-
14d BW, g	296.8^{b}	338.8^{a}	365.0^{a}	27.29	0.02
15-21d					
ADFI, g	73.11	70.24	69.23	-	-
ADG, g	44.24	40.55	39.77	8.58	0.75
21d BW, g	606.5	622.7	642.9	44.89	0.59
Overall 0-21d					
ADFI, g	42.43	42.74	44.03	-	-
ADG, g	26.75	27.59	28.47	2.86	0.71
FCR	1.60	1.44	1.44	-	-
EPEF*	180.5	205.9	212.7	-	-

[a,b]Means with different superscript within the same row differ significantly (P < 0.05).

[1]CTR = basal diet; Bac- = basal diet with supernatant filtrate of the isogenic mutant of *P. pentosaceus* FBB61-2 non producing pediocin A; Bac+ = basal diet with supernatant filtrate of *P. pentosaceus* FBB61 producing pediocin A.

[2]For BW and ADG, n = 12; for FI and FCR, n = 1.

* EPEF (European Production Efficency Factor)= [BW(kg) * Liveability(%)/Age at depletion (days) * F:G]*100

Floor Pen Study

Health Status of Animals, Intestinal Lesions, and Bacterial Counts.

Mortality was 4.2% and 6.9% for CP and PA groups, respectively, whereas it was null for the CTR unchallenged animals. Although in dead animals there were no clinical signs and typical lesions of NE, it could be presumed that the bacterial challenge may have had a negative influence on broiler health status. The necroscopic examination of animals slaughtered at 14d and 42d did not show typical lesions of NE. No statistical differences were found among treatments for coliforms, LAB and *C. perfringens* in excreta, colon, ileum, and caecum counts.

Growth performance.

Data are shown in table 3. No statistical differences were found among treatments for ADFI between 0-14d. During the same period, ADG was significantly higher for PA vs CTR and CP (+14% and +15%, respectively; $P = 0.01$), and FCR ratio tended to be numerically lower in PA vs CTR and CP (-23% and -20%, respectively; $P = 0.08$). At 14d, BW was significantly higher for PA vs CTR and CP (+17% and +18%, respectively; $P < 0.01$). In the 2nd phase, ADG of PA tended to be higher compared to CP group (+4%, $P = 0.08$), and equal to the unchallenged birds. Overall, AFI was significantly lower in the PA group compared with the CTR group, with no significant changes in overall ADG, FCR, and final BW. EPEF value was numerically higher for PA compared to CTR (+8%).

***Table 6.3**. BW, average daily gain (ADG), average daily feed intake (ADFI), and feed conversion rate (FCR) of broilers in the floor pen study*

	Treatment[1]			Statistics[2]	
Item	**CTR**	**CP**	**PA**	**SEM**	**P-value**
Initial BW, g	34.7	35.2	34.7	0.18	0.12
I phase (0-14d)					
ADFI, g	39.4	37.5	34.5	2.16	0.31
ADG, g	16.7[b]	16.5[b]	19.0[a]	0.59	0.01
FCR	2.38	2.30	1.83	0.17	0.08
14 d BW, g	269[b]	266[b]	315[a]	7.63	<0.01
II phase (15-42d)					
ADFI, g	139.4	133.3	130.5	3.39	0.20
ADG, g	71.1	68.0	70.7	0.97	0.08
FCR	1.96	1.96	1.85	0.06	0.34
Final BW, g	2266	2244	2296	27.11	0.41
Overall (0-42d)					
ADFI, g	101.9[a]	96.8[ab]	93.1[b]	2.32	0.05
ADG, g	50.7	48.4	50.6	0.93	0.17
FCR	2.01	2.01	1.85	0.07	0.19
EPEF[*]	268.4	254.8	275.0	-	-

[a,b] Means with different superscript within the same row differ significantly ($P < 0.05$).
[1] CTR = negative control group; CP = *C. perfringens* challenged control group; PA= *C. perfringens* challenged group fed diet supplemented with *P. pentosaceus* and pediocin A
[2] n = 6.
[*] EPEF (European Production Efficency Factor)= [BW(kg) * Liveability(%)/Age at depletion (days) * F:G]*100

6.4. Discussion

After the ban of antibiotic growth promoters, as of January 1st 2006, clostridial enteritis is a urgent issue in livestock production, primarely because of the loss of performance of affected animals. *Clostridium perfringens* is the main causative agent of NE, a worldwide spread multi-factorial disease that can affect birds both in acute and sub-clinical forms. Acute NE may cause 1% of daily mortality up to a 30% of mortality in the entire production cycle (Van Immersel *et al.*, 2004; Dahiya *et al.*, 2006). Subacute enteritis is very frequent, with great economic implication associated to lower digestion and absorbtion, feed efficiency and impairment of growth performance (Kaldhusdal *et*

al., 2001; Loveland and Kaldhusdal , 2001; Hofacre *et al.* , 2003; Van Immerseel *et al.*, 2004).

This study aimed to induce subacute necrotic enteritis in broiler chickens, in order to understand if the supplementation of pediocin A and its producer strain *P. pentosaceus* FBB61, could restore growth performance to regular levels.

In the pilot study, broilers challenged with *C. perfringens* showed lesions typical of NE, whereas, in the floor pen study, broilers challenged with *C. perfringens* did not produce clinical signs of NE and there was no mortality directly associated to *C. perfringens* exposure. The same results were shown by other authors, who observed no clinical signs of NE after *Clostridium* challenge in broiler chickens (Olkowski *et al*, 2006; Drew et al, 2004). Even though the challenge model used did not produce acute NE, birds challenged with *C. perfringens* had low performance, if compared to those of Ross performance objectives, and developed a subacute enteritis characterized by low ADFI and ADG.

Even though there is plenty of literature on NE challenge models and management, limited information is available about the addition of bacteriocins to bird diets, thus outlining the unicity of the present study. Lactic acid bacteria and their bacteriocins have been long studied as food preservatives and starters, as they improve food shelf-life, and organoleptic and nutritional value (Wood and Holzapfel, 1995; Leroy and De Vuyst, 2004; De Vuyst and Leroy, 2007); the most popular example of LAB derived bacteriocin is nisin, which is recognized as a GRAS ingredient by the FDA since 1988. By definition, bacteriocins are tipically active against closely related bacteria limiting their use to a selected number of pathogenic strains; pediocin A was in fact described to have a broad spectrum of activity against numerous strain of gram-positive bacteria and foodborne pathogens among which *L. monocytogenes* (Piva and Headon 1994). In the present study we found that pediocin A had strong inhibitory capacities against *C. perfringens* type A in an *in vitro* antimicrobial assay. Being *C. perfringens* the organism involved in the pathogenesis of poultry enteritis, pediocin A application in feeds seems to be innovative. Few data are available about the use of bacteriocins in feeding poultry as a method to counteract intestinal pathogens development and shedding, even though in recent times there is growing interest in such a field. Cole *et al.* (2006) described the efficacy of bacteriocins derived from a strain of *L. salivarius* against *C. coli* colonization in turkeys, as well as Stern *et al.* (2005), but no studies with *C. perfringens* are currently available.

In these experiments, we studied the effect of feeding a partially purified bacteriocin, pediocin A, and its *P. pentosaceus* producer strain to animals challenged with high doses of *C. perfringens* (10^6 to 10^8 CFU). The minimal inhibitory concentration of pediocin A against *C. perfringens* was 40 AU/ml after 48 h, and, although equal versus to higher doses were used in the *in vivo* experiments (80 AU/g of feed in the pilot study, and 40 to 60 AU/g feed in the floor pen study) no effect was found in the number of viable *C. perfringens* cells in the intestines of slaughtered chickens. This might be explained by considering the diluting effect of *in vivo* intestinal microbial content. It is well known that the intestine of birds hosts a very large bacterial population (10^4-10^5 CFU/g and 10^{10}-10^{11} CFU/g in small and large intestine, respectively;) among which a large portion is made of gram-positive (Bjerrum *et al.*, 2006). Being gram-positive organisms the target of pediocin A, it is possible that there was a sparing effect on *C. perfringens* due to the high competitiveness of the environment. Nevertheless, animals fed with pediocin A and *P. pentosaceus* had better performance in both experiments. In the pilot study, during the first period (0-9 d, before challenge) and at 14d (after challenge), birds fed with pediocin A had higher ADG by a 20% and 19%, respectively, when compared to untreated animals. The results of the first experiment were confirmed in the floor pens study, where a larger number of replicates and animals was used. In facts, birds fed with pediocin A-containing diet had higher ADG by 14% and 15% when compared to a negative and a positive control, respectively, during the period 0-14d immediately after challenge ($P = 0.01$). During the second phase (15-42d), after the bacterial challenge, the difference in ADG between infected animals with or without pediocin treatment was less pronounced (4%) without reaching significant levels ($P = 0.08$).

These results can be explained by the fact that, even if we were not able to measure appreciable reduction of *C. perfringens* counts, pediocin A might have targeted other gram-positive species, thus revealing a possibility for pediocin A to beneficially modulate bacterial balance, by favouring beneficial bacteria at the site where noxious bacterial overgrowth occurs during enteritis (Casadei *et al.*, 2009). Previous data showed that pediocin A was able *in vitro* to reduce the extent of fermentation, resulting in a long-lasting utilization of fermentable energy sources, and to control intestinal microbial metabolism (Casadei *et al.*, 2009). Even though antibiotic growth promoters mechanism of action is not still fully understood, its known effects in depressing

microflora growth, thereby increasing growth performance by 5 to 10 %, make pediocin A result in a growth promoting analogue.

Even though *C. perfringens* is the main causative agent of NE, there are many factors that can contribute to the pathology to develop, and *C. perfringens* itself is not enough to predispose to NE to rise, even when administered at 10^8 CFU/d. It is likewise evident that such an high number of *C. perfringens* cells in the intestine may disturb microflora metabolism, alter the intestinal fermentation pattern, and impair digestion and absorbtion. In both experiments, pediocin A helped birds to restore weight to levels comparable to unchallenged animals. Moreover, in the second experiment, along with pediocin A, we added *P. pentosaceus* producer strain to the diet, that could have had exerted probiotic effects, growing in the intestine of birds, and then activating in situ pediocin A production. This observation is supported by previous study results, where *P. pentosaceus* FBB61 producer strain added to an *in vitro* cecal fermentation system significantly modified the extent of fermentation by reducing ammonia concentration and isoacids molar proportions (Piva *et al.*, 1995). Recently, Lee *et al.* (2007) demonstrated the growth promoting and protecting effect of a *Pediococcus*-based probiotic in *Eimeria* challenged birds, reporting higher ADG and a decrease in fecal oocysts shedding in birds fed with probiotic when compared to challenged birds without probiotic supplementation. Mountzouris *et al.* (2007) found that feeding broiler chickens with a multibacterial species probiotic, containing *Pediococcus* strain, significantly improved growth performance and, to an extent, cecal microflora composition, when compared to avilamycin.

In conclusion, our data demonstrated that both pediocin A alone, and the combination with its producer strain *P. pentosaceus*, have *in vivo* growth promoting effects, further substantiating previous *in vitro* results (Casadei *et al.*, 2009), and that pediocin A allowed to restore optimal growth in birds challenged with the enteropathogenic *C. perfringens* (Grilli *et al.*, 2009).

7. CONCLUSIONS

In the contest of improving food safety, as required by the European Authority and consumer, the attention was focused on microbiological risk in food animal. *Clostridium perfringens* and *Salmonella* spp. were the major causes of human infection. In particular, to prevent microbiological risk is necessary decrease slaughterhouse cross-contamination. Aim of this study was to investigate the antimicrobial effect of natural compounds, such as, organic acids, nature-identical compounds, and bacteriocin, against the main foodborne pathogens *Clostridium perfringens* and *S. Typhimurium*.

The first step was to study the antimicrobial *in vitro* activity of several organic acids and flavours against *C. perfringens* and *Salmonella*, in order to find possible synergisms among those compounds. The second step was to study their possible use in animal production to screen the role of such substances in controlling *Salmonella* infection in pigs in an *in vitro* system simulating the intestinal environment and microflora; and finally, substances under investigation were tested *in vivo* to evaluate their properties as feed additive to prevent clostridial infection in broilers. In parallel, a bacteriocin and its producer strain *P. pentosaceus* were tested *in vivo* to investigate their role into control *C. perfringens* infection in broilers.

The Minimal Inhibitory Concentration results showed that organic acids in association with nature-identical compounds had a stronger antimicrobial activity compared to the same substance alone. In particular, carvacrol showed a strong antimicrobial activity when used in combination with an organic acid, both against *C. perfringens* and *Salmonella*. Carvacrol antimicrobial activity against gram-positive and gram-negative was confirmed by several authors (Dorman and Deans, 2000; Lambert *et al.*, 2001; Peñalver *et al.*, 2005). Organic acids alone were less effective than nature-identical compounds.

The fermentation study clearly confirmed that carvacrol had a better activity when compared with thymol to control *Salmonella* infection in swine intestine. In particular the use of carvacrol with an organic acid could represent an effective approach to contain *Salmonella* gastrointestinal infections in pigs.

The results obtained *in vivo* using a microencapsulated blend of organic acids and flavours to control *C. perfringens* infection evidenced that the blend helped birds to restore weight to levels comparable to unchallenged animals. The microencapsulated

blend probably acts on intestinal microflora, stimulating positive bacteria such as LAB and increasing the competitiveness for the environment. Restore normal performance conditions results in decreasing of economic lost for the farmer.

The parallel *in vivo* study to analyse the effect of pediocin A and *P. pentosaceus* to control clostridial infection, demonstrated that both pediocin A alone, and mostly the combination with its producer strain *P. pentosaceus*, have *in vivo* growth promoting effects, further substantiating previous *in vitro* results (Casadei *et al.*, 2009), and that pediocin A allowed to restore optimal growth in birds challenged with the enteropathogenic *C. perfringens* (Grilli *et al.*, 2009)

The use of a blend of carvacrol and an organic acid can be an useful instrument in controlling *Salmonella* infection, such as the use of a blend of organic acids and flavours or pediocin A and its producer strain contained clostridial infection. Reduction of foodborne pathogens shedding on farm and consequently at slaughter could improve meat safety for human consumption.

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9. ACKNOWLEDGMENTS

A tutti quelli che hanno sempre ascoltato i miei sfoghi, li ascoltano ancora, e continueranno ad ascoltarli.
A tutti quelli che mi hanno dato la forza di continuare a lottare.
A tutti quelli che mi hanno ricordato quanto valgo, quando altri affermavano il contrario.
A tutti quelli che hanno riempito le mie pause.
A tutti quelli che "sono le cinque… ape?"
A tutti quelli che prima non c'erano, ma che da quando ci sono mi hanno cambiato la vita, e spero non se ne vadano mai.
A tutti quelli che mi hanno appoggiata e hanno fatto in modo che questa avventura si concludesse positivamente, trovando una soluzione quando la soluzione sembrava non esistere.
A tutti quelli che si riconoscono nelle mie parole… GRAZIE.
Perché in realtà, chi non merita considerazione spesso ruba la scena a chi invece ci vuole realmente bene, e ci fa sprecare momenti felici con inutili chiacchiere.

Non ricordo il giorno in cui
Fissando la nebbia
Mi sembrò di scorgere qualcosa muoversi.
Era un ombra perfetta.
Ci andai incontro e potei ammirarne
La vacuità e l'immensità,
Il tepore e l'ardore,
La malinconia e l'allegria,
La rassegnazione e la passione.
Neanche una parola guardandoci.
Viaggiai aldilà delle nuvole
Per valicare cime e valli,
Prati e maggese,
Case e baracche,
Fiumi e torrenti.

V.P.