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DEVELOPMENT OF NON PHARMACEUTICAL STRATEGIES TO IMPROVE INTESTINAL HEALTH IN WEANING PIGLETS

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CHAPTER 1.

LITERATURE REVIEW

1.1. THE ECOLOGY OF GASTROINTESTINAL TRACT AT WEANING

The microbial population of young pigs develops during the first 48 h of life via the ingestion of maternal faeces (Makie et al., 1999) or by the contact with the sow skin and teats that are usually contaminated (Arboukle, 1968).

During the suckling period the dominant groups in the upper part of the gastrointestinal tract are lactobacilli and streptococci (Jensen, 1998). At birth the stomach of piglets is sterile (Sinkovics and Juhasz, 1974), but after few hours viable cells of lactobacilli, streptococci, coliforms and clostridia, can be isolated from the gastric content. During the following 5-6 days the number of lactobacilli and streptococci is around 10^5-10^7 cells per gram of content (Jensen, 1998; Pluske, 2002) ant it will be stable for all the suckling period. In the proximal small intestine there is a microflora similar to that of stomach, being therefore lactobacilli and streptococci the dominant groups (Jensen, 1998). In contrast the distal small intestine, contains an higher number of bacteria (10^8-10^9 cells per gram of digesta). Although determining the exact composition of the large intestine microflora is very hard, because of the high number of uncolturable cells, King an Kelly (2001) found 10^{10}-10^{11} cells per gr of content in the lumen of colon, with more of 400 different colturable bacterial species. Numerous studies conducted on the characterization of large intestine microflora demonstrated that the maior groups are *Streptococcus, Lactobacillus, Prevotella, Selenomona, Mitsokuella, Megasphera, Clostridia, Eubacteria, Bacteroides, Fusobacteria, Acidodaminicocci,* and *Enterobacteria* (Salanitro et a., 1977; Allison et al., 1979; Russel, 1979; Robinson et al., 1981; Moore et al., 1987; Jensen, 2001; and Pluske et al., 2002). Although detailed studies on the microflora in the hindgut have been conducted, there is evidence of a great diversity between animals and between different studies results, since the variability in number or type of cells depends on the nutrients availability in the gut, being nutrients limiting factors which support the growth of one bacterial strain rather than another one (Gaskins, 2001).

At weaning there are many changes which interfere with the normal microbial populations. Jensen (1998) investigated the effect of weaning on microbial counts, pH, and dry matter of digesta. In that study it was observed that during the first 2-4 days after weaning pH and dry matter content of fecal samples and lactobacilli counts decreased, whereas, concurrently, coliforms counts increased. This tends to make the pig susceptible to diarrhea scouring and poor growth performance. In the same study piglets were sacrificed at weaning (28 days), 6 days and 20 days after weaning, and samples from different intestinal tracts were collected to measure pH, dry matter, and microbial determination and activity through ATP concentration. The results demonstrated that the microflora of piglets after 6 days from weaning was different from the other groups, since there was an higher number of coliforms cells and a lower number of lactobacilli in each intestinal segment. Animals sacrificed 20 days after weaning presented a significantly higher microbial activity in the large intestine, as demonstrated by lower pH and higher ATP concentration. These results conducted Jensen to conclude that the microbiota of piglets is instable until 3 weeks after weaning, when the large intestine fermentative capacity is fully developed.

1.2. HEALTH IMPLICATION OF MICROFLORA IN THE HOST AND IMPACT OF ANTIBIOTIC GROWTH PROMOTERS (AGP) ON THE ECOLOGY OF THE GASTROINTESTINAL TRACT

The major health implications of the gut flora vary widely ranging from the production of toxic, carcinogenic or mutagenic metabolites from substances derived from the diet or produced endogenously, to the detoxification of dietary toxicants, to immunostimulation (Link-Amster *et al*., 1994; Marteau *et al*., 1997), to intestinal permeability (Isolauri *et al*., 1993; Mao *et al*., 1996), and to confer colonization resistance towards pathogens and a consequent prevention of diarrhoea (Raza *et al*., 1995).

Intestinal fermentations prevalently occur in the hind gut (Decuypere and Van der Heyde, 1972) where decarboxylation of several amino acids by bacteria can produce monoamines and polyamines (Guggenheim, 1951; Dierick *et al*., 1986). that can exert toxic effects in different species (Lean *et al*., 1989; Cole *et al*., 1995). Furthermore the intestinal microflora is also deeply involved in the production of ammonia. Ammonia is produced both by endogenous and bacterial enzymes within the alimentary tract. Bacterial enzymes appear to produce 75% of the alimentary tract ammonia with urea hydrolysis being the major contributor in mammals residing in conventional nongerm-free environments (Visek, 1984). Energy is the fermentation limiting factor in the hind gut (Orskov *et al*., 1970). As energy sources (starch and fermentable carbohydrates) are depleted, the fermentation becomes more and more proteolytic. This results in ammonia and amines production (Russell *et al*., 1983); ammonia destroys cells, alters nucleic acid synthesis, increases intestinal mucosal cell mass, increases virus infections, favors growth of cancerous cells over noncancerous cells in tissue culture (Visek, 1978) and reduces the villus height (Nousiainen, 1991). Absorbed ammonia must be excreted as urea with an energy cost of approximately 7% of the total energy expenditure in monogastric as well as in ruminant animals (Eisemann and Nienaber, 1990) influencing the metabolism and resulting in reduced animal performance (Visek, 1984).

The growth promoting effects of introducing antimicrobials in animal diets have been known from decades, since Stokstad and Jukes (1949) demonstrated that the presence of tetracyclin residues in poultry feed increased the growth of animals. Improved growth performance following the use of antibiotics were then described in turkeys (Stokstad and Jukes, 1950), pigs (Jukes *et al*., 1950), and ruminants (Jukes and Williams, 1953; Stokstad, 1954). From that time the use of antibiotics as growth factors has become widespread.

The major benefits derived from the use of subtherapeutic doses of antibiotics in animal feeding were: disease prevention, improved feed utilization, and increased growth rate. These effects were more evident in younger, stressed animals (Hays, 1969) and where management and hygiene conditions were worse. Especially in pigs, most feed antibiotics were used in newly weaned piglets, a critical time for

infection in these young animals, and only to a lesser extent in older pigs being raised for slaughter, where their use was generally regarded as unnecessary and not cost effective.

Feed antibiotics occasionally have been shown to reduce the number of bacteria present in the gut (Jensen, 1988) but more often to have little effect on total counts of viable bacteria.

Several researchers observed that animals receiving diets added with antibiotics showed similarities with *germ free* animals (Stutz *et al*., 1983; Nousiainen, 1991): reduced gut weight and length, thinner intestinal wall, and reduced cell turnover in the gut mucosa. Although a final explanation for these effects has not been found yet, they surely are related to changes in the composition of the intestinal microflora. The mechanism by which the antibiotic growth promoters act is not known by certainty, but several hypotesis have been made:1) a reduction in the thickness of the intestinal mucosa and, as a consequence, a more efficient absorption of nutrients; 2) energy and nutrients are spared because of a reduction of competitors microorganisms; 3) the production of discrete lesions in the cell wall of enteric bacteria and the reduction of microorganism responsible for intestinal disorders; 4) a reduction in amounts of bacterial toxins and toxic metabolites produced in the intestine; 5) an increase in intestinal alkaline phosphatase levels; 6) a decrease in the level of production of intestinal ammonia; 7) a reduction of microbial deconjugation of bile salts. (Jensen, 1998).

Since the introduction of AGP in animal husbandry, an increasing concern on the widespread of antibiotic resistant microorganisms conducted Europe toward the ban of AGP, as of January 1st , 2006; this led researchers to find alternative molecules to be used in animal production which act antibiotic-like, whithout being a threat to human health.

1.3. FEEDING STRATEGIES TO REDUCE THE IMPACT OF POST WEANING SYNDROME

Weaning is a very crucial moment in the growth of pigs; there are many factors which contribute to the rise of gut infections, which have an high economic impact in pigs husbandry, especially after the removal of antibiotic growth promoters (AGP). These infections are commonly called Post Weaning Syndrome (PWS) or Post Weaning Colibacillosis (PWC) (Pluske et al., 2002) since it seems to be *E. coli* the main cause of such syndrome. Even if it has been demonstrated that *E. coli* is necessary for diarrhea outbreaks, PWS is nowadays regarded as a multifactorial disease (Dirkzwager et al., 2005; Pluske et al., 2002) which needs many cofactors playing important roles in predisposing to its development. Among these "causative agents" we can list environmental factors, such as the separation from the mother, the regrouping of animals, the exposure to a new antigenic environment, and the "cold- stress" due to the lack of thermoregulation capacity (Wathes et al., 1989). All those factors contribute to make piglets stressed with a consequent higher production of cortisol which, via the sympathetic nervous system, alters the intestinal transit time and causes immune suppression (Pluske et al., 2002). Furthermore the piglets during weaning loose any passive antibody protection provided from the sow's milk. Anorexia and feed refusal are important consequences of stressful weaning and contribute in worsening the yet dramatic situation.

In addition to that there are other factors, such as physiological factors as the passage from liquid to solid feed. The possibilities for maximizing sow productivity and the need to more efficiently utilize the expensive farrowing facilities have led pig producers to wean piglets at three-four weeks of age. At that time the GIT and its digestive enzymes set are not fully developed (Dirzwager et al., 2003; Marion et al., 2002), the bacterial colonization is still transient and the piglets result more exposed and sensitive to anti-nutritional factors present in a vegetable diet, being therefore more susceptible to mal-absorption. The first

consequence is a larger amount of undigested feed in the intestine, which represent a good substrate for microorganism overgrowth (Dirkzwager et al., 2005; Hopwood et al., 2003).

Acid secretion in young pigs does not reach appreciable levels until 3-4 weeks after weaning (Cranwell and Moughan, 1989). The suckling pig employs several strategies to overcome the limitation of insufficient acid secretion and these have been discussed by Easter (1988). The primary strategy involves the conversion of lactose in sow's milk to lactic acid by *Lactobacilli* bacteria residing in the stomach. Secondly, the nursing pig reduces the need for transitory secretion of copious amounts of acid by frequent ingestion of small meals. Finally, diets are known to differ widely in acid-buffering capacity: this capacity is lowest in cereals and cereal by-products, intermediate or high in protein feedstuffs and very high in mineral sources, except in dicalcium and monosodium phosphates (Jasaitis *et al*., 1987).

The failure to maintain a low gastric pH has major implications for the performance of the early-weaned pig. First, an elevated pH would cause a reduction in the activation of pepsinogen which occurs rapidly at pH 2 and very slowly at pH 4 (Taylor, 1962). The pepsins have two optimal pH, 2 and 3.5, and their activity declines above 3.6 with no activity at pH > 6.0 (Taylor, 1959). As a result, feed proteins may enter the small intestine essentially intact with an eventual reduction in efficiency of protein digestion. The end-products of pepsin digestion also stimulate the secretion of pancreatic proteolytic enzymes (Rerat, 1981). Furthermore acid from the stomach is the primary stimulant for pancreatic secretion of bicarbonate (Kidder and Manners, 1978). In addition, acid leaving the stomach plays a role in the feedback mechanism in the regulation of gastric emptying, thus, decreasing the digesta load on the small intestine. Secondly, an acid gastric condition is believed to have pronounced bactericidal properties for certain microorganisms, in particular for the *Coliforms* (Sissons, 1989). Viable microorganisms entering the digestive tract via the mouth are unable to pass through the acidic conditions of the stomach and successfully colonize the small intestine. A rise in gastric pH would, therefore, allow increased proliferation of *Escherichia coli* (Smith and Jones, 1963) which has been associated with scours

and increased mortality (White *et al*., 1969; Thomlinson and Lawrence, 1981). Furthermore, evidence suggests that proliferation of *Coliforms* in the stomach may lead to further diminution of gastric acid secretion due to the release of a bacterial polysaccharide with an inhibitory effect on acid secretion (Baume *et al*., 1967; Wyllie *et al.*, 1967).

All the above cited circumstances, e.g. stress, anorexia, and physiology, cause great damages to the intestinal mucosa, which undergoes to several structural changes such as the reduction of villi height and an increase in crypts depth (Pluske et al., 1996). The reduction of the intestinal absorptive area and the appearance of a less mature enterocyte population help to explain the increased susceptibility of the pig to diarrhoea. In fact in the small intestine, nutrients, electrolytes, and water are absorbed by villus enterocytes, whereas electrolytes and water are secreted in crypt cells (Powell, 1987). Because shorter villi and deeper crypts have fewer absorptive and more secretory cells, absorption might be poorer and secretion increased. A heat-stable toxin (toxin b) produced by some *Escherichia coli* strains is known to be villus shortening (Whipp *et al*., 1986). During a study on weaned pigs, Nabuurs *et al*. (1993) recovered an *Escherichia coli* strain that produced heat-stable toxin b from all the litters affected by diarrhoea.

Genetic also could play a key role in PWC syndrome, since it has been studied that certain pigs do not express glycoproteic receptors for *E. coli* K88 fimbriae in the brush border of cells lining the intestinal villi in the small intestine (Hampson, 1994).

In this context practical feeding and management strategies can be adopted in order to ameliorate post weaning conditions and piglets health status.

1.3.1. Improvement of gut development at weaning.

Stimulate feed intake before and after weaning by supplying a palatable diet during the first 5-10 days is a very important tool in help a normal gut development; the light management can significantly influence this parameter, since during the dark period the ingestion is depressed (Dirkzwager et al., 2005).

Another strategy could be to supply the diet with substrates like butyrate, which is known to be an intestinal mucosa growth factor (Piva et al., 2002).

1.3.2. Improvement of feed digestion.

The choice of right ingredients, highly digestible nutrient sources, is a very important tool in controlling PWS associated intestinal microbial proliferation. Numerous studies investigated the correlation between the inclusion of soluble NSP in piglets starter diets and the proliferation of *E. coli*. Feeding piglets with pearl barley meal increased intestinal viscosity and altered microbial fermentation, helping *E. coli* proliferation both in small and large intestines as demonstrated by Mc Donald et al. (2001b). These data suggests that the presence of soluble NSP in weaner diets is an undesirable effect, which can be overcome by selecting cereals low in NSP content or adding exogenous enzymes, such as β-glucanases, or xilanases, to increase soluble NSP digestibility.

The source of proteins, such as vegetable or animal proteins, is another very important aspect to be evaluated, since it has been demonstrated that soybean meal is rich of anti-nutritional factors that reduce protein digestibility, and induce mucosal damages, especially in highly susceptible weanling pigs (Miller et al., 1984). Proteins of animal origins, such as milk whey or fish meal proteins, are therefore preferable to soybean proteins. Hall and Byrne (1989) observed villus stunting, loss of activity of lactase and sucrase from the mucosa and reduced live-weight gain in weaning pigs when the diet was changed from milk to a dry pelleted meal feed containing milk, soya and cereal proteins, without any interactions with microbial pathogens. They supposed, that the intestinal damage detected could be the result of antibody-mediated damage induced by antibodies to soya proteins in the meal.

Nutritionists have attempted to overcome the digestive inefficiency of early weaned pigs by incorporating milk products in cereal/oilseed meal based diets. Milk products of high quality have consistently improved postweaning growth performances (Pals and Ewan, 1978; Graham *et al.*, 1981; Cera *et al.*, 1988). These beneficial effects arise from their high content of lactose (Mahan, 1992)

which is not only more digestible than the complex carbohydrates from plant sources, but is also fermented to lactic acid to provide gastric acidity (Wilson and Leibholz, 1981) and to act as a non-digestible oligosaccharide (Piva *et al*., 1998).

Protein diet concentration is another critical factor. High proteins levels in starters diets may result in large amounts of free AA available for microflora fermentations with final production of polyamines (putrescine, cadaverine, histamine, etc..) which are implicated in PWS. Typical starters diets contains up to 25% crude proteins/dry matter with an average digestibility of 75-85% (Pluske et al., 2002). A 7 kg piglet, can eat up to 60 gr proteins/day , a 25% of which can by-pass small intestine digestion and reach the lower hindgut, where it is rapidly fermented by proteolytic microflora, such as *Bacteroides, Clostridium, Enterobacterium, Streptococcus*, to poly-amines and gas and ammonia, that seem to be implicated in PWS (Aumaitre et al., 1995; Pluske et al., 2002).

Improvement of colonization, which means to select a stable and positive microflora in the animal, by adding probiotics and prebiotics to the diet. It is possible that resident bacteria in the gut, antagonistic to other invading bacteria, may contribute to infection resistance by competitive exclusion (Lloyd *et al*., 1977) or by the production of metabolites which have antibacterial effects on the pathogens.

Although the definition of probiotics presents considerable problems and has varied over the years, the final aim of their application is that probiotics should have beneficial effects on animals by the establishment of an optimum balance of microbes in the alimentary tract. Salminen *et al*. (1996) have summarized the most important functional effects of probiotics: these include aspects such as immune modulation and strengthening the gut mucosal barrier, due to: 1) gut microflora modification; 2) adherence to the intestinal mucosa with capacity to prevent pathogen adherence or pathogen activation; 3) modification of dietary proteins by the intestinal microflora; 4) modification of bacterial enzyme capacity, especially of those suggested to be related to tumour induction; 5) influence on gut mucosal permeability.

Prebiotics or non digestible oligosaccharides (NDO) are carbohydrates that escape enzymatic digestion in the small intestine and form a substrate for the

gastrointestinal microflora. NDO can modify the composition of the intestinal microflora by selectively stimulating the growth and/or activity of one or a limited number of bacteria, as for example inulin, which can be fermented by strains of lactic acid bacteria and *Bifidoacterium* in a preferential way (Gibson and Roberfroid, 1995). Many NDO display bifidogenic properties and increase the bifidobacteria counts in different sections of the gastrointestinal tract and/or faeces (Tanaka *et al*., 1983; Mutai *et al*., 1984; Hidaka *et al*., 1986; Hayakawa *et al*., 1990). Several investigators have shown that NDO are able to decrease the concentration of ammonia and putrefactive products in the hind gut of man and monogastric animals (Gibson and Roberfroid, 1995; Piva et al., 1996; Jensen, 1998).

1.3.3. Improvement of the immune response

Adding vitamins, antioxidants, PUFA, and antibody derived from plasmatic proteins to the diet could be a tool in controlling PWS consequences.

1.3.4. Inhibition of the growth of pathogenic bacteria

Adding organic acids to the diet causes the pH decline in the gut and may inhibit the growth of *E. coli* pH-sensitive and improve stomach functionality by decreasing its empting rate, while supplementing the diet with essential oils or plant derivatives, which have well known antimicrobial properties (Burt, 2004) can reduce intestinal pathogens shedding.

1.4. REPLACING ANTIBIOTIC GROWTH PROMOTERS

1.4.1. Organic acids

Positive effects on the performance of weanling pigs have been achieved by adding organic acids or their salts to the feed. Some physical and chemical properties of organic acids that are used as acidifiers in pig diets are presented in Table 1.1.

Table 1.1. Formulas, physical and chemical characteristics of organic acids used as dietary acidifiers for pigs (Foegeding and Busta, 1991)

Acid	**Formula**	**MM[a]**	**Density**	**Form**	**pK_a**
		g/mol	g/mL		
Formic	HCOOH	46.03	1.220	liquid	3.75
Acetic	CH_3COOH	60.05	1.049	liquid	4.76
Propionic	CH_3CH_2COOH	74.08	0.993	liquid	4.88
Butyric	$CH_3CH_2CH_2COOH$	88.12	0.958	liquid	4.82
Lactic	$CH_3CH(OH)COOH$	90.08	1.206	liquid	3.83
Sorbic	$CH_3CH:CHCH:CHCOOH$	112.14	1.204	solid	4.76
Fumaric	COOHCH:CHCOOH	116.07	1.635	solid	3.02
					4.38
Malic	$COOHCH_2CH(OH)COOH$	134.09		liquid	3.4
					5.1
Tartaric	COOHCH(OH)CH(OH)COOH	150.09	1.760	liquid	2.93
					4.23
Citric	$COOHCH_2C(OH)(COOH)CH_2COOH$	192.14	1.665	solid	3.13
					4.76
					6.40

[a]MM, molecular mass expressed in grams

Dietary acidification reduces the buffering capacity of the diet and this may support a more efficient proteolysis of digesta in the stomach and therefore result in a higher protein digestibility of the diet. Addition of organic acids reduces dietary pH curvilinearly depending on the acid pK_a value and buffering capacity of the diet. The pH-lowering effect of different organic acids is reduced in the following order: tartaric acid > citric acid > malic acid > fumaric acid > lactic and formic acid > acetic acid > propionic acid. Organic salts have only a small influence on dietary pH (Partanen and Mroz, 1999).

Eckel *et al*. (1992) observed that formic acid supplementation positively influenced nutrient digestibility. Improvements were higher for protein (up to 4%) than for energy (up to 2%) and were more pronounced in a period short after weaning than at a later age.

Conversely, other researchers (Falkowski and Aherne, 1984; Bolduan *et al*., 1988; Radecki *et al*., 1988) have reported no improvements in the digestibility of dry matter or nitrogen when organic acids were fed. Mosenthin *et al.* (1992) reported that apparent ileal digestibility of nitrogen was not affected by propionic acid supplementation. However, propionic acid addition significantly improved apparent ileal digestibility of arginine, histidine, leucine, phenylalanine and valine.

Organic acids exert various, specific antimicrobial properties. Formic acid for example acts against yeasts and certain bacterial species, such as *Bacillus* spp., *Escherichia coli* and *Salmonella,* but lactobacilli and moulds are rather resistant to formic acid (Rehm, 1961). This antibacterial effect might be explained by the protons (H^+ ions) and anions ($HCOO^-$ ions) into which formic acid is divided after passing the bacterial cell wall and which have a disruptive effect on bacterial protein synthesis (Lück, 1986). There is some evidence from the literature that fumaric and propionic acid, as well as formic acid, decrease intestinal microbial growth (Bolduan *et al*., 1988; Sutton *et al*., 1991; Gedek *et al*., 1992).

While travelling through the gastrointestinal tract neutralophiles, like *Escherichia coli, Salmonella typhimurium* or *Shigella flexneri*, must endure the extreme, low pH of the stomach as well as the short chain fatty acids present in the intestine and faeces. Figure 1.1 shows how pH varies through the gastrointestinal tract; Figure

1.2 shows the amount of volatile fatty acids (VFA) in the different sections of the gastrointestinal tract.

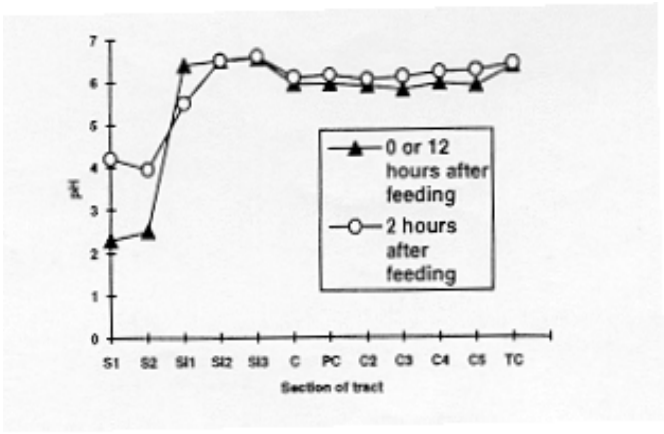

Figure 1.1. Mean pH in the gastrointestinal contents of the pig (Clemens *et al*., 1975). Symbols along the abscissa represent the sections of the tract as follows: S1, S2 = stomach; SI1, SI2, SI3 = small intestine; C = cecum; PC = proximal colon; C2, C3, C4, C5 = colon; TC = terminal colon

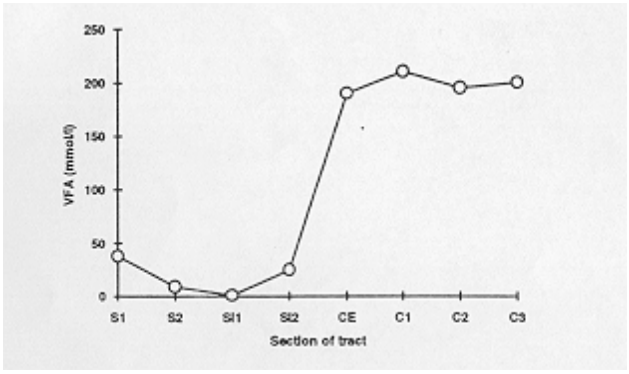

Figure 1.2. Mean volatile fatty acids concentration in segments of the pig gastrointestinal tract (Dukes, 1990).
Symbols along the abscissa represent the sections of the tract as follows: S1, S2 = stomach; SI1, SI2, = small intestine; C = cecum; C1, C2, C3 = colon

These bacteria are, therefore, exposed to acid stress, which can be described as the combined biological effect of low pH and weak organic acids present in the environment. Weak acids, including those produced as a result of intestinal fermentation, can diffuse across the cell membrane in the uncharged, protonated form and dissociate inside the cell, lowering internal pH (pH_i; Bearson *et al*., 1997). The lower the external pH (pH_e), the more undissociated weak acid will be

available (based upon pK_a values) to cross the membrane and affect pH_i. To overcome the lowering of pH_i, several amino acid decarboxylases are induced; these elevate the pH_i by consuming a proton during decarboxylation and they then exchange the decarboxylation 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). Similar inducible systems, with arginine and glutamate decarboxylases, have been described for *Escherichia coli* (Lin *et al.*, 1995).

Russel (1991a,b) observed that bacteria that can tolerate fermentation acids at low pH have relatively small transmembrane pH gradients (ΔpH).

Since organic acids are able to pass the bacterial cell wall in their undissociated more lipophilic form, their rate of dissociation is very important. The effect of pH on organic acid dissociation is given by the Henderson-Hasselbach equation, where A^- and HA are the dissociated and undissociated species, respectively

$pH_e = pK_a + \log[A^-]/[HA]$. Figure 1.3 shows how the rate of dissociation of the acid depending on the pKa.

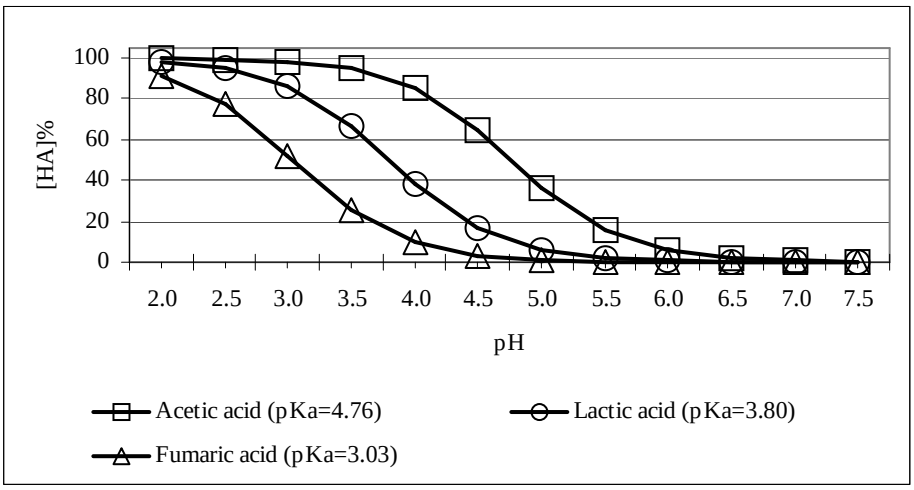

Figure 1.3 [HA] % in relation to pH

At neutral pH, there is very little HA, but HA increases logaritmically as the pH declines. Because fermentation acids are generally more toxic to bacteria at low pH, it has generally been assumed that the antimicrobial activity of fermentation acids was caused by HA molecules (Salmond *et al*., 1984). But HA is membrane-permeable and it never accumulates; only fermentation anions seem to have the ability to accumulate intracellularly depending on pH_i: by high pH_i more acid becomes dissociated and the anions accumulate (Russell and Diez-Gonzalez, 1998).

Russell and Diez-Gonzalez (1998) theorized the ΔpH-mediated model of fermentation acid toxicity, where anions accumulation is ΔpH dependent

$$\Delta pH = \log ([A^-] + [HA])_{in} / ([A^-] + [HA])_{out})$$

This model is supported by a variety of observations: intracellular acetate anion seems to be in equilibrium with ΔpH (Diez-Gonzalez and Russell, 1998); bacteria (e.g. *Escherichia coli*) that are highly sensitive to fermentation acids have high ΔpH values at acidic pH (Salmond *et al*., 1984; Wallace *et al*., 1989); bacteria (e.g. lactobacilli and streptococci*)* that are highly resistant to fermentation acids seem to have low ΔpH values (Russell and Hino, 1985; Kashket, 1987; Olsen *et al*., 1991).

Russell and Diez-Gonzalez (1998) observed that low ΔpH prevents fermentation acid anion accumulation, but there are limits to this strategy for growth, and that cells that have higher concentrations of intracellular potassium as a potential countercation (Gram-positive bacteria) would be able to accumulate higher concentrations of fermentation anions, and the decrease in intracellular pH would not have to be so great. Virtually all bacteria have a constitutive, low-affinity potassium transport system that operates as a potassium/proton symport mechanism. This electrogenic system is able to dissipate ΔpH (Kashket and Barker, 1977). Kajikawa and Russell (1992) observed that passive potassium efflux is a mechanism for increasing membrane potential and, based on this observation, theorized a potassium-dependent system of ΔpH and membrane potential interconversion. If a bacterium has a very high concentration of intracellular potassium, membrane potential remains high and ΔpH is low, and vice versa. This scheme is supported by a contrast between lactic acid bacteria and

Escherichia coli. The lactic acid bacteria, *Streptococcus bovis* and *Lactobacillus lactis*, always had very high internal potassium concentrations and never generated large ΔpH values (Cook and Russell, 1994). *Escherichia coli*, a bacterium with lower intracellular potassium levels, was able to decrease ΔpH as the environment became more acidic (Kaback, 1990). Potassium addition caused an almost immediate increase in the intracellular pH of *Escherichia coli* cells that were suspended in a medium at acidic pH (Kroll and Booth, 1983).

Among the monocarboxylic organic acids formic (Eckel *et al*., 1992), lactic (Roth *et al*., 1993) and sorbic acid (Kirchgessner *et al*., 1995) showed the best results on weanling pig performances. Supplementing weanling pig diets with acetic (Roth and Kirchgessner, 1988) or propionic acid (Kirchgessner and Roth, 1982a) improved performance to a much lower extent or not at all. Potential reasons for the variable results are: the kind of acid, the dose level, a depressive effect on feed intake and the health status of the animals. Within the dose ranges used, formic acid at 12, lactic acid at 16 and sorbic acid at 24 g/kg of feed were the most effective and may therefore be regarded as the optimal doses. Table 1.2 shows some results for monocarboxylic organic acids in piglet feeding trials.

Table 1.2. The effect of monocarboxylic acid supplementation on weanling pig performance

Acid	%[1]	BW[2]	DWG[3]	%Δ[4]	FCR[5]	%Δ[6]	Reference
Formic	0.6	6.1	463	+21.8*	1.46*	-5.6	Eckel *et al*., 1992
	1.2	6.1	468	+22.1*	1.43*	-7.5	
	1.8	6.1	401	+4.6	1.53	-1.0	
	2.4	6.1	325	-15.1*	1.60	+3.9	
Acetic	0.9	5.6	415	-2.1	1.77	+1.1	Roth and Kirchgessner, 1988
	1.8	5.6	429	+1.2	1.72	-1.7	
	2.7	5.6	441	+4.0	1.70	-2.9	
Propionic	1.0	5.6	388	-3.2	1.78	+1.1	Kirchgessner and Roth, 1982a
	2.0	5.6	385	-4.0	1.80	+2.2	
	3.0	5.6	395	-1.5	1.74	-1.1	
Lactic	0.8	6.8	489	+4.7	1.65	+1.2	Roth *et al*., 1993
	1.6	6.8	505	+8.1	1.60	-1.8	
	2.4	6.8	501	+7.3	1.60	-1.8	
Sorbic	1.2	7.2	490	+13.7*	1.63*	-4.1	Kirchgessner *et al*., 1995
	1.8	7.2	523	+21.3*	1.60*	-5.9	
	2.4	7.2	546	+26.7*	1.59*	-6.5	

[1]*inclusion level of acid in the diet*
[2]*initial body weight, kg*
[3]*average daily weight gain, g*
[4]*percentage increase/decrease in DWG relative to control*
[5]*feed conversion rate as g feed per g weight gain*
[6]*percentage improvement (-)/deterioration (+) in FCR relative to control*
**significantly different (P<0.05) from control*

Besides these monocarboxylic acids, which (apart from sorbic acid) are products of bacterial metabolism in the intestinal tract, acids which are natural intermediates of the citric acid cycle of the cells are also used for supplementing piglet feeds. Several investigators have reported improvements in starter pig performance when either fumaric or citric acid was added to the diet (Kirchgessner and Roth, 1982b; Falkowski and Aherne, 1984; Burnell *et al*., 1988). Kirchgessner *et al*. (1993) reported improved pig performances also when malic acid was used. In contrast to these results, supplementing the diets with tartaric acid did not improve pig performances (Kirchgessner *et al*, 1993). Table 1.3 shows some results for di-/tricarboxylic organic acids in piglet feeding trials.

Table 1.3. The effect of di-/tricarboxylic acid supplementation on weanling pig performance

Acid	%[1]	BW[2]	DWG[3]	%Δ[4]	FCR[5]	%Δ[6]	Reference
Fumaric	0.5	7.8	513	-8.4	1.53	-2.0	Kirchgessner and Roth, 1976
	1.0	7.8	559	+0.1	1.50	-3.9	
	2.0	7.8	625*	+11.6	1.45*	-7.0	
	4.0	7.8	581	+3.8	1.48*	-5.1	
Citric	0.5	13.9	443	+4.7	1.98	-4.8	Kirchgessner and Roth-Maier, 1975
	1.5	13.9	403	-4.7	2.19	+5.2	
	4.5	13.9	502	+18.7*	1.90*	-8.7	
Malic	1.2	6.5	465	+4.0	1.59*	-3.6	Kirchgessner *et al*., 1993
	1.8	6.5	452	+1.1	1.59*	-3.6	
	2.4	6.5	456	+2.0	1.57*	-4.8	
Tartaric	1.2	6.6	449	-7.2	1.64	-3.0	Kirchgessner *et al*., 1993
	1.8	6.6	453	-6.4	1.65	-2.4	
	2.4	6.6	433	-10.5	1.64	-3.0	

[1]*inclusion level of acid in the diet*
[2]*initial body weight, kg*
[3]*average daily weight gain, g*
[4]*percentage increase/decrease in DWG relative to control*
[5]*feed conversion rate as g feed per g weight gain*
[6]*percentage improvement (-)/deterioration (+) in FCR relative to control*
**significantly different (P<0.05) from control*

In recent years, organic salts have been coming more and more under consideration as dietary supplements because they are easier to handle, doses can be more accurately measured, and they are less corrosive than the free acids. Formates, as well as free formic acid, have been demonstrated to improve performance of weanling pigs: this improvement was reported for sodium formate (Kirchgessner and Roth, 1987a), for calcium formate (Kirchgessner and Roth,

1990), and for potassium diformate (Paulicks *et al*., 1996). However neither sodium nor calcium formate were as efficient growth promoters as free formic acid. These neutral reacting salts are more efficient if they are used in combination with the free acid (Kirchgessner and Roth, 1990). Table 4 shows some results for formates in piglet feeding trials.

Attempts to improve pig performances using inorganic acids have met with disappointing results. The use of hydrochloric acid, sulfuric acid and phosphoric acid in weaner diets was investigated by Giesting and Easter (1986). Hydrochloric acid resulted in severe depressions in intake and growth, sulfuric acid depressed feed efficiency, phosphoric acid improved growth and feed efficiency in one trial, but was without any effect in a subsequent one. Similar observations were made by Roth and Kirchgessner (1989) who reported no effect of phosphoric acid addition on postweaning performance.

1.4.2. Plant extracts and essential oils

Plant extracts are one of the oldest additives used by mankind. For thousands of years herbs and spices have provided flavorings properties to food and many have been used for their antimicrobial properties to preserve food. Some of most common plants known for their antibacterial properties are garlic, onion, thyme, basil, cumin, and bay. Among this plants there are also spices, that have been used in the past to cover unpleasant odours of unfresh meat, and, at the same time, antimicrobial, such as cinnamon and cloves. These natural microbial compounds are found in different parts of the plant, such as stems, leaves, barks, and flowers, that usually are scented (Lis-Balchin, 2003).

Plant extract contains a very wide number of active substances in variable amounts differing for composition and chemical structures, depending on the extraction method and on the plant cultivar. All the characteristic which influence plant extract composition affect their chemical properties and effect; it is generally accepted, therefore, for practical application, to work with their pure derived substances, naturally derived or chemically synthetized, e.g with essential oils (EO) or natural identical flavors (NIF).

Although EO are known for their antimicrobial properties, they seem to have additional "side" effects: 1) changes in immune function (Boyaka et al., 2001) 2) stimulatory effect on pancreatic secretions (Platel and Srivasan , 1996, 2000); 3) antifungal (Mahmoud, 1994); 4) antiviral (Garcia et al., 2003); 5) antitoxigenic activity (Sakagami et al., 2001); 5) antioxidant activity (Dorman et al., 2000b).

The antimicrobial properties of essential oils have been reviewed by many authors in the past, but only in recent times attempts to identify the bioactive principles have been made (Dorman and Deans, 2000), and following this studies a correlation between the antimicrobial activity of a compound and its % in an essential oil, its chemical structure, functional groups and configuaration has been made (Dorman and Deans, 2000). In Table 1.4 are presented the principal structures producing antimicrobial activity in plant extracts an referenced modes of action.

Table 1.4: chemical structures involved in antimicrobial action of EO and mechanism of action (adapted from Cowan, 1999)

Class	**Subclass**	**Mechanism of action**
Phenols	Phenols and phenolic acids	Enzyme inactivations (1)
		Membrane un-stabilizers (2)
	Quinones	Irreversible union to adhesins, mb polypeptides and enzymes those become inactive (3)
	Flavonoids, Flavones, and Flavonols	
	Tannins	1, 2, 3 and metal chelators
	Coumarins	Interaction with eukaryote DNA
Terpenoids		
Alkaloids		Insertion in cellular wall or in DNA structures
Lectins		Block of viral fusion and absorption
Polypeptides		Di-sulphur bridges formation
Polyacetilens		unknown

Essential oils are volatile compounds usually obtained by steam distillation, although a solvent extraction can be made also. Since they are hydrophobic substance they are insoluble in water and soluble in organic solvents.

EO comprise a great numbers of individual components, maior component constitute up to 85% of an EO, while the minor components can be present in traces although their presence seem involved in the antimicrobial activity by acting synergically with the maior constituent (Burt, 2004). The phenolic compounds are generally responsible for the greatest antimicrobial action. The composition in EO of a plant is not always constant, as it can differ among geographical regions or harvesting seasons (Cosentino et al., 1999; Marino et al., 1999; Juliano et al., 2000; Faleiro et al., 2002); it has been demonstrated that EO from plant harvested in summer, immediately after flowering, have an higher antimicrobial power (McGimpsey et al., 1994; Marino et al., 1999); the part of the plant from which the EO is extracted can also influence its composition (Delaquis et al., 2002). In Table 1.5 are presented the maior constituents of the most common EO with antibacterial properties.

Table 1.5. Maior constituents of EO (from Burt, 2004)

Common name of EO	Latin name of plant source	Major components	Approximate % composition[b]	References
Cilantro	Coriandrum sativum (immature leaves)	Linalool E-2-decanal	26% 20%	(Delaquis et al., 2002)
Coriander	Coriandrum sativum (seeds)	Linalool E-2-decanal	70%	(Delaquis et al., 2002)
Cinnamon	Cinnamomum zeylandicum	Trans cinnamaldehyde	65%	(Lens- Lisbonne et al., 1987)
Oregano	Origanum vulgare	Carvacrol Thymol g-Terpinene Cymene	Trace-80% Trace-64% 2-52% Trace-52%	(Lawrence, 1984; Prudent et al., 1995; Charai et al., 1996; Sivropoulou et al., 1996; Kokkini et al., 1997; Russo et al., 1998; Daferera et al., 2000; Demetzos and Perdetzoglou, 2001; Marino et al., 2001)
Rosemary	Rosmarinus officinalis	a-pinene Bornyl acetate Camphor 1,8-cineole	2 –25% 0 –17% 2 –14% 3 –89%	(Daferera et al., 2000, 2003; Pintore et al., 2002)
Sage	Salvia officinalis L.	Camphor a-Pinene h-pinene 1,8-cineole a-tujone	6 –15% 4 – 5% 2 –10% 6 –14% 20–42%	(Marino et al., 2001)
Clove	Syzygium aromaticum	Eugenol Eugenyl acetate	75–85% 8 –15%	(Bauer et al., 2001)
Thyme	Thymus vulgaris	Thymol Carvacrol g-Terpinene p-Cymene	10–64% l 2 – 11% 2 –31% 10–56%	(Lens-Lisbonne et al., 1987; McGimpsey et al., 1994; Cosentino et al., 1999; Marino et al., 1999; Daferera et al., 2000; Juliano et al., 2000)

[b]*Percentages of total volatiles rounded up to the nearest whole number*

There is abundant literature on EO antimicrobial activity *in vitro* against foodborne pahogens, but a standardised method to test that substances has not been developed. The minimum inhibitory concentration (MIC) is cited by most researchers as a measure of the antibacterial actitivity of EO. The definition of the MIC differs between publications and this is another obstacle to comparison between studies. Some MIC values of most common constituents of EO against foodborne pathogens are presented in table 1.6.

Table 1.6. Selected MIC of EO components against foodborne pathogens (adapted from Burt, 2004)

EO component	Species of bacteria	MIC (μl^{-1})[b]	References
a-Terpineol	E. coli	0.45– >0.9	Cosentino et al., 1999
	S. tiphimurium	0.22	Cosentino et al., 1999
	S. aureus	0.9	Cosentino et al., 1999
	L. monocytogenes	>0.9	Cosentino et al., 1999
	B. cereus	0.9	Cosentino et al., 1999
Carvacrol	E. coli	0.22– 5	Kim et al., 1995a; Cosentino et al., 1999
	S. tiphimurium	0.22– 0.25	
	S. aureus	0.17– 0.45	Kim et al., 1995a; Cosentino et al., 1999
	L. monocytogenes	0.37– 5	Cosentino et al., 199; Lambert et al.,2001
			Kim et al., 1995a; Cosentino et al., 199
Citral	E. coli	0.5	Kim et al., 1995a
	S. tiphimurium	0.5	Kim et al., 1995a
	S. aureus	0.5	Onawamni et al., 1989
	L. monocytogenes	0.5	Kim et al., 1995a
	B. cereus	0.18– 0.9	Cosentino et al., 1999
Eugenol	E. coli	1	Kim et al., 1995a
	S. tiphimurium	0.5	Kim et al., 1995a
	L. monocytogenes	>1	Kim et al., 1995a
Geraniol	E. coli	0.5	Kim et al., 1995a
	S. tiphimurium	0.5	Kim et al., 1995a
	L. monocytogenes	1.0	Kim et al., 1995a
Thymol	E. coli	0.22– 0.45	Cosentino et al., 1999
	S. tiphimurium	0.056	Cosentino et al., 1999
	S. aureus	0.14– 0.22	Cosentino et al., 1999
	L. monocytogenes	0.45	Cosentino et al., 1999
	B. cereus	0.45	Cosentino et al., 1999

[b] *In the references MICs have been reported in the units mg ml^{-1}, % (v/v), and mmol l^{-1}, Ag ml_ 1 and mmol l^{-1}. For ease of comparison these have been converted to µg ml^{-1}, whereby it was assumed that EOs have the same density as water (Burt, 2004).*

Considering the large numbers of constituents of EO, it must be supposed that their antimicrobial activities is not related to a specific mechanism of action but rather to several targets in the microbial cell (Skandamis et al., 2001; Carson et al., 2002). The sites of action of EO are illustrated from Fig. 1.3.

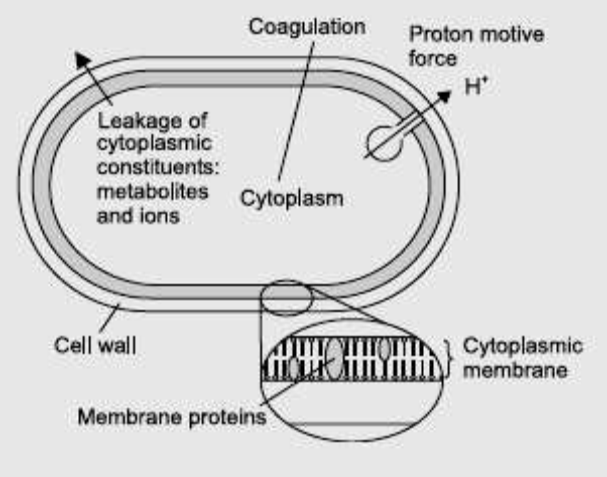

Fig. 1.3. Locations and mechanisms in the bacterial cell thought to be sites of action for EO components: degradation of the cell wall (Thoroski et al., 1989; Helander et al., 1998); damage to cytoplasmic membrane (Knobloch et al., 1989; Sikkema et al., 1994; Oosterhaven et al., 1995; Ultee et al., 2000a, 2002); damage to membrane proteins (Juven et al., 1994; Ultee et al., 1999); leakage of cell contents (Oosterhaven et al., 1995; Gustafson et al., 1998; Helander et al., 1998; Cox et al., 2000; Lambert et al., 2001); coagulation of cytoplasm (Gustafson et al., 1998) and depletion of the proton motive force (Ultee and Smid, 2001; Ultee et al., 1999). From Burt (2004.

It is supposed that EO acts through two different mechanism of action: the first is related to their hydrophobicity which allow them to insert in the phospholipid bilayer and the second is related to the inhibition of bacterial enzymes and receptors through their union with specific sites. Through their hydrophobic structure EO are able to disrupt the bacterial membrane and to change its permeability (Knobloch et al., 1989; Sikkema et al., 1994; Oosterhaven et al., 1995; Ultee et al., 2000, 2002); this causes a ion efflux from the inner cell to the external medium (Oosterhaven et al., 1995; Gustafson et al., 1998; Helander et al., 1998; Cox et al., 2000; Lambert et al., 2001). The ion leakage is usually coupled with other cytoplasmic constituents leakage, and until a certain amount of loss it can be tolerated by the bacterial cell without loss of viability, but, if the efflux is longer prolonged, it will cause cell to collapse. The importance of the hydroxyl group in determining the antimicrobial power has been confirmed, since it has been found that phenolic compounds, such as thymol and carvacrol, have the higher antimicrobial activity (Dorman and Deans, 2000; Ultee et al., 2002). Furthermore it has been established that EO can act on the proteins linked to the

cytoplasmic membrane (Knobloch et al., 1989); two possible mechanisms have been suggested to explain the interactions of phenols on such proteins. The first implies that the hydrocarbons can accumulate in the phospholipid membrane and interfere with the lipid-protein interactions; the other is that the lipohilic hydrocarbon can directly interact with hydrophobic parts of the proteins (Juven et al., 1994; Sikkema et al., 1995). Another supposed action of EO is the interference with cells enzymes: Conner and Beuchat (1984), found that the presence of EO in culture media stimulate the growth of pseudomycelia, as a result of incomplete separations of newly formed cells in yeasts, suggesting that EO may have involved in energy regulation or synthesis of structural constituents.

The mode of action of carvacrol and its analogue, thymol, two of the maior constituents of oregano and thyme essential oils, have been extensively studied.

Carvacrol and thymol are able to disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to ATP. Ultee et al. conducted different studies on *B. cereus* in order to investigate the mechanism through carvacrol exerts its antibacterial action against gram negative bacteria, and results showed that carvacrol dissolves in the phospholipid bilayer aligning between the fatty acid chains resulting in conformational changes of the membrane. A disturbance of the van der Waals interactions between the acyl chains in the membrane causes an increase in membrane fluidity, which in turn would increase passive permeability (Ultee et al., 2002). In the same study the difference between cymene, a precursor of carvacrol differing only for the absence of the hydroxyl group, and carvacrol has been investigated; to asses the lipophilicity of the two substances the $P_{o/w}$, e.g. the partition coefficient octanol/water has been measured. Since cymene resulted to have greater $P_{o/w}$ values than carvacrol and a lesser antimicrobial power it has been postulated that the disgregation of the membrane could not be the unique mechanism of action of those molecules, and that another factor must be involved. This factor is the presence of the hydroxyl group in the carvacrol molecule. The mechanism of action proposed by Ultee et al., (2002) is that carvacrol act as a transmembrane carrier of monovalent cations. The characteristic feature of a phenolic hydroxyl group is its significantly greater

acidity than that of an aliphatic hydroxyl group. Substantially it should acts as an organic acid do: undissociated phenolic compound pass through the cytoplasmic membrane toward the cytoplasm and it dissociate by releasing the proton in the cytoplasm. It re-associates by carrying a K^+ ion or any other cation and in that form is able to re-diffuse through the cytoplasmic membrane to the external environment. This hypothesis was supported by the authors by the observed efflux of K^+ and influx of H^+ in *B. cereus* during exposure to carvacrol. Similar to carvacrol, thymol contains both a hydroxyl group and a system of delocalized electrons and was found to possess strong antimicrobial activity. Juven et al. (1994) examined the activity of thymol against *S. typhimurium* and *S. aureus* and hypothesised that thymol binds to membrane proteins hydrophobically and by means of hydrogen bonds, thereby changing the permeability characteristics of the membrane; furthermore thymol was found to be more inhibitive at pH 5.5 than 6.5. At a lower pH thymol would be more undissociated and therefore more hydrophobic, thereby binding better to the hydrophobic areas of proteins and dissolve better in the lipid phase (Juven et al., 1994).

The hypothesized mechanism of action of carvacrol is illustrated in Figure 1.4.

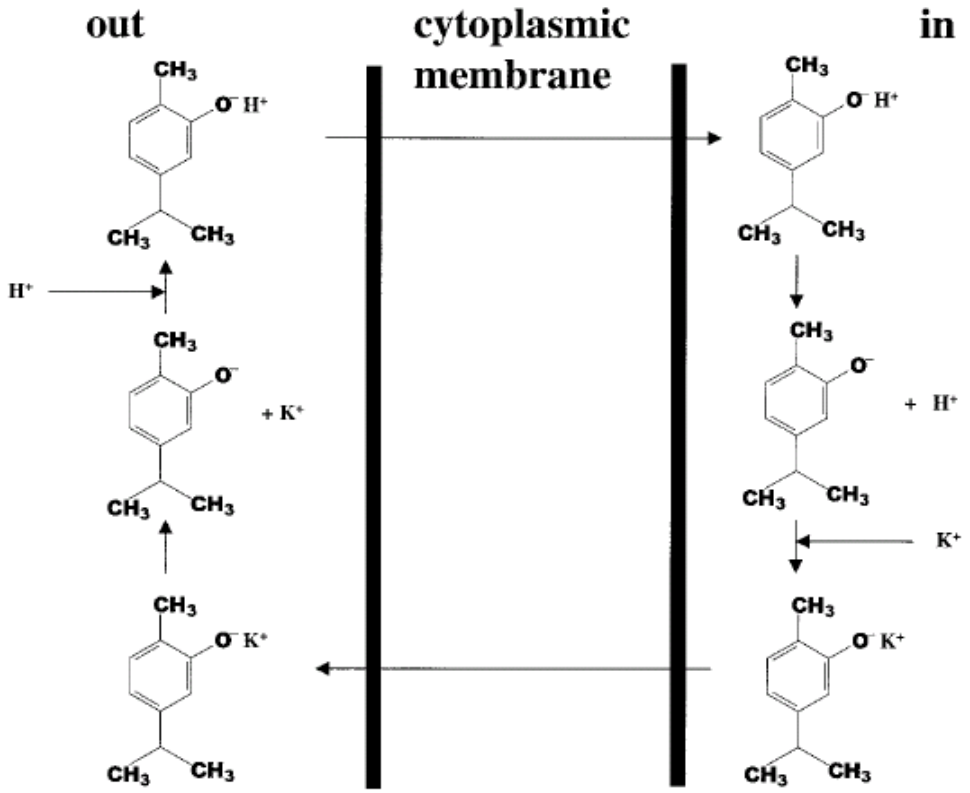

Fig 1.4. Schematic overview of hypothesized carvacrol mechanism of action. From Ultee et al., (2002a). Undissociated carvacrol pass through the cytoplasmic membrane toward the cytoplasm and it dissociate by releasing the proton in the cytoplasm. It re-associates by carrying a K^+ ion or any other cation and in that form is able to re-diffuse through the cytoplasmic membrane to the external environment.

Conversely to *in vitro* studies, little is known on the *in vivo* effect of essential oils. The application of essential oils or their aromatic compounds as alternative to AGP in animal production is too recent and there is too little information about the actual opportunities of these products. Manzanilla conducted a study (Ph.D Thesis, 2005) on the addition of a plant extract containg carvacrol, cinnamaldheyde, and capsicum oleoresin to weaner diets with different source and concentration of proteins. Although growth performance of animals was not affected by the presence of the plant extract, the lactobacilli /*Enterobacteriaceae* ratio in jejunum was shifted toward lactobacilli, thus being related to a lower diarrhea incidence. Decreases in *E. coli* excretion in chickens were obtained with the same plant extract by Jamroz et al. (2003). In another study Manzanilla et al.,

(2004) observed that the inclusion of plant extract in weaner diet determined again an increase in lactobacilli counts, while it tended to reduce *Enterobacteriaceae* counts, resulting in an increase in the lactobacilli/*enterobacteriaceae* ratio.

Antimicrobial properties of such compounds are regarded as substituting the AGP antibacterial effect, but plant extract, essential oils and flavors have other properties which may help digestive functions in the animal, first of all the stimulation of enzymatic secretions (Platel and Srinivasan ,1996, 2000). Since the piglets 3 weeks old have an enzymatic set not fully completed, this could be an interesting application at weaning (Kamel, 1999). For example it has been suggested that a low blood irrigation of stomach may be the cause of a scarce HCl secretion (Dunshea, 2003); being capsaicin a vasodilator, this properties could be used to improve stomach function of the animal. Flavors can also modify the transit time of digesta (Mikelefield et al., 2000, 2003), and, they can protect the intestinal epithelium against aggression through their antioxidant properties (Teissedre and Waterhouse, 2000).

CHAPTER 2.

OBJECTIVES

Weaning is a very crucial moment in the life of pigs; there are many factors which contribute to the rise of gut infections, which have an high economic impact in pig husbandry, especially after the removal of antibiotic growth promoters in EU, as of January 1st 2006.

In this context management and improvement of biosecurity are studied, but the development of new nutritional strategies are now the priority.

The use of organic acids and flavors as alternatives to AGP in animal husbandry has been proposed by many authors. Despite the abundant literature regarding antibacterial activities of such substances in *in vitro* experiments, little data are now available on their *in vivo* effects, especially regarding flavors.

In this scenario the objectives of this work were:

1. to screen the antimicrobial power of different organic acids and flavors against foodborne pathogens, which represent a threat to both animal productivity and human health, to find possible synergisms among those compounds;
2. to evaluate the role of such compounds and their possible synergistic effect in modulating swine intestinal microflora in an *in vitro* system which simulate intestinal environment;
3. to evaluate substances under investigation *in vivo* as feed additive to prevent intestinal disorders during weaning.

To reach these objectives 3 groups of experiments were planned:

1. determination of the Minimal Inhibitory Concentration (MIC). The first study was planned to assess the MIC of organic acids and flavors against *Clostridium perfringens* and *Campylobacter jejuni*. The second study was to assess the presence of synergism between citric acid and thymol against *Salmonella enteritidis*, *Escherichia coli* and *Clostridium perfringens*.
2. Intestinal fermentations. The first study was to evaluate the role of citric acid, thymol, and three blends of the two substances in modulating the small intestine microflora and fermentation parameters in an *in vitro* system (batch culture tecniques). The second study regarded the investigations of such compounds on the cecal microflora.

3. *In vivo* studies. In the first study it was evaluated the role of microencapsulated citric acid, thymol, and a blend of the two in improving piglets health status and productivity; the second experiment was to evaluate a dose-response effect on feeding growing doses of a microencapsulated blend of citric acid and thymol.

CHAPTER 3.

DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATION OF ORGANIC ACIDS AND NATURAL IDENTICAL FLAVORS

3.1. DETERMINATION OF THE MINIMAL INHIBITORY CONCENTRATION OF ORGANIC ACIDS (OA) AND NATURAL IDENTICAL FLAVORS (NIF) OF THE GROWTH OF *CLOSTRIDIUM PERFRINGENS* AND *CAMPYLOBACTER JEJUNI*.

3.1.1. Aim of the study

Aim of this study was to investigate the role of organic acids (OA) and natural identical flavors (NIF) as antimicrobial compounds in inhibiting *C. perfringens* and *C. jejuni* growth in order to study their possible use in animal husbandry to reduce microbial infections and microbial carcass contamination at slaughter.

3.1.2. Materials and methods

Bacterial strains and growth conditions

The bacterial strains used to test the substances in this study were *Clostridium perfringens* ATCC 13124, *Clostridium perfringens* field isolate (FI) and *Campylobacter jejuni* ATCC 33291. *C. perfringens* ATCC 13124 and *C. jejuni* ATCC 33291 were bought from American Type Culture Collection (ATCC, University Boulevard, Manassas, VA, USA); *C. perfringens* FI was received from the University of Montréal, Saint Hyacinthe, QC, curtesy of Dr. Serge Messier, Canada stored in cooked meat. After *C. perfringens* ATCC 13124 and *C. jejuni* have been revitalized according to manufacturer instructions, the master strains were preserved at room temperature in Cooked Meat (CM broth, Difco Laboratories, Division of Becton Dickinson and Company, Sparks, MD, USA) for Clostridia and at 4°C in Campylobacter Broth (CB broth, Oxoid, Unipath Ltd, Basingstoke, Hampshire, RG24 OPW) for Campylobacter. Clostridia working seeds were obtained by inoculating 100 μL of each stock culture in 10 mL of RCM and incubating all at 37°C in anaerobic conditions through two subsequent

incubations of 24 h , while Campylobacter was obtained using CB broth and incubating in microaerophilic conditions.

Chemicals

NIF and OA objects of the study were: carvacrol (Fluka), eugenol (Fluka), salicylaldehyde (Fluka), thymol (Aldrich), vanillin (Fluka) and lactic acid (Fluka), all purchased by Sigma-Aldrich Chemie Gmbh, Steinheim, Germany, acetic acid (Carlo Erba Reagents, Rodano, Milano, Italy) and, α-chetoglutaric acid, benzoic acid, caprylic acid, citric acid, formic acid, fumaric acid, D-gluconic acid, heptanoic acid, iso-butyric acid, DL-malic acid, propionic acid, sorbic acid, succinic acid, tartaric acid and n-valeric acid (Sigma-Aldrich Corporation, St. Louis, MO, USA). The molecules were diluted in RCM broth for Clostridia tests and in CB broth for *C. jejuni* tests as described below.

Broth dilution method

Each OA and NIF was diluted in RCM or CB broth in order to obtain 1M solutions (stock solutions) except for benzoic and sorbic acids which were diluted at 500 mM, caprylic acid, valeric acid and vanillin which were diluted at 250, 125, and 62.5 mM, respectively. Solutions of salicylaldehyde and heptanoic acid at 1 M, carvacrol and thymol at 125 mM, eugenol were prepared in RCM broth and CB broth using ≤ 5% (v/v) of ethanol (Merck, Darmstadt, Germany). All the solutions were adjusted to pH 6.5 with HCl or NaOH and sterilized using a filter with a membrane pore size of 0.45 µm (Stericup/Steritop Filter Unit, Millipore Corporation, Bedford, MA, USA).

Serial two-fold dilutions of each stock solutions were made in RCM and CB broth to reach final concentrations of 3.91 mM and 0.49 mM for media containg OA and NIF, respectively.

Disposable tubes containing 5 ml of test media were inoculated with 10^4 CFU/ml of each bacteria. RCM broth cultures were incubated at 37°C for 48h in anaerobic conditions; whereas CB broth cultures at 42°C for 48h in micro-aerobic conditions. As positive control, three tubes containing 5 ml of RCM or CB broth without any compound under study were inoculated with the same concentrations of microorganism and incubated as previously described, for compound solutions

prepared without ethanol; whereas, dilutions of RCM broth or CB broth with appropriate amount of ethanol but without any compounds were also prepared.

Optical density (OD) as bacterial growth index was measured after 24 and 48h of incubation using a spectrophotometer (UltroSpec 3000 Pharmacia Biotech, Biochrom, Ltd, Cambridge, UK) at lambda (λ) = 600 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 24 and 48h 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 inhibited the increase of turbidity of bacterial cultures after 24 and 48h of incubation.

Agar test

Agar plates of RCM and CB were spread with 50 µl of RCM or CB cultures from each tube after 24 and 48h of incubation. Plates were incubated for each strain at the same conditions of tubes and they were read after 24h and 48h to verify visible bacterial growth as to screen bacteriostatic or bactericidal activity of each substance under study.

The MIC were confirmed as the lowest concentration of the specific substance which prevents the visible growth of bacteria on agar.

Statistical analyses

The experiment was made in triplicate. 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 $P<0.05$.

3.1.3. Results and discussion

MIC of all the tested substances are shown in the Table 3.1.

Campylobacter jejuni provided only agar results because of its weak growth, that was not detectable by optical density reading.

Natural identical flavors

Eugenol, carvacrol, and thymol were the most effective flavors against all the bacterial strains.

The MIC of eugenol was 15.63, 3.91, and 1.95 mM for C. perfringens ATCC 13124, C. perfringens FI, and C. jejuni, respectively. Statistical analysis showed that concentrations of eugenol below the MIC influenced the extent of growth of Clostridia: after 24h it reduced the O.D. of C. perfringens ATCC 13124 (-32%, P<0.001) and of C. perfringens FI (-40%, P<0.001) at 1.95 mM.

The MIC of carvacrol was 15.63, 3.91, and 7.81 mM for C. perfringens ATCC 13124, C. perfringens FI, and C. jejuni, respectively.

The MIC of thymol was15.63, 3.91, and 31.25 mM for C. perfringens ATCC 13124, C. perfringens FI, and C. jejuni, respectively.

The MIC of salicylaldehyde was 31.25 mM for all the tested strains; even if it has been the less effective substance among flavors, it decreased the growth of both Clostridium strains (-43%, P<0.001) at 15.63 mM since from 24h of incubation.

The MIC of vanillin was 15.63 mM for C. perfringens 13124 and 62.5 for both C. perfringens FI and C. jejuni. Compared to control, vanillin at 7.81 mM depressed the growth of C. perfringens ATCC 13124 (-73%, P<0.001) and of C. perfringens FI (-79%, P<0.001) after 24h and of C. perfringens FI (-72%, P<0.001) after 48h. At 0.98 mM vanillin was able to contain the growth of C. perfringens FI (–58%, P<0.001) either at 24h at 48h.

Organic Acids

Organic acids were tested only against *C. perfringens* 13124 and *C. jejuni*. α-chetoglutaric acid was not tested against *C. jejuni.*

C. perfringens was more sensitive to acid action than *C. jejuni*. Benzoic and heptanoic acid were the most effective acids for both the bacterial strains. The MIC of benzoic acid was 31.25 mM for both *C. perfringens* and *C. jejuni*. Benzoic acid controlled the growth of *C. perfringens* ATCC 13124 even at 3.91 mM (-40%, *P*<0.001) after 24 h; after 48h, it still induced a decrease of microbial OD at 15.63 mM (-71%, *P*<0.001).

The MIC of heptanoic_acid was 7.81 and 31.24 mM for *C. perfringens* and *C. jejuni*, respectively. Hepatnoic acid showed to be inhibitory in broth against *C. perfringens* only at 31.25 after 24 and 48 h of incubation, while it was inhibitory

at 15.63 and 7.81 mM in agar after 24h and 48h, respectively, thus showing to have inhibitory effect at low concentrations during the 48h duration of the study. Heptanoic acid limited the *C. perfringens* growth until 3.91 mM by -33% and by -24% ($P<0.01$) after 24h and 48h, respectively.

.The MIC of caprilic acid was 7.81 and 62.5 for *C. perfringens* and *C. jejuni*. Caprilic acid at 3.91 mM in broth was able to reduce the growth of *C. perfringens* by -96% and -45% ($P<0.01$) after 24h and 48h, respectively.

The MIC of citric acid was 62.5 for *C. perfringens*, while it was not detected until 1000 mM for *C. jejuni*. Citric acid at 31.25 mM was able to decrese *C. perfringens* OD values after 24h of incubation, but it allowed the growth on agar thus showing to have a bacteriostatic effect at that concentration. Citric acid was able to contain *C. perfringens* growth in broth even at 3.91 mM (-71%, $P<0.001$) either at 24h and 48h.

The MIC of sorbic acid was 31.25 and 500 mM for *C. perfringens* and *C. jejuni*, respectively. sorbic acid decreased OD values of *C. perfringens* even at 3.91 mM, after 24h and 48h by -42% and -37%, respectively ($P<0.001$), compared to control.

The other acids showed a weaker action than the prevoius ones against both strains, especially against *C. jejuni* which was resistant to acetic, formic, gluconic, iso-butyrric, lactic, succinic, tartaric, and valeric acid until 1000 mM. *C. perfringens* was resistant to acetic, α-chetoglutaric, formic, and propionic acid. Nevertheless those acids were able to reduce the broth turbidity, such as the viable cells of *C. perfringens* on agar even at lower concentrations than 1000 mM.

Acetic acid significantly reduced the turbidity until 31.25 mM after 24h, (–63%, $P<0.001$); α-chetoglutaric acid at 250 mM was still able to contain the growth of *C. perfringens* (-80%, $P<0.001$) after 48h of incubation; formic acid decreased OD values until 15.63 mM either at 24h (-55%, $P<0.001$) and 48h (-35%, $P<0.001$); propionic acid limited the growth of *C. perfringens* even at 3.91 and 62.50 mM after 24h and 48h, respectively (-11%; -30%, $P<0,05$) while it was inhibiting the growth of *C. jejuni* at 62.5 mM.

The MIC of fumaric, D-gluconic, and succinic acid against *C. perfringens* was 500 mM.

The MIC of DL-malic acid was 125 and 250 mM for *C. perfringens* and *C. jejuni*, respectively, but it was able to reduce the development of *C. perfringens* even at concentrations below the MIC: at 62.50 mM after 24 and 48h (-98%, -26%, respectively; $P<0.05$) it exerted a bacteriostatic effect.

The MIC of lactic and tartaric acid for *C. perfringens* was 1000 mM. Tartaric acid was able to reduce *C. perfringens* growth until 125 mM after 24h and 48h by -78% and -80%, respectively ($P<0.001$).

The MIC of valeric acid for *C. perfringens* was 125 mM; after 24h, n-valeric acid was able to reduce its growth until 15.63 mM (-17%, $P<0.001$), whereas, at 48h, the acid was able to contain the microbial development until 7.81 mM by -10% ($P<0.01$).

The MIC of iso-butyric acid for *C. perfringens* was 500 mM. This acid showed to have a bacteriostatic effect at 125 and 250 mM after 24h and 48h of incubation by having OD values lower than MIC values; furthermore, after 24h the MIC on agar was 250 mM, thus confirming that at 250 mM it acted as a bacteriostatic allowing the growth after 48h.

There are numerous studies about bactericidal activity of plant extracts and organic acids on spoiling *bacteria,* moulds, and on pathogens such as *S. aureus*, *Salmonella* spp., *E. coli, Pseudomonas aeruginosa*, *Bacillus cereus, Listeria monocytogenes*, *Yersinia enterocolitica*, (Conner and Beuchat, 1984; Galli et al., 1985; Daouk at al., 1995; Paster et al., 1990; Paster et al., 1995; Sivropoulou et al., 1996; Manou et al., 1998; Burt and Reinders, 2003; Friedman et al., 2002; Lee et al., 2004, Peñalver et al., 2005), but few authors studied antimicrobial activity of natural inhibitory substances against *C. perfringens* and *C. jejuni* up to now (Kamel et al., 2000; Strauss et al., 2001; Friedman et al., 2002; Friedman et al., 2003).

In this study Clostridia resulted more sensitive than *C. jejuni* : this could be explained with the fewer barriers and different structural and chemical composition of the cells wall of Gram-positive *bacteria* which results usually more sensitive to inhibition by antimicrobials than Gram-negative bacteria (Nikaido and Varra, 1985; Lis-Balchin, 2003). From data of Si et al.(2006) it appears that essential oils/compounds have Gram-negative bacteria specifically as

a target, although the authors specified that further investigation is required to reach a conclusion; however there are other studies which demonstrate the lack of specificity of those compounds toward Gram-positive bacteria rather than Gram-negative ones (Lis-Balchin, 1998). Dorman and Deans (2001) and Lambert et al. (2001) reported that oregano, which contains carvacrol as the main active component, has strong antimicrobial activity against both Gram-positive and Gram-negative bacterial pathogens. The antimicrobial mechanism of essential oils compounds is poorly understood. Current researche on the antimicrobial action of phenolic compounds, such as carvacrol and thymol, focuses their attention on cell membrane permeability (Davidson, 1997; Ultee et al., 1999) which depends on the hydrophobicity of the solutes that have to cross the membrane and on its composition. Partition coefficient of essential oils in cell membrane is determinant for their effect. The hydrophobic constituents of essential oils are capable of accessing to the periplasm of Gram-negative *bacteria* through the porin proteins of the 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 (e.g. thymol and carvacrol), or the presence of hydroxylic group (e.g. thymol, carvacrol and eugenol) alter polarity and topography of a molecule, therefore changing the affinity to different binding sites in the bacteria (Si et al., 2006). Furthermore Shapiro and Guggenheim in their studies found that hydrophobicity (water solubility) and steric descriptions (molecular size and shape) have important roles in anti-bacterial activity.

Kamel (2000) found that clove extract (80% eugenol) at 500 ppm (3,05 mM) was active in killing 50% of *C. perfringens* cells. In the same study oregano extract, which contains from 60% to 76% of carvacrol, showed to be inhibiting *C. perfringens* at 800 ppm (5.32 mM), thus confirming the results of our study against *C. perfringens* ATCC 13124 and *C. perfringens* FI. *C. jejuni* also was inhibited by oregano essential oil at 400 ppm, (2,66 mM) which resulted to be a

lower value than the one obtained in this study (7,81 mM). Differently to previous studies and to the present research, Friedman et al. (2002) found that the best inhibitory substance against *C. jejuni* was carvacrol instead of eugenol and thymol. Indeed, the concentration of carvacrol that killed 50% of bacterium during 60 minutes of incubation was 0.7 mM, whereas eugenol was 1.3 mM and thymol was 1.6 mM. The comparison between the results obtained in this study and the one from other researchers is difficult because flavors are very complex mixtures of compounds with different structures and chemical composition (Lee et al., 2004). Furthermore, they are poorly soluble in water and their antibacterial activity has been tested with different methods and techniques such as type and quality of emulsifier and strains and number of cells of micro-organisms tested (Zaika, L. L. 1988, Lahlou, 2004, Peñalver et a., 2005).

Organic acids resulted less effective than flavors in terms of molar concentration, but caprylic acid showed in broth and agar the same MIC obtained for carvacrol and thymol by broth dilution method against *C. perfringens* ATCC 13124; even benzoic and heptanoic acids showed the same MIC of salycilaldehyde and thymol, and propionic acid obtained the same MIC of vanillin against *C. jejuni*. Ten among the tested acids permitted a visible *C. jejuni* growth on agar even at the greatest concentration of each acid. A recent study indicated also that fumaric or malic acids (10 to 50 mM), lactic acid (20-50 mM) and succinic acid (30-50 mM) or mixtures of all of them at 10-40 mM added to media could stimulate the growth of *Campylobacter spp*, whereas citric acid was not able to stimulate the growth of three of six *Campylobacter spp* isolates showing that those isolates could not use citric acid as a metabolite (Hinton, 2006). In our study fumaric, lactic, succinic, and citric acids permitted visible *C. jejuni* growth on agar even at the greatest concentration of each compound, whereas malic acid stopped its growth at 250 mM after 24h and 48h.

The mode of action of organic acids is primarly associated to the fact that the undissociated organic acids can penetrate the bacteria cell wall and disrupt the normal physiology of bacteria pH sensitive (Gauthier, 2002). Lambert and Stratford (1999) described that 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 (Gauthier, 2002; Piva, 2000). 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 show different levels of sensitivity to different organic acids under specific circumstances.

In conclusion, the results obtained in this study suggest that Clostridia were more sensitive than *C. jejuni* to antimicrobial effect of tested molecules; organic acids resulted less effective than flavors against tested bacteria and some of these could even stimulate the growth of *C. jejuni*. Further studies are needed to choose right combination of substances to reduce carcass contamination at slaughterhouse and increase meat safety.

Table 3.1: Experimental MIC values of all the tested substances against 10^4 CFU/mL of *C. perfringens ATCC 13124, C. perfringens FI, and C. jejuni ATCC 33291* after 48 h of incubation. The values are expressed as mM/L. (n =3)

	C. perfringens **ATCC 13124**	***C. perfringens*** **FI**	***C. jejuni ATCC*** **33291**
Carvacrol	15.63	3.91	7.81
Eugenol	3.91	3.91	1.95
Salicylaldehyde	31.25	31.25	31.25
Thymol	15.63	3.91	31.25
Vanillin	15.63	62.50	62.50
Acetic acid	NA[b]	ND[c]	NA[b]
α-chetoglutaric acid	NA[b]	ND[c]	ND[c]
Benzoic acid	31.25	ND[c]	31.25
Caprylic acid	7.81	ND[c]	62.50
Citric acid	62.50	ND[c]	NA[b]
Formic acid	[b]NA	ND[c]	NA[b]
Fumaric acid	500	ND[c]	NA[b]
D-Gluconic acid	500	ND[c]	NA[b]
Heptanoic acid	7.81	ND[c]	31.25
Iso-butyric acid	500	ND[c]	NA[b]
Lactic acid	1000	ND[c]	NA[b]
DL-Malic acid	125	ND[c]	250
Propionic acid	NA[b]	ND[c]	62.50
Sorbic acid	31.25	ND[c]	500
Succinic acid	500	ND[c]	NA[b]
Tartaric acid	1000	ND[c]	NA[b]
n-Valeric acid	125	ND[c]	NA[b]

[b]*NA = not available*
[c]*ND = not determined.*

3.2. DETERMINATION OF THE MINIMAL INIBITORY CONCENTRATION OF CITRIC ACID AND/OR THYMOL AGAINST STRAINS OF *SALMONELLA ENTERITIDIS, ESCHERICHIA COLI O157:H7* AND *CLOSTRIIDUM PERFRINGENS* AT 6, 12 AND 24 H OF INCUBATION, BY GROWTH AND ABSORBANCE MISURAMENT IN MICROTITER PLATES.

3.2.1. Aim of the study

The purpose of the study was the determination of the Minimal Inhibitory Concentration (MIC) of citric acid and thymol, alone or in combination, against *Salmonella enteritidis, Escherichia coli* O157:H7, and *Clostriidum perfringens* and the individuation of an additive, synergistic or antagonist effect, when tested in combination.

3.2.2. Material and methods

Bacterial strains and growth conditions

Bacterial strains object of the study were *Salmonella enteritidis* 3502, *Escherichia coli* O157: H7 ATCC 35150 and *Clostridium perfringens* ATCC13214. Salmonella strain, isolated from salami, was kindly provided from Dr. John B. Luchansky, USDA, Microbial and Food Safety Research Unity, Wyndmoor, PA, USA), stored in Brain Heart Infusion agar plates (BHI, Oxoid, Unipath Ltd, Basingstoke, Hampshire, RG24 OPW), while *E. coli* and *C. perfringens* were bought from American Type Culture Collection (ATCC, University Boulevard, Manassas, VA, USA). *S. enteritidis* was revitalized by transferring a colony from the agar plate to a tube containing BHI broth by mean of a sterile loop, and incubating the so-obtained culture at 37°C for 24h. After the incubation the

Salmonella stock culture was stored at -20°C. After *E. coli* and *C. perfringens* were revitalized according to manufacturer instruction, the master strains were stored at –20°C in BHI broth, and at room temperature in Cooked Meat (CM broth, Difco Laboratories, Division of Becton Dickinson and Company, Sparks, MD, USA), respectively. *S. enteritidis* and *E. coli* working seeds were obtained by inoculating 100 μL of each stock culture in 10 mL of BHI broth incubating all at 37°C for 24h through two subsequent incubations; *C. perfringens* working seed was obtained by inoculating 100 μL of the stock culture in 10 mL of Reinforced Clostridial Medium broth (RCM, Oxoid, Unipath Ltd, Basingstoke, Hampshire, RG24 OPW) and incubating all in anerobic conditions at 37°C for 24h through two subsequent incubations.

Chemicals

Thymol and citric acid were purchased by Sigma-Aldrich Chemie Gmbh, Steinheim, Germany. The molecules were diluted in BHI for *S. enteritidis* and *E. coli* and in RCM broth for *C. perfringens* tests as described below.

Broth dilutions method and MIC determination in microtiter plates

Citric acid was diluted in BHI or RCM broth in order to obtain 500 mM solutions (stock solutions); thymol was dissolved in BHI and RCM with 5% (vol/vol) ethanol to a final concentration of 62.5 mM or 23.36 mM. All the stock solutions were adjusted to pH 6.5, and sterilized by filtration (pore diameter 0.22 μm). Serial dilutions of each stock solutions were made in BHI or RCM broth with pH 6.5 to reach final concentrations in the microtiter plates of: 250, 125, 62.5, 31.25, 15.63 mM for citric acid; 1.46, 0.98, 0.73, 0.49, 0.37 mM for thymol; citric acid and thymol combinations at same concentrations (except citric acid 250 mM);

S. enteritidis, E. coli and *C. perfringens* inhibition trials were performed on 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 the citric acid/ thymol solution. The positive control reference well was inoculated in the same way with 100 μl of a 10^5 CFU/ml culture and 100 μl of broth (pH = 6.5) without the addition of any substance. Each substance was tested in duplicate. *S. enteritidis* and *E. coli* plates were incubated at 37 °C in aerobiosis, *C. perfringens* plate was incubated at the same temperature but in anaerobic conditions.

The turbidity of cultures was used as index of bacterial growth and optical density (OD) was measured after 6, 12 and 24h of incubation using a spectrophotometer (Tecan Spectra Classic, Tecan Group Ltd, Switzerland) at lambda (λ) = 630 nm.
The MIC was defined as the lowest concentration of molecule that completely inhibited the growth of the organism as detected with the absorbance measurement. 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: ΣFIC = 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 ΣFIC ≤0.5, an additive effect was defined when the ΣFIC is >0.5 to <2, Indifference was defined as a ΣFIC ≥ 2 but < 4 and antagonism was defined as a ΣFIC ≥ 4.

Statistical analyses and interpretation of results

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 $P<0.05$.

3.2.3. Results and Discussion

S. enteritidis growth after 6 h of incubation was inhibited by citric acid at 250 mM and by citric acid and thymol in combination at 15.63 and 1.46, respectively, whereas thymol at 1.46 mM did not decrease optical density values. The MIC of citric acid and thymol after 12 h and 24 h alone was not determined because the highest concentrations of the two molecules were not effective in inhibiting Salmonella growth, while the MIC of the combination at 12 h was 31.25 mM and 0.95 mM, for citric acid and thymol, respectively, and 62.5 mM and 1.46 mM at 24 h , although the combination of 31.25 mM of citric acid and 1.46 mM of thymol already reduced growth by 80% when compared to positive control. The

latter combination (15.63 mM of citric acid and 0.37 mM of thymol) reduced *S. enteritidis* growth of 30% when compared to control (Fig. 3.a).

The MIC of *E. coli* after 6 h were the same observed for *S. enteritidis*; nevertheless, after 12 h, *E. coli* resulted to be more sensitive than Salmonella to the action of citric acid and thymol individually tested, being inhibited by citric acid at 250 mM, and by thymol at 1.46 mM, while the MIC of the two molecules combined together was the same of the one observed in Salmonella. At 24 h citric acid and thymol were not able to inhibit *E. coli* growth when individually tested, but in combination the MIC values were 31.25 mM and 1.46 mM, for citric acid and thymol, respectively; similarly to what observed in Salmonella results, the combination of 15.63 mM of citric acid and 1.46 mM of thymol reduced growth by 80% (Fig. 3.b).

C. perfringens growth was measured only after 12 and 24 h. After 12 h it resulted more sensitive than *E. coli* and *S. enteritidis* to the antibacterial effect of both citric acid and thymol: the MIC values were 3.9 mM and 1.46 mM of citric acid and thymol, respectively, and 3.9 mM and 0.37 mM when tested in combination. After 24 h the MIC values were the same of that at 12 h with the exception of thymol alone which was 3.9 mM instead of 1.46 as observed at 12 h (Fig 3.c).

These findings suggest that *C. perfringens* is more sensitive than *S. enteritidis* and *E. coli* to the action of organic acids and flavors, thus confirming observations of the previous study on the inhibitory power of such molecules against Gram-positive and Gram-negative bacteria. In the experiment with *C. perfringens* an additive effect between citric acid and thymol was found, as it is reflected by the FIC index (0.7 and 0.6 at 12 and 24 h, respectively); nevertheless the combination of the two molecules allowed to reach the MIC by lowering their inclusion rate in the medium of 50 % and 90% for citric acid and thymol, respectively (Tab. 3.2.) when compared to citric acid and thymol alone. In *S. enteritidis* and *E. coli* experiments the synergistic effect is present, although FIC index were not calculated because the MIC of citric acid and thymol for *S. enteritidis* and *E. coli* were not detectable at the highest concentrations tested. Citric acid and thymol combined in the culture media allowed to reach a MIC whereas the two substances tested alone did not. Citric acid and thymol concentrations

corresponding to MIC values tested in combination for *E. coli* and *S. enteritidis*, i.e. 15.63 mM of citric acid and 1.46 mM of thymol, and the dose with 3.9 mM of citric acid and 0.37 mM of thymol for *C. perfringens*, had the same citric acid to thymol ratio (about 10:1) which suggests that citric acid and thymol act in synergy in determining their antimicrobial effect against pathogen strains when used in such a ratio.

These results are of great interest to animal husbandry, where, for many years, antibiotic growth promoters (AGP) helped to improve animal performance, exerting a control on intestinal microflora and reducing the development of pathogens, being *E. coli* and Salmonella the maior causes of mortality in piglets at weaning. The possible development of antibiotic resistant bacteria led the AGP ban in Europe as of January 1st 2006. This ban increased the interest in finding natural alternative to growth promoters, such as flavors and organic acids. Partanen and Mroz (1999) extensively reviewed organic acids positive effect on piglets performance at weaning, especially citric acid which improved feed to gain ratio. Flavors are studied because of their antimicrobial and antitoxin properties (Dorman and Dean, 2000; Burt, 2004), for their effect in stimulating enzyme activity or for immunomodulatory properties (Middleton and Kandaswami 1992; Azumiet al., 1997; Platel and Srinivasan, 2000, Manzanilla et al., 2004). Even if many researchers investigated on such alternatives, few studied interactions between different classes of molecules and the possible synergy both *in vitro* and *in vivo* (Delaquis et al., 2002; Manzanilla et a., 2004) in order to have better performance.

In the present study the MIC of thymol individually tested on *E. Coli* and Salmonella was an unknown value above 1.46 mM (0.2 µg/mL); when thymol is used in combination with an organic acid, such as citric acid, the MIC value decrease to 0.95 after 12 h of incubation, which can be converted to 0.13 µg/mL. Citric acid, at the same way, had a MIC value of 250 mM when individually tested (48 µg/mL), whereas it had 31.25 mM (6 µg/mL) after 12 h of incubation combined with thymol. The antibiotic growth promoters, such as Tylosin, were used as feed additive from 44 to 110 µg/mL, depending on the type of molecule and on the stage of production (Si et al., 2006). As the MIC values of this study

were lower than the concentration of AGP in swine production (44-110 µg/mL vs 6.1 µg/mL) it can be thought that they could be valid alternative for dietary antibiotics to be included at lower levels.

Tab. 3.2. Synergistic antibacterial effect of citric acid and thymol against *S. enteritidis*, *E. coli* O:157 and *C. perfringens* after 6, 12 and 24-hours of incubation. The MIC concentration of citric acid and thymol used in combination are expressed as per cent of MIC concentration of acid or thymol individually tested.

h	MIC Citric acid	MIC Thymol	MIC Citric acid + Thymol	Citric acid (%)	Thymol (%)	FIC
			S. enteritidis			
6	250	ND	15.63 + 1.46	6.2	100[a]	-
12	ND	ND	31.25 + 0.95	12.5[a]	65[a]	-
24	ND	ND	62.56 + 1.46	25[a]	100[a]	-
			E. coli			
6	250	ND	15.63 + 1.46	6.2	100[a]	-
12	250	1.46	31.25 + 0.95	12.5	65	0.8
24	ND	ND	31.25 + 1.46	12.5[a]	100[a]	-
			C. perfringens			
12	7.8	1.46	3.9 + 0.37	50	25.3	0.7
24	7.8	3.9	3.9 + 0.37	50	9.5	0.6

[a] *where the MIC was not detectable (ND) it was considered the highest concentration tested*

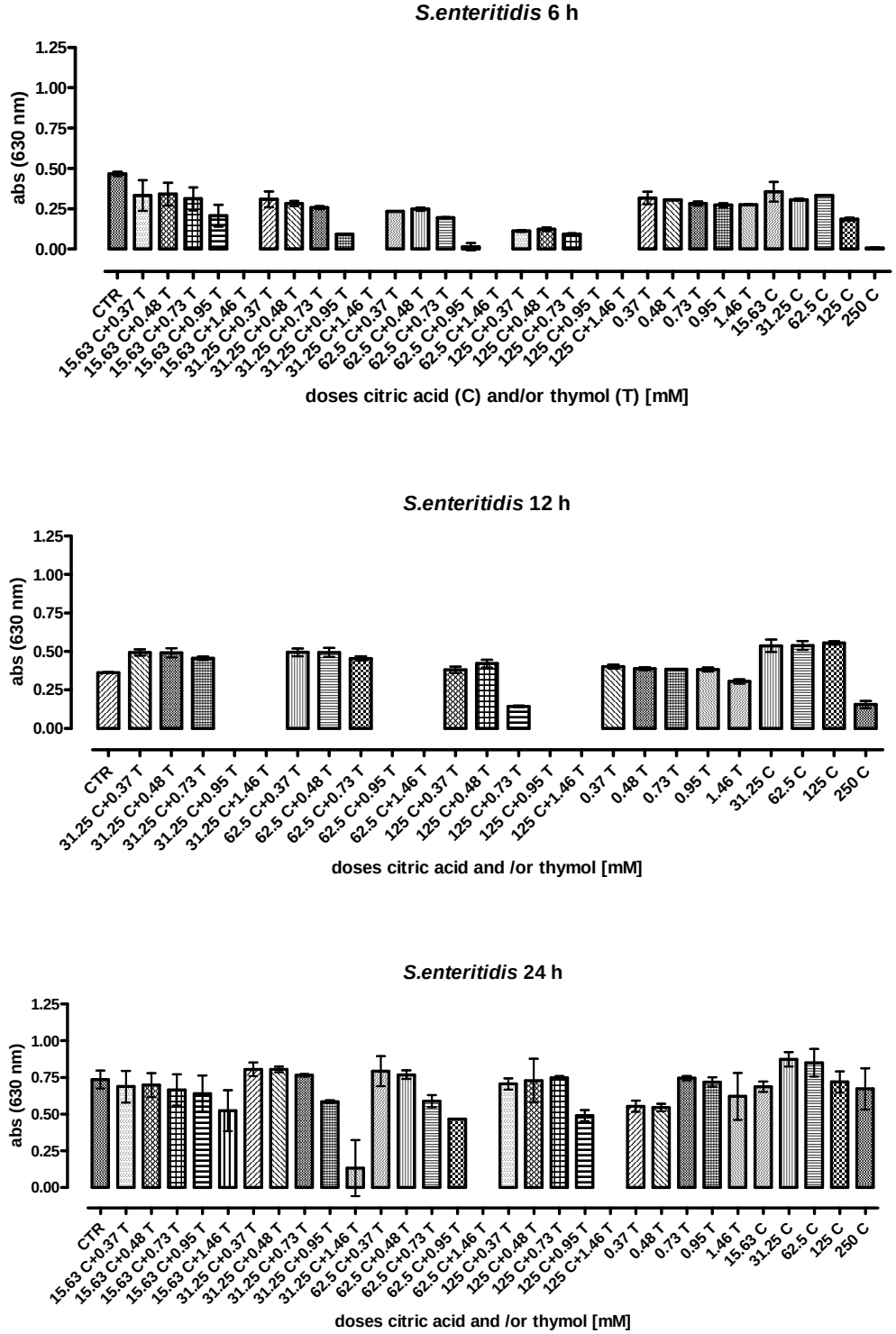

Fig. 3.a*: Absorbance (λ= 630 nm) values of S. enteritidis growth after 6, 12, and 24 h of incubation. Column bars indicate mean ± SD. Each co lumn is the mean of two values. T= thymol; C= citric acid*

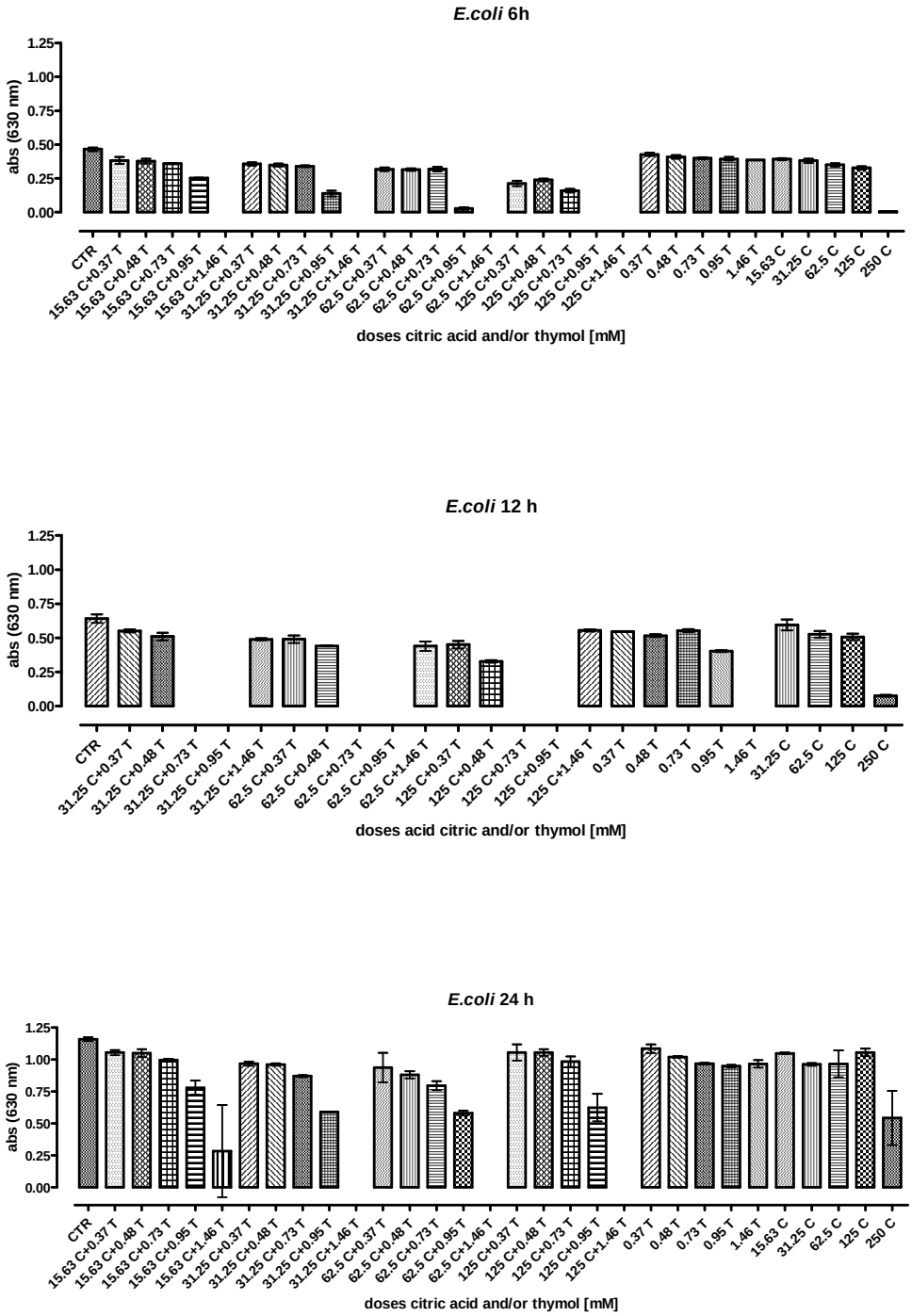

Fig. 3.b*: Absorbance (λ= 630 nm) values of E. coli O157: H7 growth after 6, 12, and 24 h of incubation. Column bars indicate mean ± SD. Ea ch column is the mean of two values. T= thymol; C= citric acid*

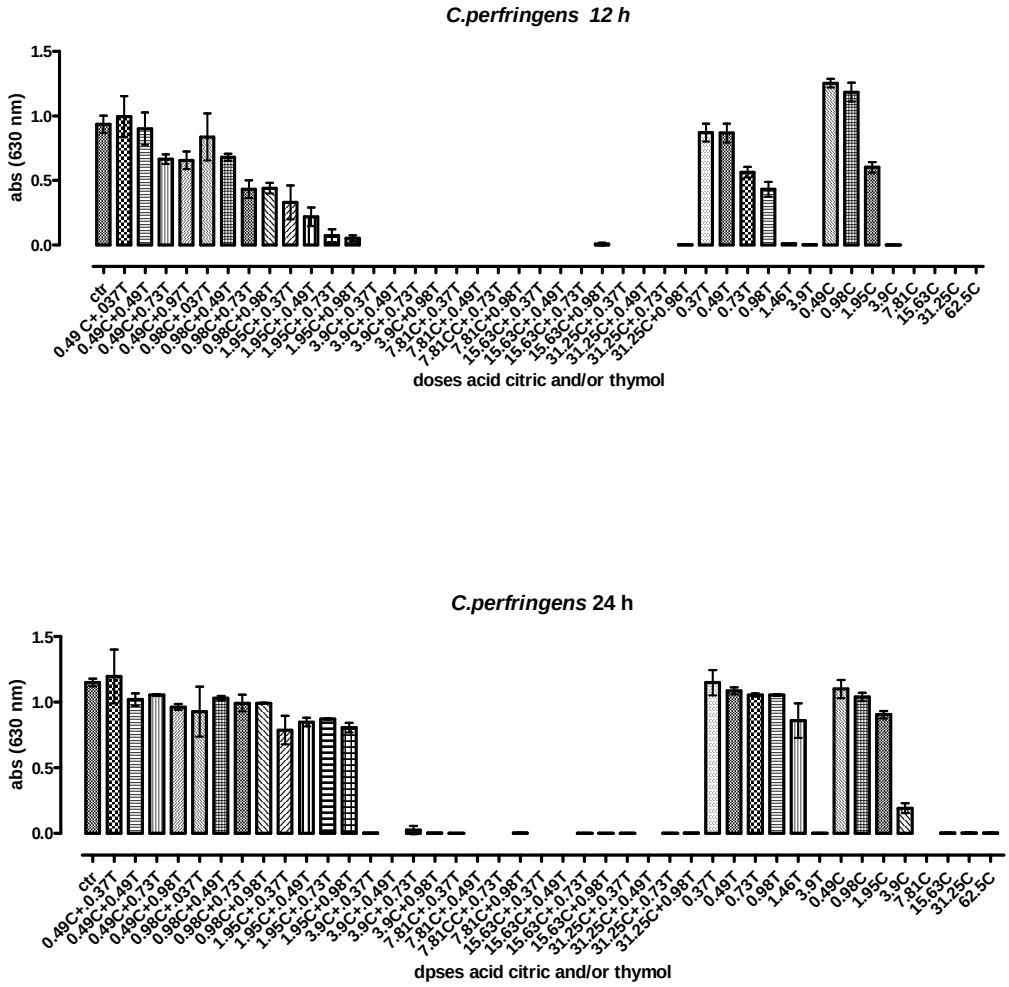

Fig. 3.c*: Absorbance (λ= 630 nm) values of C. perfringens growth after 12 and 24 h of incubation. Column bars indicate mean ± SD. Each co lumn is the mean of two values. T= thymol; C= citric acid.*

CHAPTER 4.

IN VITRO FERMENTATIONS

4.1. ROLE OF CITRIC ACID AND THYMOL IN *IN VITRO* SWINE FERMENTATION

4.1.1. Aim of the study

Purpose of this part of the study was to screen the role of citric acid and thymol in modulating the intestinal microflora of pigs in an *in vitro* fermentation system (batch-culture techniques), which simulate the intestinal environment and microflora. A two step procedure was applied: 1) **digestion** of the feed through enzymatic reactions; 2) **fermentation** of the digested diet with intestinal content and substances under investigations. It has been studied the effect of citric acid and thymol on both small intestinal and cecal parameters through two subsequent studies.

4.1.2. Materials and methods

Digestion.

A standard diet for weaner pigs (Table 4.1) was digested *in vitro* to simulate ileal 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 waterbath 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_2HPO_4, 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. Chemical analyses of the diet before and after digestion are reported in Table 4.2. The digested diet was used as the substrate in the *in vitro* fermentation study.

Fermentation.

Within 20 min after slaughter of pigs (six animals, 10 months old, live weight approximately 160 kg) small intestine or cecal contents were collected and kept in a sealed nylon bag at 37°C during transfer to the laboratory. Small intestine and cecal contents were then diluted with buffer (ratio 1:10 and 1:3, respectively) 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 $CaCl_2$•6H2O + 9.3 g Na_2HPO_4•12H_2O + 0.67 g NaCl + 0.12 g $MgSO_4$•7H_2O in 1 L of distilled water. Buffer pH was then adjusted to pH 6.7 by adding 3N HCl. The buffer solution was kept at 37°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, 15 mL of inoculum in each vessel) per treatment, containing 20 and 100 mg of predigested diet, respectively (Piva et al. 1996). Syringes and vessels were sealed and incubated at 37°C for 24 h. For small intestine fermentation the diet was digested until the first step of the digestion procedure in order to obtain the gastric digestion products only, while the diet for cecal fermentation underwent also the pancreatic digestion step.

Six dietary treatments were investigated: control diet (CTR), or control diet added with a solution of citric acid (pH 6.7) at 1875 ppm (CA), of thymol (pH 6.7) at 125 ppm (TY), citric acid and thymol at 750 and 50 ppm, respectively (CT1), citric acid and thymol at 1875 and 125 ppm, respectively (CT2), and citric acid and thymol at 3000 and 200 ppm, respectively (CT3). Citric acid and thymol were purchased from Sigma-Aldrich (Chemie Gmbh, Steinheim, Germany).

Gas production was measured as described by Menke et al. (1979), using 10 mL glass syringes and recording the cumulative volume of gas produced every 30 min. Samples of fermentation fluid were collected from each vessel at time 0, 4, 8 and 24 h after incubation in a shaking water bath for ammonia analysis; pH, microbial counts and SCFA were determined at the end of the fermentation.

*Chemical analyses of feed and fermentation fluid***.**

Analyses of the diets (crude protein, crude fiber, ether extract, ash, and starch) were performed according to AOAC standard methods (AOAC, 2000; Method 954.01 for crude protein, Method 962.09 for crude fiber, Method 920.39 for ether extract, Method 942.05 for ash, Method 920.40 for starch) .

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

The short chain fatty acids (SCFA) in fermentation fluid were analysed by gas chromatography (Varian 3400, Varian Analytical Instruments, Sunyvale, CA 94089, USA; Carbopack B-DA/4% CW 2M, 80/120, packed column; Supelco, Sigma Aldrich s.r.l., 20151 Milano, Italy). The fermentation liquid was centrifuged (3,000 × g, 15 min.) and 2 mL of the supernatant were added with pivalic acid as an internal standard (Fussel and McCalley, 1987) prior to injection.

Bacterial counts.

Viable counts of bacteria in fermentation samples were measured by plating serial 10-fold dilutions onto Rogosa agar plates (CM 627 B, OXOID, Bansingstoke, UK) for Lactic Acid Bacteria (LAB), Tryptose Sulphite Cycloserine Agar (TSC, CM 587 B, OXOID, Bansingstoke, UK) for Clostridia, and Violet Red Bile Agar (VRBA, DIFCO, Becton, Dickinson and Company, Sparks, Maryland, USA) for coliforms according to the manufacturer conditions. There were three plate-replicates per treatment. Rogosa and TSC agar plates were incubated for 48 h at 37°C under anaerobic conditions. VRBA agar plates 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 modified Gompertz equation, as suggested by Zwietering et al. (1992) with the following:

exponential phase (h) = $V_F/(\mu_m e)\{1 - \ln[(3 - \sqrt{5})/2]\}$.

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 $P < 0.05$.

Table 4.1: diet composition

Ingredients[a]	**%**
Barley	30
Wheat	22
Milk whey	20
Yogurt	5
Proteic potatoe	4.2
Gluten	4.17
Soybean oil	3.5
Yeast molasses	3.3
Lactose	3.3
Coconut oil	2.5
Lys-HCl	0.6
Vitamins and mineral premix[1]	0.4
Sweetener	0.3
NaCl	0.3
Met	0.17
Tre	0.16
Phytase	0.1
DE kcal/kg	3412
ME kcal/kg	3257
NE kcal/kg	2363

[a] *as fed*

[1] *Supplying per Kg: Vit. A: 16,000 IU; Vit. D3: 2,200 IU; Vit. E: 45 mg; Vit. B1: 1.8 mg; Vit. B2: 5.5 mg; Vit. B6: 3 mg; Vit. B12: 0.04 mg; Vit. PP: 25 mg; Vit. K3: 1.4 mg; Choline: 185 mg; Fe: 170 mg; Cu: 160 mg; Zn: 140 mg; Mn: 85 mg; I: 1 mg; Se: 0.3 mg; Co: 1 mg.*

Table 4.2: diet chemical analysis before digestion and nutrients digestibility after gastric and pancreatic *in vitro* digestion

Nutrients (% d.m.)	**Before digestion**	**Digestibility (%) gastric digestion**	**Digestibility (%) pancreatic digestion**
Crude protein	17.9	73	88
Ether extract	7.5	50	ND
Crude Fiber	3.2	50	ND
Crude Ash	4.9	74	ND
Starch	44.6	30	87

ND = not determined

4.1.3. Results and discussions

Small Intestine Fermentation.

Gas production curves were accurately described by the modified Gompertz model ($r^2 = 0.89$).

Gompertz growth model showed that compared to control the blend of citric acid and thymol at 1875 and 125 ppm (CT2) and citric acid and thymol at 3000 and 200 ppm (CT3), significantly reduced gas production by 95% for both treatments ($P<0.01$). Citric acid alone (CA) tended to reduce the maximum volume of gas produced by 29%. (Table 4.3).

Maximum rate of gas production did not exhibit any statistical difference.(Table 4.3).

The duration of the exponential phase was significantly reduced by CT2 and CT3 (-85.2% and -70.5%, respectively, $P<0.01$). CA, thymol alone (TY), and CT1 lowered the duration of the exponential phase (-31%; -27%; -25%, respectively) without reaching significant *P* values. (Table 4.3).

Gas production data showed that citric acid and thymol may inhibit small intestinal bacterial activity when used in combination: CT2 and CT3 were able to determine a significant reduction of the microflora activity reflected by the volume of gas produced, which indicates a lower bacterial metabolism and a lower duration of the corresponding exponential growth. It is noteworthy that

when the same concentrations of citric acid and thymol of CT2 were used alone, i.e. CA and TY treatments, they did not have the same effects on small intestinal microflora, thus demonstrating a synergystic effect of such molecules. CT3 had the same attitude toward intestinal microflora of CT2 treatment even if the concentration of citric acid and thymol was higher than the one present in the CT2 suggesting that there was no dose-related effect.

Ammonia concentration was measured in the fermentation fluid after 4, 8 and 24h from the beginning of the experiment. (Table 4.4). At 4h ammonia concentration in CT3 vessels was significantly reduced when compared to citric acid and thymol treatment alone (-31% for both CA and TY, $P<0.05$) but it was not modified relative to the control. After 8h of fermentation, no changes occurred in ammonia production. After 24h of fermentation, ammonia concentration was reduced by all treatments compared to control (-19.9%, -39.5%, -27.3%, -46.4%, and -48.7% for CA, TY, CT1, CT2, and CT3, respectively; $P<0.01$). Every treatment was successful in controlling ammonia throughout the 24h study even if the most probable cause of ammonia reduction was thymol, as it reduced by itself up to 39.5% of ammonia when compared to control. Again, CT2 and CT3 ammonia reduction were not significantly different each other, as previously described for gas parameters, thus confirming no dose-response effect.

Measurements of fermentation fluid pH from each treatment were assessed at the end of the experiment without showing statistical differences (Table 4.4).

No Clostridia were found after 48h of incubation. Thymol alone and combinations of citric acid and thymol at the highest concentrations significantly decreased coliforms counts (-0.7 Logs, 0.5 Logs, and -1 Log, for TY, CT2, and CT3, respectively; $P<0.01$) when compared to control. Lactic acid bacteria (LAB) were not modified by any treatment but CT3 (citric acid and thymol at the highest concentration), which significantly lowered counts by 3 Logs when compared to control. The ratio coliforms/LAB was significantly higher for CT3 treatment than for the control (1.4 vs 2.1, respectively, $P<0.05$).(Table 4.5). These findings suggest that thymol and citric acid at the highest concentrations may have a bactericidal effect on both coliforms and LAB, as it can be confirmed by results on gas and ammonia production. Furthermore, it seems that the inhibitory effect

on coliforms can be related to thymol rather than to citric acid as it may be suggested by ammonia concentrations. It can be observed that thymol can act on proteolitic bacteria and on coliforms exerting its antibacterial effect on both. The highest concentration used of the blend of citric acid and thymol shifted the microbial balance toward coliforms probably because of the lower resistance of Gram-positive bacteria than Gram-negative to flavors antimicrobial properties (Nikaido and Varra, 1985; Russel et al., 1991; Krieg, 2001; Lis-Balchin, 2003).

SCFA concentration (Table 4.6) was significantly lower in thymol (TY) containing vessels when compared to citric acid (CA) and to CT2 vessels ($P<0.01$), but it was not different from the control (Table 6). Acetic acid was increased by citric acid (CA), and CT1 (+38% and +44%, respectively, $P<0.01$) when compared to control. Propionic acid and iso-butyric acid concentrations, when compared to control, were decreased by every treatment: -38%, -85%, -61%, -84%, and -87%, for CA, TY, CT1, CT2, and CT3, respectively, for propionic acid ($P<0.0001$); -50%, -60%, -38%, -53%, and 62%, for , TY, CT1, CT2, and CT3, respectively, for iso-butyric acid ($P<0.0001$). Nor-butyric acid was significantly decreased by all the treatments (-39%, -91.4%, -40%, -92%, -95% for CA, TY, CT1, CT2, and CT3, respectively, $P<0.0001$), as well as iso-valeric acid, which was present at very small concentrations and it was absent in thymol and CT2 treatments. Nor-valeric acid was not detected in all the treatments whereas lactic acid was found only in CT2 and CT3 treatments.

SCFA data confirmed the findings of the previous results, i.e. that is thymol to formerly influence the trend of fermentation pattern in small intestine; it is noteworthy that only starting at 125 ppm it can exert its antibacterial action, alone or in combination with citric acid, and that higher concentrations of thymol did not influence data in a significantly different way. The trend of the decrease of butyric acid in all treatments is comparable to the decrease found in ammonia after 24 h; this can be explained by the fact that butyrate producing bacteria are limited to few genera, such as *Clostridium* spp, *Butyrivibrio* spp, *Eubacterium* spp, *Fusobacterium* spp, the former producing large amounts of butyrate as fermentation product (Holdeman et al., 1977); furthermore iso-butyric and iso-valeric acid are formed from the deamination of valine and leucine (Van Soest,

1982) and are indicative of the extent of protein catabolism. Although in this study Clostridia were not found in any treatment, because they were under the detection limit, it can be supposed that thymol has been able to control proteolysis during the 24h of fermentation controlling proteolitic bacteria, as indesirable microflora. Propionic acid was decreased by every treatment, but, again, thymol led the extent of this process; the molar ratio C2/C3 was appreciably increased by thymol alone and by CT2 and CT3 compared to control (3.14, 19.22, 35.19, 33.01 for CTR, TY, CT2, and CT3, respectively; $P<0.0001$). Being propionic and acetic acid final metabolic compounds of fermentation by *Propionibacteria* and cellulolytic bacteria, respectively, the increase of C2/C3 ratio has been probably caused by the inhibition of *Propionibacteria* and sugar fermenters coliforms, determining a consequent selection of a cellulolytic flora during the 24h of fermentation.

Table 4.3: Modified Gompertz equation fitted to gas production data from the 24 h *in vitro* incubation of swine small intestine inoculum*

Treatment	V_F	μ_m	Log phase
CTR	7.60 ± 1.84 [b]	1.10 ± 0.48	6.77 ± 1.89 [b]
CA	5.38 ± 2.29 [b]	1.08 ± 0.29	4.66 ± 1.43 [b]
TY	8.03 ± 1.55 [b]	1.54 ± 0.39	4.96 ± 0.35 [b]
CT1	6.91 ± 1.54 [b]	1.30 ± 0.52	5.09 ± 1.21 [b]
CT2	0.40 ± 0.004 [a]	2.66 ± 3.31	1.00 ± 1.34 [a]
CT3	0.04 ± 0.001 [a]	1.22 ± 1.50	2.01 ± 1.68 [a]

**Values means± SD of five syringes for each diet tested. Values within the same column with different superscripts are significantly different (P < 0.05). CTR= basal diet; CA =basal diet with citric acid at 1875 ppm; TY = basal diet with thymol at 125 ppm; CT1 = basal diet with citric acid at 750 ppm and thymol at 50 ppm; CT2 = basal diet with citric acid at 1875 ppm and thymol at 125 ppm; CT3 = basal diet with citric acid at 3000 ppm and thymol at 200 ppm; V_F = maximum volume of gas produced (mL); μ_m = maximum rate of gas production (mL h^{-1}); log phase = exponential phase (h).*

Table 4.4: Ammonia values (mmol L^{-1}) at 4, 8 h and ammonia and pH values at 24 h of an *in vitro* incubation of swine small intestine inoculum*

Treatment	4h	8h	24h	pH
CTR	5.55 ± 1.31 [ab]	5.87 ± 1.62	23.02 ± 1.49 [e]	6.88 ± 0.04
CA	7.27 ± 0.74 [b]	4.78 ± 1.47	18.45 ± 0.56 [d]	6.84 ± 0.03
TY	7.35 ± 0.41 [b]	4.77 ± 0.55	13.93 ± 1.41 [b]	6.78 ± 0.07
CT1	6.15 ± 1.39 [ab]	4.43 ± 1.21	16.74 ± 0.31 [c]	6.79 ± 0.02
CT2	6.10 ± 1.08 [ab]	5.03 ± 1.31	12.34 ± 1.17 [a]	6.62 ± 0.08
CT3	5.05 ± 0.24 [a]	4.23 ± 0.64	11.81 ± 0.39 [a]	6.56 ± 0.63

**Values are means± SD of five vessels for each diet tested. Values within the same column with different superscripts are significantly different (P < 0.05).*

Table 4.5: Counts of viable bacteria (log_{10} CFU mL^{-1}) at 24 h of an *in vitro* incubation of swine small intestine inoculum*

Treatment	Coliforms	LAB	Clostridia
CTR	8.45 ± 0.11^{b}	5.90 ± 0.39^{b}	ND
CA	8.97 ± 0.47^{b}	5.68 ± 0.48^{b}	ND
TY	7.71 ± 0.35^{a}	5.05 ± 0.23^{b}	ND
CT1	8.36 ± 0.28^{b}	5.33 ± 0.06^{b}	ND
CT2	7.87 ± 0.04^{a}	4.93 ± 0.03^{b}	ND
CT3	7.39 ± 0.01^{a}	2.37 ± 2.13^{a}	ND

**Values are means± SD of 3 vessels for each treatment. Values within the same column with different superscripts are significantly different ($P < 0.05$). ND = under the detection limit*

Table 4.6. Short-chain fatty acids values (mmol L^{-1}) after 24 h of an *in vitro* incubation of swine small intestine inoculum*

Treatment	Acetic acid	Propionic acid	i-Butyric acid	*n*-Butyric acid	i-Valeric acid	Lactic acid	C2/C3 ratio	Total acids
CTR	20.12 ± 0.90^{a}	6.41 ± 0.38^{d}	0.37 ± 0.05^{c}	2.28 ± 0.23^{c}	0.64 ± 0.10	ND	3.14 ± 0.12^{a}	29.82 ± 1.48^{ab}
CA	32.57 ± 1.09^{cd}	3.97 ± 1.89^{c}	0.19 ± 0.02^{a}	1.38 ± 0.12^{b}	0.08 ± 0.05	ND	8.21 ± 0.53^{a}	38.19 ± 1.08^{b}
TY	19.11 ± 0.66^{a}	0.94 ± 0.03^{a}	0.15 ± 0.02^{a}	0.19 ± 0.05^{a}	ND	ND	19.22 ± 2.19^{b}	20.12 ± 0.47^{a}
CT1	27.73 ± 0.85^{abc}	2.50 ± 0.30^{b}	0.23 ± 0.03^{b}	1.37 ± 0.12^{b}	0.07 ± 0.02	ND	11.20 ± 1.38^{a}	32.41 ± 1.50^{ab}
CT2	35.35 ± 2.43^{b}	1.01 ± 0.08^{a}	0.17 ± 0.02^{a}	0.18 ± 0.07^{a}	ND	5.10 ± 2.12	35.19 ± 2.47^{c}	41.89 ± 6.2^{b}
CT3	25.43 ± 13.35^{ab}	0.84 ± 0.34^{a}	0.14 ± 0.02^{a}	0.10 ± 0.02^{a}	0.12 ± 0.11	6.33 ± 1.68	33.01 ± 14.24^{c}	33.01 ± 15.6^{ab}

**Values are means± SD of five vessels for each diet tested. Values within the same column with different superscripts are significantly different ($P < 0.05$).*

ND = under the limit of detection as well as n-valeric acid

Cecal fermentation

Analysis of variances of V_F data was significant (P = 0.03), but the post-test failed to show differences among means of groups. However results suggested that citric acid (CA) stimulates microflora fermentation in the caecum, acting as a substrate, and enhancing maximum volume of gas produced by 59.5% when compared to control (Table 4.7). Furthermore CT1, CT2, and CT3 had the same trend of citric acid, increasing maximum volume of gas produced by 61.7%, 27.1%, and 31.9%, respectively, when compared to control, while thymol tended to have no effects on gas production. Analysis of variances of maximum rate of gas production data reflected the same tendency of results obtained from analysis of gas volume but the post test provided significant outputs (Table 4.7). Rate max was significantly increased by all treatments but the thymol one, which was not effective in increasing rate max (+62.5%, +40%,+52.5%, and +45% for CA, CT1, CT2, and CT3, respectively, P<0.01), while the duration of the exponential phase was not modified by any treatment (Table 4.7).

Ammonia concentration was significantly increased after 4 h by CT2 and CT3 by 25% and 40%, respectively, when compared to control (P<0.01); after 8 h ammonia concentration was significantly reduced by citric acid (CA, -17.9%; P<0.05) while it was significantly increased by CT3 (+25.6%; P<0.001) when compared to control; after 24 h there were no differences (Table 4.8).

Thymol significantly lowered pH values (6.6 vs 6.5 for CTR and TY, respectively, P<0.05) whereas citric acid (CA) had the opposite effect by highering the pH from 6.6 to 6.7 (P<0.001) (Table 4.8).

The highest concentration of citric acid and thymol combined together significantly lowered coliforms counts by 1.4 Logs (P = 0.01), whereas it increased LAB by 0.4 Logs (P<0.01), thus resulting in a lower ratio coliforms/ LAB when compared to control (0.9 vs 0.6 for CTR and CT3 respectively, P<0.0001) (Table 4.9).

Following gas production trend, compared to control, CT2 and CT3 significantly increased total SCFA concentration (+29% and +64%, respectively; P<0.0001) (Table 4.10); acetic acid was increased by citric acid alone and by the combination of thymol and citric acid at the two highest concentrations (+20.6%,

+43.3, and +91.8%, for CA, CT2, and CT3, respectively; $P<0.0001$); compared to control thymol decreased propionic acid (-10.4%, $P = 0.0015$), iso and nor-valeric acid amounts (-24.5% and -15.4%, respectively, $P<0.05$); iso-butyric acid concentration was not affected by any treatment, whereas nor-butyric acid was increased by CT3 when compared to control (+27.5%, $P<0.0001$). Lactic acid was not detected in any treatments.

These findings suggest that in caecum interactions between molecules under investigation and the environment are completely different from the ones in the small intestine. In caecum citric acid acts as a substrate for microflora, strongly increasing gas production from 750 to 3,000 ppm, alone or in combination with thymol. It is known that several bacterial strains can use citric acid (Medina de Figueroa et al., 2000) as an energy source: certain species of *Leuconostoc* are able to uptake citrate and to split it into oxalacetate and acetate, the former being decarboxilated to form pyruvate and subsequently reduced to lactate (Marty-Teysset et al., 1996); furthermore, *Ln. mesenteroides* maintains the inner pH around 6.5-7.0 in acidic environment (pH = 4), when using citric acid as a substrate (Belguendouz et al., 1997).

Interestingly, despite the opposite effects on gas production, after 8 h of fermentation, citric acid vessels resulted in lower ammonia concentration than in control ones when used alone at 1875 ppm, suggesting that an effective control of ammonia cecal concentration may be achieved by selecting a type of microflora which positively influences fermentation, shifting the compostition of the microflora from coliforms to LAB, (ratio coliforms/ LAB, 0.9 vs 0.6 for CTR and CT3, respectively). As citric acid is metabolized in a very fast way, the control of ammonia production is limited to the first 8 h of fermentation, while it decrease at 24 h, when data suggest that there were no differences in ammonia concentration among treatments. The higher concentration of ammonia in CT2 and CT3 vessels than in control ones is related to the higher metabolic microflora activity as suggested by gas parameters and SCFA concentration: the higher the volume of gas produced, the higher the number of cells of anaerobic fermentative microorganisms and the cellular turnover and metabolic and catabolic products concentration. Metabolism of hetero-fermentative LAB produces only 50% lactic

acid and considerable amounts of ethanol, acetic acid and carbon dioxide; the higher number of LAB found in CT3 treatments caused an higher acetic acid concentration beside the higher production of gas. Acetic to propionic ratio was significantly increased by every treatment, especially by CT3 (2.9 vs 4.9, for CTR and CT3, respectively, $P<0.001$) suggesting the role of citric acid as a substrate for a cellulolytic and "positive" bacteria in the cecum. From this analysis it could be concluded that citric acid alone, or in combination with thymol at the two highest concentrations, stimulates the growth of LAB and of an "healthy" microflora, as previously described by Drinan et al., (1976).

In caecum thymol antibacterial activity seems to be not as strong as in the small intestine. This could be explained by the fact that despite well known thymol antibacterial properties (Burt, 2004) there are several bacteria, among which *Pseudomonas* strains, capable of assimilating thymol or its analogue carvacrol, degrading it completely by a specific metabolic pathway involving the opening of the hydroxilic-phenolic ring (Chamberlain and Dagley, 1968) and leading to the release of acetate, 2-ketobutyrate, and isobutyrate. In the large intestine there are 10^{10} to 10^{11} viable cells per gram of intestinal content (Butine and Leedle, 1989), most of them still unidentified, while in the small intestine there is a lower number of microorganism (10^{7}-10^{8} CFU/g; Jensen and Jorgensen, 1994), a smaller extent of which may be able to use aromatic compounds as an energy source, resulting in a more sensitive microflora to antibacterial substances such as thymol or other phenolic compounds. Furthermore thymol had an inhibiting effect on propionic acid production as it had in the small intestine fermentation , thus confirming previous observations on thymol inhibitory power on sugar-fermentative bacteria.

Table 4.7: modified Gompertz equation fitted to gas production data from the 24 h *in vitro* incubation of swine cecum inoculum*

Treatment	V_f	μ_m	Log phase
CTR	4.02 ± 1.57	0.40 ± 0.09 [a]	8.78 ± 2.34
CA	6.41 ± 1.12	0.65 ± 0.09 [b]	8.83 ± 2.59
TY	3.78 ± 1.73	0.37 ± 0.08 [a]	8.88 ± 2.59
CT1	6.50 ± 0.46	0.56 ± 0.06 [b]	10.06 ± 0.31
CT2	5.12 ± 2.01	0.61 ± 0.15 [b]	7.38 ± 1.54
CT3	5.31 ± 0.71	0.58 ± 0.01 [b]	8.72 ± 0.86

**Values are means± SD of five syringes for each diet tested. Values within the same column with different superscripts are significantly different (P < 0.05). CTR= basal diet; CA =basal diet with citric acid at 1875 ppm; TY = basal diet with thymol at 125 ppm; CT1 = basal diet with citric acid at 750 ppm and thymol at 50 ppm; CT2 = basal diet with citric acid at 1875 ppm and thymol at 125 ppm; CT3 = basal diet with citric acid at 3000 ppm and thymol at 200 ppm; V_F = maximum volume of gas produced (mL); μ_m = maximum rate of gas production (mL h^{-1}); log phase = exponential phase (h).*

Table 4.8: Ammonia values (mmol L^{-1}) at 4, 8 h and ammonia and pH values at 24 h of an *in vitro* incubation of swine cecum inoculum*

Treatment	4h	8h	24h	pH
CTR	9.34 ± 1.99 [a]	11.93 ± 1.37 [b]	20.66 ± 1.56	6.62 ± 0.03 [b]
CA	7.79 ± 0.65 [a]	9.79 ± 1.13 [a]	20.13 ± 0.96	6.72 ± 0.03 [c]
TY	8.91 ± 1.47 [a]	11.77 ± 1.30 [b]	19.51 ± 1.92	6.54 ± 0.02 [a]
CT1	9.42 ± 1.85 [a]	11.68 ± 0.91 [b]	19.56 ± 1.24	6.61 ± 0.02 [b]
CT2	11.67 ± 0.98 [b]	12.58 ± 0.65 [b]	19.11 ± 1.30	6.57 ± 0.03 [ab]
CT3	13.07 ± 2.08 [b]	14.98 ± 0.55 [c]	19.08 ± 0.82	6.58 ± 0.07 [b]

**Values are means± SD of five vessels for each diet tested. Values within the same column with different superscripts are significantly different (P < 0.05).*

Table 4.9: Counts of viable bacteria (log_{10} CFU mL^{-1}) at 24 h of an *in vitro* incubation of swine cecum inoculum*

Treatment	Coliforms	LAB	Clostridia
CTR	6.23 ± 0.004 [b]	7.22 ± 0.11 [a]	3.45 ± 0.21
CA	6.67 ± 0.57 [b]	7.16 ± 0.02 [a]	3.75 ± 0.18
TY	6.57 ± 0.98 [b]	7.4 ± 0.14 [a]	3.36 ± 0.32
CT1	6.77 ± 0.36 [b]	7.35 ± 0.13 [a]	3.42 ± 0.39
CT2	5.86 ± 0.24 [b]	7.42 ± 0.03 [a]	3.00 ± 0.00
CT3	4.85 ± 0.4 [a]	7.67 ± 0.01 [b]	2.16 ± 1.89

[*] *Values are means± SD of 3 vessels for each treatment. Values within the same column with different superscripts are significantly different ($P < 0.05$).*

Table 4.10. Short-chain fatty acids values (mmol L^{-1}) after 24 h of an *in vitro* incubation of swine cecal inoculum*

Treatment	Acetic acid	Propionic acid	i-Butyric acid	*n*-Butyric acid	i-Valeric acid	*n*-Valeric acid	C2/C3 ratio	Total acids
CTR	50.28 ± 3.18^{a}	17.28 ± 0.72^{b}	1.83 ± 0.04	5.99 ± 0.42^{a}	1.62 ± 0.08^{d}	1.36 ± 0.05^{b}	2.91 ± 0.13^{a}	78.36 ± 4.19^{a}
CA	60.66 ± 3.68^{b}	17.76 ± 1.03^{b}	1.97 ± 0.04	5.84 ± 0.44^{a}	1.46 ± 0.13^{c}	1.31 ± 0.12^{ab}	3.41 ± 0.05^{c}	88.95 ± 5.40^{a}
TY	52.01 ± 7.25^{a}	15.48 ± 2.27^{a}	1.75 ± 0.24	5.39 ± 0.93^{a}	1.22 ± 0.15^{a}	1.15 ± 0.15^{a}	3.36 ± 0.06^{c}	77.01 ± 10.97^{a}
CT1	59.75 ± 2.55^{b}	18.43 ± 0.68^{b}	2.00 ± 0.09	6.13 ± 0.29^{a}	1.39 ± 0.06^{bc}	1.29 ± 0.05^{ab}	3.24 ± 0.03^{b}	88.99 ± 3.58^{a}
CT2	72.07 ± 7.02^{c}	18.2 ± 1.58^{b}	1.86 ± 0.23	6.27 ± 0.63^{a}	1.27 ± 0.12^{ab}	1.28 ± 0.14^{ab}	3.96 ± 0.09^{d}	101.00 ± 9.61^{b}
CT3	96.46 ± 4.64^{d}	19.73 ± 0.98^{b}	1.81 ± 0.17	7.64 ± 0.42^{b}	1.30 ± 0.08^{bc}	1.39 ± 0.07^{b}	4.89 ± 0.08^{e}	128.3 ± 6.18^{c}

**Values are means± SD of five vessels for each diet tested. Values within the same column with different superscripts are significantly different ($P < 0.05$). Lactic acid was under the detection limit*

CHAPTER 5.

IN VIVO EXPERIMENTS

5.1. ROLE OF MICROENCAPSULATED CITRIC ACID AND THYMOL TESTED INDIVIDUALLY OR COMBINED ON PRODUCTIVITY AND INTESTINAL MICROFLORA OF NEWLY WEANED PIGLETS

5.1.1. Aim of the study

Aim of the *in vivo* studies was to test the efficacy of citric acid and thymol as in feed additives alternative to antibiotic growth promoters at weaning. Two experiments were assessed: in the first study it was evaluated the role of citric acid, thymol, and a combination of the two in improving piglets health status and productivity. Since it has been proven that organic acids loose their antimicrobial power passing through the acidity of the stomach, and that only a small amount of them can reach the lower gut, we used microencapsulated substances to bypass the stomach acidity and to reach the small and large intestine.

5.1.2. Materials and methods

Animals, Housing and Dietary Treatments.

Ninety-six crossbred piglets (Landrace x Duroc) were weaned at 21 d and were transported to the barns of the DIMORFIPA, Faculty of Veterinary Medicine, University of Bologna, for a post weaning trial 42 days long. The animals began the trial at the BW of 6.7 kg (SD = 0.7) and were immediatly divided after their arrival into four groups (24 animals per group), homogenous for weight and gender and were housed in 16 cages corresponding to four replicates of six pigs. Then piglets received the basal diet with or without (control diet) the addition of microencapsulated (Vetagro srl, Reggio Emilia, Italy) citric acid or thymol at 1875 and 125 ppm, respectively, or a blend of the two providing the same amounts of citric acid and thymol. The treatments were: a) 0 ppm **(CTR)**, b) protected citric acid included at 5,000 ppm, providing acid at 1875 ppm **(CA)**, c)

protected thymol included at 5,000 ppm, providing thymol at 125 ppm **(TY)** and d) protected blend of citric acid and thymol included at 5,000 ppm and providing the same amounts of the two principle of the other treatments **(CT)**. All diets were formulated to provide the same amount of energy, protein, essential amino acids, calcium and phosphorus. No antimicrobial agents were added to diets. The composition of the base diet was changed after the first three weeks. Feed and water were provided ad libitum. Composition and estimated chemical analyses of the experimental diets are reported in Table 5.1 and Table 5.2, respectively.

Experimental Procedure and Sampling

Body weight was recorded individually at the beginning of the trial, three weeks later and at the end of the trial; feed intake was recorded daily.

On d 21 a sample of fresh faeces was collected from the rectum of six animals per treatment for viable counts of bacteria. On d 42 the same animals were killed by captative bolt and slaughtered. The gastrointestinal tract was immediately removed and samples from stomach, proximal jejunum, distal jejunum, cecum and the transverse colon were collected to determine pH, ammonia content, SCFA and microbial population. Mucosa samples from jejunum, ileum, and cecum were collected for morphological analysis. pH was measured within one hour from the sampling, while the intestinal contents for the microbiological determinations were immediately processed, whereas samples for others determinations were immediately freezed and stored at –20°C.

Animal housing and care were conducted in accordance with the published guidelines for animal welfare and protection (directive No. 86/609/EEC and Italian Law Act, Decreto Legislativo No. 116, issued on January 27, 1992).

Chemical analyses of feed and intestinal contents

Analyses of the diets (crude protein, crude fiber, ether extract, ash and starch) were performed according to AOAC standard methods (AOAC, 2000; Method 954.01 for crude protein, Method 962.09 for crude fibre, Method 920.39 for ether extract, Method 942.05 for ash, Method 920.40 for starch).

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

The SCFA in the intestinal chymus were analysed by gas chromatography (Varian 3400, Varian Analytical Instruments, Sunyvale, CA 94089, USA; Carbopack B-DA/4% CW 2M, 80/120, packed column; Supelco, Sigma Aldrich s.r.l., 20151 Milano, Italy). The digesta were diluted 1:2 with distilled water and centrifuged (3,000 x g, 15 min.) and 1 mL of the supernatant was filled in microfuge tubes and deproteinized with 50 µL perchloric acid (Merck, Darmstadt, Germany). After three hours the samples were centrifuged again (3,000 x g, 10 min.) and the supernatant were added with pivalic acid as an internal standard (Fussel and McCalley, 1987) prior to injection.

Microbial Counts

Viable bacteria counts from faeces at 21 days and intestinal digesta were performed using the ten fold dilution method and plating the samples onto Violet Red Bile Agar (VRBA), Rogosa Agar (RA) and Tryptose Sulphite Cycloserine Agar (TSC) for the enumeration and isolation of total coliforms, Lactic Acid Bacteria (LAB) and *C. perfringens*, respectively. VRBA was incubated for 24 h at 37°C in aerobiosis while RA and TSC were incubated at 39°C for 48 hours under anaerobic conditions, according to the manufacturer instructions.

Morphological evaluations

Mucosa samples from jejunum (1 m after pilor), ileum (1 m before ileo-cecal valve) and cecum have been formalin-fixed-paraffin-wax embedded and 3 mm histological sections have been stained with haematoxylin and eosin and measured by image analysis (Cytometrica, Byk Gulden, Milan, Italy) to assess 10 villous height and 10 crypt depth randomly of each section .

Statistical analyses

Data were analyzed by ANOVA using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA) in a completely randomized design. Each cage formed the experimental unit. The differences among means of groups were analyzed using the t-Student test. Differences were considered statistically significant at $P < 0.05$.

Table 5.1: Diets composition[a]

Ingredients (%)	**PHASE 1**	**PHASE 2**
Wheat	24.98	29.99
Flaked barley	23.33	30.00
Soy 48%	-	12.00
Acid milk whey10%	18.33	5.00
Biscuits	10.00	11.83
Proteic Potatoe	6.67	1.83
Yogurt	6.00	-
Gluten	3.00	2.33
Soy oil	3.00	2.00
Coconut oil	2.50	2.00
Lys HCl 98%	0.51	0.54
Vitamins and mineral Premix*	0.40	0.40
Calcium carbonate	0.38	0.88
NaCl	0.25	0.33
Fosfitalia 25/20	0.20	0.40
Alimet MHA	0.14	0.11
Tre	0.11	0.16
Choline	0.10	0.10
Phytase	0.10	0.10

[a]*as fed*

**Providing per kg: Vit. A: 16,000 IU; Vit. D3: 2,200 IU; Vit. E: 45 mg; Vit. B1: 1.8 mg; Vit. B2: 5.5 mg; Vit. B6: 3 mg; Vit. B12: 0.04 mg; Vit. PP: 25 mg; Vit. K3: 1.4 mg; Choline: 185 mg; Fe: 170 mg; Cu: 160 mg; Zn: 140 mg; Mn: 85 mg; I: 1 mg; Se: 0.3 mg; Co: 1 mg.*

Table 5.2: diet chemical analysis[a]

Nutrients (% d.m.)	**PHASE 1**	**PHASE 2**
Crude Proteins	18.54	18.1
Ether Extract	8.16	6.70
Crude Fiber	1.27	2.84
Crude Ash	4.58	4.46
Starch	32.84	38.13
Lys*	1.30	1.20
Met+Cys*	0.78	0.69
ED kcal/kg*	3807	3557
EN kcal/kg*	2643	2493

**calculated values*

5.1.3. Results

Performance of animals are showed in Table 5.3. Animals preserved a good health status all the trial long. Some sporadic event of diarrhea occurred, but there were no differences among groups.

When compared to control feed intake was significantly increased during the whole experiment by the thymol treatment by 15.4% and 11% during the first and second phase, respectively ($P<0.05$) with an overall increase of 12.6% ($P<0.05$). Growth parameters were not affected by treatments.

Viable counts, and intestinal pH, ammonia and SCFA are showed from Table 5.4 to 5.9. Clostridia populations were under the detection limit and could not be determined. At 14 d microbial fecal counts were not influenced by treatments. At 42 viable cecal coliforms were significantly reduced by the blend of citric acid and thymol by 3.9 Log (6.7 vs 2.8 Log CFU mL^{-1}, for CTR and CT, respectively; $P<0.05$). Furthermore citric acid and thymol alone were able to decrease coliforms count of 0.7 Log and 3 Log, respectively, even if they didn't reach significant *P* values. Lactic Acid Bacteria counts were not different among

treatments, but the cecal ratio coliforms/LAB was reduced by every treatment reaching significant levels (P = 0.02) only for the blend (0.8, 0.7, 0.5, and 0.3 for CTR, CA, TY, and CT, respectively.)

pH and NH_3 in the intestinal tracts were not different among treatments.

SCFA production was formerly affected at stomach level, where CT significantly increased propionic acid concentration from 0.10 mmol to 0.28 mmol L^{-1} for CTR and CT, respectively (P = 0.0001); all treatments increased n-butyric acid by 254%, 348%, and 389% for CA, TY, and CT, respectively (P = 0.0002), thus increasing the overall amount of SCFA in the stomach (+ 175%, +252%, and +297%, for CA, TY, and CT, respectively (P = 0.0001). In proximal jejunum acetic acid was significantly lowered by CA, TY, and CT, (-38%, -22%, and -26%, respectively, P = 0.01), whereas lactic acid was significantly increased by TY when compared to control (+74%, P =0.03).

Morphological results are showed in Table 5.10. Only cecal crypts were affected by treatments, having TY and CT group higher crypts depth than CA group. The control group had intermediate values between TY - CT and CA groups.

Table 5.3. Growth performances of piglets receiving a diet added or not with microencapsulated citric acid (CA), thymol (TY) or a blend of citric acid and thymol (CT) in the six weeks after weaning

	n	CTR	CA	TY	CT	*P*
Initial BW, kg	24	6.58 ± 0.85	6.69 ± 0.85	6.85 ± 0.79	6.76 ± 0.68	0.69
ADG, g d^{-1} (0-21d)	24	258.0 ± 65.2	267.4 ± 77.6	298.4 ± 65.8	288.8 ± 72.9	0.18
ADFI, g d^{-1} (0-21 d)	4	412.8 ± 24.7^{a}	405.8 ± 38.9^{a}	476.5 ± 28.1^{b}	434.5 ± 39.2^{a}	0.04
G:F (0-21 d)	4	1.60 ± 0.07	1.52 ± 0.10	1.60 ± 0.12	1.52 ± 0.14	0.51
21 d BW, kg	24	12.00 ± 1.78	12.30 ± 2.18	13.11 ± 1.87	12.82 ± 1.63	0.17
ADG, g d^{-1} (22-42) d	24	451.3 ± 129.3	481.1 ± 131.3	501.9 ± 83.5	458.0 ± 147.4	0.49
ADFI, g d^{-1} (22-42 d)	4	702.8 ± 58.5^{a}	786.8 ± 39.8^{b}	779.8 ± 24.1^{b}	741.0 ± 27.7^{a}	0.04
G:F (22-42 d)	4	1.57 ± 0.09	1.64 ± 0.05	1.56 ± 0.05	1.62 ± 0.06	0.33
Final BW, kg	24	21.02 ± 3.74	21.92 ± 3.96	23.15 ± 2.88	21.98 ± 4.10	0.27
ADG, g d^{-1} (0-42 d)	24	352.2 ± 89.6	371.5 ± 62.28	397.5 ± 62.28	371.3 ± 98.85	0.35
ADFI, g d^{-1} (0-42 d)	4	554.5 ± 26.4^{a}	591.8 ± 32.7^{a}	624.5 ± 21.1^{b}	584.0 ± 29.3^{a}	0.03
G:F (0-42 d)	4	1.58 ± 0.07	1.60 ± 0.05	1.57 ± 0.07	1.58 ± 0.07	0.95

[*] values are means ± SD. Values within the same row with different superscripts are significantly different (P < 0.05). ADG = average daily gain; ADFI = average daily feed intake;G:F = gain to feed ratio; BW = body wheight

Table 5.4: fecal bacterial counts at 21d (log_{10} CFU g^{-1})[a]

	CTR	CA	TY	CT	*P*
LAB	6.74 ± 0.25	6.75 ± 0.48	6.46 ± 0.49	6.77 ± 0.30	0.94
Coliforms	8.77 ± 0.23	8.70 ± 0.33	8.62 ± 0.25	8.74 ± 0.39	0.99

[a] *values are means ± SD of six replicates per treatments*

Table 5.5: Counts of viable coliforms (log_{10} CFU mL^{-1}) in chymus from stomach (S), proximal jejunum (PJ), distal jejunum (DJ), and cecum (CE) from piglets that had received a diet added or not with microencapsulated citric acid (CA), thymol (TY)or a blend of citric acid and thymol (CT) in the six weeks after weaning[a]

	CTR	CA	TY	CT	*P*
S	2.99 ± 0.64	3.25 ± 0.29	2.08 ± 0.95	2.65 ± 0.65	0.65
PJ	3.16 ± 0.70	3.80 ± 0.24	3.35 ± 0.72	2.90 ± 0.66	0.77
DJ	5.83 ± 0.30	6.06 ± 0.36	6.24 ± 0.22	6.04 ± 0.35	0.82
CE	6.73 ± 0.25[b]	6.03 ± 0.16[ab]	4.30 ± 1.37[ab]	2.81 ± 1.27[a]	0.04
CO	5.81 ± 1.17	5.20 ± 1.06	5.45 ± 1.17	4.81 ± 0.96	0.93

[a] *values are means ± SD of six replicates per treatments. Values within the same row with different superscripts are significantly different (P < 0.05).*

Table 5.6: Counts of viable LAB (log_{10} CFU mL^{-1}) in chymus from stomach (S), proximal jejunum (PJ), distal jejunum (DJ), and cecum (CE) from piglets that had received a diet added or not with microencapsulated citric acid (CA), thymol (TY)or a blend of citric acid and thymol (CT) in the six weeks after weaning[a]

	CTR	CA	TY	CT	*P*
S	6.38 ± 0.37	6.89 ± 0.27	6.36 ± 0.39	6.91 ± 0.42	0.57
PJ	5.67 ± 0.39	6.36 ± 0.29	6.03 ± 0.43	6.10 ± 0.35	0.62
DJ	5.89 ± 0.38	5.69 ± 0.39	5.79 ± 0.40	6.12 ± 0.48	0.89
CE	8.24 ± 0.19	8.42 ± 0.15	8.52 ± 0.34	8.52 ± 0.34	0.86
CO	8.54 ± 0.27	8.34 ± 0.37	8.82 ± 0.20	8.97 ± 0.30	0.44

[a] *values are means ± SD of six replicates per treatments. Values within the same row with different superscripts are significantly different (P < 0.05).*

Table 5.7: pH values in chymus from stomach (S), proximal jejunum (PJ), distal jejunum (DJ), and cecum (CE) from piglets that had received a diet added or not with microencapsulated citric acid (CA), thymol (TY)or a blend of citric acid and thymol (CT) in the six weeks after weaning[a]

	CTR	**CA**	**TY**	**CT**	***P***
S	4.13 ± 0.25	4.04 ± 0.19	4.11 ± 0.30	4.14 ± 0.13	0.99
PJ	5.81 ± 0.13	5.50 ± 0.28	5.70 ± 0.11	5.88 ± 0.15	0.48
DJ	6.74 ± 0.24	7.12 ± 0.10	7.05 ± 0.30	6.86 ± 0.16	0.58
CE	5.33 ± 0.05	5.35 ± 0.08	5.42 ± 0.12	5.29 ± 0.10	0.75
CO	6.00 ± 0.20	5.78 ± 0.23	5.99 ± 0.23	5.85 ± 0.06	0.82

[a] *values are means ± SD of six replicates per treatments.*

Table 5.8: ammonia levels (mmol L^{-1}) in chymus from stomach (S), proximal jejunum (PJ), distal jejunum (DJ), and cecum (CE) from piglets that had received a diet added or not with microencapsulated citric acid (CA), thymol (TY)or a blend of citric acid and thymol (CT) in the six weeks after weaning[a]

	CTR	**CA**	**TY**	**CT**	***P***
S	7.77 ± 1.32	7.40 ± 0.66	6.52 ± 1.00	6.61 ± 0.81	0.77
PJ	15.40 ± 1.64	15.10 ± 2.02	10.28 ± 2.12	16.18 ± 1.93	0.16
DJ	14.84 ± 2.60	13.78 ± 1.02	11.33 ± 1.36	14.30 ± 1.62	0.51
CE	8.00 ± 2.06	6.24 ± 0.85	6.76 ± 1.11	11.22 ± 3.23	0.33
CO	23.01 ± 4.24	15.15 ± 2.24	24.16 ± 3.47	26.01 ± 6.13	0.31

[a] *values are means ± SD of six replicates per treatments.*

Table 5.9. Short-chain fatty acids values (mmol L^{-1}) in chymus from stomach (S), proximal jejunum (PJ), distal jejunum (DJ), and cecum (CE) from piglets that had received a diet added or not with microencapsulated citric acid (CA), thymol (TY)or a blend of citric acid and thymol (CT) in the six weeks after weaning

		CTR	CA	TY	CT	*P*
S	Acetic acid	1.66 ± 1.25	1.91 ± 1.11	2.51 ± 1.71	2.69 ± 0.93	0.48
	Propionic acid	0.14 ± 0.10^{ab}	0.14 ± 0.0^{a}	0.19 ± 0.12^{ab}	0.36 ± 0.20^{b}	0.0001
	n-Butyric acid	0.96 ± 1.09^{a}	3.37 ± 1.10^{b}	4.31 ± 1.58^{b}	4.68 ± 1.20^{b}	0.0002
	Total	2.04 ± 0.55^{a}	5.63 ± 1.83^{b}	7.21 ± 2.68^{b}	8.09 ± 1.33^{b}	0.0001
PJ	Acetic acid	1.11 ± 0.24^{b}	0.68 ± 0.13^{a}	0.86 ± 0.10^{a}	0.82 ± 0.24^{a}	0.01
	Propionic acid	0.19 ± 0.07	0.09 ± 0.03	0.10 ± 0.01	0.17 ± 0.15	0.14
	n-Butyric acid	2.57 ± 0.99	2.25 ± 1.01	3.37 ± 0.87	2.53 ± 2.06	0.54
	Total	4.23 ± 1.35	3.07 ± 1.12	4.39 ± 0.95	3.73 ± 2.23	0.49
DJ	Acetic acid	4.18 ± 1.47	4.75 ± 1.20	4.04 ± 0.33	3.94 ± 1.00	0.75
	Propionic acid	0.38 ± 0.46	0.20 ± 0.10	0.13 ± 0.04	0.18 ± 0.09	0.60
	n-Butyric acid	7.60 ± 2.24	8.52 ± 2.80	11.23 ± 1.87	9.37 ± 0.70	0.30
	Total	12.34 ± 3.41	15.19 ± 4.14	17.32 ± 1.84	13.92 ± 0.77	0.33
CE	Acetic acid	43.5 ± 16.0	48.8 ± 4.01	46.5 ± 12.1	45.5 ± 8.04	0.87
	Propionic acid	26.0 ± 8.86	34.3 ± 3.53	32.0 ± 8.10	33.1 ± 3.60	0.17
	n-Butyric acid	14.6 ± 7.80	17.5 ± 1.76	15.3 ± 7.04	15.4 ± 4.52	0.83
	Total	87.74 ± 30.46	103.3 ± 7.004	97.00 ± 25.14	97.68 ± 13.70	0.80

[*] *Values are means of six animals for each treatment. Different superscripts within each row are significantly different ($P < 0.05$).*
Colon data were not available.

Table 5.10. Morphological evaluations of villi height and crypts depth (μm) of jejunum, ileum and cecum from piglets that had received a diet added or not with microencapsulated citric acid (CA), thymol (TY)or a blend of citric acid and thymol (CT) in the six weeks after weaning[a]

Treatments	**Jejunum**		**Ileum**		**Cecum**
	Villi	Crypts	Villi	Crypts	Crypts
CTR	263 ± 75	226 ± 29	231 ± 26	210 ± 30	334 ± 37 [ab]
CA	273 ± 28	206 ± 31	218 ± 20	182 ± 24	301 ± 39 [a]
TY	266 ± 36	229 ± 25	233 ± 22	207 ± 12	368 ± 56 [b]
CT	237 ± 27	251 ± 73	217 ± 18	216 ± 17	376 ± 36 [b]

[a]*Values are means of six animals* ± SD *for each treatment. Different superscripts within each column are significantly different (P < 0.05).*

5.1.4. Discussion

The increasing need to find alternatives to antibiotic growth promoters focused research attention on additives, such as organic acids and flavors, which may improve health status and growth performance of piglets at weaning. The transit to liquid "animal" more digestible feed, such as maternal milk, to "vegetal" solid feed, added to the stress due to sow dependence, to transportation and to environmental changes, are all causes of the Post Weaning Syndrome (PWS), which results in alterations in villus-crypt structure (Miller et al., 1984; Hampson 1986; Kelly et al.,1991), and consequently malabsorbtion. During weaning the free access to feed and its presence in the gut is necessary for a correct development of intestinal mucosa, being the post-weaning anorexia a factor which lead to a worse atrophy of villi and to a decrease in crypt cell production rate (Pluske, 2000). In such a way the increased feed intake in thymol fed animals found in this study is interpreted as a positive effect, probably due to its properties to stimulate pancreatic secretions and brush borders enzymes. Futhermore many investigations on broilers observed the positive effect of essential oils and flavors on feed intake and growth performance (Bassett, 2000; Jamroz and Kamel, 2002). Even if there are few data avaiable on pigs performance Isley et al., (2003) observed that feeding sows with a combination of essential oils such as capsicum,

carvacrol, and cinnamaldheyde, significantly improved piglets weights at 21 d of lactation.

Another consequence of PWS is the *E. coli* pathogenic intestinal overgrowth, resulting in diarrhea onset and high mortality rate. (Kyriakis, 1989). The growth promoting function of organic acids has been extensively reviewed by many authors (Roth and Kirchgessner, 1989; Partanen and Mroz, 1999; Canibe and Jensen, 2003) and it has been supposed that it is more related to organic acid antimicrobial effect, rather than to the decrease of digesta pH, since evidence of the latter is difficult to find (Bolduan et al.,1988; Roth and Kirchgessner, 1997; Fevrier et al., 2000). Flavors have well known *in vitro* antibacterial properties (Burt, 2004), but little is known about their *in vivo* effect. Manzanilla et al. (2004) observed that the inclusion of plant extracts to piglets diet significantly increased intestinal lactobacilli and lactobacilli to coliforms ratio. Results observed in the present study are coherent with the previously described ones, since we found that both the blend of citric acid and thymol and the individual substances strongly affected coliforms counts in cecum and shifted the coliforms/ Lactic Acid Bacteria (LAB) ratio more toward LAB rather than coliforms. It has been suggested that organic acids and phenolic compunds, such as thymol, may exert their antibacterial action both diffusing through the bacterial cell in the small intestine and cecum of animals (Lueck, 1980; Partanan and Mroz, 1999). It is therefore necessary that they can reach the lower part of the gut maintaining their structure and concentration bypassing the stomach pH, and this can be pursued by using lipid microencapsulated additives (Piva et al., 2007), that allow a release of active ingredients after they entered the duodenum as they are attacked by pancreatic lipases. This could explain why microbial counts were affected only in the distal part of the intestine, where the lipases action has been fully completed. Microbiological data are aligned to cecal fermentation data. In the fermentation study the synergism between citric acid and thymol allowed to lower coliforms counts and to increase LAB, thus lowering the colforms / LAB ratio. The concentrations of citric acid and thymol that allowed to observe those results in the *in vitro* study were a little higher than the one employed in the *in vivo* study (3000 vs 1875 ppm, for citric acid and 200 vs 125, for thymol); this discrepancy is

not numerically detectable if applied to an *in vivo* system, because it is very hard to define a linear relationship between *in vitro* and *in vivo* concentrations, the latter depending on the amount of ingested feed, on the feed passage rate in the gut, and on the volume of digesta. Despite the *in vitro* results, no acetic acid increase was observed in the cecal chymus collected from the piglets at the end of the *in vivo* trial. *In vivo*, organic acids are absorbed through the intestinal wall and SCFA accumulate in the chymus only if there is a saturation of the intestinal absorption capacity. Therefore, SCFA total production determined by the diet may not be observable due to the intestinal absorption that occurs *in vivo* (Biagi et al., 2006).

The lack of consistent effects of the blend on intestinal morphology could be explained by the fact that mucosa samples have been collected at the end of the trial, six weeks after weaning, therefore from piglets with almost a fully developed digestive system (Gabert and Sauer, 1994).

5.2. EFFECT A MICROENCAPSULATE BLEND OF CITRIC ACID AND THYMOL ON PIGLETS GROWTH PERFORMANCE, INTESTINAL MICROFLORA AND GUT MORPHOLOGY

5.2.1. Aim of the study

The second trial was to evaluate a dose-response effect on feeding growing doses of a microencapsulated blend of citric acid and thymol.

5.2.2. Materials and methods

Animals, Housing and Dietary Treatments

Ninety-six crossbred piglets (Landrace x Duroc) were weaned at 21 d and were transported to the barns of the DIMORFIPA to the Faculty of Veterinary Medicine, University of Bologna, for a post weaning trial 42 days long. The animals began the trial at the BW of 6.09 kg (SD = 0.71) and were immediatly divided after their arrival into four groups (24 animals per group), homogenous for weight and gender and were housed in 16 cages corresponding to four replicates of six pigs. Then piglets received the basal diet with or without (control diet) the addition of a microencapsulated blend containing citric acid and thymol at 1875 and 125 ppm, respectively. The blend was included in the diets at : a) 0 ppm **(CTR)**, b) 2,000 ppm **(CT2)**, c) 5,000 ppm **(CT5)** and d) 8,000 ppm **(CT8)**. All diets were formulated to provide the same amount of energy, protein, essential amino acids, calcium and phosphorus. No antimicrobial agents were added to diets. The composition of the base diet was changed after the first two weeks. Feed and water were provided *ad libitum*. Composition and estimated chemical analyses of the experimental diets are reported in Table 5.1 and 5.2, respectively.

Experimental Procedure and Sampling

Body weight was recorded individually at the beginning of the trial, two and three weeks later and at the end of the trial; feed intake was recorded daily.

On d 14 a sample of fresh faeces was collected from the rectum of six animals per treatment for viable counts of bacteria. On d 42 the same animals were killed by captative bolt and slaughtered. The gastrointestinal tract was immediately removed and samples from stomach, proximal jejunum, distal jejunum, cecum and the transverse colon were collected to determine pH, ammonia content, SCFA and microbial population. pH was measured within one hour from the sampling, while the intestinal contents for the others determinations were immediately freezed and stored at –20°C.

Animal housing and care were conducted in accordance with the published guidelines for animal welfare and protection (directive No. 86/609/EEC and Italian Law Act, Decreto Legislativo No. 116, issued on January 27, 1992).

Chemical analyses of feed and intestinal contents

Analyses of the diets (crude protein, crude fiber, ether extract, ash and starch) were performed according to AOAC standard methods (AOAC, 2000; Method 954.01 for crude protein, Method 962.09 for crude fibre, Method 920.39 for ether extract, Method 942.05 for ash, Method 920.40 for starch) and Van Soest et al. (1991) for neutral detergent fiber (NDF) and acid detergent fiber (ADF) determinations. Gross energy of the predigested diet was measured by bomb calorimetry (model 1261; Parr Instrument, Moline, IL, USA).

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

The SCFA in the intestinal chymus were analysed by gas chromatography (Varian 3400, Varian Analytical Instruments, Sunyvale, CA 94089, USA; Carbopack B-DA/4% CW 2M, 80/120, packed column; Supelco, Sigma Aldrich s.r.l., 20151 Milano, Italy). The digesta were diluted 1:2 with distilled water and centrifuged (3,000 x g, 15 min.) and 1 mL of the supernatant was filled in microfuge tubes and deproteinized with 50 µL perchloric acid (Merck, Darmstadt, Germany). After three hours the samples were centrifuged again (3,000 x g, 10 min.) and the supernatant were added with pivalic acid as an internal standard (Fussel and McCalley, 1987) prior to injection.

Microbial Counts

Viable bacteria counts from faeces at 14 days and intestinal digesta were performed using the ten fold dilution method and plating the samples onto Violet Red Bile Agar (VRBA), Rogosa Agar (RA) and Tryptose Sulphite Cycloserine Agar (TSCA) for the enumeration and isolation of total Coliforms, Lactic Acid Bacteria (LAB) and *C. perfringens*, respectively. VRBA was incubated for 24 h at 37°C in aerobiosis while RA and TSC were incubated at 39°C for 48 hours under anaerobic conditions, according to the manufacturer instructions.

Statistical analyses

Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC) in a completely randomized design. Linear and quadratic contrasts were used to determine the nature of the response exhibited to the feeding of the citric acid and thymol blend. Each cage formed the experimental unit. The differences among means of groups were analyzed using the t-Student test. Differences were considered statistically significant at $P < 0.05$.

5.2.3. Results

Performance of animals are shown in Table 5.11.

The animals preserved a good health status all the trial long. Some sporadic event of diarrhea occurred, but there were no differences among groups. No statistical evidences have been reported during the whole experiment, and no dose-related effect was found, but the CT5 (5000 mg/kg) treatment gave the best results about growth performances. Compared to control the 5,000 ppm treatment numerically increase the average daily gain and the feed intake of 14% and 17%, respectively since from the first period (0-14 d) (P= 0.7; P= 0.2); in the second period (15-42 d the average daily gain tended to be improved from the 2,000 and 5,000 ppm treatments of 20.6% and 15%, respectively (P = 0.066) when compared to control. Final body weight was 12.3% higher in 2,000 and 5,000 ppm fed animals (P = 0.087). The group receiving the blend at 8,000 ppm did not show any difference with the control, even if during the first phase there was a light, but not significant, increase in feed consumption and, as a consequence, in live weight at 14 d (+7% e +8.6%, respectively).

Viable fecal and intestinal counts, intestinal pH, ammonia, and SCFA are showed from Table 5.12 to Table 5.16, respectively.

At 14 d fecal coliforms and clostridia counts compared to control were significantly increased by all treatments (+1.8, +1.6, +1.5 Log CFU mL^{-1} for coliforms at 2,000, 5,000, and 8,000 ppm, respectively, P = 0.0005; (+3.0, +2.9, +3.2 Log CFU mL^{-1} for clostridia at 2,000, 5,000, and 8,000 ppm, respectively, P = 0.0036). At the end of the trial, coliforms and clostridia populations were under the detection limit and could not be determined; LAB counts tended to be increased in the proximal jejunum by 2,000, 5,000, and 8,000 ppm (+0.6, +2, +2.6 Log CFU mL^{-1}, respectively; P = 0.12).

Ammonia levels in the cecum tended to be affected by 5,000 and 8,000 ppm the former lowering NH_3 concentration from 7.93 to 5.03 mmol L^{-1} (CTR vs CT5, respectively; P = 0.12), and the latter highering its concentration from 7.9 to 9.6 mmol L^{-1} (CTR vs CT8, respectively; P = 0.12). No other differences were observed.

Intestinal pH at was not affected by treatments.

All treatments significantly reduced propionic acid concentration in the cecum, when compared to control (-30%, -37.5%, -28.9%, for 2,000, 5,000, and 8,000 ppm, respectively, P = 0.01). The blend at 2,000 and 5,000 ppm tended to reduce cecal total SCFA by 17% and of 22%, respectively (P = 0.12), and colonic acetic acid by 28.5% and by 10.5%, respectively (P = 0.1)

Table 5.11. Growth performances of piglets receiving a diet added or not with a microencapsulated blend of citric acid and thymol at 2,000 ppm (CT2), 5,000 ppm (CT5), 8,000 ppm (CT8) in the six weeks after weaning*

	n	CTR	CT2	CT5	CT8	*P*
Initial BW, kg	24	5.93 ± 0.69	6.21 ± 0.57	6.19 ± 0.67	6.01 ± 0.76	0.41
ADG, g d^{-1} (0-14 d)	24	241.6 ± 93.6	248.4 ± 99.1	275.3 ± 112.0	262.4 ± 108.4	0.68
ADFI, g d^{-1} (0-14 d)	4	363.7 ± 22.0	412.2 ± 25.1	426.2 ± 56.6	388.7 ± 53.0	0.22
G:F (0-14 d)	4	0.66 ± 0.06	0.57 ± 0.09	0.64 ± 0.09	0.673 ± 0.06	0.28
14 d BW, kg	24	9.31 ± 1.34	9.47 ± 1.84	10.04 ± 1.89	9.68 ± 1.53	0.47
ADG, g d^{-1} (15-42) d	24	435.1± 149.4	524.7 ± 144.8	502.0 ± 114.6	441.0 ± 140.8	0.07
ADFI, g d^{-1} (15-42 d)	4	703.3 ± 28.9	795.8 ± 80.6	756.4 ± 122.2	693.3 ± 79.4	0.32
G:F (15-42 d)	4	0.64 ± 0.01	0.63 ± 0.05	0.61 ± 0.03	0.61 ± 0.02	0.44
Final BW, kg	24	21.06 ± 4.87	23.64 ± 3.95	23.66 ± 5.12	21.58 ± 4.71	0.09
ADG, g d^{-1} (0-42 d)	24	360.3 ± 118.5	415.0 ± 89.6	416.7 ± 92.1	370.9 ± 118.3	0.15
ADFI, g d^{-1} (0-42 d)	4	590.0 ± 26.5	668.0 ± 60.9	646.3 ± 94.4	591.8 ± 70.1	0.30
G:F (0-42 d)	4	0.65 ± 0.02	0.62 ± 0.04	0.62 ± 0.03	0.63 ± 0.03	0.56

* *values are means ± SD. ADG = average daily gain; ADFI = average daily feed intake; G:F = gain to feed ratio; BW = body wheight*

Table 5.12: fecal bacterial counts at 14 d (log_{10} CFU g^{-1})[a]

	CTR	CA	TY	CT	*P*
LAB	6.54 ± 1.97	7.24 ± 0.66	7.00 ± 1.38	7.05 ± 0.91	0.92
Coliforms	4.59 ± 0.40[a]	6.41 ± 0.40[b]	6.18 ± 0.21[b]	6.13 ± 0.49[b]	0.0005
Clostridia	4.10 ± 0.17[a]	7.13 ± 0.59[b]	6.98 ± 1.56[b]	7.30 ± 0.88[b]	0.0036

[a] *values are means ± SD of six replicates per treatment. Different superscripts within each row are significantly different (P < 0.05)*

Table 5.13. Counts of viable LAB (log_{10} CFU mL^{-1}) in chymus from stomach (S), proximal jejunum, (PJ), distal jejunum (DJ), cecum (CE), and colon (CO) from piglets receiving a diet added or not with a microencapsulated blend of citric acid and thymol at 2,000 ppm (CT2), 5,000 ppm (CT5), 8,000 ppm (CT8) in the six weeks after weaning*

	CTR	2,000	5,000	8,000	*P*
S	2.96 ± 1.67	3.44 ± 0.85	3.76 ± 1.24	2.56 ± 1.49	0.48
PJ	1.71 ± 1.88	2.27 ± 2.66	3.72 ± 0.61	4.30 ± 0.3130	0.12
DJ	5.28 ± 1.03	5.42 ± 0.81	5.67 ± 0.60	4.55 ± 0.68	0.17
CE	6.10 ± 1.33	7.30 ± 0.77	6.52 ± 1.05	6.15 ± 0.80	0.24
CO	7.19 ± 1.37	7.94 ± 0.52	7.64 ± 0.70	7.64 ± 0.70	0.55

[*] *values are means ± SD of six animals for each treatment*

Table 5.14.pH in chymus from stomach (S), proximal jejunum, (PJ), distal jejunum (DJ), cecum (CE), and colon (CO) from piglets receiving a diet added or not with a microencapsulated blend of citric acid and thymol at 2,000 ppm (CT2), 5,000 ppm (CT5), 8,000 ppm (CT8) in the six weeks after weaning*

	CTR	2,000	5,000	8,000	*P*
S	3.86 ± 0.79	3.07 ± 0.66	3.10 ± 0.89	3.08 ± 0.79	0.25
PJ	5.30 ± 0.55	5.76 ± 0.59	5.55 ± 0.30	5.69 ± 0.36	0.36
DJ	6.62 ± 0.30	6.24 ± 0.56	6.24 ± 0.56	6.39 ± 0.42	0.47
CE	5.04 ± 0.08	5.05 ± 0.39	5.09 ± 0.23	5.14 ± 0.19	0.88
CO	5.61 ± 0.35	5.32 ± 0.27	5.78 ± 0.72	5.62 ± 0.43	0.41

[*] *values are means ± SD of six animals for each treatment*

Table 5.15.Ammonia levels (mmol mL^{-1}) in chymus from proximal jejunum, (PJ), distal jejunum (DJ), cecum (CE), and colon (CO) from piglets receiving a diet added or not with a microencapsulated blend of citric acid and thymol at 2,000 ppm (CT2), 5,000 ppm (CT5), 8,000 ppm (CT8) in the six weeks after weaning*

	CTR	**2,000**	**5,000**	**8,000**	**P**
PJ	18.80 ± 6.74	21.06 ± 9.53	22.76 ± 5.88	23.56 ± 4.69	0.69
DJ	20.06 ± 6.26	16.18 ± 6.82	17.91 ± 5.36	24.54 ± 5.16	0.15
CE	7.93 ± 2.99	7.36 ± 4.20	5.03 ± 2.03	9.60 ± 1.77	0.12
CO	31.53 ± 11.36	22.52 ± 16.40	39.21 ± 47.60	22.37 ± 5.27	0.56

** values are means ± SD of six animals for each treatment. Stomach ammonia concentration was not determined.*

Table 5.16. Short-chain fatty acids values (mmol L^{-1}) in chymus from stomach (S), proximal jejunum (PJ), distal jejunum (DJ), cecum (CE), and colon (CO) from piglets receiving a diet added or not with a microencapsulated blend of citric acid and thymol at 2,000 ppm (CT2), 5,000 ppm (CT5), 8,000 ppm (CT8) in the six weeks after weaning*

		CTR	2,000	5,000	8,000	*P*
S	Acetic acid	ND	ND	ND	ND	-
	Propionic acid	ND	ND	ND	ND	-
	n-Butyric acid	ND	ND	ND	ND	-
	Total	ND	ND	ND	ND	-
PJ	Acetic acid	2.64 ± 0.95	1.55 ± 1.10	1.89 ± 1.35	1.56 ± 1.00	0.37
	Propionic acid	ND	ND	ND	ND	-
	n-Butyric acid	ND	ND	ND	ND	-
	Total	2.64 ± 0.95	2.19 ± 1.54	2.09 ± 1.55	1.68 ± 1.07	0.70
DJ	Acetic acid	8.25 ± 4.35	8.35 ± 4.15	10.92 ± 4.24	5.83 ± 5.59	0.36
	Propionic acid	ND	ND	ND	ND	-
	n-Butyric acid	ND	ND	ND	ND	-
	Total	9.11 ± 4.59	9.73 ± 4.21	11.56 ± 4.46	5.97 ± 5.44	0.29
CE	Acetic acid	83.75 ± 10.41	74.24 ± 11.57	71.03 ± 13.76	82.65 ± 16.57	0.29
	Propionic acid	46.72 ± 6.65^{b}	32.86 ± 10.41^{a}	29.19 ± 6.21^{a}	33.23 ± 12.78^{a}	0.02
	n-Butyric acid	8.63 ± 3.77	8.18 ± 2.21	7.75 ± 3.73	10.45 ± 5.22	0.65
	Total	139.10 ± 10.29	115.30 ± 22.24	108.0 ± 22.32	126.5 ± 30.67	0.12
CO	Acetic acid	75.05 ± 18.46	53.63 ± 29.08	67.14 ± 20.75	86.02 ± 18.57	0.11
	Propionic acid	34.09 ± 7.89	24.21 ± 12.91	27.63 ± 10.40	31.13 ± 6.60	0.35
	n-Butyric acid	12.85 ± 3.82	11.93 ± 6.52	13.24 ± 3.93	15.90 ± 3.46	0.50
	Total	126.90 ± 27.13	93.43 ± 50.31	112.10 ± 32.37	137.20 ± 24.17	0.18

**Values are means of six animals for each treatment. Different superscripts within each line are significantly different (P < 0.05).*
ND = under the detection limit

5.2.4. Discussions

While large literature on the use of organic acids or flavors in animal production is available, little is present on the use of combinations of both kind of additives (Manzanilla et al., 2004; Piva et al., 2007) to achieve better results.

In the second *in vivo* study we presented the effects of feeding piglets with a protected blend of citric acid and thymol in a costant ratio (15:1) at growing inclusion rate in the feed, in order to find a dose-related effect on growth performance and gut microflora.

Despite a dose-related effect wasn't found, the treatments containing the additive at 2,000 and 5,000 ppm gave the best performance results, being the final body weights of piglets numerically heavier than weights of the piglets fed the control diet. Although statistical evidence was not reached, because of the low statistical power, the trend of the trial was positive, leading to the suggestion that such kind of additives could be a valid alternative to antibiotic growth promoters. Tylan 40, a commercial trademark of Tylosin, was used as swine feed additive from 44 to 110 µg/mL, depending on the age and on the stage of production (Blackwell Science Inc., 2003; Si et al., 2006). In this study the maximum concentrations of citric acid and thymol achieved at 8,000 ppm were 7.2 µg/mg and 0.5 µg/mg, respectively, therefore being under the range of antibiotic growth promoters (AGP) doses.

Regarding to microbiological aspects, although explanations of the increased amount of coliforms and clostridia in all the treated animals cannot be found, lactic acid bacteria (LAB) in proximal jejunum at the end of the trial tended to be increased by every dose. Even if the largest microbial population in pigs is located in cecum, it seem that is the small intestine microflora to determine diarrhea, especially coliforms, being *E. coli* the most probable cause of intestinal malabsorption syndrome (Kyriakis,1989; Buddle and Bolton,1992). Although it is known that the smaller the population of bacteria in the small intestine is, the more nutrients will be available for absorption by the animal (Canibe et al., 2003; Jensen, 1998), being lactobacilli the prevalent population of the small intestine, it is generally accepted that they represent an important source in mantaining

intestinal health status, as they compete with coliforms for adhesion sites on the mucosal epithelium and stimulate the immune system (Blomberg et al., 1993; Canibe and Jensen, 2003; Perdigon et al., 2001).

CHAPTER 6.

CONCLUSIONS

Weaning is a very crucial moment in the life of pigs; there are many factors which contribute to the rise of gut infections, which have an high economic impact in pig husbandry, especially after the removal of antibiotic growth promoters in EU. The use of organic acids and flavors as alternatives to AGP in animal husbandry has been proposed by many authors. Despite the abundant literature regarding antibacterial activities of such substances in *in vitro* experiments, little data are now available on their real *in vivo* effects, especially regarding flavors.

Aim of this study was to investigate the antimicrobial *in vitro* effect of organic acids and natural identical flavors against different strains of foodborne pathogens, such as Clostridium perfringens, Campylobacter jejuni, *Salmonella enteritidis*, and *Escherichia coli*, which represent a threat to both animal productivity and human health, 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 modulating the intestinal microflora of 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 intestinal disorders during weaning.

The Minimal Inhibitory Concentration results showed that gram positive bacteria were usually more sensitive than gram negative to antimicrobial effect of tested substances, and that organic acids were less effective than flavors. Furthermore, it has been observed the presence of a synergism between citric acid and thymol, especially against *S. enteritidis,* and *E. coli,* two of the maior causes of mortality in piglets at weaning.

The fermentation study clearly showed the different microbial metabolic pattern for the small and large intestine. In the small intestine, thymol led the inhibitory trend of the whole fermentation parameters analysed, decreasing gas production, ammonia concentrations and coliforms counts, while in cecal fermentations was citric acid to lead the fermentation trend acting as a substrate for positive microflora growth, increasing gas production parameters and Lactic Acid Bacteria (LAB) counts. Even if the largest microbial population in pigs is located in cecum, it seem that is the small intestine microflora to determine diarrhea, especially coliforms, being *E. coli* the most probable cause of intestinal

malabsorption syndrome (Buddle and Bolton, 1992; Kyriakis,1989). LAB are the prevalent population of the small intestine, and it is generally accepted that they stabilze the small intestine ecology, as they compete with coliforms for adhesion sites on the mucosal epithelium and stimulate the immune system (Blomberg et al., 1993; Canibe and Jensen, 2003; Perdigon et al., 2001). On the other hand, bacteria need nutrients for their own metabolism and compete with the host for nutrients in the small intestine (Canibe et al., 2001), hence, the smaller the population of bacteria in small intestine is, the more nutrients are available for absorption by the animal (Jensen, 1998). Whereas, in the presence of high number of bacteria in cecum and colon, the animal is able to utilize a part of energy from the nutrients being resistant to hydrolysis by host enzymes. Since the hypothetical mechanism of action of AGP has always been attributed to an energy saving because of the reduction of competitors microorganisms and to a reduction of microorganism responsible for intestinal disorders, a substance which should replace a growth promoter should therefore modulate intestinal microflora in two different ways, by inhibiting formerly coliforms in the small intestine and by stimulating the growth of "beneficial" cecal bacterial populations. The effect exerted by thymol and citric acid in the fermentation study was aligned with the previous observations. Furthermore, even if the results obtained could be attributed to the mechanism of action of thymol or citric acid in a preferential way in the small intestine and in the cecum, respectively, they showed to have a stronger effect when used in combinations.

Since it has been proven that organic acids and flavors loose their antimicrobial power passing through the acidity of the stomach, and that only a small amount of them can reach the lower gut, we used microencapsulated molecules to bypass the stomach acidity and to reach the small and large intestine (Piva et al., 2007) in the *in vivo* studies.

The results obtained *in vivo* are aligned with the ones from the *in vitro* experiments. The presence of a synergistic effect between citric acid and thymol has been confirmed in the first study, where the microencapsulated blend of citric acid and thymol strongly affected coliforms in cecum, decreasing the number of viable cells by 3 Logs and the cecal coliforms / LAB ratio, therefore supporting

the growth of a beneficial microflora rather than enterobacteriaceae, that are harmful for intestinal health. In the same way, in the second study the microencapsulated blend increased the number of LAB in the proximal jejunum, which is a tool in improving intestinal health.

The use of a blend of citric acid and thymol can be a useful instrument in controlling intestinal ecosystem in the absence of AGP's, since it has been able both to inhibit pathogens growth and modulate microflora *in vitro* and to improve intestinal health of piglets *in vivo*. Studies on the reduction of foodborne pathogens shedding at slaughter could be the future perspective of the use of this technology to improve meat safety for human consumption.

CHAPTER 7.

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