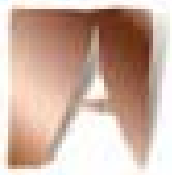

UNIVERSITAT AUTONÒMA DE BARCELONA

Evaluation of in-feed additives in early-weaned pigs: Study of the XTRACT™, a plant extracts based additive

MEMÒRIA PRESENTADA PER EDGAR GARCIA MANZANILLA

PER ACCEDIR AL GRAU DE DOCTOR DINS EL PROGRAMA DE DOCTORAT DE PRODUCCIÓ ANIMAL DEL
DEPARTAMENT DE CIENCIA ANIMAL I DELS ALIMENTS

BELLATERRA, 2005

FACULTAT DE VETERINÀRIA DE BARCELONA

Josep Gasa Gasó, catedratic del departament de Ciencia Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona

Certifica:

Que la memòria titulada "Evaluation of in-feed additives in early-weaned pigs: study of the XTRACT^{TM}, a plant extract based additive", presentada per Edgar Garcia Manzanilla per optar al grau de Doctor en Veterinària, ha estat realitzada sota la seva direcció y, considerant-la acabada, autoritza la seva presentació per que sigui jutjada per la comissió corresponent.

Y per que consti als efectes oportuns, signa la present a Bellaterra, 2 de mayo de 2005.

Dr. Josep Gasa Gasó

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RESUM

El principal objectiu d'aquest treball es avaluar els efectes d'un additiu comercial XT (5% carvacrol, 3% cinamaldehide, 2% oleoresina d capsicum) sobre els rendiments productius i diferents paràmetres de la fisiologia digestiva del garrí deslletat precoçment: digestibilitat, pH, estructura del epiteli, poblacions microbianes i les seves activitats metabòliques. Un altre objectiu d'aquest treball es avaluar les possibles interaccions del XT amb altres ingredients, nutrients i additius de la dieta. Aquest treball s'ha d'entendre com a part d'un ampli pla de recerca multicentric organitzat per la companyia Pancosma. Degut a aquest fet, les decisions sobre la investigació a realitzar estaven en molts casos condicionades pels resultants obtinguts en altres centres i equips de recerca.

En concret hem avaluat i) l'efecte del XT als paràmetres mencionats depenent del nivell i font de proteïna, ii) la possible interacció entre l'XT i l'àcid fòrmic, i la comparació de l'efecte del XT amb l'acció de l'avilamicina i el butirat sòdic. Finalment, després de tres anys de col·laboració i donat l'interès de la companyia en desenvolupar nous productes basats en extractes de plantes, hem desenvolupat un mètode *in vitro* per testar el poder antimicrobià d'un ampli ventall de substàncies derivades de plantes.

Les dues primeres proves s'han dut a terme en una segona fase propietat d'una empresa de producció en tres fases. En el primer experiment, s´ha utilitzat 240 garrins deslletats als 21 dies d'edat i les dietes experimentals s´han subministrat durant tres setmanes. En el segon experiment s'han utilitzat 216 animals deslletats als 21 dies però les dietes experimentals s'han subministrat als animals entre les 2 a 5 setmanes post-destete. La tercera prova s'ha dut a terme a les instal·lacions de la Universitat Autònoma de Barcelona utilitzant 32 animals deslletats als 21 dies d'edat i les dietes experimentals s'han subministrat durant tres setmanes. En tots els experiments, s'han registrat els paràmetres productius i s'han analitzat els paràmetres fisiològics després del sacrifici de 8 animals per tractament.

Respecte al treball *in vitro*, el nostre objectiu ha sigut desenvolupar un mètode molt simple però realista, utilitzant el contingut de diferent trams del tracte intestinal, obtingut dels porcs sense cap modificació, i incubant-lo curts períodes de temps.

Resultats productius

Els resultants productius són molt variables entre experiments. Aquestes variacions són degudes probablement a l'ús de diferent instal·lacions, edats i estats de salut dels animals i la diferent composició de les dietes bassals.

Els extractes de plantes no han produït diferencies en la primera prova, han disminuït les baixes per diarrea en la segona i han millorat el guany de pes i la ingestió d'aliment a la tercera.

Mesures fisiològiques i físiques

La digestibilitat ileal i rectal i el pH en les diferents part del tracte gastrointestinal s'han estudiat de manera sistemàtica a les tres proves *in vivo* però no s'han obtingut resultats consistents. Tanmateix, la inclusió de extractes de plantes a la segona prova va provocar un alentiment del buidament gàstric. En aquesta prova, la inclusió dels extractes de plantes també va comportar canvis al pH de l'estomac i a la població microbiana intestinal. Aquestes troballes poden estar relacionades amb canvis al "turnover" gàstric.

Poblacions microbianes

El resultat més consistent de tots els obtinguts es l'augment de lactobacillus amb la inclusió del XT a les dietes. Al primer i segon experiment aquest canvi es va trobar mitjançant l'ús de recomptes en placa a partir de contingut intestinal. Al tercer experiment, aquesta tècnica no va mostrar diferencies però utilitzant la tècnica del RT - PCR en mostres de colon es va tornar a detectar aquest increment en el numero de lactobacillus en els animals que ingerien XT. Malauradament, la rellevància d'aquest augment no està gens clara perquè no va tenir relació directa amb cap benefici productiu.

La inclusió del XT ha afectat també altres paràmetres microbians: la concentració de bases púriques i els perfils d'àcids grassos volàtils. Aquest resultats no són molt consistents i de vegades contraris.

Paràmetres epitelials

S'ha estudiat els efectes del XT a l'estructura epitelial però una vegada més els resultats són molt variables depenent de la prova. De totes maneres queda clar que l'XT exerceix

una gran influencia sobre certs paràmetres immunes del epiteli, i s'han de continuar investigant amb tècniques més especifiques.

Estudis *in vitro*

El mètode *in vitro* s'ha utilitzat amb èxit pe compara diferents additius. Les variacions de fermentació *in vitro* no només depenen del additiu utilitzat, sinó també de l'edat de l'animal i de la part del tracte gastrointestinal utilitzada d'on s'obté l'inocul. Aquestes variacions s'han d'analitzar amb deteniment.

D'aquests estudis podem concloure que les dosis comercials de XT no presenten efectes antimicrobians directes sobre les poblacions microbianes estudiades (lactobacillus i enterobacterias). Utilitzant el contingut de jejú com inocul, es necessiten dosis properes a 10000 ppm per obtenir efectes antimicrobians. D'altre banda, amb dosis més baixes de les substàncies pures s'aconsegueixen efectes similars. Aquestes dosis varien entre 500 i 3000 ppm de carvacrol i cinamaldehid, depenent de la part del tracte gastrointestinal estudiada. Amb tots els productes, la dosis mínima necessària per inhibir la fermentació es sempre menor amb el contingut del jejú.

SUMMARY

The main objective of this PhD dissertation is to evaluate the effects of the commercial additive XT (5% carvacrol, 3% cinnamaldehyde, 2% capsicum oleoresin) on productive performance and on different parameters of normal digestive physiology of the early weaned pig: digestibility, pH, epithelial structure, bacterial populations and metabolic activity. It is also an objective of this work to evaluate possible interactions of XT with other ingredients, nutrients and additives included in the diet. This work must be understood in a broader multi-centric research plan organized by the company Pancosma. As a result, the decisions about the research protocols were very often influenced by the results of the other research teams or/and centers.

In particular, the experiments here presented evaluate i) the effects of XT on the above mentioned parameters depending on the CP level and source, ii) the possible interaction between XT and formic acid, and finally the comparision of the effects of XT with the action of avylamycin and butyrate. After three years of collaboration and given the interest of the company in developing new products based on plant extracts, we developed an *in vitro* methodology to test the antimicrobial effects of a wide range of plant derived substances.

We carried out the first two experiments in a commercial second phase unit of a three phase pig producer. In the first experiment we used 240 piglets weaned at 21 days and the experimental diets were fed to the piglets for three weeks after weaning. In the second experiment we used 216 animals weaned at 21 days, but the diets were fed from 2 to 5 weeks after weaning. The third trial was carried out in the facilities of the Universitat Autònoma de Barcelona using 32 piglets weaned at 21 days and the experimental diets were fed to the animals for three weeks after weaning. In all the experiments, the performance was registered and the physiological parameters were analyzed after the sacrifice of 8 animals per treatment.

Concerning the *in vitro* method, our objective was to develop a very simple but realistic method able to obtain significant results, using crude intestinal content obtained from the pigs and incubating it for short periods.

Productive performance

The performance results were very variable among the experiments. These variations were probably due to the use of different facilities, age and health status of the animals and the different composition of the basal diets.

Plant extracts inclusion produced no productive performance differences in the first trial, decreased the casualties produced by diarrhea in the second trial, and improved weight gain and feed intake of the animals in third trial.

Physiologic and physical parameters

Ileal and whole tract digestibility and pH measurements in different parts of the gastrointestinal tract were systematically studied in the three *in vivo* trials but no consistent effects of including plant extracts were found. However, plant extracts inclusion in the diet in the second trial resulted in a decrease of gastric emptying rate. In this trial, plant extracts inclusion also produced changes in the gastric pH and in microbial populations, findings that could be related with the variation in gastric emptying rate.

Microbial populations

The most consistent result in the three experiments was an increase in lactobacilli counts with the inclusion of XT in the diets. In the first and second trials this change was found by direct agar plate count using jejunum content samples. In the third experiment, agar plate counts using jejunum content were not different among treatments, but RT - PCR in the colon content showed a higher lactobacilli content in the animals fed XT. However, the relevance of this higher intestinal lactobacilli content is not clear, since there was no direct relation with productive benefits.

XT inclusion also had an effect on other microbial parameters: purine bases concentration and VFA profile. However, those results were not very consistent and often were highly controversial.

Epithelial parameters

The effects of XT on the epithelial structure were studied and, once more, the results were very variable depending on the trial. However, a high influence of XT was found on some immune parameters measured in the epithelium and in the lamina propia,

results that encourage to continue investigating these effects with more specific techniques.

In vitro studies

The *in vitro* method was successfully used to compare different additives. Variations in *in vitro* fermentation were found not only depending on the additive used but on the age of the animal and on the gastrointestinal tract part used as source of the inoculum. These variations should be studied in more detail in the future.

From these studies, we can conclude that the recommended commercial dose of XT presents no direct antimicrobial effect on the studied populations (lactobacilli and enterobacteria). Doses of near to 10000 ppm are needed to obtain antimicrobial effects in jejunum content. However, lower doses were needed when the pure substances instead of the XT mixture were used. These doses were between 500 and 3000 ppm for carvacrol and cinnamaldehyde, depending on the gastrointestinal tract part studied. The minimum dose of all studied substances to produce antimicrobial effects was always lower for the jejunum than for stomach or cecum content.

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ABREVIATION USED

ADFI: Average daily feed intake
ADG: Average daily gain
AGP: Antibiotic growth promoter
APC: Antigen presenting cell
BW (or LW): Body or live weight
CP: Crude protein
CD: Crypt depth
DAPI: 4',6'-diamino-2-phenylindole
DM: Dry matter
EU: European Union
FA: Formic acid
FDA: Food and Drug Organization
FM: Fresh matter
FM18: Diet from experiment 1 containing Fish Meal and 18% CP level
HPLC: High performance liquid chromatography
GALT: Gut associated lymphoid tissue
G:F: Gain to feed ratio
GIT: Gastro Intestinal Tract
IEL: Intra epithelial lymphocyte
LPCD: Lamina propia cell density
MI: Mitosis index
OM: Organic matter
P: P-value
PB: Purine bases
PE: Plant extracts
SBM: Soybean meal
SBM18: Diet from experiment 1 containing Soy Bean Meal and 18% CP level
SBM20: Diet from experiment 1 containing Soy Bean Meal and 20% CP level
USA: United States of America
VC: Variation coeffiecient
V:C: Villus:crypt ratio
VFA: Volatile fatty acids
VH: Villus height
XT: XTRACT™, commercial product

CHAPTER 1.

GENERAL INTRODUCTION

In the current pig production systems, one of the main critical points is the growth and survival of the early weaning pig. The change of the piglet from the mother's environment to a completely new one produces drastic drops in productive performance and can induce severe diarrhea episodes. Many different strategies had been used to improve performance and to minimize casualties in this phase of the pig's life and, actually, performance of the piglet has been broadly improved. However, economic losses in this phase are still important, especially in the EU after the recent ban of the antibiotic growth promoters (AGP).

For the last 40 years, concerns about antibiotic resistances have increased worldwide. The modern animal production system is a big antibiotic consumer (Cromwell, 2002), and the first legislations regulating its consumption are appearing in Europe and the USA (Council Directive 70/524/EC, FDA Guidance Document GD#152). Some sectors interpret these legislations as market barriers; however, the problem of antibiotic resistances is not fiction. The ban of some AGP by EU in 1999 is just a first step to control the over-utilization of antibiotics and, in the future, the objective will be also the reduction or even elimination of the systematic therapeutic usage of antibiotics.

Management and biosecurity improvements are being studied to palliate the effect of the AGP elimination. However, the development of new nutritional strategies is the priority of this sector. The ban of most AGP in 1999 and the recent new regulations for animal feed additives (Regulation (EC) 1831/2003) have motivated a hard re-organization of the European animal nutrition market. Most companies have improved the development of new additives and ingredients, and some other nutrition or pharmacology companies have created new sections to introduce this kind of products in their catalogues. It is clear that this is a good opportunity for companies to "make money".

In this highly economic context, science stands sometimes in a second place, but progressively companies are realizing that scientific arguments are excelent arguments for selling, and they invest every day more money in R&D projects. This situation is a very good opportunity for researchers to interact with the companies and to develop their investigations, always maintaining the scientific criteria.

The animal in-feed additives group comprises acidifiers, probiotics, prebiotics, enzymes and a lot of different substances with a wide range of reported actions on animal health and production. In spite of the very good results obtained using these additives (Partanen and Mroz, 1999; Partridge 2001), they are still not comparable to those obtained using AGP and research is still very active looking for new alternatives.

Currently, some of the more “fashionable” products in this sector are plant extracts. In fact, the European food safety authority (EFSA) expert panel, on its animal feed (FEEDAP) unit has expressed their specific interest in improving research concerning these products. However, the plant extracts group comprises substances of very different origin and chemical structures, which make their study difficult. On the other hand, these products have the advantage of their “natural” origin which is a very interesting characteristic for public opinion. In any case, it is not very responsible to use plant extracts in a not controlled and properly studied way.

In the current animal production context, Kamel (1999) indicated that the use of plant extracts in animal feeding is difficult since almost no *in vivo* studies are available, although some information has recently appeared (Hermann et al., 2003; Isley et al., 2003; McIntosh et al., 2003; Molero et al., 2004; Newbold et al., 2004; Allan et al., 2005). These studies indicate that there are very good perspectives for these kinds of products, however, more studies are needed describing doses, technological characteristics, toxicity and other important characteristics.

CHAPTER 2.

LITERATURE REVIEW

Early weaning of the pig is one of the most studied problems in pig production. The abrupt transfer of the piglet from the mother's environment to a completely new one produces drastic drops in productive performance and, in the worst situations, can induce severe diarrhea. During the last 50 years a lot of different strategies have been used to improve performance and to minimize casualties in this phase of the pig's life and, actually, performance of the piglet has been broadly improved. However economic losses in this phase are still important, especially after the recent ban of the AGP in the EU, and many efforts are done to correct this situation.
Three principal strategies are used to minimize problems at weaning:

i) New preventive systems concerning management (Biosecurity rules, all in-all out, production in different phases, piglets from a single sanitary origin, Henry, 2001), pathology (improved vaccination plans, Henry, 2001) and genetics (selection against specific pathologies, Mathew 2001).
ii) Environmental improvements, such as modern installations (Henry, 2001) or a better formation of the staff (Hemsworth and Barnett, 2000).
iii) Nutritional strategies concerning the presentation of the feed, together with new or modified ingredients and additives.

In this review, only nutritional strategies, especially focused in additives, will be described. However, the other strategies are very important and do not exclude the application of nutritional strategies simultaneously (Madec et al., 1998).

2.1. WEANING, WHAT IS REALLY HAPPENING?

In normal practical conditions, the pig is stressed at weaning by different factors and this situation produces drastic reductions in the piglet's growth, and induces the appearance of pathologic problems in some cases.
Whittemore and Green (2001) proposed 100-400 g/d as a commercially acceptable ADG for pigs from 1 to 3 weeks after early weaning. However, growth rates of 500 to 800 g/d have been observed for this period in pigs fed cow's milk *ad libitum* (Williams, 2003). So we can easily conclude that commercial piglet growth rate is very low after weaning compared to the maximum potential. How could we improve this

performance? To answer this question we must understand first what is really happening at weaning.

Three key facts take place at weaning in the normal management of commercial farms:

1 - Introduction to solid, bulky food containing vegetable ingredients instead of liquid highly digestible milk.

2 - Psychological stress due to separation from the sow and mixing with other litters.

3 - Physical stress due to new environmental conditions.

Piglets must face these stressful facts with serious physiologic limitations. First, the digestive tract of the piglet is totally adapted to milk digestion, and the adaptation to solid feed does not take place in just a few hours. Other clear limitations are that thermoregulation and immune function are still underdeveloped. These two last limitations make environmental and sanitary conditions especially relevant for piglet's growth.

All these external stressors produce in the animal a critic phase for its survival, which becomes even worse by the appearance of a transitory anorexia.

2.1.1. Weaning anorexia, a key fact

As a result of all the events previously described, stress induces transitory anorexia in the piglet. Post-weaning anorexia affects most of the piglets for no longer than two days after weaning and normally 50% of the animals start eating during the first 12 hours (Bruininx et al., 2001). This can be considered the acute phase of the anorexia. When pigs finally start to eat, their feed intake is still not enough to cover their requirements. This low ingestion, especially concerning energy, is the first limiting factor for a normal recovery and development of the piglet after weaning (Thacker, 1999, Henry, 2001). Le Dividich (1994) (Figure 2.1.) showed that ME feed intake is under maintenance levels during the first 5 days after weaning (red line) and stays under pre-weaning levels for two weeks (blue line)).

This low feed intake that takes place after weaning causes new problems to the piglet, and increases the limitations of the animal to develop a rapid adaptation to the aggressions. Figure 2.2 shows the process happening after weaning. It shows the

important role of anorexia in inducing digestive epithelial damage, and in limiting immune response and termorregulation capacity of the animal.

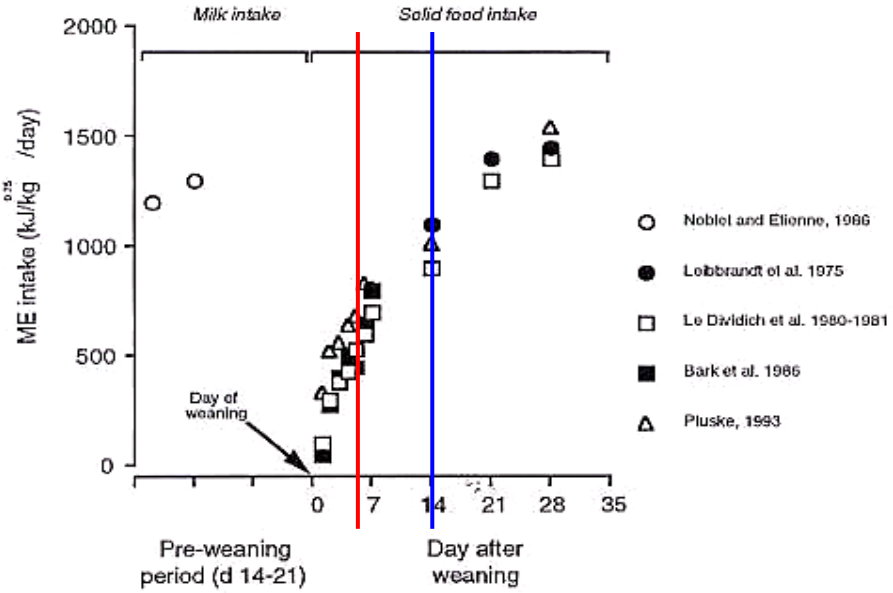


Figure 2.1. Evolution of ME ingestion of the piglet after weaning. (Le Dividich, 1994).

The success of the adaptation of the animal to the new situation depends on the complex interactions among three main components that will be explained in the next pages. These components are:

- Physiological and structural elements of the digestive tract.
- Microbial populations.
- Immune response.

2.1.2. Physiologic and structural adaptation of the epithelium

The introduction of the solid diet to the intestine when it is still adapted to milk results in a bad digestion process until the animal develops the mechanisms to digest this new diet. Basically, this adaptation may be considered in two main parts. The first part is the stomach and the small intestine. This part of the digestive tract must be adapted to digest the solid food by changing enzymatic secretion, secreting more acid and adapting the flow rate of the digestive content. The second part is the hindgut. In the adult pig,

the hindgut is in charge of the digestion of the fiber fraction of the diet. Milk has no fiber, so the hindgut is not very important before weaning. When solid diet is ingested for the first time, the digestibility is reduced and a higher proportion of the diet reaches the cecum. These new nutrients arriving to the hindgut, mainly carbohydrates, stimulate a fast development of a complex microbial ecosystem that will be present until the animal's death. Then, the hindgut increases its volume to retain fiber for a longer time and to contain all this microbial population.

Both parts of the GIT are very important for the optimum digestion of the diet and to avoid diarrhea. However, the most studied and important changes in this period take place in the small intestine.

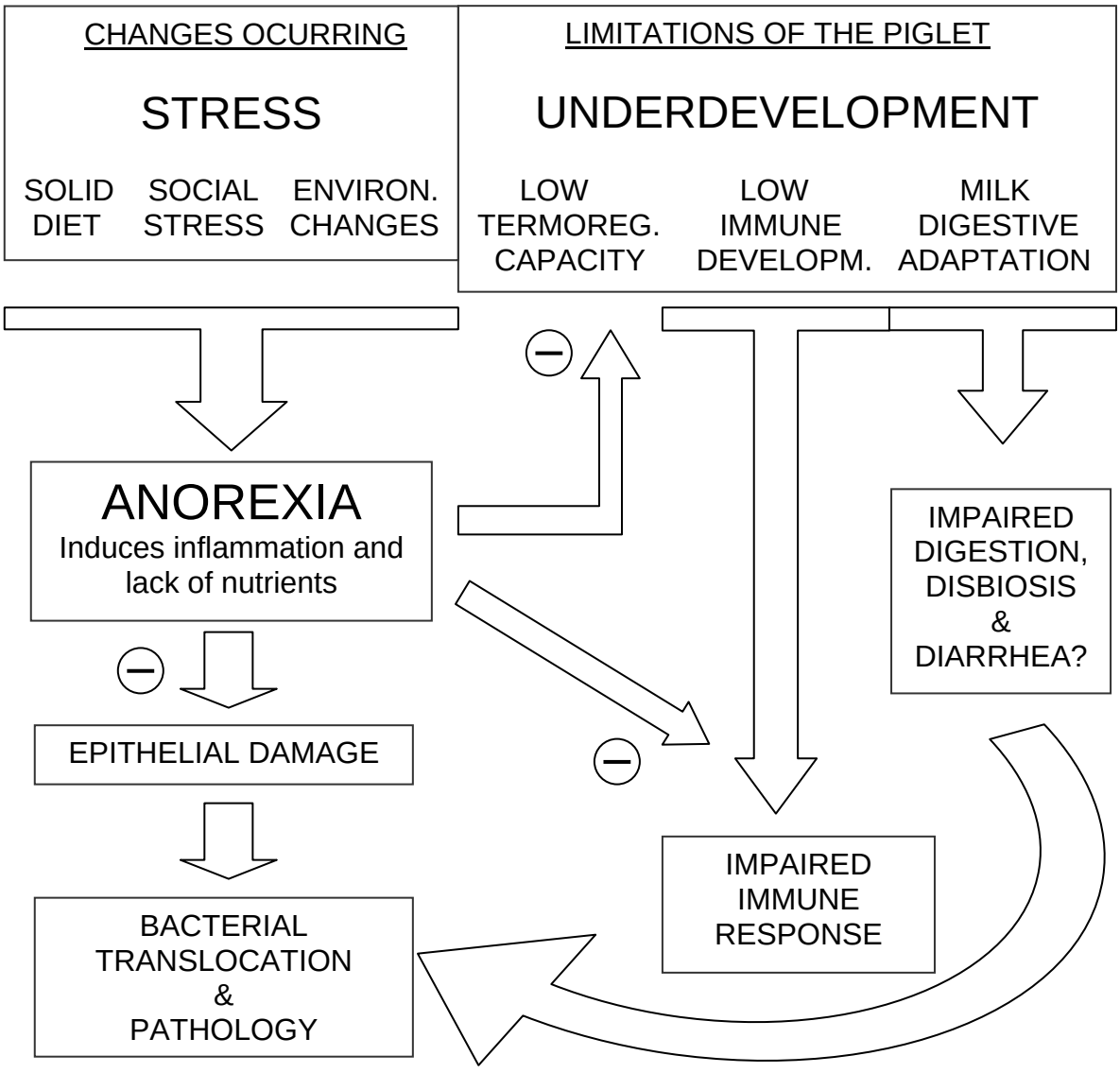


Figure 2.2. Importance of anorexia at weaning.

2.1.2.1. *Short description of the small intestine epithelium*

The intestinal mucous membrane is one of the most active systems in maintaining a good health status. It acts as a nutrient supplier for the entire organism and also as a barrier that regulates relations between the internal and external medium. As a consequence, the intestinal epithelium is the tissue presenting the fastest renewal rate in the organism (Buddle and Bolton, 1992) and the digestive tract is responsible for 30% of the total nutrient requirements of the pig (Burrin et al., 2001).

The intestinal mucous membrane is organized in a folded structure to maximize the contact surface between the external and internal media. The structural units of this system are the villus and the Lieberkühn crypt. Figure 2.3 shows the normal aspect of a villus stained with hematoxilin-eosin and a schematic drawing of the epithelial cell structure.

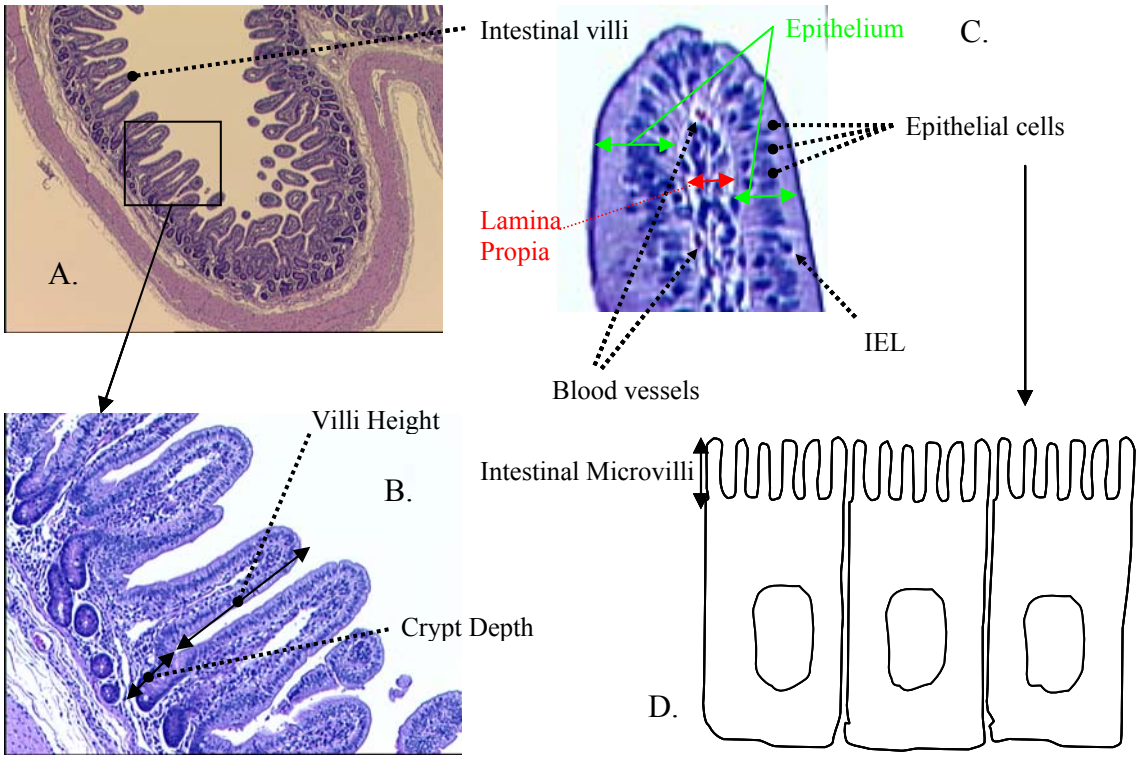


Figure 2.3. Photography of the intestinal epithelium (transversal section of the tube), A.; determination of the VH and CD, B.; real aspect of the epithelial cells in the tip of a villi, C.; scheme of the epithelial cell showing microvilli structure, D.

The intestinal villi are finger-like structures of 300-1000 micrometers, presenting a columnar epithelium which regulates the exchange of substances. This epithelium is

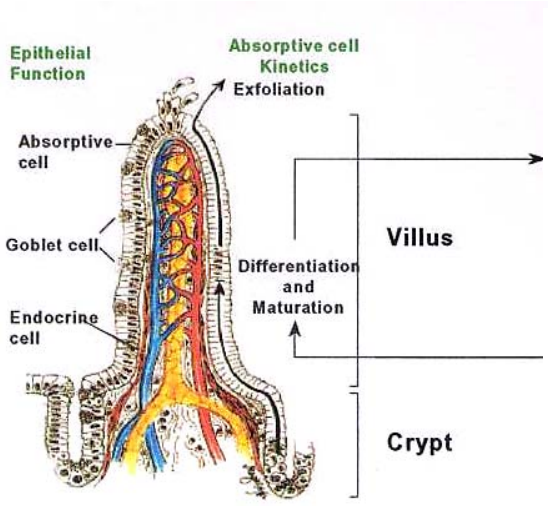
formed mostly by enterocytes but we can also find endocrine cells, immune cells and goblet cells. Enterocytes are long cells presenting crowded micro-villi (1 micrometer) on the apical region to maximize the absorption surface. Goblet cells secrete mucus (water + glycoproteins (mucins)) to protect the epithelium. This mucus layer is very important in bacterial binding to the epithelium but it is very difficult to study due to its especial characteristics.

Below the basal membrane of the epithelium we can find the lamina propia. This is the connective tissue which provides structural, vascular, lymphatic and neural support for the epithelium. Moreover, it contains a high amount of immune cells such as lymphocytes, and macrophages, which recognize and eliminate, if necessary, all external antigens.

Nutrients are transported from the intestinal lumen to the blood by different mechanisms. It is mostly done by intracellular transport and after that nutrients are released to the lamina propia and taken by the blood stream. However, water molecules cross through the intercellular space because they are small enough to pass through the tight junction between enterocytes.

2.1.2.2. *Dynamic aspects*

The intestinal epithelium is renewed via the migration of the enterocytes from the crypt to the top of the villi (Figure 2.4.).



Enterocyte functions:	**Presence of Hidrolases**	**Transport of nutrients**	**Transport of H_2O and ions**
VILLUS	+++	+++	Absorption
	+++	++	
	++	+	
	+	-	
CRYPT	-	-	Secretion

Figure 2.4. Epithelial cell renewal and function.

Normally, enterocytes show secretor function when they are in the crypt and later develop an absorptive function when they migrate to the villus. It follows that net absorption in the small intestine depends on the villi-crypt relation (Buddle and Bolton, 1992).

During the migration, the enterocyte develops its two principal functions: digestive and absorptive. In a first phase, enterocytes express digestive enzymes and their micro-villi suffer elongation (digestive function development). Enzymes will be accumulated in the microvilli of these enterocytes (Dahlqvist and Nordström, 1965). After this, the nutrient transport capacity to the lamina propia appears (absorptive function development) (King et al, 1981 y 1983, Smith, 1985).

Once on the top of the villi, enterocytes are eliminated by different mechanisms: mechanical forces, pancreatic enzymes, bile, pepsin, bacterial aggressions, etc (Clarke, 1973). Thus, the renewal of the intestinal epithelium is a consequence of a dynamic equilibrium between production of enterocytes in the crypt and desquamation in the villi (Clarke, 1973). The renovation of enterocytes is slower in the neonatal piglet (7-10d) than in adult pigs (3-4d), so, piglets need a longer time to recover their original villi height after insults (Buddle and Bolton, 1992). This difference is due to the shorter villi and deeper crypts in adult pigs (Moon, 1971). Within different parts of the small intestine, the ileum presents the fastest renewal rate because villi are shorter (Buddle and Bolton, 1992).

To understand how enterocyte renewal and functions are regulated, different mechanisms have been suggested, such as a negative feed back from villi cells to crypt cells (Galjaard et al., 1972, Eastwood, 1977; May et al., 1981), systemic positive stimuli of the development by glucocorticoids (James et al 1987a), by some peptides (James et al., 1987b) and by pancreatic secretions (Tivey y Shulman, 1991).

2.1.2.3. *Changes at weaning*

One of the most studied responses to dietary changes at weaning is the modification of the epithelium structure (Pluske et al., 1996, 1997). Comparing the mucous membrane of weaned and un-weaned animals, two main changes are observed after weaning: lower villi and deeper crypts (Pluske et al., 1991). When the pig is naturally weaned these changes take place as a progressive process of adaptation to the solid diet lasting from 9 to 12 weeks (Hall et al., 1989). A diminished height of the villi induces variations in the enzymes secreted by the epithelium (Smith, 1992) since enzymes expressed along the

villus are longitudinally different (Dahlqvist and Nordström, 1965). In particular, shorter villi secrete lower amounts of milk digestion enzymes, such as lactase, and higher amounts of carbohydrases (Smith, 1992). On the other hand, the solid vegetal diet is normally more abrasive than milk and produces an increase of the enterocyte renewal rate. Deeper crypts just reflect a higher enterocyte production to maintain villus height (Smith, 1992).

However, when the piglet is early-weaned this adaptation occurs in a very short term. A detailed description of the villus-crypt evolution during eleven days after early weaning (Figure 2.5, Hampson 1986) shows that villi height decreases until day five after weaning (circle line) and then the crypts react to compensate this decrease. Un-weaned pigs (square line) showed also increased crypt depth but of lower magnitude.

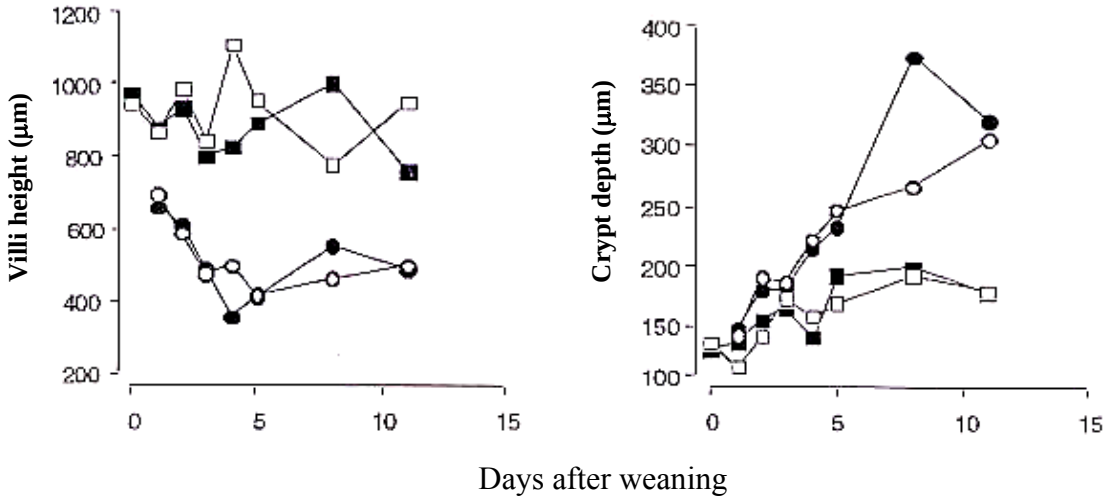


Figure 2.5. Evolution of villi height and crypt depth in 21 days weaned (circles) and un-weaned pigs (squares).

Above in the text, the villus-crypt relation has been introduced as an absorption capacity index and it is logic to expect that the rapid change in villus-crypt relation can induce transitory absorption problems. On the other hand, the piglet is in a compromised situation also because of the low ingestion. Enterocytes obtain part of the nutrients from intestinal lumen and the lack of nutrients due to transitory anorexia can induce cellular damage in these cells (Nuñez et al., 1996), losing intestinal barrier integrity, and causing the appearance of inflammatory reactions (McCracken et al., 1999). Then, this impaired absorption could become a diarrhea episode.

Nabuurs et al. (1993) showed how villi height is lower in animals suffering from diarrhea and even more when casualties appear. Moreover, crypts were deeper in these animals. Experimental fasting periods normally produce shorter villi but also shorter crypt because of the lack of enteral nutrients for enterocytes (Raul y Scheiffer, 1996, McCracken et al., 1999). Deeper crypts after weaning of the pig could indicate that enterocytes are not underfed in spite of the low ingestion and diarrhea occurrence but this aspect is not totally clear. This kind of reaction of the crypt has also been related to some allergies in humans and animals (Stokes et al., 2002). Thus, in the weaned pig, it could be induced by some components of the solid diet such as soy proteins.

Thus, the new diet and the low feed intake cause an impaired nutrient absorption and damage in the epithelial barrier. When this happens, a third factor acquires a determinant role: the intestinal microbiota.

2.1.3. Microbial populations

2.1.3.1. *Normal microbiota*

Concerning normal microbiota, high variations can be found along the gastrointestinal tract (Jensen, 1998) and radial differences are described (Gaskins, 2001) within each segment. These differences are produced by the different environmental conditions of each segment. Stomach and proximal small intestine (duodenum) contain 10^3-10^5 CFU/g of FM due to the low pH and the rapid flow. Along the small intestine, the digestive content flow rate is fast enough to avoid important proliferation of most of bacterial groups. Given the high flow rate, the binding mechanisms of bacteria to the epithelium are of special importance to allow bacterial proliferation (Anderson, 2003). The binding sites, rather than the diet, will probably condition which types of bacteria are present in the small intestine mucous membrane. In spite of the adverse conditions, counts of 10^8 CFU/g of FM are normal in distal small intestine. The normal presence of bacterial population in the small intestine consumes high quantities of nutrients competing with the animal, but is useful to avoid the colonization of the GIT by detrimental groups of bacteria.

Once in the hindgut, the flow rate is significantly lowered and the composition of the medium is stable when no insult happens. These conditions allow the development of a very complex ecosystem containing 10^{10}-10^{11} CFU/g of FM, including more than 500 species mainly anaerobic gram positive (Moore et al. 1987, Akkermans et al., 2003). This microbiota present in the hindgut is responsible for the digestion of an important

part of the diet, mainly fiber (Bach Knudsen et al., 1993) and it shows a high adaptability to substrate changes.

The microbiota is affected in all segments by some characteristics of the media such as nutrients availability, and inhibitory compounds such as VFA, H_2S, de-conjugated bile salts, NH3 and bacteriocines (Gaskins, 2001).

Concerning radial differences, mucus and presence of specific binding sites in enterocytes determine that bacteria associated to epithelium are different than those free in the lumen. Gaskins (2001) described four different niches for bacterial ecosystems: lumen, unstirred mucus, deep mucus in crypt and epithelium. These differences between lumen and epithelium populations are clear in the small intestine, where digesta flow is high and the washout of most species is higher than the proliferation rate but they are also important in the hindgut as described by Takahashi et al., (2004).

Due to all these factors, it is difficult to refer to stable bacterial groups present in each segment. Using classic culture methods, it is normally accepted that lactobacilli, enterobacterias and streptococcus are the predominant genera (Ewing and Cole, 1994) in the small intestine, but recent improvements in molecular techniques pointed out the importance of other groups. In the hindgut, it is even more complicated to determine clear groups and probably it is better to describe different populations and their general characteristics as gram stain, aerobic capacity, metabolism characteristics or substrate preferences. Molecular techniques are currently providing a lot of new information about these populations.

2.1.3.2. *Weaning disbiosis as a risk situation*

The intestinal microbiota is normally maintained in equilibrium and evolves with the age of the animal. However, abrupt changes such as weaning (Dunsford et al. 1991), fasting periods, changes in diet (Brunsgaard, 1998) and total parenteral nutrition (Ganessunker et al, 1999) can break this equilibrium and sometimes induce disbiosis and finally diarrhea.

A clear example of this case is the early weaning of the pig; during lactation, the pig eats a liquid diet coming from the sow. The abrupt stop of milk ingestion at early weaning produces a lack of nutrients not only for the animal, but also for the intestinal microbiota that, after one or two days of fasting, is totally disturbed. After this disruption, nutrient intake is recovered with solid and particulated feed partially from vegetal origin and normally contaminated with new bacterial groups. The intake of new

ingredients changes flow rates, the digestion products are totally different and fermentations happen because some microorganisms proliferate in abnormal quantities. Reid and Hillman (1999) proposed the fecal lactobacilli-coliform ratio as an indicator of the ability of the piglet to resist pathogens, since lactic acid bacteria are known to inhibit the growth of enterotoxigenic E.coli (Hillman et al., 1995). In any case, this effect has been never demonstrated in the piglet and probably this index is too simple to explain a very complex situation. The index has been helpful until now, because of the limitations of microbial analysis techniques, but current new molecular techniques allow a better understanding of the evolution in time of the microbial populations of the piglet. In this context, normally non-studied groups such as clostridium spp. bacteria appear to be more relevant than we previously thought.

2.1.4. Immature immune response

2.1.4.1. *Intestinal defense mechanisms*

The intestinal defense system includes complex interactions between epithelium. external mucous layer and immune system.

The **epithelium** acts as a physical barrier between the lumen and the lamina propia. The components of this barrier are epithelial cells, tight junctions between cells and the basal membrane of the epithelium. The epithelium presents also specialized cells which collaborate in defensive functions such as goblet cells or intraepithelial immune cells (paneth cells, that secrete antimicrobial peptides, and lymphocytes), and is able to regulate immune function through secretion of cytokines (Gaskins, 2003).

The **mucous layer** has protective, lubricant and transport functions. It is not a static barrier but it is able to adapt mucus quantity and composition to different environmental conditions (Gaskins, 2003). The mucous layer is secreted by specialized epithelial cells called goblet cells and is formed mainly of water (95%) but its characteristic properties come mainly from the glycoproteins called mucines. The carbohydrate moiety from mucines act as selective binding sites for bacteria and “decide” which bacteria remain bound to the epithelium and which ones will be dragged away by the intestinal flow.

Concerning the **immune system**, the gut is supported by non-specific immunity (Natural killer cells, mast cells, macrophages and neutrophiles) which act through chemotactic mechanisms. However, the most important immune mechanism is the one formed by specific immunity; the so-called gut associated lymphoid tissue (GALT). GALT represents 30% of total intestinal mass and accounts for the 50% of the body

lymphoid tissue (James, 1993). It is easy to imagine that any activation of this immune tissue will suppose a high nutrient waste for the animal. GALT is mainly formed by two parts, an organized part formed by peyer patches and intestinal lymph nodes, and a diffuse part which consists of immune cells disseminated along the intestinal tract (lamina propia and intraepithelial immune cells) (Stokes 2001).

Peyer patches are formed by multiple follicles (B-cells) surrounded by inter-follicular zones (T-cells).

In the lamina propia, plasma cells (mature B-cells) are mostly situated in the crypts, and T-cells (CD4+ and CD8+) in villi, following a particular spatial organization (Vega-Lopez et al., 1993; Olivier et al., 1994).

This specific immune system is divided in humoral response, normally directed to bacteria, and cellular response, normally directed to virus infected cells. Humoral immune response is started by specialized cells called M-cells which transport antigens from lamina propia to peyer patches. Once there, antigen presenting cells (APC: tissue macrophages or dendritic cells) process the antigen and stimulate T-helper cells (CD4+) that secrete lymphokines. These lymphokines induce B cells in peyer patches to become class specific for IgA production. B cells go to the epithelium where they are called plasma cells and secrete IgA after re-exposure to antigen. These IgA are excreted to the intestinal lumen where they bind the antigen (King et al., 2003).

The cellular immune response is started by the cells infected by viruses, which process the viral antigen and present it in their cytoplasmatic membrane. T-cytotoxic cells (CD8+) react and secrete a bioactive factor to destroy infected cells (King et al., 2003).

2.1.4.2. *Immune underdevelopment at weaning*

At birth, the piglet has no immune protection due to the epitheliocorial placenta, but it receives protection through the sow's calostrum (IgG, Passive immunity). This protection decreases very quickly and the piglet normally changes from passive to active immunity at three weeks of age (King et al., 2003). The active immune system of the pig takes 7-9 weeks to develop complete alimentary antigen tolerance and pathogen elimination ability. However, at early-weaning, the piglet's immune system receives high quantities of antigenic stimuli at three weeks of age and the immune system may be overwhelmed.

In particular, each part of the immune system matures as follows:

a) Unspecific immunity, macrophages and polymorphonuclear cells do not reach adult levels until 5 weeks of age (Vega-López et al. 1995) and chemotactic mechanisms are impaired in young pigs (King et al., 2003).
b) Concerning specific immunity, organized immune sites are rapidly mature but effectors sites organization takes 7 to 9 weeks and some characteristics are not achieved until 6 months of age (King et al., 2003).

i) APC cells do not reach adult levels until 5 weeks of age (Vega-López et al. 1995).
ii) B-cells are accumulated to adult levels and pass from IgM to IgA expression during first 4 weeks of age (Pabst and Rothkotter et al., 1999).
iii) Helper T-cells (CD4+) appear at 3 weeks of age and cytotoxic T cells (CD8+) at 7 weeks of age (Vega-López et al. 1995, 2001). Intraepithelial lymphocytes (IEL) significantly increase with age and represent 50% of total intestinal lymphocytes at 5 weeks of age (Vega-López et al. 1995, 2001).

Regarding this information, we conclude that piglets present a totally immature immune system when commercial early weaning occurs at 21-28 days of age. Moreover, they exist proves of the prejudicial effect of the weaning stress in the immune response by itself (Wallgren et al., 1994).

2.1.4.3. *Immune activation: high nutrient waste at weaning*

The evolution of the intestinal immune system is clearly affected by the microbiota, as shown by data from microbial effects on germfree animals. Conventional animals vs. germfree present higher cell turnover rate, higher lamina propia cells, higher IgA secretion, thicker muscular wall and bigger peyer patches with different lymphocyte populations (Gaskins, 2003). All these characteristics are present in adult animals so it is logic to deduce that microbiota plays a principal role in intestinal maturation. Moreover, these differences occur despite the fact that both conventional and germ free animals are exposed to dietary antigens. Hence, the importance of the microbiota compared to dietary antigens is clear.
On the other hand, discrimination between innocuous antigens (mostly dietary and some bacterial) and those from pathogenic bacteria is essential. Oral tolerance must be induced to these innocuous antigens. How this oral tolerance is developed is still under

discussion (Strobel and Mowat, 1998, Bailey et al., 2001) but if tolerance is not developed in a correct way, inappropriate inflammation of the GIT occurs.
Weaning exposes the piglet to new microbial and dietary antigens. After weaning, increases in different populations of T-cells in lamina propia are described and general immune system activation occurs (King et al., 2003). In addition McCracken et al. (1995) described changes related to acute-phase responses: higher plasma concentrations of IL-1, fibrinogen, glucagons and increased liver weight.
Taking into account all this information, King et al., (2003) proposed two hypotheses to explain this sudden activation of the immune system at weaning:

-Anorexia compromises the integrity of the epithelial barrier allowing luminal antigens to penetrate in the organism initiating an immune response.
-The immune system is immature and not able to discriminate between harmful and innocuous antigens and shows over response.

The first hypothesis has been reported in different studies. In particular, anorexia increases paracellular permeability rather than transcellular permeability (Vedonk et al. 2001) and a negative correlation has been described between villous height (indicator of epithelial damage) and CD8+ and CD4+ cells counts in lamina propia (Spreeuwenburg et al. 2001). However, anorexia usually produces shorter villi but also shorter crypts. The reaction of the epithelium at weaning is to produce shorter villi but deeper crypts, and this is similar to the reaction documented in humans in dietary allergies (Stokes et al., 2002). In fact, inclusion of soybean meal in weaning pig diets induces increased immune reactions. However these reactions appear after some days and activation at weaning is extremely rapid (King et al., 2003). As a summary, both hypothesis seem to be true and could occur at the same time. First, anorexia produces epithelium damage allowing bacterial translocation and rapid reaction but dietary antigens can also induce responses similar to hypersensitivity.
In any case, it is clear that all this activity related to immune activation is high nutrient consuming and reduces performance. This immune stress is able to affect nutrient metabolism, inhibit voluntary feed intake, stimulate acute-phase protein synthesis, and bring about some more systemic effects (King et al., 2003).

2.2. NUTRITIONAL STRATEGIES TO IMPROVE HEALTH AT WEANING: IN FEED ADDITIVES

From a nutritional point of view, strategies to improve the piglet's health at weaning include i) modifications of food processing or feed structure (i.e: fermented liquid feed (Canibe and Jensen, 2003)), ii) changes in ingredients (i.e. inclusion of rice (Pluske et al., 2002)) and iii) utilization of in feed additives (Jensen et al., 2003). In this review only in-feed additives will be discussed. The most important of these in feed additives are the AGP and it is of extreme importance to know how the AGP produce their benefits and how they have been used so far.

2.2.1. Antibiotics growth promoters, their ban, and the pig industry

From the introduction of aureomycin in 1949 as a growth promoter, the sub-therapeutic dosage of antibiotics in animal feed has been generalized all over the world and has produced important benefits in productive performance and in the prevention of pathologic processes (Anderson et al., 1999). However, after five decades of usage, concerns about bacterial resistance have become an important issue, and from WHO/OIE/FAO reports (http://www.who.int/foodsafety/micro /meetings/nov2003/en), it is evident the need to act against the possibility of bacterial resistances appearing. A highly restrictive legislation has been recently applied in the EU, which has introduced the progressive ban of the AGP in animal feeds from 1999 to next 2006. There is a debate about the usefulness of the EU banning AGP to avoid antibiotic resistances, especially if this fact produces an increase in the therapeutic usage of antibiotics which probably can induce resistances in an easier way. Other countries such as the USA or Australia propose a rationalization of the antibiotic usage and a continuous monitoring of the resistances (FDA - GD152). For example, they avoid coincidences in human and animal therapeutic antibiotics and try to reduce the need of AGP through better production systems. In this context, the ban of AGP makes sense only as a first step in antibiotic usage control. Concerns on resistances include all antibiotic applications, even human usage.

The imminent ban of AGP in the EU is expected to have a negative impact in animal production due to an increase in the incidence of piglet diarrhea and other digestive disturbances. The elimination of the AGP from animal feeds was applied previously in Sweden, in 1986, and in Denmark, in 1998-2000. Now we can extract the first

conclusions from this experience. In Sweden, as a consequence of the ban, digestive pathologies were increased in an important proportion in the weaning pig (Göransson, 1997) producing an increase in the use of therapeutic antibiotics. Currently, therapeutic antibiotics usage has been reduced after the application of production systems modifications. On the other hand, data coming from the Danish experience (WHO, 2003) show a less dramatic situation due to the application of management strategies previous to the ban. From both experiences we know that the ban i) did not create problems to the production of growing and finishing pigs and ii) induced problems in weaning pigs but depending on the herds. In most herds suffering diarrhea as a consequence of the ban, the introduction of new production practices corrected this problem. These differences between herds point out the importance of the application of biosanitary rules and the use of new strategies as alternatives to substitute antibiotics as growth promoters.

In this context, "additives to substitute AGP" are defined as new additives for weaning pigs diets used not only to promote growth but also to optimize their health, minimizing the risk of diarrhea. This way, the use of therapeutic antibiotics would also be reduced.

All the investigations done with antibiotics can help us in the development of these new improvers of intestinal health. The importance of the microbiota in animal performance was shown by Fuller (1979, 1983). He demonstrated how germfree animals suffered a decrease in growth after being infected with enterococcus and how this depression disappeared after inclusion of antibiotics in the diet. Moreover, inclusion of antibiotics did not produce any improvement in performance in germfree animals but it did in conventional animals, especially those living in dirty environmental conditions (Roura, 1992).

Anderson et al. (1999) observed that antibiotics used in animal feeding present different chemical structures and act on microbial population through different mechanisms. However, their effect does not seem to be affected by their particular mechanism of action. This fact is indicating that their effect is linked, at least in part, to their reduction of intestinal microbial mass in an unspecific way. Recently, interesting results have been obtained with modern molecular techniques by Collier et al. (2003), that showed that antibiotic inclusion decreases total bacterial mass and produce higher homogeneity in bacterial populations in the intestine.

Most of the investigations concerning AGP effects were done before the 80's, and four theories were proposed (Visek, 1978) to explain their growth promotion effect:

- Reduction in the growth depression produced by some bacterial metabolites.
- Reduction of the competence for nutrients between microbiota and the host.
- Improved absorption and use of nutrients due to a better functionality of the intestinal wall.
- Inhibition of sub-clinic infections.

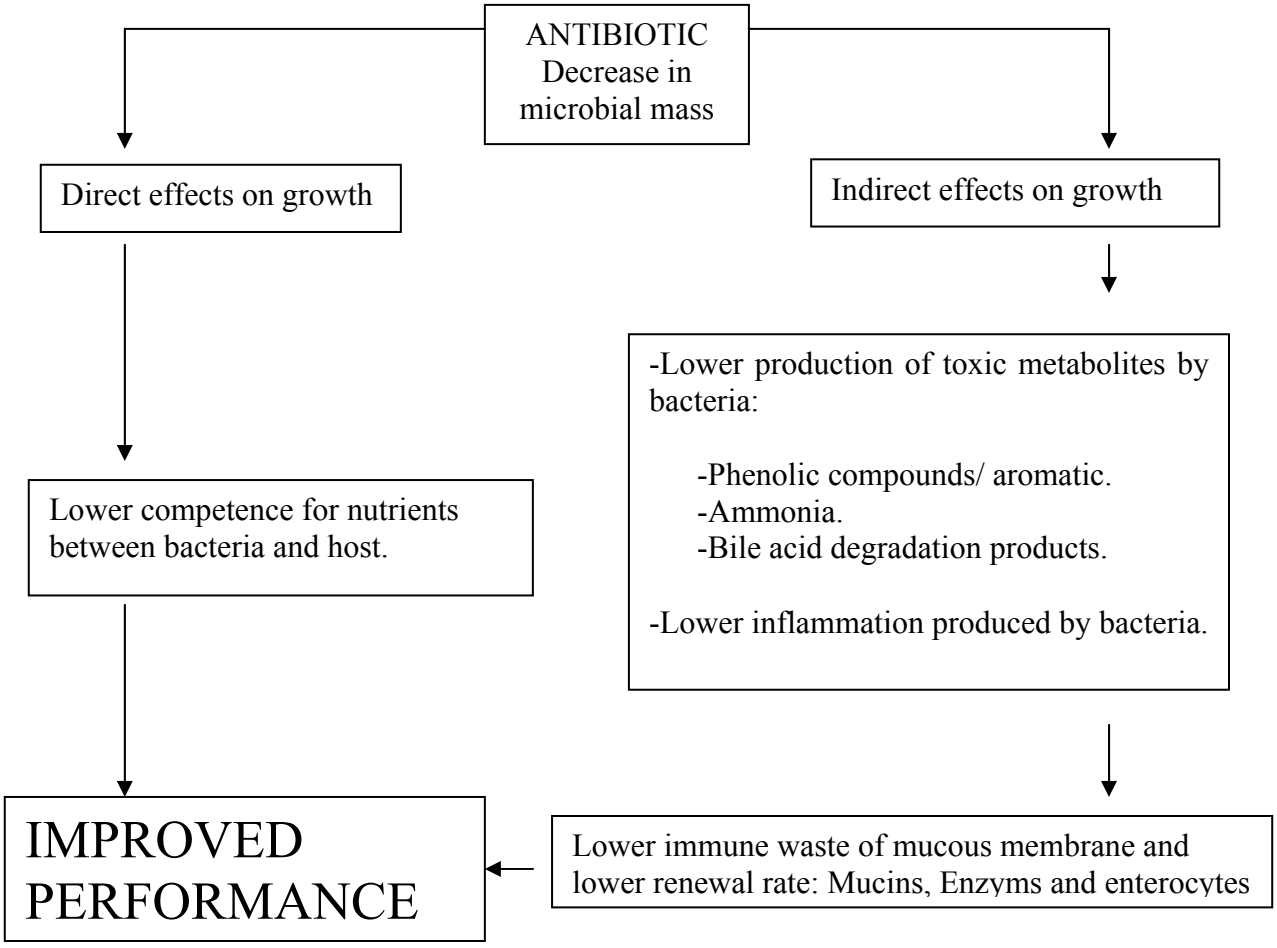


Figure 2.6. Mechanism of action of AGP (Anderson et al., 1999).

The mechanisms of action seem to be more a permission effect of antibiotics rather than a promotion effect as pointed by Anderson et al. (1999). Figure 2.6. shows the classification of AGP effects suggested by Anderson et al. (1999).

Anderson et al. divided AGP effects in direct and indirect effects. Concerning INDIRECT effects, it has been demonstrated that some toxic substances are directly related to bacterial metabolism. Jensen et al., (2003) enumerated as detrimental growth factors ammonia, amines, indoles, H2S, phenolic compounds, secondary bile acids and

SCFA. In some *in vitro* studies, Gram positive facultative anaerobic bacteria (especially important in intestine) are pointed out as the main growth depressors through the production of these metabolites. Lactobacilli and enterococci are included in this group, and these two genera are curiously proposed also as probiotics to substitute antibiotic utilization (Robertfroid, 2000).

Another indirect effect is a decrease in intestinal inflammation, but it is a decrease of the immune response rather than only inflammatory. Piglets need some immune adaptation to external aggressions, but an acute over stimulation induces an excessive nutrient waste, thus producing growth depression (Williams, 1992). Considering the direct competence between intestine and muscle determined by Reeds (1993) in piglets, a decrease of nutrient consumption by the intestine could produce an improvement in performance. In contrast, Stahly (1995) studied the nutrient utilization depending on the immune stimulation level, and a higher stimulation is not always related to lower growth rates.

This lower intestinal inflammation caused by AGP is also proposed to improve nutrient absorption because a thinner epithelium would facilitate the pass of the nutrients through the intestinal wall.

The most important DIRECT consequence of antibiotic inclusion is a higher nutrient availability to the host because they are not used by bacteria.

Once we know all these effects of AGP we have two clear options in developing new additives. We can try to mimic the AGP effects to improve animal performances or we can try to develop new products with completely different mechanisms, based in the knowledge of the piglet physiology, to obtain similar results. The different objectives of these new products were grouped by Lawrence y Hahn (2002) in:

1) Improvement of immune capacity of the piglet.
2) Improvement of digestive capacity.
3) Quantitative and qualitative modifications of intestinal microbiota.
4) Promotion of beneficial microbiota growth.
5) Avoidance of the union or adhesion of pathogenic bacteria to epithelium.
6) Preservation of cellular integrity of the epithelium.

In the last few years, reviews about alternatives to AGP have proliferated (Close, 2000; Doyle, 2001; Wenk, 2002) and all of them agree in one point: the improvement of the

performance with the use of new additives is still not comparable to that obtained with AGP, and the results are sometimes contradictory. Probably these new options are not as effective as antibiotics in growth promotion; however, it is true that currently the effect of AGP is not as strong as in the past. Some authors (Page, 2003) think that this is a logic consequence of resistances but it is probably also due to the successful application of other strategies such as management options. It is generally accepted that all additives work better when the piglet is under some challenge situation, but even in healthy situations it is not a bad idea to use new additives to help preventing eventual losses due to pathogens, especially during pig weaning.

In next parts of this review, the main alternatives to AGP will be briefly explained.

2.2.2. Pre and probiotics

Prebiotics and probiotics are now normal words in human nutrition after that many beneficial effects had been demonstrated. The utilization of these additives in animal nutrition is not very important probably because of the lack of a clear effect as growth promoters. Their mechanism of action is based on the ability of certain substrates (prebiotics), microorganisms (probiotics) or both of them (symbiotics) (Roberfroid, 1998) to create a particular intestinal microbiota beneficial for the animal.

The WHO/FAO (2001) defined probiotics as “live microorganisms which when administered in the adequate amounts confer a health benefit on the host”. Experimental results have shown the ability of probiotics to colonize the small intestine when they are included in the diet, and to develop beneficial effects once they are in the GIT (Fuller, 1992). Inclusion of probiotics has been used against digestive pathologies in piglets probably because they inhibit the adhesion of pathogen bacteria to the intestinal mucous membrane (Stewart et al., 1993; Spencer y Chesson, 1994; Mack, 1999). However, the utilization of probiotics is criticized because organisms are sometimes genetically manipulated (Sanders and Klaenhammer, 2001).

On the other hand, it is not clear if the beneficial organisms are the same for different species, since different species present very different microbiota profiles (Perez de Rozas et al., 2004a). Thus, extrapolations from humans to animals, especially the pig, are risky and more investigations must be carried out. Illustrating this idea, an interesting result was presented by Lee (2005) in poultry. She proposed the effectiveness of some clostridium species in preventing intestinal disturbances even when Clostridium perfringens is one of the main pathogens in poultry.

Prebiotics have been defined by Gibson and Roberfroid (1995) as "Non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and (or) activity of one or a limited number of bacteria in the colon, and hence improve host health". Non-digestible oligosaccharides (fructans and fructooligosacharides) are fermentable substrates for Bifidobacteria and Lactobacilli (Sghir et al., 1998) but not for other organisms. The direct consequence is that non-digestible oligosaccharides promote a stimulation of lactic acid producing bacteria (Gibson and Roberfroid, 1995). Prebiotics also have other effects, as suggested by Mouricot et al. (1990), who showed the ability of determined oligosaccharides (galactomanans) to simulate membrane receptors and to block the adhesion of different bacteria (Streptococos, Haemophilus and E. coli K99) to the gut epithelium membrane. They have effects also on the immune response through a direct effect on cell receptors or via changes in the microbiota (Buddington, 2001).

The inclusion of oligosaccharides in feed for piglets and growing pigs presents equivocal results since some good results (Buddington, 2001) have been obtained but usually no biological effects are found (Houdijk, 1998). Increases in voluntary feed intake and growth rate have been shown after weaning with prebiotics inclusion, probably by affecting the initial microbiota (Brendemuhl and Harvey, 1999) and reducing the incidence of diarrhea (Bolduan, 1993).

2.2.3. Enzymes

It is difficult to imagine a direct effect of enzymes on gastrointestinal microbiota; however there are different situations where enzymes can indirectly affect the intestinal environment. Usually when digestion is not adequate disbiosis can occur. This is the case of the weaning pig, which presents an enzymatic ability to digest milk components and is abruptly introduced to a solid diet mainly of vegetal origin. In this situation, is easy to accept that improving the digestive ability of the pig through enzyme supplementation we could minimize the microbial changes occurring at weaning. This mechanism is clearly demonstrated in chickens fed viscous diets (viscous NSP - β-glucans y arabinoxilans) (Dänicke, 2001).

Another interesting option is to use enzymes to produce prebiotic oligosaccharides in the intestine through *in situ* hydrolysis of branched-chain NSP (arabinoxylans and xyloglucans). This has been proved adequate in poultry (Bedford and Apajalahti, 2001). Unfortunately the improvement of digestion and intestinal environment achieved with

enzyme usage in pigs is not comparable to their successful use in aviculture (Partridge, 2001). Probably these differences between pigs and poultry are due to differences in their intestinal tracts. The pig has higher retention times, higher development of the hindgut and higher water content of digestive content (that produces lower viscosity). In any case few studies have been done, so more experiments are needed to determine the effect of diet composition on enzyme efficacy.

2.2.4. Acidifiers

The first utilization of acidifiers as in-feed additives for swine was done in the 60´s, using lactic acid in drinking water (Burnet and Hanna, 1963). After that, acidifiers were included also in feed and now are the most important alternative to AGP, although the responses in performance are not comparable (Edmonds et al., 1985).

Acidifiers present preservative effects in manufactured feeds but also have *in vivo* effects based on two mechanisms of action. Firstly, acidifiers can act through pH reduction. This effect is more important in weaned pigs were the acid secretion in the stomach is limited (Giesting and Easter, 1991). *In vivo* demonstration of this mechanism is difficult but it is still a good hypothesis given the importance of stomach pH for digestion, and also as a barrier (Radcliffe et al., 1998).

The second mechanism is the antimicrobial activity of the acid molecule, independently of the pH. The acid in water solution is in a state of dynamic equilibrium between dissociated and non-dissociated forms. The relative proportion of each form depends on the pH of the medium but also on the strength of the acid. The parameter which describes this concept is the pK_a (pH in which dissociated and un-dissociated forms are present in equal concentration). Strong acids (normally inorganic acids: clorhydric, sulfuric and orto-phosphoric) are mostly dissociated in aqueous solution and present a pKa of around 1. Weak acids (normally organic acids: formic, propionic, acetic, butyric, etc.) are mostly non-dissociated and present a pK_a of around 3-5. It is hypothesized that the antimicrobial form is the non-dissociated one, so a better antimicrobial effect in the intestinal environment is achieved with weak acids. This idea is suggested because non-dissociated forms are able to pass cellular membranes by passive diffusion given their non-polar nature. Once inside the cell, the higher pH of the media produces dissociation of the acid molecule and pH decreases abruptly, producing unstabilization of the cell (Partanen y Mroz, 1999).

Considering the importance of the media in the effects of the acidifiers it is logic to suppose that one of the most important factors *in vivo* affecting the effects of acidifiers is the diets since different diet present different buffer capacities. Usually, the higher buffer capacity is presented by mineral ingredients, such as carbonates, phosphates and oxides, a little lower capacity is presented by protein concentrates and the lowest capacity belongs to cereals (Jasaitis et al., 1987). Better results are obtained when the acidifiers are combined with vegetal ingredients rather than milk-by products because the latter produce lactic acid by themselves (Giesting y Easter, 1991).

Concerning productive responses, an increase in voluntary intake in piglets has been consistently reported (Partanen and Mroz, 1999) with the use of acidifiers. However, they can produce palatability problems depending on the dosage and on the type of acid used (Henry et al., 1985).

2.2.5. Minerals

The best results with the use of minerals in weaned pigs are obtained using zinc and copper in different forms, but included in doses 10 times higher than recommended for nutritional requirements. The therapeutic dose is about 2500-3000 ppm for zinc and 100-250 ppm for copper (Cronwell, 1989). Their mechanisms of action are not totally well known, but they are used due to their efficiency in preventing diarrhea, hence promoting better performance results. Regarding intestinal changes promoted by zinc oxide (ZnO), its ability to avoid diarrhea appearance is curious. For example Hojberg et al. (2004) showed that ZnO inclusion reduces lactobacilli counts along the intestine and increases coliform bacteria. Furthermore, ZnO has powerful buffer capacity. Probably these facts are pointing out that high lactobacilli/coliform ratios and lower pH are not always good indicators of intestinal health. Other mechanisms of action have been suggested for these minerals such as a decrease in the epithelial permeability (Roselli, in press) but more studies are needed to clarify them.

Unfortunately, mineral forms present high reactivity with other dietary components and competence with other minerals in metabolic pathways. Moreover, they are highly present in feces (Close, 2000). As a consequence of this high elimination to the environment, they are strictly regulated by law and their use is allowed only at nutritional doses. Now, new organic sources of these minerals are studied because they present higher availability and biological effects and thus they may be included in lower amounts.

Other minerals and vitamins have been studied but the positive results of their inclusion in diets are normally associated to an initial deficiency status. Clays such as sepiolyte have also some interest in increasing digestibility and preventing diarrhea (Castaing, 1998).

2.2.6. Immune active products

The immune system of the piglet is underdeveloped at weaning and supplemental immune elements could be useful. Ig present in spray dried porcine plasma (SDPP) and specific antibodies concentrated in the egg white from hens immunized against porcine pathogens are the main studied options. It is hypothesized that these antibodies could act against pathogens in the intestinal lumen sparing the effort to the piglet. SDPP is probably the most effective product although the results seem to be more related to effects on feed intake than to immune effects (Coffey et al., 1995, Owen 1995, Pierce, 1995, Van Dijk, 2001). The hypothesis of immune action is supported by the fact that the effect of whole plasma is also achieved by the use of the high weight fraction (containing Ig) but not by other fractions (Owen et al., 1995, Pierce et al., 1995). The results of Jiang et al. (2000) are in coincidence with this hypothesis. They found a decreased cellular density in the lamina propia in animals fed with SDPP independently of the ingestion level. The specificity of plasma in improving productive performance is demonstrated by the better results obtained when SDPP comes from farms of similar sanitary characteristics (Normantiene, 2000). In any case, the bigger the sanitary challenge, the better the results (Close, 2000).

SDPP is now forbidden in the EU. As an alternative to SDPP, there are hyper immune eggs or intestinal mucous concentrates (Owusu-asiedu et al., 2002, 2003a and b). In both cases the European legislation is not clear but their effect are still not comparable to the ones obtained with plasma.

It is important to keep in mind that these substances are more important as protein sources than as feed additives; because their mode of action is similar to an additive but their inclusion level may be between 5 and 15 % (Gatnau and Zimmerman, 1991).

Another option related to immune function is the inclusion of fatty acids as prostaglandins and leucotriens precursors (Harbige et al., 2001) or inclusion of adjuvants that improve defense mechanisms against specific antigens (Cheeke, 2000). All these kind of products present important possibilities of development but require high efforts in the future.

2.2.7. Enteric nutrients

Enteric nutrition can by defined (Gardiner et al., 1995) as nutrition of the intestinal mucous membrane from dietary ingredients. This innovative subject is based on the discovery of substances with special effects on epithelial regeneration. Some of these substances are present in the sow's milk and they disappear at weaning, so it is logical to include these substances in post-weaning diets to protect the epithelium.

EGF (Epidermic growth factors) that stimulate epithelial growth (Allee and Touchette, 1999), polyamines (putrescine, spermidine and spermine) as stimulators of growth and differentiation of mucous cells (Grant et al., 1990) and glutamine/glutamate (main energy source for enterocyte respiration and main amidic nitrogen source for nucleotide biosynthesis (Ayonrinde et al., 1995a and 1995b; Wu et al., 1996)) are the main examples of this type of substances.

Some amino acids like arginine, alanine and glycine are required at higher levels than reported in NRC 1998 (Gaskins, 2003). Nucleotides and sphingolipids (Gil and Rueda, 2002), n-3 polyunsaturated fatty acids and some SCFA (Gardiner et al., 1995) have also been suggested to preserve gut integrity at weaning.

Other interesting options without a clear defined action have also been proposed during the last years such as the use of bacteriofage viruses against bacteria (Huff et al., 2002a,b and 2003), the use of yeast extracts (White et al., 2002), or the use of plant extracts.

2.3. PLANT EXTRACTS

Plant extracts (PE) are one of the oldest additives used by mankind. However during the 20th century they were left apart because of the irruption of synthetic drugs, more effective and easier to handle. Now, doubts about the safety of some synthetic drugs, especially antibiotics, have allowed the growth of a new interest on the so-called natural products, and the most important substances in this group are PE. However, it is important to remark that, concerning PE, natural is not always synonym of safe. Important toxic reactions have been described for different normally used PE.

One of the main subjects concerning PE is their characterization (Cowan, 1999). PE contain a lot of active substances in very variable amounts and their composition is greatly affected by factors such as the method of extraction (solvent and extraction

conditions as showed by Güllüce et al. (2003)) or the niche of the plant used (especially concerning geographic location, climatic conditions, plant variety and age (Bischof-Deichnik et al., 2000)).

This characterization is important in two senses: scientific and legal.

Concerning science, it is possible to analyze the PE which we are working with, but it is difficult to obtain always a standard product in relatively big amounts. All the characteristics that influence PE composition also affect their effects. Hence, for research and for practical application of the PE, it is better to work with pure active substances, natural or synthetic, or with accurately controlled blends.

Concerning legislation, traceability, and thus characterization, is one of the main requisites to register an additive. This condition makes difficult the registration of natural PE since their standardization is always difficult.

Now, PE used in animal production as alternatives to the AGP are interesting substances which act basically as antimicrobials. However many other different effects have been reported for PE: changes in immune function (Boyaka et al., 2001; Koh et al., 1998); enzyme stimulation (Platel and Srivasan, 1996, 2000); antiparasitic (Force et al., 2000), antifungal (Mahmoud, 1994), antiviral effects (Aruoma et al., 1996; Benencia and Courrèges, 2000; Garcia et al., 2003) and anti-toxigenic activity (Azumi et al., 1997; Sakagami et al., 2001) and antioxidant activity (Aruoma et al., 1996; Dorman et al., 2000b; Teissedre and Waterhouse, 2000). Given this wide range of effects, PE must be considered one of the main candidates to study, concerning not only pig weaning but also other problems of animal production.

2.3.1. Effects of plant extracts on microbial populations

PE have been used for a long time as human medicals and food preservatives. This use was motivated by one of the most important characteristic of PE, that is, their antimicrobial activity (Didry et al., 1994). This activity has been studied in several *in vitro* studies with very good results as shown by Dorman and Deans (2000a). These results point PE as an alternative to AGP in animal production. This application is still too recent and there is too little information available about the actual possibilities of these products. However the great interest of this sector is motivating the appearance of the first studies using PE in animal models or *in vivo* (Evans and Martin, 2000; Kubena et al., 2001; Botsoglou et al., 2002; Turner et al., 2002a and b; Hermann et al., 2003

Hoffman et al., 2003; Isley et al., 2003; Jamroz et al. 2003 Lee et al., 2003; McIntosh et al., 2003; Molero et al., 2004; Newbold et al., 2004; Allan et al., 2005).

It is difficult to define what kind of antimicrobial molecules are present in PE. Usually, antimicrobial active substances in PE are of very different chemical structure, with high occurrence of phenol rings, mostly hydrophobic and some of them with similar structure to important molecules from bacterial metabolism such as receptors or enzyme substrates (Cowan, 1999). It is also known that many of these substances are secondary metabolites that plants use against predators, or with different functions such as pigmentation, aromatization or flavoring.

Table 2.1. Chemical structures implicated in antimicrobial effect of PE and referenced mechanisms of action (Cowan, 1999).

Class	**Subclass**	**Mechanism of action**
Phenol compounds	Simple phenols and phenol acids	Enzyme inactivation. (1) Membrane un-stabilizers. (2)
	Quinones	Irreversible union to adhesins, membrane polypeptides and enzymes those become inactive. (3)
	Flavonoids, flavones and flavonols	
	Tannins	1, 2, 3 and Metal quelators.
	Coumarins	Interact with eukariote DNA (antiviral)
Terpenoids		2
Alkaloids		Insertion in cellular wall or in DNA structures.
Lectins and polypeptides		Block viral fusion and adsorption Di-sulphur bridges formation
Poliacetilens		???

Table 2.1, adapted from Cowan (1999), presents the principal chemical structures producing antimicrobial activity in PE and the mechanisms of action referenced until now.

Some of the effects presented in table 2.1 need to be better investigated but some of them are well studied. This is the case of the hydroxyl group (-OH) present in phenol compounds. The importance of this group on antimicrobial activity is well known (Cowan, 1999) and any variation in its position inside the molecule, like it happens between carvacrol and thymol (figure 2.7), produces marked differences in antimicrobial power (Dorman and Deans 2000a).

It is supposed that PE act via two main mechanisms of action. The first is related to the general hydrophobicity of PE, which facilitates their union to the bacterial surface inducing unstabilization (Tsuchiya et al., 1996; Mendoza et al., 1997; Zhang and Lewis, 1997). The second mechanism is the inactivation of different molecules of the bacteria (such as enzymes or receptors) through their union to specific sites (Sharon and Ofek, 1986; Ya et al., 1988; Stern et al. 1996; Haslam, 1996;).

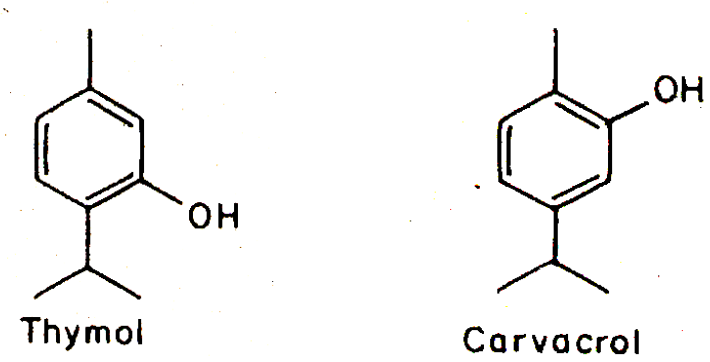


Figure 2.7. Chemical structure of thymol and carvacrol.

Some authors suggest a higher efficacy of PE against gram negative organisms (Zaika, 1988; Hussein, 1990; Smith-Palmer et al., 1998) but others did not found any difference between PE effects on gram negative and gram positive bacteria and, sometimes, even the contrary effect is proposed (Deans and Ritchie, 1987; Deans et al 1995). Actually, it is possible that some PE present specific actions and other PE do not given the different mechanism of action proposed.

In fact, as it happens for antibiotics, the chemical structure will determine the mode of action and hence a possible selective effect of PE. For instance, alkilic chains plus a phenol group seem to present better activity against gram negative bacteria, given the characteristic of their cellular wall (Pelczar et al, 1988). In any case, this specific effect of some PE could be interesting in therapeutic or preventive applications like it happens with antibiotics. Concerning growth promotion, it must be studied if a selective effect of PE would be useful, because there is a risk of producing detrimental effects (Dorman and Deans, 2000).

The effect of PE on different bacterial species has been determined in a high number of *in vitro* studies using spectrophotometry measurements or agar plate inhibition rings.

Many of these studies have explored the real antimicrobial power of classic herbal products or spices (Hili et al., 1997; Valsaraj et al., 1997; Ali-Shtayeh et al., 1998; Essawi et al., 2000). Among all these studies, only two of them will be discussed, because they study a high number of plants and bacterial groups and they also study the PE and the active substances separately.

The main results are shown in tables 2.2 and 2.3 (adapted from Friedman et al., 2002 and Dorman and Deans, 2000). In both cases thymol antimicrobial activity has been used as reference value for comparison (value = 1) due to its generalized high antimicrobial activity.

From these tables we can obtain interesting conclusions. The first one is that different bacteria show different sensitivities to different PE. For instance, *C. jejuni* shows a higher sensitivity to PE compounds than other very resistant bacteria such as *Salmonella enterica* (table 2.2).

It can also be observed how some compounds like alpha-terpinen (table 2.3) are highly effective against a very interesting target (Salmonella) but not against the other microorganisms. Finally, some PE present very different results than their main components. This is the case of thyme and thymol in table 2.2. Thymol presents a very high antimicrobial effect against all studied bacteria, but thyme present lower or higher activities depending on the bacteria. These variations are due to synergisms or interferences with other substances present in the PE.

Table 2.2. Comparision of the antimicrobial effect of 50 PE and pure components against 5 of the principal foodborne pathogens. All values are compared with thymol as value 1. Marker in yellow values equal or higher than 1.

	E. Coli	S.enterica	C. Jejuni	L.monocytogenes I	L.monocytogenes II	media
Allspice	0,43	0,23	1,00	0,89	1,00	0,71
Anethole trans	0,09	0,04	0,17	0,12	0,12	0,11
Basil	0,15	0,07	1,00	0,89	0,67	0,55
Bay leaf	0,46	0,23	0,67	1,14	1,14	0,73
Benzaldehyde	0,12	0,08	1,00	0,17	0,22	0,32
Bornyl acetate	0,09	0,04	0,20	0,12	0,12	0,11
Caraway	0,13	0,06	0,67	0,24	0,33	0,29
Carvacrol	1,00	0,60	2,00	1,00	0,89	1,10
Carvone R	0,13	0,07	0,65	0,12	0,12	0,22
Carvone S	0,12	0,08	0,50	0,23	0,47	0,28
Cineol	0,09	0,04	0,20	0,12	0,12	0,11
Cinnamaldehyde	1,00	0,75	6,67	4,00	8,00	4,08
Cinnamon bark	0,33	0,21	1,00	0,89	1,00	0,69
Cinnamon cassia	0,55	0,43	2,00	0,42	0,53	0,79
Cinnamon leaf	0,55	0,38	0,67	0,89	0,89	0,67
Citral	0,27	0,13	1,00	0,80	0,40	0,52
Citronella	0,15	0,06	0,22	0,20	0,44	0,21
Citronella R	0,09	0,04	0,09	0,12	0,18	0,10
Citronella S	0,09	0,04	0,40	0,12	0,18	0,17
Clove bud	0,46	0,23	1,00	1,14	0,89	0,74
Coriander	0,15	0,06	0,53	0,12	0,16	0,20
Cumin seed	0,20	0,08	0,20	0,22	0,32	0,20
Elemi	0,15	0,07	2,00	0,31	0,36	0,58
Estragole	0,21	0,14	2,00	0,22	0,23	0,56
Eugenol	0,55	0,33	1,00	1,33	1,00	0,84
Fir needle siberian	0,12	0,05	2,00	0,62	1,00	0,76
Geraniol	0,40	0,20	0,20	0,29	0,16	0,25
Geranyl acetate	0,09	0,04	0,59	0,12	0,12	0,19
Hyssop	0,11	0,07	0,20	0,24	0,44	0,21
Isoeugenol	0,40	0,19	0,06	0,12	0,12	0,18
Lavender	0,15	0,07	0,33	0,17	0,24	0,19
Lemon grass	0,43	0,19	1,00	0,67	0,01	0,46
Limonene	0,09	0,04	0,06	0,12	0,32	0,13
Linalool	0,15	0,08	0,06	0,12	0,12	0,11
Mentol	0,11	0,06	0,05	0,14	0,17	0,11
Nutmeg	0,11	0,07	0,11	0,30	0,40	0,20
Orange mandarin	0,15	0,05	2,00	0,44	0,80	0,69
Oregano origanum	1,20	0,60	1,00	1,00	0,80	0,92
Oregano Spanish	1,20	0,60	2,00	1,14	1,00	1,19
Palmarosa	0,50	0,21	0,29	0,47	0,30	0,35
Perillaldehyde	0,22	0,15	0,67	0,23	0,27	0,31
Rose damask	0,11	0,07	0,18	0,15	0,22	0,15
Rose French	0,14	0,06	0,40	0,18	0,28	0,21
Rose geranium	0,15	0,08	0,22	0,13	0,25	0,17
Salicylaldehyde	0,46	0,25	0,50	0,19	0,18	0,32
Spearmint	0,21	0,10	0,67	0,26	0,14	0,28
Terpienol	0,15	0,17	0,20	0,14	0,12	0,16
Thyme	1,20	0,60	1,00	0,89	0,36	0,81
Thymol	1,00	1,00	1,00	1,00	1,00	1,00
Wormwood	0,11	0,06	0,05	0,16	0,80	0,24
Mean	0,33	0,19	0,83	0,50	0,59	0,49

Table 2.3. Effects of 21 substances derived from PE against 25 bacterias representing a wide range of bacterial families. The effects of the substances are compared to thymol effects which is used as reference value (1). Marked in yellow values equal or higher than 1.

	Borneol	Carene	Carvacrol	Carvacrol methyl ester	citral	Eugenol	geraniol	geranil acetate	cis-hex-3-en-1-ol	limonene	linalool
Acinetobacter	0,23	0,34	1,52	0,00	0,27	0,52	0,20	0,35	0,27	0,00	0,31
Aeromonas	0,31	0,41	1,41	0,00	0,29	0,63	0,24	0,34	0,32	0,00	0,43
Alcaligenes	0,00	0,44	0,67	0,18	0,26	0,38	0,22	0,32	0,29	0,00	0,37
Bacillus	0,27	0,24	1,01	0,00	0,15	0,56	0,16	0,28	0,16	0,00	0,36
Beneckea	0,18	0,21	0,28	0,14	0,15	0,42	0,12	0,22	0,15	0,00	0,23
Brevibacterium	0,16	0,22	0,52	0,00	0,18	0,30	0,17	0,30	0,19	0,00	0,30
Brocothrix	0,25	0,00	0,87	0,00	0,21	0,48	0,25	0,31	0,82	0,00	0,28
Citrobacter	0,00	0,00	0,38	0,00	0,15	0,20	0,20	0,15	0,21	0,17	0,59
Enterococcus	0,00	0,43	0,81	0,00	0,82	0,38	0,49	0,29	0,34	0,00	0,63
Enterobacter	0,00	0,39	0,61	0,00	0,20	0,32	0,21	0,25	0,21	0,23	0,32
Erwinia	0,00	0,34	0,48	0,00	0,32	0,31	0,25	0,27	0,29	0,23	0,38
E.coli	0,20	0,39	0,85	0,17	0,32	0,39	0,28	0,32	0,35	0,33	0,40
Flavobacterium	0,27	0,42	1,01	0,20	0,26	0,45	0,27	0,43	0,41	0,41	0,61
Klebsiella	0,00	0,29	0,59	0,18	0,22	0,27	0,00	0,20	0,27	0,18	0,32
Lactobacillus	0,00	0,00	0,21	0,07	0,09	0,24	0,07	0,14	0,19	0,00	0,28
Micrococcus	0,00	0,21	0,50	0,00	0,13	0,22	0,11	0,15	0,24	0,00	0,25
Moraxella	0,00	0,31	0,55	0,00	0,17	0,26	0,16	0,23	0,17	0,20	0,26
Proteus vulgaris	0,00	0,33	0,79	0,19	0,21	0,25	0,17	0,29	0,24	0,22	0,36
Pseudomonas	0,00	0,79	1,94	0,00	0,49	1,16	0,43	0,49	0,63	0,00	0,00
Salmonella	0,00	0,44	0,86	0,16	0,38	0,41	0,20	0,28	0,38	0,36	0,24
Serratia	0,13	0,19	0,53	0,00	0,14	0,54	0,13	0,16	0,29	0,15	0,21
Staphylococcus aureus	0,22	0,36	0,64	0,00	0,16	0,36	0,16	0,21	0,26	0,00	0,28
Yersinia enterocolitica	0,00	0,56	0,81	0,00	0,32	0,42	0,29	0,30	0,42	0,26	0,34
Media	0,09	0,29	0,70	0,07	0,24	0,38	0,20	0,27	0,29	0,12	0,36

	menthone	nerol	Alfa pinene	beta pinene	Sabinene	terpinene	alfa terpinen	terpineol	thujone	Thymol	Mean
Acinetobacter	0,33	0,38	0,00	0,38	0,26	0,00	0,49	0,63	0,29	1,00	0,37
Aeromonas	0,26	0,29	0,00	0,26	0,00	0,00	0,91	0,62	0,46	1,00	0,39
Alcaligenes	0,19	0,22	0,00	0,24	0,24	0,00	0,66	0,59	0,42	1,00	0,32
Bacillus	0,18	0,32	0,00	0,00	0,19	0,00	0,33	0,73	0,32	1,00	0,30
Beneckea	0,12	0,23	0,00	0,13	0,15	0,13	0,60	0,35	0,19	1,00	0,24
Brevibacterium	0,00	0,28	0,00	0,00	0,15	0,15	0,25	0,44	0,23	1,00	0,23
Brocothrix	0,23	0,31	0,00	0,20	0,26	0,00	0,26	0,39	0,41	1,00	0,31
Citrobacter	0,17	0,17	0,13	0,13	0,20	0,00	0,39	0,33	0,28	1,00	0,23
Enterococcus	0,00	0,00	0,35	0,30	0,00	0,00	0,41	0,51	0,51	1,00	0,35
Enterobacter	0,21	0,24	0,00	0,00	0,24	0,00	0,57	0,71	0,35	1,00	0,29
Erwinia	0,20	0,24	0,27	0,00	0,00	0,20	0,46	0,63	0,34	1,00	0,30
E.coli	0,19	0,22	0,26	0,23	0,00	0,18	0,42	0,48	0,36	1,00	0,35
Flavobacterium	0,22	0,27	0,25	0,33	0,00	0,00	0,52	0,82	0,46	1,00	0,41
Klebsiella	0,15	0,00	0,20	0,20	0,19	0,20	0,27	0,48	0,28	1,00	0,26
Lactobacillus	0,10	0,21	0,00	0,00	0,00	0,10	0,16	0,32	0,15	1,00	0,16
Micrococcus	0,13	0,14	0,14	0,12	0,00	0,00	0,24	0,21	0,21	1,00	0,19
Moraxella	0,18	0,00	0,16	0,12	0,00	0,14	0,29	0,47	0,26	1,00	0,23
Proteus vulgaris	0,19	0,00	0,22	0,20	0,00	0,17	0,29	0,60	0,38	1,00	0,29
Pseudomonas	0,00	1,01	0,00	0,49	0,00	0,47	1,27	0,66	0,69	1,00	0,55
Salmonella	0,20	0,00	0,25	0,19	0,00	0,52	0,47	0,61	0,37	1,00	0,35
Serratia	0,17	0,20	0,00	0,13	0,00	0,00	0,36	0,28	0,18	1,00	0,23
Staphylococcus aureus	0,32	0,30	0,26	0,23	0,00	0,00	0,43	0,58	0,30	1,00	0,29
Yersinia enterocolitica	0,29	0,26	0,24	0,21	0,00	0,00	0,31	0,74	0,32	1,00	0,34
Mean	0,18	0,20	0,11	0,16	0,08	0,10	0,41	0,49	0,32	1,00	0,29

These results are not directly applicable *in vivo* but they give a good indication of which PE are more active against specific bacteria. Usually thyme (thymol), oregano (carvacrol), clove (eugenol) and cinnamon (cinnamaldehyde) extracts are the most active antimicrobial PE (active substances) (Didry et al., 1994; Kim et al., 1995a, b; Ouattara et al., 1997; Lambert et al., 2001; Chang et al., 2001).
Garlic must be mentioned separately because of the large benefits found in human medicine (the reader is directed to the supplement of the Journal of nutrition Vol. 131, 2001).

As a last remark, in the application of these PE *in vivo* it is important to consider the dose used. Compared to antibiotics, the *in vitro* dosage of PE to obtain similar results is normally 10 to 100-fold higher (Lee and Ahn, 1998; Karaman et al., 2001;). No information is available of their effective dose in complex media such as the digestive tract content, but it is well known that parameters such as pH or fat presence affect this dosage (Briozzo et al., 1989; Juven 1996; Friedman and Jürgens 2000). Thus, it is not unusual to need doses of 500-2000 ppm to obtain the expected effects. If these high doses are really needed, it could be a very important limitation for the use of PE because their strong smell and taste can result in low palatability of the diets.

2.3.2. Usefulness of plant extracts effects on digestive function

The antimicrobial capacity of the PE is their more studied effect as substitutes of the AGP. However, other effects of PE on animal physiology could help to obtain productive benefits. PE present important effects on immunity, and especially in digestive function.
The immune regulation effect of PE is scarcely known, not even in rats. Some evidences point out that the effects obtained could be interesting for future applications (Koh et al 1998; Shan et al., 1999; Kayser et al., 2001; Kim et al., 2003), however, more investigations must be carried out to clarify the possible productive performance benefits derived.
Concerning digestive function, PE have important effects upon secretions and motility of the stomach and intestine. It is well know the capacity of some PE to stimulate enzymatic secretions (Platel and Srinivasan, 1996 and 2000). Given the enzymatic limitation of the piglet at weaning, this capacity has been proposed as a possible

interesting effect at weaning (Kamel, 1999). This mechanism may be controversial if the limitation of the pig is not due to the regulation of enzyme secretion but to a real production limitation. When the secretion limitation appears by a lower development of the digestive system, i.e. carbohydrate enzymes in small intestine, the action of the PE is not useful.

However, when the secretion limitation is caused by changes in physiology, it could be useful to study PE effects. For example, changes in the irrigation of the stomach and intestine have been proposed as limiting HCl secretion and nutrient absorption respectively (Dunshea, 2003). It is known that some substances present in PE such as capsaicin increase gastrointestinal blood irrigation. This interesting property of PE can be used to improve the intestinal function of the animal.

PE can also modify the transit time of the digestive content (Mickelefield et al., 2000 and 2003), and in some cases can protect the intestinal epithelium against aggressions due to their antioxidant properties (Teissedre and Waterhouse, 2000)

CHAPTER 3.

OBJECTIVES

"Pero vamos a ver, tu que quieres medir???"

Enric Mateu

Concern on antibiotic growth promoter (AGP) usage in animal production is growing up everywhere even in countries without any legal regulation. In the European context this subject is currently regulated by strict laws, most of the AGP have been withdrawn (http://europa.eu.int/comm/food/food/animalnutrition/feedadditives/authowithdrawal_en.htm) and regulations of the process for legal registration of any sort of substance or additive clamed as an alternative to the AGP will be more exigent in the future (Regulation 1831/2003).

XTRACT™ (XT) is a commercial in-feed additive normally used in animal nutrition all over the world. XT is a blend composed by 5% of carvacrol, 3% of cinnamadehyde and 2% of capsicum oleoresin, all included in an inert carrier (hydrogenated rape seed oil). As commercial product, XT sales must rely on a commercial strategy mainly based on both, competitive prices and scientific results showing the affectivity and the inoquity of the product.

Concerning scientific data, in 1999 Pancosma, the company producing and manufacturing XT, launched a broad research multidisciplinary project involving several university departments and research centers around Europe and elsewhere. In particular, this thesis accounts for part of the program dedicated to study the use of XT on the early-weaning pig and has been carried out in cooperation with the University of Leeds, the Université d'Auvergne, Univerza v Ljubljani. In this context the objectives of this work were:

1. To evaluate the effects of XT on productive performance of the early weaned pig and on apparent ileal and whole tract digestibility of the fed diets.

2. To study the possible effects of XT on different parameters of normal digestive physiology of the early weaned pig: pH, epithelial structure, bacterial populations and metabolic activity.

3. To evaluate possible interactions of the XT with other ingredients, nutrients and additives included in the diet.

4. To develop some standard methodologies to carry out a rapid screening of this kind of products, especially concerning the antimicrobial capacity.

To reach this objectives four different experiment were planned and performed:

- Experiment 1: XT was evaluated in three different diets varying in protein, source and level. Productive performance and digestive parameter were studied.

- Experiment 2: XT was evaluated at two different doses, commercially recommended and double dosage, in combination with formic acid at 0.5%. Productive performance and digestive parameter were studied.

- Experiment 3: XT was compared with other AGP alternatives, avylamicin and sodium butyrate. Again productive performance and digestive parameters were studied.

- Experiment 4: Two in vitro methodologies were used to evaluate the effect of XT and its three components on bacterial activity.

CHAPTER 4.

EFFECTS OF PLANT EXTRACTS IN THE INTESTINAL ECOSYSTEM OF THE EARLY WEANING PIG: INFLUENCE OF DIETARY PROTEIN

"Llamalo X"

Mariola Baucells

4.0. CONTEXT

This was the first experiment that we planned with PE. Literature data concerning *in vivo* effects of PE were scarce by this time. The company, Pancosma, was interested in performing some *in vivo* experiments using their commercial product XT at commercial doses in early weaned pigs, but they allowed us to define the context. We decided to define three different diets differing on the protein source and level. We decided to use dietary protein as a source of variation because it is probably the most determining fraction affecting digestive function at weaning (Thacker, 1999) and because its well-know influence on AGP effects (François, 1962).

4.1. MATERIAL AND METHODS

The experiment was performed at a commercial second phase farm of Baucells S.A. and received prior approval from the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona. The treatment, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines.

4.1.1. Animals, Housing and Dietary Treatments

Two hundred and forty commercial crossing ((Landrace × Large white) × Pietrain) piglets excluded from receiving creep feed, 5.4 ± 0.40 kg live weight and 20 ± 1 day-old, were randomly allocated to 24 pens (10 animals per pen) in an environmentally controlled room (temperature and ventilation).

A		D	F	C
B		E	E	B
C		F	D	A
D		A	C	F
E		B	B	E
F		C	A	D

Figure 4.1. First trial was carried out in a high biosecurity transition facility (up left). The scheme (up right) shows that pens were distributed in four lines of 6 pens each. The 6 treatments were distributed at random within the pens of each line. Details of the scales and feeder (down).

Mean initial weight of the animals in each pen was adjusted, by visual choice of the pigs, to be comprised between 5.3 and 5.5 kg/piglet. Pens were organized in four lines of six pens (Fig. 4.1). Each pen, within each line, was randomly allocated to one of six experimental treatments following a 2 × 3 factorial arrangement resulting from the combination of two levels (0 or 200 mg/kg, as feed basis) of XT standardized in 5% (wt/wt) carvacrol, 3% cinnamaldehyde and 2% capsicum oleoresin (*Capsicum annum*) with 3 basal diets. The three XT components were included in an inert fatty carrier before including them in the feed. The basal diets contained the same proportion of cereals (47%), milk by-products (25%) and porcine plasma (4%), and different crude protein levels and sources.

The three diets (table 4.1 and 4.2) were FM18 diet (CP = 18%) which contained 10% of LT fish meal (FM), SBM18 diet (CP = 18%) in which a 5% of FM was isoproteically replaced by full fat extruded soybean meal (SBM) (5% FM, 9% SBM) and SBM20 diet (CP = 20%), in which a higher protein level was obtained by supplementary SBM over the 10% FM (10% FM, 6,3% SBM). $Cr_2 O_3$ was included as a digestibility marker.

Table 4.1. Composition of the diets on an as-fed basis (g/kg).

Ingredient	FM-18 diet	SBM-18 diet	SBM-20 diet
Corn	168.4	181.5	162.9
Barley	200.0	200.0	200.0
Wheat	100.0	100.0	100.0
Fish meal	100.0	50.0	100.0
Spray-dried animal plasma	40.0	40.0	40.0
Fat-enriched whey	100.0	100.0	100.0
Acid whey	150.0	150.0	150.0
Soy-bean oil	37.2	32.7	34.0
Full fat extruded soybeans	-	89.8	63.0
Dextrose	50.0	-	-
Sepiolite (a clay)	40.0	40.0	40.0
L-Lysine	4.0	5.0	1.4
DL-Methionine	2.2	2.6	1.7
L-Threonine	0.8	1.1	0.1
L-Tryptophan	0.3	0.3	0.1
Choline chloride 50%	0.06	0.05	0.03
Chromic oxide	1.5	1.5	1.5
Vitamin and mineral premix[a]	5.0	5.0	5.0

[a]Provided the following per kilogram of diet: vitamin A, 13500 IU; vitamin D_3, 2000 IU; vitamin E, 80 mg; vitamin K_3, 4 mg; thiamin, 3 mg; riboflavin, 8 mg; vitamin B_6, 5 mg; vitamin B_{12}, 40 mg; nicotinic acid, 40 mg; calcium pantothenate, 15 mg; folic acid, 1.3 mg; biotin, 150 mg; Fe, 120 mg as iron carbonate; Cu, 175 mg as copper sulfate $5H_2O$; Zn, 110 mg as zinc oxide; Mn, 65 mg as manganese sulphate; I, 1mg as potassium iodate; selenium, 0.10 mg as sodium selenite.

Table 4.2. Analyzed nutrient content of the diets on a DM basis[a].

Nutrients	Amount in FM-18 diet	Amount in SBM-18 diet	Amount in SBM-20 diet
CP, g/kg	190.4	197.2	217,6
Crude Fiber, g/kg	25.6	26.1	25.5
Fat, g/kg	66.9	66.0	65.6
Ash, g/kg	86.5	81.9	89.5
GE, Mcal/kg	4.52	4.63	4.87
Lysine, calculated value, g/kg	15.3	15.3	15.3

[a] Analyzed DM of diet = 91.8%.

4.1.2. Feeding regimen, Controls and Sampling

During 14 d, the animals were allowed *ad libitum* access to feed and performance was monitored weekly. From d 15 to 19 a controlled feed intake pattern was applied from 08:00 to 20:00 in order to standardize the digestive tract conditions at sacrifice. In

particular, 30-min periods of feeding (ingestion period) were alternated with 1-h fasting periods (fasting period). The adequacy of timing and *ad libitum* conditions were confirmed when animals in the pen moved to eat to the feeders at the start of each feeding period and finished in a 30-min period. Pigs were fed *ad libitum* the remainder of the day (from 20:00 to 8:00 of the next day). On days 18 and19, after the 12:00, 13:30, 15:00, and 16:30 ingestion period, one pig per treatment was weighted and killed by i.v. injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain; 200 mg/kg BW).

A complete and different line of pens was used at each slaughter time. Thus, one pig (the closest to the mean BW within the pen) was selected from each pen each day. The animals were bled; the abdomen opened immediately from sternum to pubis, and the whole gastrointestinal tract was removed, weighed and sampled.

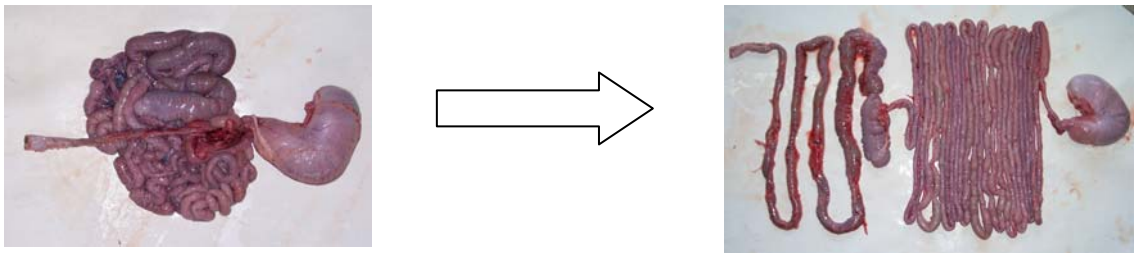


Figure 4.2. The whole gastrointestinal tract was removed and separated in the different parts.

The pH in four GIT segments was measured by insertion of a unipolar electrode (penetration pH-meter CRISON 507, electrode Crison 52-32, Net Interlab S.A.L., Madrid, Spain) through a small incision made in the wall of the organ. The pH measurements were performed in the middle of the caudal portion of the stomach, 15 cm proximal to the ileocecal valve, in the lowest part of the cecum and in the colon, 20 cm distal to the caecum.

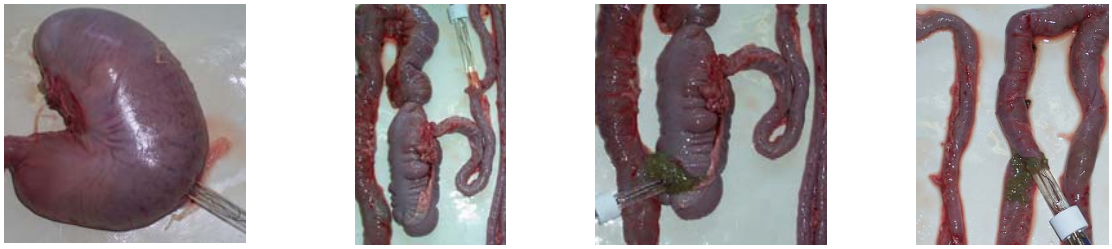


Figure 4.3. pH was measured in stomach, ileum, cecum and colon.

Samples for **histological study** were obtained from the proximal and distal jejunum wall, 75cm from the stomach and 15 cm proximal to the ileum. The samples were cut open longitudinally along the mesenteric attachment and fixed by immersion in 10% (vol/vol) buffered formalin immediately after slaughter.

A jejunum portion (25cm long), 20 cm proximal to the ileum was tied off and collected for **enterobacteria** and **lactobacilli counts**. The jejunum portion was stored at 4ºC until the culture was done later in the same day. Total contents of the ileum and rectum, and samples from homogenized caecum and colon, were collected, lyophilized, milled and stored for subsequent analysis.

Figure 4.4. Samples taken from homogenized content o stomach, ileum, cecum and colon.

A second sample was taken from homogenized cecum contents, which was acidified with H_3PO_4 (approximately 4 g fresh weight/mL of [wt/wt] H3PO4, 1% [wt/wt] of mercuric chloride and 50 mM 3-methyl valerate as an internal standard), and stored at –20ºC for **VFA analysis**.

4.1.3. Analytical Procedures

Chemical analysis of the diet was performed according to the Association of Official Analytical Chemists (AOAC, 1995) standard procedures. The **GE** was determined by an adiabatic calorimeter and **Cr concentration** in diet, ileum and feces was analyzed following the procedure described by Williams et al. (1962) by atomic absorption spectrophotometry. **Total starch** of feed and digesta samples was measured by the method of Theander (1991). Briefly, total starch was determined as glucose liberated after enzymatic incubation with thermostable α-amilase (Sigma, Ref. A-4551, Sigma, Madrid, Spain) for 1 h at 100ºC, and amyloglucosidase (Sigma, Ref. A-3514, Sigma, Madrid, Spain) for 4h at 60ºC.

Tissue samples for **histological study** were dehydrated and embedded in paraffin wax, sectioned at 3 μm, and stained with haemetoxylin and eosin. In each section we measured the villus height (VH), the crypt depth (CD), the intraepithelial lymphocytes (IEL) number in the villus, the index of mitosis (MI) in the crypt and the intravillous lamina propia cell density (LPCD). Measurements were done in ten well oriented villi and crypts from each section and the average value was used as the experimental unit. The VH and CD were measured using a linear micrometer ocular (Olympus, REF. 209-35040, Microplanet, Barcelona, Spain). VH was represented by the distance from the crypt opening to the tip of the villus (Figure 2.3.). CD was determined from the base of the crypt to the level of the crypt opening. The villus/crypt ratio (V/C) was calculated. The same villus and crypt columns were used to determine the number of IEL and mitoses (meta- and anaphases), respectively. The IEL number was expressed as number per 100 villus cells and the mitotic activity was expressed as the number of mitoses per 100 crypt cells. When VH and CD were affected by treatments IEL and MI were also presented in tables as total number in a crypt. LPCD was determined by counting total visibly stained nuclei in a total area of 2000 μm^2 from ten villi in each section using a grid ocular (Olympus REF. 209-35046, Microplanet, Barcelona, Spain). Cell density was expressed as number stained nuclei per 1000 μm^2. Goblet cell number was counted in villi and crypt, but only in distal jejunum, and expressed as number of goblet cells per 100 villus or crypt cells. All histology measurements were done by the same person.

For **bacterial counts**, one gram of sample was weighed, serially diluted, and 100 μl aliquots were plated in agar MacConkey (Oxoid, Ref. CM 115, Oxoid S.A, Madrid, Spain) for enterobacteria counts (dilutions 10^{-3} to 10^{-7}) and in agar rogosa (Oxoid, Ref. CM 627) for lactobacilli counts (dilutions 10^{-5} to 10^{-9}). Enterobacteria were counted after 24-h incubation (37ºC) and lactobacilli were counted after a 48-hour incubation period (37ºC, 5% CO_2).

Purine bases (PB; Adenine and Guanine) in lyophilized ileal, caecal, colonic and rectal contents (60 mg) were determined by HPLC (AGILENT 1100 Series) according to Makkar and Becker (1999), after their acid hydrolysis with 2 mL 2 M perchloric acid at 100ºC for 1h, including 0.5 mL of 1 mM-allopurinol as internal standard.

VFA concentration (μmol/g of fresh matter) in deproteinized caecal digesta was determined by GLC, following the procedures of Jouany (1982)**.**

4.1.4. Calculations and Statistical Analysis

Ileal and rectal apparent digestibility of each nutrient fraction (Nf) was calculated by the marker concentration (Cr) ratio method between diet (D) and digestive content (d) and using the equation:

$$\text{Digestibility coefficient} = [1-(Cr\ D / Cr\ d) \times (\%\ Nf\ d / \%\ Nf\ D)]$$

All results were analyzed by ANOVA with the GLM procedure of SAS 8.1 (SAS Institute, Cary, NC, USA), including XT and diet included as classification factors. The XT × diet interaction was included in the model.

In **productive performance analysis**, the pen was used as the experimental unit, and initial mean live weight was used as covariate.

In **slaughter data analysis**, the pig was used as the experimental unit. The day and period of sacrifice were initially included in the model but were not significant for any variables (P = 0.43 to 0.94); thus, they were ultimately excluded from the model. As suggested by Lowry (1992) for this factorial arrangement, the interaction was studied when the P-value was significant or when it was less than 0.15 without any significant principal effect. In these cases, the effect of the XT inclusion was analyzed within each diet by orthogonal contrasts, and the effect of the diet was studied, within XT at the 0 ppm rate, by mean pair-wise comparison using Bonferroni's correction (Lowry, 1992).

The alpha level used for determination of significance for all analyses and contrast was 0.05. Additionally, the REG procedure of SAS was used for regression determinations presented in the discussion section.

4.2. RESULTS

4.2.1. Productive Performance and Digestibility

Table 4.3. shows the productive performance observed during the 14-d experimental period and the ileal and whole tract digestibilities of the diets on days 18 and 19. No differences were noted among treatments for average daily feed intake (ADFI) (240 ± 6.2 g) and average daily gain (ADG) (177 ± 5.6 g). However gain to feed ratio (G:F) was decreased (P = 0.007) for animals fed FM18 diet (0.69 ± 0.014) compared to those fed SBM18 (0.75 ± 0.014) and SBM20 (0.76 ± 0.014).

An interaction was observed for ileal digestibility of OM (P = 0.032) and starch (P = 0.016). Thus, XT inclusion tended to decrease OM ileal digestibility of the FM18 (P = 0.064) and SBM18 (P = 0.071) diets. These differences are partially associated to the decrease on the ileal digestibility of the starch with XT inclusion in FM18 (P = 0.032) and SBM18 (P = 0.014) diets, but not in SBM20 diet (P = 0.227). No differences were found among dietary treatments on ileal digestibility of the protein and the whole tract digestibility of OM.

4.2.2. Morphology of the Small Intestine

Table 4.4 shows the structural characteristics of the mucous membrane in proximal and distal jejunum. Between segments, VH was higher in the proximal than in the distal jejunum (415.0 ± 10.60 vs. 331.5 ± 8.9; p = 0.001) and MI was lower in proximal jejunum (1.19 ± 0.041 vs. 4.94 ± 0.300; p = 0.001). However, CD was the same for proximal and distal jejunum (208.7 ± 5.60 vs. 213.8 ± 4.63, P = 0.784).

Among dietary treatments, an interaction was observed for VH. Diets FM18 and SBM18 determined a higher VH in proximal jejunum, compared to SBM20 when XT was not included in the diet (483.7 and 485.6 vs. 373.3, respectively). However, proximal jejunum villi were shorter with XT inclusion in diets FM18 (P = 0.0003) and SBM18 (P = 0.013) and did not vary in SBM20 diet (P = 0.714). A similar response was observed in distal jejunum, but this change was only significant in diet SBM18 (P = 0.015). CD was only affected in proximal jejunum, where XT inclusion decreased CD in diet FM18 (P = 0.0001). From all these variations, the calculated VH / CD ratio did not show differences as affected by the XT inclusion, but, in proximal jejunum, was higher (P = 0.003) for diet SBM18 (2.26 ± 0.069) compared to diets FM18 (1.95 ± 0.069) and SBM20 (1.93 ± 0.069).

Concerning IEL, MI, and LPCD, they showed different pattern between proximal and distal jejunum.

In proximal jejunum, the diet SBM20 compared to the FM18 and SBM18 increased LPCD (1.35 vs. 1.13 and 1.10, P = 0.004) and MI (11.3 vs. 9.5 and 9.33, P = 0.037), and decreased the total number of IEL when XT was not included (33.0 vs. 45.2 and 52.8). In this part, XT inclusion affected mitoses and IEL. When mitoses are expressed as total number in villus, they were lower with XT inclusion in diets FM18 (P = 0.070) and SBM18 (P = 0.095). The inclusion of XT decreased also total IEL in villus when included in diets FM18 (P = 0.002) and SBM18 (P = 0.040).

Table 4.3. Growth performance and digestibility of the pigs fed the experimental diets[a].

Diet[b]	FM18		SBM18		SBM20		SEM	P-values[b]			FM18	S18	S20
Item XT, mg/kg	0	200	0	200	0	200		XT	DIET	INT[e]	Ct vs Xt	Ct vs Xt	Ct vs Xt
Average Daily Feed Intake[cd], g/d	253	254	251	223	228	232	12.2	0.448	0.175	0.382	-	-	-
Average Daily Gain[d], g/d	177	176	187	170	178	173	11.8	0.427	0.980	0.784	-	-	-
Gain : Feed[d], g/g	0.70	0.69	0.74	0.76	0.78	0.74	0.019	0.465	0.007	0.384	-	-	-
Ileum Digestibility, %													
Organic matter	76.8	70.9	72.4	67.2	71.8	75.6	1.91	0.082	0.150	0.032	0.064	0.071	0.167
Starch	93.2	91.3	93.7	90.7	92.9	94.4	0.78	0.069	0.034	0.016	0.032	0.014	0.227
Total tract OM Digestibility, %	87.3	87.8	86.9	87.7	87.8	88.6	0.78	0.321	0.488	0.974	-	-	-

[a]Values are least square means (n = 4 for productive performance and n = 8 for digestibility).

[b] FM18 = diet without soybean meal, 18% CP level; SBM18 = diet with soybean meal, 18% CP level; SBM20 = diet with soybean meal, 20 % CP level; XT = plant extract mixture; INT = interaction (XT × DIET).

[c]As-fed basis.

[d]Initial weight included in the model as covariate.

[e]Interaction was studied when the P-value was significant or when it was lower than 0.15 without any significant principal effect. Orthogonal contrasts were used to study XT effect within each diet. Differences due to diet, when XT is not included, are indicated by different super index in the same row.

Table 4.4. Intestinal histology of pigs fed the experimental diets[a].

	Diet[b]	FM18		SBM18		SBM20		SEM	P-values[b]			FM18	S18	S20
Item	XT, mg/kg	0	200	0	200	0	200		XT	DIET	INT[c]	Ct vs Xt	Ct vs Xt	Ct vs Xt
Proximal Jejunum														
Villus height, µm (VH)		483.7^{x}	372.0	485.6^{x}	409.0	373.3^{y}	383.4	19.42	0.0008	0.004	0.011	0.0003	0.013	0.714
Crypt depth, µm (CD)		255.9^{x}	178.3	219.1^{y}	211.0	199.4^{y}	207.4	9.83	0.003	0.354	0.0002	0.0001	0.581	0.584
Villi:Crypt, µm:µm (V/C)		1.86	2.04	2.32	2.19	1.96	1.91	0.094	0.971	0.003	0.275	-	-	-
Mitoses, n/100 cells (MI)		1.09	1.17	1.15	1.05	1.32	1.37	0.092	0.870	0.037	0.608	-	-	-
Mitoses, n/crypt		0.94	0.72	0.90	0.68	0.82	0.94	0.078	0.150	0.597	0.086	0.070	0.095	0.315
IEL, n/100 cells		31.7	25.1	34.6	28.8	27.7	31.0	2.33	0.137	0.393	0.094	0.051	0.100	0.353
IEL, n/villus		45.2^{y}	26.6	52.8^{x}	39.6	33.0^{z}	42.4	3.38	0.013	0.016	0.0005	0.002	0.040	0.168
LPCD[d], n/1000µm^2		9.25	9.75	9.35	9.30	10.70	11.9	0.57	0.249	0.004	0.559	-	-	-
Distal Jejunum														
Villus heigh, µm (VH)		341.1	311.1	340.3	284.7	319.3	342.4	13.77	0.093	0.451	0.033	0.158	0.015	0.353
Crypt depth, µm (CD)		214.9	203.8	210.9	211.1	226.4	202.8	10.38	0.187	0.876	0.532	-	-	-
Villi:Crypt, µm:µm (V/C)		1.60	1.62	1.64	1.54	1.56	1.79	0.136	0.679	0.819	0.495	-	-	-
Mitoses, n/100 cells (MI)		4.31^{y}	6.79	2.56^{z}	3.99	5.95^{x}	5.53	0.540	0.015	0.0001	0.037	0.003	0.088	0.581
IEL, n/100 cells		32.5^{x}	29.6	27.8^{y}	29.9	32.8^{x}	29.0	0.62	0.140	0.185	0.066	0.107	0.284	0.041
IEL, n/villus		46.6^{x}	41.4	40.8^{y}	39.4	45.8^{x}	42.0	1.70	0.032	0.090	0.601	-	-	-
LPCD[d], n/1000µm^2		8.60^{y}	10.80	10.45^{x}	10.55	10.50^{x}	9.30	0.866	0.185	0.115	0.009	0.002	0.920	0.258

[a]Values are least square means (n = 8). Histology measurements were done in proximal jejunum, 75 cm from the stomach, and in distal jejunum, 15 cm proximal to the ileum.

[b] FM18 = diet without soybean meal, 18% CP level; SBM18 = diet with soybean meal, 18% CP level; SBM20 = diet with soybean meal, 20 % CP level; XT = plant extract mixture; INT = interaction (XT × DIET).

[c]Interaction was studied when the P-value was significant or when it was lower than 0.15 without any significant principal effect. Orthogonal contrasts were used to study XT effect within each diet. Differences due to diet, when XT is not included, are indicated by different super index in the same row.

[d]LPCD = lamina propia cell density

In distal jejunum, mitoses were different for each diet and were increased by XT when included in diets FM18 (P = 0.003) and SBM18 (P = 0.088). IEL were lower for SBM18 diet, compared to FM18 and SBM20, when XT was not included and decreased in total number with XT inclusion (P = 0.032). LPCD was lower for diet FM18 than for diet diets SBM18 and 20, and was increased by XT when included in this diet (P = 0.002).

4.2.3. pH Measurements and Microbiological Proliferations

pH measurements did not show differences among the treatments. Means obtained were 3.9 ± 0.76 in stomach, 6.6 ± 0.34 in ileum, 5.7 ± 0.34 in caecum and 6.1 ± 0.35 in colon. Table 4.5 shows the total microbiota load in the digestive tract; estimated by the purine bases (PB) concentrations in ileum, caecum, colon and rectum, together with the lactobacilli and enterobacteria counts in distal jejunum.

PB concentration increased from ileum to cecum and colon and decreased to rectum. PB concentration was higher in ileum (P = 0.0001) and cecum (P = 0.007) in pigs fed FM-18 and SBM-20 diets than SBM18. No differences were found in colon and rectum.

The inclusion of XT increased lactobacilli counts (7.6 ± 0.16 vs. 8.2 ± 0.16; P = 0.005) especially in the FM-18 (7.8 vs. 8.4) and SBM-18 (7.1 vs. 8.3) diets. In contrast, enterobacteria counts in the same diets showed a numerical decrease. As a result, the lactobacilli / enterobacteria ratio (Lact/Ent) was higher for XT treated animals compared to non-supplemented ones as well (0.84 ± 0.300 vs. 1.94 ± 0.321, p=0.017).

Table 4.5. Microbiology of the pigs fed the experimental diets.

Diet[b]	FM18		SBM18		SBM20			P-values[b]		
Item XT, mg/kg	0	200	0	200	0	200	SEM	XT	DIET	INT
PB concentration, µmol/g DM										
Ileum	17.5	15.6	8.1	10.5	14.1	13.9	1.07	0.917	0.0001	0.145
Caecum	33.5	32.2	31.4	28.4	37.3	37.2	2.14	0.428	0.007	0.806
Colon	35.1	31.0	27.0	29.9	30.9	27.7	2.24	0.431	0.105	0.251
Rectum	6.3	10.3	9.6	9.8	7.6	8.3	1.30	0.131	0.364	0.312
Microbial counts in distal jejunum, $\log_{10}$ cfu/g										
Enterobacteria	6.5	6.1	6.8	5.8	6.6	6.8	0.39	0.238	0.552	0.422
Lactobacilli	7.8	8.4	7.1	8.3	7.8	8.0	0.25	0.005	0.255	0.180
Lactobacilli:Enterobacteria	1.34	2.19	0.24	2.50	0.94	1.13	0.482	0.017	0.405	0.168

[a]Values are least square means (n = 8).

[b] FM18 = diet without soybean meal, 18% CP level; SBM18 = diet with soybean meal, 18% CP level; SBM20 = diet with soybean meal, 20 % CP level; XT = plant extract mixture; INT = interaction (XT × DIET).

4.2.4. Hindgut Fermentation

Table 4.6 shows total VFA concentrations and profile in caecum. Total VFA concentrations decreased in XT treated animals (208.6 ± 8.62 vs. 184.2 ± 8.01; P = 0.045) especially in diets FM18 and SBM18. These changes were simultaneous with a decrease in acetic acid percentage (P = 0.033) and an increase in butyric (P = 0.050) and valeric percentage (P = 0.027) (p-values of the interaction < 0.15, table 5). Among diets, acetate percentage was higher for SBM20 (P = 0.008) diet and valeric percentage was higher for SBM18 diet (P = 0.009).

Table 4.6. Volatile fatty acids concentration (μmol/g fresh matter) and profile in cecum of pigs fed the experimental diets[a].

Diet[b]	FM18		SBM18		SBM20		SEM	P-values[c]		
Item XT, mg/kg	0	200	0	200	0	200		XT	DIET	INT
Total VFA	233.1	187.1	204.2	181.3	188.4	184.2	13.87	0.045	0.236	0.346
Acetic acid, %	53.1	51.7	53.1	46.6	55.3	54.7	1.54	0.033	0.008	0.147
Propionic acid, %	31.0	30.0	29.5	30.4	28.2	29.8	1.26	0.636	0.502	0.555
Butyric acid, %	13.0	16.2	13.9	16.6	13.6	12.8	1.03	0.050	0.153	0.120
N-valeric acid, %	1.8	2.7	2.6	4.7	2.1	2.0	0.51	0.027	0.009	0.124
Branched VFA, %	0.76	0.69	0.51	0.45	0.74	0.66	0.116	0.490	0.094	0.996

[a]Values are least square means (n = 8).

[b] FM18 = diet without soybean meal, 18% CP level; SBM18 = diet with soybean meal, 18% CP level; SBM20 = diet with soybean meal, 20 % CP level; XT = plant extract mixture; INT = interaction (XT × DIET).

4.3. DISCUSSION

4.3.1. Productive Performance and Digestibility

The three diets used in this experiment were formulated to promote a range of dietary insult for piglets after weaning. The FM18 diet was initially considered the lower risk diet. The isoproteic replacement of fish meal in SBM18 diet, or the supplementation in SBM20 diet with extruded soybean were considered risk factors, by including soy protein in the diet, and further increasing the protein level, respectively. Unexpectedly, FM18 presented a worse the G:F than the other two diets. Makkink et al. (1994) showed that, during the first 3 days after weaning, fish meal protein promotes better growth

rates than soybean meal or soy protein concentrates. However, after ten days, the G:F for fish meal and soybean meal were equaland both were lower than the G:F for soy prtein concentrated Despite the lower G:F ratio in FM18 fed animals, whole tract OM digestibility was not different among diets, and ileal OM digestibility was even higher for fish meal. This data implicate that ileal digestibility differences are compensated in the large intestine. However, the use of the nutrients in the hindgut is less effective because they are partially degraded by microbiota. This incongruence could be indicating the existence of some other parameter determining performance other than digestibility of the ingredients.

4.3.2. Morphology of the Small Intestine

Despite the changes due to the treatments in the current study, VH and CD measurements were in a range comparable to data obtained by Cera et al. (1988), Zijlstra et al. (1996), and Pluske et al. (1996). Diets FM18 and SBM20 diets promoted a lower VH:CD ratio but diet SBM20 promoted lower absolute VH. This lower VH in SBM20 diet was related to an increase in MI probably to maintain the VH in a physiologic range. SBM20 promoted also a higher LPCD as well. Higher MI are related to higher cell renewal rates in the epithelium and LPCD has been used as an indicator of the immune function activation (Jiang et al. 2000). On the other hand, increases in dietary protein have been related to possible bacterial overgrowth. Thus, in the current investigations, changes in epithelium could be related to differences in the diet composition or to bacterial growth due to higher available dietary protein. These possible increases in bacterial growth could induce a higher aggression for the epithelium.

XT inclusion reduced VH and CD when included in 18% CP level diets in proximal jejunum and VH showed a positive correlation to CD ($r = 0.60$) but not to mitoses in crypt. The differences promoted by XT were lower in distal jejunum where crypt was not affected by treatment and no correlation was found between VH and CD. However, in distal jejunum, MI was increased for XT treated animals fed the 18% CP level diets. The number of mitoses in distal jejunum was not correlated either to VH but when we compared both parts, proximal and distal, we found that the MI in distal jejunum was correlated to VH in proximal jejunum ($r = - 0.60$). In coincidence with these results, ileal OM and starch digestibilities were reduced in animals fed with 18% CP level diets including XT. Lower digestibility values can be related to the reduced VH through a

lower nutrient absorption and/or consumption by the epithelium. In this sense, increases on the MI in distal jejunum can be a response to the presence in the lumen of these non absorbed nutrients or to changes in microbiota produced by these nutrients. Specific molecular regulatory mechanisms of the bacteria on the epithelium renewal are out of reach for the authors but the high influence of different bacteria on epithelium activity is well know (Bry et al., 1996).

Following this pattern in proximal jejunum, XT also promoted lower IEL number without variations in LPCD. This IEL are the first immune defense line in intestinal villi (Stokes et al., 2002). From investigations with germ free animals, it is known that most of the reported changes in the intestinal immune response to diet or microbiota are produced in immune cells present in lamina propia (King et al., 2003). However, changes in microflora can produce variations in the presence of IEL in the villi (McCracken and Lorenz, 2001).

How XT is able to produce these changes in VH and IEL in a direct way is difficult to explain and the effects produced and their interactions with protein level point out to some effect in digestive physiology or in microbial populations that induce epithelial changes.

4.3.3. Microbial Proliferations

Despite the lack of diarrhea episodes, microbial counts showed remarkable changes. It is generally accepted that the largest microbial population of the pig is localized in the large intestine; however, it has been established that the microbial population of the small intestine is the most important factor in determining diarrhea (Buddle and Bolton, 1992) and can affect animal immune function (Anderson et al., 1999). Lactobacilli represent the largest group of microorganisms in the small intestine and are important to maintain good intestinal health because of their ability to control potentially pathogenic groups, such as *E. coli* (Blomberg et al., 1993; Canibe and Jensen, 2003) and to other positive effects, as reviewed by Perdigon et al. (2001). In this sense, the ratio of lactobacilli and enterobacteria (Lact:Ent) has been used as an index of intestinal equilibrium (Hillman et al., 1995; Reid and Hillman 1999).

XT inclusion increased lactobacilli counts especially in the animals fed the diets presenting lower CP level (18%) resulting in a increase of the Lact:Ent ratio. Decreases in E. coli excretion were obtained with the same plant extract mixture used in chicken (Jamroz et al., 2003). Increases of lactobacilli on the gut microbiota have been recently

reported using spray dried animal plasma (Torrallardona et al., 2003) and different antibiotics (Collier et al., 2003), and could be related with lower diarrhea incidence.
How lactobacilli are increased in jejunum with XT inclusion is not clear. Changes observed in epithelium and lower ileum digestibility could produce the observed increase in lactobacilli by the means of higher availability of some prebiotic compounds that promote lactobacilli growth.
On the other hand, a direct antimicrobial effect of XT against determined microbial groups could allow the proliferation of lactobacilli as was observed by Collier et al. (2003) using antibiotics. The dose of carvacrol and cinnamaldehyde used in this experiment was approximately 10-fold under the antimicrobial dose determined by Dorman and Deans (2000). No previous data have been reported regarding the antimicrobial dose of these PE in vivo. However, this dose could be effective or inactive depending on the media, especially pH and/or presence of fats and proteins, as demonstrated in vitro by Juven et al. (1994).

Concerning the interaction of XT and protein effects, the influence of nutrient availability, especially protein, was reviewed by François (1962) concerning antimicrobial activity. It is hypothesized that nutrient limitation induces a harder competence between the different groups of microorganisms and the gastrointestinal tract and changes in microbiota are more marked. In fact, this mechanism can be extent to other additives influencing intestinal microbiota and can explain why the effect of XT was more related to the protein level than to protein source.
Variations in ileal digestibility can be also a consequence of microbial quantitative and qualitative changes in intestine. Important qualitative changes on the intestinal microbiota have been reported in this study. Quantification of the total microbial mass was also carried out by PB quantification in intestinal content. PB concentrations were not associated with the lower small intestine digestibilitiy obtained with the XT treatments, but were higher for diets FM18 and SBM20, which contain higher quantities of fish meal. The use of purine bases content in the digestive tract of the single-stomached animals as a marker of microbial growth is based on the assumption that only a negligible amount of dietary nucleic acids reaches the distal segments of the small intestine. McAllan (1980) determined in steers a small intestine digestibility of dietary RNA and DNA of 91-97%, which suggests that a certain amount of dietary purine bases can reach ileum or cecum. Among the ingredients used in this study, FM

shows a remarkably high content of purine bases (31.2 μmol/g DM, Perez et al., 1996). Thus, we can disregard that undigested dietary PB could contribute to the highest PB content observed in the ileum and cecum of FM18 and SBM20.

4.3.4. Hindgut Fermentation

Volatile fatty acids are the major end products of bacterial metabolism in the large intestine of swine (Bergman, 1990). In the present experiment, we used VFA concentration and profile as an index of the changes in the microbial population and of the quantity and source of products fermented in the hindgut. Present results indicate changes in the contents of the cecum from VFA concentration and profile. The most important factor affecting VFA production is the quantity and source of substrate arriving in the hindgut (Bergman, 1990). From *in vitro* studies, it has been demonstrated that fermentation of the different polysaccharides produce distinct patterns of VFA production (Macfarlane and Macfarlane, 2003). Given that ileal digestibility was decreased by XT in 18% CP level diets, it was hypothesized that more fermentable substrate would reach the cecum, and VFA production would be promoted in these animals. Results were contrary to the expected; VFA concentration in cecum decreased with XT supplementation, especially in diets containing 18% CP level. These results could indicate the persistence of the microbial influences of PE, or a carryover effect from proximal segments in the cecum microbial fermentation. This effect from proximal segments could be produced by a higher number of lactobacilli arriving to the distal segments, which produces lactic acid, not measured in the present study, instead of VFA.

However, concerning VFA profile, XT included in 18% CP level diets promoted a decrease in acetic acid percentage, and an increase in butyric acid percentage, a direct product of starch fermentation in the hindgut (Martinez-Puig et al., 2003), in accordance with the lower ileum starch digestibility of these diets.

CHAPTER 5.

EFFECT OF PLANT EXTRACTS AND FORMIC ACID ON THE INTESTINAL EQUILIBRIUM OF EARLY-WEANED PIGS[1]

"Esto con un par de remaches…"

Jose Francisco Pèrez

5.0. CONTEXT

From trials done in other research centers, Pancosma S.A. observed some synergic effect of XT combined with acidifiers. Then they suggested combining XT with a commercial acidifier to increase the effect and to differentiate their product from others. We decided to use one of the most effective commercial acidifier, formic acid. We used a dose lower than normally used for growth promotion (between 1 and 1.5 kg/Tn) because we hypothesized that combining both additives no higher dose will be required. To maximize the responses, we decided to apply an experimental management of the animals for inducing diarrhea based in social and nutritional stressing factors.

5.1. MATERIAL AND METHODS

The experiment was performed at a commercial second phase farm of Baucells S.A. and received prior approval from the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona. The treatment, housing, husbandry and slaughtering conditions conformed to current European Union Guidelines.

5.1.1. Animals, Housing, Management and Dietary Treatments

Pre-experimental Period. The pre-experimental period lasted 12 days. Two hundred and forty weaning pigs ((Landrace × Large white) × Pietrain) excluded from receiving creep feed, 6.0 ± 0.40 kg live weight (BW) and 20 ± 1 days old were allocated in 24 pens (10 animals/pen) in a environmentally controlled room. Pens were organized in 4 lines of 6 pens and each line was considered a replicate of pens for sacrifices. During the pre-experimental period pigs were fed *ad libitum* with a standard medicated (400 mg/kg of colistin sulphate 10% and Oxytetracycline 20%) pre-starter diet based on cereals, 50%; milk by-products, 20%; Soybean meal (SBM) 44, 5%; and soy concentrate, 5% (analyzed CP in DM basis = 19.0% and calculated lysine in DM basis = 14.4%).

Experimental Period. Twelve days after weaning, a stress management system (adapted from Kyriakis, 1989), based on social and dietary stress factors, was applied to the animals. In particular, the lightest animals (n = 24) were removed from the experiment and the remaining animals (216 animals; 8.1 ± 0.20 kg BW) were mixed in the same room (social stress) for 2 hours. Then animals were reorganized at random in the same 24 pens, maintaining an equal mean weight in each pen (9 animals each). A starter non-

medicated diet containing 21,5% of SBM (19% CP level, 1.29 % Lys level, Table 1) was fed (dietary stress).

Table 5.1. Composition of the diets on an as-fed basis.

Ingredient	g/kg
Corn	153.5
Barley	200.0
Wheat	250.0
Soybean meal, 44% CP	215.0
Soy-bean oil	30.0
Full-fat extruded soybeans	100.0
L-Lysine	3.0
DL-Methionine	1.5
L-Threonine	1.5
L-Tryptophan	0.1
Choline chloride 50%	0.3
Sepiolite (a clay)	13.6
Dicalcium phosphate	14.0
Calcium carbonate	6.0
Sodium chloride	5.0
Chromic oxide	1.5
Vitamin and mineral premix[a]	5.0

[a]Provided the following per kilogram of diet: vitamin A, 13000 IU; vitamin D_3, 1800 IU; vitamin E, 60 mg; vitamin K_3, 3 mg; thiamine, 2 mg; riboflavin, 6 mg; vitamin B_6, 3 mg; vitamin B_{12}, 25 mg; nicotinic acid, 25 mg; calcium pantothenate, 15 mg; folic acid, 1 mg; biotin, 130 mg; Fe, 100 mg as iron carbonate; Cu, 175 mg as copper sulphate $5H_2O$; Zn, 110 mg as zinc oxide; Mn, 55 mg as manganese sulphate; I, 1mg as potassium iodate; selenium, 0.10 mg as sodium selenite.

Table 5.2. Analyzed nutrient content of the diets on a DM basis[a].

Nutrients	Amount
Crude Protein, g/kg	190.2
Crude Fiber, g/kg	40.6
Fat, g/kg	65.5
Ash, g/kg	78.9
Gross Energy, Mcal/kg	4.19
Lysine, calculated value, g/kg	14.44

[a] Analyzed Dry Matter of diet = 89.3%.

The starter diet was supplemented with 6 different treatments following a 2 x 3 arrangement, resulting from the combination of three levels (0, 150 and 300 mg/kg) of a

commercial plant extract mixture (XT) standardized in 5% (wt/wt) carvacrol, 3% cinnamaldehyde and 2% capsicum oleoresin (*Capsicum annum*) with two levels of formic acid (FA) (0 and 5 g/kg). These treatments were distributed at random in each replicate of pens. Chromic oxide was included as an indigestible marker.

5.1.2. Feeding Regimen, Controls and Sampling

For 21 days, animals were allowed *ad libitum* access to feed and performance was monitored weekly. During the first week, the presence or absence of liquid diarrhea was monitored daily in each pen through visual observation of the slat and perianal zone of the piglets. On days 22 to 25 the same controlled feed intake pattern described in previous chapter was applied from 8:00 am to 8:00 pm in order to standardize the digestive tract conditions upon sacrifice. On days 24 and 25, after the 12:00, 13:30, 15:00 and 16:30 ingestion period, one pig per treatment was weighed and sacrificed with intravenous injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain) (200mg/kg BW) as described in previous chapter.

The pH was measured in four segments as described in previous chapter.

The stomach and hindgut were separated and weighed full and empty, and a sample of the homogenized gastric content was taken to determine **DM content**. Both digestive **organ weight** and DM measurements were done only on day 25, so that n=4 were considered for these variables.

Samples for **histological study** of the proximal jejunum, **enterobacteria and lactobacilli counts** in distal jejunum**, VFA analysis** in cecum and proximal colon and purine basis in ileum and rectum, were taken as described in previous chapter.

5.1.3. Analytical Procedures

Chemical analysis of the diet, **histological study**, **enterobacteria** and **lactobacilli agar plate counts, VFA and PB concentration** were obtained by the procedures described in the previous chapter.

5.1.4. Calculations and Statistical Analysis

Ileal and rectal apparent digestibility of each nutrient fraction (Nf) was calculated by the marker (Cr) ratio method between diet (D) and digesta (d) and using the equation:

$$\text{Digestibility coefficient} = [1-(Cr\ D / Cr\ d) \times (\%\ Nf\ d / \%\ Nf\ D)]$$

Persistence of diarrhea and registered casualties where analyzed by chi-square test of FREQ procedure of SAS (Version 8.1; SAS Institute, Cary, NC) for XT and FA factors.

Other results were analyzed by ANOVA with the GLM procedure of SAS using XT and FA included as classification factors. Interaction was included in the model.

In **productive performance analysis**, pen was used as experimental unit and initial live weight was used as a covariable.

In **sacrifice data analysis**, pig was used as experimental unit, and weight of the animals at sacrifice was included as covariable in physical digestive measurements. Day and period of sacrifice were initially included in the model but were not significant for any variables (P = 0.37 to 0.97), so were ultimately excluded from the model. As suggested by Lowry (1992) for these factorial arrangements the interaction was studied when the P-value was significant, or when it was lower than 0.15 without any significant principal effect. In particular, the linear and quadratic trend of the XT factor was studied for equally spaced levels within each level of FA, and the FA effect was studied within XT at the 0 ppm rate. Both were studied by orthogonal contrast. The alpha level used for determination of significance for all analyses and contrasts was 0.05. Additionally, the REG procedure of SAS 8.1 was utilized for regression determinations of pH with various other responses.

5.2. RESULTS

5.2.1. Productive Performance and Digestibility

First, it should be noted that on day 14 after weaning, two days after the starter diet introduction, a diarrhea episode occurred. The presence of liquid feces was observed in all pens. An enterohaemolityc E. coli K88 was identified as present agent and animals were immediately treated through intramuscularly administered amoxicylin (Hipramox, Laboratorios Hipra, S.A., Girona, Spain) during three days. The diarrhea episode persisted over two days and five casualties were registered; all casualties occurred in different pens, 4 casualties belonging to 0 ppm XT group (5.6% mortality) (Table 5.3.) and one animal belonging to 150 ppm Xt (1.4% mortality). No casualties were registered in animals treated with 300 ppm of Xt (0% mortality).

Table 5.3. shows the ADFI, ADG and G:F observed during the 21 days experimental period, and ileal and rectal OM digestibility on days 24 and 25. No differences were obtained among treatments for ADFI (648 ± 6.9 g), ADG (426 ± 6.9 g), and ileum and rectum OM digestibility (61.8 ± 1.23 % and 83.7 ± 0.29 %, respectively) but G:F was better for FA-treated animals (0% FA = 0.65 vs. 0.5% FA = 0.67, P = 0.040).

Table 5.3. Growth performance and digestibility of the pigs fed the experimental diets[a].

Formic acid, %	0			0.5				P-values[b]		
Response XT, mg / kg	0	150	300	0	150	300	SEM	XT	FA	INT
ADFI[cd], g/d	693	645	645	634	613	655	16.4	0.268	0.237	0.295
Average Daily Gain[d], g/d	452	403	423	417	411	447	11.3	0.139	0.967	0.165
Gain:feed[d], g/g	0.65	0.63	0.66	0.66	0.67	0.68	0.010	0.285	0.040	0.434
Persistence of diarrhea[ef]	2	0	1	2	1	2		0.269	0.387	-
Casualties[f]	2	0	0	2	1	0		0.038	0.615	-
OM digestibility , %										
Ileal	60.2	62.6	62.6	62.6	60.5	61.3	2.70	0.984	0.889	0.800
Total tract	82.8	83.7	83.8	84.0	83.4	84.2	0.68	0.703	0.513	0.587

[a]Values are least square means (n = 4 for productive performance and n = 8 for digestibility).

[b]XT = Plant extract mixture; FA = Formic acid; INT = interaction (XT × FA).

[c]ADFI = Average daily feed intake; As-feed basis

[d]Initial weight included in the model as covariable.

[e]Number of pens (from a total of four pens per treatment) presenting liquid feces two days after the beginning of the diarrhea episode (two days after diet change). No liquid diarrhea was detected the following days of experiment.

[f]Treatments compared for principal factors (XT and FA) by chi-squre test.

5.2.2. Digestive pool and pH measurements

No differences were observed between treatments on the digestive tract weight or content weight except on stomach. XT increased linearly stomach content (P = 0.006) when 0% of FA was included (Table 5.4.). FA also increased stomach content (P = 0.003) and DM of this content (P = 0.010) when no XT was included (Table 5.4.).

pH measurements showed differences between treatments in stomach and colon (Table 5.4.) but not in ileum and cecum. XT increased linearly stomach pH (P = 0.005) when no FA was included, a similar response as was observed for the weight of stomach content. Between this pH and the stomach content, a linear correlation was determined (r = 0.83). FA tended to increase stomach pH (P = 0.060) when no XT was included (Table 5.4.).

Table 5.4. Body weight of the sacrificed animals and variables measured in the stomach and hindgut.[a]

Formic acid, %	0			0.5				P-values[b]					
Response XT, mg / kg	0	150	300	0	150	300	SEM	XT	FA	INT[d]	XT at 0 % FA[e]	XT at 0.5 % FA[e]	FA at 0% XT[f]
BW sacrifice, kg	19.9	20.2	19.9	19.7	19.4	20.4	0.52	0.702	0.682	0.476	-	-	-
Stomach													
Empty wt[c], g	118	132	125	136	122	135	3.7	0.839	0.214	0.084	0.390	0.881	0.050
Content wt[c], g	137	275	333	308	213	257	22.2	0.113	0.698	0.003	0.006	0.280	0.003
DM content[c], %	29	33	36	37	36	34	0.9	0.310	0.019	0.014	0.089	0.226	0.010
PH	2.4	3.4	3.6	3.2	3.0	3.4	0.27	0.051	0.900	0.091	0.005	0.586	0.060
Ileum													
PH	6.4	6.6	6.5	6.4	6.3	6.5	0.09	0.484	0.098	0.234	-	-	-
Hindgut													
Empty wt[c], g	348	395	399	388	344	375	12.9	0.537	0.446	0.064	0.139	0.580	0.141
Content wt[c], g	633	628	605	612	617	668	61.3	0.984	0.884	0.874	-	-	-
PH (cecum)	5.5	5.5	5.5	5.6	5.6	5.6	0.09	0.963	0.264	0.978	-	-	-
PH (colon)	6.0	5.9	5.6	5.6	5.6	6.0	0.09	0.653	0.153	0.079	0.121	0.191	0.071

[a]Values are least square means (n = 4 for weights and DM; n = 8 for pH).

[b]XT = Plant extract mixture; FA = Formic acid; INT = interaction (XT × FA).

[c]BW of the animal at sacrifice included in the model as a covariable.

[d] Interaction was studied when the P was significant or when it was lower than 0.15 without any significant principal effect. Orthogonal contrasts were used to study XT for equally spaced levels within each level of FA, and the FA effect within XT at the 0 ppm rate.

[e]The values provided are for the linear contrast; no quadratic responses at $P < 0.10$ were noted.

[f]Specific contrast for FA effect (non-treated diet vs. 0.5% FA - 0ppm XT diet).

Table 5.5. Intestinal histology and microbiology of pigs fed the experimental diets.[a]

Formic acid, %	0			0.5				P-values[b]		
Response XT, mg / kg	0	150	300	0	150	300	SEM	XT	FA	INT
Histology[c]										
Villi height, μm	407	369	386	330	352	365	19.4	0.840	0.073	0.418
Crypt depth, μm	290	391	298	269	296	276	12.2	0.714	0.357	0.654
PB concentration, μmols/g DM										
Ileum	10.7	10.9	6.6	11.0	9.8	7.1	1.37	0.025	0.945	0.862
Rectum	10.8	10.4	11.3	7.1	10.8	8.9	1.22	0.440	0.078	0.298
Microbial Counts[c], $\log_{10}$ cfu/g										
Lactobacilli[d]	7.3	7.9	8.7	7.9	7.6	7.9	0.30	0.090	0.615	0.145
Enterobacteria	5.9	6.2	5.6	5.8	5.9	5.3	0.24	0.088	0.233	0.908
Lact:Ent	0.93	1.61	3.44	2.02	1.83	2.72	0.34	0.002	0.563	0.130

[a]Values are least square means (n = 8).

[b]XT = Plant extract mixture; FA = Formic acid; INT = interaction (XT × FA).

[c]Histology measurements were done in proximal jejunum, 75cm from the stomach, and microbial counts were determined in distal jejunum, 20 cm proximal to the ileum.

[d]Interaction was studied when the P was significant or when it was lower than 0.15 without any significant principal effect. Orthogonal contrasts were used to study XT for equally spaced levels within each level of FA, and the FA effect within XT at the 0 ppm rate. Lactobacilli counts showed linear trend for XT when included in 0% FA diets (P = 0.019).

5.2.3. Morphology of the epithelium and microbial proliferation

As far as the morphology of the epithelium is concern, means for VH and CD in the jejunum were 366 ± 10.3 and 286 ± 6.3 (μm) respectively. Proximal jejunum VH tended to be shorter in the FA groups (Table 5.5., P = 0.073) while CD was unaffected.

Microbial mass, estimated by using PB concentration in ileum and rectum content, and lactobacilli and enterobacteria counts in jejunum are presented in Table 5.5. PB concentration in ileum (9.4 ± 0.64 μmol/g DM) was not different than in rectum (9.9 ± 0.54 μmol/g DM). Among dietary treatments, lower PB concentration was observed in the ileal contents when XT was added (P = 0.025), and FA tended to diminish PB concentration in rectum (P = 0.078).

Moreover XT increased linearly lactobacilli (P = 0.019) when no FA was added (Table 5.5.). As a consequence the lactobacilli/enterobacteria ratio showed an increase (P = 0.002) due to the inclusion of XT.

5.2.4. Hindgut Fermentation

Table 5.6. shows total VFA concentration and individual profile in cecum and colon contents. The total concentration of VFA in colon was lower than measured in cecum (148 ± 4.4 vs 170 ± 4.4 μmol/g fresh matter; P = 0.001). Total colon VFA were diminished linearly by XT inclusion (P = 0.018) when FA was added.

No differences were observed between cecum and colon for the profile of acetic (55.3 ± 0.62 %), butyric (13.7 ± 0.33 %), and valeric acids (2.3 ± 0.13 %). On the other hand, branched VFA percentage increased from cecum to colon (0.66 ± 0.121 vs. 2.28 ± 0.124 %; P = 0.001) and propionic decreased (28.2 ± 0.49 vs 25.9 ± 0.50 %; P = 0.011).

Comparing treatments , XT inclusion increased acetate percentage in cecum (P = 0.018) and in colon (P = 0.025), simultaneous to a decrease on butyrate (P = 0.096 in cecum, P = 0.040 in colon) and valerate percentage (P = 0.001 in cecum, P = 0.039 in colon). Acidification did not affect the VFA proportions.

Table 5.6. VFA concentration (μmol/g fresh matter) and profile in cecum and colon of pigs fed the experimental diets. [a]

Formic acid, %		0			0.5				P-values[b]		
Response	XT, mg / kg	0	150	300	0	150	300	SEM	XT	FA	INT
Total VFA											
Cecum		162.2	168.7	167.9	182.9	171.7	167.4	10.57	0.901	0.386	0.575
Colon[c]		153.0	136.1	160.5	157.9	160.7	118.6	10.24	0.138	0.196	0.034
% Acetic acid											
Cecum		53.2	57.8	55.3	53.0	55.4	59.9	1.57	0.018	0.608	0.089
Colon		52.9	56.2	56.0	53.0	53.6	56.6	1.43	0.025	0.647	0.853
% Propionic acid											
Cecum[c]		28.6	26.5	29.2	29.8	29.4	26.1	1.25	0.411	0.730	0.058
Colon		25.7	25.3	24.7	26.7	29.0	24.2	1.17	0.142	0.185	0.501
% Butyric acid											
Cecum		14.6	12.2	13.1	13.9	12.7	12.1	0.87	0.096	0.594	0.715
Colon		15.8	13.6	13.6	14.8	13.7	13.9	0.71	0.040	0.379	0.355
% N-valeric acid											
Cecum		2.9	1.8	1.9	2.7	1.9	1.1	0.33	0.001	0.259	0.396
Colon		3.1	2.7	2.5	2.8	2.5	2.0	0.32	0.039	0.539	0.797
% Branched VFA											
Cecum		0.77	0.61	0.56	0.58	0.67	0.76	0.105	0.954	0.783	0.184
Colon		2.5	2.2	2.3	2.6	1.2	2.8	0.40	0.975	0.617	0.676

[a]Values are least square means (n = 8).

[b]XT = Plant extract mixture; FA = Formic acid; INT = interaction (XT × FA).

[c] Interaction was studied when the P was significant or when it was lower than 0.15 without any significant principal effect. Orthogonal contrasts were used to study XT for equally spaced levels within each level of FA, and the FA effect within XT at the 0 ppm rate. Colon total VFA showed linear trend for XT when included in 0.5% FA diets (P = 0.018).

5.3. DISCUSSION

Post-weaning anorexia has been described as the leading detrimental factor for the piglet health. However other factors which also occur at weaning, such as the hypersensitivity induced by the presence of soybean meal in diets (Li et al., 1990, 1991) and the re-grouping of animals (Blecha et al., 1985) may increase the incidence of digestive disturbances. In the present study a stress episode was incorporated after weaning, so it was independent of the post-weaning anorexia, in order to evaluate the effect of the studied additives under a programmed adverse situation.

In fact, stress management and/or non-medicated diet SBM level on day 12 probably induced the diarrhea episode occurred on day 14. Pens from treatments without XT registered 4 of the 5 casualties independent of the formic FA inclusion. With the mixing method used (Kyriakis, 1989) we assume an equal distribution of diarrhea between pens and observations of diarrhea agree with this. However an assurance of the homogeneity of the exposure to the pathogenic agent can not be provided because no experimental infection was done. In any case, the results here obtained can be encouraging for future studies. In this experiment the benefits obtained from the inclusion of both PE and FA were additive, lower casualties appeared in coincidence with better feed conversion. On the other hand, changes on digestive tract variables where non-additive as it is shown below.

5.3.1. Small intestine effects

Treated animals showed a higher total content and a higher %DM of this content in the stomach. Higher stomach content in animals in which we assumed a similar feed intake may reflects a lower emptying rate and consequently a more homogeneous digesta flow to the duodenum. A reduction in emptying rate due to acidifiers has been described earlier (Hunt and Knox, 1972) associated to the effects of the low pH of the lumen on the duodenum receptors (Partanen and Mroz, 1999). In this sense, the retention time in the stomach as affected by acidifiers has been suggested as a possible mechanism to improve the protein digestion in the stomach and to increase the barrier effect of the stomach against pathogenic bacteria (Partanen and Mroz, 1999). However, in the present experiment, pH was higher with the inclusion of the FA and showed a positive correlation with the stomach content ($r = 0.83$). This fact suggests that registered pH in the stomach was mainly affected by the buffering capacity of solid meal and water and probably was indirectly affected by the gastric emptying rate. In fact, when including stomach contents as a covariable in the statistical model the effect of the additives on pH was not significant. Concerning XT, influences on the gastric emptying have previously been described as in the case of capsaicin present in the capsicum (Debreceni et al., 1999; Kang et al., 1999). In fact increased mean retention time has been described using capsaicin in doses similar to used in our experiment (Chang et al., 1999). This slower emptying is due to direct effect of capsaicin on gastric motility (Gonzalez et al., 1998). In this experiment, the addition of the XT as well as the FA promoted an increase of this retention time. However no additive effect was found. An explanation

for this lack of additivity can be found in another effect of the capsaicin. The same capsaicin receptors that reduce gastric motility are present in duodenal receptors responsible of acid brake and when capsaicin is administered acid brake is abolished (Raybould and Hölzer, 1993). Due to the important influence of the stomach on the pre-digestion of the diet and as a barrier for pathogens, increasing gastric retention time without affecting ingestion could produce important beneficial effects on digestive ecosystem and more efforts should be addressed towards the likely influence and mechanisms of the PE and acidifiers on gastric emptying.

Both VH and CD are important indicators of the digestive health of the pig and directly related to the absorptive capacity of the mucous membrane (Buddle and Bolton, 1992). From a theoretical point of view, VH reflects a balance between the mitotic activity of the crypt enteric cells (Cera et al., 1988) and the desquamation produced principally by external aggressors (Nabuurs, 1995). In the present study VH and CD measurements were in a range comparable with data obtained by Cera et al. (1988), Zijlstra et al. (1996) and Pluske et al. (1996). However FA led to shorter VH without CD variation. The exact cause of this shorter VH, and if it is related with the better conversion, remains unclear but could be a consequence of an increased desquamation and/or diminished mitotic rate.

Concerning microbial populations, the XT inclusion increased lactobacilli counts in non-acidified diets and tended to decrease enterobacteria counts resulting in a increase of the Lact:Ent ratio. Similar results have been shown in the previous chapter. Despite the non existence of a direct correlation it is interesting to remark how Lact:Ent ratio followed the same pattern showed by the stomach content and pH. These results could indicate some influence of the gastric emptying rate and pH on the microbiota in caudal segments. Canibe and Jensen (2003) indicated that changes in gastric contents that reduce survival of pathogens or proliferation in the stomach also seem to reduce the presence of pathogens along the remaining of the digestive tract. This suggests that the stomach acts as a barrier against colonization of pathogens in the gastrointestinal tract, and may be modulated by feeding/management strategies which are expected to influence gastric function. In any case, a direct modulator effect of FA or XT on the microbiota cannot be ruled out.

The antimicrobial properties of some PE have been reported in numerous *in vitro* studies (Dorman and Deans, 2000) and some selective antimicrobial effect has been also shown depending on the extract used and the dose of inclusion (Zaika, 1988; Smith-Palmer et al., 1998). In the present study PB concentration in ileum digesta was diminished by the XT inclusion. The manner by which microbial mass was reduced is difficult to explain since the principal group of microbes in small intestine, i.e. lactobacilli were increased. This fact could be a consequence of the importance of other bacterial groups in the gut equilibrium not well described until now because of the limitation of the techniques (Anderson et al., 1999). In agreement with this supposition Collier et al. (2003) found lower total microbial mass in coincidence with increased lactobacilli in animals treated with antibiotics, using PCR techniques. The dose of carvacrol and cinnamaldehyde used in this experiment was approximately 10 fold under the antimicrobial one determined in different *in vitro* studies (Dorman and Deans, 2000). No previous data have been found about antimicrobial dose of this PE *in vivo.* However this dose could be effective or inactive depending on the media, specially pH and presence of fats, as demonstrated Juven et al. (1994) *in vitro*. On the other hand, higher doses of these compounds should be studied specially to avoid negative effects on the palatability, accumulation of some compounds in fat deposit, or toxic effects for the animals.

5.3.2. Hindgut effects

VFA are the major end products of bacterial metabolism in swine large intestine (Bergman, 1990). In the present experiment we used VFA concentration and profile as an index of the changes on the microbial population and of the quantity and source of products being fermented in the hindgut. Present results indicate changes in cecum and colon contents from the VFA profile. The most important factor affecting VFA production is the quantity and source of substrate arriving in the hindgut (Bergman, 1990). From *in vitro* studies it has been demonstrated that fermentation of different polysaccharides produce distinct patterns of VFA production (Macfarlane and Macfarlane, 2003). In our experiment no differences in total OM ileal digestibility were found but only from this data we can not rule out possible differences in fermentable substrates arriving to hindgut. Increases in acetic acid are normally indicating higher proportion of easily fermentable carbohydrate arriving to hindgut. The lower microbial mass described in the ileum could produce lower consumption of this substrates

allowing their arrival to hindgut. How it could happen without affecting ileum digestibility is difficult to understand for the authors. On the other hand probiotic development investigations carried out at the last years have shown that for a determined substrate, changes in microbiota can result in different fermentation products due to the different metabolism of the bacteria (Jiang and Savaiano, 1997). In this works most of bacteria studied are lactic acid bacteria and increases in total VFA and in the percentage of acetate have been reported (Sakata et al., 2003). Important increases in the lactobacilli arriving from the small intestine have been described in this experiment, however it is difficult to demonstrate an influence of the small intestine microbiota on the bacterial populations inhabiting the hindgut, and as such the beneficial or detrimental effect produced by these changes in fermentation.

The results of the VFA analysis are different than those presented in the previous chapter where decreases in acetic were reported. The authors attribute these differences to the different age of the animals, which produces differences in the development status of the cecum microbiota (Bergman, 1990).

CHAPTER 6.

EFFECTS OF BUTYRATE, AVILAMICINE, AND PLANT EXTRACTS IN THE INTESTINAL ECOSYSTEM OF THE EARLY WEANING PIG

"*El polo campero, el pollo campero…*"

Ana C. Barroeta

6.0. CONTEXT

After two trials in a commercial farm and other trials in other countries we thought that there was a strong limitation in the methodology used to detect and understand mechanisms of action. We had some results but it was difficult to define the real chain of events producing the found differences. So we decided to enlarge and complete our methodologies. In this experiment we extend the already used methodologies to more parts of the GIT, and we also applied some new methodologies. In this third experiment we compared the XT with an still allowed AGP (avilamycin) and a commercial product (sodium butyrate).

6.1. MATERIAL AND METHODS

The experiment was performed at the Experimental Unit of the Universitat Autònoma de Barcelona and received prior approval from the Animal Protocol Review Committee of the institution. The treatment, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines.

6.1.1. Animals, Housing and Dietary Treatments

Thirty two commercial crossing ((Landrace × Large white) × Pietrain) piglets excluded from receiving creep feed, 6.0 ± 0.10 kg live weight (BW) and 20 ± 2 d old were randomly allocated to 8 pens (4 animals per pen) in an environmentally controlled room.

Figure 6.1. Facilities of the Universitat Autònoma de Barcelona.

The pens were distributed among the four treatments that resulted from the inclusion of three different feed additives, avilamicine (AV), sodium butyrate (BT) and XTRACT^{TM} in a control diet (table 6.1. and 6.2.). PE were included in an inert fatty carrier before including them in the feed and $Cr_2 O_3$ was also included as a digestibility marker.

6.1.2. Feeding regimen, Controls and Sampling

Durin 14 d, animals were allowed ad libitum access to feed and performance was monitored weekly. On d 15 to 19 the controlled feed intake pattern described in chapter 4 was applied to standardize the digestive tract conditions upon slaughter.

On days 19 and 21, after the 12:00, 13:30 and 15:00, and 16:30 ingestion period, one pig per treatment was weighted and killed by i.v. injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain; 200 mg/kg BW).

Table 6.1. Composition of the diets on an as-fed basis.

Ingredient	g/kg
Corn	276.3
Barley	300.0
Soybean meal 44 % CP	40.0
Full-fat extruded soybeans	40.0
Soy-protein concentrate	60.0
Fish meal LT	50.0
Dried whey	40.0
Acid whey	150.0
Wheat gluten	6.8
L-Lysine	4.4
DL-Methionine	2.7
L-Threonine	1.9
L-triptofan	0.4
Colina 50	2.0
Sepiolite (a clay)	10.0
Dicalcium phosphate	11.0
Chromic oxide	1.5
Vitamin and mineral premix[a]	3.0

[a]Provided the following per kilogram of diet: vitamin A, 13500 IU; vitamin D_3, 2000 IU; vitamin E, 80 mg; vitamin K_3, 4 mg; thiamin, 3 mg; riboflavin, 8 mg; vitamin B_6, 5 mg; vitamin B_{12}, 40 mg; nicotinic acid, 40 mg; calcium pantothenate, 15 mg; folic acid, 1.3 mg; biotin, 150 mg; Fe, 120 mg as iron carbonate; Cu, 175 mg as copper sulfate $5H_2O$; Zn, 110 mg as zinc oxide; Mn, 65 mg as manganese sulphate; I, 1mg as potassium iodate; selenium, 0.10 mg as sodium selenite.

Table 6.2. Calculated nutrient content of the diets on an DM basis.

Nutrients, g/kg	Amount
Crude Protein, g/kg	205.9
Crude Fiber, g/kg	31.3
Fat, g/kg	57.2
Ash, g/kg	75.3
Gross Energy, Mcal/kg	4.79
Lysine, calculated value, g/kg	15.2

[a] Analyzed Dry Matter of diet = 89.3%.

Two pigs of each pen were killed each day. Animals were bled; the abdomen opened immediately from sternum to pubis, and the whole gastrointestinal tract was removed and weighted, and sampling.

The pH in five segments was measured as described in chapter 4. The pH measurements were performed in the middle of the caudal portion of the stomach, 15 cm proximal to the ileocecal valve, in the lowest part of the caecum and in the colon, 20 cm distal to the caecum and 50 cm to the anus.

Samples for **histological study** were obtained as described in chapter 4 from the distal jejunum, ileum and colon wall, 15 cm proximal to the ileum, 20 cm proximal to the ileocecal valve and 20 cm to the cecum.

Samples for **enterobacteria, lactobacilli and total bacteria counts** from distal jejunum.

Samples for **VFA analysis** were taken, as described in chapter 4, from homogenized stomach, proximal jejunum, ileum, cecum, proximal and distal colon and rectum contents.

6.1.3. Analytical Procedures

Chemical analysis of the diet, **total starch** of feed and digestive content, **histological study, bacterial agar plate counts** and **VFA concentration** were determined as described in chapter 4.

Direct quantification of total bacteria in jejunum samples was carried out by epifluorescent direct count method (Hobbie et al. 1977) using 4',6-diamidino-2-phenylindole (DAPI) staining. One gram of sample was diluted ten times with sterile saline solution and 0.5 ml of the suspension was fixed with 4.5 ml of 2 % formaldehyde. Samples were stained with DAPI at a final concentration of 1 μg/ml, and filtered

throughout polycarbonate membrane filters (0.22 μm, Whatman Ref. 110656). Bacteria were enumerated using an ocular graticule (Olympus NCWHK 10x) counting 10 random fields per filter.

6.1.4. Calculations and Statistical Analysis

Ileal and rectal apparent digestibility of each nutrient fraction (Nf) was calculated by the marker (Cr) ratio method between diet (D) and digesta (d) and using the equation:

$$\text{Digestibility coefficient} = [1-(Cr\ D / Cr\ d) \times (\%\ Nf\ d / \%\ Nf\ D)]$$

All results were analyzed by ANOVA with the GLM procedure of SAS, using treatment included as classification factors.

In **productive performance analysis**, pig (n = 8) was used as experimental unit for ADG, and pen (n = 2) for ADFI and G:F. Initial live weigh was used as covariate for productive performance results.

In **slaughter data analysis**, pig was used as experimental unit. Day and period of sacrifice were initially included in the model but were not significant for any variables (P = 0.43 to 0.94); thus, they were ultimately excluded from the model. When analysis of pooled data from different GIT parts is presented, it means that the GIT part was included in the analysis as another class factor. The alpha level used for determination of significance for all analyses and contrast was 0.05.

6.2. RESULTS

6.2.1. Average daily gain and digestibility measurements

Table 6.3. shows the production performance results observed during the 14 days experimental period together with, ileal and whole tract digestibilities on days 19 and 21. No differences were noted among treatments for ADG or ADFI, but ADG trend to be higher for the animals fed the three experimental treatments (P = 0.052) and ADFI was numerically higher for treated animals (P = 0.147). As a consequence of this variations animals fed AV and BT presented better G:F ratio (P = 0.001). BT decreased starch digestibility in both ileum (P = 0.0015) and rectum (P = 0.0018) and also decreased whole tract OM digestibility (P = 0.0004).

Table 6.3. Growth performance and digestibility of the pigs fed the experimental diets[a].

Treatment[b] / Response	CT	AV	BT	XT	SEM	P – values
BW day 0, kg/animal	6.0	6.0	6.1	6.1	0.10	0.643
0-7 days						
ADG[d], g/animal	40.4	67.8	93.3	73.5	15.33	0.158
ADFI[cd], g/animal	138.8	182.8	171.1	192.4	15.97	0.239
G:F, g/g	0.30	0.37	0.53	0.36	0.071	0.266
7-14 days						
ADG[d], g/animal	208.0	287.1	261.9	258.3	23.63	0.156
ADFI[cd], g/animal	338.4	371.3	339.9	409.6	25.03	0.288
G:F, g/g	0.62^{y}	0.77^{x}	0.77^{x}	0.63^{y}	0.023	0.013
0-14 days						
ADG[d], g/animal	124.7	177.4	177.6	165.9	14.39	0.052
ADFI[cd], g/animal	238.6	277.0	255.5	300.9	15.17	0.147
G:F, g/g	0.53^{z}	0.64^{y}	0.69^{x}	0.55^{z}	0.010	0.0009
Digestibility, %						
Ileal OM	61.8	51.8	60.0	60.0	3.379	0.225
Total tract OM	82.7^{x}	81.3^{x}	73.9^{y}	81.2^{x}	1.28	0.0004
Ileal starch	95.2^{x}	91.9^{xy}	88.1^{y}	94.6^{x}	0.99	0.0015
Total tract starch	96.2^{x}	95.6^{x}	87.3^{y}	95.0^{x}	1.26	0.0018

[a]Values are least square means (n = 8 for ADG and n = 2 for ADFI and G:F). ADFI = Average daily feed intake; ADG = Average daily gain; G:F = Gain to Feed ratio.

[b]CT = control diet; AV = avilamicyne, 400 ppm; BT = sodium butyrate, 0.3%; XT = XTRACT™, 300ppm.

[c]As-feed basis

[d]Initial weight included in the model as covariable

[x,y,z]Means within rows without a common superscript differ (P < 0.05).

6.2.2. pH and dry matter contents

Table 6.4. shows the pH and DM contents of the different parts of the GIT. No differences were found concerning pH or DM content (%) in intestinal separated locations. However, when data was pooled for the hindgut, DM and pH were higher along the hindgut for avilamycin treated animals (P = 0.037 and 0.0001).

6.2.3. Microbiological Proliferations

Table 6.5. shows the microbial populations. No differences were observed between treatments on the lactobacilli, enterobacteria or total bacteria counts.

Table 6.4. Variables measured in the stomach and hindgut.

Treatment[b] / Response	CT	AV	BT	XT	SEM	P – values
PH						
Stomach	3.6	3.4	3.8	3.1	0.42	0.711
Ileum	6.3	6.5	6.4	6.6	0.11	0.179
Cecum	5.5	5.6	5.5	5.3	0.08	0.105
Proximal colon	5.6	5.7	5.6	5.4	0.08	0.304
Distal colon	5,8	6,0	5,7	5,6	0.09	0,166
Hindgut overall[c]	5.6^{y}	5.8^{x}	5.6^{y}	5.4z	0.09	0.0001
DM, %						
Ileum content	13.3	14.2	11.5	11.1	1.44	0.412
Cecum content	12.8	15.5	10.6	15.3	0.65	0.0001
Prox col. Content	16.1	18.2	14.6	15.3	1.53	0.433
Dist col. Content	22.1	25.2	18.0	19.5	1.96	0.099
Rectum content	18.9	27.6	24.8	18.6	1.74	0.654
Whole Hindgut[c]	18.6^{xy}	21.3^{x}	17.7^{y}	18.7^{xy}	1.75	0.037

[a]Values are least square means (n = 8).

[b]CT = control diet; AV = avilamicyne, 400 ppm; BT = sodium butyrate, 0.3%; XT = XTRACT™, 300ppm.

[c] Analysis of the overall means along the cecum, colon and rectum. Treatment, location were used as classification effects and interaction was included in the model. Location P-value was 0.0001. Interaction was non significant (P = 0.832 for pH and P = 0.943 for DM content).

[x,y,z]Means within rows without a common superscript differ (P < 0.05).

Table 6.5. Microbiological counts (enterobacteria and lactobacilli) (log_{10} cfu/g fresh matter) of pigs fed the experimental diets.

Treatment[b] / Response	CT	AV	BT	XT	SEM	P – values
Lactobacilli	7.8	8.1	7.6	8.2	0.28	0.589
Enterobacteria	3.6	5.00	5.00	5.7	0.59	0.185
Relation	4.12	3.10	2.64	2.50	0.71	0.444
Total	7.88	7.80	7.59	7.86	0.130	0.448

[a]Values are least square means (n = 8).

[b]CT = control diet; AV = avilamicyne, 400 ppm; BT = sodium butyrate, 0.3%; XT = XTRACT™, 300ppm.

6.2.4. Morphlogical parameters

Table 6.6. shows the results from the histological study of jejunum, ileum and colon. VH was not affected by the treatment, crypt was increased in jejunum for AV and BT treated animals (P = 0.029) and was also numerically higher for these treatments in ileum (P =0,280). As a consequence villus:crypt ratio was lowered in jejunum and ileum

by these two treatments (P = 0.110 and 0.041 respectively). These two additives also increased the presence of goblet cells in colon (P = 0.0002).

Tabla 6.6. Histological study in of the pigs fed the experimental diets[a]

Treatment[b]	CT	AV	BT	XT	SEM	P – values
JEJUNUM						
Villus heigh, µm	397.1	409.8	414.5	412.3	24.66	0.977
Crypt depth, µm	205.44^{y}	279.5^{x}	285.6^{x}	215.92^{xy}	17.44	0.029
Villi:Crypt, µm:µm	2.00	1.49	1.52	2.03	0.154	0.110
Goblet cells villi, n/100 cells	3.11	4.43	3.45	3.54	0.933	0.898
IEL, n/100 cells	14.72^{x}	8.27^{z}	13.21^{xy}	10.27^{yz}	0.792	0.0017
Goblet cells crypt, n/100 cells	10.13	13.03	13.16	11.17	1.062	0.315
Mitoses, n/100 cells	1.61	1.14	1.33	1.8	0.218	0.391
Lymphocytes, n/1000µm2	2.69^{xy}	2.03^{y}	2.00^{y}	3.02^{x}	0.197	0.010
Nuclei, n/1000µm2	10.12	9.74	10.01	10.64	0.395	0.556
Lymp:Nuclei	0.24^{xy}	0.20^{y}	0.20^{y}	0.28^{x}	0.017	0.014
ILEUM						
Villus heigh, µm	274.3	285.2	261.6	308.4	16.02	0.394
Crypt depth, µm	192.4	227.7	213.9	185.2	15.00	0.280
Villi:Crypt, µm:µm	1.5^{xy}	1.31^{y}	1.26^{y}	1.76^{x}	0.105	0.041
Goblet cells villi, n/100 cells	5.88	4.63	8.38	6.07	0.847	0.063
IEL, n/100 cells	15.11	10.12	14.89	9.77	1.442	0.052
Goblet cells crypt, n/100 cells	16.01	19.66	18.88	18.58	1.466	0.437
Mitoses, n/100 cells	1.66	1.86	2.06	1.73	0.262	0.803
Lymphocytes, n/1000µm2	2.81	2.7	3.08	3.37	0.176	0.063
Nuclei, n/1000µm2	10.49^{xy}	9.76^{y}	11.37^{x}	10.7^{xy}	0.351	0.036
Lymp:Nuclei	0.27	0.27	0.28	0.32	0.016	0.226
COLON						
Crypt depth, µm	388.4	352.8	363.1	343.1	13.51	0.126
Goblet cells crypt, n/100 cells	9.25^{z}	13.29^{xy}	15.3^{x}	10.15^{yz}	0.925	0.0005
Mitoses, n/100 cells	0.87	1.00	1.20	1.00	0.168	0.534
IEL, n/100 cells	4.87	2.39	3.72	3.7	0.569	0.052
Lymphocytes, n/1000µm2	2.47^{y}	2.13^{y}	2.74^{y}	3.49^{x}	0.192	0.0004
Nuclei, n/1000µm2	9.79	9.23	10.64	10.36	0.426	0.132
Lymp:Nuclei	0.26	0.24	0.26	0.34	0.027	0.090

[a]Values are least square means (n = 8).

[b]CT = control diet; AV = avilamicyne, 400 ppm; BT = sodium butyrate, 0.3%; XT = XTRACT™, 300ppm.

[x,y,z]Means within rows without a common superscript differ (P < 0.05).

Mitotic index was not affected by treatments in any studied part of the GIT. Concerning immune cells, IEL were decreased in jejunum (P = 0.0017) and ileum (P = 0.052) for animals fed AV and XT, and XT promoted a numerical increase for lymphocytes in lamina propia of jejunum (P = 0.010), ileum (P = 0.063) and in colon (P = 0.0004).

6.2.5. Hindgut Fermentation

Figure 6.2. shows total VFA concentrations along the GIT. Total VFA concentration was lower in proximal colon of animals fed BT.

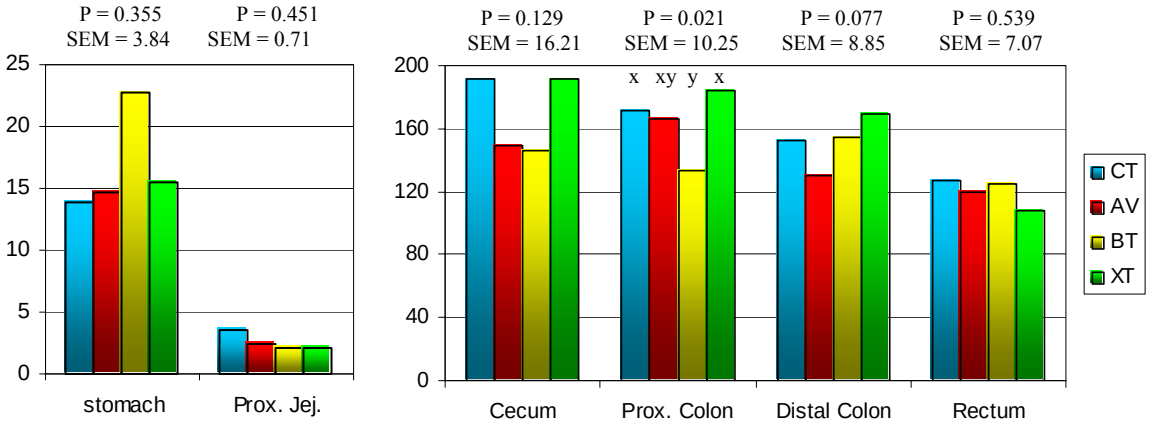


Figure 6.1. Total VFA concentrations (µmol/g FM) along the GIT of pigs fed the experimental diets. Means significantly different are shown by different letters.

Table 6.7. shows the VFA profile along the GIT. Butyrate was increased in stomach of BT treated animals (P = 0.0001), but this increase disappeared in proximal jejunum (P = 0.706). AV promoted a decrease in propionic percentage along the hindgut.

When data from hindgut was pooled and analyzed, AV promoted a decrease in propionic (P = 0.001). As a consequence, acetic and branched VFA percentage was increased (P = 0.044 and 0.0005, respectively). On the other hand, XT increased butyric percentage in the hindgut (P = 0.001) and increased propionic acid (P = 0.001).

Table 6.7. VFA concentration and profile in GIT of pigs fed the experimental diets.

Treatment[b] / Response	CT	AV	BT	XT	SEM	P – values
ACETIC, %						
Stomach	91.7^{x}	93.3^{x}	81.6^{y}	95.3^{x}	0.99	0.0001
Proximal jejunum	87.3	91.6	85.7	95.0	3.48	0.301
Cecum	53.0	56.1	54.8	52.5	2.08	0.665
Proximal colon	53.8	55.9	54.4	53.2	1.59	0.709
Distal colon	52.1	56.0	52.8	51.4	1.43	0.219
Rectum	50.8	53.3	53.0	50.0	1.53	0.446
Hindgut[f]	**52.5^{y}**	**55.3^{x}**	**53.8^{xy}**	**52.1^{y}**	**1.66**	**0.044**
PROPIONIC, %						
Stomach	2.89	1.41	2.48	1.37	0.460	0.059
Proximal jejunum	4.78	1.58	5.30	2.56	2.238	0.633
Cecum	30.2	28.1	32.5	29.4	1.32	0.163
Proximal colon	28.9^{xy}	25.9^{y}	31.5^{x}	28.3^{xy}	1.17	0.022
Distal colon	26.8^{x}	20.9^{y}	27.5^{x}	24.9^{x}	0.91	0.0005
Rectum	26.6^{x}	21.4^{y}	27.2^{x}	24.2^{xy}	1.08	0.004
Hindgut[f]	**27.9^{y}**	**24.1^{z}**	**29.7^{x}**	**27.3^{y}**	**1.16**	**0.001**
BUTYRIC, %						
Stomach	4.87^{y}	5.11^{y}	15.54^{x}	2.98^{y}	0.970	0.0001
Proximal jejunum	4.18	3.10	4.52	1.54	5.180	0.706
Cecum	14.2	13.8	11.1	15.8	1.49	0.229
Proximal colon	13.6	15.2	11.7	15.4	1.33	0.223
Distal colon	15.6	17.7	15.7	19.2	1.24	0.214
Rectum	15.6	18.1	14.9	18.8	1.02	0.055
Hindgut[f]	**14.7^{yz}**	**16.2^{xy}**	**13.4^{z}**	**17.2^{x}**	**1.28**	**0.001**
BRANCHED, %						
Cecum	0.31	0.41	0.12	0.26	0.080	0.123
Proximal colon	0.84	1.07	0.60	0.77	0.147	0.178
Distal colon	2.58	3.11	1.74	1.86	0.417	0.160
Rectum	3.78	4.55	2.45	4.14	0.570	0.105
Hindgut[f]	**1.70^{yz}**	**2.24^{x}**	**1.33^{z}**	**1.90^{xy}**	**0.266**	**0.0005**

[a]Values are least square means (n = 8).

[b]CT = control diet; AV = avilamicyne, 400 ppm; BT = sodium butyrate, 0.3%; XT = XTRACT^{TM}, 300ppm.

[c]Values fro stomach and duodenum were 0.

[f] Analysis of the overall means along the cecum, colon and rectum. Treatment, location were used as classification effects and interaction was included in the model. Location P-value was lower than 0.0002 for total concentration and percentages but for acetic (P = 0.154). Interaction was only significant in branched VFA analysis.

[x,y,z] Means within rows without a common superscript differ (P < 0.05).

6.3. DISCUSSION

6.3.1. Productive Performance and Digestibility

The three additives used in this experiment promoted numerically better ADG and ADFI, and AV and BT also improved the G:F ratio. It is normally accepted that the AGP and the acidifiers, reduce the detrimental effect on the gut microbial populations through a direct action on intestinal microbiota (Partanen and Mroz 1999; Anderson 2003). However, it is difficult to understand how it is possible to improve performance of animals fed BT when their digestibility was drastically decreased. It could be related to some extent with the marker behavior in the gut and could be indicating that chromium oxide flow is affected by the treatment. However, it does not seem the case since starch content at ileum and feces was also clearly affected by this treatment. This improvement in productive performance could be also related to an improvement in the health status of these animals. Butyrate, as a salt, is not just an acidifier but a very important nutrient for the epithelium (Scheppach et al., 1996). It has been hypothesized that this kind of additives could help to maintain the epithelium integrity protecting the animal against pathogenic agents (Gardiner et al., 1995). In contrasts to this hypothesis the analysis of the butyrate present in proximal jejunum content didn't show a significant amount of this product in treated animals.

PE antimicrobial effects has been proposed (Cowan 1999), but not demonstrated *in vivo*. In the present experiment a small increase in ingestion and growth without affecting G:F was found. This higher ingestion can be also related to an improvement in health status of the animals or to palatability effects of the XT used (Wenk, 2005). Unfortunately, studies concerning the different acceptance and palatability studies of these products by the pigs are not available.

6.3.2. Morphology of the Small Intestine and microbial proliferations

Despite the variation due to the treatments in the current study, VH and CD measurements were again in a range comparable to data obtained by Cera et al. (1988), Zijlstra et al. (1996), and Pluske et al. (1996). Both, AV and BT decreased V/C through an increase in crypt depth. This is, the only coincidence among all the foregut parameters studied between this two treatments. However the cause of this variation seems to be different depending on the treatment. Avilamycin is normally used as an

AGP (Kyriakis, 1989), and produce effects on microbial populations which could also affect the epithelium. In this treatment animal showed a decreased number of IEL. These lymphocytes have been demonstrated to have important functions in the regulation of the epithelium renewal, both inhibiting and promoting this renewal. Thus this difference in IEL number may be a response of the epithelium to changes in microbiota (McCracken and Lorenz, 2001) produced by AV and could be related to an up-regulation of the epithelial renewal. In the BT treated animals IEL where not altered, but Goblet cells in the villi were increased in ileum This response in mucus productive cells has been also related to changes in intestinal microbiota (Deplancke and Gaskins, 2001).

XT promoted a decrease in IEL in jejunum and ileum, but this variation was accompanied by an increase of lymphocyte presence on the lamina propia which was also present in colon. Jiang et al. (2000) related a higher number of nuclei in lamina propia to a higher activation of the immune system. In this case, total number of nuclei is not altered, but the lymphocytes. IEL and lamina propia lymphocytes are in constant dialogue in the intestine and they can migrate from lamina propia to IEL during the early maturation of the intestine (Stokes et al., 2002). Thus, the different disposition of the lymphocytes in epithelium and lamina propia is indicating differences of the luminal stimuli among treatment, which produce different maturation patterns. In this case it is known that luminal bacteria can produce variations in epithelial immune responses (McCracken and Lorenz, 2001). However, cinnamaldehyde has also effects in lymphocyte proliferation and maturation (Koh et al., 1998) and can be causing this change in epithelium immune cells.

The results do not show differences in the studied microbial populations in the jejunum. However, some of the variations in epithelial parameters may be related with changes in non studied bacterial populations. In a parallel study, which was done with the same animals (Perez de Rozas et al., 2004b, Castillo et al., in press) using molecular techniques, more intestinal segments were studied and more bacterial groups were identified. Using RT-PCR no differences were found in total bacteria, lactobacilli and enterobacteria (Castillo et al., in press), which agree with the results presented here. However, bacterial profile (Perez de Rozas et al., 2004; Castillo et al., in press) showed important divergences among treatments and the three additives promoted and increased biodiversity in the GIT compared to control. This so called biodiversity is a measure of the quantity of different microorganisms detected and his respective frequency, and has

been proposed as an indicator of stability of the intestinal microbiota (Zoetendal et al., 2004). Butyrate specially increased biodiversity of microbiota and particularly in the distal parts of the gut. This higher biodiversity in hindgut populations can explain a lower efficiency of these populations to ferment the substrates and then producing a lower digestibility. However it seems to produce a beneficial effect on animal performance.

This high biodiversity probably will not be recommended for older pigs, where productivity is extremely related the profitability of the diet. But compared to growing and finishing, weaning is a critical phase devoted to guaranty the health of the animal and, for this propose, other characteristics than digestibility could be important. In this sense, higher biodiversity can avoid the proliferation of a simple bacterial group which induces easy disbiosis. How these additives, and specially butyrate, produce this higher biodiversity is difficult to understand with the presented results.

6.3.3. Hindgut Fermentation

Volatile fatty acids are the major end products of bacterial metabolism in swine large intestine (Bergman, 1990). In the present experiment, we used VFA concentration and profile as an index of the changes on the microbial population and of the quantity and source of products been fermented in the hindgut. Two principal changes are shown in these results.

The first important change is the effect of the lower digestibility found in BT fed animals. This lower digestibility could be reflected in i) the lower fermentation reflected as a lower VFA concentration in the cecum and proximal colon, ii)the lower branched VFA produced, as direct product of protein fermentation. Along the cecum and the colon, carbohydrates resistant to foregut digestion are degraded, percentage of protein fermented is higher in distal parts and branched fatty acids, direct product of protein fermentation, represent higher percentage. Here starch seems to be less digested and remains available for bacteria whole along the hindgut, reducing/minimizing protein fermentation.

The second important change is the higher production of propionic acid in AV treated animals. It is normally a consequence of non-digested easily fermentable carbohydrates arriving to the hindgut (Bergman, 1990). On the other hand, carbohydrate digestion of the AV treated animals seems to be very effective since it presents the higher quantity of branched VFA. However it has been also shown that different bacteria fermenting the

same substrate are able to produce differences in fermentation products (Jiang and Savaiano, 1997). In the case of propionic bacteria, it has been related to the fermentation produced by some not cultivated bacterial genera, which have been related to a very stable intestinal microbial ecosystem (Skene et al., 2004).

XT did not produce significant variations of the VFA concentration but it was numerically higher than other two treatments in cecum and colon. This fact could be realated to a higher fermentation in the hindgut, however it did not agree with PB results which will be discussed in the general discussion.

On the other hand, XT produced an increase in butyric acid production as happened in the first trialusing animals of the same age.

CHAPTER 7.

IN VITRO ACTIVITY OF CARVACROL, CINNAMALDEHYDE AND CAPSAICIN AGAINST DIFFERENT INTESTINAL BACTERIA IN COMPLEX INTESTINAL MEDIA AND IN THE TNO IN VITRO MODEL 1 (TIM - 1).

"Olgaaaaaaaaaaaaaaaaaaaaa!!!!!!!!!!!!!!!!!!"

Roser Sala

7.0. CONTEXT

In the above presented *in vivo* experiments, we observed some effects of the PE on pig physiology, but these effects were very variable.

On the other hand, the legal context in Europe is changing. Legislation will be stricter with new products and some products currently allowed will be revised and probably forbidden until their risk is defined. This may be the case of some PE, whose toxicity and effects are not totally defined.

Given the non-consistent effects of XT upon piglet health and performance and the new future legal context, new additives need to be designed to substitute and/or improve XT. There are many substances to choose when working with PE, but only a low number of studies are available about their effects in animal production. Testing a high number of PE *in vivo*, using different doses, etc, can be extremely time consuming and expensive. On the other hand, the testing of PE in pure culture media is not always representative of the real effect of the products under *in vivo* conditions. Thus, we decided to develop an *in vitro* method in order to test a high number of products in a rapid way, but using a medium similar to the real intestine media. The results found in the three previous *in vivo* experiments and the interest of the company was particularly directed to changes in the microbiota, so this *in vitro* method was specifically designed to test the effect of PE on microbial populations present on the intestine.

In this chapter, we also present an experiment carried out with a dynamic *in vitro* model as another possibility to study the effect of different additives upon intestinal bacterial population. The usefulness of this method will be discussed.

7.1. MATERIAL AND METHODS

Three *in vitro* experiments were carried out in a closed system using piglet intestinal content. A fourth experiment was performed using the TNO dynamic *in vitro* model simulating the stomach and the small intestine (TIM-1, Minekus et al., 1995). This last experiment was carried out in the Faculté de Pharmacie de l'Universite d'Auvergne (Clermont-Ferrand, France).

The main objective of the first three experiments was to propose a very simple and repeatable *in vitro* method, which could allow us to test a high number of substances in

the same experiment, using a culture media as similar as possible to the pig's intestinal content.

The particular objectives concerning the closed *in vitro* system were:

- To study the evolution of the fermentation along the gastrointestinal tract.
- To study the evolution of the fermentation along five weeks after weaning.
- To use the model to test the effects of the different studied substances (XT and its components) upon both the microbial fermentation and some bacterial populations.

The objective of the experiment using the TIM-1 dynamic simulator was to propose this method as adequate to create a defined intestinal microbiota, in order to test the effect of PE additives on these bacteria.

7.1.1. Closed *in vitro* system

The *in vitro* method here used is an adaptation of the methods used by Robinson et al., (1989), Menke and Steingrass, (1988), and Theodorou et al., (1994) for bacterial fermentation measurement. Briefly, it pretends to study of the evolution of bacterial populations in intestinal content, maintained in anaerobic incubation, through gas production measurement.

7.1.1.1. *Animals to obtain the inoculums*

Experiment 1. Six three-week old piglets were weaned, and fed a commercial pre-starter diet (45% corn, 17% milk by products, 30% potato and soy protein; 19% CP level and no additives) during two weeks, and afterwards the animals were killed.

Experiment 2. Six three-week old piglets were weaned, and fed a commercial pre-starter diet (45% corn, 17% milk by products, 30% potato and soy protein; 19% CP level and no additives) during one week, and afterwards the animals were killed.

Experiment 3. Six piglets weaned at three weeks of age, were fed a pre-starter diet during four weeks (45% corn, 17% milk by products, 30% potato and soy protein; 19% CP level and no additives) and a commercial soy-cereal diet (66% corn, 30% soy bean meal; 20 % CP) along one week. After that, the piglets were killed.

The animals were sacrificed with intravenous injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain) (200mg/kg BW), the abdomen was opened and the gastrointestinal tract was extracted. Total content of stomach, jejunum

(described as the small intestine segment comprised 1m from stomach and 1m to ileo-cecal valve) and cecum was separately collected in three different refrigerated bottles.

7.1.1.2. *Treatments*

Table 7.1 shows the products and doses used in each experiment and for each part of the GIT. For all experiments, the concentrations of the products used are expressed in a fresh matter basis. XT, a standardized mixture of 5% (wt/wt) carvacrol, 3% cinnamaldehyde and 2% capsicum oleoresin, was the only product tested in experiment 1. The used doses were 0, 100, 1000 and 10000 ppm.

The treatments in experiment 2 for stomach and jejunum were formic acid, carvacrol, cinnamaldehyde, capsicum oleoresin (*capsicum anuum*) and XT at the concentrations indicated in table 7.1. Only one product (XT) was tested in cecum content.

The treatments in experiment 3 were carvacrol, cinnamaldehyde and capsicum oleoresin used at different doses depending on the product and on the part of the digestive tract studied.

Table 7.1. Products and doses (ppm) used in each experiment and part of the GIT.

GIT part / Treatment	Stomach			Jejunum			Cecum		
	Exp1	Exp2	Exp3	Exp1	Exp2	Exp3	Exp1	Exp2	Exp3
XTRACT™	100	100		100	100		100	100	
	1000	1000		1000	1000		1000	1000	
	10000	10000		10000	10000		10000	10000	
								10^5	
Carvacrol		100	1000		100	500			1000
		1000	2000		1000	1000			2000
		10000	3000		10000	2000			3000
			4000			3000			4000
Cinnamaldehyde		100	1000		100	500			1000
		1000	2000		1000	1000			2000
		10000	3000		10000	2000			3000
			5000			3000			5000
Capsicum oleoresin		100	5000		100	500			5000
		1000	8000		1000	1000			8000
		10000	11000		10000	10000			11000
			14000			15000			14000
Formic acid		100			100				
		1000			1000				
		10000			10000				

7.1.1.3. *Preparation of the tubes and measurements*

All samples were transported to the laboratory, pooled in continuous CO_2 perfusion and 15 ml of the final sample were placed in 25 ml glass tubes, which already contained the product to test (Figure 7.1). The O_2 was eliminated from the tubes through the injection of CO_2. The tubes were hermetically closed with rubber caps and incubated for 6 hours. Each different treatment and dosage comprised a minimum of two replicated tubes for gas measuring and microbial counts.

Figure 7.1. Tubes containing the media (left). Measuring the accumulated gas (right).

Total gas production was measured in all experiments and bacterial populations by agar-plate conts only in experiment 3. Total gas production measurement was done using a 20 or 50 ml glass syringe (Ruthe, Portugal) and a 0.60 × 25 mm needle (Sterican Ref. 4657667, Braun medical AG, CH6021, Emmenbrücke) (Figure 7.1.). The needle was maintained in the tube for 10 seconds to allow all the gas to be measured. The total gas volume was measured in each tube at different times: 0, 2, 4 and 6 hours for experiments 1 and 2 and 0, 1, 3, 6 hours for experiment 3. The fermentation of the stomach content of experiment 3 was only maintained until 3 hours.

The effects of the different treatments on lactobacillus and enterobacteria counts were studied in experiment 3 for stomach and jejunum contents. The samples used for bacterial counts were the initial gastrointestinal content (0 hours of incubation) and the content of the tubes containing the higher and the lower doses at the end of the incubation. The tubes were opened and the content was sampled. One gram of sample was weighted and serially diluted. Aliquots of 100 µl were plated in agar MacConkey

(Oxoid, Ref. CM 115) for enterobacteria (dilutions from 10^{-3} to 10^{-7}) and in agar rogosa (Oxoid, Ref. CM 627) for lactobacilli (dilutions from 10^{-4} to 10^{-7}). Enterobacteria were counted after a 24-hour incubation (37º C) and lactobacilli after a 48-hour incubation (37º C, 5% CO_2).

7.1.2. Dynamic in vitro simulator

Experiment 4 was carried out using the TNO *in vitro* model 1 (TIM-1) (Minekus et al., 1995) which is a dynamic simulator of the gastric and intestinal function (figure 7.2) and has been used in different bacterial survival studies (Marteau et al., 1997; Blanquet et al., 2003).

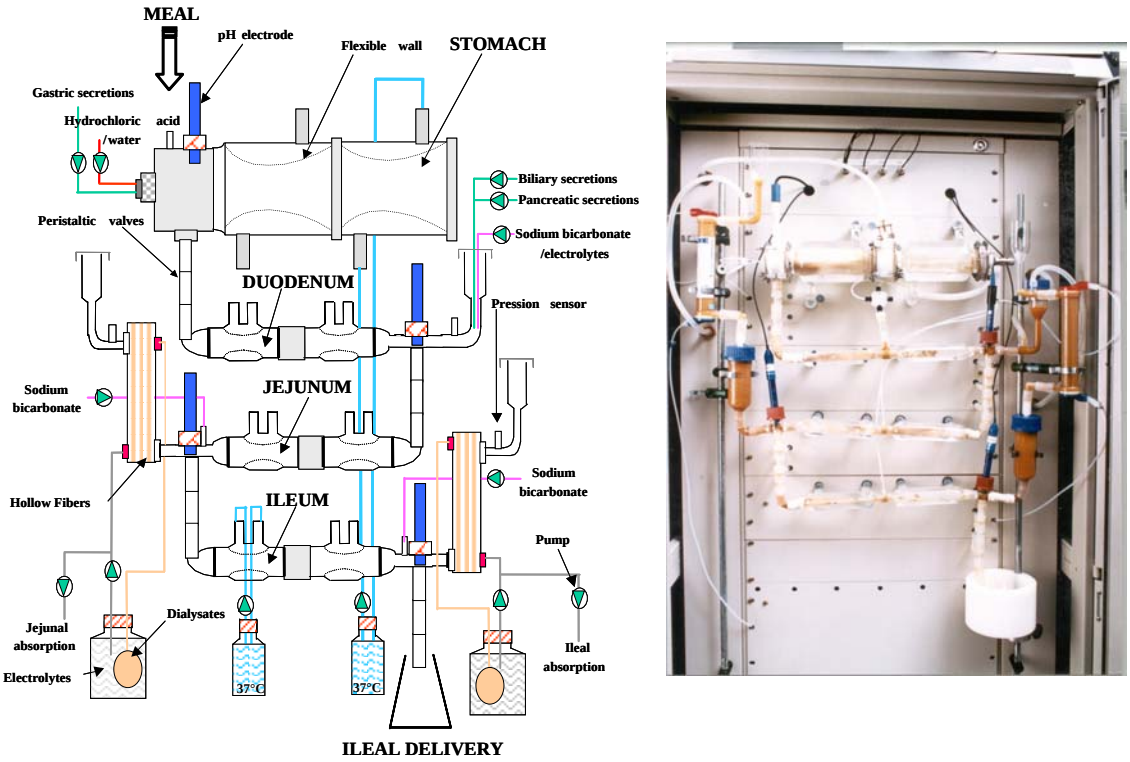


Figure 7.2. Scheme (left) and photograph (right) of the TIM-1 dynamic simulator.

Briefly, the model is composed of four different compartments simulating stomach, duodenum, jejunum and ileum and allows a continuous dynamic simulation of monogastric digestion controlling: gastrointestinal flow rate, through a valve system (Elashoff curve); temperature, maintained by a hot water circuite (37ºC), pH, enzyme and bile salts secretion, fixed controlled quantities; and anaerobic conditions; through N_2 infusion. The pH is controlled by the secretion of HCl in stomach, or sodium bicarbonate in duodenum, jejunum and ileum, to maintain the pH in a prefixed level

(Figure 7.3.). This pH level evolves with time for the stomach (t = 0, pH = 6; t = 30', pH = 3.5; t = 120', pH = 3; t = 180', pH = 2.5; t = 240, pH = 2) and is fixed for duodenum (pH = 5), jejunum and ileum (pH = 6.5).

Moreover the model includes a dialysis system to simulate the absorption of the digested nutrients (pore filter = 5000 Dalton).

For bacterial studies, the model is previously sterilized by means of 1 hour of steam circulation and afterwards controlled bacterial populations are introduced.

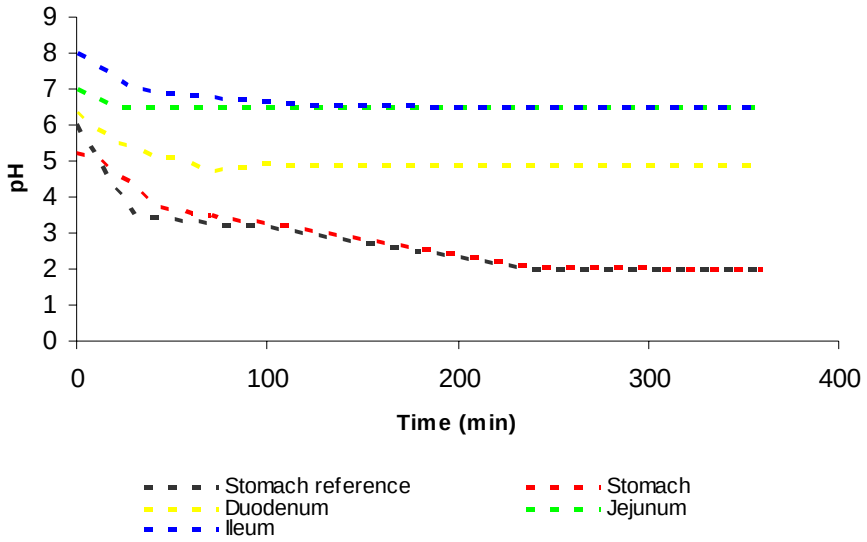


Figure 7.3. Theoretical evolution of the pH in stomach and real evolution of the pH measured in the four compartments of the TIM-1.

In the present experiment, two digestion simulations 6 hours long were done for each treatment (0 and 10000 ppm of XT) and content samples were taken from stomach, duodenum and ileum at different times: 0, 30', 1h, 2h, 4h and 6h.

Bacterial populations. Bacterial strains used in the model were a pure E. coli culture (CECT 515NT) and lactobacilli spp. isolated from intestinal contents used in experiments 2. For isolation of lactobacilli spp., small intestine content was plated in agar rogosa and the colonies obtained were serially plated in agar rogosa for three times. E. coli was grown in BHI (Oxoid Ref. CM 0225) for 12h and lactobacillus in MRS liquid media (Fluka ref: 69966, Steinheim, Switzerland) for 36 hours. The cultures were stored in 1 ml eppendorf tubes and maintained at -80ºC until their use. The day before each digestive simulation, the content of two freezed eppendorf tubes, one for lactobacilli and one for enterobacteria, was introduced in 750 ml of MRS media and in 25 ml of BHI media, respectively, and incubated at 37ºC with agitation overnight. Next

morning, bacterial populations were prepared as follows: for lactobacilli, total MRS media culture was standardized to 0.350 nm of optical density using sterile MRS media as a blank. 750 ml of the standardized culture were centrifuged 20 min, 4ºC, 3000 g and the pellet obtained was recovered with 50 ml of PBS. For E.coli, the BHI culture was also standardized to 0.830 nm of optical density. The total pellet obtained for lactobacilli and 1 ml of the E.coli culture was used as initial bacteria to introduce in the stomach of the TIM-1.

7.1.3. Statistics

Gas results are always shown as cumulative production along time.

All data were analyzed using SAS 8.1. (SAS Institute, Cary, NC, USA). The data were analyzed using Proc GLM and comparing the results for each GIT part, time and product separately. Dose was used as a class variable.

Means were compared applying Tukey's correction in the test. Alpha levels were 0.05 for all comparisons.

7.2. RESULTS

7.2.1. Gas production measurements

7.2.1.1. *Gas production along the GIT (no treatment)*

The gas production measurement followed different evolutions depending on the GIT part studied (Figure 7.4.). Gas production in stomach was reduced with the age of the animal. In fact, no gas production was observed in 5 week-old animals. Total gas production was always higher in jejunum than in stomach and cecum, and increased with age. In cecum, the gas production was also increased with age.

7.2.1.2. *Effect of XT on gas production*

XT affected the gas production measurement in a different way depending on the GIT part studied (Figure 7.5.). XT did not produce any difference in gas production in stomach and cecum ($P > 0.171$). However, XT caused a reduction in jejunum gas production when included at the higher dose (10000ppm) ($P < 0.001$ and $P < 0.005$ for 2, 4, and 6h measurements in experiment 1 and 2, respectively). 100000 ppm were required to decrease gas production in cecum content ($P < 0.001$).

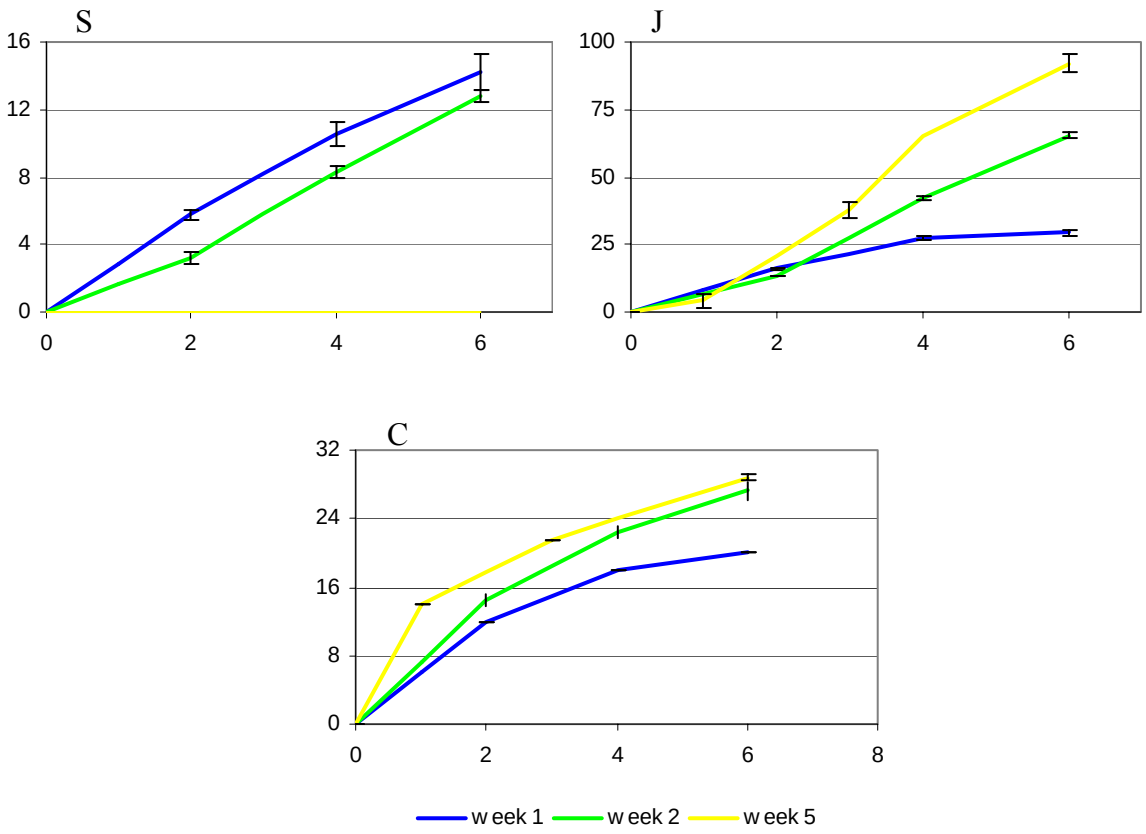


Figure 7.4. Total ml of gas production of the control replicates in stomach, S, jejunum, J, and cecum, C, depending on the age of the animals (experiments 1, 2 and 3).

7.7.1.3. *Effect of carvacrol, cinnamaldehyde, capsicum oleoresin and formic acid on gas production*

In both experiments, 2 and 3, the effects of the different additives were more pronounced in jejunum than in stomach or cecum content.

In experiment 2 (Figure 7.6), carvacrol produced total inhibition of the gas production in the stomach only at 10000 ppm ($P < 0.0001$ for 2, 4 and 6 hours), cinnamaldehyde and formic acid induced a partial inhibitory effect of the gas production at 10000 ppm ($P < 0.0002$ for 2, 4 and 6 hours) and capsicum had no effect at any concentration ($P > 0.09$ for 2, 4 and 6 hours).

In jejunum, 1000 ppm of carvacrol, cinnamaldehyde or formic acid produced some partial inhibitory effect on gas production, 10000 ppm of carvacrol produced a total inhibition of the gas production and 10000 ppm of cinnamaldehyde or formic acid produced gas production values near to 0 ($P < 0.0001$ for 2, 4 and 6 hours). In this part of the GIT, 10000 ppm of capsicum oleoresin produced also a partial inhibition of the microbial gas production ($P < 0.05$ for 4 and 6 hours).

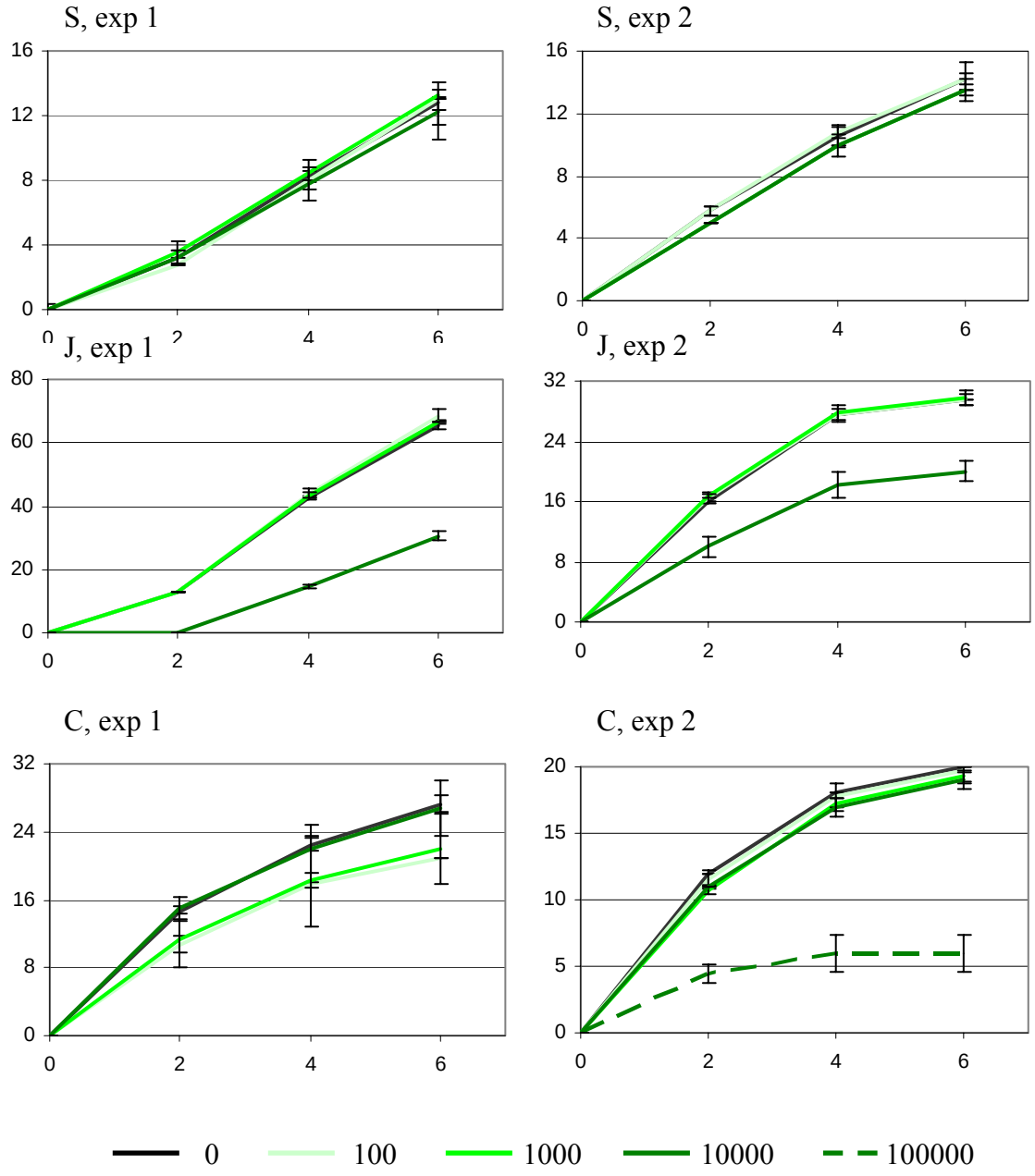


Figure 7.5. Total ml of gas production in stomach, S, jejunum, J, and cecum, C, depending on the XT dose in experiments 1 and 2 (animals 2 and 1 weeks old, respectively).

In experiment 3 (Figure 7.7), no gas production was registered from the fermentation of the stomach content. Carvacrol at 1000 ppm and cinnamaldehyde at 500 ppm were enough to inhibit partially the gas production in the jejunum, and doses of 2000 and 1000 ppm respectively were necessary to obtain total inhibition or near to 0 values of gas production ($P < 0.0001$ for 3 and 6 hours). Capsicum oleoresin produced partial inhibition of the gas production only at 6 hours and used at 15000 ppm ($P = 0.013$).

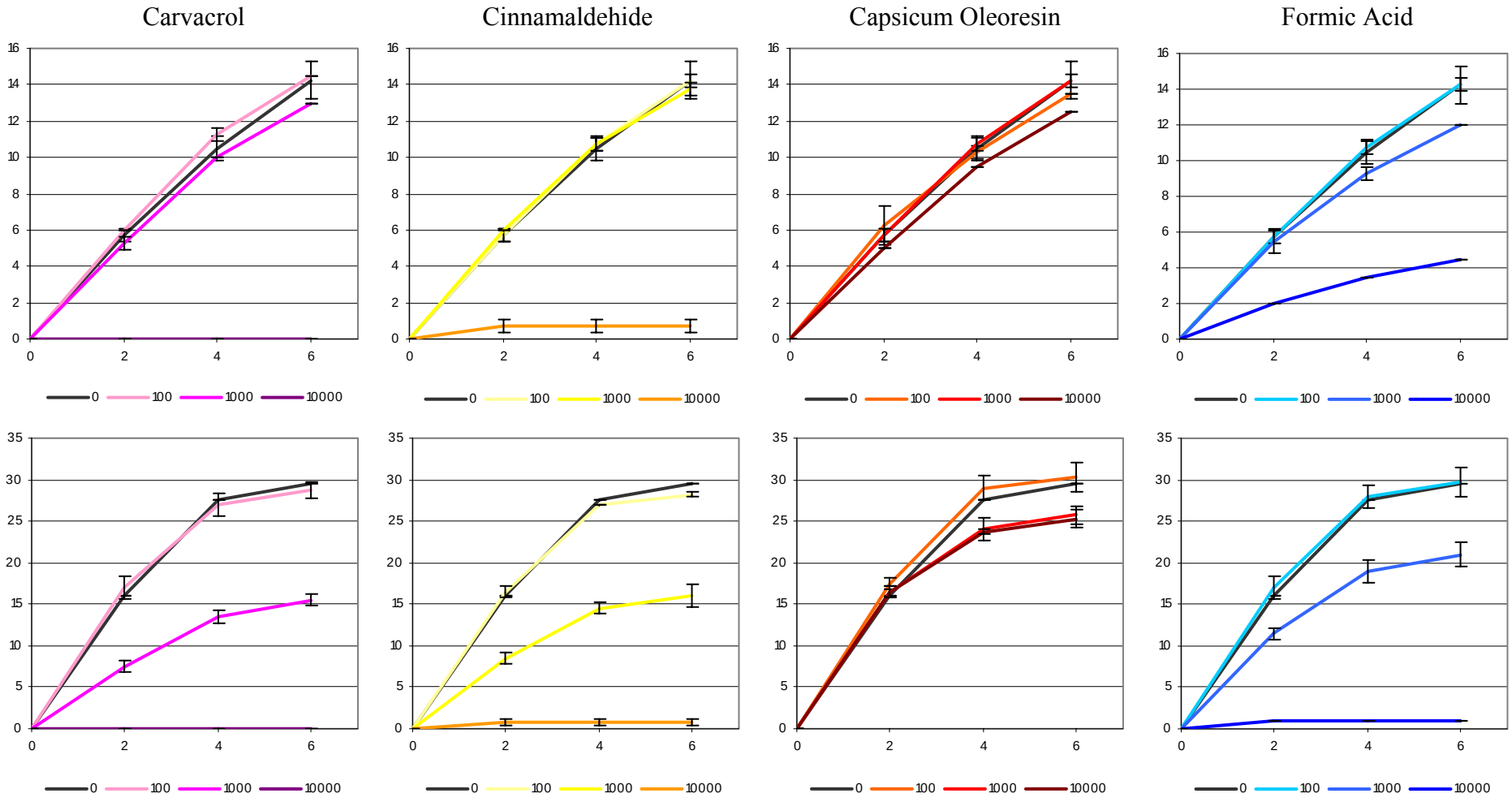


Figure 7.6. Total ml of gas production in stomach (upper row) and jejunum (lower row) depending on the inclusion dose of carvacrol, cinamaldehyde, capsicum oleoresin or formic acid (experiment 2, animals were 1-week old).

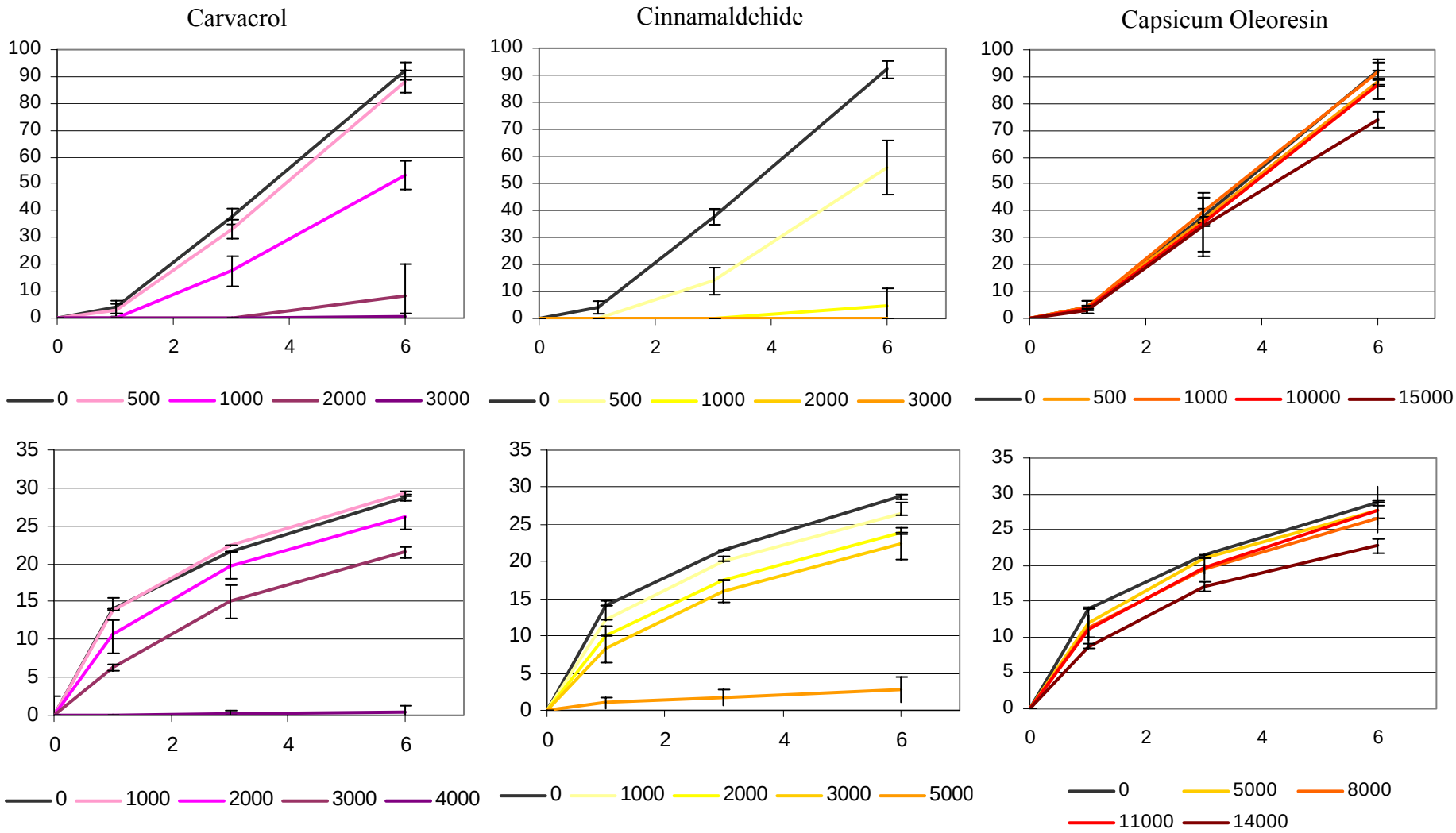


Figure 7.7. Total ml of gas production in jejunum (upper row) and cecum (lower row) depending on the inclusion dose of carvacrol, cinamaldehyde, or capsicum oleoresin (experiment 3, animals were 5 week old).

Carvacrol at 3000 ppm and cinnamaldehyde at 2000 ppm produced partial inhibition of the gas production in the cecum, and doses of 4000 and 5000 ppm respectively produced inhibition almost total of the gas production ($P < 0.002$ for 1, 3 and 6 hours). Capsicum oleoresin produced partial inhibition of the gas production in cecum only from 3 hours of incubation and when used at 14000 ppm ($P = 0.025$).

7.2.2. Microbial counts

7.2.2.1. *Closed in vitro system.*

Concerning microbial counts (Figure 7.8), no enterobacteria growth was registered in the stomach after the 3h incubation period.

In jejunum, the enterobacteria growth was only affected by cinnamaldhyde at 5000ppm ($P < 0.001$).

Lactobacilli grew in both stomach and jejunum content and this growth was reduced by carvacrol and cinnamaldehyde at the higher dosage ($P <0.1$ for stomach and $P < 0.001$ for jejunum). However, lactobacillus growth, in jejunum, was also lowered by cinnamaldehyde at 500 ppm ($P = 0.001$), as happened with total gas production.

7.2.2.2. *TIM-1.*

Figure 7.9 shows the evolution of lactobacillus and E.coli populations (log CFU/ml) in the stomach, duodenum and ileum compartment of the TIM-1. E. coli was present in the stomach until 2h of digestion but was detected in the duodenum and jejunum during the 6-hour simulation. Lactobacillus resisted during the 6 hours in all compartments. E coli population was affected by the XT from $t = 0$ in the stomach and this effect was present along the different compartments of the model.

Lactobacillus was not affected by the XT inclusion but the counts were numerically higher in stomach and duodenum from 2h.

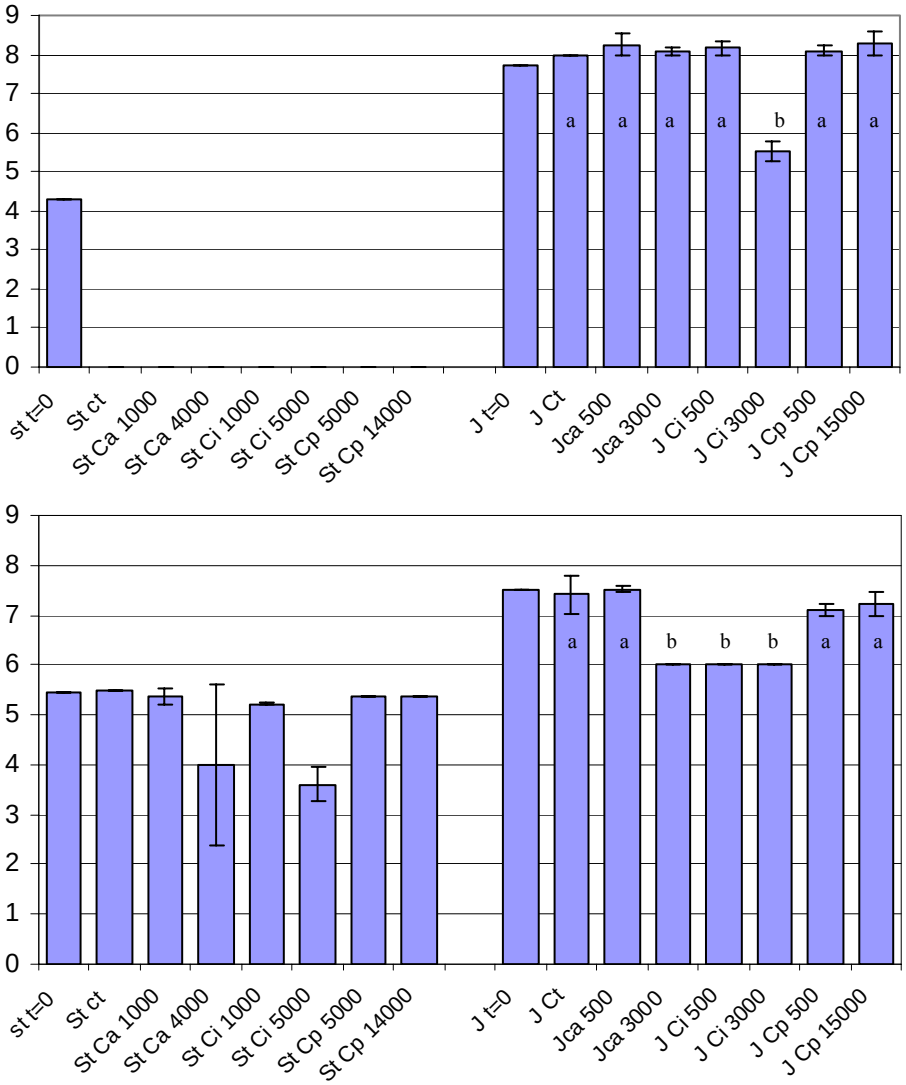


Figure 7.8. Enterobacteria (upper) and lactobacilli (lower) counts measured in stomach and jejunum content after 3 and 6 hours of incubation respectively. The concentrations of additive used were the control group, the higher and the lower concentration used for carvacrol (Ca), cinnamaldehyde (CI) and Capsicum oleoresin (Cp).

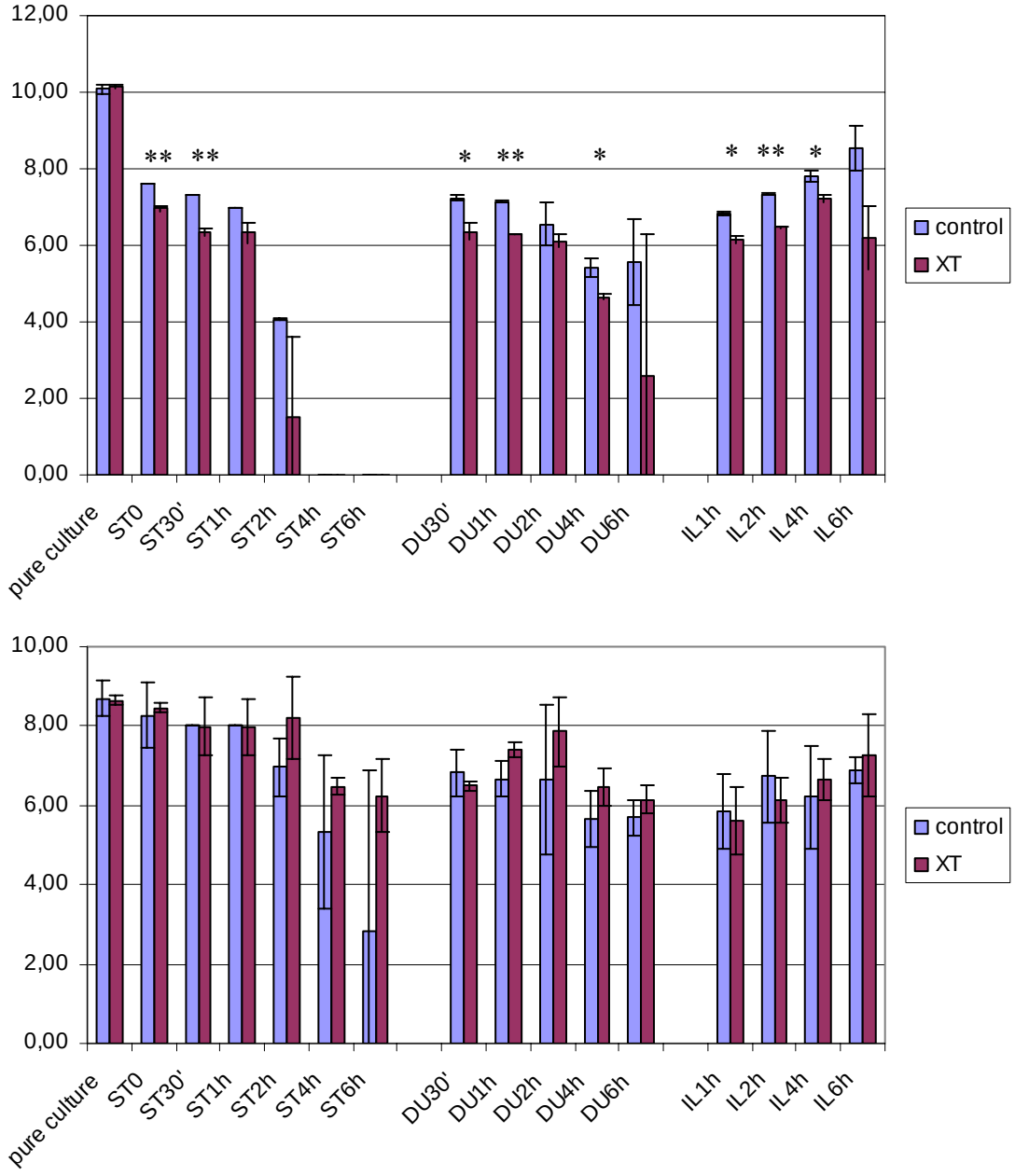


Figure 7.9. Evolution of the bacterial counts (enterobacteria, upper and lactobacillus, lower) in stomach, duodenum and ileum compartment of the TIM-1 during 6 hours of simulation.

7.3. DISCUSSION

7.3.1. Closed system

7.3.1.1. *Evolution of the fermentation along the GIT.*

The method used in these experiments were similar to the one used by Robinson et al. (1989) but here the intestinal content was used as obtained, without any dilution. Looking at the obtained results the fermentation in the piglet GIT after weaning seems to follow a defined pattern depending on the GIT part. Among the three GIT studied parts (figure 7.9), the stomach presented the lower gas production and jejunum the higher. Robinson et al. (1989) did not use the stomach context as an inoculum source, but they found that small intestine produced, in two hours of incubation, a higher non-significant quantity of gas than the cecum. This difference could be increased with longer incubation times as happened in our experiments.

According to the results, fermentation is present for at least two weeks after weaning in the stomach. Probably, the low acid secretion capacity of the animal's stomach after weaning is allowing some fermentative microbes to grow. However, when the animal passed the critic initial period, the fermentation in stomach became negligible. This finding could be indicating that the barrier effect of the stomach evolves during the first weeks after weaning and does not totally limit bacterial growth in the first two weeks. It is remarkable that, in parallel experiment we found (non published data) that the inoculum of 1-week old animals eating diets containing zinc oxide presented no fermentation in stomach at all, even though the pH of the inoculum did not decrease.

In contrast to stomach, jejunum and cecum gas production increased with age, especially in jejunum. A higher gas production has usually been related mainly to a higher substrate availability or, secondly, to a more adapted microbiota (Williams et al., in press). This very marked increase in jejunum fermentation with age can be due to the different diet in the case of the difference between 2 and 5 weeks. The growing phase diet contains more complex carbohydrates not easy to digest for the animal. These carbohydrates could remain in the intestinal lumen for a longer time and the microbiota would be able to produce higher quantities of gas due to the higher available substrate. In any case, the rapid adaptation of the microbiota to ferment these carbohydrates in the small intestine remains unexplained for the author. On the other hand, gas production was also increased from 1 to 2 weeks after weaning and the diet was the same. This difference could be due to the lower ingestion presented by animals in the first week

after weaning and/or by an evolution in the efficiency of the intestinal microbiota. In any case, the *in vitro* study of the evolution of the gas production of GIT content seems to provide us with very valuable information to understand what is happening in the pig gut at weaning (Williams et al., in press).

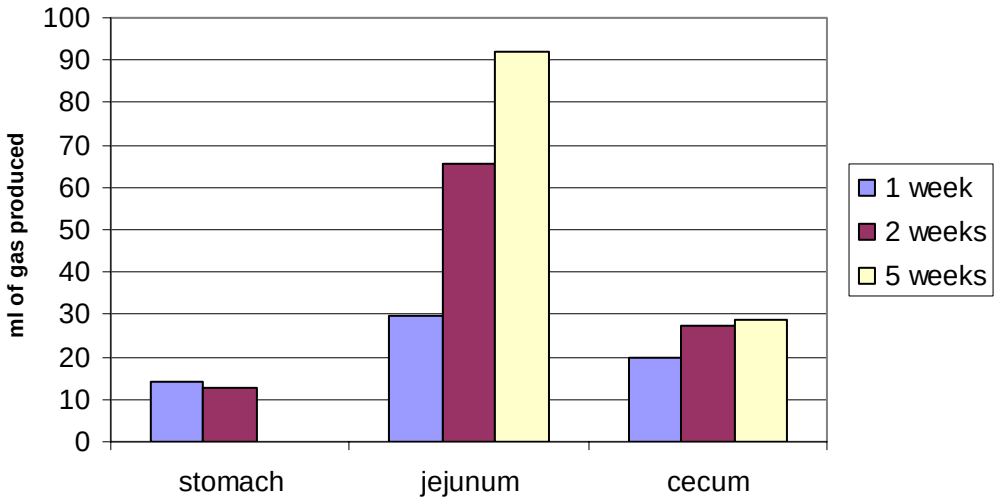


Figure 7.10. Total gas production of the stomach, jejunum and ileum content from animals 1, 2, and 5 weeks after weaning at 21 days.

In jejunum, high quantities of gas production were found, even higher than in cecum. It is a surprising result when we think about what happens in the animal, where fermentation in jejunum is not as important as in cecum. However, in the animal, the rapid transit and absorption of digested nutrients probably result in lower possibilities to ferment and a lower availability of the substrates. In any case, this fact can be suggesting that fermentation in small intestine is higher than it is supposed as suggested by Williams et al. (in press).

In cecum, gas production was probably lower than expected, because there was no continuous supply of fermentable substrates and/or because the production of VFA could reduce the pH of the media in a rapid way, inhibiting bacterial metabolism.

For future studies, it could be also very interesting to know the differences of the fermentation products and the pH in each part of the GIT.

7.3.1.2. *Effect of additives on fermentation and cultures*

The experiments using pure cultures are very useful in testing the antimicrobial effect of different PE (Dorman and Deans, 2000; Friedman et al., 2002). However, when PE are

used in complex media, the presence of fats, proteins and variations in pH or O_2 concentrations affects greatly the antimicrobial power of these substances (Juven et al., 1994; Cowan, 1999). PE are hydrophobic substances that show affinity for cellular membranes of bacteria in pure cultures, but fats and proteins present in complex media attract the PE, and lower their antimicrobial effect (Juven et al., 1994). The pH variations and O_2 concentrations in the environment produce changes in bacterial sensitivity to antimicrobials (Juven et al., 1994).

To take the effect of the complex media into account, it is important to have methods simulating the intestinal content of the animals for testing additives. The incubation of the intestinal content has been used many times to study fermentation processes in the gut of different animals (Menke and Steingrass, 1988; Robinson et al., 1989; and Theodorou et al., 1994). However, the method here used presents some differences to the techniques normally used for fermentation studies. In particular, the inoculum was used as obtained without the inclusion of diluents, buffers or added nutrients. On the other hand, fermentation processes are studied during periods of 24 to 72 hours and we used periods of only 6h. These differences result in a lower durability of the media conditions but it is more realistic to study the influence of the media in the antimicrobial effect of the additives.

The results found using the closed system were carried out simultaneously to the development of the method. Thus, the experiments should be repeated to check the validity of the exact values obtained. However, these results provide us with very valuable information about the effects of PE in intestinal content.

The first experiment was planned just to make a first approach to the effective dosage of XT. From this experiment we concluded that the dose of XT to produce bacterial inhibition was higher than expected. Then, in the second experiment we decided to try the effect of formic acid, an additive alternative to the AGP usually used in animal production, and to compare its effect to the one of the XT components. The range of doses used was very wide in order to determine a slimmer range to be studied in more detail in a third experiment.

Formic acid and the XT components were found to be effective at doses of around 1000 ppm (0.1%). We must consider that 10-fold dilutions are normal in the intestine and, when formic acid is included in the diet at commercial doses (1-1.5%), this dilution effect produces concentrations of 0.1-0.2% of the acid once in the GIT. Although XT components are effective at the same concentrations than formic acid, their doses of

inclusion in the diet are normally lower (between 0.01 and 0.05%). The effect of the PE at doses of 0.1% or 1% is not easy to imagine. PE could produce strong palatability problems used at these high doses and could even be toxic (Wenk, 2005).

The third experiment was used to make a more accurate approach to the effective dose of the three components of the XT in inhibiting gas production. Carvacrol and cinnamaldehyde, as components of oregano and cinnamon respectively, or alone, have been described as two of the most effective substances in inhibiting bacterial growth (Dorman and Deans, 2000; Friedman et al., 2002). Normal doses producing inhibitory effects in pure culture media are between 80 ppm and 800 ppm depending on the bacteria studied (Friedman et al., 2002). However, in the present experiment the inhibitory effects were found to be effective from 500 to 5000 ppm depending on the GIT part studied.

Concerning capsicum oleoresin, no data have been found about its antimicrobial power but doses here found as effective are too high to be applied in animal practice. However, it is know that capsaicin, the component of this oleoresin, produces strong effects on normal physiology of the animals through nervous system alterations (Chang et al., 1999; Debreceni et al., 1999).

XT is commercially used at 300 ppm as-feed basis. This represents a concentration of the active substances of 15, 9 and 6 ppm for carvacrol, cinnamaldehyde and capsicum oleoresin respectively. From these concentrations, no general antimicrobial effects can be expected as has been shown in these experiments. Some selective effects for these PE have been proposed (Zaika, 1988; Hussein, 1990; Smith-Palmer et al., 1998) but probably they are not appearing at these very low concentrations. However, other effects not studied in here, like changes in the blood flow or in the digestive content flow rate, can be expected from the commercial dose of capsaicin (Kang et al., 1993; Gonzalez et al., 1998; Chang et al., 1999; Debreceni et al., 1999). Perhaps we must start to consider other effects of the PE to avoid the possible complications associated with the antimicrobial doses, such as palatability problems.

Concerning the agar plate culture, the results are of limited value due to the low number of repetitions. On the other hand, more bacterial genera could and should be studied in future experiments. However, the agar plate cultures done from the closed system samples showed that variations in gas production are related, at least, with the studied bacterial genera (even when the total inhibition of gas production is not always indicating total death of all the bacterial population).

The closed method used in these investigations seems to be useful for rapid evaluation of the short time effects of additives on intestinal microbial populations, maintained in a medium very similar to real intestinal content. However, these very simple closed methods are only indicative, and results must be confirmed in the animal or using more complex models.

7.3.2. TIM-1

Until now, TIM-1 had been used to study the survival of single bacteria genera (Marteau et al., 1993 and 1997; Blanquet et al., 2003). The successful use of TIM-1 to study the effect of the PE on an artificial complex microbiota points this method as an interesting option to study the antimicrobial effects of additives on a complex intestinal microbiota.

In the experiment presented here, the number of digestion simulations done (two for treatment) was probably too low to produce more marked differences, or to show differences at lower dose ranges. However, the reduction in E. coli populations with 10000 ppm of XT is in coincidence with the results obtained for the gas production in the closed system.

This method is especially interesting to study without any other interference the relations between the different bacteria and the effect of the additives on these bacteria. Obviously, many responses present in the animal like the immune response are not represented in this method. However, TIM-1 presents the advantage of the continuous dialysis system, compared to other *in vitro* methods. This fact, even being just passive dialysis, avoids i) *ad libitum* nutrient supply for the bacteria and ii) saturation of the media with bacterial metabolites. These characteristics promote a competitive situation among bacteria very similar to the one produced in the animal GIT.

In the future this method should be improved to add more bacterial groups to the artificial bacterial population used.

CHAPTER 8:

GENERAL REMARKS AND DISCUSSION ON THE METHODS

"A veces, la razón no hay que darsela al que la tiene sino al que la necesita"

El joven

The particular effects of XT in each experiment have been discussed in previous chapters. However, the author considers that one important part of the work presented here concerns the methodology used. This kind of experiments becomes sometimes real *in vivo* screening trials. Thus it is very important to optimize the efforts and to use the most successful criteria in detecting the effects of the additives. The validity of the results obtained by each methodology will be discussed, especially regarding the meaning of some of the "indexes" used.

A lot of different physiological parameters have been proposed as indicators of the health status of the piglet when using AGP (François, 1962). However, none of these parameters give, by themselves, a clear idea of the ability of the animal to face an eventual stressing situation. Jensen et al. (2003) enumerated many important variables to study in the GIT in this kind of investigations, such as: morphology, motility, mucus production and characteristics, trans-epithelial permeability, immunity, enzymes, pancreatic secretion and microbiota. We have used only some of these variables to evaluate the effect of XT added to the diet of the weaned pig.

The main characteristic of the results of these experiments is their low consistency among experiments. Results obtained using these PE are very affected by dietary factors, or by the conditions surrounding the experiment. However, this variability is a permanent characteristic when we evaluate alternatives to AGP. Furthermore, from both the literature and our own experience, it appears that, so far, no substance shows the same effects presented by antibiotics.

8.1. PRODUCTIVE PERFORMANCE

Two facts are very clear, when AGP are not used lower growth is obtained and more therapeutic antibiotics have to be used. Thus, the main indicators of the success of any alternative to the AGP must be better growth rates and an improved health status of the animals. In fact, both growth rates and health status can be considered as one because healthy animals are growing at higher rates. Thus, production performance must be recorded in any study of alternatives to AGP. However, the production performance validity of one, two or three experiments is questionable even when results are positive. In the present investigations with the XT, one experiment showed effects on productive performance, one showed an effect on diarrhea occurrence and the other one showed no effect on productive performance at all. With such data is difficult to conclude that XT has a growth promoter effect but encourage to continue studying and improving XT to

achieve better results in all kind of situations. In general, to show significant production performance improvements caused by the inclusion of an additive in the diet is not always easy, since many times the number of animals or the replicates are insufficient or the environment is not the adequate.

Normal differences in productive performances due to antibiotic inclusion are 5-10% of ADG increase, and normal variation coefficients (VC) for piglet's weight are 10-15% (Aaron and Hays (2000)). If we use the table presented by Aaron and Hays (2000) to calculate the number of replicates needed for this kind of experiments (table 8.1), we can see that when the VC of ADG is 10% we need 64 replications / treatment to detect differences of 5% in this variable. When we review the typical experiments analyzing the effects of alternatives to AGP, we can see that this high amount of replicates is rarely used. Then, only some experiments show differences in productive performance and the conclusions should be obtained from accumulation of data from different experiments through techniques as meta-analyses.

Table 8.1. Estimated number of replications needed in growing pig experiments. A randomized complete block design with four treatments, two-tailed test of significance, and an 80% of power is assumed. 4 to 8 animal per replicates considered. Results presented only for $\alpha = 0.05$. Adapted from Aaron and Hays (2000).

	Expected difference in rate of gain or feed efficiency (% mean)		
Average coefficient of variation	2.5	5	10
15	565	99	36
10	252	64	17
7.5	142	36	10

Concerning the number of animals, it is interesting to remark also the importance of the experimental unit. The first and the second experiments were done with more than 200 animals, but in fact only four replicates were used for each treatment. This is a very dangerous design because the intra-group variability can be hiding important effects. For example, in the case of the experiment presenting diarrhea, the animals suffered it in very different intensities and as a consequence the intra-group variability was increased even if the group mean was the same. Thus, the validity of this result is relative. On the

other hand, in the third experiment the differences became significant, which could be a result of the individual weight control, which gives us 8 replicates for each treatment.

Concerning the environment it is interesting to remark that, in normal commercial practice, the worst the health and environmental status of the animals the better the improvements obtained using AGP (Page, 2003). It is normal to obtain lower effects of the AGP on animals in research institutes or university facilities than in commercial ones (Page, 2003). It could be due to the better environmental conditions of the research facilities resulting in a lower pressure to challenge animal health. In our case, the first and second experiments were carried out in commercial facilities and the third one was done in the university facilities. Why we obtained significant differences in the third experiment despite being carried out in the university can be explained not only by the higher number of replicates but also by an accessory environmental observation. The first and second experiments were done with animals coming from one farm presenting some sporadic diarrhea episodes and were allocated to commercial, relatively new, facilities with strict biosecurity rules. The animals used in the third experiment were obtained from a farm presenting a very good health status (no diarrhea episodes registered and serology negative to Aujeszky, PRRS, Influenza, *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis*) and coming from a zone of low density of pig production. However, they were allocated to old metallic facilities with no biosecurity rules. This change to a worst environment could produce and health stress to the pigs maximizing their response to additives.

Table 8.2. Means for ADG and G:F of animals in experiments 1 and 3 separated as control or treated animals. The means represent the productive results of animals weaned at 21 days of age and studied during the 14 days after wenaing.

	Experiment 1, data from table 4.3.		Experiment 3, data from table 6.3.	
	Control	Treatments	Control	Treatments
ADG, g	177, 187, 178	176, 170, 173	125	177, 177, 166
G:F	0.70, 0.74, 0.78	0.69, 0.76, 0.74	0.53	0.64*, 0.69*, 0.55

*This means were significantly different to control.

Table 8.2 summarizes the means for ADG and G:F of experiments 1 and 3 separated as control or treated animals. No statistical analysis has been applied to these data because they are difficult to compare directly. No differences due to treatments were found in experiment 1, and the means of control and treated animals in experiment 1 were similar

to those of treated animals in experiment 3. However, experiment 3 showed differences between treated and control animals. It seems that the control group in experiment 3 was worse than the other animals and that is why differences appeared. This fact is indicating the importance of controlling environment in this kind of experiments.

8.2. PHYSICAL MEASUREMENTS AND DIGESTIBILTY

Some physical measurements (organ weights, pH, DM) have been used in these experiments. In spite of being very simple and somehow crude techniques, they detected changes which have great relevance on the animal performance and, in particular, on the intestinal ecosystem. For instance, the relation between full stomach weight and pH found in the second experiment is very relevant for the intestinal environment and is probably related to changes found in lower parts of the GIT. On the other hand, low DM content of the hindgut content has been related to animals suffering diarrhea. This fact is just a field observation not analyzed in the experiments discussions but one can see how animals presenting lower DM contents present alterations in other studied parameters systematically.

Digestibility is also a parameter which has been normally related to feed efficiency in healthy animals and, provided the same feed intake, higher digestibility coefficients are reflected in better performances. However, in the case of the piglet, the digestibility by itself is not determining the growth rate as much as the health status of the animal. For example, in experiments 1 and 3 the animal performance was studied in the first two weeks after weaning and ADG of 170-180 g/day and ADFI of 240-250 g/day were obtained. In these situations where feed intake is still very low, small health problems can represent a very important nutrient consumption as a percentage of the total ingestion. This fact makes digestibility a parameter of relative importance.

On the other hand, it must be accepted that the techniques used in these experiments to measure digestibility coefficients may be thoroughly criticized (Jagger et al., 1992; Yin et al., 2000) because of the variable recovery of the marker. Even with the homogenization of the pattern of intake applied in the experiments, the variability is high and the validity of the sample is not sure. Furthermore, the efficiency of the analytical method for measuring chromium in feces and digesta has been questioned and several new techniques have been proposed (Fenton and Fenton, 1979; Aguilera et al., 1988). Even if we accept chromium oxide as an indigestible digestibility marker very

often, only low amounts of ileal samples were obtained (2-5 g of DM). These low quantities could not be representative of the total content.

8.3. MICROBIAL POPULATIONS

One of the most consistent results presented in the experiments was the increase in lactobacilli counts with XT inclusion. This change was observed in the first two experiments by agar plate counts but not in the third one. However in the third experiment, this change was found by quantitative PCR in the cecum by Castillo et al. (in press). It is not clear how this change is produced, but it is not necessarily a direct effect of the XT on microbiota.

In any case, the important question is, how relevant is the increase of lactobacilli for the animal growth? Lactobacillus count is one of the most studied parameters concerning intestinal environment in the piglet and many species of this genera are used as probiotics (Fuller, 1992). This is a parameter imported from human medicine, where it has been related to some beneficial effects. However, no clear evidences exist of the benefits of high lactobacilli populations for animal growth. On the other hand, enterobacterias are normally assumed as deleterious bacteria because some pathogens (E.coli, Salmonella) belong to this group. This conception can be extremely wrong since total enterobacteria do not have to be related to the presence of pathogenic enterobacteria. Only the presence of these pathogenic bacteria can be assumed as deleterious by itself. In fact, the first experiments with AGP showed increases in enterobacterias as a favorable change as reviewed by François (1962).

Comparing the means of the different experiments here presented (table 8.3) one can observe how the mean lactobacilli counts is similar for all experiments. However, enterobacteria varied depending on the experiment.

Table 8.3. Means for lactobacilli, enterobacterias and their relation in the three *in vivo* experiments.

	Experiment 1, data from table 4.5.	Experiment 2, Data from table 5.5.	Experiment 3, Data from table 6.5.
Lactobacilli	7.9	7.9	7.9
Enterobacteria	6.4	5.8	4.8
Relation	1.5	2.1	3.1

Enterobacteria counts were similar in experiments 1 and 2, and lower in experiment 3, even though the animals from experiments 1 and 3 were of the same age and were fed similar diets. In fact, control animals in experiment 3 presented a very low enterobacterias counts compared with all other animal groups. These animals presented also the worst growth rates.

Probably too much importance has been given to only two or three bacterial groups, even when they are not the most important in number. In contrast, groups of high relevance, such as clostridium, are rarely studied. Perhaps, lactobacilli and enterobacteria or E. coli have become an acceptable commercial index, but their validity is highly questionable.

Currently, new molecular techniques minimize the role of the main groups of bacteria studied by classic methods. New important populations are being described and the first molecular studies about the metabolic role of some bacterial groups are appearing (Zoedental et al., 2004). New microbiological parameters indicating the health status of the animal are also appearing, i.e. biodiversity (Zoodental et al., 2004; Castillo et al., in press) or the fermentation pattern.

Although it is not a properly defined parameter yet, different approaches to biodiversity indicate that a high biodiversity make the intestinal ecosystem stronger against pathogenic aggressions (Zoodental et al., 2004). It is necessary to check the relevance of this parameter on production performances, however, the results obtained by Castillo et al. (in press), using the same animals of the third experiment, illustrate this possible relation. In figure 8.1 we can see how animals presenting better performances and better conversion ratios, treated with butyrate and avilamycin, showed also a higher microbial biodiversity in jejunum samples.

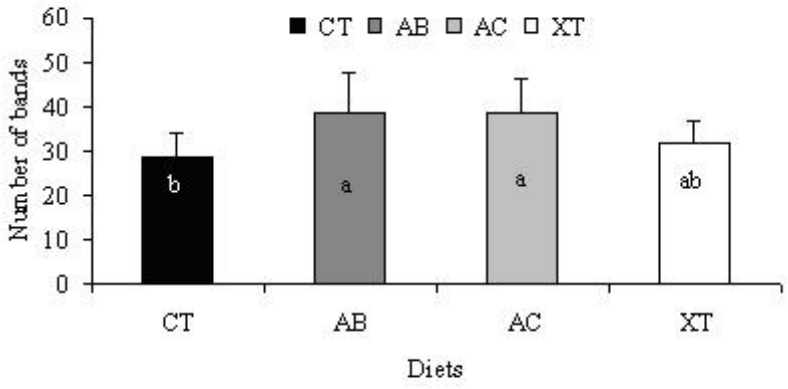


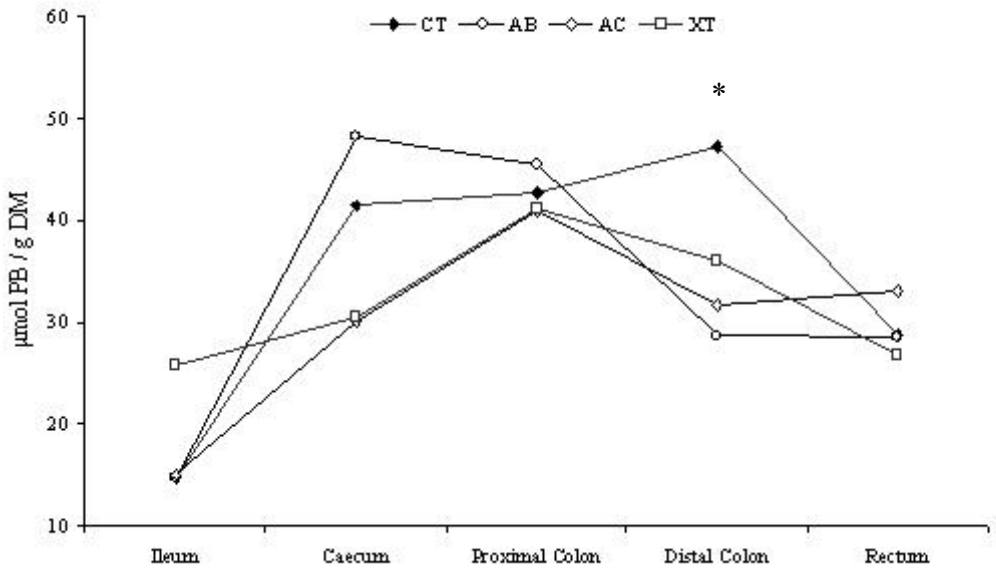
Figure 8.1. Biodiversity degree in samples of jejunum digesta, measured as total number of restriction bands obtained by PCR-RFLP in early-weaning pigs receiving a control diet (CT) or the same diet with 0.04 % avilamycin (AB); 0.3 % butyric acid (AC) and 0.03 % plant extract mixture (XT). (With permission of the authors)

Concerning fermentation patterns, it is illustrated i.e. by the evolution of the purine bases concentration in the animals of the third experiment. Animals with better performances, butyrate and avilamycin treated animals, presented lower purine bases in distal colon indicating lower bacterial activity (figure 8.2.). This pattern is similar to that observed in adult animals by Martinez-Puig et al. (2003) and can be related to a more mature hindgut.

Classic measurements of fermentation such as VFA can also provide us with interesting information about changes in fermentation of intestinal microbiota, and in fact some interesting changes have been described in these experiments. VFA are very useful indicating changes in fermentable substrates given a common microbiota (Fernandes et al., 2000), however, it has not been studied in depth how changes in microbiota affect fermentations, given a particular substrate.

In any case, what it is clear in these investigations is the strong effect of the different additives on the bacterial profile of the animals. This fact is shown in Figure 8.3. In this figure one can observe how bacterial populations are grouped depending on the additive present in the diet (data from experiment 3) even when other important parameters such

as the sow are included in the experimental design. More efforts describing what is exactly the change produced by AGP can help us in mimicking their effect.



(*) Shows that diets within an intestinal section differ significantly in value($p < 0.05$). Proc Mixed analysis showed significant differences between intestinal parts ($p<0.0001$) and in diet*intestinal section interaction ($p < 0.01$).

Figure 8.2. Purine bases (adenine + guanine) concentration (µmol/g DM) in digesta samples from ileum, caecum, proximal colon, distal colon and rectum in early-weaning pigs receiving a control diet (CT) or the same diet with 0.04 % avilamycin (AB); 0.3 % butyric acid (AC) or 0.03 % plant extract mixture (XT).

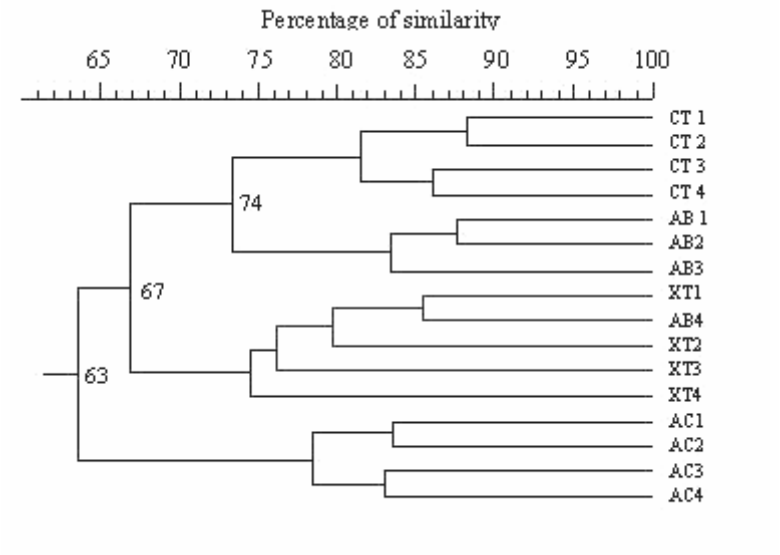


Figure 8.3. Dendogram (percentage of similarity) obtained by PCR-RFLP in 4 early-weaning pigs/treatment (experiment 3) receiving a control diet (CT) or the same diet with 0.04 % avilamycin (AB); 0.3 % butyric acid (AC) and 0.03 % plant extract mixture (XT). (With permission of the authors)

As a general remark, microbiological parameters will be extensively developed in the future because of the enormous advances of molecular biology, even though a great mathematic effort is required to integrate the obtained information. This evolution in microbiological parameters will change the current conception of the intestinal microbiology. However, microbiology by itself is not always a parameter indicating health or illness and need to be complemented with other parameters, in this case especially with intestinal epithelium structure and immune system.

8.4. EPITHELIAL INTEGRITY AND IMMUNITY

Villi or crypt length are very important parameters measured in most of additive evaluations since it was related to the weaning process by Hampson (1986) and to diarrhea intensity by Nabuurs (1993). These parameters can indicate changes in epithelium dynamics but can also drive us to wrong conclusions. A clear example is to assume that deeper crypts indicate a reaction of the epithelium to compensate villi erosion. This is true only when no variations in mitosis are occurring (Smith, 1992).

Cell production rate can by unaffected if we found deeper crypts but lower mitosis index.

On the other hand, the cause-effect relation of these parameters and diarrhea appearance is not totally defined, at least as a direct positive correlation. It seems that decreases in villi length and increases in crypt depth are normal at weaning within some limits (Vente-Spreeuwenberg et al., 2003). When diarrhea appears these limits are trespassed, but it is not clear if changes in villi and crypt are a cause of diarrhea or just a consequence. These parameters can be complemented by some other descriptive parameters related with functionality of the epithelium such as measurements of inflammation or permeability, which show the consistency of epithelial barrier. Some new parameters have appeared in the last years, i.e. translocation measurements (Taylor et al., 1995) or expression of binding sites for bacteria (Jeyasingham et al., 1999), which gives a more relevant description of the epithelium in diarrhea predisposition.

In this relation between the intestinal microbiota and the animal, also immune parameters seem to be a very useful tool to understand how the piglet is reacting against the aggression (weaning). Moreover, these parameters are altered even when no pathology is present and can indicate a possible sub-clinic problem. The interest of the immune parameters is different from microbiology measurements because immunity is the response of the animal and thus is indicating an active effort which can be directly translated to nutrient consumption and lower G:F records. However, changes in immune parameters may not be considered good or bad by themselves, but depend on the other variations in the immune response. For instance, a lower IEL presence must be understood as a re-organization in front of a change situation but a generalized unspecific response could be translated to an excessive nutrient waste.

IEL and lamina propia lymphocytes numbers (used in these experiments) have been shown as parameters related to the local immune response of the animal (Jiang et al., 2000) in an experiment studying the effect of spray dried porcine plasma (SDPP) in the piglet. These changes could also be the indirect consequence of the variations in microbiota or diet. However, these two parameters are still too crude, and more developed techniques must be applied in these experiments as CD markers or immune mediators. As an example, in a parallel study using animals from experiment 3 (table 8.4), we studied the blood distribution of white cells and some tissular (Peyer patches and mesenteric nodes) marked cells in control (Nofrarias, personal communication), XT and an extra SDPP treatment groups. The SDPP treatment showed a decrease in the %

of monocytes in blood and also in the SWC3 marker in the intestine. The cellular markers allow us to relate the variation to a particular immune response, in this case, SDPP treated animals presented a lower inespecific immune response. This change was accompanied by a lower lamina propia cell density as found by Jiang et al. (2000) (result not shown).

Table 8.4. Hemogram, and flow cytometry measurements of different marked cells (% of cells) from immune ileocecal node and ileal peyer patches from animals used in experiment 3 and a parallel group fed control diet but containing spray dried porcine plasma instead of soycomil protein concentrate. (with permission of the author)

	Control	Xtract	Spray Dried Porcine Plasma
Blood			
Leucocyte number	20125 ± 7690	17260 ± 2823	19388 ± 4782
% limfocyte	50,8 ± 5,7	43,8 ± 5,9	50,8 ± 13,2
% monocyte	7,3 ± 2,2 **a**	5,2 ± 1,8 **ab**	3,8 ± 1,6 **b**
% neutrofile	40,3 ± 5	49,6 ± 6,4	44,3 ± 13,7
% eosinofile	1,8 ± 0,5	1,4 ± 0,5	1,3 ± 0,7
Intestinal Node			
CD45	99,2 ± 0,5	99,5 ± 0,2	99,2 ± 0,4
CD3	58,7 ± 8,5	57,3 ± 9,1	58,1 ± 7,5
SWC3	15,6 ± 3,5 **a**	15,7 ± 2,5 **a**	12,6 ± 2,1 **b**
CD21	41,6 ± 6,3 **a**	32 ± 7,7 **b**	33,2 ± 8,1 **b**
Peyer Patches			
CD45	98,4 ± 2,3	98,7 ± 0,8	99 ± 0,6
CD3	6,5 ± 3	5,6 ± 1	7,1 ± 2,1
SWC3	18,4 ± 6,2 **a**	21,6 ± 10,5 **a**	10,5 ± 2,6 **b**
CD21	34,6 ± 16	37,2 ± 16,2	38,2 ± 22,4

Marker used are CD45, for Leucocyte; SWC3, for Monocyte and macrophage; CD21, for B cell; for CD3 T cells.

8.5. *IN VITRO vs. IN VIVO* STUDIES AS A MODEL FOR ADDITIVES EFFECT IN THE EARLY-WEANING PIG

In this thesis only one closed *in vitro* system method to study some microbiological parameters has been used. There are other *in vitro* methods as presented by Oomen et al. (2002) which are applicable in different situations for microbiology studies and different methods also exist to study separately effects of additives in epithelium, as cell cultures (Roselli et al., in press) or Ussing chamber (Boudry et al., in press).

Given the complexities of the interactions occurring in the animal itself, *in vitro* systems are very useful to understand the effect of an additive in each part of the intestinal ecosystem. These methods are a necessity in human studies because of obvious ethic

limitations but to some extent when it is possible ethics induce us to use these methods in animal science instead of *in vivo* studies. This is the case of exploratory studies, dose determinations for antimicrobial effects, etc. However, *in vitro* systems are too often directly imported from human studies and are not adapted enough to animal studies. One clear example is the use of fecal inocula for fermentation experiments. This is a normal practice for humans but in animals it has no sense because there is the possibility of cannulation or sacrifice. In the method here used we tried to simulate more closely the intestinal situation in the pig, like Macheboeuf (2004a and b) did recently for ruminants. Ethic conditions were respected because animals were sacrificed for other experiments and we obtained very valuable results.

Methods highly sofisticated such as TIM 1 are sometimes a very useful tool given their accuracy and flexibility but benefits are limited by the complexity of the method.

8.6. USING PLANT EXTRACTS. What have we learned?

We have studied only some of the possible effects of the PE by using only some of these substances. It remains unclear i.e. what is the effect of this substances on enzyme secretion, which could be very important. We must do more systematic efforts in characterizing these effects, which could give really new alternative ways to improve animal performance.

Concerning the experiments here presented we must discuss two main points: dose and commercial form. From the data obtained we can see influences in very different parameters, which are indicating the wide effect of these substances even at very low dosage. From the *in vitro* experiments, it is known that usual commercial concentrations have no antimicrobial properties, so probably no direct effect is obtained in the microbiota with animals fed XT. These effects may be related to other changes observed as discussed above. We showed that antimicrobial concentrations acting in intestinal content are higher than 500 ppm but concentrations needed to obtain other effects are lower, as happens with the higher retention time of the stomach due to capsaicin (Chang et al., 1999).

Higher doses than the ones used are too expensive for farm animal utilization so companies normally have no interest in studies using these doses. These higher doses must be studied to know the real possibilities of these products as antimicrobials, their

effects on animal ingestion, etc. Fortunately, these doses appear in recent experiments in scientific journals (Oetting et al., 2004; Son et al., 2004).

Concerning commercial forms, many companies are currently producing different PE products for animal production. However, PE are products with difficult commercial differentiation because they are natural products and everyone can sell the same product from a known composition. The most used commercial differentiation strategies are:

- Secret composition
- Mixtures of different substances, other extracts or other alternatives.
- Encapsulations or other pharmaceutical forms.

From a scientific point of view, the benefit for the scientific community of papers presenting results of products of secret composition is very relative. Mixtures of different substances with very different effects (i.e. capsaicin in these experiments) need to be studied by itself in a separate way firstly, when no bibliography data are available. In fact, this kind of mixtures will be avoided in the future by EU authorities. Finally, encapsulation or other pharmaceutical forms are good options, especially for PE because of their hydrophobic characteristics. Through this method it can avoid losses of the substances due to association with fats, it can delivery the substance in a particular place and time being more effective, and it can even avoid negative palatability problems.

CHAPTER 9.

CONCLUSIONS

"Si es así, es así"

Marga Martín

1. Weaning pig diets supplementation with XT induce variations in performance, digestibility, microbial populations and VFA production, epithelium structure and immune cells organization. However, most of the changes are not consistent between experiments and/or experimental conditions.

2. The effects of XT on intestinal bacterial populations, epithelial structure and digestibility are affected by protein level in a range between 18 and 20 % of CP. The higher CP level, the lower the effects. On the other hand, protein source (fish meal vs. soybean) produce no effects in this parameters.

3. Formic acid at undercommercial doses (0.5 %) and XT at commercial levels diminish the gastric retention time of the feed in coincidence with a transitory increase of gastric pH in early weaned pig. The effect is not additive when both products are used in combination. However the coincidence of a lower severity of diarrhea produced by XT togheter with a better conversion rate brought about by formic acid recommends the use of both additives combined.

4. In our experimental conditions, commercial doses of XT included in the diet during two weeks before weaning, improve the ADG without affecting growth:feed ratio. This growth:feed ratio is significantly improved by avilamycin and sodium butyrate

5. *In vitro* results show that concentrations of XT normally added to the feed at commercial conditions have no direct antimicrobial effects measured both, as gas production or enterobacteria and lactobacilli counts in the contents of the stomach, jejunum and cecum. Doses have to increase up to 10.000 ppm to found any antimicrobial effect.

6. Effects of carvacrol, cinnamaldehyde and capsicum oleoresin studied in incubated intestinal content are more marked in jejunum than in stomach and cecum content. Carvacrol and cinnamaldehyde show a higher antimicrobial effect than capsicum oleoresin.

CHAPTER 10.

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