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EVALUATION OF EFFICACY OF AN ADDITIVE BASED ON ESSENTIAL OILS IN FRONT OF AN *ESCHERICHIA COLI K88* ORAL CHALLENGE IN WEANLING PIGLETS

EVALUACION DE LA EFICACIA DE UN ADITIVO BASADO EN ACEITES ESCENCIALES FRENTE A UN DESAFIO ORAL DE *ESCHERICHIA COLI* K88 EN LECHONES DE DESTETE

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Summary

Phytogenic feed additives are characterized to contain botanic compounds biologically active with organoleptic and medical properties. Globally their use in feeding strategies or as alternative to growth promoters for animal production has increased, especially during the weaning period. The high stress and the abrupt feeding changes experienced by the piglet in this period have a negative impact on voluntary feed intake, which may promote the presence of enteropathies that affect piglet performance and increase mortality rates.

The main objective of this study was to evaluate the potential of two phytogenic feed additives in weanling pigs to enhance gut health at early life stages and to fight intestinal pathogens.

To achieve the objective, a trial was designed to evaluate the potential of two different phytogenic feed additives in weanling piglets in front of an Enterotoxigenic *Escherichia coli* K88 (ETEC K88) oral challenge. In brief, 96 weanling piglets were transported to the University facilities from commercial farm with mothers that did not received *E. coli* vaccination. Animals were distributed in 4 rooms of 8 pens each (32 pens, three animals per pen). The experiment included four treatment groups with eight replicates per each including: (i) a control group with plain diet (T1); (ii) the same plain diet supplemented with ZnO (2500 ppm Zn) (T2); (iii) supplemented with phytogenic 1 (1kg/tm) (T3); (iv) or supplemented with phytogenic 1 (1kg/tm) plus phytogenic 2 (1.5 kg/tm) (T4). Pigs were fed over 16 days *ad libitum* with the experimental diets. After an adaptation period, animals were orally challenged with ETEC K88 and one animal per pen was euthanized at day 8 post-inoculation (PI). In this experiment, main parameters assessed were animal performance, clinical signs, bacterial loads in the gut, immune response and intestinal morphology.

In general terms, we were not able to find significant differences in performance comparing the phytogenic diets to the plain diet, although some trends could be found. ZnO supplementation increased (P = 0.03) average daily gain (ADG) in the 0-4 PI period compared to pigs fed treatment T1, and treatments T3 & T4 showed intermediate

values. The same pattern was observed with gain:feed ratio (G:F; P = 0.03) in the same period. Additionally, some significant effects were found in other parameters. The numbers of lactobacilli in feces and colon digesta on day 8 PI were the highest (P = 0.0007; P = 0.03, respectively) with diets supplemented with phytogenics (T3 & T4) and the lowest with the diet including ZnO (T2). The ratio *lactobacilli:coliformrs* in feces on day 8 PI was significantly increased (P=0.002) by the inclusion of the T3 treatment when compared to T1 and T2, showing T4 intermediate values. Phytogenics did not affect the serological concentration of TNF-α. There was a trend at day 4 PI in Pig-MAP to decrease with T4 (P=0.07). Villus:crypt (VH:CD) ratio was significantly increased (P=0.009) by the inclusion of ZnO and also by the T4 treatment compared to T1, showing T3 intermediate values.

In conclusion, both tested phytogenics could help the piglet to fight the ETEC challenges after weaning considering the numerical improvements observed in performance immediately after the challenge. This better response could be due to an improved microbiota balance suggested by the increased ratio of *lactobacilli:coliforms* observed in feces on day 8 PI, particularly in the T3 treatment. Additionally the phytogenic T4 shows enhanced anti-inflammatory properties with reductions in Pig-MAP serological levels concomitant with an improvement in the intestinal architecture (VH:CD ratio).

Resumen

Los aditivos fitogénicos se caracterizan por contener compuestos botánicos biológicamente activos con propiedades organolépticas y medicinales. A nivel mundial, su uso como estrategia nutricional o como alternativas a los promotores de crecimiento en la producción animal ha incrementado, especialmente durante la etapa del destete. El alto nivel de estrés y el cambio abrupto de alimentación experimentados por los lechones en este periodo tiene impactos negativos en la ingesta voluntaria de alimentos, lo que pueden promover la presencia de enteropatías que afectan al rendimiento de los lechones y aumentan las tasas de mortalidad.

El objetivo principal de este estudio fue evaluar el potencial de dos aditivos fitogénicos en lechones destetados para mejorar la salud intestinal en temprana edad de vida y combatir patógenos intestinales.

Para lograr este objetivo, se diseñó un estudio para evaluar el potencial de dos diferentes aditivos fitogénicos en lechones destetados frente a un desafío oral por *Escherichia coli* Enterotoxigenica K88 (ETEC K88). En resume, 96 lechones destetados fueron transportados a las instalaciones de la Universidad desde una granja comercial con madres que no recibieron la vacuna contra E. *coli.* Los animales fueron distribuidos en 4 salas de 8 corrales cada una (32 corrales, tres animales por corral). El experimento incluyó cuatro grupos de tratamientos con ocho réplicas de cada uno, incluyendo: (i) un grupo control con la dieta base (T1); (ii) la misma dieta base suplementada con ZnO (2500 ppm Zn) (T2); (iii) suplementada con fitogénico 1 (1kg/tn) (T3); (iv) o suplementada con fitogénico 1 (1kg/tn) más fitogénico 2 (1.5 kg/tn) (T4). Los lechones fueron alimentados durante 16 días *ad libitum* con las dietas experimentales. Después de un periodo de adaptación, los animales fueron desafiados oralmente con ETEC K88 y un animal por corral fue sacrificado al día 8 post-inoculacion (PI). En este experimento, los principales parámetros evaluados fueron el rendimiento animal, los signos clínicos, las cargas bacterianas en el intestino, la respuesta inmune y la morfología intestinal.

En términos generales, no se observó diferencias significativas en rendimientos productivos comparando las dietas que incluían fitogénicos con la dieta base, aunque se

pudieron encontrar algunas tendencias. La suplementación con ZnO incremento (P=0.03) la ganancia media diaria (ADG) en el periodo 0-4 PI comparado con lechones alimentados con el tratamiento T1 y los tratamientos T3 & T4 mostraron valores intermedios. Se observó el mismo patrón con la relación gain:feed (G:F) (P=0.03) en el mismo periodo. Adicionalmente se observaron algunos efectos significativos en otros parámetros. El recuento de lactobacilos en heces y en la digesta de colon en el dia 8 PI fueron los más altos (P = 0.0007; P = 0.03, respectivamente) con las dietas suplementadas con fitogénicos (T3 & T4) y los más bajos con la dieta que incluía ZnO (T2). El ratio *lactobacilos:coliformes* en heces en el día 8 se incrementó significativamente (P=0.002) por la inclusión del tratamiento T3 en comparación con T1 y T2, mostrando valores intermedios el tratamiento T4. Los fitogénicos no afectaron la concentración serológica de TNF-α, aunque se encontró una reducción numérica (P=0.12) en el día 4 PI. Hubo una tendencia en el día 4 PI en Pig-MAP para disminuir con el T4 (P=0.007). El ratio vellosidad:cripta aumento significativamente (P=0.009) con la inclusión de ZnO y también con el tratamiento T4 en comparación con el T1, y el T3 mostro valores intermedios.

En conclusión, ambos aditivos fitogénicos probados podrían ayudar al lechón a combatir los desafíos de ETEC después del destete, considerando las mejoras numéricas observadas en el rendimiento inmediatamente después del desafío. Esta mejor respuesta podría deberse a un mejor equilibrio de la microbiota sugerido por el aumento de la proporción de *lactobacilos:coliformes* observados en heces el día 8 PI, particularmente en el tratamiento T3. Además, el aditivo fitogénico T4 muestra propiedades antiinflamatorias con la reducción en los niveles serológicos de Pig-MAP concurrente con una mejora en la arquitectura intestinal (ratio VH:CD).

Résumé

Les additifs phytogéniques sont caractérisés par le fait qu'ils contiennent des composés botaniques biologiquement actifs aux propriétés organoleptiques et médicinales. Au niveau mondial, son utilisation en tant que stratégie nutritionnelle ou en tant qu'alternative aux facteurs de croissance de la production animale a augmenté, en particulier au stade du sevrage. Le niveau élevé de stress et de brusques changements de régime subis par les porcelets au cours de cette période ont des effets négatifs sur la consommation alimentaire volontaire, ce qui peut favoriser la présence d'entéropathies qui affectent les performances des porcelets et augmentent les taux de mortalité.

L'objectif principal de cette étude était d'évaluer le potentiel de deux additifs phytogéniques chez les porcelets sevrés pour améliorer la santé intestinale à un âge précoce et pour lutter contre les agents pathogènes intestinaux.

Pour atteindre cet objectif, une étude a été conçue pour évaluer le potentiel de deux additifs phytogéniques différents chez des porcelets sevrés contre une provocation orale par *Escherichia coli* Enterotoxigenica K88 (ETEC K88). En résumé, 96 porcelets sevrés ont été transportés vers les installations de l'Université depuis une ferme commerciale avec des mères n'ayant pas reçu le vaccin contre E. *coli.* Les animaux ont été répartis dans 4 chambres de 8 enclos chacune (32 enclos, trois animaux par enclos). L'expérience comprenait quatre groupes de traitement avec huit répétitions de chacun, comprenant : (i) un groupe témoin avec le régime de base (T1); (ii) le même régime alimentaire de base supplémenté en ZnO (2500 ppm de Zn) (T2); (iii) supplémenté avec un phytogène 1 (1kg / tn) (T3); (iv) ou supplémenté avec phytogénique 1 (1kg / tn) plus phytogénique 2 (1,5 kg / tn) (T4). Les porcelets ont été nourris pendant 16 jours ad libitum avec les régimes expérimentaux. Après une période d'adaptation, les animaux ont été soumis à une provocation orale avec ETEC K88 et un animal par enclos a été sacrifié au huitième jour après l'inoculation (PI). Dans cette expérience, les principaux paramètres évalués étaient les performances des animaux, les signes cliniques, les charges bactériennes dans l'intestin, la réponse immunitaire et la morphologie intestinale.

En termes généraux, aucune différence significative n'a été observée dans les rendements de production comparant les régimes comprenant des phytogéniques au régime de base, bien que certaines tendances puissent être trouvées. La supplémentation en ZnO a augmenté (P = 0,03) le gain quotidien moyen (ADG) au cours de la période allant de 0 à 4 PI par rapport aux porcelets nourris avec le traitement T1 et les traitements T3 et T4 ont présenté des valeurs intermédiaires. La même tendance a été observée avec le rapport gain: aliment (G: F) (P = 0,03) au cours de la même période. De plus, certains effets significatifs ont été observés dans d'autres paramètres. Le nombre de lactobacilles dans les matières fécales et dans le digesta du côlon au jour 8 était le plus élevé (P = 0,0007, P = 0,03, respectivement) avec les régimes supplémentés en phytogéniques (T3 et T4) et les plus bas avec le régime qui comprenait ZnO (T2). Le rapport lactobacilles: coliformes dans les matières fécales au 8ème jour augmentait significativement (P = 0,002) en raison de l'inclusion du traitement T3 par rapport à T1 et T2, le traitement T4 montrant des valeurs intermédiaires. La phytogénétique n'a pas d'effet sur la concentration sérologique de TNF-α, bien qu'une réduction numérique (P = 0,12) ait été constatée au jour 4. Au 4ème jour, l'IP du Pig-MAP avait tendance à diminuer avec la T4 (P = 0,007). Le rapport villosité: crypte a augmenté de manière significative (P = 0,009) avec l'inclusion de ZnO et également avec le traitement à la T4 par rapport à la T1 et la T3 a présenté des valeurs intermédiaires.

En conclusion, les deux additifs phytogénétiques testés pourraient aider le porcelet à relever les défis posés par ETEC après le sevrage, compte tenu des améliorations numériques observées dans les performances immédiatement après le défi. Cette meilleure réponse pourrait être due à un meilleur équilibre du microbiote suggéré par l'augmentation de la proportion de lactobacilles: coliformes observés dans les matières fécales au 8ème jour PI, en particulier dans le traitement T3. De plus, l'additif phytogénique T4 présente des propriétés anti-inflammatoires avec réduction des niveaux sérologiques de Pig-MAP concomitant avec amélioration de l'architecture intestinale (rapport VH: CD).

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Abbreviations

- ADFI: average daily feed intake.
- ADG: average daily gain.
- AGPs: antibiotic growth promoter.
- ANOVA: analysis of variance.
- BPW: buffered peptone water.
- BHI: brain heart infusion.
- BW: body weight.
- CFU: colony formed units.
- CE: European Commission.
- D: days
- DM: dry matter.
- EO: Essential oils.
- ETEC: enterotoxigenic *Escherichia coli.*
- FM: fresh matter.
- GC: goblet cells.
- G:F : Gain-Feed (ratio).
- GLM: general linear model.
- IEL: intraepithelial lymphocytes.
- PI: post inoculation.
- Pig Map: Pig Major Acute-phase Protein.
- PWD: post weaning diarrhea
- ROS: reactive oxygen species.
- RSD: residual standard deviation.
- TNF–α: Tumor Necrosis Factor.
- T1: control group with plain diet.
- T2: group with the same plain diet supplemented with ZnO.
- T3: group with the same plain diet supplemented with *phytogenic 1.*
- T4: group with the same plain diet supplemented with *phytogenic 1* plus *phytogenic 2.*

1. Literature review

1.1. Current situation in swine production

Weaning is one of the most critical periods of pigs' life, which has severe consequences on productive performance through their full productive life. In commercial conditions piglets are weaning around 3 or 4 weeks of life because economical reasons. However, at this early age, weaning represents a big challenge for the pig, because they are exposed to new social partners, change of diet and decrease in immunology (Lallès et al., 2007). This is maybe the period which causes the biggest stress for the piglet, and because of that we can see a decrease on feed intake. This decrease entails low weight gain and high diarrhea incidence, which could even lead to death (Bolhuis et al., 2009). All in all, weaning is a difficult period for piglets to learn to eat a different food from the one they are used to. Moreover, this period causes a decrease in nutrients supply and their digestive capacity.

Factors mentioned above can trigger, *Escherichia coli* post weaning diarrhea (PWD), also called post weaning enteric colibacilosis, as an important cause of death in weaned pigs and occurs worldwide. Enteric *E. coli* infection in weaned piglets may also manifest as a diarrhea which usually occurs during the first week of post weaning and often results in decreased weight gain. This may be because of several factors, such as the stress of weaning, lack of antibodies originating from the sow's milk, and dietary changes. All together contribute to the severity of this disease. Post weaning diarrhea caused by *E. coli* is associated with an enormous proliferation of *E. coli,* and then their colonization in the small intestine through bacterial attachment to receptors on the small intestinal epithelium or in the mucus coating the epithelium. The PWD due to *E. coli* is caused primarily by enterotoxigenic *Escherichia coli* (ETEC), a pathotype that is characterized by production of adhesins that mediate bacterial adherence to the intestine and enterotoxins that cause diarrhea.

In general, pigs infected with ETEC can show watery diarrhea which lasts about 1 to 5 days after ETEC infection. Some pigs show sudden death without diarrhea symptoms but with intestinal edema. Diarrhea usually results in a significant dehydration, either due to a failure of the intestine to reabsorb or absorb fluid or due to a great increase in fluid secreted into the intestine (Sun and Kim, 2017).

Due to all the factors mentioned above, antimicrobial compounds have been extensively used in the past normally for three main purposes: at therapeutic and prophylactic doses to cure or prevent diseases, and as antibiotic growth promoters (AGPs) at sub-therapeutic quantities to increase animal growth rates and to improve feed efficiency. However, the strong political and social pressure to prevent antibiotic resistance in pathogenic microbiota, entailed in 2006 to European Union and subsequently several countries globally, to ban the supplementation of AGPs in animal feed. This decision lessens the productivity and profitability of animal production system (Tallard, 2015), but also provided an opportunity to implement better zootechnical practices and fundamental research to develop new feed additives (i.e. probiotic, prebiotic, acidifiers, enzymes, phytogenic. etc.) as alternative to AGPs in animal production (Karásková et al., 2015).

1.2. General Aspects of Phytogenic Feed Additives

Phytogenic feed additives (often called phytobiotics or botanicals) are plant-derived compounds which may have positive effects on animal growth and health due to their antibacterial, anti-inflammatory, and antioxidant properties (Yan et al., 2010; Zeng et al., 2015). In addition, their aromatic and oily characteristics have been shown to increase the palatability of feed with no flavor or aroma difference in the finished meat product (Holden and Mckean, 2001). Phytogenic substances utilized in phytogenic feed additives include herbs, spices, essential oils and non-volatile extracts, from, for example, clove, anise, thyme, fennel or Melissa, and many others (Table 1) (Máthé, 2007). Herbs, such as garlic, oregano, and thyme, are classified as non-woody flowering plants. Spices are herbs with a prolific smell or taste, such as cinnamon and ginger (Jacela et al., 2010). Essential oils are volatile lipophilic compounds derived by cold expression or by alcohol and steam distillation of herbs and other plants, and oleoresins are extracts derived from non-aqueous solvents (Windisch et al., 2008).

Commercially available phytogenic supplements can vary considerably in composition. Numerous studies have been conducted between 90´s until today with the objective of studying the effect of phytogenic in swine diets and to evaluate their ingredient

composition. Oregano, thyme, and cinnamon derivatives have been the most studies phytogenic, followed by ingredients derived from garlic, pepper, and citrus fruit. However, several additional plant sources have been also used, including clove, rosemary, anise, fenugreek, nettle, yam, and/or chicory powder. Due to their great diversity in chemical properties, low inclusion levels in diets, and relatively unknown stability through feed processing, studies involving phytogenic supplementation in swine are widely varied in results (Zeng et al., 2015).

The phytogenic effects on animals may be very diverse and mostly unknown, as they may depend on many factors, e.g. the dosage, botanical source, concentration of bioactive compounds, age of animals, duration of supply, feed composition of the diet, and the health status of the animals. The composition and qualities of the extracts from the same botanical family's plant will also depend on factors like, e.g. botanical and environmental manage, method of extraction, properties of the used extraction solvent, etc. While purified extracts contain only one active compound, unpurified extracts may contain several different molecules extracted with a certain solvent. Thus, the challenge to ensure a successful use of phytogenic feed additives in animal production is to know the chemical nature of their compounds, as well as, investigate the multitude modes of actions and implications on health, physiology and nutritional animal status (Doughari, 2012).

The [European Commission (CE) 1831/2003] defined feed additives as products used in animal nutrition for purposes to influence favorably the features of feedstuff and animal products, as well as, improve the performance and health of animals, without adverse effects on human health and environment. In addition, feed additives are categorized according their functions and properties in a) technological; b) sensory; c) nutritional and d) zootechnical. For their peculiar characteristics, phytogenic compounds are used in animal production mainly as sensory and zootechnical additives, especially to those critical phases when feeding activity, health and performance of the animals are highly compromised; like in weaning piglets, but also at early life span of poultry or restocking at young bull fattening.

Table 1. Herbs and parts thereof used in feed additives.

Common name	Latin name	Parts utilized[a]
Anise	*Pimpinella anisum*	Seeds
Caraway	*Carum carvi*	Seeds
Cinnamon	*Cinnamomum verum*	Bark
Chamomile	*Matricaria recutita*	Flowers
Citrus	*Citrus sp.*	Peel
Clove	*Syzygium aromaticum*	Buds
Fennel	*Foeniculum vulgare*	Seeds
Garlic	*Allium sativum*	Bulb
Ginger	*Zingiber officinale*	Rhizome
Melissa	*Melissa officinale*	Leaves
Onion	*Allium cepa*	Bulbs
Oregano	*Origanum vulgare*	Leaves
Peppermint	*Mentha piperita*	Leaves
Rosemary	*Rosmarinus officinalis*	Leaves
Sage	*Salvia officinalis*	Leaves
Thyme	*Thymus vulgaris*	Leaves
Valerian	*Valeriana officinalis*	Root, rhizome

[a] parts either used in complete or ground form to obtain extracts.

1.3. Phytogenics as source of bioactive compounds to animal nutrition and health

Therapeutic use of plants and their botanical compounds are not new, as they certainly go back in about third millennium BC. Hippocrates (ca. 460-377 BC), described a wide natural product of plants and animal origins for medicinal purposes (Doughari, 2012). Phytogenic or phytobiotic have been considered as bioactive, mainly to contain pharmaceutical and organoleptic properties to enhance or influence important physiological and metabolic effects in the organisms.

Phytogenics constitutes an important source of bioactive compounds that would explain the effect found for the different phytogenic feed additives, e.g. glycosides, polyphenols, terpenoids, flavonoids, alkaloids, polypeptides, and essential oils, etc. They are produced as secondary metabolites by plants to modify pigmentation, growth, reproduction, resistance to pathogens, attraction or signaling, and numerous other functions in the plant (Doughari et al., 2009).

General knowledge about the structure and function of bioactive molecules found in the most phytogenic feed additives, are summarized below (frankic et al., 2009)

a) Glycosides

Glycosides are defined as the condensation products of sugar (including polysaccharides) which, are colorless, crystalline carbon, hydrogen and oxygen containing (some contain nitrogen and sulfur) water-soluble phytoconstituents, found in the cell. The main groups of glycosides comprise saponins, cyanogenic and cardiac glycosides, and glucosinolates. Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin). Some compounds of this group have been related to properties that promote appetite and aid digestion. Glycosides commonly possess an intensely bitter taste. The bitter acts on gustatory nerves which results in increased flow of saliva and gastric juices (Firn, 2010).

b) Flavonoids

Generally, flavonoids are widely distributed among the plants and are responsible of plant resistance to photo-oxidation of ultraviolet light. Other important function in plants is the defense against herbivores, and as attraction element to pollinizer animals through the color and smell that give to plants. Structurally, they are made of more than one benzene ring (a range of C15 aromatic compounds) and numerous reports support their uses as antioxidants, antimicrobials and anti-carcinogenic. In addition, several flavonoids are pigments and induce the flavor in higher plants. Other group of flavonoids includes flavones, dihydroflavons, flavans, flavonols, anthocyanidins and proanthocyanidins (Doughari, 2012).

c) Phenolics

Phenolics, or polyphenol extracts are chemical components that occur ubiquitously as natural color pigments responsible for the color of fruits and plants. Phenolics in plants are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase. The most important role in plants is the defense against pathogens and herbivore predators (i.e. - their likely applications in the control of human and animal pathogens). They are classified into (i) phenolic acids, (ii) flavonoid polyphenolics, flavonones,

flavones, xanthones and catechins, and (iii) non-flavonoid polyphenolics (Kar, 2007). Phenolics essentially represent a host of natural antioxidants, with high potential to combat cancer, and as anti-inflammatory agents.

d) Terpenoids

According to Firn, (2010) terpenes are unsaturated hydrocarbons derived from the union of isoprene units (5 atoms of carbon). They exist commonly in liquid form, as essential oils, resins, or oleoresins. Terpenoids are insoluble in water and, depending on the number of carbon atoms and number of isoprene units involved in the formation of these compounds, are classified as; mono-, di-, tri- and sesquiterpenoids. Important monoterpenes include camphor, eugenol, limonene and menthol. Diterpenes (C20) are classically considered resins. Taxol, the anticancer agent, is the common example. The triterpenes (C30) include steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal or cytotoxic activity. The sesquiterpene lactones have been isolated and broadly, they have antimicrobial and neurotoxic action.

e) Essential oils

Essential oils (EO) are odorous and volatile products of various plant and animal species. They mostly contribute to the odoriferous constituents or (essences) of the aromatic plants that are used abundantly in enhancing the aroma of some spices (Martinez et al., 2008). Essential oils are secreted directly by the plant protoplasm or by the hydrolysis of some glycosides and plant structures, which have been associated with their secretion such as leaves, stems, flowers, roots or rhizomes. Essential oils can be prepared from various plant sources either by direct steam distillation, expression, extraction or by enzymatic hydrolysis. Chemically, a single volatile oil comprises of more than 200 different chemical components, and mostly the traces are solely responsible for attributing its characteristic flavor and odor (Firn, 2010).

Table 2. ***Summary table representing common aromatic plant extracts, their utilized parts, main active substances and reported properties.***

Chemical group	Main active compounds	Vegetal source	Utilized parts	Properties
Glycoside	Allicin	Garlic	Bulb	Digestion stimulant, antiseptic, antimicrobial
Glycoside	Allyl isothiocyanate	Mustard	Seed	Digestion stimulant
Phenolic	Anethol	Anise	Fruit	Digestion stimulant, galactagogue
Phenolic	Apiol	Parsley	Leaf	Appetite and digestion stimulant, antimicrobial
Phenolic	Zingerone	Ginger	Rhizome	Gastric stimulant
Terpenoide	Cineol	Cardamom	Seed	Appetite and digestion stimulant
Terpenoide	Eugenol	Clove	Cloves	Appetite and digestion stimulant, antimicrobial
Terpenoide	Linalol	Coriander	Leaf, seed	Digestion stimulant
Terpenoide	Sabinene	Nutmeg	Seed	Digestion stimulant, antidiarrheaic
Terpenoide	Cineol	Bay laurel	Leaf	Appetite and digestive stimulant, antiseptic
Terpenoide	Menthol	Peppermint	Leaf	Appetite and digestion stimulant, antimicrobial
Terpenoide	Cineol	Rosemary	Leaf	Digestion stimulant, antimicrobial, antioxidant
Terpenoide	Thymol	Thyme	Whole plant	Digestion stimulant, antimicrobial, antioxidant
Aldehyde	Cinnamaldehyde	Cinnamon	Bark	Appetite and digestion stimulant, antimicrobial
Aldehyde	Cuminaldehyde	Cumin	Seed	Digestive, carminative, galactague
Alkaloide	Trigonelline	Fenugreek	Seed	Appetite stimulant
Alkaloide	Capsaicin	Capsicum	Fruit	Antidiarrheaic, antiinflammatory, antimicrobial, stimulant
Alkaloide	Piperine	Pepper	Fruit	Digestion stimulant

Adapted from Doughari (2012)

1.4. Phytogenic as growth promoters in piglets

Phytogenic feed additives have attracted increasing interest as an alternative feeding strategy to replace antibiotic growth promoters after the completely banned of AGPs. Many of the phytogenic mixture used in nursery pig trials that produced positive results in growth performance contained active components from cinnamon, clove, thyme, and/or anise from 0.01 to 1% of the diet. The following phytogenic mixtures and inclusion levels produced increased average daily gain (ADG) in newly weaned pigs compared to control diets with no additives: 0.03% fenugreek, clove, and cinnamon essential oils (Cho et al., 2006); 0.1 and 0.3% ginseng, Chinese yam, sunflower, Chinese licorice, and ballon flower (Huang et al., 2012); 0.2% citrus fruit and chestnut tree extract (Hong et al., 2004); 0.01% thymol and cinnamaldehyde (Li et al., 2012); and 0.025% cinnamon and thyme (Zeng et al., 2015). For the study of Huang et al. (2012), the improvements in ADG and gain:feed ratio (G:F) occurred in the first two weeks after weaning, and nor in the total observed 4 weeks period. Straub et al. (2005) study also included diets with 0.5% and 1% Chinese rhubarb, however these inclusion levels did not create any significant differences in growth performance compared to the control diet. Most of studies in pigs show that the improvement of performance was on average 2% increase in weight gain and 3% in feed conversion efficacy, ranging from -5 to 9 % for weight gain. These figures are comparable to the potential of conventional growth promoters (antibiotics, organic acids, probiotics), where increases of roughly 4% have been described (Castillo et al., 2006).

Contrasting with the above studies, few studies in nursery pigs produced negative responses in growth performance when phytogenic were added to the diet. An essential oil mixture of anise, citrus, and oregano that was added to the diets at 0.1% inclusion level decreased G:F 3 to 4 weeks post-weaning compared to pigs that were fed a diet with no additives (Kommera et al., 2006). The same effect was observed in a 6 weeks study with 1.8% inclusion of purple coneflower (Maass et al., 2005). Liu et al. (2013) found that 10 mg/kg of garlic and 10 mg/kg of turmeric oleoresins tended to decrease average daily feed intake (ADFI) in the first 2 weeks following weaning compared to pigs fed no additives. ADG and ADFI were decreased in the third week post-weaning in a study by Namkung et al. (2004) for pigs fed a diet containing 0.75% cinnamon, thyme,

and oregano herbs, and a tests carried out by Jugl-Chizzola et al. (2005) showed, a depression of palatability in pigs fed essential oils from fennel and caraway, as well as from the herbs thyme and oregano. Therefore, the assumption that herbs, spices, and their extracts improve the palatability of feed does not seem to be justified in all cases.

It is important to note the considerable number of nursery pig studies that reported no differences in at least 3 of the 4 growth parameters (final BW, ADG, ADFI, or G:F) between pigs fed diets supplemented with phytogenic and diets with no additives. Maass et al. (2005) found no differences in BW, ADG, and ADFI for pigs fed 0 or 1.8% purple coneflower, and Jugl-Chizzola et al. (2005) found no differences in BW, ADG, and G:F for pigs fed 0 to 10 g/kg of thyme. No differences between the control diet and phytogenic treatment for ADG, ADFI, and G:F were found in studies using 1,000 or 10,000 mg/kg of garlic (Horton et al., 1991), 0.1% cinnamon, oregano, thyme, rosemary, and cloves (Huang et al., 2010), and 0.03% carvacrol, cinnamaldehyde, and capsicum (Manzanilla et al., 2006; Nofrarias et al., 2006). Finally, with the following phytogenic mixtures and inclusion levels researchers reported no effect of phytogenic in all 4 growth parameters: 0.1, 9.5, or 1% thyme herb and essential oil (Hagmuller et al., 2006); 40 mg/kg of oregano, anise, citrus, and chicory powder essential oils (Kroismayr et al., 2008); 150 or 300 mg/kg of carvacrol, cinnamon, and capsicum (Manzanilla et al., 2004); 0.5, 0.1, or 0.15% carvacrol, thymol, or chestnut meal tannins (Muhl and Liebert, 2007); 700, 1.400, or 2.100 ppm of thyme, clove, oregano, eugenol, and carvacrol (Oetting et al., 2006). Collectively, these studies covered a wide range of inclusion levels and ingredients; which makes it difficult to confirm any direct effects of phytogenic supplementation on growth performance in weaning pigs.

Altogether, studies in swine evaluating the effects of phytogenic supplementation have reported results in growth performance, the efficacy of these studies in improving feed intake and efficiency may be subject to several different factors that vary from study to study. Accordingly, a closer analysis of the action of phytogenic on the internal gastrointestinal tract and microbial environment is necessary to understand how plant extracts can influence growth performance.

Table 3. ***References regard the effects of phytogenic compounds on weaned piglet´s perform***

Phytogenic compounds	Dose, mg/kg	Test time, days	Effects (compared with control)			References
			[1]ADFI	ADG	FCR	
Stevia rabaudiana 10 - 20%	4000	28	_	_	_	Clouard and Val-Laillet. 2014
Citrus sinensis 60 - 80%	31		_	_	_	
Extracts of hot-flavoured spices 5 - 15%	400		_	_	_	
Fenugreek 40%, clove 12.5%, cinnamon 7.5% and carrier 40%	300	14	↑			Cho et al., 2006
Cinnamon verum, Origanum vulganum spp, Syzygium aromaticum, Rosmarinus	1000	14	_	_	_	Huang et al., 2010
Anis oil, citrus oil, oregano oil, and natural flavors	1000		↑	↑	↓	Kommera et al., 2006
Thymol and cinnamaldehyde 18%	50	7	_	_	_	Li et al., 2012
Carvacrol 5%, cinnamaldehyde 3% and capsicum oleoresin 2%	150	21	_	_	_	Manzanilla et al., 2004
Anise oil 4.44 g, clove oil 1.30 g and cinnamon oil 2.0 g/kg of additive	300		↑	↑	↓	Maenner et al., 2011
Cinnamon, thyme, oregano and a carrier	7500	21	↓	↓	↑	Namkung et al., 2004
Fennel oil	100	21	_	_	_	Schöne et al., 2006
Caraway oil	100		_	_	_	
Buckwheat, thyme, curcuma, black pepper and ginger	250	7	↓	_	↓	Yan et al., 2012
Cinnamaldehyde 4.5% and thymol 13.5%	250	28	_	↑	↓	Zeng et al., 2015

[1] ADFI average daily feed intake, ADG average daily body weight gain, FCR feed conversion rate. (-) no effects.

1.5. Potential modes of action of phytogenic

The exact mechanisms of action through which bioactive compounds from phytogenics exert their positive effects are not well understood. However, several *in vitro* and *in vivo* studies in many animal species have allowed suggesting different modes of action, including increasing palatability, digestive secretions and intestinal enzyme activity, antioxidant and anti-inflammatory activities, as well as, antimicrobial, antiviral, anti-parasitic activities. All these together contribute to the prevention of post weaning diarrhea (Holden and Mckean, 2001). Some of these are briefly discussed below.

Influence of phytogenic on palatability

Many aromatic herbs and essential oils are used to improve flavor and palatability of feed, as well as performance in livestock production. However, the responses are variable and the observed effect of supplemented phytogenic to pig diets on feed intake is not consistent (Neil et al., 2006; Stelter et al., 2013; Zeng et al., 2015). It is believed that the increased feed palatability associated with the supplementation of phytogenic could also be due to their antioxidative properties that can preserve the qualities of diets and prevent the release of unfavorable odors from the diets (Franz et al., 2010; Sola-Oriol et al., 2011).

Improving palatability of a new feed by the use of feed additives might enable to increase feed intake in piglets. Based on the assumption that a preferred feed is consumed in greater quantity, sensory additives may turn out to be useful method to modulate appetite and feed intake in pigs (Clouard et al., 2012). Currently, in swine production aromatic herbs and essential oils are often claimed to improve the flavor and palatability of feedstuffs, thus enhancing zootechnical performance.

Anti-oxidative and anti-inflammatory activity of phytogenic

According to Omonijo et al. (2018) oxidative stress represents an important chemical mechanism that leads to biological damage, which in turn can affect growth performance and health in pigs, especially in modern high-performance swine production systems. Pigs are frequently exposed to several stressors including weaning, malnutrition, disease challenge, heat stress, in-feed mycotoxin contaminations, transportation and overcrowding. These stressors are known to increase the production of Reactive Oxygen Species and when the antioxidant system is overwhelmed by the production of Reactive Oxygen Species, oxidative stress occurs. A drop-in performance, compromised immunity, muscle degeneration, increased risk of stroke in fast growing pigs, reduced appetite, diarrhea, and destruction of liver tissue might be associated with oxidative stress. Inflammation is closely related to oxidative stress. White blood cells and many other cells produce ROS in large amounts during inflammation. Thus, a common aspect of inflammation is oxidative stress, characterized by accumulation of

non-enzymatic oxidative damage (Franz et al., 2010). In fact, inflammation is one of the major sources of oxidative stress in the body (Blomhoff, 2010).

Plants in response to oxidative stress produce many antioxidants in nature. In contrast, animal cells have a limited production of antioxidants, and oxidative damage can therefore easily accumulate in animal cells when the critical balance between ROS and antioxidant defense is unfavorable.

Plant extracts and essential oils with anti-oxidative properties have been used successfully in animal diets (Zou et al., 2016; Liu et al., 2017). Different bioactive compounds of phytogenics are behind these antioxidant properties. Phenols are of great importance in plant physiology. Most phenols have antioxidant properties and are the most abundant antioxidants in mammal's diet. Flavonoids are the most common phenol compound in vegetables and fruits. The carotenoids are other class of antioxidant compound and represent a large group of pigments that are widespread in nature and responsible for the yellow, orange, red or purple colors of many vegetables, fruits and flowers. They can be absorbed and stored in the animal body, giving color to animal tissues. Essential oils may also affect lipid metabolism in the animal: a dietary supply of thyme oil or thymol to ageing rats showed a beneficial effect on the antioxidative enzymes, as well as on polyunsaturated fatty acid composition in various tissues (Youdim and Deans, 2000). Different in vivo studies with piglets have also evidence the antioxidant properties of phytogenic. Koh et al. (1998) showed that capsicum and cinnamaldehyde, two common ingredients in phytogenic mixtures, have been shown to exert anti-inflammatory properties by inhibiting the action and proliferation of T cells.

Some studies have evaluated the potential antioxidative properties associated with phytogenics supplementation in swine. Total antioxidant capacity was reported to be increased by additions of 0.01 to 0.025% thymol and cinnamaldehyde in the diets of newly weaned pigs (Li et al., 2012; Zeng et al., 2015). In the study of Zeng et al., (2015), activities of superoxide dismutase and glutathione peroxidase were reportedly increased by phytogenic addition to the diets, whereas in the study by Li et al. (2012), there were no changes. Frankic et al. (2010) support these results, he found no effect of adding 271.2 mg/kg of carvacrol, cinnamaldehyde, and capsaicin to the diets of nursery

pigs on glutathione peroxidase levels. Further research on the individual modes of action of phytogenic ingredients should be completed to reach further conclusions.

Liu et al. (2013). Showed that levels of serum TNF-α and IL-1β were unchanged in nursery pigs supplemented with 10 mg/kg of capsicum, garlic, or turmeric oleoresins, but decreased when the same pigs were inoculated with porcine reproductive and respiratory disease syndrome (PRRS). This indicates that phytogenics may provide an antiflammatory response in an immunosuppressed state. Namkung et al. (2004) also reported no changes in pigs fed 0 or 0.75% cinnamon, thyme, and oregano on the levels of plasma TNF-α and IL-1β. Interestingly, a study using 0.01% thymol and cinnamaldehyde reported an increase in plasma TNF-α post-weaning (Li et al., 2012). Lastly, Kroismayr et al. (2008) found that pigs supplemented with 40mg/kg of oregano, anise, citrus peels, and chicory powder essential oils had decreased NF-κB in the ileum and tendency for decreased NF-κB in the colon compared to pigs fed no phytogenic.

Effects on intestinal morphology and Intestinal Function

Phytogenics supplementation potentially influences the gut morphology of newly weaned pigs. Improvement in villus height and reduction in crypt depths can increase the ability of a pig to absorb nutrients and reduce energy losses (Oetting et al., 2006). In addition, villus atrophy is primarily caused by an increased rate of apoptosis and a decreased rate of enterocyte proliferation (Zeng et al., 2015). Therefore, the action of phytobiotics on the morphology and proliferation of intestinal cells is an important factor to consider when evaluating the overall effect of phytobiotics on swine intestinal health.

Some additives, such as aromatic herbs or volatile oils, may relieve animals from having to mount an immune response to critical offenses, increasing the intestinal availability of essential nutrients for absorption and, thus, assisting the animal to grow better within its genetic potential. This may also be due to the stimulation of digestive secretions, e.g. saliva, bile, mucus, as well as enhanced enzyme activity, being a core of beneficial nutritional actions (Platel and Srinivasan, 2004).

Manzanilla et al. (2004) reported that feeding a combination of essential oils (carvacrol, cinnamaldehyde and capsaicin) to piglets, increased gastric retention time of ingested feed, resulting in a better nutrient absorption and favoring intestinal stability against digestive disorders. In addition, Jang et al. (2004) suggested that essential oils used as feed additives for broilers enhanced the activities of trypsin and amylase in tissue homogenates of the pancreas, as well as the jejunal chyme content. A mixture of carvacrol, cinnamaldehyde and capsaicin also stimulated the intestinal secretion of mucus. The increased release of large amounts of mucus and the creation of a thick layer of mucus on the glandular stomach and jejunum wall in chicks fed with the above mixture could be responsible for the reduced adherence of pathogens (*E. coli, Clostridium perfringens* and others) to the gut epithelium.

Most of the reported positive effects by phytogenic supplementation on gut morphology are found in the jejunum. A 0.025% mixture of cinnamaldehyde and thymol (Zeng et al., 2015) and either 0.1 or 0.3 % mixture of ginseng, Chinese yam, sunflower, Chinese licorice, and ballon flower (Huang et al., 2012) were found to increase the villus height in the jejunum of nursery pigs compared to pigs that received no phytogenic. Huang et al. (2012) also reported a decrease in jejunal crypt depth, an increase un jejunal villus height:crypt depth ratio, and an increase in duodenal and ileal villus height, although the latter two results were only significant for the 0.3% phytogenic mixture. A combination of thymol and cinnamaldehyde was also reported to increase jejunal villus height:crypt depth ratio in another study (Li et al., 2012).

In all the above studies, there does not appear to be a tendency of one specific type of phytogenic or inclusion level to have a more significant result than other, although this can be difficult to determine due to the vast variety of ingredients, but most of them indicate that the addition of phytogenic to the diets of pigs generally can help improve the health of the intestinal tract and, overall, improve the growth performance of pigs.

Antimicrobial activity of Phytogenic and control of diarrhea incidence

Herbs and spices are well known to exert antimicrobial action in vitro against important pathogens, including fungi (Adam et al., 1998; Smith-Palmer et al., 1998; Hammer et al., 1999; Dorman and Deans, 2000; Burt, 2004; Si et al., 2006; Ozer et al., 2007). In *in vivo*

studies, essential oils were an important tool to inhibited growth of *Clostridium perfringens* and *E. coli* in the hindgut and ameliorated intestinal lesions and weight loss than the challenged control birds (Mitsch et al., 2004; Jamroz et al., 2006; Jerzsele et al., 2012).

Regarding to swine production there are many studies which reported the effect of phytogenic supplementation on the total count of aerobic and anaerobic bacteria in the gut found either a decrease in bacteria or no difference between pigs supplemented with phytogenic and pigs that received no additives (Muhl and Liebert, 2007; Kroismayr et al., 2008; Li et al., 2012; Zeng et al., 2015).

Bacteria from the order Lactobacillales, including the commonly known lactobaccili, are generally accepted to be beneficial in maintaining a healthy intestinal environment due to their ability to control pathogenic bacteria (Manzanila et al., 2004). Multiple studies with a variety of phytogenic mixtures (thymol, cinnamaldehyde, carvacrol, capsicum, ginseng, Chinese yam, Chinese licorice, sunflower, balloon flower) and inclusion levels (0.015 to 0.3%) reported increased levels of *Lactobacilli* in the jejunum, ileum, colon, rectum, and feces (Manzanilla et al., 2004; Maenner et al., 2011; Huang et al., 2012; Li et al., 2012; Zeng et al., 2015). Several other authors, however, with similar phytogenic combinations and inclusion levels, reported no change in *Lactobacilli* concentration in the stomach, ileum, cecum, colon, rectum, and feces (Namkung et al., 2004; Muhl and Liebert, 2007; Kroismayr et al., 2008; Maenner et al., 2011; Li et al., 2012; Zeng et al., 2015).

Enterobacteriaceae is a large family of gram-negative bacteria that includes familiar pathogens such as *Salmonella* and *Escherichia coli* (*E. coli*). Manzanilla et al. (2004) reported no differences in cumulative levels of *Enterobacteria* in the jejunum of newly weaned pigs fed 0, 150, or 300 mg/kg of carvacrol, cinnamaldehyde, and capsicum. However, 0.1, 0.5 and 1% thyme herb and essential oil (Hagmüller et al., 2006) and 50, 100, and 150 g/t of thymol and cinnamaldehyde (Li et al., 2012) were found to decrease the amount of *E. coli* fecal shedding in weaned pigs compared to non-supplemented. In addition, the supplementation of phytogenic mixtures containing low inclusion levels (0.01 to 0.025%) of thymol, cinnamaldehyde, or both resulted in decreased prevalence

of *E. coli* in the cecum, colon, and rectum (Jugl-Chizzola et al., 2005; Li et al., 2012; Zeng et al., 2015). Only a few authors reported no differences in *E. coli* counts in the stomach, ileum, cecum, or colon (Namkung et al., 2004; Maenner et al., 2011; Zeng et al., 2015).

Antimicrobial properties of the diverse botanical compounds in a phytogenic additive are not the result of one specific mode of action; instead, it is the cumulative effect on many different targets in various parts of the microbial cell. It has been reported for example that their antimicrobial effect might depend on the pH, chemical structure, concentration of specific bioactive compounds, along with the population and types of microorganisms.

Essential oils, which are hydrophobic, can disperse and disrupt bacterial cell and mitochondrial membranes (Li et al., 2012). This may contribute to antibacterial properties. Burt (2004) surmises that the antibacterial mechanism of some phytogenic compounds, such as thymol, eugenol and carvacrol, is the disruption of the cellular membrane promoted by its hydrophobic property, the inhibition of ATPase activity and release of intracellular ATP. However, it is generally accepted that the largest common antibacterial mode of action is related to the phenolic compounds founds in a large variety of phytogenic compounds (Lambert et al., 2001). This commonly includes, but is not limited to, plants from the *Labiatae* family (e.g. mint, oregano, thyme), from the *Umbelliferae* family (e.g. anise, coriander), and plants with flavonoids (e.g. garlic, onion).

Some compounds may also modulate gene expression and signal transduction pathways, producing the destruction or inactivation of genetic material (Surh, 2003). And the disturbance of cytoplasmic membrane, disruption the proton motive force, electron flow, decrease active transport and coagulation of cell contents (kotzekidou et al., 2008).

Several authors have published work on the effect of phytogenic supplementation on diarrhea incidence and faecal consistency in newly weaned pigs. Faecal score was improved in pigs fed 0.01% or 0.025% inclusion levels of thymol and cinnamaldehyde compared to pigs that were fed no additive (Li et al., 2012; Zeng et al., 2015). Kantas et al. (2015) and Liu et al. (2017) carried out studies where it was observed an increased weigh gain and decreased diarrhea incidence in nursery and growing pigs by using the

extracts of *Macleaya cordata* (Wild) as feed additives at the concentration ranging from 15 to 50 mg/kg. Dietary supplementation of blend extracts of cinnamon, thyme, and oregano inhibited colonization of pathogenic *E. coli* in the intestine of nursery pigs (Namkung et al., 2004). A reduction in diarrhea incidence was reported in studies involving the following phytogenic mixtures and inclusion levels: 0.1 or 0.2% citrus fruit and chestnut tree extract (Hong et al., 2004); 0.3% ginseng, Chinese yam, sunflower, Chinese licorice, and balloon flower (Huang et al., 2012); and 50, 100, or 150 g/t of thymol and cinnamaldehyde (Li et al., 2012). Interestingly, both Hong et al. (2004) and Huang et al. (2012) reported a significant reduction in diarrhea incidence only in the first few days or first week post-weaning, but this could be due to the fact that as weaned pigs age their overall gut health improves and diarrhea incidence decreases. Additive mixtures containing 0.05 to 1.8% of ingredients such as fenugreek, clove, cinnamon, oregano, thyme, rosemary, anise, citrus, capsicum, chestnut meal, and purple coneflower were involved in studies that did not find any effect of phytogenic supplementation on reducing diarrhea incidence or improving faecal consistency (Manzanilla et al., 2004; Maass et al., 2005; Cho et al., 2006; Kommera et al., 2006; Muhl and Liebert, 2007; Huang et al., 2010). The varying results in the effects of phytogenic mixtures on diarrhea incidence may be related to the individual active components' ability to modulate the gut microbiota and digestibility.

1.6. Future consideration

The level of standardisation of phytogenic feed additives has increased over the last 10 years. However, the different additives available in the market vary greatly in their composition, and thus in their in vivo effects as well. Further researches on phytogenics are needed to focus on their modes of action and aspects of their application, for these reasons the following project is proposed.

2. Experimental Part

2.1. Hypothesis

Increasing antimicrobial resistance in pathogenic bacteria has created the need for the development of novel preventive and therapeutic agents in the animal industry.

Thus, the hypothesis established in this study is the following:

- It is possible to improve the response of piglets to weaning and to reduce the incidence of post-weaning diarrhea by including phytogenic blends in the diet as an alternative to antimicrobials.

2.2. Objectives

To test the previous hypothesis, the main objective of this study was:

- To evaluate in weaning piglets the potential of two new blends of phytogenics as feed additives in front of an oral ETEC K88 challenge, analyzing their effects on performance, clinical response, immune system and gut health.

2.3. Considerations

The present trial was carried out under the frame of a confidentiality agreement with private company, which restrict a full description of the tested products.

2.4. Material and Methods

The trial was conducted by the Animal Nutrition and Welfare Service (SNiBA) of the Department of Animal and Food Science of the Universitat Autònoma de Barcelona.

The experiment was performed at the *Servei de Granges i Camps Experimentals* of the *Universitat Autònoma de Barcelona (UAB)* and received prior approval from the Animal and Human Experimental Ethical Committee of this institution and the competent Authorities (Permit No. CEEAH: 4026 DMAH: 10118). The treatment, management,

housing, husbandry and slaughtering conditions conformed to European Union Guidelines (Directive 2010/63/EU).

2.4.1. Animals, housing and experimental design

The trial was conducted as a Level 2–High Risk Biosecurity Procedure, with appropriate training of the personnel involved. A total of ninety-six male piglets from a commercial farm with mothers that did not receive *E. coli* vaccination were weaned at 21 days of age and at an average body weight (BW) of 4.8 ± 0.62 kg. Piglets were transported to facilities of the Universitat Autònoma de Barcelona, weighted and distributed in four rooms of eight pens each (thirty-two pens, three animals per pen). Each pen of three animals was readjusted by weight (a lower, an intermediate and a bigger weight) to obtain a final homogenous weight among the pens. Each pen (3 m^2) had a feeder and a water nipple to provide food and water for *ad libitum* consumption. The weaning rooms were equipped with automatic heating, forced ventilation and an individual heat-light per pen. The experiment was conducted during the winter season (January), with an average room temperature of 28ºC (± 4ºC).

The experiment included four treatment groups with eight replicates per each including: (i) a control group with plain diet (T1); (ii) the same plain diet supplemented with ZnO (2500 ppm Zn) (T2); (iii) supplemented with phytogenic 1 (1 kg/tm;T3); (iv) or supplemented with phytogenic 1 (1 kg/tm) plus phytogenic 2 (1.5 kg/tm;T4). All the animals were subjected to an oral challenge with ETEC K88.

2.4.2. Experimental diets

Additives under study were two different blends of phytogenic products containing extracts and essential oils from many plants. Whereas treatment T3 included a blend of different commonly used essential oils, T4 also included additionally other more novel phytogenics with putative antioxidant, antinflamatory and immunomodulatory properties.

ZnO was supplemented as ZINCOTRAX at 3100 mg /kg of feed (equivalent to 2500 mg of zinc/kg of feed).

The experimental diets were manufactured in Pinsos Molinet SL (Gaia, Barcelona) under the SNiBA supervision. Basal diet was initially made in a single batch (Table 4) and subsequently divided into four batches to include the different additives to conform the four experimental diets.

Table 4. Ingredients and nutrient composition of the experimental diets as-fed basis, g/kg.

Ingredients (g/kg FM)	
Maize	207.3
Wheat	180.0
Barley 2 row	170.0
Extruded soy bean	149.0
Sweet whey powder (cattle)	100.0
Soybean meal 47	80.0
Fishmeal MT	60.0
Whey powder 50% fat	25.0
Monocalcium phosphate	6.8
Cacium carbonate (CaCo3)	3.9
L-Lysine HCL (78)	4.5
Vit-Min Gplus [1]	4.0
DL-Methionine 99	2.6
Sodium chloride	2.5
L-Threonine	2.3
L- Valine	1.5
L-Triptophan	0.6

Estimated composition (g/kg FM)	
Dry Matter	914
Ash	74
Crude fat	62
Crude protein	198
Neutral detergent fiber	91.4
Acid-detergent fiber	34.2

[1] Provided per kilogram of complete diet: 510,0 mg Butilhidroxitolueno (BHT); 3.750,0 mg of Mn (glycine manganese chelate, hydrated); 15.000,1 mg of Fe (chelate glycine irons); 6.250,0 mg of Zn (Zinc glycine glycine chelate, solid); 20,0 mg of L-Selenometionina; 5.000,1 mg of Cu (Copper chelate (II) of soluble glycine hydrate); 27.500,0 mg of Cu (copper sulphate II pentahydrate); 17.500,3 mg of Zn (zinc oxide); 6.250,2 mg of Mn (manganese oxide); 175,0 mg of I (iodine calcium anhydrous); 50,0 mg of Se (sodium selenite); 500,1 mg of Vit K3; 1.750,4 mg of Vit B2; 749,7 mg of Vit B1; 1.875,0 mcg of Vit D; 9.098,8 UI of Vit E; 3.000.000,0 UI of Vit A; 975,0 UI of Vit E/acetate; 3.750,0 UI of Vit E/acetate; 1.833,5 mg of Vit B6; 15,0 mg of Vit B12; 11.248,8 mg of Nicotinic Acid; 4.250,0 mg of Pantothenic Acid; 50,0 mg of Biotina; 374,8 mg of folic acid; 350.000,0 UI of Vit D.

2.4.3. Bacterial strain

The enterotoxigenic *Escherichia coli* (ETEC) K88 strain was COLI30/14-3 and it was isolated from feces of 14-week old pigs and provided by the Veterinary Laboratory of Diagnosis of Infectious Diseases of UAB. This strain presented virulence factors K88ab, K88ac, LT, STb and EAST1 and was negative to K99, F6, F18, F41, STa, VT1, VT2, and EAE.

The oral inoculums were prepared by an overnight incubation at 37ºC in Brain Heart Infusion (BHI) with slow agitation (1 x g) in an orbital incubator to obtain $2x10^8$ cfu/mL. The final culture broth was used as the oral inoculums by preparing 96 doses of 6 mL. To quantify the inoculums (cfu/mL) serial dilutions were cultured in TSA plates (overnight, 37ºC). Serial dilutions of placebo inoculums were also seeded in TSA plates to confirm sterility.

2.4.4. Experimental Procedure

The length of the study was of 16 days. Mortality rate was registered, and no antibiotic treatment was administered to any animals in the trials. After an adaptation period of 7 days. One animal of each pen was euthanized on day 8 post-inoculation (PI).

Animals were identified (by ear tag), weighted and distributed among the pens on arrival. In addition, fecal samples for microbiological analysis of lactobacilli and enterobacteria were obtained from the medium weight piglets of each of the 32 pens of the experiment.

Animals received the experimental diets over 16 days *ad libitum.* Feed intake by pen was registered at days 0, 7, 8, 9, 10, 11, 14, 15 and individual weight on days 0, 4 and 8 PI. The ADG, ADFI and G:F were calculated by pen.

After one week of adaptation to the diets the animals were orally challenged with the pathogen. A single 6 mL oral dose ($1x10^9$ cfu) of ETEC K88 was administered to the total of animals. In order to ensure that the stomach was full at the time of inoculation, and thus facilitate bacterial colonization, feed withdrawal was performed at 21:00 h of the previous day and provided back 30 min before inoculation.

After the ETEC K88 challenge, animals were checked daily for clinical signs to evaluate their status post-inoculation (i.e. dehydration, apathy and fecal score), always by the same person. Fecal score was measured using a scale: 1 = solid and cloddy, 2 = soft with shape, 3 = very soft or viscous liquid and 4 = watery or with blood. Rectal temperature was assessed on day 0 PI (before the challenge) with a digital thermometer and 1 and 2 PI. Fecal consistency was registered on days 0, 1, 2, 3, 4 and 7 PI. Blood samples were taken on days 4 PI from the initial heaviest BW and 8 PI from the initial intermediate BW piglet from each pen.

For microbiological analysis faecal samples were taken before inoculation and after the challenge on days 0, 4, 8 PI. Fecal samples were taken aseptically from 32 animals after spontaneous defecation associated with the manipulation of the animal or by rectal stimulation. On day 8 PI samples were collected directly from rectum after euthanasia (see below). Fecal samples were always taken from the animal with intermediate initial BW of each pen (N=32).

On day 8 PI, one pig per pen was euthanized. The animal selected was the one with the intermediate weight on arrival. Animals were euthanized and sequentially sampled during the morning (between 8:00 to 14:00). Prior to euthanasia, a 10 mL sample of blood was obtained by venipuncture of the cranial vena cava using 10 mL tubes without anticoagulant (Aquisel; Madrid, Spain). Immediately after blood sampling, piglets received an intravenous, lethal injection of sodium pentobarbital (200 mg/kg BW; Dolethal, Vetoquinol S. A.; Madrid, Spain). Once dead, the animals were bled, the abdomen was immediately opened and the whole gastrointestinal tract excised.

After the gastrointestinal tract was excised, fecal samples were obtained directly from the rectum for traditional microbiology. Digesta (approximately 40 mL) from the ileum and proximal colon (considered to be 0.75 m from the ileocecal junction) was collected for bacterial counts. It was homogenized and classified their consistence. The pH of the contents was immediately determined with a pH-meter calibrated before it was used (Crison 52-32 electrode, Net Interlab; Barcelona, Spain).

For histological study, 3-cm section from the proximal ileum were removed, opened longitudinally, washed thoroughly with sterile PBS and fixed by immersion in a formaldehyde solution (4 %).

Blood samples were centrifuged (3, 000 x for 15 min at 4 ^{O}C) to obtain serum, and the serum obtained was divided into different aliquots and stored at -80 ^{O}C.

2.4.5. Analytical procedures

For microbial counts, ileum and colon contents (day 16 (8 PI)), ileum scrapings (day 16 (8 PI)) and feces (day 0, 8, 12 and 16) samples were suspended in Phosphate Buffer Saline (1:10) and subsequently homogenized during 5 minutes. Thereafter, 10-fold serial dilutions were made in PBS to seed in chromogenic agar for the counting *E. coli* and Rogosa agar for lactobacilli. Counts were read after 24 h incubation at 37 ^{O}C.

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin. Morphological measurements of ileal sections were performed with a light microscope (BHS, Olympus) using the technique described in Nofrarias et al. (2006). Measured parameters included: Villus height, Crypt depth, ratio Villus: Crypt, Intraepithelial lymphocytes (IEL), Globet cells (GC) and Mitosis.

Concentrations of Tumor Necrosis Factor-alpha (TNF-α) in serum samples were determined by Quantikine Porcine TNF-α kits (R&D Systems). Pig major acute-phase protein (Pig-MAP) concentration was determined by a sandwich-type ELISA (Pig MAP Kit ELISA, Pig CHAMP Pro Europe S.A.), according to the manufacturer's instructions.

2.4.6. Statistical analyses

The results are expressed as lsmeans with their standard errors. A one-way ANOVA was used to examine the effect of treatments. All statistical analyses were performed using the generalized linear model and mixed procedure. Statistical Analysis package used was SAS version 9.2 (SAS Institute Inc.). When treatment effects were established (P = 0.05), treatment least squares means were separated using the probability of differences function adjusted by Tukey–Kramer test. The statistical trend was also considered for P values in the range 0.05- 0.10.

3. Results

A total of 6 casualties were registered in the days following inoculation. Three of these deaths were in the group T1 on day 4 PI, two in the group T4 (one on day 3 PI, and the other one on day 4 PI), and one in the group T3 on day 4 PI. All of them were attributed to post-weaning stress and subsequent bacterial challenge. These animals had previously shown symptoms of apathy. No antibiotic treatment was administered to any of the animals on the experiment.

Unexpectedly in three pens animals did not eat at all along the whole study. Table 5 shows the amount of feed intake registered in terms of g/day per animal. Feed intake was always below 8 g/day, amount that could be compatible with the waste of feed associate to the movement of animals around the feeder. The three pens belonged to the treatments including experimental phytogenics (T3 or T4). They were pen 5 (T3, room 1), pen 25 (T3, room 3), and pen 31 (T4, room 4). Considering that animals did not receive the experimental additives due to lack of feed intake, we decided to remove these replicates from the study. Results are therefore presented removing these animals what means that T1 and T2 have 8 replicates each, T3 have 6 replicates and T4 have 7 replicates.

Table 5. *Average daily feed intake (ADFI) (g/animal·day) registered in removed pens from the study.*

	Pens		
ADFI (g)	**5 (T3)**	**25 (T3)**	**31 (T4)**
Wk adap.	6.8	4.5	4.4
1 PI	6.7	4.3	3.3
2 PI	6.7	0.0	0.0
3 PI	2.0	4.7	5.3
4 PI	4.3	3.7	2.0
5-7 PI	-0.9	-1.1	-0.7
8 PI	4.3	7.3	8.0

3.1. Animal performance

Results of the evolution of body weight (BW), average daily feed intake (ADFI), average daily gain (ADG) and gain:feed ratio (G:F) are showed in table 6.

Table 6. *Effect of the experimental treatments on growth performance.*

	Treatments [A]					
	T1	**T2**	**T3**	**T4**	**RSD** [B]	**P-value**
BW [C] **(kg)**						
Initial	4.85	4.85	4.87	4.58	*0.062*	*0.959*
Final	7.10	7.36	7.26	7.15	*0.844*	*0.931*
ADFI [D] **(g/d)**						
Adap [E]	84.4	117.5	144.7	85.6	*59.13*	*0.217*
0-4 PI [F]	197.1	284.1	251.8	238.7	*74.51*	*0.162*
4-8 PI [G]	391.7	436.2	442.9	425.1	*110.83*	*0.813*
0-8 PI [H]	259.6	333.6	309.6	313.2	*86.82*	*0.391*
Overall [I]	177.8	232.8	232.7	207.0	*63.56*	*0.302*
ADG [J] **(g/d)**	(g)					
Adap	28.8	52.4	21.8	33.6	*37.99*	*0.466*
0-4 PI	96.3 [b]	249.2 [a]	170.4 [ab]	156.9 [ab]	*97.28*	*0.035*
4-8 PI	341.6	286.7	358.6	308.7	*115.20*	*0.646*
0-8 PI	219.0	268.0	264.5	232.8	*93.55*	*0.689*
Overall	130.2	167.4	151.2	139.8	*58.86*	*0.632*
G:F [K] **(g/d)**						
Adap	0.08	0.48	0.15	0.39	*0.462*	*0.300*
0-4 PI	0.33 [b]	0.88 [a]	0.70 [ab]	0.58 [ab]	*0.359*	*0.039*
4-8 PI	1.09	0.75	0.99	0.79	*0.255*	*0.045*
0-8 PI	0.79	0.80	0.87	0.72	*0.196*	*0.587*
Overall	0.67	0.72	0.67	0.66	*0.184*	*0.897*

[A] Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. [B] Residual standard deviation. [C] Body weight. [D] Average Daily Feed Intake. [E] Experimental Days 0 to 7. [F] Experimental Days 8 to 11. [G] Experimental Days 12 to 16. [H] Experimental Days 8 to 16. [I] Experimental Days 0 to 16. [J] Average Daily Gain. [K] Gain:feed ratio. T1 and T2 had an n=8, for T3 was n=6, and for T4 was n=7.

No statistical differences related to the experimental treatments were found in the final body weight of the animals.

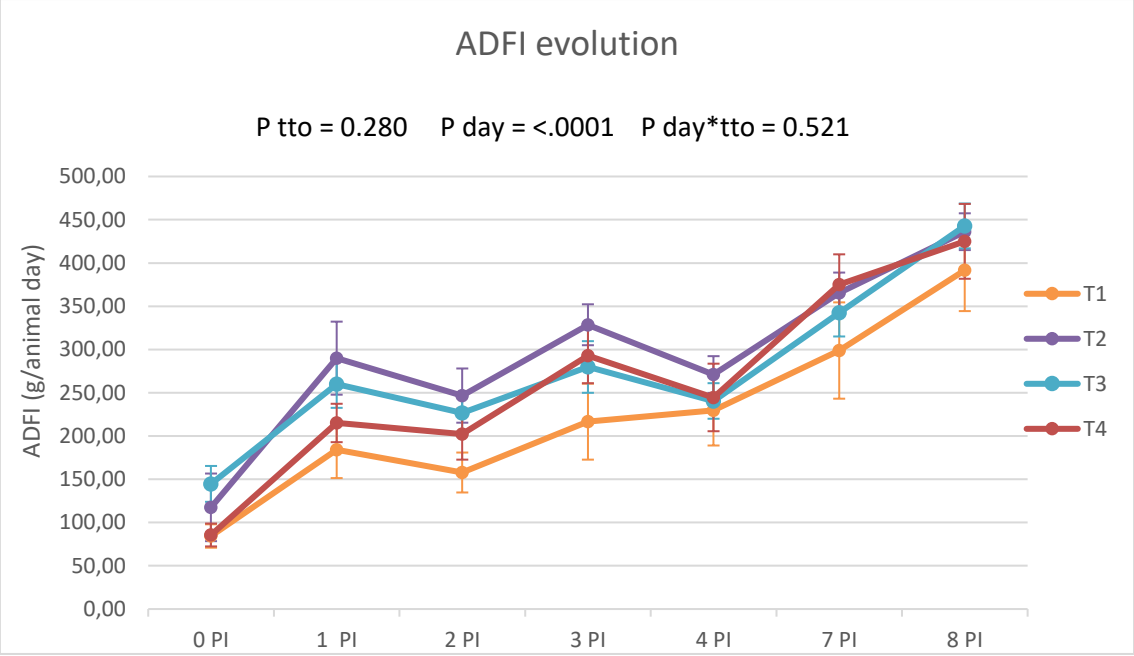
Regarding ADFI no significant differences were found due to the experimental diets, however a numerical increase was observed in the animals receiving ZnO (T2) or phytogenics (T3 & T4) compared to the control diet (T1) in the post inoculation period.

The ADG showed statistical differences immediately after the challenge (0-4 PI period). Animals receiving the ZnO supplementation (T2) showed the highest growth being significantly different from those animals receiving control diets (T1) (P = 0.035). Animals receiving phytogenics (T3 & T4) showed intermediate values.

The impact of the supplemented diets in ADG, was also reflected in the gain:feed ratio (G:F) along the 0-4 days PI, that again showed the highest values in T2 and the lowest in T1 (P = 0.039) with intermediate values for T3 & T4.

After the challenge, feed intake was also registered in a daily pattern. The ADFI evolution is therefore presented in Figure 1. Data were analyzed using a mixed linear model.

Figure 1. *Average daily feed intake of the post-Inoculation period.*



Experimental Days 8 to 16 (0 to 8 PI). Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. Groups T1 and T2 had an n=8, for the group T3 had n=6, and T4 n=7.

As could be expected feed intake changed significantly along the first week PI (P day < 0.001). However no significant changes were observed due to the experimental treatments (P = 0.280) nor interaction (P = 0.521). It is fair to highlight that mean values for animals receiving the plain diet (T1) were always the lowest and animals receiving ZnO (T2) exhibited the highest values from day 1 to 4 PI.

3.2. Clinical signs

3.2.1. Fecal consistency

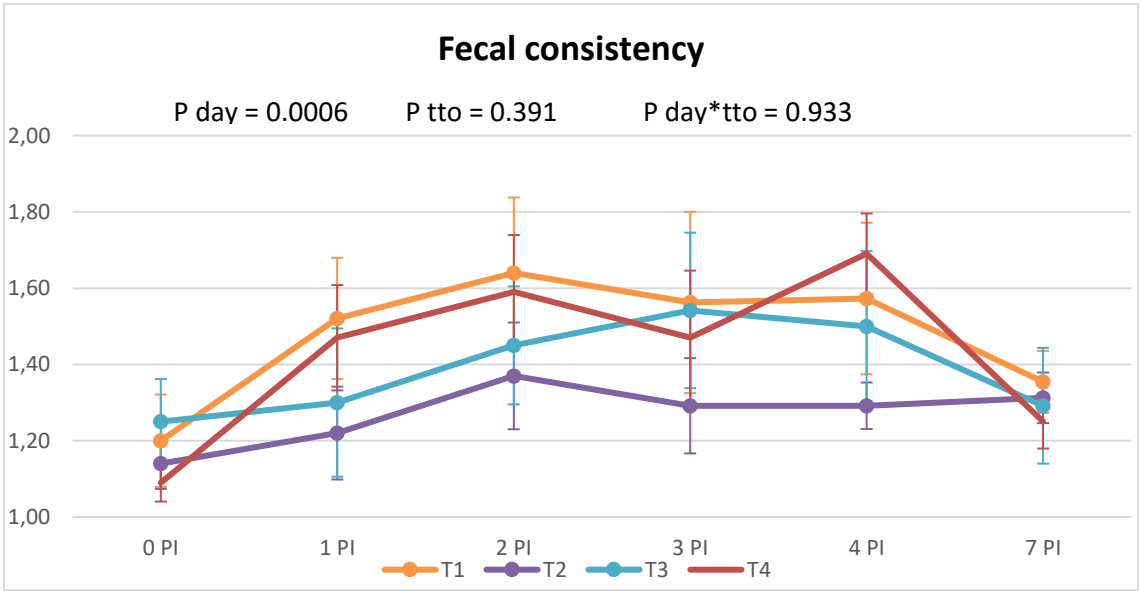
The fecal score was recorded in the days post challenge and classified using a scale ranging from 1 to 4 as following:

1	Normally shaped feces
2	Shapeless soft feces
3	Thin or liquid feces
4	Very liquid feces (translucid) or with blood

The fecal scores registered on the post challenge period are shown in figure 2.

The oral challenge promoted a mild course of diarrhea that was translated into a significant increase in fecal scores after the challenge (P day = 0.0006). Differences in fecal consistency due to treatments were not significant (P = 0.391) and neither the interaction with time (P = 0.933). However it should be highlighted that animals receiving the ZnO treatment (T2) showed always the lowest scores in the 1-4 PI period.

Figure 2. *Evolution of the mean fecal scores in the different experimental groups during the post challenge period. Values from 1 to 4 ranging from dry faces until very liquid excreta*

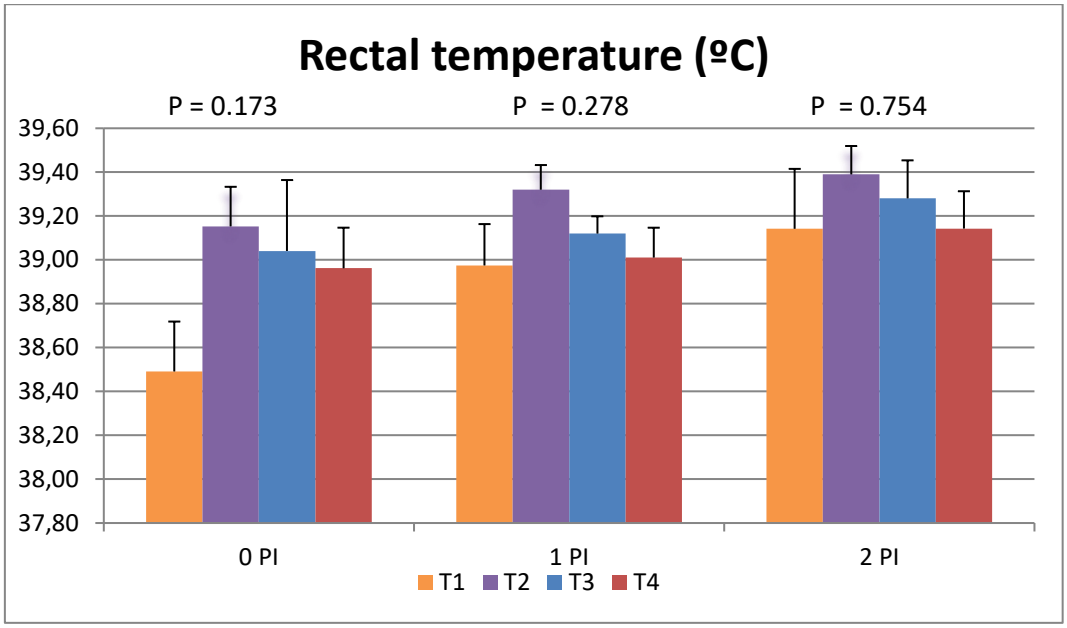


Experimental Days 8 to 15 (0 to 7 PI). Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. Groups T1 and T2 had an n=8, for the group T3 had n=6, and T4 n=7.

3.2.2. Rectal temperature

Values for the rectal temperature before inoculation and 24 and 48 h post-challenge are shown in figure 3.

Figure 3. *Evolution of mean rectal temperatures of the different experimental groups.*



Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. Groups T1 and T2 had an n=8, for the groups T3 n=6, and T4 n=7.

Rectal temperature was in all cases within the physiological range (there was no fever) and no differences were seen between groups.

3.3. Microbial analysis

All microbiological plate counts were always done in the middle size animal of each pen. Animal feces were tested for total coliforms and lactobacilli on their arrival (day 1), before the oral challenge of *ETEC K88* (day 0 PI) and on days 4 and 8 PI. Ileal and colon digesta, and Ileal scrapings were also tested for coliforms and lactobacilli at day 8 PI.

For the plate counts, detection limit was 10^5 cfu/g of FM.

Table 7. *Effects of the experimental treatments on the plate counts of total coliforms and lactobacilli in fecal samples, ileal digesta, colon digesta, and ileal scraping (log CFU/g FM).*

	Treatments [A]					
	T1	**T2**	**T3**	**T4**	**RSD [B]**	**P-value**
Total coliforms (cfu/gFM)						
Feces						
Arrival [C]	8.04	8.04	8.61	7.11	*0.939*	*0.072*
Day 0 PI [D]	7.66	7.44	6.79	7.39	*1.337*	*0.677*
Day 4 PI [E]	7.90	8.43	8.28	8.33	*0.896*	*0.673*
Day 8 PI [F]	8.59	7.91	7.27	8.20	*1.106*	*0.187*
Ileal scrapings						
Day 8 PI	5.19	5.17	5.10	6.17	*1.396*	*0.440*
Ileal digesta						
Day 8 PI	7.88	7.43	8.43	8.55	*0.947*	*0.124*
Colon digesta						
Day 8 PI	8.15	6.69	7.51	7.25	*1.689*	*0.396*
Total lactobacilli (cfu/gFM)						
Feces						
Arrival	7.11	7.26	7.18	6.92	*0.748*	*0.879*
Day 0 PI	8.76	8.78	8.53	8.84	*0.528*	*0.747*
Day 4 PI	8.22	7.37	7.27	8.05	*1.252*	*0.389*
Day 8 PI	7.55 [ab]	6.26 [b]	8.71 [a]	8.27 [a]	*1.026*	*0.0007*
Ileal scrapings						
Day 8 PI	4.66	5.42	5.06	5.29	*1.059*	*0.513*
Ileal digesta						
Day 8 PI	7.80	7.58	7.04	7.38	*1.316*	*0.773*
Colon digesta						
Day 8 PI	8.65	7.86	8.79	8.82	*0.674*	*0.0336*

[A] Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. [B] Residual standard deviation. [C] Experimental Day 0. [D] Experimental Day 8. [E] Experimental Day 12. [F] Experimental Day 16. Groups T1 and T2 had an n=8, for the group T3 had n=6, and T4 n=7.

Regarding total coliforms in fecal samples, ileal and colon digesta, and ileal scrapings, no significant changes were detected due to the treatments. However, on day 8 PI the number of lactobacilli was the lowest with the diet supplemented with ZnO and the highest with diets supplemented with phytogenics (T3- T4) with a difference of more than 2 log units (P diet = 0.0007). A decrease in the lactobacilli population with the supplementation of ZnO was also observed in colon digesta on day 8 PI (P diet=0.0336).

The balance in microbiota was also assessed by means of the *lactobacilli:coliforms* ratio as difference of log CFU (see Table 8).

Table 8. *Effects of the experimental treatments on the latobacilli:coliforms ratio in fecal samples, ileal digesta, colon digesta, and ileal scraping.*

	Treatments [A]					
	T1	**T2**	**T3**	**T4**	**SEM [B]**	**P-value**
Ratio *lactobacilli:coliforms*						
Feces						
Arrival [C]	-0.88	-0.78	-1.51	-0.18	*0.569*	*0.382*
Day 0 PI [D]	1.09	1.34	1.74	1.45	*0.586*	*0.864*
Day 4 PI [E]	0.32	-1.05	-1.00	0.0007	*0.598*	*0.201*
Day 8 PI [F]	-1.03 [b]	-1.64 [b]	1.44 [a]	0.07 [ab]	*0.558*	*0.002*
Ileal scrapings						
Day 8 PI	-0.53	0.24	-0.04	-0.88	*0.582*	*0.449*
Ileal digesta						
Day 8 PI	-0.07	0.15	-1.38	-1.16	*0.707*	*0.222*
Colon digesta						
Day 8 PI	0.49	1.17	1.27	1.56	*0.811*	*0.759*

[A] Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. [B] Standard error of mean. [C] Experimental Day 0 . [D] Experimental Day 8. [E] Experimental Day 12. [F] Experimental Day 16. Groups T1 and T2 had an n=8, for the group T3 had n=6, and T4 n=7.

No differences due to the treatments were found in the *lactobacilli:coliforms* ratio in ileal scraping, ileal or colon digesta samples at day 8 PI. In fecal samples however we were able to detect statistical differences on day 8 PI (P=0.002) showing the diet supplemented with phytogenic T3 higher values than the basal diet (T1) and the diet supplemented with pharmacological doses of ZnO (T2). Animals receiving phytogenic T4 showed intermediate values.

3.4. Ileal and colonic consistency

The ileal and colonic consistency of the animals that were euthanized was visually evaluated. Scores were defined as follow:

1	Liquid/Watery
2	Watery with solid particles
3	Juicy (similar to melted chocolate)
4	Semisolid or pulpy (that sticks to the spoon)

Results are shown in Table 9. In general terms no significant differences were found related to experimental treatments.

Table 9. *Ileal and colonic digesta consistency scores for the different experimental groups on day 8 PI.*

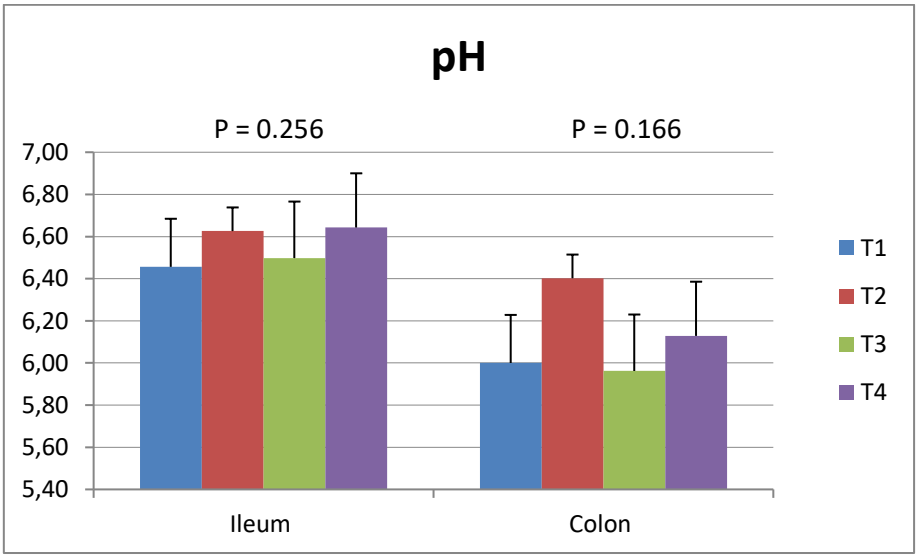
	Treatment [A]					
	T1	**T2**	**T3**	**T4**	**RSD [B]**	***P-value***
Ileal consistency	3.62	3.50	3.80	3.57	*0.718*	*0.904*
Colonic consistency	3.25	3.62	3.66	3.57	0.810	*0.743*

[A] Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. [B] Residual standard deviation. Groups T1 and T2 had an n=8, for the group T3 had n=6, and T4 n=7.

3.5. Effects on digesta pH

Values for the pH of ileum and colon digesta on day 8 PI are shown in figure 4.

Figure 4. *Represent pH values in ileal and colonic digesta on day 8 PI.*



Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. Groups T1 and T2 had an n=8, for the group T3 had n=6, and T4 n=7.

3.6. Inflammatory response

Table 10. Shows the serum levels for Pig-Map and TNF-α in the different treatment groups at days 4 and 8 post Inoculation.

Table 10. *Effect of the experimental treatments on serum levels of pro-inflammatory cytokine TNF-α and acute phase protein Pig-MAP in weaning piglets 4 and 8 days after an oral challenge with ETEC K88.*

	Treatments [A]					
	T1	**T2**	**T3**	**T4**	**RSD [B]**	**P-value**
Pig-MAP	(mg/mL)					
Day 4 PI	3.13	2.60	3.02	1.10	*1.527*	*0.070*
Day 8 PI	2.22	2.66	2.93	1.44	*2.707*	*0.756*
TNF-α	(pg/mL)					
Day 4 PI	99.66	75.58	86.44	85.78	*19.227*	*0.124*
Day 8 PI	71.01	79.68	83.58	75.00	*16.552*	*0.522*

[A] Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. [B] Residual standard deviation. Groups T1 and T2 had an n=8, for the group T3 had n=6, and T4 n=7.

At day 4 PI it was found a trend in Pig-MAP to decrease with T4 (P=0.070). Actually, T4 was the only treatment that showed mean values below 2 mg/mL that reported as a reference value for "high levels" and inflammatory response (Piñeiro et al., 2009).

No significant changes were detected in TNF-α.

3.7. Intestinal morphology

The following histomorphological parameters were analyzed on ileal tissue samples: villus height, crypt depth, intraepithelial lymphocytes, goblet cells and mitosis in crypts. The results of the analysis can be seen in table 11.

Table 11. *Treatment effects on histomorphological parameters at day 8 post challenge.*

	Treatment [A]					
	T1	**T2**	**T3**	**T4**	**RSD [B]**	***P-value***
Villus height (µm)	312.9	336.1	331.8	338.6	*41.57*	*0.612*
Crypt depth (µm)	214.6	179.0	191.8	184.1	*34.73*	*0.209*
Ratio Villus:Crypt	1.49^{b}	1.90^{a}	1.73^{ab}	1.84^{a}	*0.233*	*0.009*
IEL [C] (Nº cel/ 100 µm)	6.04	5.33	5.23	4.81	*1.529*	*0.482*
GC [D] (Nº cel/ 100 µm)	3.36	3.32	2.93	2.89	*1.301*	*0.853*
Mitosis [E] (Nº cel/ 100 µm)	1.38	1.27	1.21	1.24	*0.323*	*0.764*

[A] Treatments: T1, plain diet; T2, plain diet + ZnO; T3, plain diet + phytogenic 1; T4, plain diet + phytogenic 1 and phytogenic 2. [B] Residual standard deviation. Groups T1 and T2 had an n=8, for the group T3 had n=6, and T4 n=7. [C] IEL= Villus intraepithelial lymphocytes; [D] GC= Villus goblet cells/100 µm; [E] Number of mitosis in crypts.

The supplementation with ZnO (T2) significantly improved the villus/crypt ratio compared to the plain diet (T1) and also the T4 treatment (P diet =0.009). Diets T3 showed intermediate values.

4. Discussion

Discussion

The aim of this study was to determine if the administration of phytogenic feed additives was able to enhance health at early life stages and, moreover, if it confers protection against common opportunistic digestive pathogens such as ETEC K88. To assess this objective, two different phytogenic feed additives were tested in this trial. The phytogenics used in this study contains extracts and essential oils from many plants. Whereas T3 included a blend of different commonly used essential oils, T4 potentially benefits of also including other more novel phytogenics with putative antioxidant, anti-inflammatory and immunomodulatory properties.

In general terms performance was not significantly affected by the dietary inclusion of any of the phytogenics (T3 or T4) compared to the plain diet before or after the challenge. The limited number of replicates in this kind of controlled-challenge-trial, probably precluded us to detect significant differences. Despite this, some numerical trends were detected.

According to our data, overall ADFI intake was not significantly modified (P=0.30) by any of the experimental treatments, neither by T2 including pharmacological doses of ZnO. Platel and Srinivasan (2004) described how many botanical compounds and spices can improve food intake in humans and other mammal species. A digestive stimulant action mediated by an increase of salivary, gastric or bile secretions, and reduction in food transit time, may result in a stimulation of appetite. In this regards Cho et al. (2006), Kommera et al. (2006) and Maenner et al. (2011) reported an enhanced ADFI with the use of phytogenic containing fenugreek, clove, cinnamon; anis oil, citrus oil, oregano oil, natural flavor; and menthol, cinnamon aldehyde respectively. However, in the literature there can been found several studies with weaning piglets that were not able to find changes in feed intake. Manzanilla et al. (2004), and Zeng et al. (2015) did not observed effects on ADFI by the supplementation of 5% of carvacrol, 3% of cinnamaldehyde, 2% of capsicum oleoresin, or with 4.5% of cinnamaldehyde and 13,5% of thymol respectively in weaned piglets. Similarly Clouard and Val-Laillet (2014) and Huang et al. (2010) did not observed effects of phytogenic compounds on the ADFI. Moreover authors like Namkung et al. (2004) and Yan et al. (2012) observed a decrease of the ADFI

in weaned piglets due to the supplementation of cinnamon, thyme, oregano, carrier; and buckwheat, thyme, curcuma, black pepper and ginger respectively. The results found in the literature are therefore controversial and probably respond to different products, blends and doses tested.

Results regarding the ADG did not show statistical differences between treatments in the adaptation week but differences were found in the period immediately after the challenge (0-4 PI period). In this period differences in ADG between the basal diet (96 g/d) and diet T2 including ZnO (249 g/d) reached statistical difference (P = 0.035) and animals receiving phytogenics (T3 & T4) showed intermediate values. Other authors, such as Kommera et al. (2006), Maenner et al. (2011) and Zeng et al. (2015) described increases on the ADG of weaned piglets by the supplementation with anise, citrus, oregano oils and natural flavors, as well as, anise oil 4.44 g/kg, clove oil 1.30 g/kg and cinnamon oil 2.0 g/kg respectively. In contrast, Namkung et al. (2004) described a decrease of the ADG in piglets, which were offered a diet with an herbal extract containing cinnamon, thyme and oregano. No differences on the ADG were reported by Manzanilla et al. (2004) Huang et al. (2010) Clouard and Val-Laillet (2014) and Schöne et al. (2006). Which is concurrent with others nursery pig studies involving phytogenic feed additives. Studies that included 10 g/kg of thyme were reported do not affect BW or ADG (Jugl-Chizzola et al., 2005), and 0.1, 0.5, or 1% thyme herbs and essential oils did not affect BW, ADG, or ADFI (Hagmüller et al., 2006). Pigs given 40 mg/kg of a phytogenic blend containing anise (Kroismayr et al., 2008) or diets containing 0.1 to 1.0% garlic (Holden and Mckean, 2001) also promoted no differences in growth performance. Huang et al. (2010) reported no changes in ADG and ADFI for pigs given a 0.1% blend of cinnamon, thyme, rosemary, oregano, and cloves.

The increase of ADG in the supplemented diets, was also reflected in the gain:feed ratio (G:F) that also increased in the 0-4 PI period in the piglets receiving the T2 diet (0.88) compared to T1 with the lowest (0.33). Intermediate values were found again for T3 & T4. In this respect, Kommera et al. (2006) also reported an improvement of the G:F in weaned piglets that were offered diets containing anise, citrus, and oregano oils. In contrast, Namkung et al. (2004) attributed negative feed efficiencies found for piglet during the first post-weaning week to the strong smell of cinnamon, thyme, and

oregano. These results are consistent with studies carried out by Zeng et al. (2015) and Li et al. (2012) that also reported lower G:F in groups of piglets offered diet with essential oils (4.5% cinnamaldehyde and 13.5% thymol) and 0.005 to 0.03% anise (Maenner et al., 2011; Charal et al., 2016). In our case increases in ADG and G:F observed with T2 and numerically with T3 & T4 were only found in the period immediately post-challenge, but not the week before and neither in the 4-8 PI period. These changes can be therefore interpreted as an improvement of the animal response to the pathogen challenge.

As we see, there is a wide variability on the performance results reported among authors due to the supplementation of phytogenic compounds in the swine diets. Clouard and Val-Laillet (2014) suggested that several factors, such as animal characteristic (i.e. age, sex, physiological status), experimental conditions, time of exposure, dosage, and biochemical features of phytogenic, might be decisive factors in the development of performance of pigs. Until now, there is limited knowledge regarding the mode of action of each phytogenic compound in the animals, and their likely functional effects in the digestive tract and metabolism.

Post-weaning diarrhea is one of the many interdependent factors causing the high mortality rate in piglets. The addition of phytogenic feed additives has been reported to reduce the incidence of diarrhea (Li et al., 2012). The reasons for such improvement are most likely associated with the reduction of *E. coli* load in the gut, especially when piglets are raised under relatively poor environmental conditions. Lee et al. (2004) reported that both thymol and cinnamaldehyde have antimicrobial and anti-inflammatory effects. After studying the influence of 13 essential oils compounds on the *in vitro* growth inhibition of members from the intestinal microbiota, Ouwehand et al. (2006) found that beneficial microbes such as lactobacilli and bifidobacteria were less sensitive to essential oils than potentially pathogenic bacteria such as *E. coli, Clostridium perfringens* or *Salmonella.* According to this observation, in the present study the numbers of *lactobacilli* in feces and colon digesta on day 8 PI were the highest with diets supplemented with phytogenics (T3 & T4) and the lowest with T2 treatment. The *lactobacilli:coliforms* ratio, as a potential index of the microbiota balance, was found to be increased at day 8 PI in feces by the T3 treatment when compared to the basal diet, showing diet supplemented with T4 intermediate values. However we were not able to

detect a significant impact on total coliforms in any of the days or type of samples analyzed.

The oral challenge promoted a mild course of diarrhea that was translated into a significant increase in fecal scores after the challenge, however the addition of the experimental phytogenic feed additives to the diet did not influence this parameter. Other authors also have failed to demonstrate a reduced diarrhea incidence or an improved fecal score with phytogenic mixtures (Manzanilla et al., 2004; Maass et al., 2005; Cho et al., 2006; Kommera et al., 2006; Muhl and Liebert et al., 2007; Huang et al., 2010) that probably is related to the limitations of models of study. Despite this it is also possible to find works evidencing the potential of phytogenic to control clinical diarrhea (Li et al., 2012; Zeng et al., 2015, including 0.005 to 0.025% thyme and cinnamon).

Young pigs are particularly susceptible to pathogenic infection after weaning due to a stress-induced greater intestinal permeability that lead to mucosal inflammation (Moeser et al., 2007). The immune system response is chiefly mediated and executed by proteins such as cytokines and immunoglobulins. Cytokines are cell signalling proteins commonly secreted by immune cells to modulate cellular immune response (Ibergaufts, 2016). Two of these cytokines, TNF-α and IL-6, are primarily involved in activities regarding activation of NF-κB, inflammatory regulation, and cellular apoptosis (Wajant et al., 2003; Hoene and Weigert, 2008). Some agents in phytogenics are speculated to exert immune response properties. However, the inclusion of phytogenics in the current diets did not affected the serological concentrations of TNF-α. Absence of changes in serological TNF-α levels also has been reported in nursery pig study with a PRSSV challenge by Liu et al. (2013). In swine, Pig-MAP is a major acute-phase protein, and higher serum concentrations have been related to acute inflammatory processes and also to the extent of tissue injury, expressing strong and protective responses to bacterial infections (Piñeiro et al., 2009). Previous works (López-Colom et al., 2019) have demonstrated the usefulness of this biomarker to determine the intestinal injury degree and barrier integrity in recently weaned pigs. In this work we found a trend at day 4 PI in Pig-MAP to decrease with T4 ($P=0.07$) that would suggest that this phytogenic blend could exhibit anti-inflammatory properties in front of the tissue injury promoted by the ETEC challenge.

During the post-weaning phase, the intestinal tract of the weanling pig undergoes several changes in gut morphology, including a transient reduction in villus height and an increase in crypt depth (Piva et al., 2002). These changes may decrease feed efficiency because they reduce the absorptive capacity of the intestine (Campbell et al., 2013). Therefore, improvements in villus height and reductions in crypt depth can increase nutrient absorption and reduce energy losses (Oetting et al., 2006). From this point of view the villus:crypt ratio has been frequently used as an index of epithelium integrity and functionality. In our study the villus:crypt ratio was significantly increased by the inclusion of ZnO and also by the T4 diet compared to T1. The T3 showed intermediate values. These results would suggest that, particularly the second blend of phytogenics (T4), could had prevented the intestinal damage probably mediated by a better controlled inflammatory response according to the reduction also observed in Pig-MAP values.

Other authors also have found improvements of epithelium integrity with phytogenic feed additives (Huang et al., 2012; Li et al., 2012; Zeng et al., 2015). On the other hand, findings of Manzanilla et al. (2004), Namkung et al. (2004), Manzanilla et al. (2006), Nofrarias et al. (2006), oeatting (2006), and Kroismayr et al. (2008) reported that nursery pigs supplemented with phytogenics feed additives had no changes in epithelium integrity.

In summary results of this study would suggest that both tested phytogenics could help the piglet to fight the ETEC challenges commonly faced after weaning. This efficacy would be supported by the numerical increases observed in ADG and gain:feed ratio in the period immediately after the challenge, with intermediate figures between the plain diet and the diet including pharmacological doses of ZnO. In the case of the phytogenic T3 this better response could be due to an improved microbiota balance suggested by the increased *lactobacilli:coliforms* ratio in feces registered at day 8 PI when compared to plain diet. Regarding phytogenic T4 (that also included plant extracts with anti-inflammatory and antioxidant properties), this could help to modulate the inflammatory response induced by the pathogen (lower Pig-MAP), leading to the observed improved intestinal architecture (higher VH:CD ratio). Findings of this study support therefore the

usefulness of both additives during the weaning period when piglets are extremely sensitive to disease infection.

5. Conclusions

According to the results presented in this dissertation, it is concluded that:

1. When compared to a plain diet, both tested phytogenics (T3 & T4) promote numerical increases in average daily gain and gain:feed ratio immediately after the ETEC K88 challenge (0-4 days PI) that could be regarded as an improved response in front of the pathogen, however improvements do not reach the significant increases observed for pharmacological doses of ZnO (3000 ppm).

2. The addition of phytogenic T3 in the diet of weaning pigs is able to improve the microbiota balance, increasing the fecal *lactobacilli:coliforms* ratio shortly after the ETEC K88 challenge (day 8 PI).

3. The second tested phytogenic (T4) promote a higher villus:crypt ratio after the ETEC K88 challenge (day 8 PI), reaching similar values to those promoted by pharmacological doses of ZnO. This mitigation of the intestinal damage induced by the challenge could be mediated by a better-controlled inflammatory response according to the reduction (P=0.07) observed in Pig-MAP values.

6. References

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