ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 5 (2): 137-142, 2006
ISSN 1682-296X

Natural Plant Extracts and Organic Acids: Synergism and Implication on Piglet's Intestinal Microbiota

[1]M.A. Calvo, [2]E. Angulo, [3]P. Costa-Batllori, [1]C. Shiva, [1]C. Adelantado and [4]A. Vicente
[1]Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària
Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
[2]Departament de Producció Animal, Escola Tècnica Superior d´Enginyeria Agrària, Universitat de Lleida
[3]Escola Superior d'Agricultura de Barcelona, Universitat Politècnica de Catalunya, Barcelona, Spain
[4]Iproa, Pol. Ind. Sud Oest, Tagomago nº15, 08280 Sant Quirze del Vallès (Barcelona), Spain

Abstract: Two trials were carried out to study how a compound formed by natural extracts from *Rutaceae* plants, cinnamon oil and organic acids (RUTEX® PG) could have an effect on intestinal microorganisms on piglets. To obtain this synergistically combination first of all natural extracts and organic acids were separated individually to see its inhibitory action on bacterial growth *in vitro*. Secondly, the aim was to search the best natural extract and organic acid combination between all the possible ones. RUTEX® PG was the combination that was used *in vivo* trials with piglets. To study RUTEX® PG effects on piglets two trials were designed. The trials consisted in a comparison between the effect of adding RUTEX® PG to the feed on the intestinal microorganisms of piglets and the effect of antibiotics in feed. The *in vitro* experiments results showed a synergistic effect of the *Rutaceae* plant extract and the organic acids tested (citric, formic, lactic and propionic) against different microorganisms: growth inhibition was observed for the organic acid-natural extract combination, while the same concentrations did not show any inhibition when the products were tested separately. Inhibitory activity for cinnamon oil was not tested, because it was added in a very low concentration just to confer an aromatic effect. It was observed, as well, lower bacterial counts at the intestinal segments (duodenum, jejunum, ileum and colon) in comparison with the negative control group. The conclusion is that we can say RUTEX® PG can be active all along the gastro-intestinal segments.

Key words: Intestinal microorganisms, organic acids, piglets, plant extracts, *Rutaceae*

INTRODUCTION

Antibiotic growth promoters are being prohibited by the European Union and the consequence of this ban is a dramatically change in livestock management and feeding. This prohibition has been debated among meat producers, especially pork and poultry producers and alternatives to antibiotics as growth promoters are needed.

The use of organic acids as mould and bacteria inhibitors, their role as enzyme activators and their ability to modify the microbial population in the intestinal tract of piglets has been reported by several authors because of its improving performance (Eidelsburger, 1998; Roth and Kirchgessner, 1998; Overland *et al*., 2000). In this way, Freitag *et al*. (1998) reported improvements from 3.1 to 22.1% in weight gain and from 1.6 to 14.5% in feed to gain ratio in piglets by including formic acid up to 1.25% in the feed. In comparison with several trials done with the addition of Carbadox, these authors showed an average improvement in weight gain and feed to gain ratio of 18.2 and 7.1%, respectively, thus concluding that formic acid in that case could nearly compensate for the effects of an antibiotic growth promoter.

Plant extracts have been studied as well because of its demonstrated beneficial effects, although scientific literature is very little. Compounds such as phenols, polyphenols, terpenoids, alkaloids, lectins and polypeptides that are found in plant extracts could cause an antimicrobial and antifungal action (Basilico and Basilico, 1999; Cowan, 1999). *In vitro* studies have shown that rosemary and cinnamon extracts and garlic, onion and oregano (as well as other *Labiateae*) essential oils exhibit considerable antimicrobial activity against *Listeria monocytogenes*, *Escherichia coli, Pseudomonas*

Corresponding Author: M.A. Calvo, Departament de Sanitat i d'Anatomia Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain Tel: 0034935811748 Fax: 0034935812006

aeruginosa, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Salmonella* sp. and *Vibrio parahaemolyticus* (Chang *et al*., 2001; Del Campo *et al*., 2003; Kim *et al*., 2004). Nevertheless, Turner *et al*. (2001) reported contradictory or inconsistent results when comparing several trials with the addition of peppermint, garlic, clove, cinnamon or *Echinacea* (alone or mixed) in weanling pig diets.

Information about the combination of organic acids and plant extracts to study its action is little. The objective of the present report was to study the combined effect of natural plant extracts (*Rutaceae* family) with organic acids on *in vitro* bacterial growth inhibition and to test the best combination *in vivo* on intestinal microbial population.

MATERIALS AND METHODS

***In vitro* bacterial growth inhibition:** In order to assess the bacterial growth inhibition by the substances tested, the method of radial diffusion in paper disk (Navarro *et al*., 1996) was performed. These substances were: propionic acid 99%, formic acid 85%, lactic acid 80%, citric acid 100%, *Rutaceae* extract (from *Citrus limonium*, *C. aurantium*, *C. bergamia*, *Barosma betulina)*, combinations of acids and *Rutaceae* extracts and RUTEX® PG, which components are:

- Hydroglycolic extract from *Citrus limonium*, *C. aurantium*, *C. bergamia* and *Barosma betulina.*
- *Cinnamomum* spp. and *Cymbopogon* spp. essential oils.
- Organic acids and their salts: Calcium formiate, Citric acid, Lactic acid and Ammonium propionate.
- Excipients: E-551a and E-562.

Microorganisms tested were: *Escherichia coli* FVB 467, *Staphylococcus aureus* FVB 13, *Enterococcus faecalis* FVB 389, *Clostridium perfringens* FVB 68 and *Salmonella* sp. FVB 576 (FVB: Veterinary Faculty of Barcelona collection). These assays were carried out at Veterinary Faculty of Barcelona all along 2005.

Briefly, this method starts by checking the solubility of the substances prior to their study. After that, dilutions from 50 to 6000 ppm of each product to be tested are prepared and assayed by impregnating sterilized 6 mm diameter filter paper disks with them and placing these disks onto petri dishes seeded with each of the microorganisms mentioned above. The culture medium used in these petri dishes was Mueller Hinton Agar and incubation temperature was set at 37°C during 24 h.

Table 1: Basal feed composition and estimated nutrient content (as-fed) of the feed used in the trial to determine microbial population of piglet's intestine

Ingredient (%)	
Cereals	47.342
Fish meal LT	8.000
Animal plasma	5.000
Milk serum	24.000
Sucrose	5.000
Animal fat	3.500
Soybean protein concentrate	5.000
Sodium chloride	0.300
Dicalcium phosphate	0.600
Calcium carbonate	0.780
Lysine	0.150
Methionine	0.128
Premix	0.200
Chemical composition	
Net energy (MJ/kg)	10.57
Crude protein (%)	18.00
Ether extract (%)	5.80
Ash (%)	6.70
Crude fiber (%)	1.80
Lysine (%)	1.40
Methionine + cysteine (%)	0.80
Threonine (%)	0.80
Tryptophan (%)	0.25

Premix (per kg feed): 10.000 IU vitamin A, 2.000 IU vitamin D3, 20 mg vitamin E, 1 mg vitamin K, 1 mg thiamine, 4 mg riboflavin, 10 mg d-panthotenic acid, 0.5 mg niacin, 0.03 mg cobalamin, 600 mg choline chloride, 0.1 mg biotin, 0.2 mg Co, 0.3 mg Se, 0.5 mg I, 100 mg Fe, 10 mg Cu, 40 mg Mn and 100 mg Zn

Table 2: Basal feed composition and estimated nutrient content (as-fed) of the feeds used in the trial for performance of piglets

Ingredient (%)	Prestarter	Starter
Corn	24.270	12.721
Wheat + barley	34.990	52.100
Fish meal	5.030	3.260
Blood meal concentrate	5.710	0.000
Milk serum	21.980	10.000
Soybean meal 48	2.000	8.690
Extruded full fat soya	0.000	6.000
Soybean protein concentrate	0.890	0.000
Potato protein concentrate	0.000	2.000
Soybean oil	3.000	3.200
Sodium chloride	0.300	0.300
Dicalcium phosphate	0.700	0.649
Calcium carbonate	0.540	0.521
Lysine	0.250	0.239
Methionine	0.140	0.120
Premix	0.200	0.200
Chemical composition		
Net energy (MJ/kg)	10.47	10.46
Crude protein (%)	17.76	18.84
Ether extract (%)	5.02	6.49
Ash (%)	5.05	4.07
Crude fiber (%)	1.87	2.71
Lysine (%)	1.43	1.30
Methionine+ cysteine	0.80	0.76

Premix prestarter (per kg feed): 10.000 IU vitamin A, 2.000 IU vitamin D3, 20 mg vitamin E, 1 mg vitamin K, 1 mg thiamine, 4 mg riboflavin, 10 mg d-panthotenic acid, 0.5 mg folic acid, 12 mg pyridoxine, 15 mg niacin, 0.03 mg cobalamin, 600 mg choline chloride, 0.1 mg biotin, 0.2 mg Co, 0.3 mg Se, 0.5 mg I, 100 mg Fe, 10 mg Cu, 40 mg Mn and 100 mg Zn. Premix starter (per kg feed): 8.000 IU vitamin A, 1.000 IU vitamin D3, 10 mg vitamin E, 0.5 mg vitamin K, 1 mg thiamine, 3 mg riboflavin, 8 mg d-panthotenic acid, 0.5 mg folic acid, 10 mg pyridoxine, 10 mg niacin, 0.03 mg cobalamin, 500 mg choline chloride, 0.05 mg biotin, 0.1 mg Co, 0.1 mg Se, 0.2 mg I, 80 mg Fe, 10 mg Cu, 40 mg Mn and 100 mg Zn Diets were prepared according to adapted FEDNA indications

All plates were incubated under aerobic growth conditions except the ones with *Clostridium perfringens*, that were placed under anaerobic conditions. To assess repeatability of the trial, assays were performed by triplicate and to check the viability of the microorganisms used, blank controls were also included. Results were expressed as Inhibition Value in mm (IV). This parameter was calculated as follows:

$$IV = (\text{Inhibition Diameter} - \text{Disk Diameter})/2$$

Microbial population of piglets intestine (*in vivo*): This experiment received approval from the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona and the treatment, housing and slaughtering conditions conformed to current European Union Guidelines.

In order to carry on this study, 40 piglets (Landrace×Large White) weaned at 21 days of age were randomly selected and distributed in 4 pens (2×1 m^2) containing 10 piglets each one. These pens were provided with completely slatted floor and this space also had automatic lighting and environmental control system. Treatments were added to a commercial basal feed formulation (Table 1and 2 they were applied in a 2×2 factorial design as explained in Statistical Analysis. There were four treatment in base of inclusion or not antibiotic (120 ppm colistine and 300 ppm amoxicyline) and inclusion or not RUTEX® (2 kg/MT). Feed and water were provided *ad libitum*.

The trial's length was 18 days and after that time, 2 piglets per treatment were sacrificed by intracardiac injection of T-61 euthanasic (Intervet International B.V.). With the aim of separating duodenum, jejunum, ileum and colon, ligatures were performed and all these parts were collected and immediately refrigerated to be sent to the microbiological laboratory (Veterinary Faculty of Barcelona). From each sample, serial dilutions using sterilized Ringer ¼ were performed but for *Salmonella* spp. counts. According to Pascual and Calderón (2000) procedures, adequate growth media and seeding techniques were selected and detection and isolation techniques for *Salmonella* spp. were carried out.

Variables studied were the following: 1) Total mesophil aerobic bacteria counts; 2) total anaerobic bacteria counts; 3) total *Enterobacteriaceae* counts; 4) total fungi counts and 5) *Salmonella* spp. detection.

The objective of this study was to check RUTEX® PG activity all along the intestinal study. It was based on the hypothesis that if this treatment showed different bacterial counts than negative control, it could be assumed that it might be due to an external factor action, because all the other conditions related to animals did not change.

RESULTS

***In vitro* bacterial growth inhibition:** No growth inhibition was observed (IV = 0) when testing lactic and citric acid (up to a concentration of 6000 ppm) on any of the microorganisms tested.

On the other hand, higher growth inhibition was reached by propionic acid at concentrations from 2000 ppm to 6000 ppm on all the microorganisms assayed (Table 3). It was also assessed, that formic acid performance on *Salmonella* spp. and *Clostridium perfringens* was quite similar to propionic acid results, but had a weaker activity against *Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus*.

Rutaceae extract, however, showed significantly higher values at even 20 times lower concentrations than the acids (from 200 to 300 ppm, $p<0.001$).

Different authors, under several conditions and substrates (Cattaneo *et al*., 1979; Cherrington *et al*., 1991; Kwon *et al.*, 1998; Ricke, 2003) had already determined *in vitro* antimicrobial activity of organic acids and their salts, but no reports for *Rutaceae* extract have been suggested yet. However, an antifungal *in vitro* activity for *Citrus paradisa* and *Citrus sinensis* (both plants belonging to the *Rutaceae* Family) was once reported (Stange *et al*., 1993).

A synergistic effect (Table 4) was observed when combinating each acid (individually at 1000 ppm) with the *Rutaceae* extract (at 100 ppm). With this organic acid-natural extract combination, growth inhibition was observed, while no inhibition occurred when testing separately both products. It must be mentioned that this inhibitory action was only observed on *E. faecalis*, *S. aureus* and *C. perfringens*, but not on *Salmonella* spp. and *E. coli*.

Having these data into account, several combination of both organic acid and *Rutaceae* extracts were assayed and results are presented in Table 5 for the best synergistic combination, RUTEX® PG. Inhibition Values for RUTEX® PG were higher at the highest concentration tested (3000 ppm), finding *S. aureus* and *C. perfringens* more sensitive than the other bacteria.

Inhibitory activity for cinnamon oil was not tested, because it was added in a very low concentration just to confer an aromatic effect.

Microbial population of piglets intestine (*in vivo*): No incidence of diarrhoea while the trial length was reported, so all results on microbial counts were considered on healthy animals. In Table 6, results on variables studied

Table 3: Microbial growth inhibition (Inhibition Value, in mm; means±SEM) of formic and propionic acids and *Rutaceae* extract (each analysis tested by triplicate)

Item	*Salmonella* spp.	*Escherichia coli*	*Enterococcus faecalis*	*Staphylococcus aureus*	*Clostridium perfringens*
Propionic acid					
1000 ppm	0.0±0.00^{e}	0.0±0.00^{h}	0.0±0.00^{d}	0.0±0.00^{f}	0.0±0.00^{f}
2000 ppm	0.5±0.03^{d}	1.0±0.00^{f}	2.5±0.06^{c}	2.5±0.00^{d}	2.5±0.00^{d}
4000 ppm	1.0±0.03^{c}	2.5±0.00^{c}	2.5±0.06^{c}	2.5±0.03^{d}	2.5±0.00^{d}
6000 ppm	1.0±0.03^{c}	2.5±0.06^{c}	2.5±0.00^{c}	5.0±0.06^{b}	3.0±0.00^{c}
Formic acid					
1000 ppm	0.0±0.00^{e}	0.5±0.06^{g}	0.0±0.00^{d}	2.0±0.03^{e}	2.0±0.00^{e}
2000 ppm	0.5±0.00^{d}	1.0±0.00^{f}	0.0±0.00^{d}	2.0±0.00^{e}	2.0±0.06^{e}
4000 ppm	1.5±0.00^{b}	1.5±0.00^{e}	0.0±0.00^{d}	3.0±0.15^{c}	2.5±0.00^{d}
6000 ppm	2.0±0.00^{b}	2.0±0.00^{d}	2.5±0.00^{c}	3.0±0.00^{c}	3.0±0.00^{c}
Rutaceae extract					
50 ppm	0.0±0.00^{e}	0.0±0.00^{h}	0.0±0.00^{d}	0.0±0.00^{f}	0.0±0.00^{f}
100 ppm	0.0±0.00^{e}	0.0±0.00^{h}	0.0±0.00^{d}	0.0±0.00^{f}	0.0±0.00^{f}
200 ppm	2.5±0.06^{a}	3.5±0.03^{b}	5.0±0.00^{b}	5.0±0.00^{b}	4.5±0.06^{b}
300 ppm	2.5±0.00^{a}	6.5±0.03^{a}	7.6±0.03^{a}	9.5±0.06^{a}	8.0±0.00^{a}
p>F	***	***	***	***	***

$^{a, b, c, d, e, f, g, h}$ values within columns with no common superscript are significantly different, *** p<0.001

Table 4: Microbial growth inhibition (Inhibition Value, in mm; means±SEM) of the combination of each acid tested (at 1000 ppm) and *Rutaceae* extract (at 100 ppm) (each analysis tested by triplicate)

Acid in the mixture	*Salmonella* spp.	*Escherichia coli*	*Enterococcus faecalis*	*Staphylococcus aureus*	*Clostridium perfringens*
Citric	0.0±0.00	0.0±0.00	3.5±0.00^{a}	2.5±0.06	2.5±0.03
Formic	0.0±0.00	0.0±0.00	2.5±0.03^{b}	2.5±0.06	2.5±0.00
Lactic	0.0±0.00	0.0±0.00	2.5±0.00^{b}	2.5±0.06	2.5±0.00
Propionic	0.0±0.00	0.0±0.00	2.5±0.06^{b}	2.5±0.00	2.5±0.00
P>F	-	-	***	1.0	0.441

$^{a, b}$ values within columns with no common superscript are significantly different, *** p<0.001

Table 5: Microbial growth inhibition (Inhibition Value, in mm; means±SEM) of the best synergistic combination of acids and *Rutaceae* extract (RUTEX® PG; each analysis tested by triplicate)

Item	*Salmonella* spp.	*Escherichia coli*	*Enterococcus faecalis*	*Staphylococcus aureus*	*Clostridium perfringens*
1000 ppm	0.5±0.00^{b}	2.5±0.00^{b}	2.5±0.06	2.5±0.00^{c}	2.5±0.00^{c}
2000 ppm	1.5±0.06^{a}	2.5±0.00^{b}	2.5±0.03	5.1±0.03^{b}	3.0±0.06^{b}
3000 ppm	1.5±0.00^{a}	3.5±0.03^{a}	2.5±0.00	8.5±0.06^{a}	5.1±0.03^{a}
p>F	***	***	0.787	***	***

$^{a, b, c}$ values within columns with no common superscript are significantly different, *** p<0.001

Table 6: Microbial population (total counts: log CFU/g; means±SEM) of piglet's intestine content (2 piglets/treatment), according to feed treatment (antibiotics = 120 ppm colistine + 300 ppm amoxicyline)

Item	Duodenum	Dejunum	Ileum	Colon
Mesophil aerobic bacteria				
Negative control	5.0±0.00	4.0±0.00	3.0±0.00	5.0±0.00
Antibiotics	4.0±0.00	3.0±0.00	5.0±0.00	6.0±0.00
RUTEX® PG	3.0±0.00	3.0±0.00	3.0±0.00	3.0±0.00
RUTEX® PG + Antibiotics	3.0±0.00	3.0±0.00	3.0±0.00	3.0±0.00
p<F	-	-	-	-
Anaerobic bacteria				
Negative control	2.0±0.00^{b}	2.0±0.00	2.0±0.00^{b}	2.0±0.00
Antibiotics	6.0±0.00^{a}	6.0±0.00	6.0±0.00^{a}	6.0±0.00
RUTEX® PG	1.8±0.03^{c}	2.0±0.00	1.3±0.03^{c}	4.0±0.00
RUTEX® PG + Antibiotics	1.0±0.00^{d}	2.0±0.00	2.0±0.00^{b}	2.0±0.00
p>F	***	-	***	-
Enterobacteriacea				
Negative control	3.0±0.00	4.0±0.00	5.0±0.00	5.0±0.00
Antibiotics	3.0±0.00	3.0±0.00	5.0±0.00	5.0±0.00
RUTEX® PG	2.0±0.00	2.0±0.00	3.0±0.00	3.0±0.00
RUTEX® PG+ Antibiotics	2.0±0.00	2.0±0.00	3.0±0.00	3.0 ± 0.00
p>F	-	-	-	-
Moulds and yeasts				
Negative control	2.0±0.00^{b}	2.0±0.00	2.0±0.00	4.0±0.00^{b}
Antibiotics	2.5±0.00^{a}	3.3±0.00	4.3±0.00	4.3±0.03^{a}
RUTEX® PG	2.3±0.03^{b}	2.5±0.00	2.5±0.00	3.3±0.03^{d}
RUTEX® PG+ Antibiotics	2.6±0.03^{a}	4.0±0.00	4.3±0.00	3.7±0.00^{c}
p>F	***	-	-	***

$^{a, b, c, d}$ values within columns with no common superscript are significantly different, -Means are different but as SEM=0 no statistics can be performed, ***p<0.001

Table 7: Effect of RUTEX® PG on performance of piglets (n = 56)

Item	BW initial (kg)	BW initial (kg)	ADG (g)	ADFI (g)	BW final FCR
Prestarter period (21-42 days of life)					
Control	5.71±0.15	10.00±0.21	204±8	264±10	1.263±0.013
RUTEX® PG	5.73±0.10	10.41±0.26	223±12	275±11	1.216±0.026
p>F	NS	NS	NS	NS	*
Starter period (42-63 days of life)					
Control	10.00±0.21	20.09±0.50	480±18	654±24	1.455±0.098
RUTEX® PG	10.41±0.26	21.17±0.55	512±16	708±21	1.385±0.014
p>F	NS	*	*	NS	NS
Whole period (21-63 days of life)					
Control	5.71±0.15	20.09±0.50	342±11	459±15	1.359±0.048
RUTEX® PG	5.73±0.10	21.17±0.55	368±12	491±13	1.300±0.014
p>F	NS	*	*	NS	NS

p<0.05; NS: Non Significant, BW: Body Weight, ADG: Average Daily Gain, ADFI: Average Daily Feed Intake, FCR: Feed Conversion Rate

(total mesophil aerobic bacteria counts, total anaerobic bacteria counts, total *Enterobacteriaceae* counts and total fungi counts) for each part of the intestine (duodenum, jejunum, ileum and colon) are shown. It must be mentioned that *Salmonella* spp. was not detected in any animal.

Piglets that were fed with RUTEX® PG (either with or without antibiotics) showed significantly lower anaerobic bacteria counts when checking duodenum and ileum ($p<0.001$). In any case, total counts for any of the variables studied were lower in piglets with RUTEX® PG supply than those only fed with the control diet (exception was found for mesophil aerobic bacteria at ileum and anaerobic bacteria count at jejunum).

Those piglets that were fed with only antibiotics included in their diet, showed a significantly higher duodenal and ileac anaerobic bacteria than piglets in the negative control group ($p<0.001$). Apart from these results, values were also higher for mesophil aerobic bacteria at ileum and colon and for anaerobic bacteria at jejunum and colon. Nevertheless, in all of the cases mentioned, even with different means, no p-values could be obtained, as means standard errors were 0, as explained above. The increase of anaerobic bacteria counts, could be explained by the change of microbial population due to the diet.

DISCUSSION

Growth promotion mechanisms by antibiotics are quite unknown, but it is commonly accepted that this kind of substances may help to control undesirable bacteria population in the intestine and besides allow better nutrients absorption, thus creating a bacterial balance within the intestinal microbiota. However, most antibiotics have only effectiveness against certain bacteria types, so it has been suggested that they could also disturb the normal enteric microbiota (CVM, 2002), for instance increasing certain bacterial groups, as reported in our work for anaerobic bacteria.

On the other hand, there is a high variability when considering animal response to antibiotic growth promoters and it may be dependent upon the environment in which the animals are raised and the kind of diet (Bedford, 2000).

As previously described, the main objective of this work was to demonstrate that this combination (RUTEX®) PG consisting in organic acids plus *Rutaceae* extracts, remain active all along the intestinal tract. Since lower bacteria counts were observed at the different intestinal tract levels in relation to the negative control group (when considering any of the studied groups), we can state that the product exerted an action by means of the whole product, a part of it, or even its metabolites. However, this action could reach all the intestinal levels taken into account for this study.

When talking about fungi, higher counts were found in all treatments all along the intestinal tract when comparing to controls, although only P statistically highly significant values were obtained for duodenum ($p<0.001$). It may be due to the fact that total fungi counts include both moulds and yeasts, but what really increase these counts are yeasts, presumably caused by the lower pH values reached as a direct consequence of the additives supplied to the diet. The colon was an exception, because animals supplemented with RUTEX® PG (or even the same combination with antibiotics) had significantly lower counts than piglets fed with diets containing only antibiotics or the negative control.

CONCLUSIONS

With the results obtained, it is assessed that exists a synergistic effect of the *Rutaceae* plant extract and the different organic acids assayed (citric, formic, lactic and propionic) against different microorganisms. In addition, taking into account the counts of microorganisms obtained for both treated and control groups, it has been observed that the effectiveness of RUTEX® PG lasts all along the gastro-intestinal segments.

ACKNOWLEDGEMENTS

Authors greatly appreciate the help of Dr. Jordi Valero (Escola Superior d'Agricultura de Barcelona, Universitat Politècnica de Catalunya) for the statistical analysis.

REFERENCES

Basilico, M.Z. and J.C. Basilico, 1999. Inhibitory effects of some spice essential oils on *Aspergillus ochraceus* NRRL 3174 growth and ochratoxin A production. Lett. Applied Microbiol., 29: 238-241.

Bedford, M., 2000. Removal of antibiotic growth promoters from poultry diets: Implications and strategies to minimize subsequent problems. World's Poult. Sci. J., 56: 347-365.

Cattaneo, P., S. D'Aubert and C. Cantoni, 1979. Attività biocida di acidi organici (Ac. Propionico, Ac. Formico e Ac. Acetico) contro le Salmonelle (Biocide activity of organic acids against *Salmonella*). Arch. Vet. It., 30: 33-35.

CVM, Food and Drug Administration, 2000. Effect of the Use of Antimicrobials in Food-producing Animals on Pathogen Load: Systematic Review of the Published Literature. US Department of Health and Human Services.

Chang, S.T., P.F. Chen and S.C. Chang, 2001. Antibacterial activity of leaf essential oils and their constituents from *Cinnamomum osmophloeum*. J. Ethnopharmacol., 77: 123-127.

Cherrington, C.A., M. Hinton, G.C. Mead and I. Chopra, 1991. Organic acids: Chemistry, antibacterial activity and practical applications. Adv. Microbial. Physiol., 32: 87-108.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev., 12: 564-582.

Del Campo, J., C. Nguyen-The, M. Sergent and M.J. Amito, 2003. Determination of the most bioactive phenolic compounds from rosemary against *Listeria monocytogenes*: Influence of concentration, pH and NaCl. J. Food Sci., 68: 2066-2071.

Eidelsburger, U., 1998. Feeding Short-chain Organic Acids to Pigs. In Recent Advances in Animal Nutrition (Garnsworthy, P.C. and J. Wiseman, Eds.), Nottingham University Press, Nottingham, United Kingdom, pp: 93-106.

Freitag, M., H.U. Hensche, H. Schulte-Sienbeck and B. Reichelt, 1998. The effects of feed additives as substitutes for performance enhancers in pig production. Report nº 8 Forschungsber. FH Soest, Faculty of Agriculture, Germany.

Kim, J.W., Y.S. Kim and K.H. Kyunga, 2004. Inhibitory activity of essential oils of garlic and onion against bacteria and yeasts. J. Food Prot., 67: 499-504.

Kwon, Y.M., S.D. Ha and S.C. Ricke, 1998. Growth response of a *Salmonella typhimurium* poultry isolate to propionic acid under aerobic and anaerobic conditions. J. Food Safety, 18: 139-149.

Navarro, V., M.L. Villarreal, G. Rojas and X. Lozoya, 1996. Antimicrobial evaluation of some plants used in Mexican traditional medicine for the treatment of infectious diseases. J. Ethnopharmacol., 53: 143-147.

Overland, M., T. Granli, N.P. Kjos, O. Fjetland, S.H. Steien and M. Stokstad, 2000. Effect of dietary formates on growth performance, carcass traits, sensory quality, intestinal microflora and stomach alterations In: Growing-finishing pigs. J. Anim. Sci., 78: 1875-1884.

Pascual, M.R. and V. Calderón, 2000. Microbiología alimentaria (Food Microbiology). 2nd Edn., Díaz de Santos, Madrid, Spain.

Ricke, S.C., 2003. Perspective on the use of organic acids and short chain fatty acids as antimicrobials. Poult. Sci., 82: 632-639.

Roth, F.X. and M. Kirchgessner, 1998. Organic acids as feed additive for young pigs: nutritional and gastrointestinal effects. J. Anim. Feed Sci., 7: 25-33.

SPSS, 2001. SPSS for Windows (internet manual), Version 11.0.1.

Stange, R.R., S.L. Midland, J.W. Eckert and J.J. Sims, 1993. An antifungal compound produced by grapefruit and Valencia orange after wounding of the peel. J. Nat. Prod., 56: 1627-1629.

Turner, J.L., S.S. Dritz and J.E. Minton, 2001. Review: Alternatives to conventional antimicrobials in swine diets. Prod. Anim. Sci., 17: 217-226.