



***In vitro* effects of some organic acids on swine cecal microflora**

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ABSTRACT

The objective of this study was to evaluate the effects of different organic acids on bacterial growth and ammonia production by swine cecal microflora in an *in vitro* fermentation system. Formic, acetic, propionic, lactic, butyric, sorbic, fumaric, malic, citric, α -ketoglutaric, and benzoic acids were used at different concentrations (60, 120 and 240 mmol/l) to assess if any effect on cecal microflora could be shown. Cecal microbial growth was evaluated using the cumulative gas production technique. Fermentation fluid was analyzed for ammonia concentration. Lactic acid was the only acid that enhanced bacterial fermentation at every concentration tested (+30%, 73%, and 35% gas final volume at 60, 120, and 240mmol/l, respectively; $P<0.05$). Citric acid strongly increased gas final volume when used at 60 and 120mmol/l (+92% and 52%, respectively; $P<0.05$) and almost completely inhibited bacterial activity at 240mmol/l (-85% gas final volume; $P<0.05$). Sorbic acid showed the strongest antibacterial activity and was the only acid that reduced final gas volume when used at 60 mmol/l (-34%; $P<0.05$). Only sorbic (-34%, 47%, and 67%), fumaric (-57%, 35%, and 26%), citric (-31%, 18%, and 28%), and benzoic (-33%, 29%, and 38%) acids reduced ammonia after 24h of fermentation at any given concentration ($P<0.05$). Other acids, such as formic, acetic, propionic, butyric, and malic acids failed to exert an effective control of ammonia levels in the fermentation liquor. After the first set of fermentations, sorbic, α -ketoglutaric, and benzoic acids were further tested at the final concentration of 7.5, 15, and 30mmol/l. At these lower levels of inclusion, sorbic acid was the only acid that reduced final gas volume at every concentration tested (-31%, 35%, and 39% at 7.5, 15, and 30mmol/l, respectively; $P<0.05$). Compared with control, ammonia was reduced after 24h of fermentation by all treatments (with an average reduction of 34%, 20%, and 24% for sorbic, α -ketoglutaric, and benzoic acids, respectively; $P<0.05$). In conclusion, gas production data showed that organic acids inhibit or enhance cecal bacterial activity in relation to the type and concentration of acid used. Furthermore, sorbic, α -ketoglutaric, and benzoic acids can positively influence swine cecal microflora *in vitro* fermentation reducing ammonia concentrations, even when used at low concentrations.

Key words: Ammonia, Cecal microflora, *In vitro*, Organic acids, Swine.

RIASSUNTO

EFFETTI *IN VITRO* DI ALCUNI ACIDI ORGANICI SULLA MICROFLORA CECALE DI SUINO

L'obiettivo del presente studio era la valutazione degli effetti di diversi acidi organici sulla crescita batterica e sulla produzione di ammoniaca da parte della microflora cecale di suino in un sistema di fermentazione in vitro. Gli acidi formico, acetico, propionico, lattico, butirrico, sorbico, fumarico, malico, citrico, α -chetoglutarico e benzoico sono stati impiegati a diverse concentrazioni (60, 120 e 240 mmoli/l).

L'attività microbica è stata monitorata mediante la misurazione della produzione cumulativa di gas e si è inoltre proceduto alla determinazione delle concentrazioni di ammoniaca nel liquido di fermentazione. L'acido lattico è stato l'unico acido capace di stimolare la produzione di gas a tutte le concentrazioni testate (+30%, 73% e 35% di volume finale di gas rispettivamente a 60, 120 e 240 mmoli/l; $P < 0,05$). L'acido citrico ha determinato un forte aumento del volume finale di gas quando impiegato a 60 e 120 mmoli/l (rispettivamente +92% e 52%; $P < 0,05$) ed ha quasi completamente inibito l'attività batterica a 240 mmoli/l (-85% del volume finale di gas; $P < 0,05$). L'acido sorbico ha evidenziato l'attività antibatterica più forte, risultando l'unico acido capace di ridurre il volume finale di gas quando impiegato a 60 mmoli/l (-34%; $P < 0,05$). Tra gli acidi oggetto della prova, solamente gli acidi sorbico (-34%, 47% e 67%), fumarico (-57%, 35% e 26%), citrico (-31%, 18% e 28%), e benzoico (-33%, 29% e 38%) hanno ridotto ad ogni dose testata le concentrazioni di ammoniaca dopo 24h di fermentazione ($P < 0,05$). Al contrario, gli acidi formico, acetico, propionico, butirrico e malico non hanno determinato alcuna riduzione delle concentrazioni di ammoniaca. Dopo la prima serie di fermentazioni, gli acidi sorbico, α -chetoglutarico e benzoico sono stati ulteriormente studiati alle concentrazioni di 7,5, 15 e 30mmoli/l. A queste concentrazioni, l'acido sorbico è stato l'unico a ridurre il volume finale di gas ad ogni dose impiegata (-31%, 35% e 39% di volume finale di gas rispettivamente a 7,5, 15 e 30 mmoli/l; $P < 0,05$). Nonostante ciò, rispetto alla tesi di controllo, l'ammoniaca a 24h è stata ridotta da tutti i trattamenti (con una riduzione media del 34%, 20% e 24% rispettivamente per gli acidi sorbico, α -chetoglutarico e benzoico; $P < 0,05$). In conclusione, i dati relativi alla produzione di gas dimostrano che gli acidi organici possono avere un effetto inibente o stimolante sulla microflora cecale di suino, a seconda dell'acido utilizzato e della dose d'impiego. Inoltre, gli acidi sorbico, α -chetoglutarico e benzoico possono influenzare positivamente le fermentazioni cecali in vitro di suino riducendo le concentrazioni di ammoniaca anche quando impiegati a basse dosi.

Parole chiave: Ammoniaca, Microflora cecale, In vitro, Acidi organici, Suini.

Introduction

Organic acids play an important role in the preservation of foods, as they are very effective inhibitors of microbial growth (Dziezak, 1986; Knochel and Gould, 1995; Brul and Coote, 1999). While some foods are naturally acidic, sometimes acids are directly added to the food or produced inside it by organisms such as lactic acid bacteria.

Adding organic acids, such as citric, formic, fumaric, lactic or propionic acid, to the diet of pigs has been reported to be helpful in overcoming problems of the post-weaning lag period (Falkowski and Aherne, 1984; Partanen and Mroz, 1999). Among the organic acids, formic (Overland *et al.*, 2000; Partanen *et al.*, 2002), lactic (Roth *et al.*, 1993), sorbic (Kirchgessner *et al.*, 1995), and malic acid (Kirchgessner *et al.*, 1993) improved weanling pig performances. Tsiloyiannis *et al.* (2001) observed that

dietary organic acids, and especially lactic acid, are effective in reducing the incidence of porcine post-weaning diarrhea. The supplementation of fumaric acid to diets for piglets during the 3-4 weeks after weaning reduced the concentration of bacteria in the ileal digesta (Blank *et al.*, 2001).

It has been demonstrated that the use of antibiotics as growth promoters can increase the risk of diffusion of antibiotic resistance (Jacobs, 1997; Witte, 1998; van den Bogaard *et al.*, 2002). This potential risk and consumer demand for drug-free foods resulted in the decision of the European Commission to eliminate all antibiotic growth promoters. As a consequence, there is an increasing need to achieve a better understanding of the various roles that organic acids could play in controlling the microbial activity in the gastrointestinal tract of nonruminant animals.

In past years, in order to reduce the need for experimental fistulated animals, *in vitro*

gas production techniques have been developed to study the bacterial fermentations that take place along the gastrointestinal tract of animals (Bauer *et al.*, 2001; Williams *et al.*, 2001). The basic principle of all gas production techniques is that the *in vitro* fermentation of feeds by microorganisms is accompanied by the production of gas which is measured at each time point and can be considered as an index for fermentation activity (Groot *et al.*, 1996).

The objective of this study was to evaluate the effects of different organic acids on microbial growth and ammonia production by swine cecal microflora in an *in vitro* fermentation system. Formic, acetic, propionic, lactic, butyric, sorbic, fumaric, malic, citric, α -ketoglutaric, and benzoic acids were used at different concentrations to assess if any effect on cecal microflora could be shown.

Material and methods

In vitro fermentation

A commercial standard diet for growing pigs was predigested *in vitro* to simulate gastric and ileal digestion as described by Vervaeke *et al.* (1989). The feed (2g; particle size <1mm) was incubated in 40ml of pepsin solution (2g/l, HCl 0.075N; Sigma Chemical, St. Louis, MO, USA) in a shaking waterbath at 37°C for 4h. Then, the pH

was adjusted to 7.5 with NaOH (1mol/l), 40 ml of pancreatin solution (10g/l in a phosphate buffered solution pH7.5; Sigma Chemical, St. Louis, MO, USA) were added and the mixture was incubated in a shaking waterbath at 37°C for 4h. Composition of the phosphate buffered solution was as follows: 26.2mM Na_2HPO_4 , 46.7mM NaHCO_3 , 3.3mM NaCl, 3.1mM KCl, 1.3mM MgCl_2 , 0.7mM CaCl_2 (Martillotti *et al.*, 1987).

After enzymatic digestion, the preparation was centrifuged (3,000×g, 10min., 4°C),

Table 1. Composition of the diet before and after *in vitro* digestion.

| Ingredients (g/kg, as fed): | |
|-------------------------------------|-------|
| Yellow maize | 570.0 |
| Barley | 160.0 |
| Soybean meal, 47% CP | 140.0 |
| Wheat bran | 100.0 |
| Calcium carbonate | 13.5 |
| Calcium phosphate | 7.5 |
| Vitamin/mineral premix ^a | 4.0 |
| Sodium chloride | 3.5 |
| Sodium carbonate | 1.0 |
| L-lysine-HCl | 0.5 |

Chemical composition:

| | Feed, g/kg DM | Predigested feed, g/kg DM |
|-------------------------|---------------|---------------------------|
| Crude protein | 175.9 | 41.9 |
| Ether extract | 41.0 | 19.8 |
| Crude fiber | 42.3 | 63.5 |
| Neutral detergent fiber | 140.8 | 272.3 |
| Acid detergent fiber | 49.7 | 112.7 |
| Starch | 494.2 | 500.4 |
| Ash | 37.3 | 29.5 |

^aPremix provided per kg of diet: vitamin A 9600 U; vitamin D₃ 800 U; vitamin E 7.2 mg; vitamin K₃ 3.2 mg; thiamin 3.2 mg; riboflavin 4.8 mg; pyridoxine 1.6 mg; vitamin B₁₂ 0.016 mg; nicotinic acid 16 mg; d-pantothenic acid 8 mg; biotin 0.06 mg; choline 320 mg; Fe (as $\text{FeSO}_4 \cdot \text{H}_2\text{O}$) 3.6 mg; Zn (as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) 3.6 mg; Cu (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) 7.2 mg; Mn (as $\text{MnSO}_4 \cdot \text{H}_2\text{O}$) 1.44 mg; Co (as $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$) 0.016 mg; I (as KI) 0.1 mg; Se (as Na_2SeO_3) 0.02 mg; butylated hydroxytoluene 0.6 mg.

washed twice with distilled water, centrifuged (3,000×g 5min., 4°C) and dried at 60°C overnight. Diet composition and chemical analyses of the diet before and after predigestion are reported in Table 1.

The predigested diet was used as the substrate in the *in vitro* fermentation study (Piva *et al.*, 1996).

Samples of cecal content were removed from six animals (10 months old, live weight 160kg) within 20 min from slaughter, pooled, and kept in a sealed nylon bag at 39°C during transfer to the laboratory. Then, the cecal content was diluted with buffer (ratio 1:2) and filtered through four layers of cheese cloth. The filtered liquid was used as inoculum. The buffer composition (McDougall, 1948) was as follows: 116.7mM NaHCO₃, 7.6mM KCl, 0.4mM CaCl₂*6H₂O, 26.0mM Na₂HPO₄*12H₂O, 11.5mM NaCl, 0.5mM MgSO₄*7H₂O. Buffer pH was adjusted to 6.7 by adding 3N HCl. The buffer solution was kept at 39°C and flushed with CO₂ for 20 minutes before use. The inoculum was dispensed into five 10ml glass syringes (5ml of inoculum in each syringe) and five 50ml vessels (previously flushed with CO₂, 25ml of inoculum in each vessel) per treatment, containing 20 and 100mg of predigested diet (used as control diet), respectively. Syringes and vessels were sealed and incubated at 39°C for 24h.

Treatments were the predigested diet with or without (control diet) the addition of a solution (adjusted to pH6.7) containing an organic acid used at the final concentration of 60, 120, and 240mmol/l. The organic acids used were formic (F-0507, Sigma, St. Louis, MO, USA), acetic (401424, Carlo Erba Reagenti, Carlo Erba s.r.l., Rodano, Italy), propionic (P1386, Sigma, St. Louis, MO, USA), lactic (sodium salt, 71723, Fluka Chemika AG, Buchs, Switzerland), butyric (6036, J. T. Baker Chemicals B.V., Deventer,

The Netherlands), sorbic (potassium salt, S1751, Sigma, St. Louis, MO, USA), fumaric (F-2752, Sigma, St. Louis, MO, USA), malic (M0875, Sigma, St. Louis, MO, USA), citric (C0759, Sigma, St. Louis, MO, USA), α -ketoglutaric (K1750, Sigma, St. Louis, MO, USA), and benzoic acid (potassium salt, B6416, Sigma, St. Louis, MO, USA). Six *in vitro* fermentations were necessary to test all acids at the three different concentrations. The control diet was used in every fermentation as the internal standard.

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

After the first set of fermentations, it was decided to test sorbic, α -ketoglutaric, and benzoic acids also at the final concentration of 7.5, 15, and 30mmol/l. Two *in vitro* fermentations were necessary to test the three acids. The procedure and predigested diet were the same used for the first set of fermentations.

Chemical analyses of feed and fermentation fluid

Analyses of the diets (CP, crude fiber, ether extract, ash and starch) were performed according to AOAC standard methods (AOAC, 2000; Method 954.01 for CP, Method 962.09 for crude fiber, Method 920.39 for ether extract, Method 942.05 for ash; Method 920.40 for starch and Van Soest *et al.* (1991) for neutral detergent fiber (NDF) and acid detergent fiber (ADF) determinations. Neutral detergent fiber was assayed with a heat stable amylase and expressed inclusive of residual ash; acid detergent fiber was expressed inclusive of residual ash.

Ammonia in fermentation fluid and plasma urea were measured according to Young (1997) using an enzymatic colorimetric test (Urea/BUN - Color, BioSystems S.A., Barcelona, Spain).

Statistical analysis

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

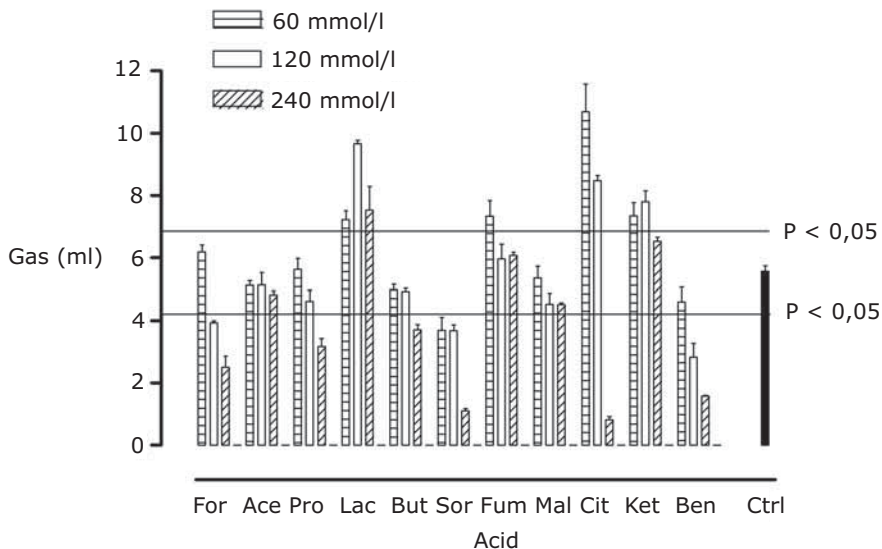
$$V = V_F \exp \{ - \exp [1 + (\mu_m e / V_F)(\lambda - t)] \}$$

where symbols have the meanings assigned by Zwietering *et al.* (1990): V = volume of gas produced at time t , t = fermentation time, V_F = maximum volume of gas produced, μ_m = maximum rate of gas production, which occurs at the point of inflection

of the gas curve and λ = the lag time, as the time-axis intercept of a tangent line at the point of inflection.

Curve fitting and statistical analysis were carried out using the program GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Fermentations with organic acids at high (60, 120, and 240mmol/l) and low (7.5, 15, and 30mmol/l) levels were considered independent trials and results were analyzed separately for high and low organic acid concentrations. Differences between maximum volume of gas produced, maximum rate of gas production, and ammonia data were analyzed by ANOVA in a completely randomized design. Differences between the control diet and the experimental treatments were analyzed using the Dunnett test. Differences were considered statistically significant at $P < 0.05$.

Figure 1. Final gas volume after 24h of fermentation when organic acids were added to the control diet.



Control diet was a pig predigested diet incubated with swine cecal microflora. Treatments were: For = formic acid, Ace = acetic acid, Pro = propionic acid, Lac = lactic acid, But = butyric acid, Sor = sorbic acid, Fum = fumaric acid, Mal = malic acid, Cit = citric acid, Ket = α -ketoglutaric acid, Ben = benzoic acid, Ctrl = control diet. Bars are means \pm SEM of five syringes. P of the model = < 0.001 . Bars under the lower line and over the upper line differ from control ($P < 0.05$).

Results and discussion

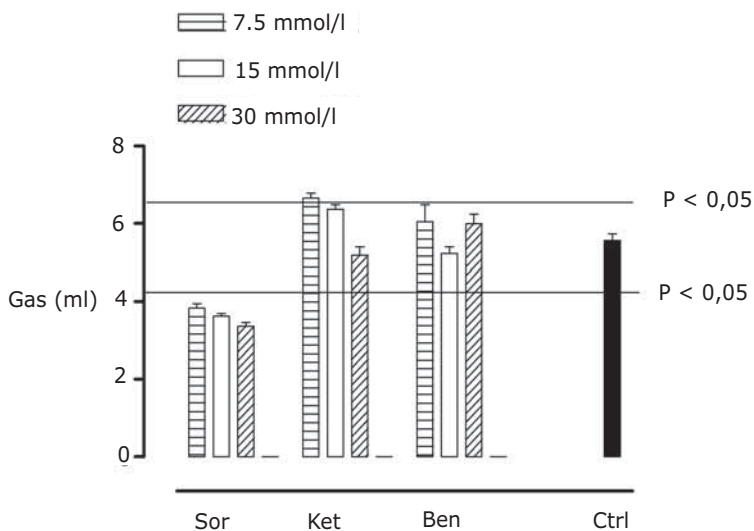
Gas production curves were accurately described by the modified Gompertz model ($r^2 > 0.99$ for all experimental treatments).

Compared with control diet, final gas volume was increased ($P < 0.05$) by lactic acid at all tested concentrations (+30%, +73%, and +35% at 60, 120, and 240mmol/L, respectively; Figure 1), fumaric acid at 60mmol/l (+32%), citric acid at 60 and 120mmol/l (+92% and +52%, respectively), and α -ketoglutaric acid at 60 and 120mmol/l (+32% and +40%, respectively). Conversely, compared with control, final gas volume was reduced ($P < 0.05$) by formic acid at 120 and 240mmol/l (-30% and -55%, respectively), propionic acid at 240mmol/l (-43%), butyric acid at 240mmol/l (-33%), sorbic acid at all tested concentrations (-34%, -34%, and -80% at 60, 120, and 240mmol/l, respectively), citric acid at 240mmol/l (-85%), and benzoic acid at 120 and 240mmol/l (-49% and -72%, respectively).

When organic acids were used at 7.5, 15, and 30mmol/l, sorbic acid reduced final gas volume at all concentrations tested (-31%, -35%, and -39% at 7.5, 15, and 30mmol/l, respectively; $P < 0.05$; Figure 2). Benzoic acid did not affect final gas volume, whereas α -ketoglutaric acid at 7.5mmol/l resulted in higher final gas volume than control (+20%; $P < 0.05$).

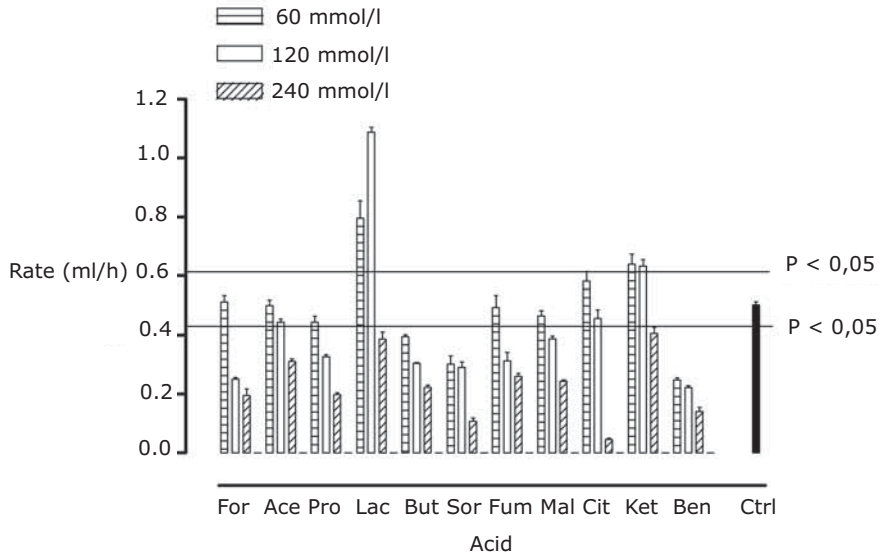
Compared with control, the maximum rate of gas production was increased ($P < 0.05$) by lactic acid at 60 and 120 mmol/l (+58% and +117%, respectively; Figure 3) and by α -ketoglutaric acid at 60 and 120mmol/l (+27% and +26%, respectively). When added at the concentration of 240mmol/l, all acids reduced ($P < 0.05$) the rate of gas production compared with the control diet. The dose of 120mmol/l resulted in a maximum rate of gas production lower than control ($P < 0.05$) for formic (-50%), propionic (-35%), butyric (-39%), sorbic (-42%), fumaric (-38%), malic (-23%), and benzoic acid (-56%) treatments. At the dose of 60mmol/l, only butyric, sorbic and benzoic acids determined a maximum rate of gas production lower than control (-21%, -40%, and -51%, respectively; $P < 0.05$).

Figure 2. Final gas volume after 24h of fermentation when organic acids were added to the control diet.



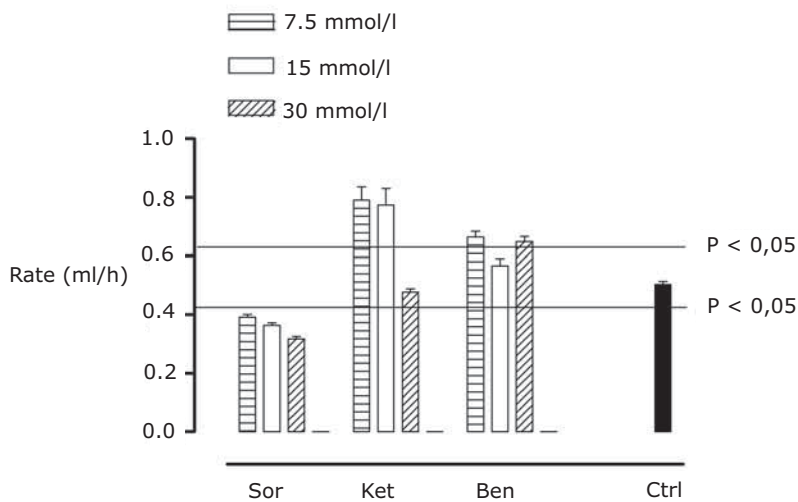
Control diet was a pig predigested diet incubated with swine cecal microflora. Treatments were: Sor = sorbic acid, Ket = α -ketoglutaric acid, Ben = benzoic acid, Ctrl = control diet. Bars are means \pm SEM of five syringes. P of the model = <0.001. Bars under the lower line and over the upper line differ from control ($P < 0.05$).

Figure 3. Maximum rate of gas production during 24h of fermentation when organic acids were added to the control diet.



Control diet was a pig predigested diet incubated with swine cecal microflora. Treatments were: For = formic acid, Ace = acetic acid, Pro = propionic acid, Lac = lactic acid, But = butyric acid, Sor = sorbic acid, Fum = fumaric acid, Mal = malic acid, Cit = citric acid, Ket = α -ketoglutaric acid, Ben = benzoic acid, Ctrl = control diet. Bars are means \pm SEM of five syringes. P of the model = <0.001 . Bars under the lower line and over the upper line differ from control ($P < 0.05$).

Figure 4. Maximum rate of gas production during 24h of fermentation when organic acids were added to the control diet.



Control diet was a pig predigested diet incubated with swine cecal microflora. Treatments were: Sor = sorbic acid, Ket = α -ketoglutaric acid, Ben = benzoic acid, Ctrl = control diet. Bars are means \pm SEM of five syringes. P of the model = <0.001 . Bars under the lower line and over the upper line differ from control ($P < 0.05$).

When organic acids were used at 7.5, 15, and 30mmol/l, the maximum rate of gas production was reduced by sorbic acid at every concentration tested (-22%, -28%, and -36% at 7.5, 15, and 30mmol/l, respectively; $P < 0.05$; Figure 4) and increased by α -ketoglutaric acid at 7.5 and 15mmol/l (+58% and +54%, respectively; $P < 0.05$) and benzoic acid at 7.5 and 30mmol/l (+32% and +30%, respectively; $P < 0.05$).

Gas production data show that organic acids may inhibit or enhance cecal bacterial activity in relation to the type and concentration of acid used. It is known that several bacterial strains can use citric acid (Medina de Figueroa *et al.*, 2000) and lactic acid (Martin, 1998) as an energy source. In our study, lactic acid was the only acid that enhanced bacterial fermentation at every concentration tested, including the highest concentration used (240mmol/l). Citric acid strongly increased gas production when used at 60 and 120mmol/l but almost completely inhibited bacterial activity at 240mmol/l. Interestingly, despite the opposite effects on gas production after 24h of fermentation, citric acid resulted in lower ammonia than control when used both at 60 and 240mmol/l. This finding suggests that an effective control of ammonia cecal concentration may be achieved by inhibiting bacterial fermentation and, as such, protein catabolism like antibiotics do or by enhancing microflora activity and shifting the ecosystem towards a more anabolic status. A similar effect was observed when benzoic acid was used. Benzoic acid reduced bacterial activity when used at high concentrations (from 60 to 240mmol/l), but increased rate of gas production when added at 7.5 and 30mmol/l. Nevertheless, ammonia concentration at 24h was reduced both by high and low benzoic acid inclusion levels.

While lactic acid at 60 and 120mmol/l increased both final gas volume and rate of

gas production, citric acid at the same concentrations did not affect the latter, suggesting that citric acid is fermented at a lower rate than lactic acid. In *in vivo* conditions, a rapid fermentation of substrates in the intestine could result in intestinal distension and abdominal pain, affecting animal feed intake, as reported by Houdijk *et al.* (1997) and Mul (1997) when pigs were fed high levels of non digestible oligosaccharides. However, when fed to animals, most organic acids are absorbed in the upper gastrointestinal tract so that is difficult to estimate how much of the acid will still be available to the hindgut microflora. In an experiment with weaned pigs, Roth *et al.* (1993) observed that feeding diets containing up to 2.4% of lactic acid improved growth performance with no detrimental effects on animal health. Similarly, feeding pigs with 4.5% of citric acid strongly improved animal daily gain (Kirchgessner and Roth-Maier, 1975) and did not reduce feed intake.

When used at 60mmol/l, lactic acid was fermented at a higher rate than control and this may explain why ammonia was reduced by lactic acid already after 8h of fermentation, while citric acid at 60mmol/l reduced ammonia concentration only after 24h of fermentation. Furthermore, at 24h, ammonia was reduced by citric acid but not by lactic acid at 60mmol/l, suggesting that the rapid fermentation of lactic acid limited its efficacy in controlling ammonia levels throughout the 24h fermentation.

The final gas volume was increased also by fumaric acid at 60mmol/l and α -ketoglutaric acid at 7.5, 60 and 120mmol/l. In a previous *in vitro* study, Piva *et al.* (2002) observed that a blend of organic acids (providing phosphoric, citric, fumaric, and malic acid at 1.53, 0.78, 2.59, and 1.12mmol/l, respectively) increased the rate of gas production when added to swine cecal digesta.

A positive modulation of the energy metabolism of some strains usually residing in the hindgut by fumaric acid (Tran *et al.*, 1997; Tielens and Van Hellemond, 1998) and α -ketoglutaric acid (Dimroth and Schink, 1998) had already been reported.

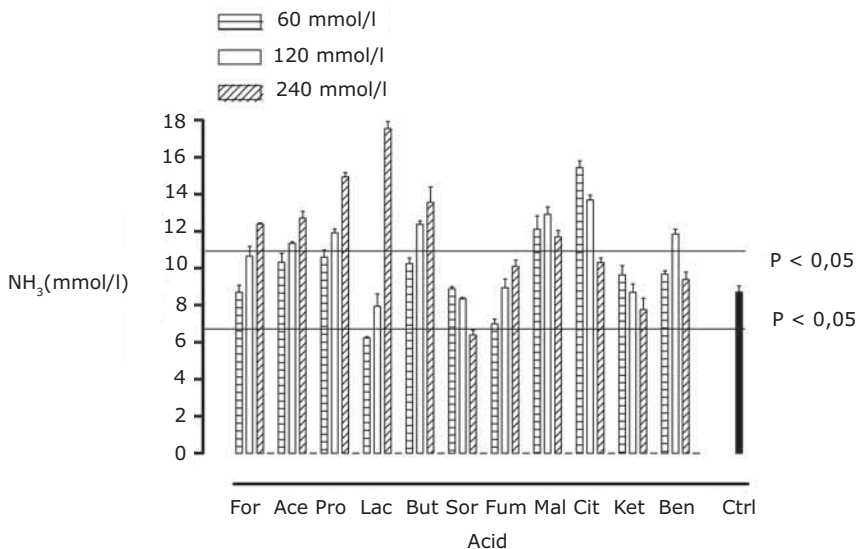
Despite the fact that malic acid can stimulate the activity of some bacterial strains (Renault *et al.*, 1988; Loubiere *et al.*, 1992), in this study, malic acid failed to enhance bacterial fermentation. Conversely, when used at 120 and 240mmol/l, malic acid inhibited bacterial fermentation reducing the rate of gas production.

Because of its antibacterial properties, sorbic acid is commonly used as a food preserving agent. The inhibiting effects of sorbic acid against several pathogens, such as *Clostridium botulinum* (Lund *et al.*, 1987), *Escherichia coli*, *Pseudomonas aeruginosa*,

and *Staphylococcus aureus* (Eklund, 1983), and *Campylobacter jejuni* (Shin *et al.*, 2001) have been demonstrated. In this study, sorbic acid showed the strongest antibacterial activity among the acids tested and was the only acid that reduced final gas volume when used at 60mmol/l. Moreover, sorbic acid resulted in lower final gas volume than control also when used at concentrations down to 7.5mmol/l. Conversely, acetic, lactic, fumaric, malic, and α -ketoglutaric acid did not reduce gas production even when used at the highest concentration tested (240mmol/l).

After 8h of fermentation, ammonia concentration was lower than control ($P < 0.05$) when lactic acid (-29% at 60mmol/l; Figure 5) and sorbic acid (-27% at 240mmol/l) were added to the vessels. At the same time point, ammonia was higher than control

Figure 5. Concentration of ammonia in the cecal inoculum after 8h of fermentation when organic acids were added to the control diet.



Control diet was a pig predigested diet incubated with swine cecal microflora. Treatments were: For = formic acid, Ace = acetic acid, Pro = propionic acid, Lac = lactic acid, But = butyric acid, Sor = sorbic acid, Fum = fumaric acid, Mal = malic acid, Cit = citric acid, Ket = α -ketoglutaric acid, Ben = benzoic acid, Ctrl = control diet. Bars are means \pm SEM of five vessels. P of the model = < 0.001 . Bars under the lower line and over the upper line differ from control ($P < 0.05$).

($P < 0.05$) with formic acid at 240mmol/l (+42%), acetic acid at 120 and 240mmol/l (+30% and +46%, respectively), propionic acid at 120 and 240mmol/l (+37% and +71%, respectively), lactic acid at at 240mmol/l (+101%), butyric acid at 120 and 240mmol/l (+42% and +56%, respectively), malic acid at all concentrations tested (+39%, +48%, and +34% at 60, 120, and 240mmol/l, respectively), citric acid at 60 and 120mmol/l (+77% and +57%, respectively), and benzoic acid at 120mmol/l (+36%).

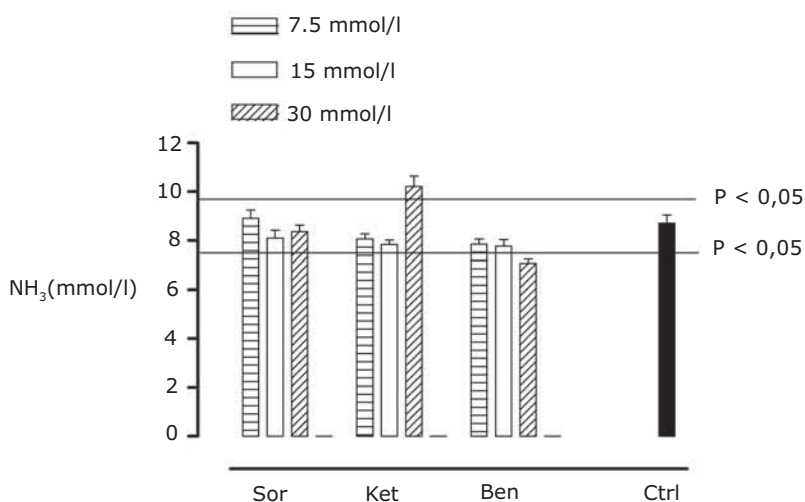
When organic acids were used at 7.5, 15, and 30mmol/l, after 8h of fermentation, ammonia concentration was lower than control (-19%; $P < 0.05$; Figure 6) only when benzoic acid at 30mmol/l was used. On the contrary, α -ketoglutaric acid at 30mmol/l resulted in higher ammonia than control (+17%; $P < 0.05$).

After 24h of fermentation, ammonia concentration was lower than control

($P < 0.05$) with lactic acid at 120 and 240 mmol/l (-52% and -54%, respectively; Figure 7), sorbic acid (-34%, -47%, and -32% at 60, 120, and 240mmol/l, respectively), citric acid (-31%, -18%, and -29% at 60, 120, and 240mmol/l, respectively), fumaric acid (-54%, -35%, and -26% at 60, 120, and 240mmol/l, respectively), α -ketoglutaric acid at 120 and 240mmol/l (-35% and -34%, respectively), and benzoic acid (-33%, -29%, and -37% at 60, 120, and 240mmol/l, respectively). At 24h, ammonia was higher than control ($P < 0.05$) with formic acid at 240mmol/l (+37%), acetic acid (+28%, +28%, and +30% at 60, 120, and 240mmol/l, respectively), butyric acid at 120 and 240mmol/l (+20% and +42%, respectively), and malic acid at 60 and 120mmol/l (+19% and +23%, respectively; $P < 0.05$).

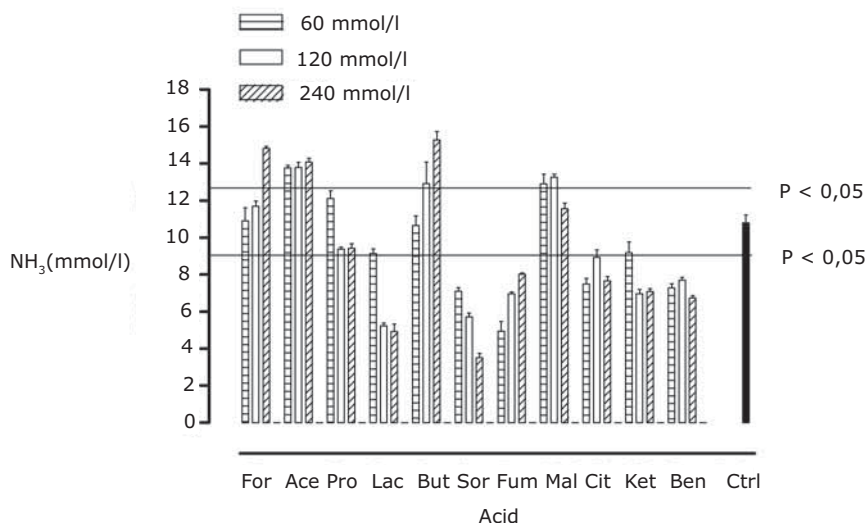
When organic acids were used at 7.5, 15, and 30mmol/l, after 24h of fermentation, compared with control, ammonia concentra-

Figure 6. Concentration of ammonia in the cecal inoculum after 8h of fermentation when organic acids were added to the control diet.



Control diet was a pig predigested diet incubated with swine cecal microflora. Treatments were Sor = sorbic acid, Ket = α -ketoglutaric acid, Ben = benzoic acid, Ctrl = control diet. Bars are means \pm SEM of five vessels. P of the model = < 0.001 . Bars under the lower line and over the upper line differ from control ($P < 0.05$).

Figure 7. Concentration of ammonia in the cecal inoculum after 24h of fermentation when organic acids were added to the control diet.



Control diet was a pig predigested diet incubated with swine cecal microflora. Treatments were: For = formic acid, Ace = acetic acid, Pro = propionic acid, Lac = lactic acid, But = butyric acid, Sor = sorbic acid, Fum = fumaric acid, Mal = malic acid, Cit = citric acid, Ket = α -ketoglutaric acid, Ben = benzoic acid, Ctrl = control diet. Bars are means \pm SEM of five vessels. P of the model = <0.001 . Bars under the lower line and over the upper line differ from control ($P < 0.05$).

tion was reduced by all treatments (-31%, -37%, and -34% for sorbic acid, -20%, -21%, and -20% for α -ketoglutaric acid, -22%, -23%, and -30% for benzoic acid, at 7.5, 15, and 30mmol/l, respectively; Figure 8).

Sorbic, fumaric, citric, and benzoic acid were the only acids able to reduce ammonia at 24h at all concentrations tested (from 7.5 to 240mmol/l for sorbic and benzoic acid, and from 60 to 240mmol/l for fumaric and citric acid).

The inhibitory effect of organic acids has been reported to be determined by their undissociated anion. Undissociated organic acids can pass across the cell membrane and dissociate in the more alkaline interior where they accumulate (Russell and Diez-Gonzalez, 1998). Since pH determines the proportions of the dissociated and non-dissociated forms of an acid (depending on the acid pK_a), it also has a strong influence on

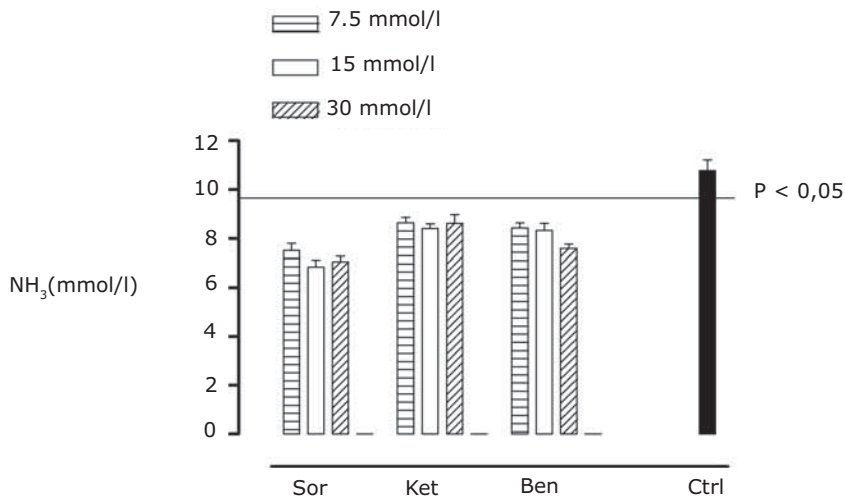
the minimum inhibitory concentrations of the acids (Eklund, 1983).

Lund *et al.* (1987) showed that sorbic acid added to foods to prevent food spoilage at 9mmol/kg was able to inhibit the growth of proteolytic strains of *Clostridium botulinum* when pH was lower than 5.5. In our study, sorbic acid reduced ammonia compared with control even when used at 7.5mmol/l at pH 6.7. Considering that sorbic acid pK_a is 4.76, the concentration of acid that is in the undissociated form and therefore able to cross the bacterial cell wall (Russell and Diez-Gonzalez, 1998) can be obtained from the Henderson-Hasselbach equation, where A^- and HA are the dissociated and undissociated species, respectively and pH_e is the environmental pH

$$pH_e = pK_a + \log[A^-]/[HA]$$

At pH5.5, 15.4% of sorbic acid is in the

Figure 8. Concentration of ammonia in the cecal inoculum after 24h of fermentation when organic acids were added to the control diet.



Control diet was a pig predigested diet incubated with swine cecal microflora. Treatments were: Sor = sorbic acid, Ket = α -ketoglutaric acid, Ben = benzoic acid, Ctrl = control diet. Bars are means \pm SEM of five vessels. P of the model = <0.001 . Bars under the line differ from control ($P < 0.05$).

undissociated form while at pH6.7 only 1.1% of the acid is undissociated. Therefore, the amount of free sorbic acid in our study when acid was added at 7.5mmol/l was much lower than the amount of free acid in the study of Lund *et al.* (1987; 0.08 vs 1.39mmol/l). The strong reduction of the ammonia concentration that was observed in our study confirms that sorbic acid acts as an effective inhibitor of the intestinal proteolytic microflora even when used at very low concentrations.

Other acids, such as formic, acetic, propionic, butyric, and malic acid, even if used at very high concentrations, failed to exert an effective control of ammonia concentrations in the fermentation liquid and in some cases determined higher ammonia concentration than in control vessels. It has to be considered that the tested concentrations were applied directly to fermentation vessels, without considering the possible different solubility and catabolization rate of various acids along the

gastrointestinal tract, and as such it is difficult to extrapolate the effective concentrations to the doses to be applied in feed to achieve a given effect on the cecal microflora.

Conclusions

The current study produced evidence that organic acids have different effects on in vitro pig cecal microflora. Despite the fact that some acid concentrations that were used in this experiment are very unlikely to be reached in the hindgut of organic acids-fed animals, these results showed that some organic acids can positively influence bacterial fermentations reducing ammonia concentration.

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REFERENCES

- AOAC, 2000. Official Methods of Analysis. 17th ed. Association of Official Analytical Chemists, Gaithersburg, MD, USA.
- Bauer, E., Williams, B.A., Voigt, C., Mosenthin, R., Verstegen, M.W.A., 2001. Microbial activities of feces from unweaned and adult pigs, in relation to selected fermentable carbohydrates. *Anim. Sci.* 73:313-322.
- Blank, R., Sauer, W.C., Mosenthin, R., Zentek, J., Huang, S., Roth, S., 2001. Effect of fumaric acid supplementation and dietary buffering capacity on the concentration of microbial metabolites in ileal digesta of young pigs. *Can. J. Anim. Sci.* 81:345-353.
- Brul, S., Coote, P., 1999. Preservative agents in foods. Mode of action and microbial resistance mechanisms. *Int. J. Food Microbiol.* 50:1-17.
- Dimroth, P., Schink, B., 1998. Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria. *Arch. Microbiol.* 170:69-77.
- Dziezak, J.D., 1986. Preservatives: antimicrobial agents. A means toward product stability. *Food Technol.* 40:104-111.
- Eklund, T., 1983. The antimicrobial effect of dissociated and undissociated sorbic acid at different pH levels. *J. Appl. Bacteriol.* 54:383-389.
- Falkowski, J.F., Aherne, F.X., 1984. Fumaric and citric acid as feed additives in starter pig nutrition. *J. Anim. Sci.* 58:935-938.
- Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for in vitro fermentation of ruminant feeds. *Anim. Feed Sci. Technol.* 64:77-89.
- Houdijk, J.G.M., Hartemink, R., Van Laere, K.M.J., Williams, B.A., 1997. Fructooligosaccharides and transgalactooligosaccharides in weaner pigs' diet. pp 69-78 in *Proc. Int. Symp. Non-digestible oligosaccharides: healthy food for the colon?*, Wageningen, The Netherlands.
- Jacobs, C., 1997. Life in the balance: cell walls and antibiotic resistance. *Science* 278:1731-1732.
- Knochel, S., Gould, G., 1995. Preservation microbiology and safety: quo vadis? *Trends Food Sci. Tech.* 6:127-131.
- Kirchgesner, M., Roth-Maier, D.A., 1975. Zum Einsatz von Zitronensäure in der Ferkelaufzucht. *Züchtungskunde* 47:329-334.
- Kirchgesner, M., Roth, F.X., Eidelsburger, U., 1993. Nutritive efficiency of tartaric acid and malic acid in the rearing of piglets. *J. Anim. Physiol. Anim. Nutr.* 70:216-224.
- Kirchgesner, M., Roth, F.X., Paulicks, B.R., 1995. Zur nutritiven Wirksamkeit von Sorbinsäure in der Ferkelaufzucht. *J. Anim. Physiol. Anim. Nutr.* 74:235-242.
- Loubiere, P., Salou, P., Leroy, M.J., Lindley, N.D., Pareilleux, A., 1992. Electrogenic malate uptake and improved growth energetics of the malolactic bacterium *Leuconostoc oenos* grown on glucose-malate mixtures. *J. Bacteriol.* 174:5302-5308.
- Lund, B.M., George, S.M., Franklin, J.G., 1987. Inhibition of type A and type B (proteolytic) *Clostridium botulinum* by sorbic acid. *Appl. Environ. Microbiol.* 53:935-941.
- Martillotti, F., Antongiovanni, M., Rizzi, L., Santi, E., Bittante, G., 1987. *Metodi di analisi per la valutazione degli alimenti d'impiego zootecnico*. Ed. CNR-IPRA, Roma, Italy.
- Martin, S.A., 1998. Manipulation of ruminal fermentation with organic acids: a review. *J. Anim. Sci.* 76:3123-3132.
- McDougall, E.I., 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem. J.* 43:99-109.
- Medina de Figueroa, R., Alvarez, F., Pesce de Ruiz Holgado, A., Oliver, G., Sesma, F., 2000. Citrate utilization by homo- and heterofermentative lactobacilli. *Microbiol. Res.* 154:313-320.
- Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, H., Schneider, W., 1979. The estimation of digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor in vitro. *J. Agr. Sci.* 93:217-222.
- Mul, A.J., 1997. Application of oligosaccharides in animal feed. Page 106 in *Proc. Int. Symp. Non-*

- digestible oligosaccharides: healthy food for the colon?, Wageningen, The Netherlands.
- Overland, M., Granli, T., Kjos, N.P., Fjetland, O., Steien, S.H., Stokstad, M., 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.
- Partanen, K.H., Mroz, Z., 1999. Organic acids for performance enhancement in pig diets. *Nutr. Res. Rev.* 12:117-145.
- Partanen, K.H., Siljader Rasi, H., Alaviuhkola, T., Suomi, K., Fossi, M., 2002. Performance of growing-finishing pigs fed medium- or high-fibre diets supplemented with avilamycin, formic acid or formic acid-sorbate blend. *Livest. Prod. Sci.* 73:139-152.
- Piva, A., Casadei, G., Biagi, G., 2002. An organic acid blend can modulate swine intestinal fermentation and reduce microbial proteolysis. *Can. J. Anim. Sci.* 82:527-532.
- Piva, A., Panciroli, A., Meola, E., Formigoni, A., 1996. Lactitol enhances short-chain-fatty acid and gas production by swine cecal microflora to a greater extent when fermenting low rather than high fiber diets. *J. Nutr.* 126:280-289.
- Renault, P., Gaillardin, C., Heslot, H., 1988. Role of malolactic fermentation in lactic acid bacteria. *Biochimie* 70:375-379.
- Roth, F.X., Kirchgessner, M., Eidelsburger, U., 1993. Zur nutritiven Wirksamkeit von Milchsäure in der Ferkelaufzucht. *Agribiol. Res.* 46:229-239.
- Russell, J.B., Diez-Gonzalez, F., 1998. The effects of fermentation acids on bacterial growth. *Adv. Microb. Physiol.* 39:205-234.
- Schofield, P., Pitt, R.E., Pell, A.N., 1994. Kinetics of fiber digestion from in vitro gas production. *J. Anim. Sci.* 72:2980-2991.
- Shin, S.Y., Hwang, H.J., Kim, W.J., 2001. Inhibition of *Campylobacter jejuni* in chicken by ethanol, hydrogen peroxide, and organic acids. *J. Microbiol. Biotechnol.* 11:418-422.
- Tielens, A.G., Van Hellemond, J.J., 1998. The electron transport chain in anaerobically functioning eukaryotes. *Biochim. Biophys. Acta* 1365:71-78.
- Tran, Q.H., Bongaerts, J., Vlad, D., Unden, G., 1997. Requirement for the proton pumping NADH dehydrogenase I of *Escherichia coli* in respiration of NADH to fumarate and its bioenergetic implications. *Eur. J. Biochem.* 244:155-160.
- Tsiloyiannis, V.K., Kyriakis, S.C., Vlemmas, J., Sarris, K., 2001. The effect of organic acids on the control of porcine post-weaning diarrhoea. *Res. Vet. Sci.* 70:287-293.
- van den Bogaard, A.E., Hazen, M., Hoyer, M., Oostenbach, P., Stobberingh, E.E., 2002. Effects of flavophospholipol on resistance in fecal *Escherichia coli* and enterococci of fattening pigs. *Antimicrob. Agents Chemother.* 46:110-118.
- Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74:3583-3597.
- Vervaeke, I.J., Dierick, N.A., Demeyer, D.L., Decuypere, J.A., 1989. Approach to the energetic importance of fiber digestion in pigs. II. An experimental approach to hindgut digestion. *Anim. Feed Sci. Technol.* 23:169-194.
- Williams, B.A., Verstegen, M.W.A., Tamminga, S., 2001. Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutr. Res. Rev.* 14:207-227.
- Witte, W., 1998. Medical consequences of antibiotic use in agriculture. *Science* 279:996-997.
- Young, D.S., 1997. Effects of drugs on clinical laboratory tests. 3th Ed. AACC Press, Washington, DC, USA.
- Zwietering, M.H., Jongenburger, L., Rombouts, F.M., van't Riet, K., 1990. Modelling the bacterial growth curve. *Appl. Environ. Microbiol.* 56:1875-1881.
- Zwietering, M.H., Rombouts, F.M., van't Riet, K., 1992. Comparison of definitions of the lag phase and the exponential phase in bacterial growth. *J. Appl. Bacteriol.* 72:139-145.