

**HEALTHYPIGUT EU PROJECT Workshop 2**  
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**Contribution of *in vitro* devices in evaluating alternatives to in-feed antibiotics for young pigs and comparisons with *in vivo* data.**

**Abstracts**

- **Communication 1. Effect of substrate adaptation on the gas production kinetics and microbial fermentation end-product profile of faecal microflora studied *in vitro*.** By Awati A., Williams B.A., Bosch M., Verstegen M.W.A., Wageningen University, The Netherlands

An *in vivo* study was conducted, to test two diets: one (FER) containing added fermentable carbohydrates-including sugarbeet pulp (SBP) and wheat starch (WST) and a control diet (CON). Neither contained antibiotics nor added copper or zinc. Twenty-four piglets selected from 12 litters (2 per litter) were weaned at 4 weeks of age (no creep feeding), and introduced to one of two diets (12 per diet). After 9 days on the diets, faecal samples were collected from selected animals, and tested *in vitro* for their activity in terms of gas production kinetics, and end-products such as VFA, ammonia, and DM disappearance of the two test substrates SBP and WST.

Faeces from piglets fed the FER diet had a tendency to produce more total gas for both substrates, compared with the faeces from piglets fed the CON diet. Piglets on the FER diet had both substrates in their diet, but faeces from piglets fed the FER diet showed rapid fermentation with SBP as a substrate compared with WST though in general, WST is considered to be a faster fermenting carbohydrate compared to SBP. Also in case of faeces from piglets fed CON diet, WST was remarkably faster fermented than SBP. This indicates that the microflora from faeces from piglets fed FER diet were better adapted to fermentation of SBP than WST *in vitro*. This may be because in the *in vivo* situation, the faster fermenting WST (and possibly digestible) might not have reached the end of GIT (anus). This suggests that alterations in diet composition lead to adaptation of GIT microflora in terms of its activity, depending upon where in the GIT, the ingredient is “processed”, be it by digestion or fermentation. It was concluded that the presence of fermentable carbohydrates in the diet, can change the GIT microbial activity in terms of its utilization of that substrate, and that can be demonstrated using this *in vitro* technique.

(Key words: *in-vitro* fermentation, gas production, microbial activity, prebiotic)

- **Communication 2. Molecular microbiological approaches for the assessment of microbiota responses during *in vitro* evaluation schemes.** By Smidt H., Konstantinov S.R., Awati A., Zhu W-Y., Williams B.A., Tamminga S., De Vos W.M., Akkermans A., Wageningen University, The Netherlands

In vitro studies based on the application of e.g. *in vitro* fermentation, tissues, and intestinal cell cultures, can be used for the assessment of novel nutritional approaches, such as the addition of probiotics and prebiotics, aiming at the replacement of in-feed antibiotics in livestock production. The development of such dietary strategies requires multi-disciplinary expertise, including a detailed evaluation of their effects on the composition and activity of the indigenous microbial communities throughout the GI tract. Molecular microbiological methods can serve the detection and identification of microorganisms, and the characterization of shifts in microbial community structure and activity, thus providing critical information on the impact that nutritional interventions have on the microbiota. As an example, *in vitro* fermentation of sugarbeet pulp (SBP) using faeces of weaning piglets as inoculum was carried out to assess enrichment of microbial populations by use of this carbohydrate source. The microbial diversity of the prominent bacteria before and after this *in vitro* fermentation was analysed using denaturing gradient gel electrophoresis (DGGE) of PCR amplicons of 16S rDNA. Before fermentation, the DGGE profiles showed differences between cultures inoculated with faeces from different piglets, though some bands were common to all piglets. After fermentation of SBP, three dominant bands appeared, of which two bands appeared in all samples and one for both replicates of one piglet.

- **Communication 3. Treatment of Caco-2 cells with *Lactobacillus casei* and *Bifidobacterium animalis* reduced the ETEC-induced neutrophil transmigration.** By M. Roselli, A. Finamore, M.S. Britti, and E. Mengheri., INRAN, Roma, Italy

Weaning period in pigs is frequently associated with infection disease and diarrhea, mainly caused by enterotoxigenic *Escherichia coli* (ETEC) K88. Antibiotics have been used over decades to reduce pig infection, but many microorganisms are becoming resistant to antibiotics, and thus there is an urgent need to find alternatives to them. Probiotics may induce various beneficial effects, including prevention of gastrointestinal diseases, maintenance of equilibrated microflora, increase of host resistance against pathogen infection, stimulation of intestinal immune response and modulation of cytokine expression. In this study, we have investigated whether *Lactobacillus casei* GG and *Bifidobacterium animalis* were able to protect intestinal cells against the inflammatory challenge induced by ETEC K88, using an *in vitro* model of human intestinal cell line. We have studied the neutrophil transmigration across Caco-2 cells grown and differentiated as inverted monolayers on semipermeable filters, and the expression of pro-inflammatory cytokines and chemokines involved in neutrophil migration. Neutrophils, freshly prepared from human blood, were added to the basolateral compartment ( $2 \times 10^6$ /ml), whereas ETEC ( $1 \times 10^7$ /ml) and/or probiotics ( $1 \times 10^8$ /ml) were added to the apical compartment. A strong neutrophil transmigration, measured as myeloperoxidase assay, was observed after 2 hours of ETEC infection. When *L. casei* GG or *B. animalis* were added together with ETEC, this migration was markedly reduced. Analysis of cytokine and chemokine expression, assayed by RT-PCR, in Caco-2 cells infected with ETEC showed an increase of pro-inflammatory cytokines tumor necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$ , interleukin (IL)-8, and of chemokines epithelial neutrophil-activating protein (ENA)-78 and growth related oncogene (GRO)- $\alpha$ . On the other hand, a decrease of anti-inflammatory transforming growth factor (TGF)- $\beta$  was observed after ETEC infection. These alterations were not induced when the cells were treated with *L. casei* GG or *B. animalis* together with ETEC. These results suggest that probiotics can be considered a valid antibiotic alternative against pathogen infection.

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- **Communication 4. Porcine epithelial intestinal cells as a tool to study the effect of food contaminant : the mycotoxin Fumonisin B<sub>1</sub>.** By Oswald I.P., Bouhet S., Hom M.P., INRA Toulouse, France

Gastrointestinal epithelium is the first tissue exposed to food contaminants, toxins and pathogens. Young weaned pigs are a sensitive target to mycotoxins present in feedstuffs. Using a pig small intestinal epithelial cell line (IPEC-1), which differentiates and mimics an intestinal epithelium, we investigated the effects of Fumonisin B<sub>1</sub> (FB<sub>1</sub>), a mycotoxin currently found in maize. We first demonstrated that this toxin inhibited the proliferation of cells by blocking their division in G0/G1 phase of the cell cycle. FB<sub>1</sub> also impaired the formation of a cellular monolayer and disrupted an already established one. Indeed a long exposure to FB<sub>1</sub> decreased the trans-epithelial electrical resistance (TEER) of IPEC-1, in a time-, dose- and partially reversible- dependent manner. The effect of FB<sub>1</sub> was then analyzed on inflammatory cytokine production. Using semi-quantitative RT-PCR and ELISA techniques, we demonstrated that FB<sub>1</sub> decreased IL-8 mRNA and protein synthesis. The effects observed *in vitro* were also detected *in vivo* during experimental intoxication of piglets. Indeed, ingestion of FB<sub>1</sub> also altered the intestinal barrier as demonstrated by the increased susceptibility to pathogenic strain of *Escherichia coli* in the treated piglets. Similarly, the decrease IL-8 level observed *in vitro* was also detected at the mRNA level in the intestine of piglets receiving the toxin. In conclusion, the porcine IPEC-1 cell line is a useful tool to study the effects of contaminants on intestinal functions in pigs.

- **Communication 5. *In vitro* permeability in the small intestine of weaned piglets fed isoenergetic rations in liquid or dry forms.** By Verdonk J.M.A.J., Vente-Spreeuwenberg, Mroz Z., ID TNO Animal Nutrition, Lelystad, The Netherlands

*In vitro* permeability and its correlation with morphological and immunological indices in the small intestine was measured with 28-day old piglets fed isocaloric diets in dry versus liquid forms. On days 0, 2 and 7 post-weaning, from the proximal and mid part of the small intestine were taken 5-cm slices to assess: 1) the *in vitro* epithelial permeation routes, i.e., transepithelial and paracellular, using radiolabelled dipeptide glycyl-L-sarcosine ( $[^{14}\text{C}]$  GlySar) and mannitol ( $[2\text{-}^3\text{H}]$ ) as mimetics, respectively; 2) gut morphological indices (villi/crypt sizes); 3) the mRNA expression levels of proinflammatory IL-8 in the mucosa. The *in vitro* transcellular transport was modulated ( $P < 0.05$ ) by the physical form of diets, whereas only numerical differences were found in the paracellular transport. On day 7 post-weaning, the dry diet resulted in a 35%-greater ( $P < 0.05$ ) transcellular transport compared to the liquid diet. Pearson's correlates between the permeation biomarkers, morphological indices and the mRNA expression levels of proinflammatory IL-8 are summarized below:

Response parameter	GlySar ( $10^{-6}$ cm/s)	Villous height ( $\mu\text{m}$ )	Crypt depth ( $\mu\text{m}$ )	Villous height / crypt depth ratio	$\log_{10}$ (IL8 mRNA)	DM intake (g/day/pig)	
						days 0-2	days 3-7
Mannitol (10 <sup>-6</sup> cm/s)	0.64***	ns	ns	ns	ns	ns	ns
GlySar (10 <sup>-6</sup> cm/s)		ns	-0.44*	-0.42*	ns	ns	ns
Villous height ( $\mu\text{m}$ )			ns	0.78***	-0.42*	ns	ns
Crypt depth ( $\mu\text{m}$ )				-0.68***	ns	ns	ns
Villous / crypt ratio					-0.46**	ns	ns
$\log_{10}$ (IL8 mRNA)						ns	ns

<sup>†</sup> Mannitol indicates paracellular transport; GlySar indicates transcellular transport.

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; ns= not significant.

In general, the results documented that the ageing factor exerted more evident impact on the measurements than the physical form of the diet.

Keywords: piglets, intestine, permeability, morphology, IL-8

- **Communication 6. Anti-secretory effect of soybean on piglet small intestine demonstrated in Ussing chambers.** By Boudry G., Lallès J.P., Grondahl M.L., Unmack M., Skadhauge E., INRA, Saint-Gilles, France, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark

Recent evidences suggest intestinal anti-secretory properties for soybean but mechanisms are still unknown. Thus, effects of soybean products on small intestine Cl<sup>-</sup> secretion were investigated *in vitro*. Pieces of jejunum and ileum (exp. 1) from 6-8 week-old piglets were mounted in Ussing chambers. Extracts of soybean products [raw (RSF) or heated (HSF) flour ; ethanol-extracted protein concentrate (SPC)] were added to the mucosal side (1mg protein.mL<sup>-1</sup> final), with mannitol as a control. After 45min., Na<sup>+</sup>-dependent glucose absorption was measured by addition of 16 mM glucose to the mucosal side (16 mM mannitol on the serosal side). cAMP-dependent secretion was tested 10 min. later (2.5mM theophylline, both sides). Nervous regulatory pathways of jejunal Cl<sup>-</sup> secretion were tested (exp. 2) by addition of 10<sup>-4</sup>M 5-HT, 10<sup>-8</sup>M substance P or 10<sup>-7</sup>M VIP on the serosal side. Soybean extracts induced a slight increase in basal short-circuit current (Isc) that was bumetanide insensitive and, therefore, not due to Cl<sup>-</sup> secretion. RSF and HSF decreased jejunal Na<sup>+</sup>-glucose absorption compared to control ( $\Delta$ Isc: 59 ± 6, 66 ± 6 vs 82 ± 6  $\mu$ A.cm<sup>-2</sup>, respectively, p<0.05). Theophylline-induced jejunal secretion was decreased by RSF, HSF and SPC vs. control ( $\Delta$ Isc: 50 ± 5, 53 ± 5, 50 ± 5 vs 69 ± 5  $\mu$ A.cm<sup>-2</sup>, p<0.05). Ileal secretion was never affected. Soybean extracts did not influence responses to the other secretagogues (exp. 2), except HSF which reduced jejunal secretion to substance P, vs. control ( $\Delta$ Isc: 34 ± 5 vs 52 ± 5  $\mu$ A.cm<sup>-2</sup>, p<0.05). In conclusion, soybean contains a heat-resistant/ethanol-sensitive and an ethanol-resistant/heat-resistant compounds that decrease jejunal glucose absorption and cAMP-dependent Cl<sup>-</sup> jejunal secretion, respectively, in piglets. These compounds remain to be identified.

- **Communication 7. Effects of formic acid and plant extracts on gastric parameters measured *in vivo* and *in vitro*.** By Manzanilla<sup>1</sup> E.G., Meunier<sup>2</sup> J.P., Pérez<sup>1</sup> J.F., Kamel<sup>3</sup> C. and Gasa<sup>1</sup> J., <sup>1</sup>Departament CAiA, Universitat Autònoma de Barcelona, Spain, <sup>2</sup>ERT-CIDAM, Faculté de Pharmacie, CBRV, France and <sup>3</sup>AXISS, France.

In a *in vivo* experiment, 108 pigs ( $32 \pm 2.0$  d,  $8.1 \pm 0.20$  kg LW) weaned at 21 days were randomly distributed in 12 pens, and fed at random one of three experimental diets: control diet (CP: 19.46%, Lys: 1.29; Ct) and Ct plus 0.5% of formic acid (Ac) or 300 ppm of a plant extract mixture (oregano, cinnamon and capsicum; Xt). After a 21 days production experiment, a controlled feed intake pattern was applied during four days, from 8:00 am to 8:00 pm, in order to approach to a steady state situation in the gastrointestinal tract. In particular, 30-minute periods of feeding were alternated with one-hour fasting periods. Pigs were fed *ad libitum* the remaining of the day (from 8:00pm to 8:00 am). At days 24 and 25, eight piglets per treatment were sacrificed (15 minutes after a feeding period) and gastrointestinal compartments sampled. The same diets were digested for 8 hours in the TIM-1 *in vitro* model developed by the TNO (The Netherlands). In the *in vivo* experience Ac and Xt diets vs. Ct; increased pH (3.2 and 3.6 vs. 2.4,  $P = 0.03$ , RSD = 0.81), the “pool” of FM (g) (307 and 332 vs. 136,  $P = 0.007$ , RSD = 56.7) and %DM (35 and 34 vs. 30,  $P = 0.087$ , RSD = 3.8) in the stomach. Regression between DM content and pH in the stomach was  $r = 0.78$  indicating the influence of the emptying rate on the pH. Using the TIM-1 model, emptying rate is fixed and stomach pH is maintained in a fixed level by HCl infusion. The quantity of 1M-HCl necessary to maintain the stomach pH in the model during the first forty minutes was lower for Ac and Xt (5.9 and 5.6 ml) in contrast to Ct (7.7 ml). The decrease of pH brought about by Ac and Xt *in vitro* compared to Ct, was hindered *in vivo* by a higher stomach DM “pool” due to a lower emptying rate.

- **Communication 8. The influence of probiotics on growth performance, immunity and intestinal flora in piglets.** By Lessard M., Matte J., Roy D., Gagnon N., Dupuis M., Fairbrother J.M., Asselin C., Farnworth E., Durand H., Goulet J., Bernier J., Agriculture et Agroalimentaire Canada, Lennoxville, Québec.

This study was performed to evaluate the influence of two probiotics, *Pediococcus acidilactici* (Bactocell) and *Saccharomyces cerevisiae ssp. Boulardii*, (Levucell) on growth performance, immunity, intestinal flora and bacterial translocation of newborn piglets. Litters were allocated to one of the five following treatments: 1) Control without antibiotic in basal diet; 2) Control with antibiotic in basal diet; 3) Bactocell; 4) Levucell; and 5) Bactocell and Levucell. During lactation, probiotics ( $1 \times 10^9$  CFU) were administered three times a week to piglets. Piglets were weaned at 21 days of age and probiotics were then incorporated into feeds ( $1 \times 10^9$  CFU/kg). Blood samples were taken at different times to evaluate vitamin B status, antibody response to ovalbumin (OVA) and to characterize circulating leukocyte populations. Piglets were slaughtered either at 18, 24 or after *E. coli* challenge at 56 days of age. The intestine was excised. Tissue samples from ileum and mesenteric lymph nodes and digesta samples from caecum and mid colon were taken for immunological and microbial analysis, and pH determination. Preliminary results showed that growth performance and antibody response of piglets treated with Bactocell, Levucell or both were similar to those of control piglets fed with or without antibiotics. Bacterial translocation in mesenteric lymph nodes was increased in control piglets fed basal diet compared to other groups after *E. coli* challenge. These results suggest that probiotics added to feed prevent bacterial populations to translocate toward other tissues. Upcoming results on the influence of these probiotics on intestinal bacterial populations and immune measurements will provide further information on possible mechanisms by which they promote health of the host.