Histol Histopathol (2004) 19: 49-58

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Structural patterns of swine ileal mucosa following L-glutamine and nucleotide administration during the weaning period. An histochemical and histometrical study

C. Domeneghini, A. Di Giancamillo, G. Savoini, R. Paratte, V. Bontempo and V. Dell'Orto

Department of Veterinary Sciences and Technologies for Food Safety, University of Milan, Milan, Italy

Summary. Dietary supplementations with L-glutamine and/or nucleotides were screened for their effects on intestinal mucosa in 16 female weaning piglets. The animals were transported to the university's facilities 24 hours after weaning. They were grouped four to a pen in controlled environmental conditions and fed one of the following four diets for 28 days: control diet (C); C+0.5% L-glutamine (G); C+0.05% "nucleotides" (N); and C+0.5 % L-glutamine+0.05% "nucleotides" (GN). Individual body weights and feed intake per group were recorded at the beginning and the end of the study as well as weekly during it. There were no significant performance differences among the groups. After 28 days the animals were slaughtered and the distal ileum and liver were examined histologically. Anti-proliferating cell nuclear antigen (PCNA) as well as anti-human macrophage immunostaining, and a modified TdT-mediated dUTP nick-end labeling technique (TUNEL) were performed, and intraepithelial lymphocyte percentage was evaluated to assess morpho-functional aspects of the ileum. Histometry was performed by assessing cell indices and counts of immuno-reactive structures.

Feeding G and/or N resulted in an increase in villi (V) height, crypt (C) depth, and a decrease in V:C ratio ($P<0.01$). In addition, feeding G and/or N resulted in an increase in mitotic mucosal cells (M), and a decrease in apoptotic mucosal cells (A), thus decreasing the A:M index ($P<0.01$). The percentages of mucosal macrophages were greater in G and/or N groups ($P<.001$) than in control piglets, and similarly among the groups the percentages of intraepithelial lymphocytes varied ($P<0.01$).

Our data showed that the diet supplementation with G and/or N had positive effects on some morpho-functional characteristics of piglet ileal mucosa. These ameliorative effects may potentially be linked to a good responsiveness of piglets to a stressful period, like a precocious weaning is in this species.

Key words: Gut, Histochemistry, Histometry, Glutamine, Nucleotides.

Offprint requests to: C. Domeneghini, Department of Veterinary Sciences and Technologies for Food Safety, University of Milan, Via Trentacoste n. 2, Milan I-20134, Italy. e-mail: cinzia.domeneghini@unimi.it

Introduction

Weaning is a crucial phase in animal husbandry, above all if it is precocious as is usually done when rearing pigs. Piglets are nowadays weaned around three weeks of age because this procedure is commercially advantageous, but thus they often suffer altered gastrointestinal conditions, in part because of the sudden alteration in the intestinal microflora consequent to the rough passage from mother's milk to a solid food. This in addition may cause a strong reduction in the length of intestinal villi height and the depth of the intestinal crypts, which consequently reduces the gut digestive as well as absorptive capacities (Pluske et al., 1997; Van Beers-Schreurs et al., 1998). This in turn may cause negative effects on average daily gain (ADG), as well as on defensive responses against infectious diseases. Actually, recent scientific evidence suggests that weaning piglets at three to four weeks of age may overwhelmingly challenge a substantially immature immune system, conducting it to a possible paralysis condition and creating an ideal environment for diseases to develop (Ladekjær-Mikkelsen et al., 2002). In a special way, viral pathologies such as porcine reproductive and respiratory disease (PRRS) (Loula, 1991; Wensvoort et al., 1991; Christianson et al., 1992) and post-weaning multisystemic wasting syndrome (PMWS) (Van Beers-Schreurs et al., 1998) may constitute an important risk in the weaning and post-

weaning periods of reared piglets. The economical impact of these syndromes can be severe because of poor piglet performances and elevated therapy costs also due to secondary bacterial diseases.

Functional feed additives (also called "nutraceuticals") can be useful alternatives to the use of antibacterial agents and chemotherapeutics during weaning, as they can stimulate the defensive responses, favorably influence gastrointestinal microflora, and improve nutrient digestion and absorption (Bustamante et al., 1994). This in addition appears a very promising possible goal in the view of single EC countries applying the recent Directive 70/524/CEE (2002/C 329 CE) of the European Parliament regarding the gradual blockage in the use of antibiotics as alimentary additives for food animal species.

The diet supplementation with L-glutamine and nucleotides may be regarded as particularly useful in these respects (Sanderson and Walker, 1991). Glutamine, a non-essential aminoacid, is the preferred oxidative substrate for intestinal epithelial cells and an important source of carbon atoms for gluconeogenesis (Smith and Wilmore, 1990; Piva and McEvoy-Bowe, 1998; Matés et al., 2002). It is reputed to support the recovery of the intestinal mucosa integrity in response to injury and infection (Souba et al., 1990), as well as to gut atrophy (Chow, 1998; Remillard et al., 1998). Glutamine is also important in nucleotide synthesis (Windermueller and Spaeth, 1980; Windermueller, 1982; Boza et al., 2000; Pierzynowski et al., 2001), as well as in regulating cell renewal in both epithelial cells (Collins et al., 1998) and lymphocytes (Matés et al., 2002). It also improves macrophage activity (Newsholme, 2001). As regards intestinal cell proliferation, glutamine has been recently shown to activate protein kinases, possibly regulating signal transduction pathways for the cellular proliferation following apoptosis (Rhoads et al., 2000). Nucleotides are essential for protein synthesis (Barness, 1994; Uauy et al., 1990) and the demand for them is high in rapidly proliferating tissues with a high rate of cell turnover such as the intestinal mucosa in a young animal. Nucleotides are also required in phospholipid synthesis, are essential coenzyme components, facilitate iron absorption, and may enhance the cell-mediated immune response (Yamamoto et al., 1997). Nucleotides, like glutamine, are described to act in the in vitro modulation of cell proliferation and apoptosis (Schlimme et al., 2000).

Both glutamine and nucleotides are usually present in food, but their entry into the gut with nutrients may be necessary in a larger quantity than usual when an animal is in a stressful condition. For the above mentioned reasons the piglet weaning period is a stressful period, and it is within this time that glutamine and nucleotides may became nutraceuticals, and the oral administration of them as supplements in the diet constitute a valid help to successfully go beyond the weaning period itself.

The aim of the present work was to investigate whether the oral treatment with L-glutamine and/or nucleotides could have an impact upon structural patterns which may be utilized for evaluating the development of articulate intestinal functional roles during a stress period in which infectious diseases may frequently occur together with low rates of growth. The observed selected parameters were those related to the morpho-functional evaluation of GALT (gut-associated lymphoid tissue) and those involved in the rates of cellular proliferation and apoptosis in order to anatomically study intestinal functions during the early weaning in "physiologically" stressed piglets. In addition, we have paid attention to relating the data obtained in both treated and control piglets with their growth performances, because these latter aspects in food animal species are as important in judging the efficacy of a treatment as the ones referring to the microanatomy of the gut. We have finally compared our results with those obtained by other authors on other mammalian species with regard to the oral administration of these nutraceuticals.

Material and methods

Animals and dietary treatments

Sixteen weaning female Suffolk piglets (average age: 21 days) were selected coming from multiparous sows. The piglets were transported from the farrowing facility to the university facilities 24 hours after the milk intake stopped (this may be regarded as causing a condition of "physiological" stress). The first 2 days in the university facilities were characterised by an irregular feeding by the piglets, likely as a consequence of the "physiological" stress. Piglets were housed in stainless steel metabolism cages (1.5x0.8 m), four per cage, under environmentally controlled conditions (temperature 28 °C, relative humidity 70%) and fed one of the following four diets for 28 days: control (C); C + 0.5% L-glutamine (G) (Merck, Darmstadt, Germany); C + 0.05 % "nucleotides" (N) (Prosol S.p.A., Madone, BG, Italy); and C + 0.5% L-glutamine + 0.05% "nucleotides" (GN). The amounts of supplementation were chosen according Touchette et al. (2000) and Lackeyram et al. (2001). The time length of administering supplemented diets was decided with the aim of showing possible gut structural changes. The control (C) diet was formulated following the NRC (Nutrient Requirements of Swine; 1998) (Table 1). The "nucleotides" were a mixture of nucleosides, nucleotides and bases.

The animals were cared for and were sacrificed at the end of trial in accordance with the European Union guidelines (86/609/EEC) approved by the Italian Ministry of Health.

Growth performances

Individual body weights were recorded at the beginning, at the end, and weekly over the study period. Feed intake per group was determined weekly.

Histology - microscopic anatomy of liver and ileum

The distal ileum (2 samples for each animal, total number of samples = 32), 2 cm before its opening to the coecum, and a small portion of the liver near the hilus were collected immediately after the sacrifice of each animal. The samples were promptly fixed in 4% paraformaldehyde in 0.01M phosphate-buffered saline (PBS) pH 7.4 for 24 h at 4 °C, dehydrated in graded alcohols, cleared with xylene and embedded in paraffin. After dewaxing and re-hydration, serial microtome sections (4 μm-thick) of both ileum and liver were stained with a sequential haematoxylin/eosin (HE) stain to evaluate the structural aspects of the organs.

Ileum sections were in addition stained with Alcian blue 8GX pH 2.5/periodic acid Schiff (AB/PAS) reaction to demonstrate neutral and acidic glycoconjugates and to evaluate the epithelial mucous cells and adherent mucous gel.

Other ileum sections were processed for visualizing mucosal cells, which were in the S-phase of the cell cycle, by immuno-staining (peroxidase-antiperoxidase method, PAP) with a monoclonal antiserum against proliferating cell nuclear antigen (PCNA) (clone PC10, Sigma, Italy). The sections were immersed in a freshly prepared 3% H_2O_2 solution in distilled water for 10 min to block the endogenous peroxidase activity. For the antigen retrieval, the slides were heated in a microwave oven at 700 W for 10 min (2x5 min) in a 0.01M citrate buffer, pH 6.0 (Foley et al., 1991; Greenwell et al., 1991, Cattoretti et al., 1993; Shi et al., 1995). After cooling at room temperature for 15 min, these sections were rinsed in TBS (Tris-buffered saline, pH 7.5) and pre-incubated with normal swine serum (NSS, Dako, Italy) diluted 1:5 in TBS containing 1% bovine serum albumin (BSA) for 20 min. The primary antibody was applied at a dilution of 1:3000 in TBS+1% BSA for 45 min at room temperature in a humid chamber (Hall et al., 1990). The sections were then incubated with rabbit anti-mouse immunoglobulin (Dako), diluted 1:25 in TBS (30 min), followed by incubation with mouse PAP complex (Dako), diluted 1:50 in TBS. Immuno-reactive sites were visualized using a freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma), 10 mg in 0.5M Tris-HCl buffer, pH 7.6, 15 ml, containing 0.03% H_2O_2. Sections were briefly counterstained with Mayer's haematoxylin, dehydrated and permanently mounted with Eukitt (Bio-Optica, Italy). In the following parts of this paper the PCNA-immuno-reactive nuclei will for brevity be designed as belonging to "mitotic cells".

Other ileum sections were immuno-histochemically (PAP) processed to identify mucosal macrophages using a monoclonal anti-human macrophage serum (clone LN-5, Sigma, Italy) diluted 1:400 in TBS. The steps before and after incubation with this primary antiserum (overnight at 4 °C in a humid chamber) were as for anti-PCNA processing, except for the antigen retrieval which was not performed.

The specificity of immuno-staining was in both cases tested by incubating sections with normal mouse serum (Dako) instead of the primary antisera: this procedure always gave negative results. As positive controls, alimentary canal samples from calf and dog were tested: in all cases the expected positive reactions were observed.

Other ileum sections were processed for identifying mucosal cells which were in apoptosis. Apoptotic cells were localized using a modified (DeadEnd™ Colorimetric TUNEL System, Promega, U.S.A.) TdT-mediated dUTP Nick-End Labeling (TUNEL) technique: DNA strand breaks generated during apoptosis were biotinylated and then identified by using horseradish-peroxidase-labeled streptavidin (Streptavidin HRP). Using this procedure, apoptotic nuclei were stained brown. Sections were briefly counterstained with Mayer's haematoxylin, dehydrated and permanently mounted with Eukitt.

Sections from all the four piglet groups were stained

Table 1. Percentage composition of the diet (as fed).

INGREDIENT	BASAL
Corn	27.6
Soybean meal (44% CP)	17
Barley	13
Flaked barley	10
Flaked maize	10
Whey powder	8.5
Wheat bran	4
Soy bean oil	2.5
Milk powder	3.5
Limestone	1
Monocalcium phosphate	0.9
Acidifiers	0.5
Vitamin and trace minerals premix[1]	0.5
Lysine-HCl	0.35
ZnO	0.3
Salt	0.15
Aroma	0.1
Threonine	0.05
DL-methionine	0.05
Calculated nutrient composition	
Crude protein	18.08
Crude fat	5.52
NDF	5.82
Lysine	1.19
Met + Cys	0.68
Threonine	0.69
Tryptophan	0.21
Calcium	1
Available phosphorus	0.51
Net Energy, kcal/kg	2432

[1]: The vitamin and trace minerals premix provided the following per kilogram of diet: vitamin A, 150.000 IU; vitamin D3, 10.000 IU; vitamin E, 200 mg; thiamine, 25 mg; riboflavin, 50 mg; pyridoxine, 25 mg; vitamin B12, 200 μg; choline, 2000 mg; biotin, 250 μg; Co, 2.3 mg as cobalt sulfate; Mn, 16 mg as manganese oxide; Fe, 200 mg as ferrous carbonate; Cu, 130 mg as copper sulfate; Zn, 375 mg as zinc oxide; K, 6 mg as potassium iodate; Se, 500 μg as sodium selenite, respectively, with barley and calcium carbonate as the carrier.

together in the same staining run for each histochemical/immunohistochemical test.

Histology – Histometry in ileum samples

The height of intestinal villi (V), the depth of intestinal crypts (C), and the ratio of villi and crypths measurements (V:C ratio; 10 per section) were determined upon HE-stained sections.

Table 2. Effects of added glutamine (G), nucleotides (N) and glutamine plus nucleotides (GN) on growth performance of weaning piglets.

	CONTROL	G	N	GN	POOLED SE
Initial wt, kg	4.93	5.00	4.90	5.06	0.43
Day 0 to 7					
ADG, g	19	48	45	79	7.09
ADFI, g	132	137	131	175	6.06
Gain:feed	0.14	0.35	0.34	0.45	0.04
Day 7 to 14					
ADG, g	219	221	164	269	12.39
ADFI, g	271	297	253	327	9.30
Gain:feed	0.81	0.74	0.65	0.82	0.02
Day 14 to 21					
ADG, g	310	321	319	342	3.91
ADFI, g	471	486	458	512	6.69
Gain:feed	0.66	0.66	0.70	0.67	0.01
Day 21 to 28					
ADG, g	267	343	329	338	10.19
ADFI, g	646	549	590	671	15.48
Gain:feed	0.41	0.62	0.56	0.50	0.03
Final wt, kg	10-33	11.53	10.90	12.27	0.38

ADG: average daily gain; ADFI: average daily feed intake

Apoptosis (A), mitosis (M) and apoptotic cell/mitotic cell index (A:M index) were evaluated by counting nuclei which were either PCNA-immuno-reactive or TUNEL-reactive. This was done in two lymphatic follicles of the GALT (lymphocytes), and in ten well-oriented villi/crypts (enterocytes) for each section (Burrin et al., 2000).

Mucosal macrophages were evaluated by counting cells which were anti-human macrophage-reactive in zones of diffuse lymphatic tissue (DLT) of the GALT; for each section, the immuno-reactive cells were counted in 10 fields (at x200 each field represented a tissue section area of about 0.015 mm^2) (Sozmen et al., 1996).

The number of intraepithelial lymphocytes (IEL, as identified in HE-stained sections) per 100 enterocytes was also recorded (Tang et al., 1999).

All the observations were conducted by a blind observer utilizing an Olympus BX51 microscope equipped with a DP software (Olympus, Italy).

Statistical analysis

The data were analyzed by ANOVA using the General Linear Model procedure of the SAS Institute, Inc (1985). The different cell type counts were co-variated for the number of cells recorded.

Results

Growth performance

There were no significant differences among the four groups in terms of growth during the 28 days of the trial (Table 2). From weaning to 7 days post-weaning average daily gain was slightly higher in the piglets of

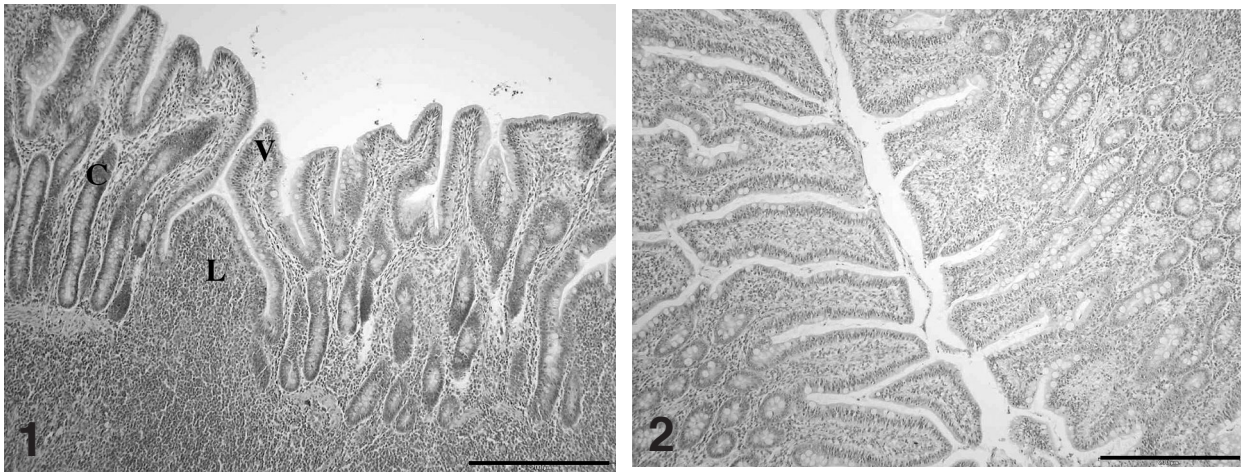

Fig. 1. Control (C) piglets diet. HE. Normal aspect of the intestinal mucosa. Note the numerous villi (V) and crypts (C), as well as the GALT (L). Scale bar: 200 μm.

Fig. 2. Diet supplementation with L-glutamine and nucleotides (GN). HE. The intestinal villi are regularly arranged and shaped. Scale bar: 200 μm.

GN group. L-glutamine and nucleotides had no effect on daily feed intake, so there were no significant differences among the groups as to gain:feed ratio.

Histology – Microscopic Anatomy of Liver and Ileum

In all cases the microscopic anatomy of the liver was normal; no differences among the four groups concerning the liver cytology were observed (data not shown).

The microscopic anatomy of the ileum in G-, N-, and GN-supplemented animals did not differ from that of control (C) piglets (Fig. 1). Regularly-arranged and shaped intestinal villi were specially noticed in GN animals (Fig. 2). Gut-associated lymphoid tissue (GALT) was present and was similarly organized in both control and treated piglets (Fig. 1).

The AB/PAS histochemical staining revealed that the content of intestinal mucous cells was prevalently acidic (AB-positive, moderately PAS-reactive) in all the examined animals (Figs. 3, 4). The adherent mucous gel showed a similar reactivity (AB-positive, moderately PAS-reactive) in all piglets, but its quantity was markedly different in the treated animals in comparison with the controls. In C-group piglets the adherent mucous gel was scarce (Fig. 3) whereas in G, N (Fig. 4), and GN groups it was revealed in larger quantities.

Anti-PCNA immuno-reactivity was prominent in ileum samples from all groups. Immuno-stained nuclei were detectable in both epithelial cells of intestinal

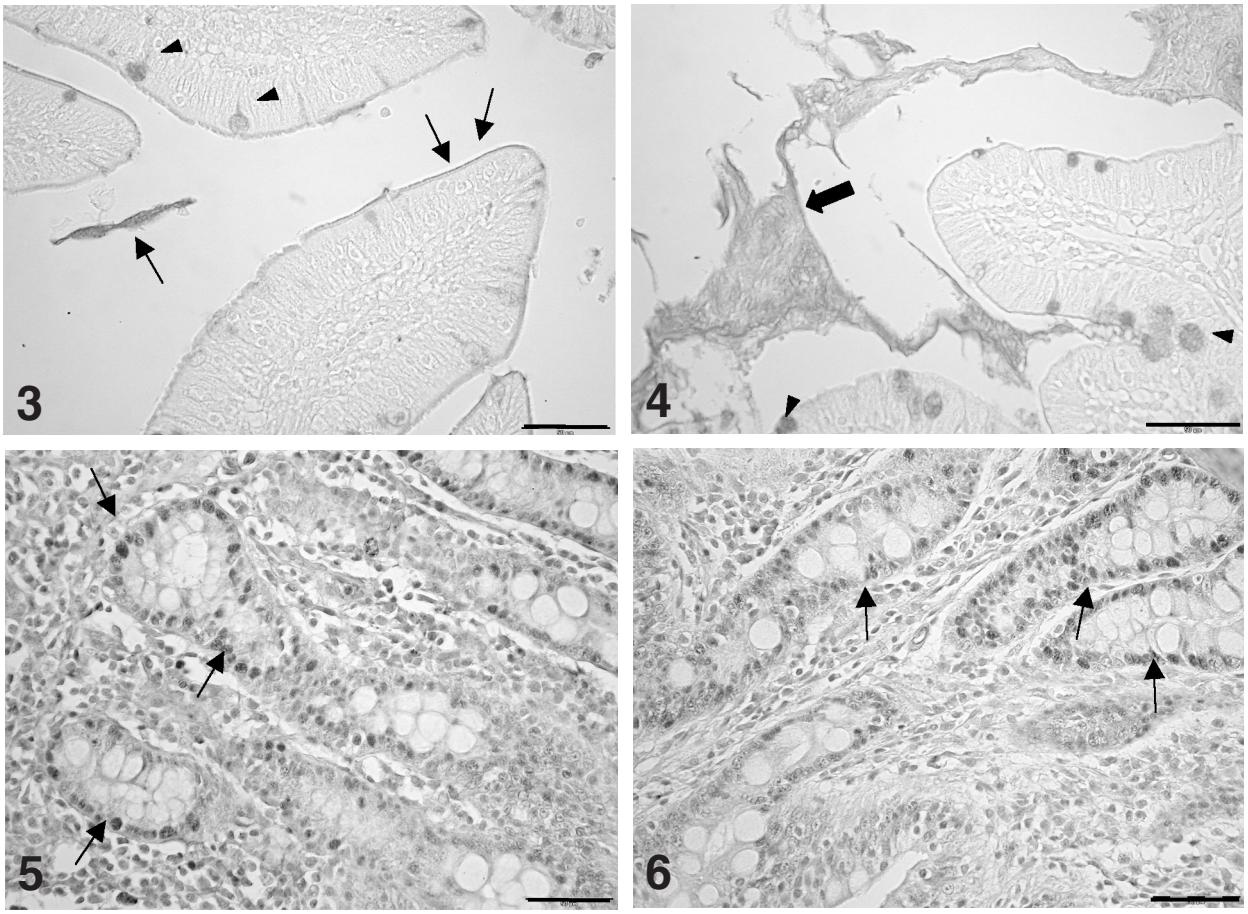

Fig. 3. C diet. AB pH 2.5/PAS. Intestinal mucous cells are strongly AB-positive (arrowheads). The adherent mucous gel shows a similar reactivity, and it is very scarce (thin arrows). Scale bar: 50 μm.

Fig. 4. Diet supplementation with nucleotides (N). AB pH 2.5/PAS. Intestinal mucous cells are strongly AB-positive (arrowheads). The adherent mucous gel is very thick (arrow). Scale bar: 50 μm.

Fig. 5. C diet. Anti-PCNA immuno-reactivity. Immuno-reactive nuclei are evident in epithelial cells of the intestinal crypts (thin arrows). Scale bar: 50 μm.

Fig. 6. GN diet. Anti-PCNA immuno-reactivity. Immuno-reactive nuclei are numerous in intestinal crypts (thin arrows). Scale bar: 50 μm.

crypts (Figs. 5, 6) and mucosal cells of GALT (Figs. 7, 8).

Immuno-reactive mucosal macrophages were in all cases detectable in both lymphatic follicles (Fig. 9) and DLT (Fig. 10).

Nuclei of apoptotic cells were TUNEL-labeled in epithelial localizations, in C (Fig. 11) G, N, and GN (Fig. 12) groups. Furthermore, TUNEL-labeled apoptotic bodies could be detected in mucosal cells of the GALT (presumably macrophages) both in control (Fig. 13) and treated piglets (Fig. 14).

Histology – Histometry in ileum samples

Histometric analysis, counts and cell indices are summarized in Table 3.

Oral feeding with L-glutamine and/or nucleotides resulted in an increase in villi (V) height ($P<0.01$), crypt (C) depth ($P<0.01$), and a consequent decrease in V:C ratio ($P<0.01$) in comparison with control animals, with the differences between the C and GN groups being the most pronounced.

In lymphatic follicles the oral feeding with L-glutamine and/or nucleotides revealed no statistical differences in mitotic cells, but a decrease in apoptotic cells ($P<0.01$), and a consequent decreasing of the A:M index ($P<0.01$), in comparison with the control group.

In epithelial cells the counts performed in G-, N- and GN-treated animals were statistically different for apoptosis, mitosis and A:M index, when compared to the C-group ($P<0.01$).

The numbers percentages of mucosal macrophages

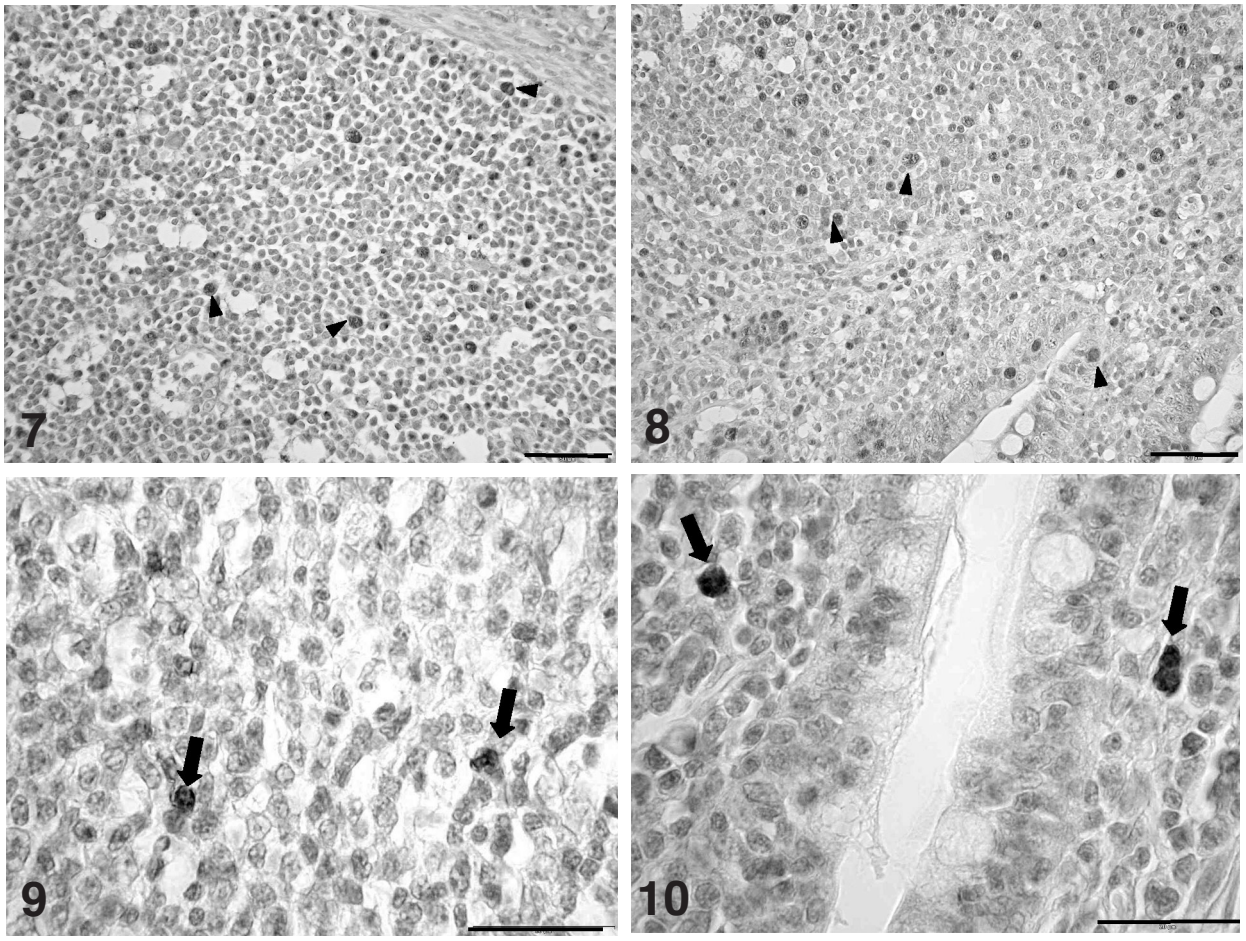

Fig 7. C diet. Anti-PCNA immuno-reactivity. Some nuclei in lymphatic tissue are immuno-positive (arrowheads). Scale bar: 50 μm.

Fig. 8. N diet. Anti-PCNA immunoreactivity. The lymphatic tissue contains immuno-reactive nuclei (arrowheads). Scale bar: 50 μm.

Fig. 9. N diet. Anti-human macrophage immuno-reactivity. Some roundish cells, presumably macrophages, are immuno-positive in the lymphatic tissue (GALT) (arrows). Scale bar: 20 μm.

Fig. 10. Diet supplementation with L-glutamine (G). Anti-human macrophage immuno-reactivity. Some roundish cells, presumably macrophages, are evidenced (arrows) in the diffuse lymphatic tissue within the tunica propria. Scale bar: 20 μm.

were larger in the G, N, and GN groups (P<0.01) than in the C piglets.

The intraepithelial lymphocytes (IEL) were also in higher percentages in the treated animals than in controls (P<0.01).

Discussion

In this paper we have shown that liver and ileum maintained their normal structure after L-glutamine and nucleotide administration, indicating that these dietary supplementations do not produce detrimental effects upon these tissues in swine species.

We focused our attention upon different aspects of the ileum micro-anatomy, because the ileum is highly susceptible to gut pathological events which may concern weaning piglets, and so its possible structural changes linked to nutraceutical administration are of a predictive value in the view of judging intestinal defensive responsiveness. In addition, the ileum is extremely rich in GALT structures. Furthermore, pilot evaluations of the entire small intestine in the rat have not evidenced differences with respect to the consequences of glutamine administration in its different tracts (Potsic et al., 2002).

The histochemical reactivity of intestinal mucous cells and adherent mucous gel does not appear to be influenced by the dietary treatment, and so we can

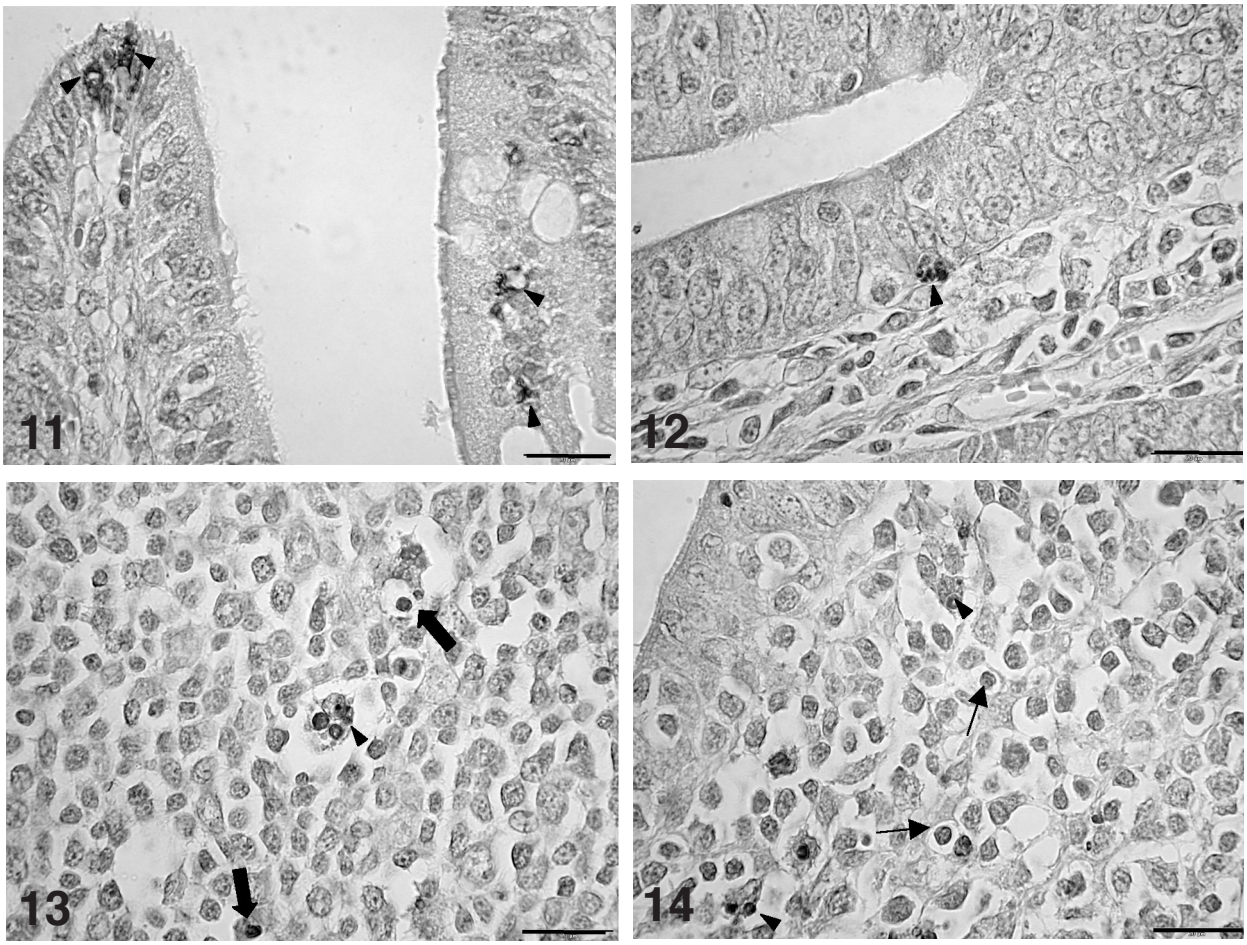

Fig. 11. C diet. TUNEL-labelling. Some nuclei belonging to enterocytes appear apoptotic (arrowheads). Scale bar: 20 μm.

Fig. 12. GN diet. TUNEL-labeling. One nucleus shows signs of chromatic condensation (arrowhead) in the surface intestinal epithelium. Scale bar: 20 μm.

Fig. 13. C diet. TUNEL-labeling. Apoptotic bodies (arrowhead) and chromatic condensation signs (arrows) are evident in cells, presumably macrophages, of the GALT. Scale bar: 20 μm.

Fig. 14. GN diet. TUNEL-labeling. Apoptotic bodies (arrowheads) and chromatic condensation signs (arrows) are evident in cells, presumably macrophages, of the GALT. Scale bar: 20 μm.

hypothesize that the highly protective functions displayed by mucous secretions has not been modified by the administration of these nutraceuticals. We can only underline that AB/PAS staining showed a much thicker adherent mucous gel in treated animals than in controls. The small intestine mucous layer constitutes a highly protective barrier which prevents penetration of potential pathogens into the epithelium (Tang et al., 1999; Atuma et al., 2001). The thicker adherent mucous gel showed in the treated animals may signify an increase in protection as well as in absorption of nutrients, which both are possibly of a favorable significance.

These histological data appear to be supported by our histometrical observations on the ileum. The significantly greater villi height and crypt depth, as well as the significant consequent decrease in V:C ratio in supplemented animals in comparison with control piglets enable us to hypothesize that the studied nutraceuticals are potentially able to promote a restoration of the mucosal thinning that occurs at weaning (Van Beers-Schreurs et al., 1998). This in turn possibly causes a greater intestinal efficiency towards absorptive and digestive processes, as also suggested by Hernández et al. (2000). Other studies in which the rat (Uauy et al., 1990), and piglet (Touchette et al., 2000) diets were supplemented with glutamine, and the mouse diet with nucleotides (Yamamoto et al., 1997) also reported similar beneficial effects on intestinal mucosa. Similar effects, but more limited than those here presented, were found by Bustamante et al. (1994) in the swine after dietary supplementation with nucleotides. Glutamine is a precursor of purine and pyrimidine nucleotides (Windermueller and Spaeth, 1980; Windermueller, 1982) and this may explain why animals supplemented with glutamine plus nucleotides had greater villi height and crypt depth than piglets administered either of these nutrients alone.

Our histological and histometrical results appear to be better explainable if we relate them to our results concerning the immunolocalization of both nuclei which were in the S-phase of the cell cycle (and thus, near mitosis) and nuclei which were on the contrary shown apoptotic. Piglets given L-glutamine plus nucleotides showed a significant increase in cell proliferation rates compared to the other groups, as was also observed in human ileum by Scheppach et al. (1994). This higher proliferation rate in treated than in control piglets is appreciable in epithelial cells (enterocytes). The PCNA-immuno-reactive nuclei of epithelial cells were exclusively localized within the intestinal crypts, and especially at the bottom. Enterocytes of the small bowel are constantly replaced by cells from the intestinal crypt, with a rate of regeneration matching the normal loss of villous epithelium (Pluske, 2001). Apoptotic nuclei examination (by TUNEL signaling) revealed that glutamine and/or nucleotide supplementation is linked to a decrease in the susceptibility of both epithelial (enterocytes) and lymphatic cells to apoptosis in comparison with control animals, as also observed by other authors (Exner et al., 2002; Masuko, 2002; Matés et al., 2002; Mendenoca et al., 2002). These two data taken together may surely explain the higher villi and more profound crypts previously described. In addition, the components of GALT also showed higher proliferation rates (even if not significant) and decreasing apoptosis signaling in treated than in control piglets. Chang et al. (2002) have recently shown that glutamine administration significantly decreases caspase activities in T-cells. We can hypothesize that mucosal defensive components are potentially more efficient as a consequence of administration of nutraceuticals. Manhart et al. (2001) demonstrated that oral glutamine supply may constitute a suitable approach for improving

Table 3. Effects of added glutamine (G), nucleotides (N) and glutamine plus nucleotides (GN) on villus height (V), crypt depth (C), V:C ratio; apoptosis, mitosis and A:M index within GALT (lymphocytes) and epithelial cells (enterocytes); mucosal macrophages; IEL (intra-epithelial lymphocytes).

	CONTROL	G	N	GN	POOLED SE
villus height, μm (V)	147.78^{A}	200.26^{B}	188.58^{C}	215.00^{D}	0.17
crypts depth, μm (C)	80.31^{A}	152.47^{B}	139.16^{C}	179.79^{D}	0.16
V:C ratio	1.84^{A}	1.31^{B}	1.35^{B}	1.20^{C}	0.02
lymphocytes (cell nr: 1738)					
apoptosis	91^{A}	57^{B}	65^{B}	59^{B}	2.41
mitosis	186	194	196	193	3.25
A:M index	0.47^{A}	0.31^{B}	$0.32B^{a}$	$0.30B^{b}$	0.003
enterocytes (cell nr: 3292)					
apoptosis	92^{Aa}	88^{Bb}	87^{B}	80^{C}	0.97
mitosis	1272^{Aa}	1323^{b}	1310^{b}	1361^{B}	18.52
A:M index	0.072^{A}	0.066^{B}	0.066^{B}	0.058^{C}	0.0001
macrophages (cell nr: 830)	34^{A}	38^{B}	37^{B}	39^{B}	0.52
IEL (mean cell nr: 100)	3.35^{A}	6.40^{B}	5.30^{C}	6.70^{B}	0.10

A,B,C,D: means lacking a common superscript differ significantly ($P<0.01$). a,b: means lacking a common superscript differ significantly ($P<0.05$).

intestinal immunity in immuno-compromised patients.

In this paper we have shown a higher number of intraepithelial lymphocytes in supplemented groups compared with control piglets. Intraepithelial lymphocytes may play a role in the modulation of immune responses as well as in the elimination of damaged or infected cells (Cerf-Bensussan and Guy-Grand, 1991). In addition, we have immuno-histochemically detected numerous macrophages localized within the diffuse lymphatic tissue and lymphatic follicles of the ileum. The treated piglets showed a higher number of macrophages within the GALT than control piglets, so it is conceivable that a greater number of them may work in conjunction with other components of the immune system in supplemented animals in comparison with control piglets (Hernández et al., 2000). Macrophages act as the first line of the host defence against viral infections (Kosugi et al., 2002) and so it may be that future field studies may evidence a protective action of the studied nutraceuticals towards these pathologies (eg., previously mentioned PRRS and PMWS).

Taken together our micro-anatomical data show that the diet supplementation with L-glutamine and nucleotides has noticeable positive effects on some morpho-functional characteristics of piglet ileal mucosa. These changes potentially enable the piglet ileum to display its secretive, absorptive as well as defensive functions with a higher efficiency in treated animals than in controls. Feeding piglets with L-glutamine and nucleotides during the weaning period did not significantly affect growth performance in our study, even if body weight was higher in the treated groups both during the first seven days of the trial and at the end. The transportation of the animals to our facility 24 hours after weaning is likely to have markedly stressed the animals, and this in turn may have caused both slowing growth in the early days of weaning and the lack of significant performance differences between treated and control animals. It is also possible that the dosages used in this study were efficient in affecting structural aspects of the ileum, but not so effective on growth performances of the piglets, so further studies are required in order to show an effect upon growth performances in the swine. However, this study is sufficient to show in weaning piglets appreciable gut morpho-functional effects, possibly directed towards its greater efficiency in both absorptive/secretive and defensive responses.

Acknowledgements. Authors wish to thank Prosol S.p.A. (Italy) for providing the nucleotides. This work was supported by grants from the University of Milan (FIRST 2002).

References

Atuma C, Strugala V., Allen A. and Holm L. (2001). The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am. J. Physiol. Gastrointest. Liver Physiol. 280, G922-G929.

Barness L.A. (1994). Dietary sources of nucleotides - From breast to weaning. J. Nutr. 124, 128-130.

Boza J.J., Moennoz D., Bournot C.E., Blum S., Zbinden I., Finot P.A. and Ballavre O. (2000). Role of glutamine on the de novo purine nucleotide synthesis in Caco-2-cells. Eur. J. Nutr. 39, 38-46

Burrin D.G., Stoll B., Jiang R., Petersoen Y., Elnif J., Buddington R.K., Schmidt M., Holst J.J., Hartmann B. and Sangild P.T. (2000). GLP-2 stimulates intestinal growth in premature TPN-fed pigs by suppressing proteolysis and apoptosis. Am. J. Physiol. 279, G1249-1256.

Bustamante S.A., Sanchez N., Crosier J., Miranda D., Colombo G. and Miller M.J.S. (1994). Dietary nucleotides: effects on the gastrointestinal system in swine. J. Nutr. 124, 149S-156S.

Cattoretti G., Pileri S., Parravicini C., Becker M.H.G., Poggi S., Bifulco C., Key G., D'Amato L., Sabattini E., Feudale E., Reynolds F., Gerdes J. and Rilke F. (1993). Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. J. Pathol. 171, 83-98.

Cerf-Bensussan N. and Guy-Grand D. (1991). Intestinal intraepithelial lymphocytes. Gastroenterol. Clin. North Am. 20, 549-576

Chang W.K., Yang K.D., Chuang H., Jan J.T. and Shaio M.F. (2002). Glutamine protects activated human T cells from apoptosis by up-regulation glutathione and Bcl-2 levels. Clin. Immunol. 104, 151-160.

Chow A. (1998). Glutamine reduces heat shock-induced cell death in rat intestinal epithelial cells. J. Nutr. 128, 1296-1301.

Christianson W.T., Collins J.E., Benfield D.A., Harris L., Gorcyca D.E., Chladek D.W., Morrison R.B. and Joo H.S. (1992). Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. Am. J. Vet. Res. 53, 485-488.

Collins C.L., Masafumi W., Souba W.W. and Abcouwer S.F. (1998). Determinants of glutamine dependence and utilization by normal and tumor-derived breast cell lines. J. Cell. Physiol. 176, 166-178.

Exner R., Weingartmann G., Eliasen M.M., Gerner C., Splitter A., Roth E. and Oehler R. (2002). Glutamine deficiency renders human monocytic cells more susceptible to specific apoptosis triggers. Surgery 131, 75-80.

Foley J.F., Dietrich D.R., Swenberg J.A. and Maronpot R.R. (1991). Detection and evaluation of proliferating cell nuclear antigen (PCNA) in rat tissue by an improved immunohistochemical procedure. J. Histotechnol. 14, 237-241.

Greenwell A., Foley J.F. and Maronpot R.R. (1991). An enhancement method for immunohistochemical staining of proliferating cell nuclear antigen in archival rodent tissues. Cancer Lett. 59, 251¯256.

Hall P.A., Levison D.A., Woods A.L., Yu C.C.-W., Kellock D.B., Watkins J.A., Barnes D.M., Gillett C.E., Camplejhon R., Dover R., Waseem N.H. and Lane D.P. (1990). Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J. Pathol. 162 , 285-294.

Hernandez J., Sorbolla A., Mendoza R. and Garcia G. (2000). Glutamine stimulates the synthesis of immunoglobulin IgG in infected early weaned pigs. J. Anim. Sci. 78 (Suppl. 1), 182.

Kosugi I, Kawasaki H, Arai Y. and Tsutsui Y. (2002). Innate immune responses to cytomegalovirus infection in the developing mouse brain and their evasion by virus-infected neurons. Am. J. Pathol. 161, 919-928.

Lackeyram D., Yue X. and Fan M.Z. (2001). Effects of dietary supplementation of crystalline L-glutamine on the gastrointestinal tract and whole body growth in early-weaned piglets fed corn and

soy-bean meal-based diets. J. Anim.Sci. 79 (suppl. 1) abstr.

Ladekjær-Mikkelsen A.-S., Nielsen J., Stadejek T., Storgaard T., Krakowka S., Ellis J., McNeilly F., Allan G. and Botner A. (2002). Reproduction of postweaning multisystemic wasting synndrome (PMWS) in immunostimulated and non-immunostimulated 3-week-old piglets experimentally infected with porcine circovirus type 2 (PCV2). Vet. Microbiol. 89, 97-114.

Loula Y. (1991). Mystery swine diseases. Agri. Pract. 12, 23.

Manhart N., Vierlinger K., Splitter A., Bergmeister H., Sautner T. and Roth E. (2001). Oral feeding with glutamine prevents lymphocyte and glutathione depletion of Peyer's patches in endotoxemic mice. Ann. Surg. 234, 92-97.

Masuko Y. (2002). Impact of stress response genes induced by L-glutamine on warm ischemia and reperfusion injury in the rat small intestine. Hokkaido Igaku Zasshi. 77, 169-183

Matés J.M., Pérez-Gomez C., Nunez de Castro I., Asenjo M. and Marquez J. (2002). Glutamine and its relationship with intracellular redox status, oxidative stress and cell proliferation/death. Int. J. Biochem. Cell. Biol. 34, 439-458.

Mendonca R.Z., Arronzio S.J., Antoniazzi M.M., Ferreira J.M. and Pereira C.A. (2002). Metabolic active-high density VERO cell cultures on microcarriers following apoptosis prevention by galactose/glutamine feeding. J. Biotechnol. 97, 13-22.

Newsholme P. (2001). Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? J Nutr. 131, 2515S-22S.

N.R.C. (1998). Nutrient Requirements of Swine. 10th ed. Subcommitee on Swine Nutrition, Commitee on Animal Nutrition, National Research Council. The National Academy Press

Pierzynowski S.G., Valverde P.J.L., Hommel-Hansen T. and Studzinski T. (2001). Glutamine in gut metabolism. In: Gut Environment of Pigs. Piva A., Bachludsen K.E. and Lindberg J.E. (eds). Nottingham University Press. UK. pp 43-62.

Piva T.J. and Mc Evoy-Bowe E. (1998). Oxidation of glutamine in HeLa cells: role and control of truncated TCA cycles in tumor mitochondria. J. Cell. Biochem. 68, 213-225.

Potsic B., Holliday N., Lewis P., Samuelson D., DeMarco V. and Neu J. (2002). Glutamine supplementation and deprivation: effect on artificially reared rat small intestinal morphology. Pediatr. Res. 52, 430-436.

Pluske J.R. (2001). Morphological and functional changes in the small intestine of the newly-weaned pig. In: Gut environment of pigs. Piva A., Bachludsen K.E. and Lindberg J.E. (eds). Nottingham University Press. UK. pp. 1-27.

Pluske J.R., Hampson D.J. and Williams I.H. (1997). Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51, 215-236.

Remillard R., Guerino F., Dudgeon D.L. and Yardley J.H. (1998). Intravenous glutamine or limited enteral feeding in piglets: amelioration of small intestinal disuse atrophy. J. Nutr. 128, 2723S-2726S.

Rhoads J.M., Argenzio R.A., Chen W., Graves L.M., Licato L.L., Blikslager A.T., Smith J., Gatzy J. and Brenner D.A. (2000). Glutamine metabolism stimulates intestinal cell MAPKs by a cAMP-inhibitable, Raf-independent mechanism. Gastroenterology 118, 90-100.

Sanderson I.R. and Walker W.A. (1991). Nutrition and immunity. Gastroenterology 7, 463-470.

SAS Institute, Inc. (1985). SAS/STAT User's Guide, release 603, 1985 edition. SAS Institute, Inc., Cary, NC, USA.

Scheppach W., Loges C., Bartram P., Christl S.U., Richter F., Dusel G., Stehle P. and Fuerst P. (1994). Effect of free glutamine and alanyl-glutamine dipeptide on mucosal proliferation of the human ileum and colon. Gastroenterology 107, 429-34.

Shi S.-R., Imam S.A., Young L., Cote R.J. and Taylor C.R. (1995). Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J. Histochem. Cytochem. 43, 193-201.

Schlimme E., Martin D. and Meisel H. (2000). Nucleosides and nucleotides: natural bioactive substances in milk and colostrum. Br. J. Nutr. 84, S59-68.

Smith R.J. and Wilmore D.W. (1990). Glutamine nutrition and requirements. J. Parent. Ent. Nutr. 14, 94S-98S.

Souba W.W., Klimberg V.S., Plumpley D.A., Salloum R.M., Flynn T.C., Bland K.I. and Copeland E. (1990). The role of glutamine in maintaining a healthy gut and supporting the metabolic response to injury and infection. J. Surg. Res. 48, 45-52.

Sozmen M., Brown P.J. and Cripps P.J. (1996). IgA, IgG and IgM immunohistochemical staining in normal dog salivary glands and in cases of chronic sialoadenitis. Vet. Res. 27, 285-294.

Tang M., Laarveld B., Van Kessel A.G., Hamilton D.L., Estrada A. and Patience J.F. (1999). Effect of segregated early weaning on postweaning small intestinal development in pigs. J. Anim. Sci. 77, 3191-3200.

Touchette J., Allee G.L., Watanabe K., Toride Y., Shinzato I. and Usry J.L. (2000). The effect of arginine and glutamine on post-weaning performance and intestinal morphology of pigs. J. Anim. Sci. 78 (Suppl. 1), 182.

Uauy R., Stringel G., Thomas R. and Quan R. (1990). Effect of dietary nucleosides on growth and maturation of the developing gut in the rat. J. Pediatr. Gastroenterol. Nutr. 10, 497-503.

Van Beers-Schreurs H.M.G., Nabuurs M.J.A., Vallenga L., Kalsbeek-Van der Valk H.J, Wensing T. and Breukink H.J. (1998). Weaning and the weanling diet influence the villus height and crypt depth in the small intestine of pigs and alter the concentrations of short chain fatty acids in the large intestine and blood. J. Nutr. 128, 947-953.

Wensvoort G., Terpstra C., Pol J.M.A., Terlaak E.A., Blooemrad M., Dekluyver E.P., Kragten C., Van Buiten L., Den Besten A., Vagenaar F., Broekhuijsen J.M., Moonen P.L.J.M., Zerstra T., De Boer E.A., Toiben H.J., De Jung M.F., Vantvelt P., Groenland G.J.R., Van Jennep J.A.M., Voets M.T.H., Verheijden H.H.M. and Braamskamp J. (1991). Mistery swine disease in the Netherlands: The isolation of Lelystad virus. Vet. Q. 13, 121-130.

Windmueller G. (1982). Glutamine utilization by the small intestine. Adv. Enzymol. Relat. Areas Mol. Biol. 53, 201-237.

Windmueller G. and Spaeth A.E. (1980). Respiratory fuels and nitrogen metabolism in vivo in small intestine of fed rats. Quantitative importance of glutamine, glutamate, and aspartate. J. Biol. Chem. 255, 107-112.

Yamamoto S., Wang M., Adjei A. and Ameho C.K. (1979. Role of nucleosides and nucleotides in the immune system, gut reparation after injury, and brain function. Nutrition 13, 372-374.

Accepted September 16, 2003