Postnatal morphological development and production of short-chain fatty acids in the digestive tract of gnotobiotic piglets

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ABSTRACT: The present study examined the impact of *Enterococcus faecium* on morphological development and production of short-chain fatty acids in the digestive tract of gnotobiotic piglets during milk nutrition and weaning. The experiment was carried out on (18) gnotobiotic piglets. The piglets were non-colostral and the feeding ration consisted of autoclaved milk substitute (Sanolac Ferkel, Germany). From the first day of life a probiotic strain of *Enterococcus faecium* was administered continually at a dose of 2 ml of inoculum (1 ml contained 1×10^4 CFU). The animals were weaned on Day 28. Gastrointestinal tract was collected from 18 gnotobiotic piglets slaughtered at three hours after birth and at the age of 2, 7, 14, 21, 28 and 35 days. The level of short-chain fatty acids was determined in the contents of jejunum, ileum and colon. Samples of intestinal mucosa (from duodenum, jejunum and ileum) were subjected to morphological analysis. We characterized regional variations in morphological and functional responses of the small intestine. The jejunal part of the intestinal tract of gnotobiotic piglets was characterized by relatively short crypts, extremely long villi and narrow *lamina propria* which contained only few cells up to Day 14 of life. Morphological examination showed that jejunal and ileal villi were significantly lower at 21 days of age ($P < 0.05$ and $P < 0.001$, resp.). Depending on age, the concentration of both acetoacetic acid and acetic acid was higher in the jejunal contents. The difference was significant on Day 7 of age ($P < 0.05$) for acetoacetic acid and on Day 28 of age ($P < 0.01$) for acetic acid. The concentration of acetic acid in the colonal content of gnotobiotic piglets was significantly higher on Day 7 ($P < 0.05$) and 21 of age ($P < 0.01$). The study demonstrated that the respective bacterial species affected differently the intestinal morphology and concentration of short-chain fatty acids and suggested that postnatal bacterial colonization patterns may have long-term effects on intestinal health and development.

Keywords: gnotobiotic piglets; intestinal morphology; short-chain fatty acids; *Enterococcus faecium*; development; weaning

Quality nutrition and optimum development of the digestive tract are essential for proper growth, high production and good health of livestock. These relations are determined by digestive juice and enzyme secretion, morphological development and microbial colonization of the digestive tract as well as by absorption capacity of the latter. The pig gut is exposed to a variety of stress factors particularly in the early postnatal period and just after weaning. This is the period of significant growth, morphological changes and maturation of the gastrointestinal tract (Le Dividich and Seve, 2000; Xu et al., 2000; Trahair and Sanglid, 2002; Godlewski et al., 2005).

Prior to birth, the alimentary tract is exposed to substances from the ingested amniotic fluid

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which seems to be of importance to its development (Trahair and Harding, 1992). The colostrum, however, differs from the amniotic fluid by the density of nutrients and high immunoglobulin, enzyme, hormone, growth factor and neuroendocrine peptide levels (Koldovsky and Thornburg, 1989). Widdowson and Crabb (1976) were the first to demonstrate the effect of the colostrum upon development of the alimentary tract by comparing the colostrums-suckling piglets with watered animals. Maternal colostrums contained high levels of several hormones and growth promoting peptides like insulin, epidermal growth factor (EGF), insulin-like growth factor-I and II (IGF-I and II), transforming growth factor-β (TGF-β), glucagon-like peptide-2 (GLP-2) and leptin. It was proved that colostral growth factors play an important role in the postnatal development of the digestive tract in newborn animals (Xu et al., 2000; Burrin et al., 2001; Guilloteau et al., 2002; Wolinski et al., 2003). During the several initial days of life of newborns, their small intestine increases its weight by about 70%, length by approx. 20%, diameter by 15%. Its absorption area increases by about 50% during the first postnatal day and by 100% during the first 10 postnatal days (Xu et al., 1992). A large luminal surface area with optimal enterocyte functional maturity is important to young growing pigs so they may attain maximum digestive and absorptive capability. Consequently, suboptimal or adverse environmental factors, influencing the morphological development of intestinal tissue, may have critical functional consequences for the young growing pig. The marked and abrupt morphological responses to weaning in the small intestine, characterized by the transformation from a dense finger-like villi population to a smooth, compact, tongue-shaped luminal villi surface may indicate critical consequences for the young pig digestive capacity and subsequent use of nutrients during the starter phase (Cera et al., 1988). The changes at weaning which include shortening of villi, hyperplasia of crypts, decrease in absorption capacity and certain loss of carbohydrate activity may, in combination with changes in the number and type of enterobacteria, induce various degree of post-weaning diarrhoea (Hampson and Kidder, 1986; Pluske et al., 1997).

Probiotics as natural bioregulators assist the maintenance of the homeostasis of the gastrointestinal tract ecosystem and, during the critical periods of animal life, can play an important role in prevention of diarrhoeic diseases of dietetic and bacterial origin (Vanderbergh, 1993). Enterococci belong to those lactic-acid bacteria which inhabit human and animal intestines (Devriese et al., 1991). Strains with a probiotic character were also detected among enterococci (Audisio et al., 1999). In terms of exactitude and interpretability of results, gnotobiotic piglets are an ideal experimental model for the study of digestive processes and their development.

The aim of the present study was to investigate the impact of *Enterococcus faecium* on postnatal morphological development and production of short-chain fatty acids (SCFA) in the digestive tract of gnotobiotic piglets.

MATERIAL AND METHODS

The experiment with gnotobiotic growing and weaned piglets was carried out at the Institute of Microbiology and Gnotobiology, University of Veterinary Medicine, Kosice, Slovakia. The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocol and the animals were handled and sacrificed in a humane manner in accordance with the guidelines established by the respective commission.

Animals, housing and diets

The experiment was carried out on 18 gnotobiotic piglets of Slovak white × Landrace breed. Gnotobiotic sucklings were obtained using the method of open hysterotomy on Day 112 of pregnancy and were reared in gnotobiotic isolators. A routine microbiological control of gnotobiotic isolators was performed throughout the experiment. Microbiological swabs were taken from isolator walls, surface of animals and from their rectum. The samples were cultivated in PYG medium (Imuna, Slovak Republic). The microbiological control was verified every fifth day on TSA agar with 5% of ram's blood (BBL, Microbiology systems, Cockeysville, USA). Sucklings were non-colostral and were fed autoclaved milk substitute (Sanolac Ferkel, Germany, in 1 kg dry matter: fat 18.0%, N-free extract 20.0%, lysine 1.7%, Ca 0.9%, P 0.7%, Na 1.0%, Mg 0.2%, fibre 1.5%, ash 10.0%, ME 17.5 MJ, vitamin A 50 000 IU, vitamin D_3 5 000 IU, vitamin E 100 mg, biotin 200 µg, Fe 100 mg, vitamin B_1 4 mg, vitamin B_2 4 mg, vitamin B_6 2 mg, vitamin B_{12} 20 µg, calcium pantothenate 10 mg, nicotinic acid 20 mg,

folic acid 1 mg, vitamin C 100 mg, choline chloride 250 mg, *Enterococcus faecium* 0.1×10^4 CFU – data analysed in the laboratory of the Department of Gnotobiology), diluted 1 : 5 with distilled water.

The milk substitute was fed to piglets individually from a glass bottle six times daily (2, 6, 10, 14, 18, 22 h) *ad libitum.* From the first day of life, a probiotic strain of *Enterococcus faecium* isolated from (Sanolac Ferkel, Germany) was applied continually at a dose of 2 ml of inoculum; 1 ml contained 1×10^4 CFU. From the 5th day of life, autoclaved water was available to piglets *ad libitum* and they were fed irradiation-sterilized rations intended for early weaning of piglets. At the age of 28 days, the suckling piglets were weaned and fed irradiation-sterilized starter feedstuff *ad libitum* (Table 1).

Three hours after birth and at the age of two and seven days, two piglets of each indicated age were sacrificed by intra pulmonary euthanasia with T61 at a dose of 1 ml/kg body weight (Holland). Three piglets of each indicated age were sacrificed at the age of 14, 21, 28 and 35 days. The gastrointestinal tract was immediately removed from the sacrified piglets and divided into six segments as follows: stomach, three equal segments of the small intestine, caecum and colon. The total content of each segment was weighed, pH was immediately measured, and short-chain fatty acid (SCFA) were determined in the contents of jejunum, ileum and colon.

Table 1. Ingredient (g/kg) and chemical composition (g/kg DM) of the diet for early weaned piglets (Day 5 to Day 28) and starter feedstuff (Day 28 to Day 35)

Item	Early weaned pigs	Starter feedstuff
CP (g)	200	180
ME (MJ)	13.3	13.0
Fibre (g)	40	45
Lysine (g)	14	11.5
Methionine and cysteine (g)	6.3	6.3
Threonine (g)	9.1	7.5
Choline (mg)	300	600
Vitamin A (IU)	8 000	8 000
Vitamin D_3 (IU)	1 000	1 000
Vitamin E (mg)	20	20
Vitamin B_2 (mg)	3	3
vitamin B_{12} (μg)	20	20
Ca (g)	8	7
P (g)	6.7	5.8
Na (g)	2	1.5
Cu (mg)	10	10
Fe (mg)	125	125
Zn (mg)	100	100
Mn (mg)	30	30

Biochemical analysis

After the collection, 1 g of digesta (jejunum, ileum, colon) was diluted with 50 ml of deionized H_2O and a 30 μl aliquot was used for analysis of short-chain fatty acids. The concentration of formic, acetoacetic, lactic, succinic, acetic, propionic, butyric and valeric acids in the intestinal content was determined by capillary isotachophoresis (ITP). The measurements were done on an "Isotachophoretic analyser ZKI 01" (Slovak Republic). A leading electrolyte of the following composition was used in the pre-separatory capillary: 10^{-2} M HCl + 2.2×10^{-2} M ε-aminocaproic acid + 0.1% methylhydroxyethylcellulosic acid, pH =4 .3. A solution of 5×10^{-3} M caproic acid + 2×10^{-2} M histidine was used as a finishing electrolyte. This electrolytic system worked at 250 μA in the pre-separatory and 50 μA in the analytic capillary. Determination of pH was carried out using a MS-20 pH meter (LP Prague, Czech Republic).

Light microscopy and morphometry

Samples of mucosa (1 cm^2) were taken from the duodenum (5 cm distal to the orifice of the pancreatic duct) and the medial part of both the jejunum and ileum. The samples were fixed in 4% formalin solution. After rinsing with water, the samples were dehydrated in a graded series of absolute ethanol (30%, 50%, 70%, 90%), cleared with benzene, saturated with and embedded in paraffin. Sections of 7 μm thickness (10 slices of each sample) were stained with haematoxylin/eosin and observed under a light microscope. The length of 10 villi and depth of 10 crypts was determined by a computer operated Image C picture analysis system (Intronic GmbH, Berlin, Germany) and the IMES analysis software, using a colour video camera (Sony 3 CCD) and a light microscope (Axiolab, Carl Zeiss Jena, Germany).

Statistical analysis

All data are presented as means ± SEM. To estimate the effect of age on the concentration of SCFA and morphological development, the data were evaluated statistically by one-way analysis of variance (ANOVA) followed by a multiple comparison Tukey's test.

RESULTS

Acidity and production of organic acid along the intestinal tract of gnotobiotic piglets

The pH in the digestive tract of gnotobiotic piglets (Figure 1) showed an increase from the stomach to ileum and a subsequent decrease in the colon with the lowest values measured on Days 14 and 35 of life (6.44 and 5.60, resp.). A non-significant decrease in jejunal pH was observed on Day 14 of age (5.45).

The concentration of acetoacetic acid in the jejunal content of gnotobiotic piglets (Figure 2) reached the highest level at the age of 7 days, in the period of milk nutrition ($P < 0.05$), in comparison with the concentration recorded three hours after birth (3.35 ± 1.25 mmol/l). The production of acetoacetic acid decreased gradually from Day 14 of age (17.70 ± 8.72 mmol/l) up to the end of the observation. A more pronounced post-weaning decrease in the level of acetoacetic acid was recorded one week after weaning ($P < 0.05$). The proportion of lactic acid (Figure 2) was relatively stable from Day 7 to 28 of life and ranged between 19.96 and 23.59 mmol/l. Afterwards, in the 5th week of life, the lactic acid level increased to 31.25 ± 4.17 mmol/l. Similarly, acetic acid level (Figure 2) was relatively stable during first two weeks of life of piglets, than increased significantly ($P < 0.01$) at the age of 28 days (22.11 ± 3.39 mmol/l) compared to the level at 3 hours after birth (6.57 ± 1.62 mmol/l) and decreased again one weak after the weaning (15.95 mmol/l).

The course of the concentration of formic and acetic acid in the ileal content (Figure 3) was similar with the exception of Day 21 when acetic acid reached the highest recorded level (32.08 ± 13.23 mmol/l). Likewise, the concentration of both acetoacetic and lactic acid in the ileal content was similar reaching the highest level at 7 (13.42 ± 2.98 mmol/l and 24.56 ± 7.22 mmol/l, resp.) and 21 days of age (13.08 ± 3.02 mmol/l and 24.40 ± 14.87 mmol/l, resp.). Then the level of acetoacetic acid decreased up to the end of the observation while lactic acid decreased up to 28 days of life of the piglets (Figure 3).

The most important production of acids in the colon of gnotobiotic piglets was the production of acetic acid (Figure 4) which remained relatively stable between Days 7 and 35 of life and the difference between its concentration recorded at three hours after the birth (6.94 ± 3.92 mmol/l) and that determined on Days 7 and 21 of life (41.98 ± 2.38 mmol/l and 44.20 ± 1.97 mmol/l, resp.) was significant ($P < 0.05$ and $P < 0.01$, resp.). A significant difference in concentration of lactic acid was recorded on Day 28 of age (26.34 ± 1.17 mmol/l, $P < 0.05$) compared to the level on the second day (8.18 ± 1.22 mmol/l). Subsequently, we recorded a non-significant, 2-fold increase in the level of this acid at five weeks of age (to 76.30 mmol/l) (Figure 4). A similar tendency was recorded for acetoacetic and propionic acids in the colonal content (Figure 4) with the exception

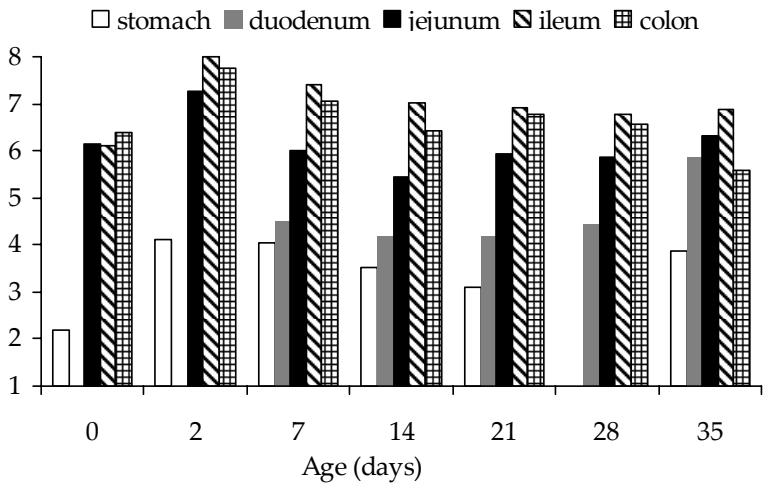

Figure 1. The pH along the gastrointestinal tract of gnotobiotic piglets

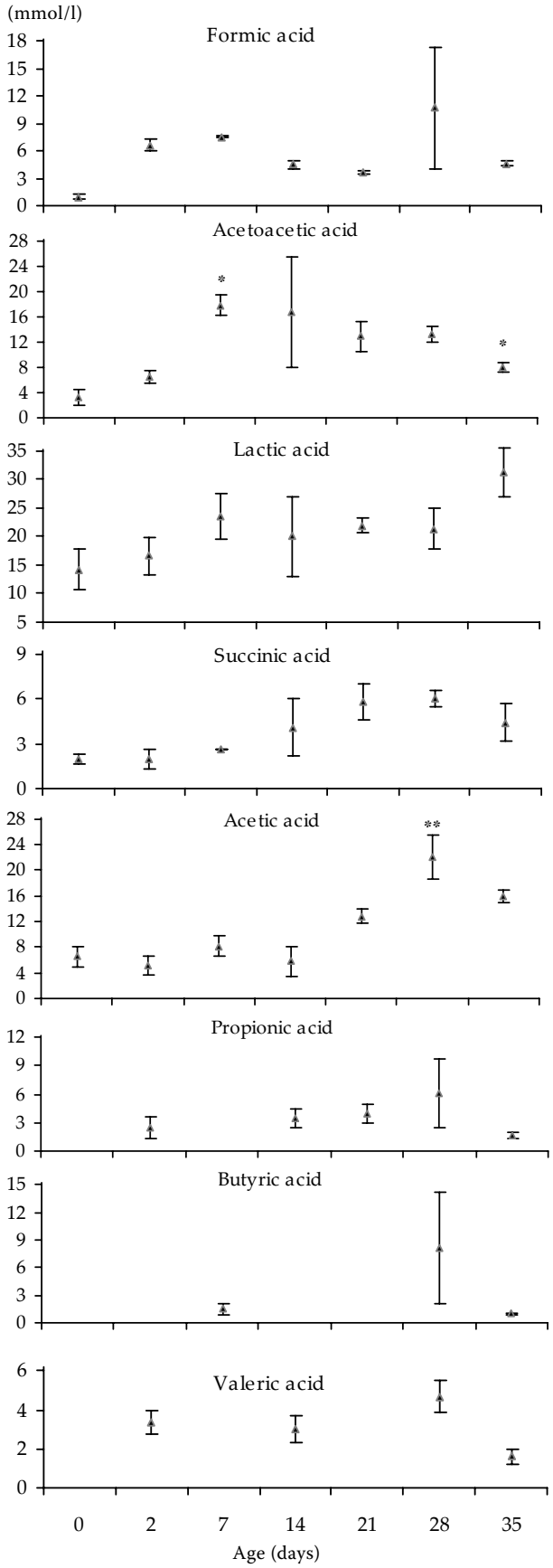

Figure 2. Concentration of SCFA in the content of the jejunum of gnotobiotic piglets (*$P < 0.05$, **$P < 0.01$)

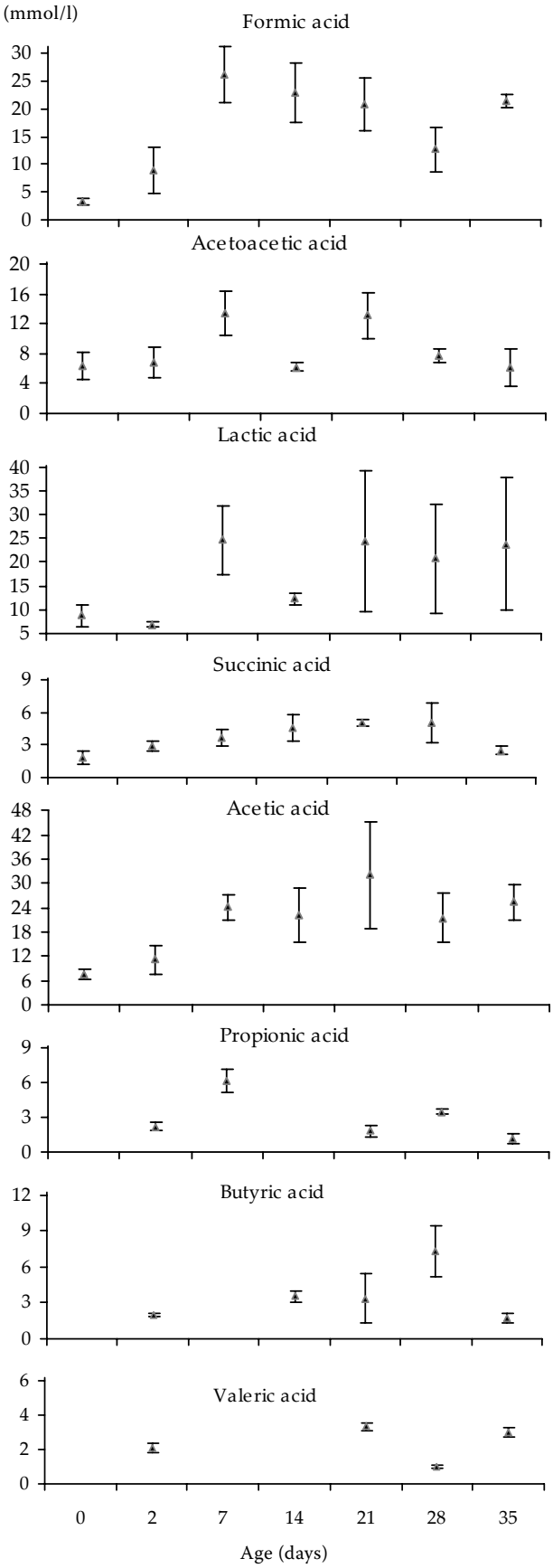

Figure 3. Concentration of SCFA in the content of the ileum of gnotobiotic piglets

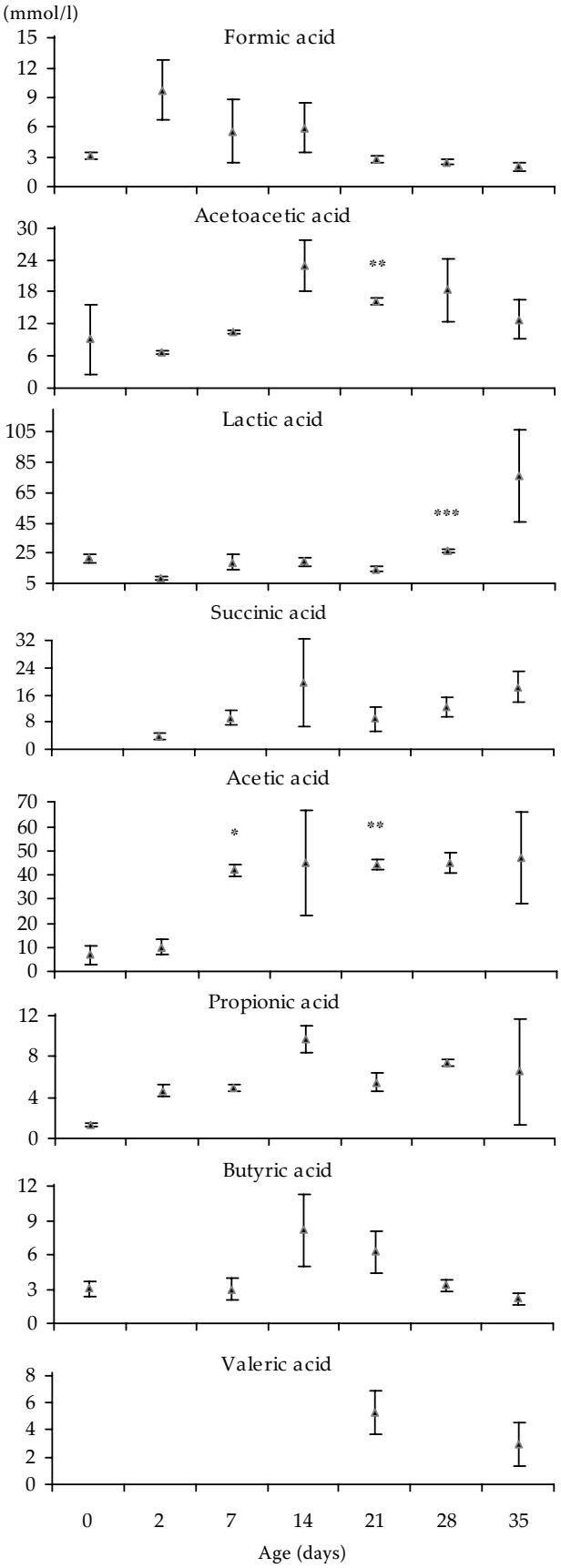

Figure 4. Concentration of SCFA in the content of the colon of gnotobiotic piglets (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

of the level recorded 3 h after the birth. The level of acetoacetic acid was significantly higher on Day 21 of age (16.22 ± 0.62 mmol/l, $P < 0.01$) compared to the second day (6.71 ± 0.30 mmol/l).

Development of digestive organs and small intestine morphometry

From Day 2 of age we recorded a gradual increase in body weight of gnotobiotic piglets from 0.75 kg up to 3.90 kg ($P < 0.05$) on Day 21 of age. On Day 28 (day of weaning) and one week after weaning the body weight of piglets was decreased insignificantly (Table 2).

The increase in relative weight of the small intestine resembled that of the large intestine (Table 2) throughout the period of investigation with the exception that the relative weight of the large intestine in comparison with the weight on Day 2 of age was increased significantly on Day 21 ($P < 0.05$), 28 ($P < 0.05$) and 35 of age ($P < 0.001$). One week after weaning we observed a non-significant decrease in the relative weight of lungs, liver and adrenals in comparison with their weight on the day of weaning.

The development of the height of villi in individual segments of the small intestine of gnotobiotic piglets is shown in Figure 5. In the duodenum we observed a gradual increase in the length of villi from 371 μm on Day 0 to 605 μm on Day 14 of life. On Day 21, the height of villi was decreased significantly ($P < 0.05$). A decrease in their length was also recorded on Day 28 but the difference was insignificant. By one week after weaning we observed that the length of villi started to increase again but did not exceed 400 μm. Completely different development of villi was observed in the jejunal segment of the small intestine. The length of jejunal villi increased from Day 0 up to Day 2 of life reaching maximum of 725 μm. In the subsequent five weeks the length of villi decreased gradually down to 387 μm in the week after weaning and the decrease was significant on Day 14 ($P < 0.05$) and 21 of life ($P < 0.001$). The initial length of villi in the ileal segment reached on average 587 μm and this length decreased to 393 μm during the first week of life. On Day 14 of age a slight increase in the length of villi was observed to the value of 424 μm which was replaced by gradual slight decrease to the value of 336 μm in the first post-weaning week.

Table 2. The organ dimensions in gnotobiotic piglets

	Age (days)					
	2	7	14	21	28	35
Body weight (kg)	0.75 ± 0.04	1.29 ± 0.02	1.80 ± 0.04	3.90 ± 0.71*	3.65 ± 1.77	3.25 ± 0.21
Small intestine(g/kg)	42.47 ± 4.96	52.59 ± 4.89	53.02 ± 6.86	64.68 ± 3.50	55.41 ± 5.40	60.67 ± 11.26
Large intestine(g/kg)	13.89 ± 2.77	18.31 ± 2.16	20.07 ± 1.39	47.41 ± 5.82*	42.10 ± 3.73*	85.63 ± 8.56***
Heart (g/kg)	8.25 ± 0.25	6.29 ± 0.32	8.52 ± 0.98	6.90 ± 0.14	6.25 ± 0.84	7.94 ± 1.175
Lungs (g/kg)	19.17 ± 0.08	16.19 ± 0.59	14.01 ± 1.07	12.96 ± 1.15	16.60 ± 0.06	16.35 ± 2.47
Liver (g/kg)	20.86 ± 1.28	21.92 ± 4.06	35.71 ± 0.67*	28.33 ± 1.96	35.66 ± 0.66*	29.51 ± 2.75
Spleen (g/kg)	0.77 ± 0.05	1.00 ± 0.09	1.23 ± 0.05	1.23 ± 0.185	1.69 ± 0.26*	1.97 ± 0.02**
Kidney (g/kg)	3.44 ± 0.33	2.95 ± 0.47	3.90 ± 0.07	4.07 ± 0.09	3.54 ± 0.94	4.50 ± 0.04
Adrenals (g/kg)	0.24 ± 0.01	0.32 ± 0.02	0.19 ± 0.01	0.12 ± 0.01	0.17 ± 0.04	0.09 ± 0.004

Significantly different from Day 2: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

The postnatal changes in the depth of crypts (Figure 6) were the same in all small intestine segments. From the birth up to Day 35 of life the depth of crypts gradually deepened and reached 164 μm on Day 35 of life. Similar development was observed also in the duodenal segment of the small intestine, where, with the exception of slight decrease on Day 14 of age, the depth of crypts gradually deepened throughout the observation period with significant difference on Day 21 of age ($P < 0.05$). One week after weaning the depth of crypts in the duodenum reached 205 μm. A significant deepen in the depth of crypts ($P < 0.05$) was recorded on Day 21 of age also in the ileal segment of gnotobiotic piglets.

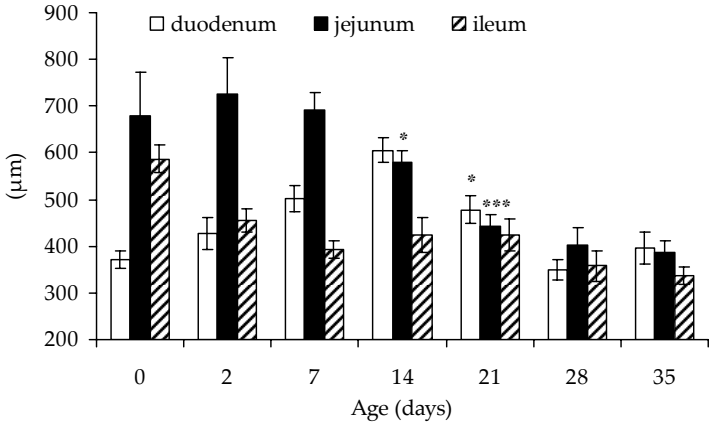

Figure 5. Comparison of villi height (μm) among gnotobiotic piglet age

*$P < 0.05$, ***$P < 0.001$

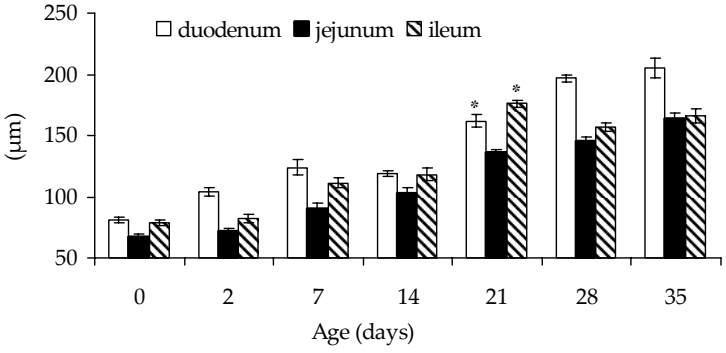

Figure 6. Comparison of crypts depth (μm) among gnotobiotic piglet age

*$P < 0.05$

Staining with haematoxylin/eosin showed that the small intestine mucosa of gnotobiotic piglets at 3 h after birth (Figure7a) and on Day 2 of age (Figure 7b) was covered by population of dense, finger-like villi. On their surface we were able to observe enterocytes with apically located nucleus. The fibrous base of intestinal villi was poorly differentiated and the intestinal crypts were small. By Day 7 of age (Figure 7c) the height of villi decreased but their diameter increased.

At the time of weaning (Day 28 of age), the differentiated basis of intestinal villi consisted of thin fibrous tissue containing fascicles of smooth muscle cells. On the surface of the villi we were able to observe goblet cells interspersed among enterocytes (Figure 7d). The enterocyte nuclei were located in the medial part of cytoplasm. The villi stroma was infiltrated with small number of lymphocytes and plasmatic cells. By Day 35 of life, the jejunal villi acquired tongue-like shape (Figure 7e).

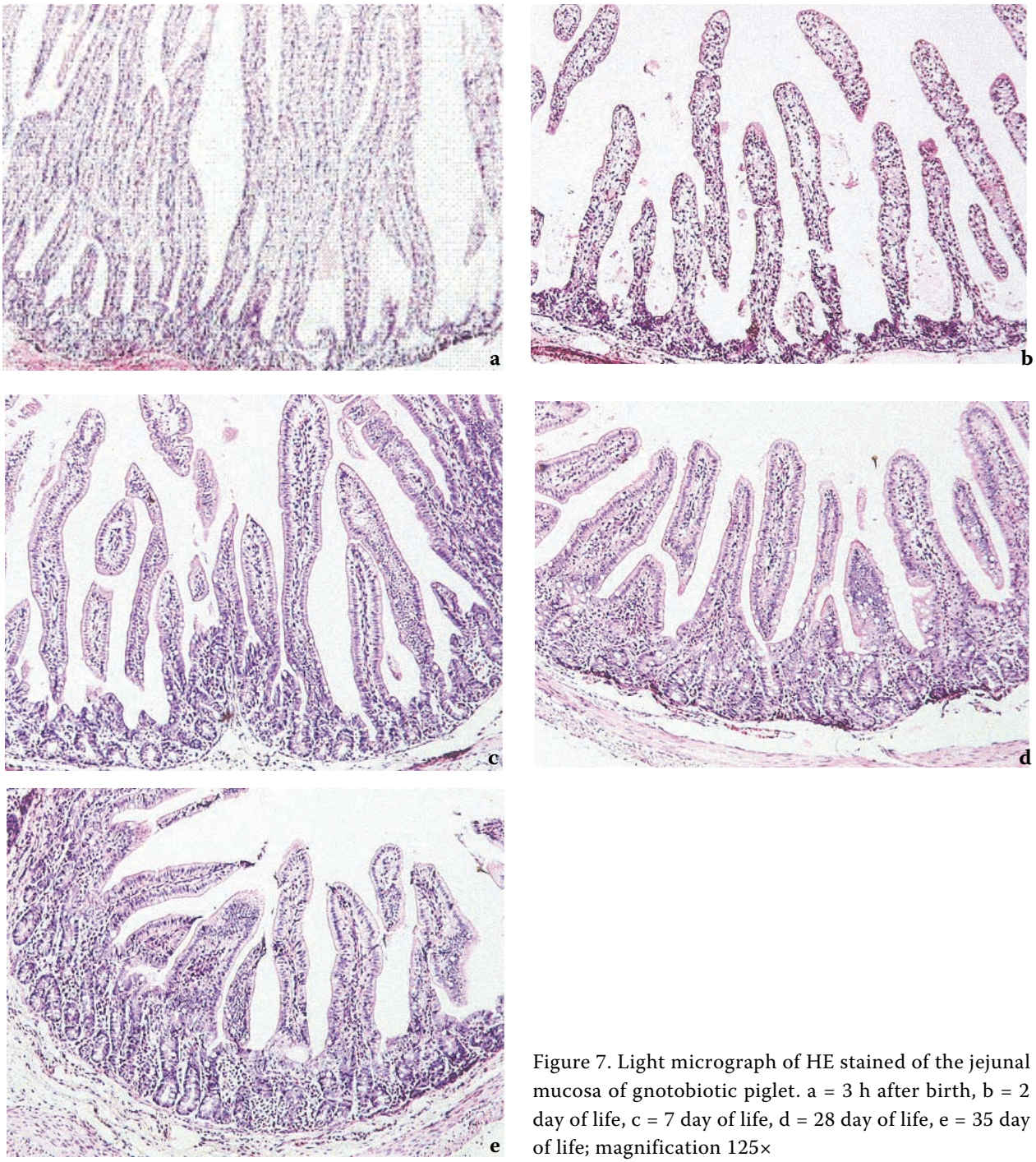

Figure 7. Light micrograph of HE stained of the jejunal mucosa of gnotobiotic piglet. a = 3 h after birth, b = 2 day of life, c = 7 day of life, d = 28 day of life, e = 35 day of life; magnification 125×

DISCUSSION

Diseases of the gastrointestinal tract can be considered the most important health and economic problem of rearing young livestock, since they may cause extremely high losses due to morbidity, mortality, cost of treatment and weight loss. At an early age, diseases debilitate the animal organism and cause delays in development which can subsequently become evident as health problems and productivity decrease. For this reason it is extremely important to ensure optimum development of the digestive tract in young animals. Recent research provided extensive possibilities to carry out thorough studies and to acquire new knowledge on the physiological and functional development of the gastrointestinal tract of animals. Management of gnotobiotic techniques and the use of gnotobiotic animals for experimental purposes have substantially influenced the methodological approach of scientists to the respective topic. Microflora is of great importance to the development of the digestive tract. The use of gnotobiotic animals in experiments has enabled to study the role of microorganisms in the process of morphological and functional development of the digestive tract.

Gnotobiotic animals typically display remarkable morphological and physiological properties resulting from a total or partial absence of microflora. In the first phase, changes occur in those organ systems which come into direct contact with the microflora. Primary morphological deviations develop in the digestive tract and the lymph organs (Kruml et al., 1969) and later secondary changes occur in the blood-making system, the liver and other organs. In gnotobiotic animals, morphological changes are accompanied by physiological changes of which digestive processes and immune reaction changes are the most typical (Abrams and Bishop, 1967; Havell et al., 1970; Cebra et al., 1999; Cukrowska et al., 2001).

The overall mass of the small intestine in germ-free species, however, is decreased, and its surface area is smaller, whereas the villi of the small intestine are unusually uniform in shape and appear slender, with crypts, which are shorter and less populated than in the respective conventional control animals (Meslin et al., 1973).

Our study showed that the jejunal part of the intestinal tract in monoassociated pigs (Figure 7a,b,c) was characterized up to 14 days of life by relatively short crypts, extremely long villi and narrow *lamina propria* containing few cells. Reduced crypt depth and increased villus length agree with the previous observations in germ-free pigs (Kenworthy, 1970; Thompson, and Trexler, 1971; Wostmann, 1975; Shurson et al., 1990; Shirkey et al., 2005). In the present study villi were the longest in the jejunum and shortest in the duodenum and ileum, whereas crypt depth was shortest in the jejunum and deepest in the duodenum throughout the observation period (Figures 5 and 6). These morphological characteristics suggested that the rates of enterocyte proliferation and exfoliation were the highest in the proximal small intestine, as indicated by deep crypts and shorter villi, respectively, with rates decreasing distally along the small intestine (Hampson and Kidder, 1986). In agreement with our morphological findings, Miniats and Valli (1973) reported longer jejunal villi in germ-free pigs but did not measure villi in other regions. On the contrary, Shurson et al. (1990) reported that germ-free pigs had longer ileal and duodenal villi but shorter jejunal villi compared to their conventional counterparts. In the germ-free chicken (Rolls et al., 1978) and rodents (Komai and Kimura, 1979; Ishikawa et al., 1986; Meslin et al., 1992) villus length was reported to decrease progressively along the small intestine from the duodenum to the distal ileum. This regional response was suggested to be the result of regional variation in microbial species diversity and abundance. Although bacterial colonization is lower in the proximal regions (ranging from 10^1 to 10^4 CFU/g of contents) compared with the distal regions (ranging from 10^9 to 10^1 l CFU/g of contents) of the small intestine in the conventional pigs (Stombeck and Guilford, 1990), regional colonization variation (species number or diversity) cannot explain the marked regional variation in small intestinal morphology observed here in the germ-free pigs. Shirkey et al. (2005) suggested that regional variation in morphology, especially in the proximal small intestine, is not entirely dependent on microbial colonization but is also influenced by such non-microbial factors as bile salts, pancreatic secretions, and compounds of dietary origin which would be expected to be in higher concentration and have more contact with mucosal surface in the duodenum.

The adaptive response of the small intestine to bacterial colonization is also evident upon examination of the relatively small intestinal length and weight. In germ-free and monoassociated pigs (Shirkey et al., 2005), the relative small intestine

length was reduced compared with conventional pigs. The mechanisms affecting intestinal length are unknown, however, it can be hypothesized that increased small intestine length in conventionalized pigs is a compensatory response to the decreased absorptive capacity associated with decreased surface area (decreased villus length) and/or to direct competition with the microbiota for dietary nutrients. Shirkey et al. (2005) observed that in the proximal region of the small intestine, the relative weights for segments from conventional pigs tended to be higher than those from germ-free and monoassociated pigs. This is consistent with the previous reports indicating that compared with germ-free animals, conventionally reared animals experience intestinal "thickening" associated primarily with increased lamina propria cellularity (Miniats and Valli, 1973) as well as thickening of the submucosa and muscular layers (Shurson et al., 1990; Furuse and Okumura, 1994; Gaskins, 1997). Throughout our observations (Table 2), the relative weight of the small intestine increased only slightly while the relative weight of the large intestine showed a significant increase on Day 21 ($P < 0.05$), 28 ($P < 0.05$) and 35 of age ($P < 0.001$) compared to Day 2 of piglet life.

Bomba et al. (1998) studied the intestinal metabolism in two groups of gnotobiotic piglets (one germ-free and one inoculated only with *Lactobacillus plantarum*) during the first three weeks of their life. At the age of one week and three weeks, the actual acidity of the jejunal content of gnotobiotic piglets inoculated with *Lactobacillus plantarum* was significantly lower ($P < 0.05$ and $P < 0.01$, resp.) in comparison with that in the non-inoculated animals. The pH value of ileal contents of inoculated piglets was also lower, however, the differences were insignificant. Zitnan et al. (2001) observed the pH of the jejunal and ileal contents in conventional suckling piglets of identical age. The mentioned investigations of the actual acidity of the individual small intestine segments allowed us to state that the pH of the jejunal contents in germ-free piglets aged one and three weeks (7.49 and 7.12, resp.) was increased significantly when compared to the values recorded in conventional piglets of the same age (6.23 and 6.19). In contrast, the pH of the jejunal content in gnotobiotic piglets inoculated with *Lactobacillus plantarum* was moderately lower (5.63 and 5.84, resp). We recorded (Figure 1) similarly low pH of the jejunal content in piglets of the same age inoculated with *Enterococcus faecium* (6.02 and 5.95).

Organic acids are the main metabolites of intestinal fermentation. The degree of their concentration in the digesta reflects the level of intestinal fermentation (Jensen, 2001; Knarreborg et al., 2001; Piva et al., 2002).

Morphological changes in the digestive tract are also influenced by SCFA (Goodlad et al., 1989) which play an important role in bacterial interactions of the alimentary tract. The ability to generate organic acids, particularly lactic and acetic acids, present one of the mechanisms by which lactobacilli perform their inhibitory effect upon pathogens (Piard and Desmazeaud, 1991). With decreasing pH values, the inhibitory activity of the above acids increases (Daly et al., 1972), their molecular form being toxic for bacteria. The increased toxicity of acetic acid is attributed to its higher pKa in comparison to lactic acid. Increased lactic acid levels intensify the toxicity of acetic acid (Adams and Hall, 1988).

Comparison of lactic acid levels in the jejunal and ileal contents of one week old gnotobiotic piglets (Bomba et al., 1998) and conventional suckling piglets (Zitnan et al., 2001) revealed that the highest levels were found in conventional animals (27.50 and 26.90 mmol/l, resp.) and in *Lactobacillus plantarum* inoculated gnotobiotic piglets (26.60 and 14.20 mmol/l, resp.). The lowest levels of lactic acid in the jejunal and ileal contents were seen in germ-free piglets (4.40 and 6.45 mmol/l, resp.). Our study (Figures 2 and 3) performed on piglets inoculated with *Enterococcus faecium* at the age of one week showed similar levels of lactic acid in the content of jejunum and ileum (23.59 and 24.56 mmol/l, resp.). At the age of three weeks the level of lactic acid in the jejunum of piglets inoculated with *Enterococcus faecium* was lower in comparison with that in jejunum of piglets inoculated with *Lactobacillus plantarum* (21.94 and 33.15 mol/l, resp.) but in the ileum of *Enterococcus faecium* inoculated piglets we found the highest level of this acid (24.39 mmol/l) in comparison with all other groups of piglets.

Our study showed that at one week of age the maximum acetic acid levels in the jejunal and ileal contents were found in conventional piglets (33.05 and 21.81 mmol/l, resp) and lower in gnotobiotic piglets inoculated with lactobacilli (11.80 and 11.85 mmol/l, resp.) and in those inoculated with *Enterococcus faecium* only in their jejunal content (8.18 mmol/l, resp.) (Figures 2 and 3). Similarly low levels were detected also in germ-free piglets (13.15 and

3.9 mmol/l, resp.). On the contrary, at the age of three weeks, we recorded higher levels of acetic acid in the jejunal and ileal content of gnotobiotic piglets inoculated with *Enterococcus faecium* (12.86 and 32.08 mmol/l, resp.) in comparison with both conventional piglets (10.7 and 25.9 mmol/l, resp.) and gnotobiotic piglets inoculated with *Lactobacillus plantarum* (11.85 and 14.2 mmol/l, resp.).

The marked and abrupt morphological response to weaning in the small intestine, characterized by transformation from a dense finger-like villi population to a smooth, compact tongue-shaped luminal villi was observed also in our study. The decreased intestinal fermentation in the digestive tract of gnotobiotic piglets in the post-weaning period (Day 35 of age) was reflected in slight or more pronounced decrease in SCFA. A more marked increase (by 50 mmol/l) in comparison with the day of weaning (Day 28 of age) was observed only for lactic acid in the colon segment. We also observed a moderate post-weaning increase in the level of lactic acid in the jejunal and ileal segment, acetic acid in the jejunum and ileum and formic and valeric acid in the ileal segment and succinic acid in the colon segment, however, none of them exceeded the level of 10 mmol/l.

The results presented show that the complexity of the intestinal microflora affects the production of the investigated organic acids in the alimentary tract of piglets.

CONCLUSION

Gnotobiotic animals are a very useful model in studying the physiology of the digestive tract. The gnotobiotic model allowed us to carry out systematic examination of the effect of a defined microbial population on postnatal intestinal development. We characterized regional variations in morphological and functional responses of the small intestine. We also identified that morphological and functional responses were affected differently by respective bacterial species, supporting the assumption that postnatal bacterial colonization patterns play an important role in neonatal intestinal development. Very good application of gnotobiotic animals is anticipated in the field of study of mutual interaction of natural microflora and pathogens in the digestive tract, mechanisms of probiotic effects of micro-organisms, properties of probiotic micro-organisms and all aspects affecting their efficacy. Gaining new knowledge in the respective areas may allow one to develop more effective probiotic products that can support health and prevent diseases of the digestive tract of animals.

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