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Running head: Rearing effect on M cells in the pig's GALT

Effect of artificial rearing of piglets on the volume densities of M cells in the tonsils of the soft palate and ileal Peyer's patches

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Article history:
Received 9 September 2016
Received in revised form 12 December 2016
Accepted 22 December 2016

http://dx.doi.org/10.1016/j.vetimm.2016.12.009

Abstract

The high prolificacy of modern hybrid sows has increased the mean litter size during the last decades. However, rearing large litters is challenging and has increased the use of alternative management strategies such as euthanasia of weak piglets, cross-fostering, supplementing piglets with milk, split-nursing and split-weaning. The latter includes artificial rearing on brooders where piglets have *ad libitum* access to milk replacer. The effect of this artificial rearing on the immune system of the piglet is the subject of various studies. The present study focused on the M cells in the tonsil of the soft palate and in the ileal Peyer's patch (iPP). These epithelial cells are specialized in antigen sampling and play a pivotal role in the induction of adaptive immune responses. The volume densities of the M cells were assessed by stereological analysis of tissue samples from piglets of 0, 3, 8 and 19 days of age. During the first three days, piglets suckled the sow, permitting them to ingest colostrum. At the third day, the piglets were either allowed to continue to suckle the sow or were transferred to brooders. The six experimental groups, each containing six piglets, thus consisted of newborn piglets, 3-day-old sow-suckled piglets, and conventionally and artificially reared piglets of 8 and 19 days of age. To identify M cells, tissue samples were immersed in 4% phosphate-buffered paraformaldehyde and paraffin sections were immunohistochemically stained against cytokeratin 18. The volume densities of M cells in both the crypt epithelium of the tonsils of the soft palate and the follicle-associated epithelium of the iPPs did not show any difference between the various age groups of conventionally reared piglets. However, values were twice as high in the iPPs compared to the tonsils of the soft palate. In contrast, a decrease in volume densities of M cells was observed in the iPPs of piglets after they had been transferred to commercial brooders ($P = 0.05$), resulting in significantly lower values ($P = 0.04$) in comparison with the age-matched sow-suckled groups. However, this observation did not translate to values of the tonsils where M cell volume densities remained the same in all age and rearing groups. Based on these results, it appears that antigen sampling is possible from birth onwards and is more advanced in the small intestine than in the oropharynx, but possibly lags behind in artificially reared piglets.

Keywords: artificial rearing; gut-associated lymphoid tissue; ileal Peyer's patch; M cells; piglet; tonsil of the soft palate

1. Introduction

The increased use of hyperprolific sows is responsible for the high number of supernumerary and low birth weight piglets that is seen in modern pork production (Quiniou et al., 2002; Kirkden et al., 2013). Because these piglets show higher morbidity and mortality rates, the profitability of these modern sows is undermined (Tuchscherer et al., 2000). An important causative factor for the affected health status is the malnutrition of the piglets (Kirkden et al., 2013). Not only the ingestion of sufficient amounts of colostrum by the neonatal piglets, which are born agammaglobulinemic (Aumaitre and Seve, 1978; Butler and Brown, 1994; Rooke and Bland, 2002), but also the level of subsequent milk uptake play key roles in the growth and vitality of the young animals (Kirkden et al., 2013).

To be able to rear large, heterogeneous litters, alternative rearing strategies are explored. These most often include cross-fostering (Ferrari et al., 2014), supplementing the piglets with milk formula and split-weaning (Rutherford et al., 2011). The latter includes artificial rearing, denoting that piglets are transferred to commercial brooders with ad libitum access to formulated milk, after sufficient ingestion of colostrum was assured (De Vos et al., 2014a; Levast et al., 2010). However, these novel concepts might influence the piglets' normal development since the used formulated milk is lower in specific factors that stimulate the immune system (De Vos et al., 2014b; Hamosh, 2001; Li et al., 2012). Thus, it is hypothesized that the development of the gut-associated lymphoid tissue (GALT) is different in artificially reared piglets.

The GALT is the largest compartment of the mucosa-associated lymphoid tissue (MALT) (Brandtzaeg, 2009). In the pig, its major components are the oropharyngeal tonsils and Peyer's patches (PPs) (Nickel et al., 1979; Pabst, 1987). The porcine tonsils that are located in the oropharynx, and thus belong to the GALT, are the lingual tonsil (*tonsilla lingualis*) and the tonsil of the soft palate (*tonsilla veli palatini*). The latter is by far the largest oropharyngeal tonsil and plays a pivotal role in the first line of defense against antigens that are taken up by the oral route (Brandtzaeg and Halstensen, 1992).

The PPs (*lymphonoduli aggregati intestinales)* play an important second defense line. The PPs of the pig are composed of the discontinuous jejunal PPs and the continuous ileal PP (iPP), which both contain secondary lymphoid tissue that mounts immune responses against ingested antigens (Binns and Licence, 1985; Pabst et al., 1988). This is in contrast to ruminants in which the iPP functions as a primary lymphoid organ (Sinkora et al., 2011). Since mucosal lymphoid tissues lack afferent lymph vessels, immune responses can only be induced against antigens that make contact with the epithelium (Casteleyn et al., 2014). Specialized epithelial cells, called M cells, sample these luminal antigens (Owen and Jones, 1974). They are characterized by a low number of short, irregular microvilli overlaid by a thin glycocalix and contain cytokeratin (CK) 18 in the pig, which can be visualized immunohistochemically (Gebert et al., 1994; Corr et al., 2008).

In order to induce immune responses against ingested antigens, sufficient numbers of M cells that translocate luminal antigens to the underlying lymphoid tissue are required (Hathaway and Kraehenbuhl, 2000). Any disturbance in the development of the GALT and thus the ability to sample luminal antigens may result in an impaired induction of adaptive immunity. The aim of the present study was to investigate the potential variability in M cell

abundancy in the epithelium that lines the crypts of the tonsil of the soft palate and the dome epithelia of the iPP in piglets aged between 0 and 19 days, which were either conventionally or artificially reared.

2. Materials and methods

2.1 Experimental groups

Thirty-six neonatal piglets (Topigs® (Topigs Norsvin, Sint-Katelijne-Waver, Belgium) × Piétrain), of either gender, that were born on a local farm and presented a normal birth weight, i.e. a birth weight ranging within 1 SD of the mean litter birth weight (Paredes et al., 2012; Willemen et al., 2014), were included in the study. Dams (n = 36), with a parity between two and five were kept under conventional Belgian commercial pig housing conditions. All animals received an intramuscular iron injection (Iron(III) Dextran, 200 mg/piglet, Uniferon, Pharmacosmos, Holbaek, Denmark) on day 3, none of the selected animals received any antibiotic treatment prior or during the experiment and boars were not castrated. Six gender-matched piglets were euthanized within 24 h after birth and another group of six piglets at the age of 3 days. On the third day, the remaining 24 piglets were assigned to conventional or artificial rearing. The conventionally reared piglets continued to suckle the sow until the age of 8 days (n = 6) or 19 days (n = 6). The artificially reared animals were transferred to commercial brooders (Rescue Decks®, S&R Resources LLC, Mason, USA). In these brooders they had *ad libitum* access to milk formula (Piggylac®, Vitamex, Drongen, Belgium). These 12 artificially reared piglets were euthanized at the age of 8 days (n = 6) or 19 days (n = 6).

All piglets had free access to water and were maintained under standard environmental conditions (12 h/12 h light/dark cycle, temperature adjusted to age). Animals were observed daily to document their general health status. Their body weights were recorded both at birth and before they were sacrificed for sampling. The composition and nutritional value of sow milk and the provided milk formula are presented in Table 1. All experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp, Belgium (2010-13).

2.2 Tissue samples

Piglets from the different experimental groups were euthanized at the ages of 0, 3, 8 or 19 days by exsanguination via transection of the jugular veins and carotid arteries, subsequent to i.p. injection of sodium pentobarbital (200 mg/kg, Kela, Hoogstraten, Belgium). After the tonsil of the soft palate had been removed, a sample measuring 10 mm by 5 mm was taken and immersed overnight in 4% phosphate-buffered paraformaldehyde (PFA; pH 7.4) at room temperature. In addition, the intestinal tract was dissected and a cross-sectional 15 mm sample of the ileum was taken at 95% of the total length of the small intestine. Intestinal samples were fixed in 4% PFA (pH 7.4) for 2 h at room temperature. All tissue samples were routinely processed to paraffin-embedded tissue blocks after rinsing in phosphate-buffered saline solution (PBS; 0.01 M, pH 7.4).

2.3. Immunohistochemical staining for M cells

Transversal tissue sections, 5 µm thick, were made randomly according to stereological principles (Gundersen and Jensen, 1987). After deparaffinizing and rehydrating the sections, antigen retrieval was performed. Sections of the ileum were exposed to proteinase K (Dako, Glostrup, Denmark) for 3 min at 37°C. In contrast, heat-induced epitope retrieval was applied for the tonsillar sections that were immersed for 30 min in Tris-EDTA (10 mM Tris, 1 mM EDTA, 0.05% Tween 20 (Acros Organics, Geel, Belgium), pH 9) that was heated to 99°C in a Pascal pressure chamber (Dako). The sections were subsequently allowed to cool down for 20 min at room temperature. After three washing steps in Tris-buffered saline solution (TBS; 0.05M, pH 7.4), endogenous peroxidase activity was depleted by 10 min incubation of the sections in 3% H_2O_2 (Merck, Darmstadt, Germany) in TBS. Afterwards, the sections were incubated for 1 h at room temperature with 10% normal goat serum (Dako) in TBS to block aspecific binding sites. The M cells in the epithelium of the tonsillar crypts and ileal follicle-associated epithelium (FAE) were immunohistochemically stained by incubating the sections overnight at 4°C with a primary murine antibody directed against human CK 18 (clone CY-90, Sigma Aldrich, Diegem, Belgium). The primary antibody was diluted 1/400 in TBS with 1% Triton-X-100 (Sigma-Aldrich) and 0.1% BSA (Sigma-Aldrich) to enhance tissue penetration and decrease aspecific binding, respectively. After three washing steps in TBS, tissue sections were incubated for 1 h at room temperature with a horseradish peroxidase-bound goat anti-mouse secondary antibody (Dako), which was diluted 1/200 in the same dilution buffer as described above. A final washing step consisted of rinsing the sections twice with TBS and once with distilled water, each time for 5 min. The immunohistochemical reaction was visualized by adding 3,3'-diaminobenzidine (DAB, Dako). All sections were counterstained with hematoxylin.

To differentiate the M cells from CK18 immunoreactive goblet cells in the iPPs (Gebert, 1997), the ileal sections were counterstained by the Periodic Acid Schiff (PAS) procedure (Rode et al., 1982; Yamabayashi, 1987). In brief, the histological sections were incubated for 5 min in 0.5% periodic acid (VWR International, Leuven, Belgium), 5 min in Schiff's reagent (Carl Röth GmbH & Co, Karlsruhe, Germany), rinsed for 5 min with running tap water, counterstained with hematoxylin followed by a final rinse with running tap water.

2.4. Stereological analysis

The sections were analyzed using a conventional light microscope (BX50, Olympus, Aartselaar, Belgium) equipped with a motorized stage (ProScan, PRIOR Scientific, Rockland, USA) and a digital camera (DP70, Olympus). The volume densities (V_v) of the M cells in the crypt epithelium of the tonsil of the soft palate and in the FAE of the ileum were determined using the software Computer-Assisted Stereological Toolbox 2 (CAST2, Visiopharm, Hoersholm, Denmark). To this purpose, a point grid was superimposed on the microscopic views (magnification 200x). The number of points hitting the M cells was divided by the number of points hitting the reference volume. For the tonsil of the soft palate, the reference volume was the volume of the crypt epithelium, whereas the FAE of the ileum was regarded as the reference volume for the ileal M cells. The density of the point

grid and the number of sections that had to be counted were determined by calculating the coefficient of error that needed to be below 5% (Gundersen and Jensen, 1987).

2.5. Statistical analysis

The potential influence of rearing method on the body weight was evaluated with analysis of covariance (ANCOVA). As the interaction term between age and rearing strategy was statistically significant, data was split by age to identify differences between both rearing strategies. A *P*-value smaller than or equal to 0.05 was considered statistically significant. The same test was used to detect potential differences in volume densities of M cells between the rearing strategies. Here, no significant interaction terms were detected. The Kruskal-Wallis (mean rank) test was used to detect age-related differences in volume densities of M cells. A Bonferroni corrected *P*-value for pairwise comparisons between age groups was obtained. Before the analysis, a non-parametric Levene's test was utilized to verify the equality of variances in the samples (homogeneity of variance) ($P > 0.05$). Statistical analyses were performed with IBM SPSS statistics (version 22; IBM Business Analytics, Armonk, NY, USA).

3. Results

3.1. Zootechnical performance

Analysis of the body weights showed an interaction between age and rearing method ($P = 0.002$; Table 2). As a consequence, the difference in body weight between both rearing groups was not identical at all time points. Splitting the data into different age groups showed that the body weights of the 8-day-old piglets did not differ between both rearing groups ($P = 0.599$). However, this changed at day 19 when formula-fed piglets weighed more than their sow-fed counterparts ($P = 0.002$).

3.2. Volume density of M cells in the tonsil of the soft palate

The histological sections of the tonsil of the soft palate demonstrated the presence of several crypts, which were lined by non-keratinized stratified squamous epithelium devoid of goblet cells (Fig. 1A). CK18-stained M cells, which had an oval to slender shape, were scattered throughout the epithelium (Fig. 1B).

Rearing strategy had no effect on the volume densities of M cells in the tonsil of the soft palate ($P = 0.277$; Fig. 2). In addition, no statistically significant differences were detected in the volume densities of M cells between the different age groups of piglets that were either conventionally ($P = 0.379$) or artificially ($P = 0.915$) reared.

3.3. Volume density of M cells in the iPP

The continuous iPP contained lymphoid follicles that were separated by interfollicular lymphoid tissue. The lymphoid follicles protruded into broad villi that were lined by a single columnar FAE or dome epithelium, which contained numerous M cells and

only a few goblet cells (Fig. 3A). The latter, which stained positive for both CK18 and PAS, were abundant in the epithelium lining the villi that were not associated with a lymphoid follicle. The CK18-stained M cells that were present in the FAE had a slender shape and laid parallel to each other (Fig. 3B). Some M cells displayed lymphocytes in their basolateral pockets.

At the level of the FAE, the volume densities of the M cells in the conventionally reared piglets appeared not to be influenced by age ($P = 0.067$; Fig. 4). During the colostrum phase, M cell densities seemed to rise from 28.9 ± 2.6% at day 0 to 38.2 ± 6.2% at day 3. However, this increase was not statistically significant ($P = 0.057$). In contrast, artificially reared piglets showed lower densities at day 8 (28.5 ± 3.5%) compared to day 3 (38.2 ± 6.2%) ($P = 0.050$), a difference that was not surmounted by the age of 19 days. Given that the density at day 8 is significantly lower when compared to the end of the colostrum phase, transferring piglets to a brooder leads to a change in the distribution of the M cells. Indeed, artificially reared piglets had lower ($P = 0.040$) M cell densities (28.5 ± 3.5% at day 8 and 29.7 ± 7.5% at day 19) than conventionally reared piglets (34.8 ± 4.4% at day 8 and 33.7 ± 4.9% at day 19), irrespective of their age.

4. Discussion

The present study determined the volume densities of M cells in the tonsil of the soft palate and the iPP in piglets that were either conventionally or artificially reared. It was hypothesized that artificial rearing affects the presence of M cells. These epithelial cells, which are specialized in antigen sampling, are characterized by a low number of short, irregular microvilli covered by a very thin glycocalix (Corr et al., 2008). Although M cells play a pivotal role in the induction of adaptive immune responses, their occurrence had not been examined before in the young piglet. After birth, passive immunity provided by the translocated maternal colostral and lactogenic antibodies wanes and is gradually replaced by active immunity during the first weeks of life (Butler and Sinkora, 2007). Particularly in artificially reared piglets, the steady development of the adaptive immune system is of primordial importance as these piglets lack lactogenic antibodies that neutralize potentially harmful antigens that have entered the digestive tract (Salmon et al., 2009).

Gebert and co-workers (1994) concluded that CK18 is a specific marker for porcine intestinal M cells because cells which possessed large amounts of this intermediate filament transcytosed yeast particles. Antigen uptake, the M cell's major function, is correlated to the presence of this filament. In addition, this intermediate filament has more recently been detected in a subset of epithelial cells that line the crypts in the porcine tonsil of the soft palate (Harada et al., 2013). Immunohistochemical stainings against CK18 were therefore performed to visualize the M cells in the tonsils of the soft palate and iPPs of differently reared piglets, aged between 0 and 19 days. However, some crypt epithelial cells and goblet cells also contain CK18. Despite the typical shape of goblet cells that contrasts with the cylindrical cytoskeleton of intestinal M cells, the sections were counterstained by means of the PAS reaction to unequivocally identify the CK18 and PAS-positive goblet cells. Enteroendocrine cells also stain positive for CK18 (Höfer and Drenckhahn, 1996). However, they occupy only 1% of the total epithelial cell population of the gastrointestinal tract

(Harrison et al., 2013) and are absent or rarely observed in the FAE (Gebert and Cetin, 1998). Goblet cells were not observed in the crypt epithelium of the tonsil of the soft palate. In contrast, Belz and Heath (1996) identified some goblet cells in the lymphoepithelium that lined the branched crypts of the tonsils of the soft palate of pigs aged between 6 and 10 weeks. The lack of lymphocyte infiltration of the crypt epithelium and the absence of goblet cells might be explained by the young age of the investigated piglets.

In the present study, not numbers of M cells but volume densities were determined. The volume density is a measure for the volume of a region / cell type of interest in relation to the reference volume (Thompson, 1930; Howard and Reed, 1998). As a result, the volume density reflects the proportion of M cells in the epithelial layer of the tonsillar crypts and the FAE of the iPP. The measurements of the volume density of the M cells revealed that 15 to 20% of the tonsillar crypt epithelium and 30 to 40% of the FAE consists of M cells. Our result regarding the iPP is in agreement with the previous observation by Gebert and coworkers (1994) that approximately 40% of the FAE in the pig consist of M cells. Because the volume density of M cells and the capacity to translocate luminal antigens correlate, determining volume densities of M cells provides indirect information on the possibilities to mount adaptive immune responses against ingested pathogens.

To gain insight into the potential influence of artificial rearing on the development of the GALT, volume densities of M cells were determined in piglets of 0, 3, 8 and 19 days of age, which were either conventionally or artificially reared. Comparing the volume densities of M cells that were recorded at the day of birth and at the age of 3 days allowed to assess the normal postnatal development during the colostrum phase. Not only the ingestion of sufficient amounts of colostrum by the neonatal piglets, which are born agammaglobulinemic (Aumaitre and Seve, 1978; Butler and Brown, 1994), but also the level of subsequent milk uptake plays a key role in the growth and vitality of the young animals (Kirkden et al., 2013). After three days of suckling the sow, the piglets either continued to suckle the sow or were raised on milk formula. The last time point that was included was day 19, which represented the average weaning age at the selected farm that applied a four-weekly batch farrowing system. The inclusion of day 8 enabled to intermediately evaluate the influence of artificial rearing. The absence of any age effect in the first days after birth on the volume densities of M cells in the tonsillar crypt epithelium indicates that colostrum intake has no influence on the proliferation of M cells. It is suggested that colostrum is of essence for the proper development of the immune systems in piglets (Aumaitre and Seve, 1978; Rooke and Bland, 2002). Consequently, a colostrum-driven increase of M cells could be expected, but was not observed. However, relative proportions were determined and not absolute numbers, meaning that M cell numbers are higher in larger tonsils. Additionally, the type of ingested milk or rearing environment did not seem to influence M cell abundance in all analyzed tonsils of the soft palate.

As in the tonsils, no effect of age was observed in the iPPs of conventionally reared piglets. However, normal M cell proliferation was affected in artificially reared piglets since they presented lower volume densities of M cells compared to their conventionally reared littermates. It might be that these artificially reared piglets are less exposed to pathogens due to a cleaner environment, since pathogenic bacteria most probably contribute to the

generation and maintenance of M cells (Savidge et al., 1991; Borghesi et al., 1999). It could also be hypothesized that sow milk, directly or indirectly, stimulates the proliferation of M cells, because it has been demonstrated that such milk contains immunostimulating substances that are low in artificial milk (Li et al., 2012). Studying the effect of e.g. transferring maternal fecal material into the brooders or supplying the piglets with sow milk instead of formula could further support this hypothesis. However, formulated milk does not seem to influence lymphocyte counts in the gut wall in piglets (Prims et al., 2016). In addition, only lower numbers of natural killer cells have been observed in peripheral blood of infants after formula feeding (Tarcan et al., 2004). This observation is important since maintenance of M cells in PPs relies on close interaction of the cell with B cells (Mach et al., 2005; Miyazawa et al., 2006). Although the ontogeny of M cells remains unclear, it is hypothesized that M cells develop differently depending on their anatomical localization (Mach et al., 2005). This might also explain why artificial rearing only affects volume densities in the iPPs and not in the tonsils.

Even though artificially reared piglets presented lower values in the iPP, they performed well. According to the manufacturer of the commercial brooders that were used, artificial rearing of piglets reduces the average mortality rate of 13% with 3 to 5%, increases the uniformity of litters and may increase the average body weight at weaning with up to 500 g (Provimi, 2014; Vlaamse Overheid, 2014). Additionally, breeders who use the commercial brooders report that artificially reared piglets do not display a post-weaning weight loss. Furthermore, this rearing strategy limits the use of foster sows leading to a rise of the cycle-index with 0.05% on average (Provimi, 2014). The piglets in our study also appeared to be heavier than their conventionally reared counterparts, which is in line with previous findings (De Vos et al., 2014a). This observation might have an effect on the observed reference volume, i.e. the epithelium of the iPPs. Larger and heavier animals may present larger iPPs and consequently possess an equal, more diffuse, amount of M cells, since not absolute numbers but ratios are determined. However, an additional study showed that there is no correlation between the volume of iPPs and body weight. Moreover, an equal volume of iPPs was observed in both artificially and conventionally reared piglets (Jurgens et al., 2016). This observation suggests that the difference in M cell volume densities between both rearing strategies is not related to an altered reference volume, but to a lower number of M cells in artificially reared piglets.

The presented results indicate that artificial rearing lowers the volume densities of M cells at the level of the small intestine, but not in the tonsil of the soft palate. The knowledge obtained from the present study could be valuable in the development and application of oral vaccines. Currently, an oral vaccine to be administered to piglets only exists against porcine proliferative enteropathy caused by *Lawsonia intracellularis*. However, it has already been demonstrated that oral vaccines against *Haemophilus parasuis* causing Glässer's disease and Shiga toxin-producing *Escherichia coli* could be effective when dosed to piglets aged 3 to 7 days (IPVS, 2014). The interference of the maternal immunity, which is defined as the inhibition of the endogenous immunoglobulin synthesis by maternal factors present in sow's milk (Klobasa et al., 1981), can be by-passed by adequate adjuvant addition (Salerno-Goncalves and Sztein, 2006). Fortunately, maternal interference is limited in artificially

reared piglets. However, the possible effect of the lower volume densities in the iPP has to be further investigated.

5. Conclusion

From this morphological study it is concluded that antigen sampling in the piglet could be possible from birth onwards and might be more advanced in the small intestine. However, artificially reared piglets possibly present a decreased capacity to induce adaptive immune responses after oral immunization, since they possess lower volume densities of M cells in the epithelium of the iPP. Functional studies should, however, be conducted to affirm this hypothesis. Given the fact that artificially reared piglets may profit from restricted maternal interference, vaccinating these piglets within the first weeks after birth could nevertheless be successful.

Conflict of interest statement

The authors have no conflict of interest to declare.

Acknowledgements

The authors are indebted to Katty Huybrechts and Gunther Vrolix for their technical assistance. This study was supported by the special research fund of the University of Antwerp (grant number BOF/DOCPRO4 28364). The authors are members of COST Action BM1308 ‘Sharing Advances on Large Animal Models (SALAAM)’.

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Tables.

Table 1. Composition and nutritional value of sow milk and formulated milk

	Sow milk[1]	**Milk formula[2]**
Composition		
Vitamin A (IU/kg)	3067	55000
Vitamin D3 (IU/kg)	360	5500
Vitamin E (IU/kg)	3.80	300
Vitamin C (IU/kg)	906	110
Ca (%)	0.18	0.89
P (%)	0.14	0.73
Lysine (%)	7.00	1.70
Methionine + Cysteine (%)	3.10	0.80
Tryptophan (%)	1.60	0.30
Threonine (%)	4.10	1.10
Nutritional value		
Protein (g / L)	55	28
Lipid (g / L)	76	23
Lactose (g / L)	53	56
Gross Energy (kcal / L)	1290	590

[1]According to Xu (2003); [2]as provided by the manufacturer (Piggylac®, Vitamex, Drongen, Belgium)

Table 2. Average body weights ± SD (kg) of piglets that were sow- or formula-fed, measured at 4 different time points

	Day 0	Day 3	Day 8	Day 19
Conventional	1.31 ± 0.09 (n = 36)	1.73 ± 0.15 (n = 30)	2.88 ± 0.58 (n = 12)	4.69 ± 0.92* (n = 6)
Artificial			2.73 ± 0.34 (n = 12)	6.40 ± 0.44* (n = 6)

*P = 0.002

Figure captions.

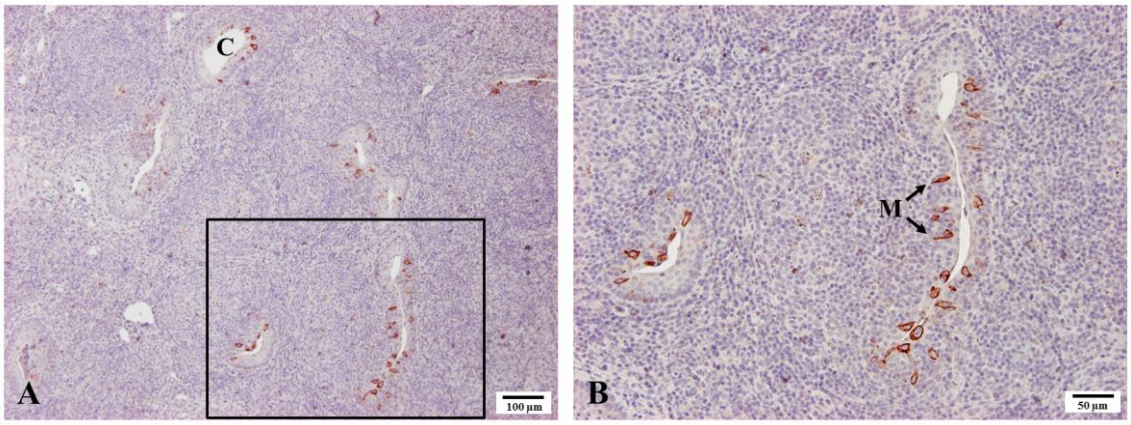

Figure 1. Cytokeratin (CK) 18 immunoreactivity in the tonsil of the soft palate of a 19-day-old artificially reared piglet. **A.** Several crypts (C) that contain CK18-stained (brown) M cells are embedded in the tonsillar lymphoid tissue. **B.** Higher magnification of a crypt (boxed area in A) that is lined by non-keratinized stratified squamous epithelium. CK18-stained M cells (M) that have an oval to slender shape lie scattered in the epithelium.

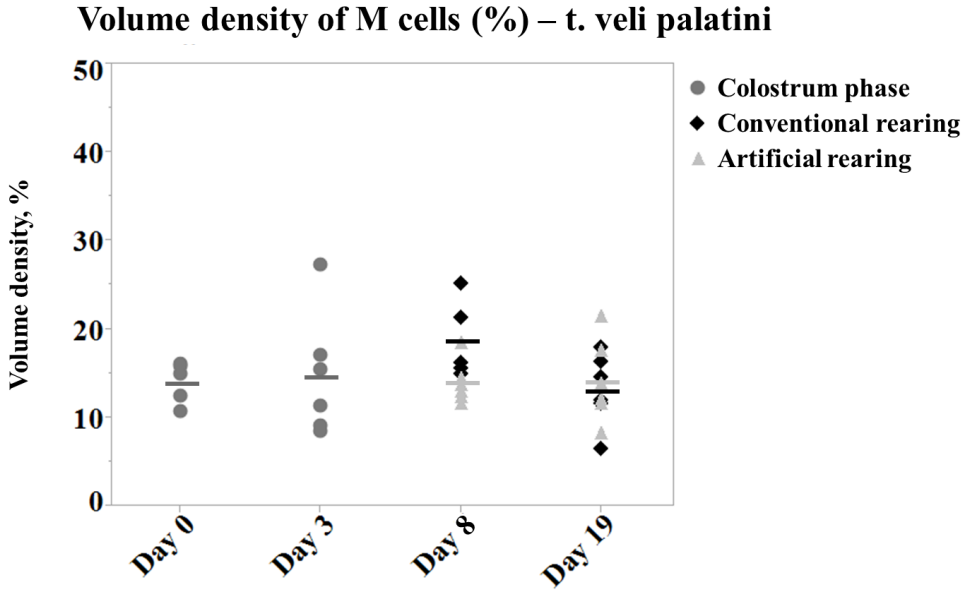

Figure 2. Dot plot presenting age- and rearing-related individual volume densities (%) of M cells in the crypt epithelium of the tonsil of the soft palate (t. veli palatini) (n = 6/group). The means of the different groups are indicated by horizontal bars. No statistically significant differences were observed.

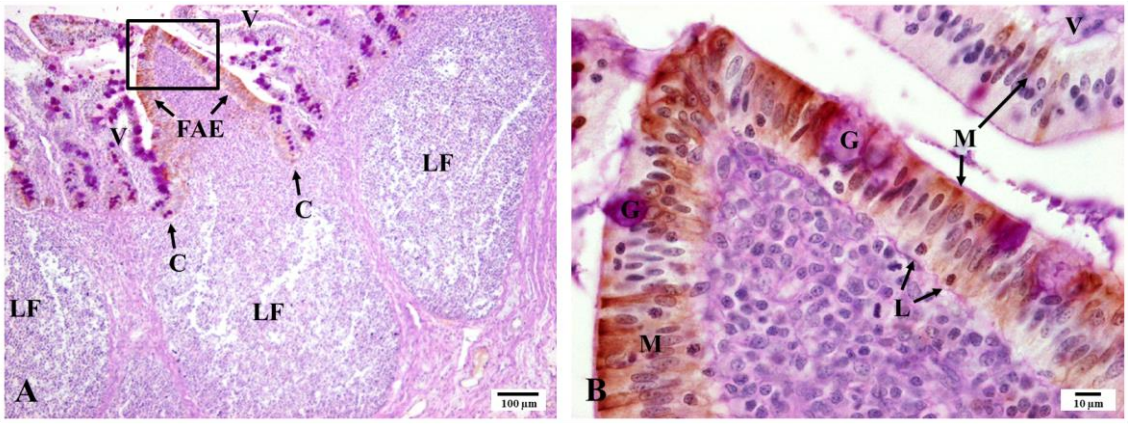

Figure 3. Cytokeratin (CK) 18 immunoreactivity in the ileal Peyer's patch of a 19-day-old artificially reared piglet. **A.** The follicle-associated epithelium (FAE), lining the villi into which a lymphoid follicle (LF) protrudes, contains numerous CK18-stained (brown) M cells, enterocytes and only a few goblet cells. The latter are more abundant in the villi (V) that are not associated with a lymphoid follicle and can be recognized by their Periodic Acid Schiff (PAS)-positive (magenta), mucus-containing goblet. The crypts (C) in between neighboring villi can contain enteroendocrine cells that are CK18-positive. **B.** Higher magnification of the FAE (boxed area in A) showing slender, CK18-stained M cells (M) that lie parallel to each other. Lymphocytes (L) are present in the intraepithelial spaces at the base of the M cells. CK18- and PAS-positive goblet cells (G) are also present. Only a few CK18-stained M cells are present in the epithelium of the neighboring villus (V).

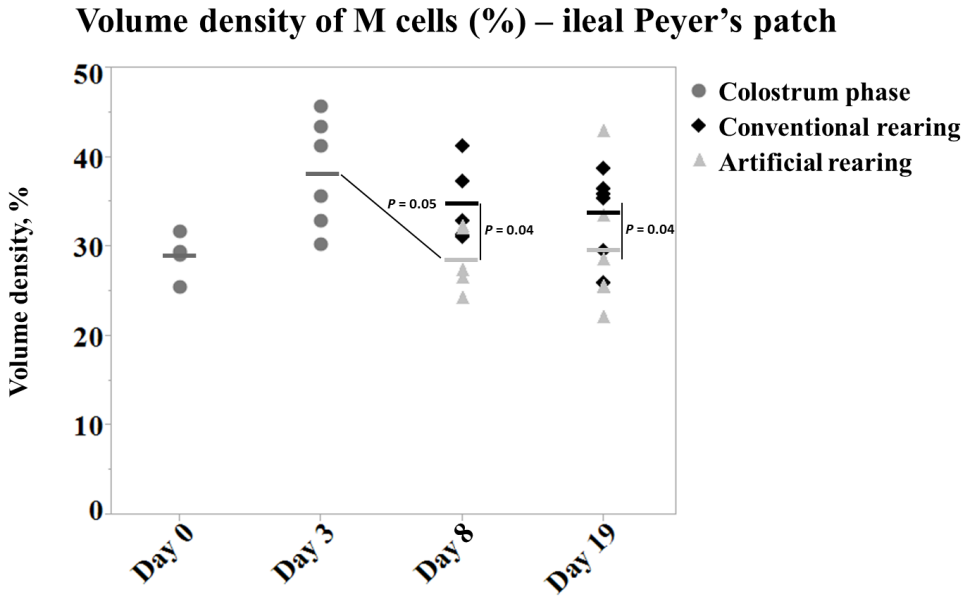

Figure 4. Dot plot presenting age- and rearing-related individual volume densities (%) of M cells in the follicle-associated epithelium of the ileal Peyer's patches (n =

6/group). The means of the different groups are indicated by horizontal bars. Statistically significant differences between experimental groups are indicated by the bars associated with a *P*-value.