# Intestinal immune cell quantification and gram type classification of the adherent microbiota in conventionally and artificially reared, normal and low birth weight piglets

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## Abstract

To be able to raise larger litters, the influences of alternative rearing strategies on piglets are currently assessed. This study compared the predominant Gram type of the adherent intestinal microbiota between conventionally and artificially reared piglets and quantified their major mucosal immune cells populations. In addition, the potential influence of the piglets' birth weights was examined. To this purpose, 40 neonatal piglets consisting of 20 normal birth weight (NBW) and 20 low birth weight (LBW) piglets suckled the sow for three days after which 20 piglets (10 NBW and 10 LBW) continued to suckle the sow and 20 piglets (10 NBW and 10 LBW) were transferred to brooders and raised on milk formula. At the age of 10 or 28 days, five piglets of each group (birth weight x rearing strategy) were euthanized and the jejunum ileum was sampled. The presence of adherent $Gram^+$ and $Gram^-$ bacteria was scored and the volume densities of $CD8^+$ cells, $CD4^+$ cells and $CD172a^+$ myloid cells were determined. Irrespective of birth weight, sow-fed piglets possessed a predominant $Gram^+$ microbiome at 10 days of age, whereas formula-fed animals had more $Gram^-$ microbiota. With increasing age, however, $Gram^+$ microbiota took the upper hand in these animals. The volume densities of $CD8^+$ and $CD4^+$ cells rose with increasing age and were consistently lower in LBW piglets. Both rearing strategies had a similar influence on the volume densities of these cell populations. In contrast, the volume densities of $CD172a^+$ myloid cells did not differ significantly between the birth weight, rearing strategy and age groups. Three observations allow to conclude that artificial rearing could be a valuable alternative for suckling the sow. First, within each birth weight category, the mean weight of the 28-day-old piglets was similar for both rearing groups. Secondly, the effect of milk formula on the composition of the intestinal

48 microbiome was only temporary since formula-fed piglets restored the more beneficial
49 Gram$^{+}$ microbiome by the end of the artificial rearing period. Finally, artificial rearing
50 did not influence, either positively or negatively, the volume densities of CD8$^{+}$, CD4$^{+}$
51 and CD172a$^{+}$ immune cells when compared to conventional rearing.
52
53 *Keywords: piglet; artificial rearing; birth weight; intestinal microbiota; intestinal*
54 *immune cells*

## 1. Introduction

Genetic selection for larger litters has substantially increased the number of live born piglets per litter (Su et al., 2007). However, larger litters are characterized by a decreased average birth weight (Bérard et al., 2010). Moreover, low birth weight (LBW) piglets are overrepresented in larger litters (Quesnel et al., 2008). These piglets in particular suffer from increased mortality and impaired growth, resulting in a higher slaughter age and poorer meat quality (Bérard et al., 2008; Kilbride et al., 2012). As a consequence, the genetic capacity of these more prolific sows is not fully exploited.

In order to improve the profitability of piglet production, pig farmers show interest in alternative rearing strategies. 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, consisting of weaning the piglets after they had been suckling colostrum, and subsequent feeding with milk formula (De Vos et al., 2014a; Levast et al., 2010).

Since conventional weaning induces significant changes in gastrointestinal physiology (Lallès et al., 2007), it is expected that any change in rearing strategy also affects digestive functioning. The $Gram^+$ gut microbiota that, amongst others, provide the suckling piglet with a colonization resistance against pathogenic bacteria could thus be disturbed at weaning (Pluske et al., 2002). This results in a higher susceptibility to gastrointestinal infection and diarrhoea (Konstantinov et al., 2006). At the moment however, the effect of artificial rearing on the adherent intestinal microbiota has only been studied fragmentary (D'Inca et al., 2010; Van Haver et al., 2009).

In addition, weaning also profoundly influences the maturation of the intestinal immune system, which is shaped by a dynamic interplay between diet and the local

microbiota (Juul-Madsen et al., 2010). First at birth and again at weaning the piglet's gut is exposed to foreign antigens and is colonized by specific bacterial populations, which play a major role in the trafficking of lymphoid cells to the intestinal mucosa (Pabst and Rothkötter, 1999; Rothkötter et al., 1991). The potential influence of artificial rearing on intestinal lymphocyte numbers is, however, ambiguous. Indeed, some authors have demonstrated an increase in the number of intestinal lymphocytes (Orgeur et al., 2001; Vega-López et al., 1995) after early weaning, whereas others observed smaller Peyer's patches (Helm et al., 2007). In contrast, there is consensus in literature that a low birth weight impairs the development of the immune system (D'Inca et al., 2011; Tuchscherer et al., 2000; Wang et al., 2008; Zhong et al., 2012). However, the potential influence of birth weight on the gut-associated lymphoid tissue (GALT) has not been determined. Furthermore, to our knowledge, this is the first study that focused on the presence of $CD172a^{+}$ myloid cells in the GALT of differently reared piglets of various birth weight categories, i.e. normal birth weight (NBW) piglets vs. LBW piglets.

The ~~major~~ aim of this study was to compare conventional rearing with artificial rearing on milk formula with regards to the adherent gastrointestinal microbiota and presence of the major mucosal immune cell populations in the jejunum ~~ileum~~. Both parameters where determined in the same intestinal region to shed light on the interplay between the microbiota and the local immune cells. Given the fact that formula is low in specific factors to adequately stimulate the immune system and gut microbiota (Helm et al., 2007; Li et al., 2012), it is hypothesized that the development of the different components of the GALT is impaired in artificially reared piglets. Therefore, a quantification of key-cells in the jejunal effector site of the GALT, i.e. the epithelium and lamina propria mucosae, and a Gram type classification of the intestinal adherent

103 microbiota were performed. To examine whether piglets of distinct birth weight
104 categories respond differently to artificial rearing, both NBW and LBW piglets were
105 included.
106
107 **2. Materials and methods**
108 *2.1. Animals*
109 Forty neonatal piglets ((Finnish Yorkshire × Belgian Landrace) × Piétrain), born
110 on a local farm, were designated as NBW or LBW piglets when their birth weights
111 ranged within 0.5 standard deviation (SD) or were below 1.5 SD of the mean litter birth
112 weight, respectively (Paredes et al., 2012; Willemen et al., 2014).
113 After three days of suckling the sow, 10 gender- and sow-matched pairs of NBW
114 and LBW piglets were transferred to commercial brooders (Rescue Decks®, S&R
115 Resources LLC, Mason, USA) where they were artificially reared on milk formula,
116 which was *ad libitum* provided until the age of 10 days (n = 10, viz. 5 NBW and 5 LBW
117 piglets) or 28 days (n = 10, viz. 5 NBW and 5 LBW piglets). The dietary control group,
118 consisting of 10 gender- and sow-matched pairs of NBW and LBW piglets, suckled the
119 sow on the farm until the age of 10 days (n = 10, viz. 5 NBW and 5 LBW piglets) or 28
120 days (n = 10, viz. 5 NBW and 5 LBW piglets). The composition and nutritional value of
121 sow milk and the milk formula is presented in Table 1.
122 Piglets in both systems had free access to water and were maintained under
123 standard environmental conditions (12h/12h light/dark cycle, temperature adjusted to
124 age). Their body weights were recorded both at birth and before they were sacrificed for
125 sampling. Animals were observed daily, with focus on general behaviour, body
126 condition and faecal composition, to document general health status. All experiments

were approved by the Ethical Committee for Animal Experiments of the University of Antwerp, Belgium (2014-01).

*2.2. Tissue samples*

Piglets from the different experimental groups were euthanized at the age of 10 or 28 days by exsanguination via transection of the jugular veins and carotid arteries after intraperitoneal injection of sodium pentobarbital (200 mg/kg, Kela, Hoogstraten, Belgium). The intestinal tract was dissected and samples were taken at the distal part of the jejunum. Samples intended for (immuno)histochemistry on paraffin sections (analysis of adherent microbiota) were fixated for 2h in 4% phosphate-buffered paraformaldehyde (pH 7.4) at room temperature. They were routinely processed to paraffin-embedded tissue blocks after rinsing in phosphate-buffered saline solution (PBS; 0.01 M, pH 7.4). Those samples intended for cryosection-immunohistochemistry (analysis of mucosal immune cells) were embedded in KP-Cryocompound® (Klinipath, Olen, Belgium) prior to snap-freezing in liquid nitrogen. All frozen samples were stored at -80°C until further processing.

*2.3. Adherent microbiota*

To detect adherent $Gram^+$ bacteria in the intestinal samples, 4 µm thick paraffin sections were Gram-stained according to the modified Brown and Brenn stain described by Taylor (1966). $Gram^-$ bacteria were analysed by means of immunohistochemical staining for lipopolysaccharides (LPS) according to the protocol of Van Haver and co-workers (2009). In brief, tissue sections were treated with Target Retrieval Solution (Dako, Glostrup, Denmark) combined with microwave heating (10 minutes 90 Watt +

20 minutes cool down) prior to incubation with a goat polyclonal anti-LPS antibody (1:600, clone O157, Acris, Herford, Germany), a biotin-conjugated polyclonal rabbit anti-goat antibody (1:200, Dako) and streptavidin-horseradish peroxidase (HRP, 1:200, Dako), consecutively. Diaminobenzidine (DAB, Dako) was used as chromogen to detect positive cells. Sections were counterstained with hematoxylin. Each stain obtained a positive score when at least one colony (≥ 2 bacteria) was found at the lower (66% of villous height) intervillous part of a whole circumferential gut transection (Van Haver et al., 2009).

*2.4. Mucosal immune cells*

To detect intestinal mucosal immune cells, frozen tissue was sectioned into 10 µm thick slices that were air-dried overnight and subsequently fixated at 4°C in 100% acetone and air-dried. $CD8^+$ cells, $CD4^+$ cells and $CD172a^+$ myloid cells were immunostained with mouse monoclonal antibodies for porcine CD8a (1:10, clone MIL12), CD4a (1:10, clone MIL17) and CD172a (1:200, clone BL1H7) (Serotec, Kidlington, UK), respectively. Subsequently, sections were incubated with a biotin-conjugated polyclonal goat anti-mouse IgG (1:200, Dako) and streptavidin-HRP (1:200). Immune cells were visualized using DAB (Dako). Sections were counterstained with malachite green (0.5% in 0.1 M sodium acetate at pH 4.2).

The sections were analysed 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 $CD8^+$ and $CD4^+$ cells, and $CD172a^+$ myloid cells were determined by means of a point counting method using the Computer-Assisted Stereological Toolbox 2

(CAST2) imaging software (Olympus). The volume density, which is defined as the ratio of the number of points hitting the immune cells (i.e. $CD8^+$, $CD4^+$ or $CD172a^+$ cells) to the number of points hitting the reference volume (i.e. the total lamina propria mucosae and epithelial layer), was for each individual assessed in that number of fields that resulted in a coefficient of error smaller than 5% (Gundersen and Jensen, 1987). One counting field was composed of a sequence of images, reconstructing at least one entire villus. Results were noted as mean volume density ± SD.

*2.5. Statistical analysis*

To detect potential differences in body weight gains and volume densities of the studied immune cell populations between the rearing strategy, age and birth weight groups, an ANCOVA was used. Prior to analysis, a Levene's test was used to verify the equality of variances of the results (homogeneity of variance) ($p > 0.05$). When significant interaction terms were detected, data tables were split and further analysed using the Mann-Whitney U (mean rank) test. For none of the investigated parameters, except body weight, a significant interaction term between age, birth weight category and rearing strategy was detected. A *p*-value less than 0.05 was considered statistically significant. Statistical analyses were performed with IBM SPSS statistics (version 22; IBM Business Analytics, Armonk, NY, USA).

## 3. Results

*3.1. Zootechnical performance of the piglets*

Since no changes in general behaviour, body condition and faecal composition were observed, it was concluded that all piglets remained healthy during the experiment.

New-born NBW and LBW piglets weighed 1.46 ± 0.10 kg and 0.87 ± 0.09 kg, respectively (Table 2). Analysis showed that the interaction term between age and rearing group was statistically significant ($p = 0.0002$), meaning that the difference in body weight between both rearing groups was not identical at both time points. Splitting the data into two age groups showed that at the age of 10 days the sow-fed piglets weighed on average 0.82 kg more than the piglets that were raised on milk replacer ($p = 0.043$). At the age of 28 days, the statistically significant difference in body weight between both rearing groups had disappeared. The age-related increase in body weight was statistically significant for both rearing groups ($p < 0.0001$), regardless of birth weight category. At all time points, the difference in body weight between birth weight categories stayed statistically significant ($p < 0.0001$).

*3.2. Adherent microbiota*

The analyses of the Gram stains (Fig. 1) showed that 4/20 and 3/20 10-day-old piglets had only $Gram^-$ and $Gram^+$ colonies, respectively. In addition, 7/20 piglets were devoid of adherent microbiota and 6/20 presented mixed populations (Fig. 2). In contrast, at 28 days, 2/20 and 8/20 piglets had only $Gram^-$ and $Gram^+$ bacteria, respectively. Only one piglet was devoid of adherent microbiota. The remaining animals presented mixed populations. Within the 10-day-old group, four artificially reared piglets vs. none of the sow-fed piglets possessed only $Gram^-$ bacteria. This difference between both rearing groups at day 10 disappeared by day 28, when in both groups a predominant $Gram^+$ adherent microbiome had been established. No birth weight effect was observed.

223 *3.3. Mucosal immune cells*

224 $CD8^+$ cells were mainly, but not exclusively, found in the epithelium. In contrast,
225 the $CD4^+$ cells and the $CD172a^+$ myloid cells resided more in the lamina propria
226 mucosae (Fig. 3).

227 The volume densities of the $CD8^+$ and $CD4^+$ immune cell populations increased
228 with the age of the piglets ($p < 0.0001$ and $p = 0.019$, respectively; Fig. 4),
229 irrespectively of birth weight and rearing method. This effect of age was absent for the
230 volume densities of the $CD172a^+$ myloid cell population ($p = 0.110$). The volume
231 density of $CD8^+$ cells ($p = 0.584$), $CD4^+$ cells ($p = 0.107$) and $CD172a^+$ myloid cells ($p$
232 $= 0.722$) did not differ significantly between rearing methods.

233 When assessing volume densities in relation to birth weight, those of the $CD8^+$
234 and $CD4^+$ cells were significantly lower ($p = 0.043$ and $p = 0.049$, respectively) in LBW
235 piglets compared to the NBW piglets, regardless of age or rearing type. In contrast, birth
236 weight did not influence the density of $CD172a^+$ myloid cells ($p = 0.195$).

237

238 **4. Discussion**

239 This study analysed the influence of birth weight and rearing method on the
240 Gram type of the adherent microbiota and presence of mucosal immune cells in the
241 jejunum of 10- and 28-day-old piglets. The conventionally reared, sow-fed group served
242 as the control group. In contrast, the artificially reared group had been exposed to
243 several interventions, such as a diet change, transport to another facility and regrouping
244 of piglets. The influence of these interventions may be reflected in the lower body
245 weights that were observed at day 10 in the artificially reared group. In the long term
246 however, the average body weight of the formula-fed group reached the level of the

247 sow-fed group, meaning that the piglets adapted to the artificial rearing method. The
248 cause of this catch-up in body weight in formula-fed piglets most probably is the *ad*
249 *libitum* availability of milk formula. Previously, our research group showed that the
250 relative energy intake of formula-fed piglets is higher compared to sow-fed animals (De
251 Vos et al., 2014b).

252 Irrespective of birth weight, all sow-fed animals possessed an adherent
253 microbiome that was predominantly composed of $Gram^+$ bacterial populations. This is
254 in agreement with literature data (Pluske et al., 2002). In contrast, one week after the
255 piglets had been transferred to the brooders, the jejunum of most formula-fed animals
256 displayed a bacterial colonization dominated by $Gram^-$ strains. However, more $Gram^+$
257 bacteria colonized the jejunum over time since almost all animals tested positive for
258 $Gram^+$ bacteria at day 28. The preponderance of the $Gram^-$ microbiota in the 10-day-old
259 formula-fed piglets could be explained by the low amount of specific factors that
260 promote the growth of $Gram^+$ bacteria in artificial milk (Helm et al., 2007; Li et al.,
261 2012). This is in agreement with the larger number of $Gram^+$ microbiota that are present
262 in the small intestine of breast-fed children, whereas bottle-fed infants have more $Gram^-$
263 colonies (Harmsen et al., 2000; Penders et al., 2005). Nevertheless, by the age of four
264 weeks the formula-fed piglets had restored the more beneficial $Gram^+$ microbiota that is
265 present in sow-suckling piglets.

266 To gain insight into the development of the piglet's intestinal immunity, the
267 volume densities of $CD8^+$ cells, $CD4^+$ cells and $CD172a^+$ myloid cells were determined
268 in the jejunal mucosa. The CD172 molecule plays a pivotal role in the activation of the
269 innate immunity in response to LPS (Borghetti et al., 2006). It is abundantly expressed
270 on the cell membrane of monocytes and macrophages, but is also present on neutrophils

and conventional and plasmacytoid dendritic cells (Piriou-Guzylack and Salmon, 2008). Since no statistically significant differences in the volume densities of $CD172a^+$ myloid cells were observed between the different experimental groups, the number of intestinal cells with phagocytic capacity seems not to be influenced by rearing method or birth weight. In addition, the population of the $CD172a^+$ cells remained constant in the neonatal period. However, our study did not attempt to identify the various subsets of $CD172a^+$ myloid cells, which means that differences in the composition of this cell population could be present between the groups.

The volume densities of the $CD8^+$ and $CD4^+$ cells varied between the experimental groups. Since the number of intestinal mucosal CD4/CD8 double positive lymphocytes increases with age to reach levels up to 4% in adult pigs, the proportion of these cells in the studied populations of young piglets is negligible (Piriou-Guzylack and Salmon, 2008; Zuckermann and Gaskins, 1996). Therefore, we examined both populations separately. From day 10 to day 28, the number of mucosal $CD8^+$ lymphocytes almost doubled. In contrast, the $CD4^+$ cells showed a less dramatic, albeit statistically significant age-related increase. This observation is in line with previous reports (Rothkötter et al., 1991), although Bailey and coworkers (2001) mention that $CD8^+$ cells only start to infiltrate the villi at the age of four weeks. Thus, $CD8^+$ and $CD4^+$ cells are attracted from the blood stream to the intestinal mucosa with increasing age. Furthermore, the volume densities of the $CD8^+$ and $CD4^+$ cells remained lower in the LBW piglets. This observation is supported by the fact that a low birth weight leads to lower numbers of T cells in the thymus and consequently in the peripheral blood (Contreras et al., 2001; Schuit et al., 1982). As a result, the effector arm of the intestinal mucosal immune system seems less pronounced in LBW piglets. However, since the

$CD8^+$ and $CD4^+$ cell densities did not differ between rearing strategies, artificial rearing seems not to disturb the intestinal expansion of these immune cell populations.

## 5. Conclusion

Reallocating piglets at the age of three days to commercial brooders and subsequent feeding on milk formula resulted in the predominance of a $Gram^-$ microbiome. This contrasted with the more $Gram^+$ microbiome that was present in piglets that continued to suckle the sow. Nevertheless, since formula-fed piglets obtained the more beneficial $Gram^+$ microbiota at the end of the rearing period, the observed effect of milk formula on the composition of the intestinal microbiome was only temporary.

The predominant Gram type of the microbiome seemed not to influence the volume densities of $CD8^+$, $CD4^+$ and $CD172a^+$ cells. Indeed, the volume densities of $CD8^+$ and $CD4^+$ cells increased with age in the neonatal piglet. In contrast, the volume density of $CD172a^+$ cells remained constant. In addition, this cell population did not present the differences in volume densities between LBW and NBW piglets, which was observed for the $CD8^+$ and $CD4^+$ cell populations. LBW piglets are thus characterized by a slower attraction or local proliferation of these immune cells. Furthermore, it was found that formula feeding did not influence the population dynamics of the three examined intestinal mucosal immune cells. These observations together with the fact that the mean weight of the 28-day-old piglets was similar for both rearing groups imply that artificial rearing on milk formula could be a valuable alternative for suckling the sow.

## Conflict of interest statement

The authors have no conflict of interest to declare.


## Acknowledgments

The authors are indebted to Olga Zdanchuk, Katty Huybrechts and Gunther Vrolix for their practical assistance. This study was supported by the special research fund of the University of Antwerp (grant number BOF/KP2013 28062). The authors are members of COST Action BM1308 ‘Sharing Advances on Large Animal Models (SALAAM)'


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## Figures

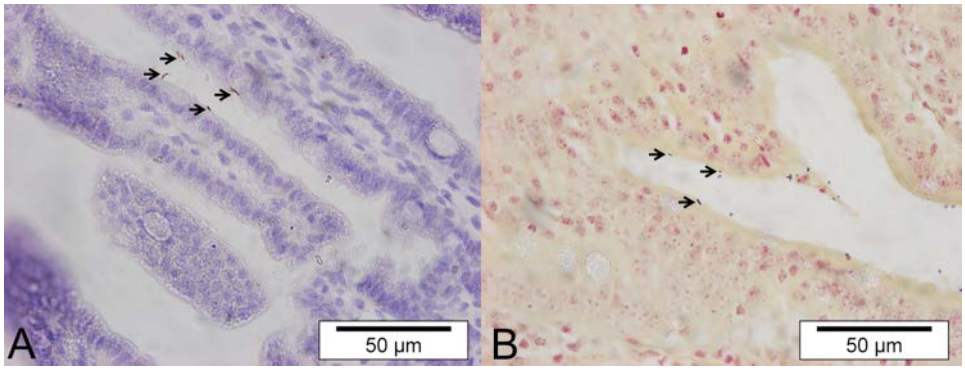



**Fig. 1.** Histological sections of the jejunum of a 28-day-old formula-fed NBW piglet showing the presence of the adherent intestinal microbiota. A: Immunohistochemical stain for LPS (DAB, brown) showing Gram$^-$ bacteria (arrows), combined with a hematoxylin nuclear stain. B: Brown and Brenn stain showing purple-stained Gram$^+$ bacteria (arrows).

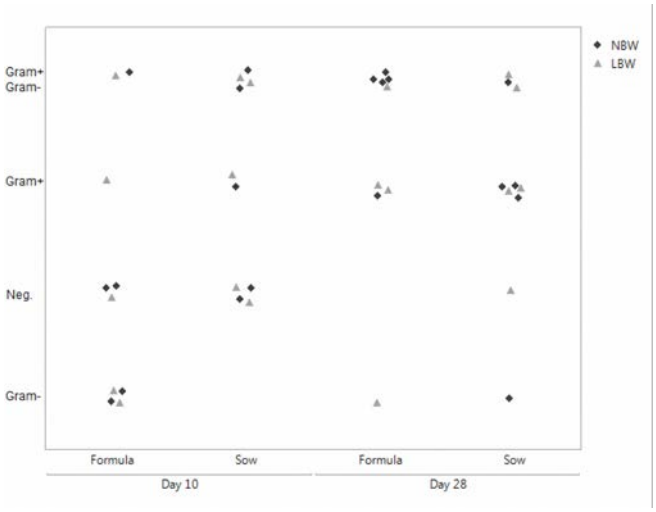
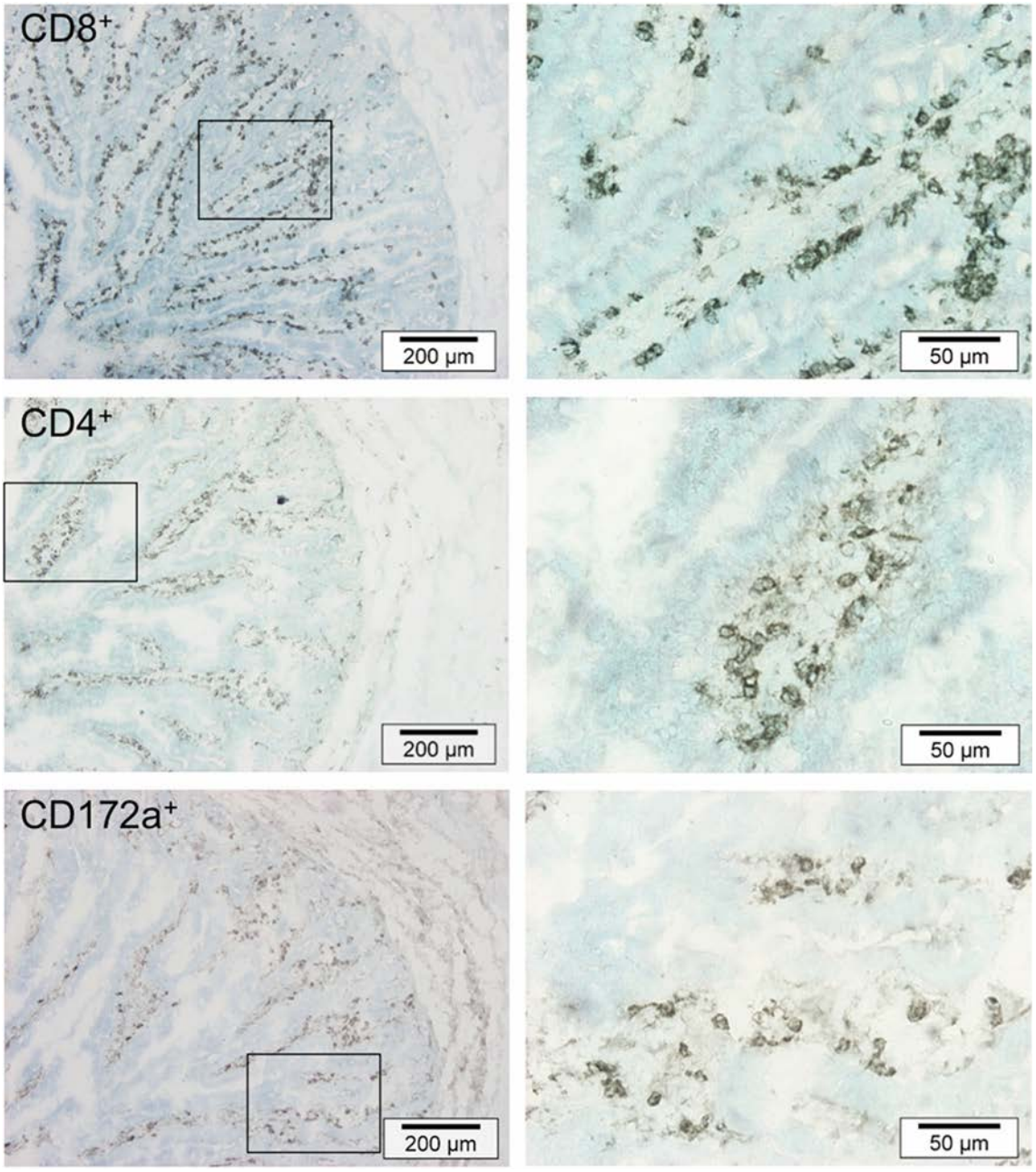


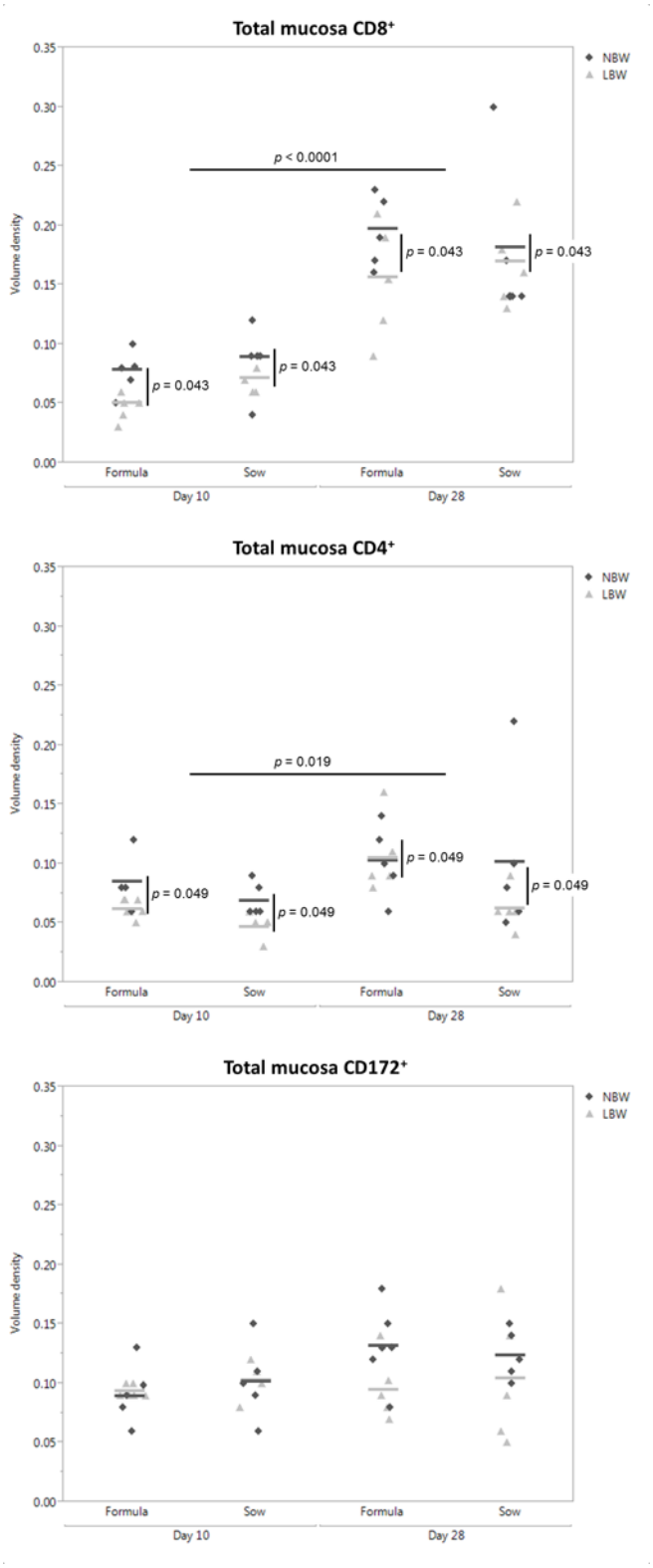

**Fig. 2.** Dot plot showing the number of piglets per birth weight, age and rearing group that tested positive for the presence of adherent Gram$^+$ and/or Gram$^-$ bacteria in the jejunum.




478

479 **Fig. 3.** Immunohistochemically stained cryosections (DAB, brown) of the jejunum of a
480 28-day-old formula-fed NBW piglet showing the presence of brown-stained $CD8^+$ cells
481 (upper panels), $CD4^+$ cells (middle panels) and $CD172a^+$ myloid cells (lower panels),
482 counterstained with Malachite green. Higher magnifications of the boxed areas in the
483 left panels are presented at the right.

484




485

486 **Fig. 4.** Dot plots showing the age-, feed-, and birth weight-related volume densities of
487 $CD8^+$ cells (upper panel), $CD4^+$ cells (middle panel) and $CD172a^+$ myloid cells (lower
488 panel) in the jejunal mucosa. The means of the different groups are indicated by the

489 horizontal bars. Statistically significant differences between experimental groups are
490 indicated by bars (n = 5).

491 **Tables**

492 **Table 1** Composition and nutritional value of sow milk and formulated milk

| | Sow milk[†] | Milk formula[‡] |
|---|---|---|
| **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 (%) | 7.0 | 1.70 |
| Methionine + Cysteine (%) | 3.1 | 0.80 |
| Tryptophan (%) | 1.6 | 0.30 |
| Threonine (%) | 4.1 | 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 |

493

494 [†]According to Xu (2003); [‡]as provided by the manufacturer

**Table 2** Average body weights ± SD (kg) of NBW and LBW piglets that were sow- or formula-fed, measured at different time points. The mean body weight of the piglets increased significantly over time ($p < 0.0001$; indicated by the different superscript letters a vs. c and b vs. d). However, at day 10, formula-fed piglets weighed less than those that suckled the sow ($p = 0.043$; indicated with the different superscript numbers 1 vs. 2). At each time point, LBW piglets presented significantly lower body weights than NBW piglets, irrespective of rearing strategy ($p < 0.0001$; indicated by the different superscript letters a vs. b and c vs. d).

| | | Day 10 | Day 28 |
|---|---|---|---|
| **Formula** | **LBW** | $1.77 \pm 0.37^{a1}$ | $6.17 \pm 1.31^{c}$ |
| | **NBW** | $2.76 \pm 0.43^{b1}$ | $9.16 \pm 1.27^{d}$ |
| **Sow** | **LBW** | $2.40 \pm 0.47^{a2}$ | $5.31 \pm 0.84^{c}$ |
| | **NBW** | $3.77 \pm 0.23^{b2}$ | $8.21 \pm 0.95^{d}$ |