INFLUENCE OF COLIFORM BACTERIA INFECTION ON INTESTINAL GOBLET CELLS SECRETORY ACTIVITY OF GERM-FREE PIGLETS

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Abstract

Recently, influence of bacteria colonization on development and maturation of gut wall is getting more into the focus of gastrointestinal research. For years, the main interest and research were aimed to the development and maturation of gut wall and its functional properties in normal conditions, less attention has been paid on the germ-free animals. Germ-free (GF) piglets have clear microbiological background and are reared in sterile environment. GF piglets are regarded as clinically relevant models for studying of human diseases, as these piglets' manifest similar clinical symptoms to humans. In this study we briefly summarised the main characteristics in the distribution of goblet cells in the wall of jejunum and colon of GF piglets as healthy control (HC) group and piglets, which were experimentally infected by *E. coli* O149:K88 as ECK group. Neutral mucins were stained with periodic acid-Shiff (PAS) whereas acidic mucins are stained with Alcian blue. Numbers of goblet cells containing total acidic mucins in both, the jejunum and colon, differed significantly between HC and ECK piglets and in the colon, a similar trend was also observed. In the ECK piglets, jejunal goblet cells exhibited decrease in neutral mucins. This change in mucin profile in response to bacterial colonization suggests a potential role as a protective mechanism against pathogenic invasion of the intestinal mucosa during of gut mucosa development in piglets.

Key words: germ free environment, piglet, Escherichia coli, intestine, goblet cells

INTRODUCTION

The pig is very similar to humans in terms of anatomy, genetics and physiology. Choosing the right breed and age allows various surgical and non-surgical procedures typically used in human medicine, including catheterization, heart surgery, valve manipulation, endoscopy and broncho-alveolar lavages (1, 2). These procedures are particularly difficult or impossible to perform in many animal models including rodents. In terms of genetics, the size and the composition of the porcine genome are comparable to those of humans. During the past half century, even though the germ-free (GF) pig has been increasingly recognized as a very valuable experimental animal model system in the inves-

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tigation of pathogens (1), the number of functional GF pig facilities remains low, mainly because of the high costs and the technical complexity in establishing and maintaining the facility (2). Despite this financial difficulty, the advantages of the piglet's models highlighted by these studies include physiological similarity to the human gut as well as to the pathological mechanisms of human diseases. Piglets, have also proven usefulness in the study of intestinal barrier function, surgical manipulation, and tissue intervention, as well as in biomaterial implantation and tissue transplantation (3). Available literature sources show that microbiota is necessary for the normal postnatal development of the structures of the gut wall. It has been suggested that pigs possess a "developmental window" in which the developing host–gut microbiota interactions are the easiest to manipulate and during which time the gut is the most susceptible to major disturbances (4). Therefore, the examination of GF animals before introduction of bacterial colonization, may provide us with better understanding of the communication that occurs between the host and its bacterial residents in the future.

Escherichia coli is an important enteric pathogen of weaned pigs, causing postweaning diarrhoea and edema disease worldwide (5). Despite the progress that has been observed in modern pig farms during the last decade to prevent infectious diseases and improve global animal health, postweaning diarrhoea remains a problem that causes significant economic losses in pig production. The age of affected pigs depends on the age at weaning. Disease normally occurs within the first 14 days after weaning, but can also be encountered after transfer to fattening premises (6). Pathogenic strains colonize the small intestine by means of specific adhesion factors (fimbriae) and produce one or several exotoxins responsible for disease (6, 7). The fimbria types as (*E. coli* O149: K88) are commonly found in pathogenic *E. coli* isolated from weaned pigs (6, 8). These strains produce an outer membrane protein (intimin) which is involved in the intimate attachment of the bacteria to enterocytes (9).

Nowadays, the main focus of attention has been directed to study distribution of bacteria and their influence on development and maturation of piglet´s gut, but a smaller number of information is obtained from GF environment. Our study is focused on investigation of *E. coli* influence on distribution and secretory activities of goblet cells (GCs) in epithelial lining of jejunum and colon of GF piglets as healthy control group (HC) and GF piglets in which at 5th day their gut was colonized with *E. coli* bacteria as ECK group. In this study, we briefly evaluated the differences in distribution of neutral and acidic mucins in the goblet cells in the wall of jejunum and colon in HC piglets and ECK piglets, which were infected by *E. coli* O149:K88.

METHODS

The structure of the small intestine is very similar in humans and piglets, including macroscopic features such as the ratio of intestinal length *per* kg bodyweight (10). Therefore, the experiment was carried out on group (n=4) of clinically healthy GF piglets (cross-bred – Yorkshire × Pietrain) – healthy control group (HC). The piglets were obtained by the open hysterotomy method, kept in sterile insulators and fed with a sterilized milk replacer (Sanolac, Ferkel, Sano, Germany). The piglets were sacrificed using T61 (Intervet International B.V., Boxmeer, The Netherlands, dose: 0.3ml/kg body weight) intracardially on 5th day. All piglets were fed every 4 hours, i.e. 6 times daily. On the day 5 after birth piglets in the infected experimental group (ECK group, n=5) were infected by the dose 2 ml of prepared culture of *E. coli* O149: K88ac (1×10^4 CFU/ml). The *E. coli* O149: K88ac strain without enterotoxin production was obtained from the Danish Institute of Agricultural Science, Denmark. Overnight culture of *E. coli* (1 ml) was inoculated into 50 ml Trypticase soy broth (Oxoid Unipath, Ltd., Basingstoke, UK) and cultivated at 37 ºC in a water bath shaker (JULABO SW 2C, Labor Technic GMBH Selbach, Germany) for approximately 2 h to optical density 0.5 at 640 nm (corresponded to 1×10^8 CFU/mL). Subsequently, the bacterial

culture was diluted in isotonic saline solution to obtain a final concentration of 1×104 CFU/ml. The purity of the broth culture was verified by spread plating on MC agar and TSA agar with sheep blood respectively (Oxoid Unipath). Piglets were examined daily for clinical signs, including lethargy, pyrexia, diarrhoea and anorexia. The infected experimental piglets were sacrificed using T61 (Intervet International B.V., Boxmeer, The Netherlands, dose: 0.3ml/kg body weight) intracardially on 8th day. The presented experiment with protocol number 3629/05-221, was approved by the State Veterinary and Food Administration of the Slovak Republic. The animals were handled and sacrificed in a human manner in accordance with the guidelines established by the relevant Ethics Committee of the University of Veterinary Medicine and Pharmacy in Košice.

Gastrointestinal (GI) tract was removed from the sacrificed piglets immediately. Small intestine (jejunum) and large intestine (colon) 1–2 cm long bioptic samples were obtained and washed with cold saline and fixed in 4% paraformaldehyde. Histological sections (4–5 μm) were deparaffinized and rehydrated. The population of GCs present in the intestinal mucosa of jejunum and colon was detected using the PAS - reaction and Alcian blue histochemical staining method. For neutral mucosubstances and mucins detection the PAS-reaction modified according to McManus was used. Fresh-made 1% solution of PAS was prepared for the PAS-reaction. For further differentiation of cell nuclei, Mayer hematoxylin staining was performed. The goblet cells and glycocalyx were stained magenta by PAS-reaction, and cell nuclei were stained dark blue by Mayer's hematoxylin (SIGMA-ALDRICH, Co). Alcian blue 8GX solution (pH 2.5) (Sigma–Aldrich, St. Louis, MO, USA) stains both sulfated and carboxylated acidic mucopolysaccharides and sulfated and carboxylated sialomucins (glycoproteins). Excessive amounts of non-sulfated acidic mucosubstances were visible in the cytoplasm of secretory GCs. Strongly acidic mucosubstances in the cytoplasm were stained blue, while nuclei were counterstained pink to red by nuclear red stain. Alcian blue/nuclear red stained tissues were acquired and the number of Alcian blue positive GCs was determined in 10 intestinal villi and corresponding intestinal crypts in each sample. All histochemically stained tissue sections were cover-slipped with Pertex (Histolab Products AB; Göteborg, Sweden).

All measurements were performed in order to ensure objectivity in blind conditions, by two observers (unaware of the experimental groups) for all experimental groups and methods, carrying out the measures of control and experimental samples of each segment of the gut under the same conditions. For the quantitative analyses of PAS positive and Alcian Blue positive GCs, we used five sections from both gut segments in all animals' groups. All measurements were done using magnification 200x. For quantitative and qualitative analyses of histochemical and histological methods for detection of mucin in GCs, light microscope OLYMPUS BX50 with a digital camera OLYMPUS SP350 (Olympus; Tokyo, Japan) and Quick PHOTO Industrial 2.3 image analyser software (Promicra; Prague, Czech Republic) were used. The statistical analysis was performed in GraphPad InStat ver. 3.10 for Windows (GraphPad Software Inc., San Diego, CA, USA). Quantitative evaluation of studied markers is expressed as mean ± SEM (standard error of the mean). The significance of the differences between experimental groups was analysed using one-way analysis of variance ANOVA test followed by a Tukey-Kramer multiple comparison test. The value of $p<0.05$ was considered to be statistically significant.

RESULTS

Mucins possess potential binding sites for both commensal and pathogenic organisms and may perform a defensive role during establishment of the intestinal barrier. In this study, the effects on intestinal GCs mucin production in the gut of HC piglets and ECK piglets were examined. Numbers of GCs containing total acidic mucins in both, the jejunum and colon, differed significantly between HC and ECK piglets (Fig. 1).

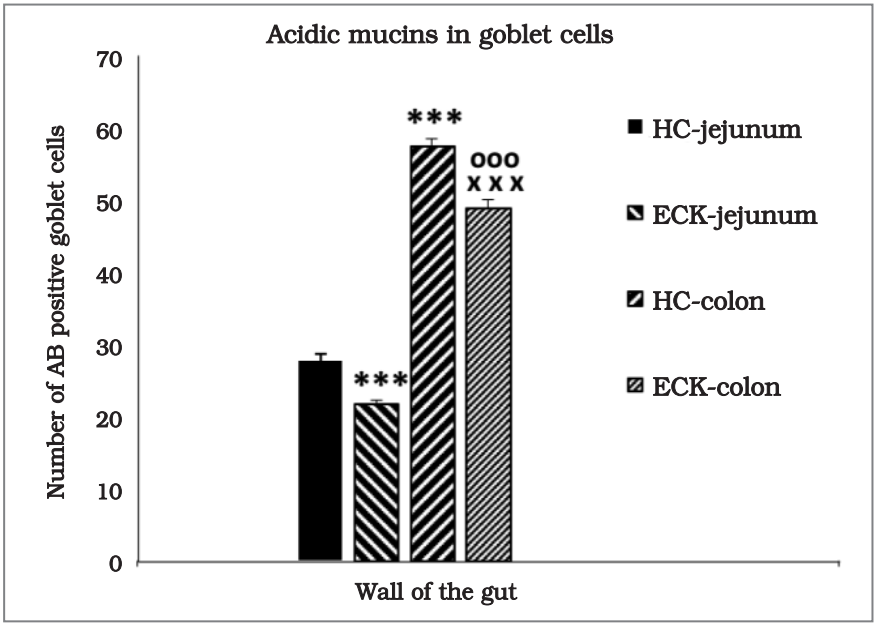


Fig. 1 Graph illustrating the average number of alcian blue (AB) positive GCs in gut for each condition. *Note that AB positive GCs were decreased in the jejunum of ECK piglets as well as in the colon of ECK piglets (***indicates the values differ significantly from the from the jejunum of HC piglets at p<0.001; [xxx] indicates the values differ significantly from the colon of HC piglets at p< 0.001). Greater numbers of AB positive GCs were observed in colon of both groups of piglets (***indicates the values differ significantly from the jejunum of HC piglets at p< 0.001; [ooo]indicates the values differ significantly from the jejunum ECK piglets at p< 0.001). Data are expressed as mean ± S.E.M., ANOVA and Tukey–Kramer tests were used.*

In the epithelium of jejunum of ECK piglets, the epithelial lining exhibited a marked decrease in number of GCs which produced acidic mucins (***indicates the values differ significantly from the jejunum of HC piglets at $p<0.001$) and they were positive for Alcian blue (AB) histochemically. In the colon, a similar trend was also observed, occurring ECK piglets ([xxx]indicates the values differ significantly from the colon of HC piglets at $p<0.001$). Overall, there were greater numbers of GCs containing acidic mucins in the colon compared with the jejunum in HC piglets (***indicates the values differ significantly from the jejunum of HC piglets at $p<0.001$) and also in the ECK piglets ([ooo]indicates the values differ significantly from the jejunum ECK piglets at $p<0.001$). Representative microphotographs show distribution of AB positive GCs in jejunum (Fig. 2. A_1, A_2) and colon (Fig. 2. B_1, B_2) of both group of piglets.

Number of PAS positive GCs (detection of neutral mucins in goblet cells) in the HC piglets was significantly different in comparison to the number of PAS positive cells in the ECK piglets (Fig. 3).

In the ECK piglets, jejunal GCs exhibited decrease in neutral mucins (**indicates the values differ significantly from the jejunum HC piglets at $p<0.01$). Further analysis of numbers of PAS positive GCs showed a marked increase in neutral mucins in the epithelium of colon in the HC piglets (***indicates the values differ significantly from the jejunum HC piglets at $p<0.001$) and also in the ECK piglets ([ooo]indicates the values differ significantly from the jejunum ECK piglets at $p<0.001$) in comparison to jejunum of both group of piglets. Significant increase of PAS positive GCs was observed between colon of HC and ECK group of piglets ([xxx]indicates the values differ significantly from the colon of HC piglets at $p<0.001$). Representative microphotographs show distribution of PAS positive GCs in jejunum (Fig. 4. A_1, A_2) and colon (Fig. 4. B_1, B_2) in both group of piglets.

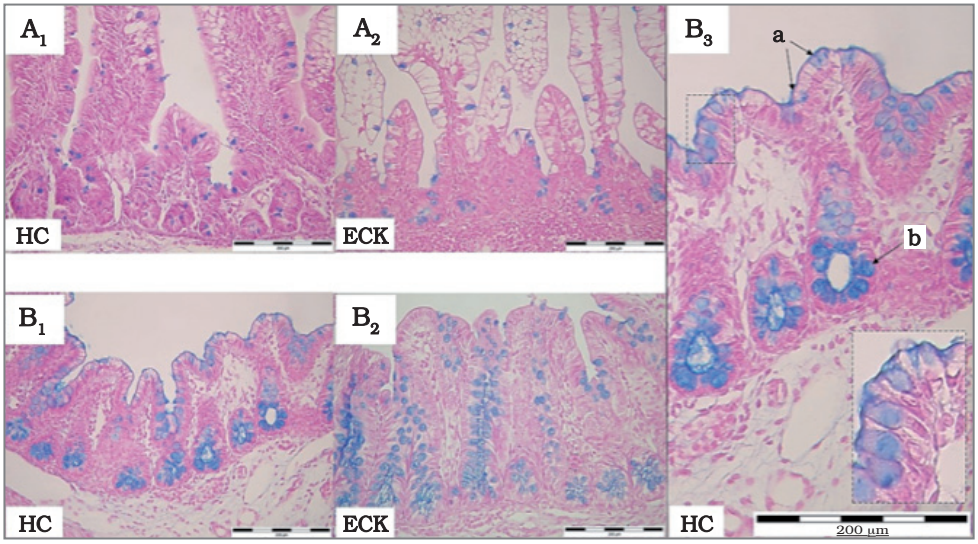


Fig. 2 Representative microphotographs of histochemical analysis of GCs producing acidic mucins by Alcian blue staining in the gut for each condition. *(HC – healthy control A_1 –jejunum and B_1 - colon; ECK – infected experimental group, A_2 – jejunum and $B_{2,3}$ – colon, a – positive thin mucus layer, b – strongly positive cytoplasm of GCs). Scale bar = 200 µm.*

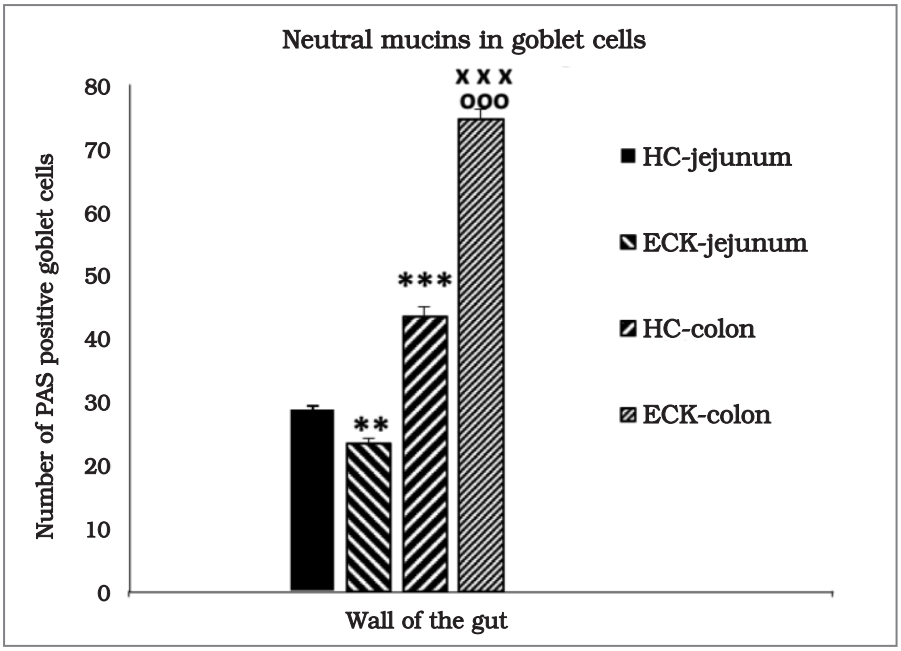


Fig. 3 Graph illustrating the average number of PAS positive GCs in gut for each condition. *Note that PAS positive GCs were decreased in the jejunum of ECK piglets (**indicates the values differ significantly from the from the jejunum of HC piglets at p<0.01). Greater numbers of PAS positive GCs were observed in colon of both groups of piglets (***indicates the values differ significantly from the jejunum of HC piglets at p< 0.001; ^{ooo}indicates the values differ significantly from the jejunum ECK piglets at p<0.001). Significant increase of PAS positive GCs was detected also in colon of ECK piglets in comparison to HC piglets (^{xxx}indicates the values differ significantly from the colon of HC piglets at p<0.001). Data are expressed as mean ± S.E.M., ANOVA and Tukey–Kramer tests were used.*

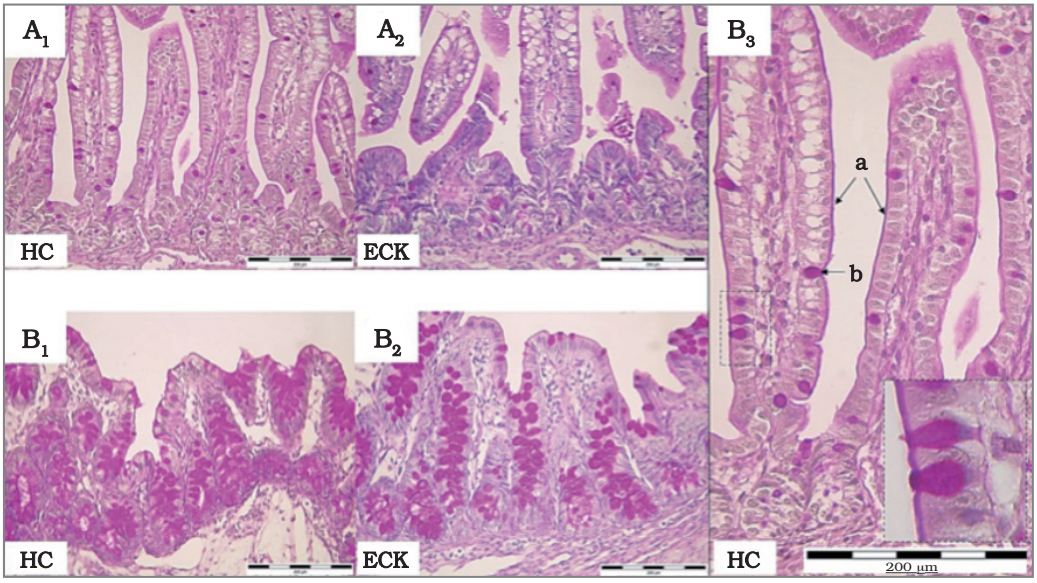


Fig. 4 Representative microphotographs of histochemical analysis of GCs producing neutral mucins by PAS-reaction in the gut for each condition. *(HC – healthy control, A_1 – jejunum and B_1 – colon; ECK – infected experimental group, $A_{2,3}$ – jejunum and B_2 – colon a – positive glycocalyx, b – strongly PAS positive cytoplasm of GCs). Scale bar = 200 µm.*

DISCUSSION

Enteric infections with pathogenic bacteria play an important role in animal health with the initiation and perpetuation of diseases such as diarrheal disease caused by enterotoxigenic *Escherichia coli* in new born pigs, calves, and lambs (11) and are responsible for reducing growth rates and consequent economic losses in animal production (5, 11).

Goblet cells are important cells of intestinal epithelial lining because they play important role in synthesis and secretion of mucus. Its major function is to protect the intestinal epithelium from damage caused by food and digestive secretions. The overlying mucus gel layer is the first line of defence that foreign bacteria and other pathogens encounter when they attempt to traverse the intestinal mucosa (12). However, simultaneously, mucin provides a desirable environment for proliferation of specific microflora due to its high carbohydrate content (13). Thus, the true chemical composition of mucus is essential for establishment of the intestinal barrier. Mucins can be classified into two broad categories: neutral and acidic depending on sugar type in the chains. These terms are derived from the chemical nature of the oligosaccharide sugar moieties (14).

Currently, small number of information is available describing the effects of bacterial colonization on the secretory histochemical pattern of intestinal mucins in piglets. Reference to numbers of GCs containing acidic mucins compared with neutral mucins in conventionally reared poultry has been reported (15). However, it has not been described in piglets from GF environment. Thus, the aim of the current study was to investigate the effects of bacterial colonization on mucin production in jejunal and colon´s GCs of piglets.

In the current study, we found that piglets infected with *E. coli* (ECK piglets) showed a significant increase in the production of neutral mucins by GCs in the colon compared to HC piglets, whereas we obtained the opposite results in the production of acidic mucins

by colon GCs in the ECK piglets compared to HC. Mucus gel is in a dynamic balance between mucin synthesis and secretion from GCs and erosion on the luminal surface. An increase in GCs number in the epithelial lining of villi and crypts in the gut may potentially increase the mucin secretion capacity of the mucosa and, consequently, improve gut health. Goblet cells are the most abundant secretory epithelial cells in the gastrointestinal (GI) tract. Their primary function involves the secretion of mucins that self-assemble into a protective mucus layer, coating the apical surface of epithelial cells. This mucus layer not only limits commensal microbe contact with the epithelium but also reduces mechanical stress on the epithelium through lubrication of the luminal bolus comprised of food contents (16). The change in mucin profile in response to bacterial colonization suggests a potential role as a protective mechanism against pathogenic invasion of the intestinal mucosa during early development. The goblet cells play role as key regulators of intestinal homeostasis at the host-microbe interface within the GI tract (17). Once released into the lumen, these mucins expand to form a dense, carbohydrate-rich matrix, assembling into homo-oligomers that give mucus its viscous properties. The mucus barrier is dynamically regulated, not only by continuous low-level mucin release by GCs but also by its continuous degradation via the mucinolytic actions of commensal, as well as pathogenic, bacteria. The oligosaccharide content in the mucus layer can influence the microbiota of the GI tract by the enhancement or inhibition of adherence of specific bacteria (18, 19, 20). *E. coli* uses fimbrial adhesion factors to bind to the mucus layer and colonize the intestine. *E. coli* fimbriae has affinity for sialic acid residues on mucin glycopeptides and glycolipids found in the pig small intestine (21). Our results support the idea that GCs play key role in host defence by responding to their luminal environment through the composition of their secretory granule (the production ratio of neutral to acidic mucins) during exocytosis. We supposed that decrease of AB positive GCs (secretory granules rich in acidic mucins) may lead to prepare small number of opportunities to colonise the gut with *E. coli* by fimbriae, which has high affinity for sialic acid residues.

This change in mucin profile in response to bacterial colonization suggests a potential role as a protective mechanism against pathogenic invasion of the intestinal mucosa during of gut mucosa development in piglets.

CONCLUSION

It can be concluded that colonization by *E. coli* generates morphological alterations reflected in changes of GCs population and distribution in the intestine. The results of current study help to understand the role of mucins in maintenance of intestinal integrity during early life period of piglets. These findings underline the need of further studies to understand the mechanisms involved in the regulation of intestinal mucin secretion. The mucus-pathogen interactions are complex and depend on microbe and host species; therefore, in order to achieve such high advancements of understanding, more knowledge is required in this topic. It warrants further investigation of gut development of immunity and its interactions with mucin dynamics and bacterial colonization.

Therefore, using of piglets from GF environment as animal models may contributed to the acquisition of new knowledge to improve both animal and human health.

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