

Importance of breast milk in the development of intestinal microbiota

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Abstract

At birth, mammals make the transition from aseptic surroundings to a pathogen-filled environment, it is known that different factors modulate immune responses, but in the digestive tract breast milk (BM) may play an important role in the maturation of the immune response, mainly in relation to the gut. In this sense, it has been demonstrated that besides nutrients and antibodies, BM contains cells, hormones, cytokines, and growth factors. The objective of this work was to evaluate the importance of BM in the development of both the piglet and the intestinal microbiota. For this, two groups of piglets were formed; one suckled with the saw and the other suckled with BM substitute. Stool samples were taken for bacterial identification, this was done through the VITEK-system, and subsequently, the serotype was identified. The results showed that the piglets suckled with BM substitute 18 (100%) presented diarrheal episodes from the 2nd day, of this group two piglets presented bloody diarrhea, and the group fed with the mother only 7 (38.8%) had diarrheic episodes from the 5th day. All isolates 285 were *Escherichia coli*. The largest number of isolates obtained of piglets suckled with BM belonged to serotype O103:H28 and largest number of isolates obtained of piglets suckled with milk substitute belonged to serotype O166:H21. Serotypes belonged to bloody diarrhea were O103:H28 and O166:H21. The present work showed the importance of BM both in the body development of the piglet and in the protection against infections.

Key words: Piglets suckled with the saw. Piglets suckled with breast milk substitute. *Escherichia coli*. Serotypes.

Introduction

At birth, piglets are devoid of gamma globulins, because the epithelial chondrial placenta of the sows, does not allow the passage of immunoglobulins to the fetus, and passive immunity can be acquired through colostrum milk during the first 36 h of life¹. To the birth, the gastrointestinal tract of the pig is sterile, but within a few hours, it is colonized by microorganisms. The colonizing bacteria are proposed to be mainly acquired from maternal feces, skin, and teats. *Escherichia coli* cells, together with Streptococci of Lancefield groups D

and K and *Clostridium perfringens*, are among the earliest bacteria to colonize the gut in piglets². The composition of the intestinal *E. coli* microbiota in pigs is very complex; these bacteria are considered normal inhabitants of the intestinal tract, but specific serotypes being an important cause of diarrhea². It is known that breastfeeding protects against acute infections of the digestive and respiratory tracts^{3,4} and that intestinal flora is modified by feeding.

At birth, mammals make the transition from aseptic surroundings to a pathogen-filled environment. The mucosal epithelial layer forms the interface between the

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external and internal environments of the gastrointestinal tract (GIT). This area is the site for digestion and absorption of various essential nutrients, yet it must also function as a barrier against various harmful agents and infectious pathogens²⁻⁴. It is known that different factors modulate immune responses, but in the digestive tract breast milk (BM) may play an important role in the maturation of the immune response, mainly in relation to the gut. In this sense, it has been demonstrated that besides nutrients and antibodies, BM contains cells^{5,6}, hormones, cytokines, and growth factors^{7,8} as a proline-rich protein⁹ and an epidermal growth factor (EGF)¹⁰. All of these biologically active factors not only facilitate the development of essential digestive functions but also regulate the maturation of the intestinal mucosal barrier. A colostral proline-rich protein induces the growth and differentiation of resting B-cells. The capacity of this protein to induce both B-cell growth and differentiation may provide evidence that its central role is the establishment of an immune response in neonates⁹. EGF is a peptide that produces a variety of biological responses, most of which involve regulation of cell replication, cell movement, and cell survival. In the GIT, EGF not only enhances proliferation and differentiation of epithelial cells but also has significant effects on healing damaged mucosa or on intestinal adaptation after injury¹⁰. The amino acid, L-glutamine is also present in BM and plays a dual metabolic role in new-born pig enterocytes acting as both a primary metabolic fuel and as an essential source of carbon and nitrogen for biosynthetic processes¹¹. In addition, L-glutamine is important for lymphocyte proliferation and generation of lymphokines¹².

The etiology, epidemiology, and pathogenesis of *E. coli* causing pre- and post-weaning diarrhea in pigs have been the subject of many studies. However, there is relatively little information about the gut microbiota and their relationship with *E. coli* in piglets suckled with BM and piglets suckled with a milk substitute.

E. coli is a Gram-negative microorganism belonging to the *Enterobacteriaceae* family, which is usually found, as commensal, in the enteric tract of the human and of many animal species. However, several *E. coli* strains can cause a wide range of clinical symptoms in humans and animals, due to the expression of particular virulence factors, whereby *E. coli* strains have been classified in different pathogroups¹³.

E. coli is a common porcine enteric pathogen, causing diarrhea in newborn and weaned pigs and edema disease in piglets after weaning. Enterotoxigenic *E. coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC) are

the main categories of diarrheagenic *E. coli* that cause enteric infections in pigs⁴.

ETEC is an *E. coli* that produces at least one member of two defined groups of enterotoxins, which are able to exert their action on the intestinal epithelium. They are heat-labile (LT) and heat-stable enterotoxins (STs). STs are classified as STa (also called STI) and STb (called (STII)¹³. Most ETEC isolates from diarrheic pigs can produce one or more of the following fimbriae: F4 (K88), F5 (K99), F6 (987P), F17, F18, and F41¹⁴⁻¹⁶. Fimbriae are surface proteins that are responsible for adhesion to intestinal epithelial cells^{15,16}. The fimbrial adhesins allow the union of the bacteria to the recipients of the microvilli of the enterocytes; this is followed by the secretion of toxins that are responsible for the production of diarrhea. Once *E. coli* has colonized the intestine, diarrhea occurs, this may be watery or inflammatory, watery diarrhea is due to the production of one or more enterotoxins and inflammatory diarrhea to the production of cytotoxins¹³.

Diarrhea associated to ETEC belong to a limited number of serogroups of *E. coli*, O8, O9, O20, O64, O101, O138, O141, O147, O149, and O157 being the most commonly found in several countries^{16,17}.

The second pathotype of *E. coli* is Shiga toxin-producing (STEC) also called verotoxin-producing *E. coli*, it causes inflammatory diarrhea. *E. coli* O157:H7 produce the toxin STx which is absorbed from the intestine and enters the bloodstream where it causes systemic damage, resulting in edema disease. However, in addition to *E. coli* O157:H7, other kinds of STEC, called "non-O157 STEC," cause human diseases. The Centers for Disease Control and Prevention estimate that non-O157 STEC are responsible for about 1579 confirmed cases of illness annually¹⁸, but cause relatively fewer cases of the hemolytic uremic syndrome (HUS) than *E. coli* O157:H7. Of these serotypes, serotype O103 was the first non-O157 STEC strain identified as a suspected cause of sporadic cases of HUS, in 1975 in France.

Materials and methods

Feeding protocol

Piglets of the race (hybrids of York X Landrace) they were randomly distributed into two groups; the first one suckled with the sow formed by 18 pigs and the second was suckled with BM substitute formed by 18 pigs.

Milk substitute Lacto Spray Pig (Humelco, The Netherlands) contains all the nutrients in the same

concentrations that the milk of the sow. Piglets were suckled the first 2 weeks each hour at *libitum* (30-40 ml approximately) of Lacto Spray Pig, during 24 h, the 3rd and 4th weeks each hour at *libitum* (60-70 ml approximately). As much the water where upon prepared milk as the baby's bottles for each taking, were previously sterilized. As of the 2nd day of life, was added to the feeding Pedialyte (ABBOT) at *libitum*. Unique dose, 0.5 ml of oxytetracycline (Pfeizer, México) intramuscular shot was applied to all the piglets (with or without diarrheic evacuations) at the 3rd and 5th days of life.

***E. coli* isolates and their characterization**

Samples were obtained from two piglets group from rectal swabs in piglets suckled with BM and suckled with BM substitute. Samples of feces were obtained of piglets with diarrhea and without diarrhea.

A total of 285 samples were isolated. The collected specimens from each pig were transported in Cary and Blair medium (DIBICO, S.A. de C.V, México), stored to 4°C and spread in MacConkey agar, *Salmonella* *Shigella* agar, Lactose Sacarosa Xilosa agar, and Green Brilliant agar. Plates were incubated at 37°C aerobically overnight. Five lactose-fermenting colonies with the appearance of *E. coli* were randomly selected of each agar milieu and cultured on sheep blood agar plates. Plates were incubated aerobically overnight. *E. coli* isolates were stored on Tryptic soy agar, and biochemical characteristics were established by VITEK-system (Bio Mérieux, Vitek, Inc. Missouri USA). Then, the isolates were serotyped and kept on Dorset Milieu at RT.

Serotyping

The determination of O and H antigens was carried out in the *E. coli* Reference Laboratory, Departamento de Salud Pública, Facultad de medicina UNAM, employing all available O (O1-O181) and H (H1-H56) antisera. Antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove the non-specific agglutinins.

Statistical analysis

The square Chi-square test was used to compare diarrhea episodes of in both piglets groups, feeding with BM and feeding with milk substitute; as well as the association between the type of lactation and the presence of *E. coli* virulence genes. The statistical analysis was examined by SPSS for Windows 20.0.



Figure 1. Photo that shows the piglets fed with a breast milk substitute. A student is standing and she is feeding a piglet and other piglets surround her waiting for her food.

Results

The results showed that the piglets suckled with BM substitute (Fig. 1), 18 (100%) presented diarrheal episodes from the 2nd day, of this group two piglets presented bloody diarrhea on day 10 of age; and the group fed with the mother (Fig. 2), only 7 (38.8%) had diarrheic episodes from the 5th day. VITEK system showed that all 285 isolated were *E. coli*.

The 95% confidence interval was 0.24-0.62, which suggests that feeding with maternal milk represents a protective factor for the presence of diarrhea in piglets; the fact that they were fed by the mother reduced the risk of diarrhea by 61%. The number needed to treat value of 1.6 indicates that all the piglets that received maternal milk, avoided early diarrhea.

Isolates 285, 164 (57.5%) come from piglets suckled with BM, which 69.5% were obtained of piglets healthy and 30.5% of piglets with diarrhea. Isolates obtained from piglets suckled with milk substitute 121 (42.5%) were obtained of piglets with diarrhea, which 10% isolates belonged to bloody diarrhea. The statistical analysis shows significant differences in diarrheic episodes between piglets fed with the mother and piglets fed with maternal milk substitute ($p = 0.000$).

It is important to note that at 1 month of age, the difference in the size of the suckling pigs with the



Figure 2. Photo that shows the piglets fed with the mother.



Figure 3. Photo that shows the piglets of 1 month of age: the small one fed with breast milk substitute and the other one fed with the mother.

mother in relation to the piglets suckled with BM substitute. The piglets suckled with the mother had normal size and the lactates with BM substitute gained very little weight (Fig. 3).

Serotypes

Two hundred and eighty-five isolates of *E. coli* were obtained from piglets with and without diarrhea. The largest amount of isolates obtained of piglets suckled with BM belonged to serotype O103:H28 (62.3%), O166:H21 (15.8%), O98:H? (14.9%), and O109:H40 (7.0) preferentially and the largest number of isolates obtained of piglets suckled with milk substitute belonged to

O166:H21 (59.6%), O109:H40 (26.6%), and O103:H28 (13.8%) serotypes preferentially. Serotypes belonged to bloody diarrhea were O103:H28 (1.0%) and O166:H21 (1.0%) (Fig. 4).

Discussion

The present report describes what is believed to be the first study in México and probability to Mundial level. This is because, at birth, piglets are devoid of gamma globulins, because the epithelial chondrial placenta of the sows does not allow the passage of immunoglobulins to the fetus, and passive immunity can be acquired through colostral milk during the first 36 h of life. The lack of immunological protection causes the death of piglets¹.

At present, there is a little information regarding isolates of *E. coli* of piglets suckled with BM and piglets suckled with a maternal milk substitute. In the present study, we work with two piglets groups: the first one was suckled with BM and the second was suckled with BM substitute.

The study was followed for a month, during which the body development of the group of piglets suckled with the mother was normal, unlike the group of piglets suckled with BM substitute, which showed little body development (Fig. 3). Similarly, the group breastfed with the mother only had 7 (38.8%) episodes of diarrhea, as opposed to the one breastfed with milk substitute, which presented 18 (100%) episodes of diarrhea, two with inflammatory diarrhea.

This could be because colostrum and BM are a complex physiological fluid with multifunctional roles within the gastrointestinal tract that facilitate the successful postnatal adaptation of the newborn by stimulating cellular growth and digestive maturation. Simultaneously, BM provides nutrients and bioactive factors that facilitate the adaptive functional changes required for the optimal transition from intrauterine to extrauterine life.

It is known that besides nutrients and antibodies, BM, Diaz-Jouanen and Williams⁵ contains cells and bioactive factors, as hormones and cytokines⁷ as well as growth factors such as cortisol, insulin, prolactose, tyrosine, EGF, and transforming growth factor¹⁹. These factors present in maternal milk can play a role in the regulation of the growth and differentiation of several neonatal tissues, including the immune and neuroendocrine systems. The EGF participate in the development of the intestinal mucosa, indeed, represents the site where is carried out the digestion and absorption of the nutrients¹⁰. All these factors participate

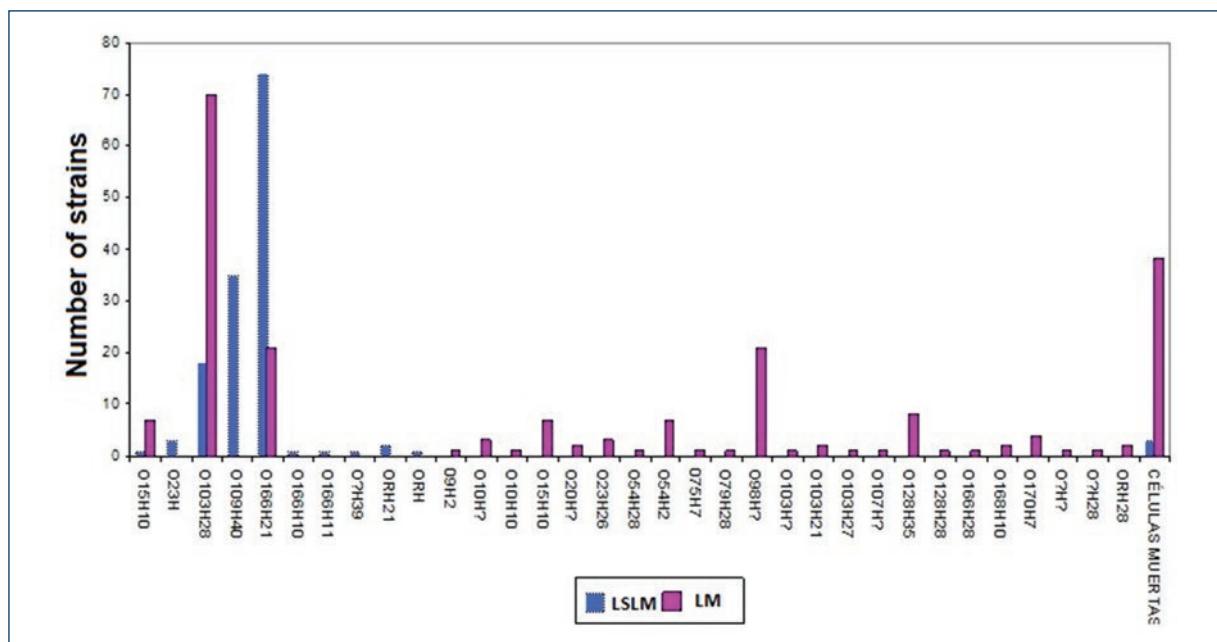


Figure 4. Serotypes of *Escherichia coli* isolated from piglets lactated with the mother and with breast milk substitute.

in the maturation of the immune system and in particular of the Gut-associated lymphoid tissue, and this favors the protection of the individual in the presence of pathogens.

On the other hand, the early weaning in the piglet can affect the physiological development, the corporal growth, the behaviors, cortisol levels, regulation of the hypothalamus-pituitary-adrenal axis, and modify the immune response. These changes must lead to the separation of the dam to a great extent, to coexist with piglets of other litters, as well as to the deficiency of the factors with the immunological potential present in maternal milk.

All isolates of piglets suckled with BM as the suckled with BM substitute by VITEK showed that 100% of isolated belonged to *E. coli*. It has been reported that suckling piglets eat considerable amounts of their sow's feces and *E. coli* cells are among the earliest Gram-negative bacteria to colonize the gut in pigs. Diarrhea episodes of piglets suckling with BM were on the 5th day of births, which is able to acquire from maternal feces, skin, and teats¹. Diarrhea episodes of piglets suckling with BM substitute were on the 2nd day of births this could be doubt to the type of lactation, or infections³. Infections at this age could be due to this piglets group lack of immunological protection, because it did not breastfeeding, and we know that piglets protection is acquired from colostrum and BM⁶.

The predominant *E. coli* serotypes in the 285 isolates were O103:H28 for isolates from the group breastfed with the mother and O166:H21 from the group breastfed with BM substitute (Fig. 4). The isolates of piglets with bloody diarrhea, belonged to serotypes O103:H28 and O166:H21. The piglets suckled with BM, without diarrhea, the prevalent serogroup it was O103:H28.

It is noteworthy that the serotype of *E. coli* O103:H28 identified both in the group of piglets suckled with BM substitute and in the group suckled with the mother, only caused inflammatory diarrhea in the piglets of the group suckled with substitute BM, this indicates that *E. coli* from both groups of piglets belonged to the *E. coli* STEC pathotype. The fact that the genes of *E. coli* producing Shiga toxins have been expressed only in the group of suckled piglets with BM substitute, this could be due to the microenvironment of the intestine of the piglets suckled with BM substitute, which favored the expression of STx genes, giving rise to bloody diarrhea.

These serotypes could belong to non-O157 serotypes; this, taking into account that serogroup O:103 was the first to be identified as *E. coli* no O157²⁰. Although serotypes differ in their virulence, the incidence and severity of infections cannot be attributed solely to the virulence factors of the pathogen are the result of the interaction of the pathogen with host and environmental factors.

E. coli producing Shiga toxin (STEC) has been associated with hemorrhagic colitis and hemolytic uremic

syndrome in humans, however, in pigs has a high involvement in the disease of edema. Regarding the serotypes of *E. coli* O103: H28, O166:H21, and O:109:H40, these are not found in the literature for *E. coli*, producing Shiga toxin, they could belong to the serotypes of *E. coli* No O157:H7¹⁸.

The present work showed the importance of BM both in the body development of the piglet and in the protection against infections.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflicts of interests.

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