Gut response to pasteurized donor human milk in a porcine model of the premature infant

A. Socha-Banasiak[1], S.G. Pierzynowski[2,3,4], P. Szczurek[5], J. Woliński[6], A. Wesołowska[7], E. Czkwianianc[1] and K. Pierzynowska[2,4,6]

[1]Department of Gastroenterology, Allergology and Pediatrics, Polish Mother's Memorial Hospital-Research Institute, Lodz, Poland; [2]Department of Biology, Lund University, Lund, Sweden; [3]Department of Medical Biology, Institute of Rural Health, Lublin, Poland; [4]Consortium SGP+Group, Trelleborg, Sweden; [5]Department of Animal Nutrition and Feed Science, National Research Institute of Animal Production, Balice, Poland; [6]Department of Animal Physiology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jablonna, Poland; [7]Laboratory of Human Milk and Lactation Research at Regional Human Milk Bank in Holy Family Hospital, Medical University of Warsaw, Department of Medical Biology, Warsaw, Poland

Received May 14, 2020 – Accepted October 14, 2020

This study investigated the tolerance and safety of pasteurized donor human milk (PDHM) given either alone or together with commercially-used supplements in a porcine model of premature infants. A porcine model, mimicking human neonates at 30-32 weeks of gestational age, was used. The 7-day experiment was performed on 20 piglets. After birth, the piglets were infused with porcine immunoglobulins via the umbilical artery and surgically fitted with a stomach port. The piglets were then randomized into five groups and fed either PDHM, different variants of fortified PDHM or 'raw' human milk (RHM). Preterm piglets fed PDHM showed signs of gastrointestinal intolerance. Four piglets across the various PDHM-fed groups died, none of them were from the group fed PDHM supplemented with long-chain polyunsaturated fatty acids (LC PUFA). In all groups fed PDHM, macroscopic features of enterocolitis were observed, however, these pathological gut changes were less manifested in piglets receiving PDHM supplemented with LC PUFA. The piglets fed RHM had no specific signs of gut damage. The poor tolerance to PDHM suggests changes in milk composition caused by the Holder pasteurization. The supplementation with LC PUFA probably improves tolerance to PDHM.

Considering all the immunological and nutritional benefits of breast milk, the best way to feed preterm infants is with their own mother's milk (OMM) (1). Early enteral nutrition (EN) is preferable to total parenteral nutrition (TPN) due to the decreased risk of complications (i.e. sepsis, vascular catherization and other adverse effects of TPN) (2). Although various feeding strategies have been proposed for premature infants, the results of studies concerning the optimal timing for the introduction of EN and the safety of using donor milk fortifiers (DMF) are controversial (3, 4).

However, literature and clinical data indicate a decreased risk of necrotizing enterocolitis (NEC) and sepsis in preterm neonates fed with OMM, thus strong efforts to promote lactation are being made (5). Although premature infants have higher nutritional

Key words: milk pasteurization; milk fortification; preterm piglets; preterm infants

Corresponding Author:
Dr Kateryna Pierzynowska,
Department of Biology, Lund University, Solvegatan 35,
22362 Lund, Sweden
Tel.: +46 73 387 96 26
Fax: +46 (0) 462222 42 06
e-mail: katerina.goncharova@biol.lu.se

0393-974X (2020)

requirements in comparison to those born at term, their mothers' milk at the usual feeding volumes is not rich enough in macro- and micronutrients, including long chain polyunsaturated fatty acids (LC PUFA). Therefore, fortification of human milk is recommended to ensure the optimal growth and development of preterm neonates (6). Moreover, when OMM is not available or not sufficient in quantity, pasteurized donor human milk (PDHM) is a recommended alternative (7). Although milk pasteurization, via the Holder method, guarantees the destruction of bacteria and viruses, the high temperatures which the milk is exposed to also leads to a considerable reduction in the levels of important immune molecules, enzymes, hormones and other biologically active compounds (8-10). Moreover, it was previously shown that pasteurization increases casein resistance to infant digestion (11). The negative influence of the Holder pasteurization process on the composition of donor human milk (DHM) may contribute to impaired growth and development of preterm infants (12, 13).

The above facts encouraged us to continue with research focused on investigating the safety of PDHM and human milk fortification in the case of prematurity. Therefore, the general aim of this study was to test pasteurized human milk variants: PDHM *vs* PDHM enriched with LC PUFA *vs* PDHM supplemented with commercial fortifiers in a model of premature human infants. Considering the similarities between preterm piglets and human infants including body size, immaturity of the gastrointestinal tract and impaired respiratory, nutritional, immunological, and metabolic status after birth, the current study made use of an established preterm porcine model supplemented with immunoglobulins intravenously. Intravenous immunoglobulin supplementation increases the similarities between preterm piglets and human neonates at approximately 30-32 weeks of gestational age (14).

MATERIALS AND METHODS

Animals

The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All efforts were made to minimize animal suffering. The study was approved by the Second Local Ethics Committee for Animal Experimentation in Warsaw, Poland (approval no. WAW2/15/2017).

The study made use of 20 piglets (Polish synthetic line 990, male: 11, female: 9) delivered by caesarean section, from two sows inseminated by the same boar semen, 8 days before full gestation (107 days, 93% gestation, corresponding to 30-32 weeks human gestation). All piglets that were delivered were used in the study. During the experiment, piglets were maintained on a 12-hour day-night cycle, with lights on from 06.00-18.00 (6am-6pm). In order to ensure the proper development of the preterm piglets immediately after delivery, they were infused with porcine immunoglobulins (Ig) (1540 mg/kg bwt – 25 ml of sterile Ig preparation/kg bwt) via the umbilical artery (15). After delivery, the sucking reflex was absent in the animals. The insertion and maintaining of orogastric tubes were recognized as procedures which cause more suffering for animals than the insertion of gastric tubes. Jugular vein catheters and gastric ports were inserted under isoflurane anesthesia, in aseptic conditions. After a long incision (5 cm), the stomach was isolated and a gastric port catheter (Silastic, Laboratory Tubing 508-002, Dow Corning, Auburn MI, USA) was implanted via the *curvatura major* and fixed with purse string catgut sutures (KRUUSE Chromic Catgut, USP 4-0; Kruuse Svenska AB, Uppsala, Sweden). The abdomen was then stitched up using 3 layers of sutures, absorbable sutures for the muscle layers and non-absorbable sutures for the skin (Silon Monofil 2/0; CHIRANA, Prague, Czech Republic). Ampicillin (250-500 mg, Doktacillin, Astra Läkemedel, Södertalje, Sweden) was administrated at the incision site. A jugular vein catheter (Silastic, Laboratory Tubing 508-001, Dow Corning, Auburn MI, USA) for infusions was also implanted during the same anaesthesia (14). After surgery, all piglets received a 5% glucose infusion via the jugular vein catheter, at a rate of ~3 mL/h, for a period of 8 hours. Enteral feeding via the stomach port was initiated immediately following the first initial 8-h period after surgery. Parenteral nutrition was not used.

The piglets were individually housed in special sampling cages for preterm piglets, equipped with heating pads to maintain a temperature of 35-37°C. The humidity in the room was ~60%. Piglets were allowed to move around freely within their cages and had visual contact

with each other. The health status of the piglets and their feeding tolerance were monitored daily throughout the study period.

Study design and enteral feeding

A total of 20 piglets were randomized into 5 groups fed with different variants of milk feeds for a period of 7 days. Piglets were fed every 2 hours. The milk feeds were freshly prepared and heated to 37ºC each time before being administered to the piglets. During the first day after birth, the piglets were fed between 2-5 mL every 2 h (depending on the piglet's body weight, piglets <1kg - 2mL, piglets >1kg - 5mL). From the second day, the volume of milk was adjusted to the piglets' body weight, caloric requirements and health status. The daily target volume of milk ingested by the piglets according to the ESPGHAN recommendation was at least 135 mL/kg/day (1). The different experimental groups (n = 4 each) were as follows:

1. PDHM: Pasteurized donor human milk, thawed after freezing (-20°C), obtained from a local milk bank (Warsaw, Poland).
2. PDHM+LC PUFA: PDHM enriched with Arachidonic acid (ARA) (Raw oil/TG-ARA, Lot 6700003994, Nu-Chek Prep, USA) and Docosahexaenoic acid (DHA) (Raw oil/TG-DHA, Lot 880 000 4666, Nu-Chek Prep, USA).
3. PDHM+LC PUFA+PF: PDHM enriched with ARA and DHA, supplemented with powder fortifier (Enfamil Human Milk Fortifier, Mead Johnson, USA).
4. PDHM+LC PUFA+LF: PDHM enriched with ARA and DHA, supplemented with liquid fortifier (Similac Human Milk Fortifier hydrolyzed protein concentrated liquid, Abbott, USA).
5. RHM: Raw human milk (non-pasteurized human donor milk), thawed after freezing (-20°C), obtained from the same local milk bank as the PDHM (Warsaw, Poland) – control group.

All samples of PDHM were pooled prior to administration to ensure that each experimental group received a representative aliquot. Details of the milk feed preparations are presented in Table I.

Feeding tolerance observations

Feeding tolerance observations were based on visual identification of the general health of the piglets, as well as on the identification of potential symptoms including abdominal distention, vomiting, abnormal stool consistency, blood in the stool and abnormal autopsy findings. Visual observations of the piglets were classified descriptively based on the following:

Days 2-4. General well-being of piglets: playful, alert "+"; not playful or not alert "0".

Days 4-7 scoring. 1) Unwillingness to stand. 2) Cold limbs. 3) Abdominal distension. 4) Dehydration syndrome (dry skin, dry oral mucosa and/or no passing of urine). 5) Vomiting.

The piglets were assessed for the above-mentioned abnormalities every 2 hours before the feeding procedure.

Stool observations were also performed and the presence of meconium was noted. The consistency of the faeces was assessed using the Brussels Infant and Toddler Stool Scale (BITSS) (16):

type 1 - "separate hard lumps"
type 2 - "lumpy and sausage-like"
type 3 - "sausage-shaped with cracks on the surface"
type 4 - "stool in the form of a smooth, soft sausage"
type 5 - "soft blobs with clear-cut edges" or "loose stools"
type 6 - "mushy consistency"
type 7 - "entirely liquid consistency with no solid pieces".

Post-mortem examination and euthanasia

Piglets which died during the study and those euthanized with sodium pentobarbiturate (100 mg/kg) at the end of experiment were examined *post-mortem*. The autopsies of the animals that died during the experiment were performed within the same day. The gastrointestinal tract was examined for pathological abnormalities, especially features of necrotizing enterocolitis (NEC) (intestinal edema and hemorrhage, intestinal pneumatosis).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). All analyses were carried out using Prism, version 8 (GraphPad Software, Inc, San Diego, CA,USA). To assess data distribution, a Shapiro-Wilk normality test was performed. An ANOVA followed by a Tukey post-hoc test or Kruskal-Wallis test for non-Gaussian distributed data was used to assess statistical differences between groups. Differences were considered significant if $p \leq 0.05$.

RESULTS

Body weight gain and milk feeding volume/energy

Baseline characteristics of piglets associated

with growth and milk intake are presented in Table II. Body weight gain during the first week of life was observed in piglets from both the PDHM+LC PUFA+PF and PDHM groups. Piglets in all other groups displayed a moderate decline in body weight (less than 15%) over the first week of life. Feeding volume increased slowly from an initial 70 mL/kg/day (day 2) to 110 mL/kg/day on the last day of the study (day 7). There were no statistical differences in mean volume milk intake among the groups.

Daily observations on the well-being and overall health of the piglets

Between day 2 and day 4 general well-being scores were recorded for most of the piglets. On day 3 a decrease in vitality was observed in individual piglets from the PDHM, PDHM+LC PUFA+PF and RHM groups. A decrease in vitality was also observed in piglets from the PDHM+LC-PUFA+PF group on day 4. Almost all piglets had distended abdomens after the volume of milk was increased

Table I. *Supplementation details for each group.*

	Preparation (volumes/amounts were scaled to meet needs)	**volume**
PDHM	PDHM (mL)	QS
PDHM +LC PUFA	ARA oil - 42% ARA (μL)	302
	DHA oil - 43% DHA (μL)	206
	PDHM - (mL)	100
PDHM +LC PUFA+PF	ARA oil - 42% ARA (μL)	302
	DHA oil - 43% DHA (μL)	206
	PF (No. of packets)	4
	PDHM (mL)	100
PDHM +LC PUFA+LF	ARA oil - 42% ARA (μL)	362
	DHA oil - 43% DHA (μL)	247
	LF (No. of packets)	4
	PDHM (mL)	100
RHM	RHM (mL)	QS

QS – quantum satis, PDHM- pasteurized donor human milk, LC PUFA - long chain polyunsaturated fatty acids, ARA-Arachidonic acid, DHA - Docosahexaenoic acid, PF-powder fortifier, LF- liquid fortifier, RHM-raw human milk.

Table II. Baseline characteristics of piglets, body weight gain and milk intake during the 7-day study period.

Groups	Sex	Initial BW (g)	Final BW (g)	BW gain (g)	Food volume (mL/kg/day)	Food energy (kcal/kg/day)
PDHM	2M, 2F	1349 ± 187	1390 ± 221	41	88.1 ± 11.9	61.7 ± 8.31
PDHM+LC PUFA	3M, 1F	1250 ± 295	1160 ± 214	-90	92.7 ± 17.0	68.6 ± 12.6
PDHM+LC PUFA+PF	1M, 3F	1318 ± 169	1445 ± 7	127	88.9 ± 17.1	78.2 ± 15.0
PDHM+LC PUFA+LF	3M, 1F	1463 ± 162	1430 ± 113	-33	91.6 ± 16.2	88.9 ± 15.7
RHM	2M, 2F	1157 ± 287	1123 ± 198	-34	93.8 ± 14.8	65.7 ± 10.3

Data are mean values±SD for each group. BW - body weight, PDHM- pasteurized donor human milk, LC PUFA - long chain polyunsaturated fatty acids, PF-powder fortifier, LF- liquid fortifier, RHM – raw human milk, M - males, F- females.

to 95 mL/kg/day on day 4. On days 5-7, an inability/unwillingness to stand (especially in piglets from the PDHM+LC PUFA group), cold limbs (in piglets from the PDHM, PDHM+LC PUFA+PF, PDHM+LC PUFA+LF groups), abdomen distension (in most of the piglets except for those from the RHM group) were observed. The total number of abnormalities observed between day 4 and day 7, including an unwillingness to stand, cold limbs, abdominal distension, dehydration syndrome and vomiting, was highest for piglets from the PDHM, PDHM+LC PUFA+PF and PDHM+LC PUFA+LF groups (scores 42, 39 and 26, respectively). On the other hand, piglets from the RHM and PDHM+LC PUFA groups had the lowest total scores (2 and 8, respectively) (Table III).

After delivery, all piglets passed meconium. From day 4 some piglets started passing watery stools (type 7 according to the BITSS scale). This abnormality was noted mainly in piglets from the groups fed PDHM. Bloody diarrhea was observed in some of the piglets from the PDHM, PDHM+LC PUFA+PF and PDHM+LC PUFA+LF groups. Almost all of the piglets from the PDHM+LC PUFA+LF group and a half of the piglets from the PDHM+LC PUFA group passed no faeces except for

Table III. *Daily observation on the overall well-being of the piglets during the 7-day study period.*

Groups	Piglet #	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Total# recorded^	Totals number recorded/group
PDHM	1	+	0	+1,2,3	D	D	D	9	42
	2	+	+	+	3	1,3	3	9	
	3	+	+	+,3	3	3	3	7	
	4	+	+	+,3	1,2,3	2,3	3	17	
PDHM+LC PUFA	1	+	+	+,3	1	-	-	1	8
	2	+	+	+,3	1	1	1	3	
	3	+	+	+,3	1	-	-	2	
	4	+	+	+,3	1	-	-	2	
PDHM +LC PUFA+PF	1	+	+	+,3	1,2,3	1,2,3,4, 5	D	29	39
	2	+	+	+,3	3	-	3	6	
	3	+	0	0,2,3	D	D	D	D	
	4	+	+	+,3	-	-	3	4	
PDHM+LC LUFA+LF	1	+	+	+	2,3	1,2,3,4, 5	D	13	26
	2	+	+	+,3	-		3	4	
	3	+	+	+,3	-	-	3	4	
	4	+	+	+,3	-	-	3	5	
RHM	1	+	+	+,3	-	-	-	0	2
	2	+	0	+,3	-	-	3	2	
	3	+	+	+	-	-	-	0	
	4	+	+	+	-	-	-	0	

+: positive well-being (playful, alert); 0: not playful, alert; ^Refers to a total count of the number of time points between days 4-7 that one of the numbered items was observed/recorded.

1: unwilling to stand; 2: cold limbs; 3: abdominal distension; 4: dehydration syndrome; 5: vomiting; PDHM: pasteurized donor human milk; LC PUFA: long chain polyunsaturated fatty acids; PF: powder fortifier; LF: liquid fortifier; RHM: row human milk.

meconium. Only piglets in the RHM group passed stools that were considered normal for neonates (type 5/6 according to the BITSS scale) throughout the whole experimental period (Table IV).

During the study period two piglets died on day 4 (one from the PDHM group and another from the PDHM+LC PUFA+PF group). Prior to their death, the piglets were lethargic and had passed no stools except for the meconium. The piglet from the PDHM group presented with an unwillingness to stand, cold limbs and abdominal distension. The second piglet had advanced bowel distention. No vomiting was observed. Two piglets from the groups that obtained fortified milk (PDHM+LC PUFA+PF and PDHM+LC PUFA+LF) died on day 7. These piglets were lethargic, unwilling to stand, had cold limbs, abdominal distension and features of dehydration syndrome. The piglets were passing watery, bloody stools. From day 6 vomiting was also observed in these piglets.

Post-mortem examination

All piglets that died during the experimental period had clinical symptoms of NEC (Table III, Table IV). Various abnormalities were observed upon autopsy of the piglets that died on day 4. In case of the piglet from the PDHM group, features of classical enterocolitis (large intestine and distal part of ileum with advanced hemorrhages) were noted (Fig. 1 a, b). Macroscopic examination of piglet number 3 (PDHM+LC PUFA+PF group) indicated extensive hemorrhage of the stomach (Fig. 1c). Intestinal

Table IV. *Summary of piglet stool quality records during the 7-day study period.*

Groups	Piglet #	First abnormal stool observed (day of study period)	Stools according to BITSS scale
PDHM	1	-	Died on Day 4
	2	7	7
	3	5	7 with blood
	4	-	No stools after meconium
PDHM+LC PUFA	1	5	7
	2	-	5
	3	-	No stools after meconium
	4	-	No stools after meconium
PDHM+LC PUFA+PF	1	5	7 with blood/died on Day 7
	2	-	5
	3	-	Died on Day 4
	4	-	No stools after meconium
PDHM+LC PUFA+LF	1	4	7 with blood/died on Day 7
	2	-	No stools after meconium
	3	-	No stools after meconium
	4	-	No stools after meconium
RHM	1	-	5/6
	2	-	5
	3	-	5/6
	4	-	5

The days on which the piglets started passing pathological stools are noted in the table. "-" – means no stools were passed after the meconium or that the piglets were passing normal stools (type 5) throughout the experiment period.
PDHM: pasteurized donor human milk; LC PUFA: long chain polyunsaturated fatty acids; PF: powder fortifier; LF: liquid fortifier; RHM: raw human milk.

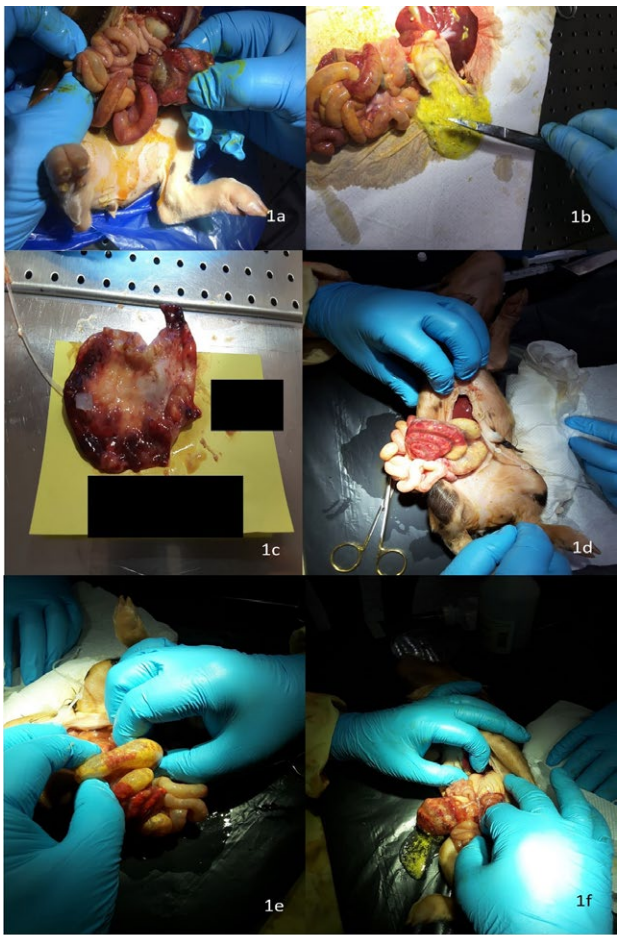

Fig. 1. *Post-mortem examination of piglets that died on day 4 and day 7. Piglets that died on day 4 of the 7-day study period: Piglet No.1 from the PDHM group (**a, b**); Piglet No. 3 from the PDHM+LC PUFA+PF group (**c**). Piglets that died on day 7 of the 7-day study period: Piglet No. 1 from the PDHM+LC PUFA+PF group (**d, e**); Piglet No. 1 from the PDHM+LC-PUFA+LF group (**f**).*

edema and hemorrhage of the colon with a bloody stool in the intestinal cavity were also observed. In the case of the two piglets from the PDHM+LC PUFA+PF and PDHM+LC-PUFA LF groups, features of enterocolitis (huge hemorrhagic areas of the colon) were noted (Fig. 1 d, e, f). Moreover, gas was observed within the bowel wall in the colon of the piglet from the PDHM+LC PUFA+LF group. In the stomach of all of the piglets that died during the experimental period, yellow, jelly-like content with a lot of mucus was observed (Fig. 1b). Bleeding from the upper part of the digestive system, as well as bloody mucus in the stomach, was observed in the piglets from the PDHM+LC PUFA+PF group.

A summary of the macroscopic observations made during *post-mortem* examination of the piglets that died during the experiment, as well as those euthanized at the end of the study is shown in Table V. Abnormal gastric content (gummy, jelly like, with mucus) was generally observed in all the piglets, except those in the RHM group. Additionally, bloody mucus was observed in the stomach of the piglets that were euthanized from the PDHM and PDHM+LC PUFA+PF groups. Piglets from the RHM group had normal, physiological stomach content (normal, soft, cheese-like type digesta). Macroscopic features of enterocolitis were observed in piglets from the PDHM, PDHM+LC PUFA+PF and PDHM+LC PUFA+LF groups (Fig. 2). Almost all the piglets had intestinal edema in different

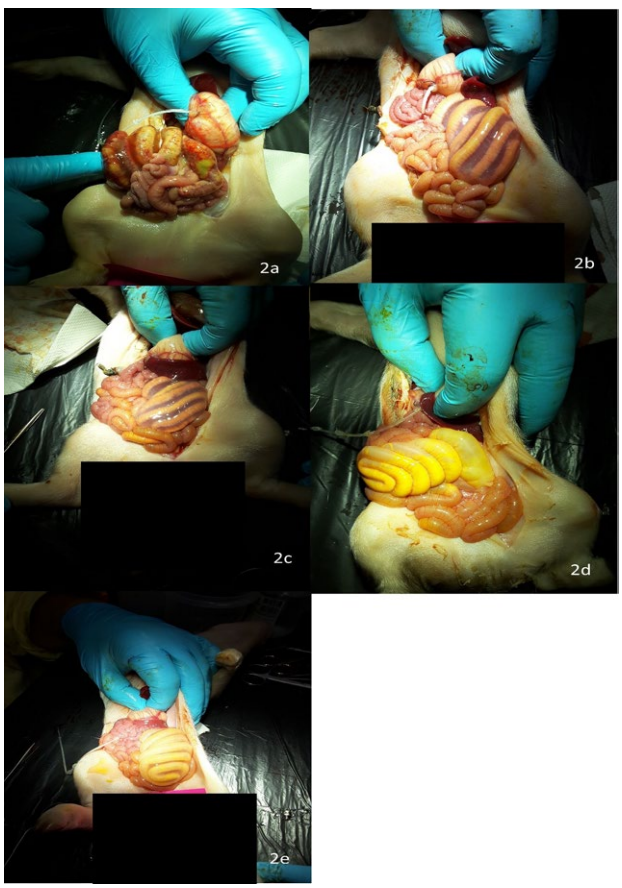

Fig. 2. *Examples of autopsy examination of euthanized piglets. Pig No. 4 from the PDHM group (a); Pig No. 4 from the PDHM+LC PUFA+PF group (**b**); Pig No. 4 from the PDHM+LC PUFA+LF group (**c**); Pig No. 1 from the PDHM+LC PUFA group (**d**); Pig No. 2 from the RHM group (**e**).*

stages which could be described as either slight (S) or large (L). Moreover, in some of the piglets the presence of small areas of intestinal hemorrhage was observed (groups: PDHM, PDHM+LC PUFA+PF, PDHM+LC PUFA+LF). Pneumatosis intestinalis was noted in the two piglets from the PDHM group. In the piglets from the RHM and PDHM+LC PUFA groups, only intestinal edema was detected. The respiratory tract of almost all of the piglets displayed features of immature lung development. The results of the *post-mortem* examination of the piglets that died during the 7-day study period and those that were euthanized at the end of the 7-day study period are shown in Fig. 1 and Fig. 2, respectively.

DISCUSSION

The current study aimed to compare the gastrointestinal effects of PDHM alone, PDHM supplemented with fat rich in LC PUFA and PDHM supplemented with LC PUFA with either liquid or powder fortifiers, in a porcine model of preterm infants. Our results may suggest some adverse effects of the traditional milk pasteurization process. This is of meaningful clinical importance since prematurity is accompanied by a general immaturity of the gastrointestinal tract, and thus the development of a milk feed that is well-tolerated by premature neonates is one of the major challenges in neonatology.

Almost all of the piglets displayed an intolerance to the milk feed, except for those in the RHM group. The feeding volume was below the targeted milk feed intake of 135 mL/kg/day (1). As a consequence, body weight gain of the piglets was generally low. However, it should be noted that due to the very

Table V. *Results of the post-mortem examination of the piglets.*

Groups	Piglet #	Stomach content	Intestinal Edema	Pneumatosis	Hemorrhage within gastrointestinal tract	Heart	Lungs	Day on which piglet died
PDHM	1	2	L	-	X			4
	2	2	S	-	-		I	
	3	2	L	X	-		I	
	4	3	L	X	X		I	
PDHM+LC PUFA	1	2	-	-	-		I	
	2	2	S	-	-			
	3	2	L	-	-		I	
	4	2	S	-	-		I	
PDHM+LC PUFA +PF	1	3	L	-	X			7
	2	2	L	-	-		I	
	3	3	S	-	X	hypertrophy	I	4
	4	2	S	-	X		I	
PDHM+LC PUFA +LF	1	2	L	X	X			7
	2	2	-	-	-			
	3	2	S	-	-		I	
	4	2	L	-	-		I	
RHM	1	1	S	-	-		I	
	2	1	-	-	-		I	
	3	1	S	-	-		I	
	4	1		-	-		I	

Stomach content classifications: 1) normal, soft, cheese-like digesta; 2) gummy, jelly-like, with mucus; 3) gummy, jelly-like, with bloody mucus

S: slight intestinal edema; L: large intestinal edema; Lungs: I-immature respiratory tract; PDHM: pasteurized donor human milk; LC PUFA: long chain polyunsaturated fatty acids, PF: powder fortifier; LF: liquid fortifier, RHM: raw human milk

short experimental period (7 days), we did not expect significant increases in the piglets' body weight at the end of the study. The less than 15% loss of body weight observed in some groups, seems to be a physiological phenomenon during the first week of life (17). The slight body weight gain in the PDHM and PDHM+LC PUFA+PF groups may be as a result of the significantly higher mass of digesta in the gastrointestinal tract (18). Previous studies comparing the effects of OMM and DHM on the general development of preterm neonates are scarce. However, some authors showed that PDHM does not promote the growth and development of preterm neonates as efficiently as OMM (13, 19).

DHM is usually prepared using the Holder pasteurization method (62.5°C for 30 minutes), which may have an adverse effect on the intestinal health of neonates and their resistance against bacterial infections (13). Similar observations were made in the present study using a porcine model of prematurity, where significant signs of the development of NEC were observed. Holder pasteurization may modify the physical properties of milk proteins, such as casein, and inactivate physiologically important molecules including erythropoietin and IL-10, which may lead to gastrointestinal intolerance and decreased protective effect of human milk against NEC (8, 9). Moreover, the Holder pasteurization process may decrease the antioxidant capacity of human milk, which may be important in the context of protection of immature infants against intestinal inflammation (13). In an observational study which focused on the analysis of the transcriptome in exfoliated epithelial intestinal cells from preterm infants, OMM was more efficient in inducing a differential expression of specific genes that may contribute to a more efficient response to a pro-oxidant challenge early in the postnatal period, compared to DHM (20). Moreover, it was shown that feeding with OMM is associated with a favorable gut microbiome colonization with a greater presence of *Bifidobacteriaceae* compared to that observed in preterms fed with DHM (21). The negative impact of the Holder pasteurization to milk composition encourages to search the new methods to better preserve the nutritional and biological properties of human milk, while assuring at least the same microbiological safety. The techniques include High-Temperature-Short-Time (HTST) pasteurization, High Pressure Processing (HPP), and Ultraviolet-C (UV-C) irradiation which are under tests (22).

It should be noted that thermally modified casein, after Holder pasteurization, becomes difficult to digest by pepsin (11). Thus, forms of insoluble conglomerates with milk fat remain in the stomach, as was clearly observed on autopsy in our piglets. These insoluble conglomerates may injure the gut mucosa, when they eventually pass into the small intestine, since they are not easily digested and serve as substrates for bacteria overgrowth which would cause bleeding and inflammation within the intestine. This theory seemed to be confirmed by the results of the present study, since jelly-type globules of undigested milk were observed in the stomach of all piglets fed PDHM, sometimes together with bloody mucus. This may suggest that these jelly-like conglomerates are not easily digested and thus could not be easily evacuated from the stomach of the preterm piglets. Poor PDHM tolerance, in comparison to RHM, was also confirmed by clinical observation of the piglets. All groups of preterm piglets fed PDHM displayed symptoms such as the unwillingness to stand, cold limbs, abdominal distension or dehydration syndrome more frequently compared to those in the RHM group. Moreover, only the piglets fed RHM passed normal stools, even though the RHM was from a foreign species - human. It would be interesting to repeat the study using similarly treated sow milk in preterm piglets. Upon *post-mortem* examination of the piglets that were euthanized following the 7-day study period, only in piglets from the RHM and PDHA+LC PUFA groups were features of pneumatosis and/or intestinal hemorrhage not detected.

Upon learning that feeding exclusively with OMM may not be sufficient to meet the needs of preterm infants, various efforts have been made to develop liquid or powder human milk fortifiers (23). Milk fortification is even more essential in the case of preterm neonates fed with PDHM due to the late lactational stage of the donor milk, which is less concentrated in nutrients than early milk

(24). Literature encourages starting human milk fortification when the volume reaches 50-100 mL/kg/d (6). In the current study, the milk fortification was started at the beginning of enteral feeding and was related to both the study aim and the assessment of food tolerance in premature neonates right after birth and throughout the short duration of the experiment. Although "Individualized Fortification" to optimize nutrient intake is now being recommended by the European Milk Bank Association, the "Standard Fortification" is still the most utilized regimen in neonatal intensive care units (6).

In the present study, bovine milk-based fortifiers, both in liquid and powder form, were used. The type of milk fortifier determines its nutritional composition. However, a study performed on a group of 150 preterm human infants showed no differences in the tolerance of preterm infants to liquid and powder milk fortifiers (25). No differences in the general health and well-being of the piglets (activity, symptoms) were observed between the groups fed PDHM with liquid fortifier and those fed PDHM with powder fortifier. However, upon analysis of stool consistency and frequency of defecation, an interesting phenomenon was observed. Almost all the piglets from the group supplemented with a liquid milk fortifier (with casein hydrolysate) did not pass any stools except for meconium.

It should be noted that the gastrointestinal tract of newborn pigs is probably even more immature (gut opening). Piglets born naturally only acquire the capacity to digest diets other than OMM much later (26). Moreover, during the study, the parenteral nutrition was not used to avoid the necessity of extension of the experimental period. The animals obtained the daily increased volume of the foreign species milk that may lead to gastrointestinal intolerance. On the other hand, our previous experiment showed no significant adverse effect of early enteral nutrition in the similar increase milk amounts (14).

During *post-mortem* examination, differences in the incidence of gastrointestinal hemorrhage and pneumatosis intestinalis among the groups were detected. Pneumatosis intestinalis, pathognomonic for NEC, was observed only in one piglet from the group fed PDHM+LC-PUFA LF and in two piglets from the PDHM group. Areas of intestinal hemorrhage were noted in both groups fed with fortified PDHM (liquid and powder). However, two piglets from the group fed PDHM+LC PUFA+PF died before the end of the 7-day study period. Potential side effects associated with the use of bovine milk-based fortifiers have been previously described (2, 23) and the use of human milk-based fortifiers are preferable over the bovine milk-based fortifiers due to the reduced mortality and morbidity of newborns (27). It has been shown that neonates exclusively receiving a human milk-based diet (OMM or DHM with human milk-based fortifier) have significantly lower rates of NEC compared to those fed preterm formula or human milk with a bovine milk-based fortifier (5, 27). Additionally, the use of PDHM with a bovine milk-based fortifier did not reduce the rates of NEC and other infections in a group of extremely premature infants when compared to preterm formula as a substitute for OMM (28). Unfortunately, in many countries, including Poland, human milk-based fortifiers are not available.

In the current study, the milk feeds were all supplemented with LC PUFA (DHA/ARA), except for the PDHM and RHM groups. DHA/ARA are essential to ensure proper growth, as well as brain and retinal development, including proper signal transduction, neurotransmission and neurogenesis (29). Infant formulas, as well as PDHM lack active bile salt-stimulated lipase (BSSL) leading to fat malabsorption including that of the crucial LC PUFAs - DHA/ARA. For these reasons, the FDA has recommended that infant formulas be supplemented with DHA and ARA at levels comparable to that found in human milk (29). However, the DHA and ARA in infant formulas, as well as in the milk feeds used in the present study, are not in the form of free fatty acids but rather as triglycerides, which could make the supplementation relatively ineffective since, as previously mentioned, infant formulas lack lipase making the neonate unable to digest lipids efficiently. Preterm infants obtain not more than 50% of the DHA that is normally accreted *in utero* (30). In the case of preterm piglets, another limitation for DHA/ARA absorption is the level

of plasma immunoglobulins. In a recent study on newborn, agammaglobulinemic pigs, Pierzynowska et al. (31) showed that ARA/DHA absorption is positively correlated with IgG level in the blood. Our piglets received a preparation of Ig directly after their delivery by caesarean section, ensuring the level of IgG was similar to those measured in full term piglets after gut closure. Considering the results of clinical observations and autopsy, we believe that the initial treatment with the Ig preparation improved DHA/ARA absorption, even though it was offered in the form of LC PUFA, which in turn improved the tolerance of the piglets to PDHM. Moreover, it was previously shown on the rat model that LC PUFA may indicate the protective properties on enterocytes by inhibition of intestinal platelet-activating factor receptor (PAFR) and Toll-like receptor (TLR) 4 gene expressions which are the key factors involved in experimental NEC pathogenesis (32). In our study, the piglets fed exclusively with RHM and piglets from the PDHM+LC-PUFA group revealed no severe symptoms of gastrointestinal inflammation.

Our results should however be interpreted with care due to the limited number of animals per group, although it has been previously shown that the porcine preterm model used in this study corresponds to preterm human neonates. The immunoglobulin infusion after birth should assure appropriate growth and development of the animals also in the case of prematurity (14).

In conclusion, it should be emphasized that the RHM was well-tolerated by the premature piglets, even though it is essentially from a foreign (human) species. The poor tolerance of piglets to pure PDHM and PDHM supplemented with fortifiers, suggests that the changes in milk composition which occur during the pasteurization process and the possible adverse effects of the post-Holder milk fortification process require further research. However, treatment with Ig and supplementation of the PDHM milk with LC PUFA may ameliorate the negative effects of pasteurization on the porcine model.

ACKNOWLEDGEMENTS

The authors would like to express their thanks to Dr Janine Donaldson for her constructive feedback and valuable input with regards to the writing of the manuscript.

The present study was supported by grants from Consorcium SGP + Group, Trelleborg, Sweden and The Royal Physiographic Society of Lund, Sweden.

REFERENCES

1. Agostoni C, Buonocore G, Carnielli VP, et al. ESPGHAN Committee on Nutrition. Enteral nutrient supply for preterm infants: commentary from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition Committee on Nutrition. J Pediatr Gastroenterol Nutr 2010; 50:85-91.
2. Dutta S, Singh B, Chessell L, et al. Guidelines for feeding very low birth weight infants. Nutrients 2015; 7:423-42.
3. Jain S, Mukhopadhyay K, Jain V, Kumar P. Slow versus rapid enteral feed in preterm neonates with antenatal absent end diastolic flow. J Matern Fetal Neonatal Med 2016; 29:2828-33.
4. Choi A, Fusch G, Rochow N, Fusch C. Target fortification of breast milk: predicting the final osmolality of the feeds. PLoS One 2016; 11(2):e0148941.
5. Cristofalo EA, Schanler RJ, Blanco CL, et al. Randomized trial of exclusive human milk versus preterm formula diets in extremely premature infants. J Pediatr 2013; 163:1592-5.
6. Arslanoglu S, Boquien CY, King C, et al. Fortification of human milk for preterm infants: update and recommendations of the European Milk Bank Association (EMBA) working group on human milk fortification. Front Pediatr 2019; 22:7:76.
7. Arslanoglu S, Corpeleijn W, Moro G, et al. ESPGHAN Committee on Nutrition. Donor human milk for preterm infants: current evidence and research directions. J Pediatr Gastroenterol Nutr 2013; 57:535-42.
8. Untalan PB, Keeney SE, Palkowetz KH, Rivera A, Goldman AS. Heat susceptibility of interleukin-10 and other cytokines in donor human milk. Breastfeed Med 2009; 4:137-44.
9. Baro C, Giribaldi M, Arslanoglu S, et al. Effect of two pasteurization methods on the protein content of

human milk. Front Biosci (Elite Ed) 2011; 3:818-29.
10. Henderson TR, Fay TN, Hamosh M. Effect of pasteurization on long chain polyunsaturated fatty acid levels and enzyme activities of human milk. J Pediatr 1998; 132:876-8.
11. Dupont D, Mandalari G, Mollé D, et al. Food processing increases casein resistance to simulated infant digestion. Mol Nutr Food Res 2010; 54:1677-89.
12. Casper C, Carnielli VP, Hascoet JM, et al. rhBSSL improves growth and LCPUFA absorption in preterm infants fed formula or pasteurized breast milk. J Pediatr Gastroenterol Nutr 2014; 59:61-69.
13. Hård AL, Nilsson AK, Lund AM, Hansen-Pupp I, Smith LEH, Hellström A. Review shows that donor milk does not promote the growth and development of preterm infants as well as maternal milk. Acta Paediatr 2019; 108:998-1007.
14. Socha-Banasiak A, Pierzynowski S, Woliński J, et al. The pig as a model for premature infants - the importance of immunoglobulin supplementation for growth and development. J Biol Regul Homeost Agents 2017; 31:87-92.
15. Goncharova K, Arevalo Sureda E, Wolinski J, Lozinska L, Weström B, Pierzynowski SG. Importance of maternal immunoglobulin transfer for cognitive function and neuronal plasticity – study on newborn piglet model. Plos One 2017**;** 28 2(6):e0180002.
16. Vandenplas Y, Szajewska H, Benninga M, et al. BITSS Study Group. Development of the Brussels Infant and Toddler Stool Scale ('BITSS'):protocol of the study. BMJ Open 2017; 7:e014620.
17. Anchieta LM, Xavier CC, Colosimo EA, Souza MF. Weight of preterm newborns during the first twelve weeks of life. Braz J Med Biol Res 2003; 36:761-70.
18. Moößeler A, Schwarzmaier T, Gregory PC, Piechotta M, Beyerbach M, Kamphues J. Pancreatic exocrine insufficiency affects not only digestibility of nutrients and growth, but also body composition and endocrinological parameters –study on piglets used as a model for children. Pancreat Disord Ther 2015; 5:S5.
19. Lund A, Löfqvist C, Pivodic A, Lundgren, P, Hård AL, Hellström A, Hansen-Pupp I. Unpasteurised maternal breast milk is positively associated with growth outcomes in extremely preterm infants. Acta Paediatrica 2019;10.1111.
20. Parra-Llorca A, Gormaz M, Lorente-Pozo S, et al. Impact of donor human milk in the preterm very low birth weight gut transcriptome profile by use of exfoliated intestinal cells. Nutrients 2019; 11:2677
21. Parra-Llorca A, Gormaz M, Alcántara C, Cernada M, Nuñez-Ramiro A, Vento M, Collado MC. Preterm gut microbiome depending on feeding type: significance of donor human milk. Front Microbiol 2018; 9:1376.
22. Moro GE, Billeaud C, Rachel B, et al. Processing of donor human milk: update and recommendations from the European Milk Bank Association (EMBA). Front Pediatr 2019;7:49.
23. Koletzko B, Poindexter B, Uauy R. Nutritional care of preterm infants: scientific basis and practical guidelines. World Rev Nutr Diet Basel 2014; 110:215-27.
24. Valentine CJ, Morrow G, Reisinger A, Dingess KA, Morrow AL, Rogers LK. Lactational stage of pasteurized human donor milk contributes to nutrient limitations for infants. Nutrients 2017; 9:302.
25. Moya F, Sisk PM, Walsh KR, Berseth CL. A new liquid human milk fortifier and linear growth in preterm infants. Pediatrics 2012; 130:e928-35.
26. Sangild PT, Thymann T, Schmidt M, Stoll B, Burrin DG, Buddington RK. Invited review: the preterm pig as a model in pediatric gastroenterology. J Anim Sci 2013; 91:4713-29.
27. Sullivan S, Schanler RJ, Kim JH, et al. An exclusively human milk-based diet is associated with a lower rate of necrotizing enterocolitis than a diet of human milk and bovine milk-based products. J Pediatr 2010; 156:562-67.
28. Schanler RJ, Lau C, Hurst NM, Smith EO. Randomized trial of donor human milk versus preterm formula as substitutes for mothers' own milk in the feeding of extremely premature infants. Pediatrics 2005; 116:400-406.
29. Martin CR, Cheesman A, Brown J, Makda M, Kutner AJ, DaSilva D. Factors Determining optimal fatty acid absorption in preterm infants. J Pediatr Gastroenterol Nutr 2016; 62:130-36.
30. Lapillonne A, Groh-Wargo S, Gonzalez CH, Uauy R. Lipid needs of preterm infants: updated recommendations. J Pediatr 2013; 162:37-47.
31. Pierzynowska K, Wolinski J, Weström B, Jazwiec R, Shmigel H, Pierzynowski SG. Absorption of

Polyunsaturated Fatty Acid (PUFA) is related to IgG blood levels of neonatal pigs during the first 48 hours postpartum. J Immunol Res 2020; 6,2020:3813250.

32. Lu J, Jilling T, Li D, Caplan MS. Polyunsaturated fatty acid supplementation alters proinflammatory gene expression and reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Pediatr Res 2007;61:427-32.