

# Effects on gastroesophageal reflux of donkey milk-derived human milk fortifier versus standard fortifier in preterm newborns. Additional data from Fortilat study.

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# Abstract

**Background :** Feeding intolerance, defined as the inability to digest enteral feeding, is a frequent diagnosis in very preterm infants. It is characterized by abdominal distension, delayed gastric emptying and increased frequency and severity of gastroesophageal reflux (GER). As seen in the FortiLat trial, human milk fortification with the new donkey milk-derived human milk fortifier (DF) seems to improve feeding tolerance in these infants. The aim of this ancillary study of the FortiLat trial was to evaluate the effects of using the DF compared with bovine milk-derived fortifier (BF) on GER in VLBW infants.

**Methods :** Over a total of 156 preterm infants enrolled into the FortiLat trial (gestational age <32 weeks and/or birth weight <1500 g) and randomized into BF-arm or DF-arm we selected all infants with clinical signs of GER and cardiorespiratory (CR) symptoms at day 21 of fortification. All the infants underwent CR and multichannel intraluminal impedance and pH (MII/pH) monitoring associated with gastric ultrasound to evaluate GER characteristics, GER-CR temporal associations and gastric emptying time.

**Results :** 10 infants were enrolled, 5 in the DF-arm. At MII/pH infants enrolled into the DF-arm showed a lower GER frequency than BF-arm infants: 2.02(1.95-3.26) vs 4.82(2.84-5.94) GER/hour ( $p=0.036$ ). No infant had a significant symptom association probability index between GER and CR events. Half gastric emptying time was similar in DF and BF-arm infants: 45.03(42.74-47.02) vs. 48.57(44.73-48.77) min. ( $p=0.744$ )

**Conclusions :** The use of donkey derived human milk fortifier reduced the GER frequency and consequently should be recommended in infants with feeding intolerance.

**Trial Registration :** ISRCTN -ISRCTN70022881. Registered 01May 2014 - Retrospectively registered, <http://www.isrctn.com/ISRCTN70022881>

## Background

Very preterm and very low birth weight (VLBW) infants frequently experience feeding intolerance (FI) in the first weeks of life. FI related to gastrointestinal anatomical and functional immaturity and decreased intestinal motility(1,2) and it is variably defined as the presence of emesis, visible bowel loops, increased abdominal girth and distension, presence of an abnormal gastric residual and gastro-esophageal reflux (GER).(3) GER is a physiological condition in preterm infants and as FI is related to immaturity of the gastrointestinal tract, expressed through lower esophageal sphincter incontinence. The presence of symptoms of FI as increased gastric residual and abdominal distension, together with lower esophageal sphincter immaturity, can increase the frequency and worsen the characteristics of GER. Among symptoms of FI regurgitation and vomiting are also characteristic signs of GER. However, in about 5% of cases infants manifest cardiorespiratory (CR) events (apnea, bradycardia, blood oxygen desaturation) as atypical signs of GER.(4–6) In this population a significant association between GER and CR events has been recently demonstrated in 11% of cases.(7) Even if the temporal and causal association is still controversial, recent studies have demonstrated that in some patients with particular GER characteristics,

there is a significant and causal association between these two events. The GER events involved in these associations are mainly weakly acidic suggesting that the empirical treatment with antacids is, in most of these cases, inappropriate.(4,7)

The proper approach to the problem of GER in preterm infants should be aimed at improving feeding tolerance by adopting techniques and behaviors involving nutrition strategies (thickening of feeds, positioning, slow feeding) and able to reduce the frequency of refluxes. Among these strategies the choice of the most proper nutrition can also influence GER. The use of breast or human milk is recommended but in preterm infants there is the need to supplement it with additional nutrients to meet their nutritional requirements.(8–11) Most of these supplements are fortifiers derived from bovine milk but concerns are arising as cow milk protein intake in the first month of life seems to be associated with allergies and intestinal inflammation in preterms.(12,13)

A new donkey milk-derived human milk fortifier (DF) was developed as an alternative to bovine milk-derived human milk fortifiers (BF). We tested it through an RCT performed in our Center (FortiLat trial). This study suggests that DF improves feeding tolerance when compared with standard BF showing a lower number of failures (necrotizing enterocolitis, FI, death) and lower risk of FI episodes.(14) The evaluation of GER and gastric emptying, as a marker of FI, was evaluated by multichannel intraluminal impedance and pH monitoring (MII/pH) and gastric ultrasound (US) as secondary endpoints of the FortiLat trial.(14,15)

Aim of this study is to evaluate the effects of the DF on VLBW and very preterm infants enrolled in the FortiLat trial with symptoms of GER and CR events.

## Methods

### Study design

All 156 infants included in the FortiLat trial (gestational age <32 weeks and/or birthweight  $\leq$ 1500 g., exclusively fed with human milk) were considered for the present study.

After informed written parental consent was obtained, infants were randomized 1:1 by a software-generated list in one of the following groups: the control group (BF-arm) underwent adjustable fortification with fortifier derived from bovine milk; the FortiLat group (DF-arm) underwent adjustable fortification with fortifier derived from donkey milk. The different composition of the two types of fortifiers can be observed in table 1.

**Table 1. Composition of BF and DF – Values per 100 g of product**

	BF	DF
Protein g (Nx6.25)	20.0	22.5
Carbohydrate (g)	66.0	59.0
Of which:		
Lactose (g)	6.0	59.0
		59
Maltodextrine (g)	60.0	0.0
Fat (g)	0.4	3.6
Kcal	385	390
Kcal/g protein	18.8	15.6
Calcium (mg)	1500	938
Phosphate (mg)	900	734
Osmolality (mosm/kg)	453	441
pH	6.8	6.7
Buffering capacity (mmol l-1/dph)	12.2	9.2

BF: bovine milk-derived human milk fortifier

DF: donkey milk-derived human milk fortifier

Details on eligibility criteria, sample size calculation, settings and randomization and are specified in the FortiLat trial.(15) This study followed the Consolidate Standards of Reporting Trials.

The detailed protocol of the FortiLat trial is described in our previous paper.(15)

At day 21 since beginning of fortification, infants showing typical (excessive regurgitations, vomiting, blenching) signs of GER and CR symptoms (apneas/desaturations/bradycardia, paleness, cyanosis)

were enrolled into the present study. Enrolled infants underwent synchronized MII/pH and CR monitoring and then gastric emptying US assessment as specified below.

## **MII/pH monitoring**

The evaluation of GER events was performed through MII/pH monitoring (Sleuth monitoring system). The recommendations for the procedure and the identification of GER events have already been described in a previous work by our group.(16) A MII-GER event was defined as a retrograde drop of impedance to 50% of the basal value for at least 5 seconds, starting in the most distal channel, proceeding to one or more proximal channels and followed by a recovery of the impedance baseline values. A reflux reaching the two most proximal channels is defined as proximal GER; pH-GER events, defined as drop of pH value below 4 longer than 5 seconds and not associated with a MII-GER event, were considered in the analysis as well.

The following features were evaluated:

- MII-GER frequency, expressed as reflux events/h;
- Bolus reflux extent (BRE): the proximal extent reached by the refluxate, indicated by the number of channels sequentially involved, expressed as number of channels;
- Bolus clearance time (BCT): duration of a reflux from the drop to 50% of the impedance baseline value to its recovery recorded in the distal impedance channel, expressed as seconds;
- Bolus exposure index (BEI): total percentage of time bolus reflux was detected by MII, expressed as a percentage;
- pH-GER frequency, expressed as reflux events/h;

Reflux index (RI): total percentage of time with pH <4, expressed as a percentage. Each MII-GER event was defined as weakly acidic ( $4 \leq \text{pH} < 7$ ), acidic ( $\text{pH} < 4$ ), or weakly alkaline ( $\text{pH} \geq 7$ ) according to the minimum pH value reached during each event.(16–18)

## **CR monitoring**

The VitaGuard VG3100 system (Getemed Medizin und Information stechnik AG, Teltow, Germany), equipped with Signal Extraction Technology (Masimo Corp. Irvine, CA, USA), was used to perform CR monitoring, heart rate, transcutaneous blood oxygen saturation, and respiratory rate were measured during synchronized CR and MII/pH monitoring by a pulse-oximetry sensor placed on the right wrist or foot and three cardiac electrodes placed on the chest. CR tracings were “visually” analyzed by a trained operator blinded to the MII/pH tracings using VitaWin3® evaluation software.

CR events were defined as episodes of apnoea lasting more than 20 seconds or over 5 seconds if followed by desaturation or bradycardia, episodes of desaturation with blood oxygen saturation below 80%, and episodes of bradycardia with heart rate below 80 beats per minute.(7,19) Minimum duration of bradycardia and blood oxygen desaturation events to be considered for the analysis was 4 seconds.

## Synchronized MII/pH and CR monitoring

CR and MII/pH monitors were synchronized using an external reference (i.e. Internet time) and digitally marking each tracing at the beginning of the study. The specific times of each CR event were adjusted for the offset between the clocks of the CR and MII/pH monitors. The symptom association probability (SAP) index was calculated to evaluate temporal associations between GER and CR events.(20) SAP index value >95% identified patients with a significant number of GER-CR associations.

The primary outcome of the study was the MII-GER frequency, secondary outcomes were reflux characteristics, half gastric emptying time (T/2) and the SAP index.

## Gastric US

Gastric antral transit was used as a proxy of gastric emptying. This was determined by measuring ultrasonically the changes in the antral cross-sectional area (ACSA) which occur after a feed (21). Serial measurements of ACSA were made before, during and after administration of feeds every 10 minutes for 90 minutes.

The gastric emptying curve was calculated from the ultrasound series and represented by the best fitting polynomial function ( $R^2 > 0.98$ .  $P < 0.001$ ) of the ACSA values and on these bases the half gastric emptying time (T/2), calculated as the time required to achieve a 50% reduction in ACSA values was obtained.(21) Half gastric emptying time was evaluated as a secondary outcome.

## Statistical analysis

Statistical analyses were performed using the STATISTICA software package for Windows (StatSoft, Inc., Tulsa, Oklahoma, USA). Results are expressed as median and interquartile range (IQR) if not otherwise specified, and the p value was set at 0.05. The distribution of the variables was evaluated by the Shapiro-Wilk test. The Student's *t*-test was used to evaluate differences between normally distributed variables, and the Mann-Whitney test for unpaired data was used for non-normally distributed variables.

## Results

Out of 156 newborns enrolled in the FortiLat trial,(22) 11 (7.05%) met the inclusion criteria. Six were allocated in the BF-arm and 5 in the DF-arm. One infant, allocated in the BF-arm, was excluded because of his weight (1120 g) at 21 days of fortification, considered too low to perform MII/pH safely. A total of 10 (6.4%) infants were enrolled and underwent synchronized MII/pH and CR monitoring in order to evaluate GER and gastric US in order to evaluate gastric emptying. A flow chart is available in Figure 1.

These 10 infants included in the analysis had a mean±SD gestational age of 30±1.3 weeks and birth weight of 1215±424 grams. No adverse effects associated with MII/pH and gastric US have been

reported in any patients enrolled.

The anthropometric characteristics of infants included in this study are summarized in Table 2.

**Table 2. Anthropometric characteristics per arm at enrollment, expressed in mean±sd.**

	BF-arm	DF-arm
Cases (no.)	5 (50%)	5 (50%)
Gender (male)	3 (60%)	2 (40%)
Gestational age (wks)	30.0 ±0.7	30.2 ±1.8
Birth weight (g)	1398 ±469	1032 ±320.2
Birth length (cm)	37.9 ±4.8	36.9 ±1.4
Birth head circumference (cm)	27.9 ±2.5	26.8 ±2.1
Age (days)	35.2 ±6.8	39.6 ±9.1
Post menstrual age (wks)	35 ±0.9	36 ±2.3
Weight (g)	1908±449	1644±386
Length (cm)	42.6 ±4.3	40.56 ±3.6
Head circumference (cm)	29.52 ±2.5	29.5 ±1.9

BF-arm: arm treated with bovine milk-derived human milk fortifier

DF-arm: arm treated with donkey milk-derived human milk fortifier

Overall, MII showed a median MII-GER frequency per hour of 3.05 (2.22-4.43) with a BCT of 19.29 (16.29-23.36) seconds, a BRE of 3.42 (3.26-3.71) cm and a BEI of 1.46 (1.18-3.04) %. The median frequency of MII-GER was 2.47 (1.50-4.04), 0.50 (0.14-0.99) and 0.08 (0.00-0.21) GER/hours, for weakly acidic, acid and weakly alkaline GER respectively. pH-metry showed a median GER frequency per hour of 1.49 (0.61-2.14). The median half gastric emptying time was 46.02 (43.24-48.72) minutes.

DF-arm infants had a significant lower frequency of MII-GER with a median of 2.02 (1.95-3.26) versus 4.82 (2.84-5.94) in BF-arm infants (p=0.036). Detailed MII/pH and gastric emptying data are reported in Table 3. We found a total of 73 CR events, 6 (2-14) events per patient: 7 (9.59%) apneas >20 seconds, 12 (16.44%) apneas <20 seconds associated with desaturations and/or bradycardias, 49 (67.12%) isolated desaturations, and 5 (6.58%) isolated bradycardias. The median value for blood oxygen desaturation events was 75 (71-78%), the lowest 54%. Median value for bradycardia was 67 (63-78) beats per minute with a lowest of 58 beats per minute. The median duration of apnea events was 12 (8-22) seconds, the longest apnea recorded was 38 seconds long. Detailed CR monitoring data are reported in Table 3.

**Table 3. MII/pH monitoring, gastric US and CR monitoring data. Comparison between BF-arm and DF-arm.**

			BF	DF	p
		Cases (no.)	5 (50%)	5 (50%)	-
MII-GER	MII-GER	GER Frequency (GER/h)	4.82 (2.84-5.94)	2.02 (1.95-3.26)	0.036
		proximal GER Frequency (GER/h)	1.97 (0.94-2.82)	0.95 (0.79-1.42)	0.246
		BCT (sec)	17.91 (16.45-24.14)	20.66 (16.24-21.01)	0.895
		BRE (cm)	3.44 (3.20-3.71)	3.41 (3.26-3.71)	0.705
		BEI (%)	3.41 (1.28-3.73)	1.44 (0.96-1.47)	0.064
	MII-WA-GER	GER Frequency (GER/h)	4.50 (2.68-4.82)	1.43 (1.35-2.47)	0.024
		proximal GER Frequency (GER/h)	1.89 (0.94-2.10)	0.48 (0.43-1.26)	0.156
		BCT (sec)	18.90 (16.70-25.04)	20.37 (19.77-21.64)	0.774
		BRE (cm)	3.37 (3.21-3.93)	3.40 (3.24-3.91)	0.865
	MII-ACID-GER	GER Frequency (GER/h)	0.08 (0.05-1.13)	0.53 (0.47-0.78)	0.611
		proximal GER Frequency (GER/h)	0.06 (0.00-0.08)	0.32 (0.11-0.48)	0.435
		BCT (sec)	18.60 (16.70-20.30)	21.88 (11.25-23.62)	0.825
		BRE (cm)	3.37 (3.00-3.73)	3.33 (3.33-4.00)	0.335
	MII-WALK-GER	GER Frequency (GER/h)	0.13 (0.00-0.24)	0.06 (0.00-0.11)	0.359
		proximal GER Frequency (GER/h)	0.00 (0.00-0.00)	0.00 (0.00-0.05)	0.397
		BCT (sec)	11.40 (0.00-14.10)	8.70 (0.00-10.32)	0.981
		BRE (cm)	2.33 (0.00-2.67)	2.00 (0.00-2.67)	0.895
pH-GER	pH-GER	GER Frequency (GER/h)	0.55 (0.05-0.77)	1.89 (1.79-2.22)	0.122
		RI (%)	1.29 (0.04-2.23)	3.89 (3.60-6.35)	0.388
Gastric US	Gastric emptying time T/2 (min)		45.03 (42.74-47.02)	48.57 (44.73-48.77)	0.744
CR-monitor	CR events	Apnea (no.)	4	3	-
		Apnea + Desaturation +/- Bradycardia	6	6	-
		Desaturation	28	21	-
		Bradycardia	2	3	-

BF: bovine milk-derived human milk fortifier; DF: donkey milk-derived human milk fortifier; MII: multichannel intraluminal impedance; GER: gastroesophageal reflux; BCT: bolus clearance time; BRE: bolus reflux extent; BEI: bolus exposure index; WA: weakly acidic; WALK: weakly alkaline; RI: reflux index; US: ultrasound; CR: cardiorespiratory

No infant had a significant association between GER-CR events (SAP =  $0.86 \pm 0.07$ ) and there were no differences in SAP between DF-arm and BF-arm infants.

The post-hoc analysis based on the obtained sample size considering an  $\alpha$  values of 0.5, revealed a power of 80.6% referred to the primary outcome (MII-GER frequency) values observed in the two arms.

## Discussion

This research represents an ancillary study of FortiLat trial,(15) which involved infants born at gestational age < 32 weeks and/or birthweight  $\leq 1500$  grams, exclusively fed with fortified human milk, randomly assigned to DF-arm or BF-arm. In the present study we evaluated by “state-of-the-art” techniques a research question not addressed in the FortiLat trial main paper, that was the effect of donkey milk-derived human milk fortifier on gastroesophageal reflux in infants with GER typical symptoms and cardiorespiratory events. The main outcome of the study was the reflux frequency and we found a significant reduction of the frequency of all MII-GER and of weakly acidic-MII-GER in the DF-arm compared to BF-arm infants.

In preterm infants the incidence of the diagnosis of GER based on symptoms is highly variable in literature and ranges between 2 and 26%.(23) In our neonatal intensive care unit we found that the burden of preterm infants with GER symptoms was 11%, and almost 38% of them showed respiratory symptoms associated with GER typical symptoms. (16) Thus, we expected to find a population with typical GER symptoms and cardiorespiratory showed symptoms of approximately 6.5%. According with these data, over the 156 enrolled in the FortiLat trial we found 10 (6.4%) infants eligible for this study. Although the sample studied was very small, a post-hoc analysis showed that the study achieved a power of 80% considering our main outcome.

Since the two fortifiers are isoproteic and isocaloric, we speculate that the quality of donkey milk protein could be responsible of this result.

The lower frequency of refluxes in the DF group, mainly affects the weakly acidic ones, which are the most frequent in infants and could be symptomatic,(16) on the contrary, the incidence of acid refluxes in this arm resulted higher. The RI evaluated with pH-metry, although not pathological, was higher in the DF-arm infants. We speculate this is probably due to the lower buffering capacity of DF compared to BF (Table 1). We previously reported that the low molecular weight of the proteins contained in hydrolyzed milk formula produced an increased buffer capacity respect to the same not-hydrolyzed milk formula.(24) The lower buffering capacity of DF could be explained by the different composition of the two fortifiers since DF is composed by protein macromolecules while BF by free amino acids.

Previous studies demonstrated an increased risk of necrotizing enterocolitis due to the neutralization of the antimicrobial effect of the physiological low gastric pH in preterm infants treated with antacid. In this context, the minor buffering effects on gastric pH of DM could be a protective factor, prevent infections and necrotizing enterocolitis, and should be studied in further larger prospective trials. Despite the

expectations, we failed to detect any differences in gastric emptying time between the two arms. However, it should be considered that the method used is characterized by a great variability between the infants evaluated, a much larger sample should be studied to detect significant differences between the two groups.

Finally, we have evaluated GER-CR temporal associations. GER and CR events are very frequent in preterm infants, but patients with a significant temporal association between these events are very rare. The execution of the MII/pH synchronized with CR monitoring allowed us to exclude this association in all the subjects studied, avoiding unnecessary and potentially harmful therapies.

It is known that GER is one of the signs of FI and our data are in line with the FortiLat trial results, suggesting a favorable effect of the donkey milk fortifier on it.

## **Conclusions**

The use of donkey derived human milk fortifier could reduce the frequency of refluxes and consequently be recommended in infants with feeding intolerance as the VLBW ones.

## **Abbreviations**

VLBW – Very Low Birth Weight

FI - Feeding Intolerance;

GER - Gastroesophageal Reflux;

CR – Cardiorespiratory;

DF – Donkey Fortifier;

BF – Bovine Fortifier;

MII/pH - Multichannel Intraluminal Impedance and pH Monitoring;

US - Ultrasound

BRE – Bolus Reflux Extent

BCT – Bolus Clearance Time

BEI – Bolus Exposure Index

RI – Reflux Index

SAP – Symptom Association Probability

## Declarations

### Ethics approval and consent to participate

This study was performed in the Neonatal Intensive Care Unit of Turin University and it was approved by its Ethics Committee (AN: 0025847. 27/05/2014) and registered (<http://www.isrctn.com/ISRCTN70022881>) after the trial starting date. The study protocol was evaluated by JPGN Editorial Office. Recruitment period was 27/11/2014 to 22/12/2016. Written informed consent was obtained from the parents of all included newborns before enrollment.

### Consent for publication

Not applicable

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The full trial protocol is available on <http://www.isrctn.com/ISRCTN70022881>.

### Competing interests

The authors declare that they have no competing interests

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### Authors' contributions

FC, PT, AC, LC, MG, GM, CP and EB participated in designing the study. CP and AC equally contributed in coordinating the clinical trial. FC, EME and AP performed MII-PH and gastric ultrasonography assessment. FC wrote the first draft and conducted statistical analysis. CR and EM wrote final the manuscript. All authors read and approved the final manuscript.

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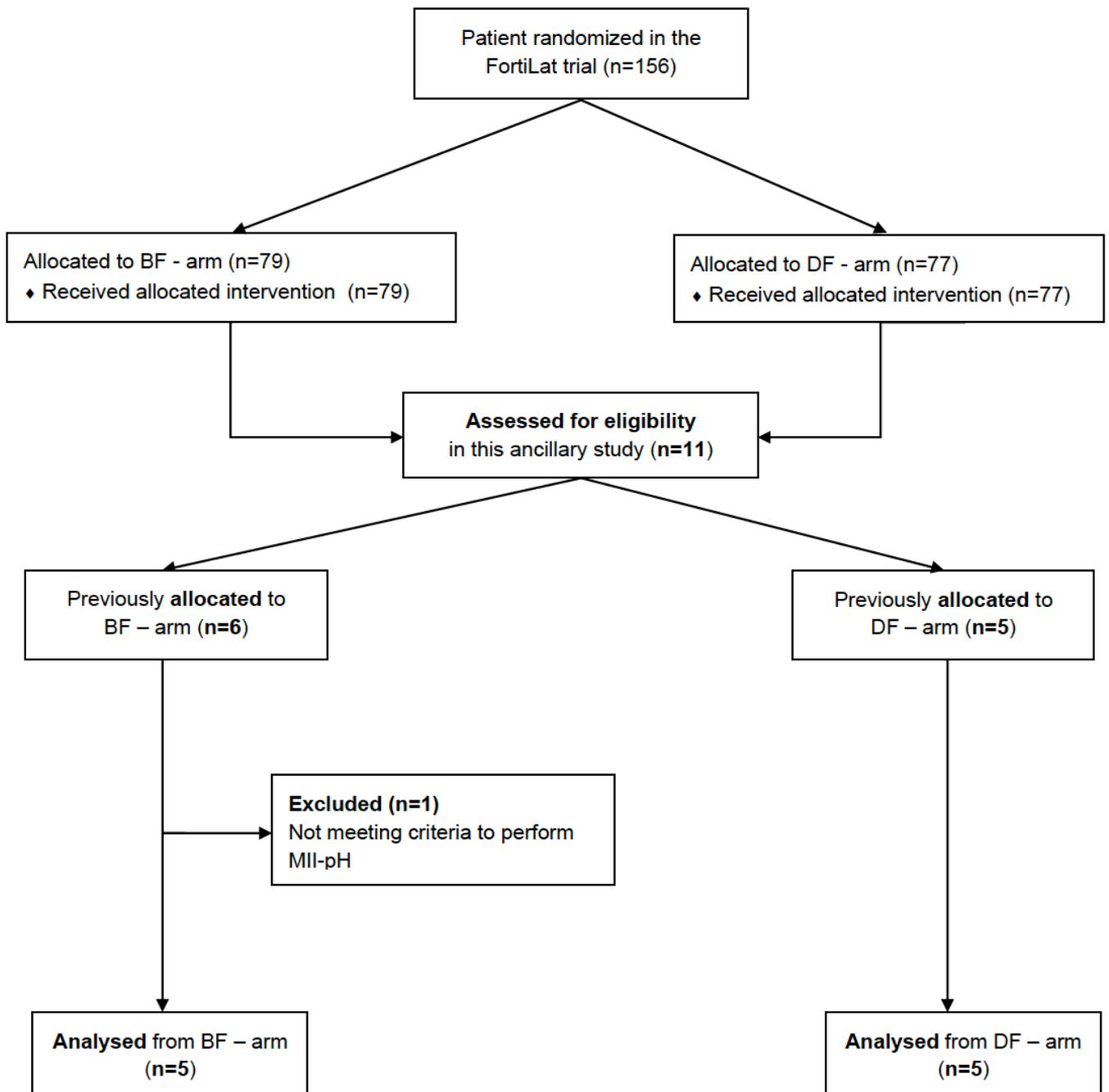
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## Figures



**Figure 1**

Diagram of patients' allocation. BF-arm: arm treated with bovine milk-derived human milk fortifier. DF-arm: arm treated with donkey milk-derived human milk fortifier. MII-pH: multichannel intraluminal impedance and pH monitoring.

## Supplementary Files

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