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Randomized Control Trials

Bovine colostrum as a fortifier to human milk in very preterm infants – A randomized controlled trial (FortiColos)☆

Agnethe May Ahnfeldt [a], Lise Aunsholt [a, b], Bo Moelholm Hansen [c], Bente Hoest [d], Valdís Jóhannsdóttir [e], Susanne Soendergaard Kappel [a, b], Anja Klamer [f], Sören Möller [g, l], Bertha Kanijo Moeller [h], Per Torp Sangild [a, b, e, l], Ann Lawaetz Skovgaard [i], Gerrit van Hall [j], Louise Dyrberg Vibede [k], Gitte Zachariassen [e, g, l, *]

[a] Section of Comparative Pediatrics and Nutrition, University of Copenhagen, Frederiksberg, Denmark
[b] Department of Neonatology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark
[c] Department of Pediatrics, Copenhagen University Hospital North Zealand, Hilleroed, Denmark
[d] Department of Pediatrics, Aarhus University Hospital, Aarhus, Denmark
[e] Hans Christian Andersen Children's Hospital, Odense University Hospital, Odense, Denmark
[f] Department of Pediatrics, Hospital Lillebaelt, Kolding, Denmark
[g] Open Patient Data Explorative Network, Odense University Hospital, Odense, Denmark
[h] Department of Pediatrics, Copenhagen University Hospital Hvidovre, Hvidovre, Denmark
[i] Department of Pediatrics, Hospital Soenderjylland, Aabenraa, Denmark
[j] Clinical Metabolomics Core Facility, Clinical Biochemistry, Rigshospitalet, Copenhagen & Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark
[k] Department of Pediatrics, Copenhagen University Hospital Herlev, Herlev, Denmark
[l] Department of Clinical Research, University of Southern Denmark, Odense, Denmark

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SUMMARY

Background: Human milk for very preterm infants need fortification for optimal growth and development but the optimal fortification product remains to be identified.
Aims: To investigate feasibility, safety and preliminary efficacy on growth and blood biochemistry when using intact bovine colostrum (BC) as a fortifier to human milk in very preterm infants.
Methods: In an open-label, multicenter, randomized controlled pilot trial (infants 26–31 weeks' gestation), mother's own milk or donor human milk was fortified with powdered BC ($n = 115$) or a conventional fortifier (CF, bovine-milk-based, $n = 117$) until 35 weeks' postmenstrual age. Fortifiers and additional micronutrients were added to human milk according to local guidelines to achieve optimal growth (additional protein up to +1.4 g protein/100 mL human milk). Anthropometry was recorded weekly. Clinical morbidities including necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) were recorded. Clinical biochemistry included plasma amino acid (AA) levels to assess protein metabolic responses to the new fortifier.
Results: A total of 232 infants, gestational age (GA) 28.5 ± 1.4 (weeks + days), fulfilled inclusion criteria. Birthweight, GA and delta Z scores from birth to end of intervention on weight, length or head circumference did not differ between groups, nor between the subgroups of small for gestational age infants. Likewise, incidence of NEC (BC: 3/115 vs. CF: 5/117, $p = 0.72$, unadjusted values), LOS (BC: 23/113 vs. CF: 14/116, $p = 0.08$) and other morbidities did not differ. BC infants received more protein than CF infants (+10%, $p < 0.05$) and showed several elevated AA levels (+10–40%, $p < 0.05$).
Conclusion: Infants fortified with BC showed similar growth but received more protein and showed a moderate increase in plasma AA-levels, compared with CF. Adjustments in protein composition and micronutrients in BC-based fortifiers may be required to fully suit the needs for very preterm infants.

Abbreviations: Conventional Fortifier, (CF); Bovine Colostrum, (BC).

☆ All authors are listed alphabetically by their last names.

* Corresponding author. Hans Christian Andersen Children's Hospital, Odense University Hospital, University of Southern Denmark, Odense, Denmark
E-mail address: Gitte.Zachariassen@rsyd.dk (G. Zachariassen).

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1. Introduction

Very preterm infants (<32 weeks of gestational age (GA)) are born with immature organs and high nutrient metabolism, which together with their increased risk of postnatal morbidities, predispose them to extra-uterine growth restriction [1]. Many studies have demonstrated that poor growth, resulting in extra-uterine growth restriction during hospitalization, is correlated with impaired neurological outcomes later in life [2]. It is therefore crucial to optimize nutrition supply in very preterm infants to improve their growth conditions and brain development [3,4]. Mother's own milk (MOM) is considered the best choice as the first enteral feed in these infants, and donor human milk (DHM) as the second-best choice [5]. However, neither MOM nor DHM can meet the relatively high nutritional requirements, especially for protein and minerals. It has therefore become widely accepted to fortify human milk (HM, e.g. MOM or DHM) with nutrient fortifiers to meet the nutritional requirements. Increasing feed osmolality by adding (pre-hydrolyzed) nutrient fortifiers may disturb gut motility and increase feeding intolerance [6–8], and there have been concerns that processed bovine milk products may predispose to necrotizing enterocolitis (NEC) and late-onset sepsis (LOS), similar to the risks induced by formula feeding [9–11].

Bovine colostrum (BC) is the first milk from cows produced within the first 24 h after parturition. BC is closely adapted to provide nutrition, immunization and microbial protection to newborn calves due to its high levels of proteins that include bioactive components such as immunoglobulins, insulin-like growth factors, lactoferrin, lysozyme and lactoperoxidase [12,13]. Many of these bioactive milk components may be active across different mammalian species [12,14–16] and remain active in BC even after low-temperature pasteurization, gentle spray-drying and irradiation [17–19]. In studies using preterm piglets as a model for preterm infants, exclusive BC feeding improved growth and protected against NEC and LOS [20–23]. In pilot studies on preterm infants, BC to supplement HM during the first 1–2 weeks had no adverse effects, but plasma tyrosine (Tyr) levels were elevated when BC was fed as the main part of the diet during the first days of life [24,25]. BC used as a fortifier, may after weeks continue to supply the gut with digestible nutrients and protective milk factors compared to fortification based on concentrated bovine or human milk products.

On this background, we hypothesized that an intact, powdered BC product is a feasible fortifier for HM to very preterm infants, will induce similar growth as a conventional bovine-milk based fortifier, is clinical (including blood biochemistry) safe and without any plasma AA imbalances. Our aim was to investigate the preliminary efficacy on growth and investigate blood biochemistry including AA-values when using intact BC as a fortifier to human milk in very preterm infants. Considering the possibility that the most preterm (below 29 weeks GA) and small for gestational age (SGA) infants could be particularly sensitive to fortification of HM, sub analyses of the data were done for these subgroups of infants.

2. Material and methods

2.1. Trial design and sample size

An open-label, randomized controlled trial was conducted between November 2017 and October 2020 at eight neonatal units in Denmark; four in Western Denmark (Aarhus University Hospital, Aarhus; Hospital Soenderjylland, Aabenraa; Hospital Lillebaelt, Kolding; Odense University Hospital, Odense) and four in Eastern Denmark (Copenhagen University Hospital Herlev, Herlev; Copenhagen University Hospital Hvidovre, Hvidovre; Copenhagen University Hospital North Zealand, Hilleroed; Copenhagen University Hospital Rigshospitalet). The trial was approved by the Scientific Ethical Committee of the Region of Southern Denmark (S-20170095) and the Danish Data Protection Agency (17/33672). An independent data safety monitoring board (DSMB) reviewed trial data and safety during the enrolment period, incorporating preliminary assessment of key outcomes and potential adverse events.

The protocol was registered at ClinicalTrials.gov (NCT03537365) and was considered a pilot trial, considering that combined NEC and LOS incidence as primary outcome would require approximately 1500 infants, as noted in our published protocol [26]. Since this study was considered a pilot trial, a conventional sample size calculation, using only one primary outcome, was not required. The aim was to include 200 infants (100 per group), which was expected to give sufficient strength to demonstrate effects on the chosen outcomes on blood biochemistry including AA and growth. For growth outcomes, a change in Z score of −0.25 (0.5 SD) from birth to end of intervention was considered clinically relevant, based on reported growth variability in Danish very preterm infants. Using a power of 90%, alpha value of 5% (two-sided) and correction for dropouts and twins, this resulted in a total estimated sample size of 204. Statistical analyses will be performed blindly on both intention-to-treat and per protocol basis. Data on feeding tolerance and bowel habits have been published elsewhere [27].

2.2. Trial participants

Eligible participants were very preterm infants born between gestational age (GA) 26+0 and 30+6 (weeks+days), who were in need of nutrient fortification, as judged by the responsible neonatologists, and hospitalized at one of the participating units until at least 34+6 weeks and days' postmenstrual age (PMA = GA + weeks and/or days since birth). Written parental consent from both parents were obtained prior to intervention. Infants with known major congenital anomalies and birth defects, who had gastrointestinal surgery or received formula feeding prior to enrolment were not included. Infants who never initiated fortification due to severe diseases or death were also excluded from the study. If parents withdrew their consent during the intervention, data were still collected and used in the analyses if allowed by the parents, otherwise the infant was excluded from the study.

2.3. Enrolment and randomization

Neonatologists and trained staff recruited the infants and obtained oral and written informed parental consent before the infants reached 100 mL/kg/day of enteral feeding. Participants were randomized using an online randomization program, REDCap [28] in a server at the Region of Southern Denmark with a 1:1 allocation, random block sizes of 4–6, and stratified by SGA (defined as a birth weight (BW) Z score ≤ -2 standard deviations (SD) for GA). Multiple birth infants were assigned to the same group randomized by the first-born sibling in order to save the sparse amount of MOM with no waste when adding fortification to MOM for multiple birth infants. Included infants were randomly assigned to receive either BC (BC group, ColoDan powder, Biofiber-Damino, Gesten, Denmark) or a conventional bovine milk-based fortifier (CF group, PreNan FM85 powder, Nestlé, Switzerland). Both fortifiers were provided to the investigators at no costs. Personnel were not blinded to the treatments due to the different ways fortifier powders had to be handled and mixed into HM. After mixing with HM, the solutions could also be distinguished by their color and texture, thereby

preventing blinding of feeding procedures. Safety and sterility of the BC product were achieved by gentle spray-drying, low-temperature pasteurization (63 °C, 30 min) and gamma irradiation, controlled by food safety authorities. Both fortifier products were powder based and did not displace any volume of HM.

2.4. Intervention

Units followed their local nutrition guideline on supplementation with micro and macro nutrients based on international recommendations [5]. Recommended feeding in relation to achieve growth targets followed guidelines at each unit and were not fixed by the protocol. All units initiated enteral tube feeding with HM (MOM or DHM) just after birth. Mothers were encouraged to express MOM as soon as possible after birth and frozen holder pasteurized pooled DHM was provided from two Danish HM banks if MOM was limited or absent. Fortification of HM was initiated when enteral feeding volumes reached 100–140 mL/kg/day and when blood urea nitrogen (BUN) value was <5 mmol/L, as required according to our ethical approval. When BUN values were ≥5 mmol/L, fortification was delayed until levels were below 5 mmol/L (even if enteral feeding volumes >140 mL/kg/day). No BUN value was targeted after start of fortification. Parenteral nutrition (PN) was provided to infants according to local guidelines. PN-volume was reduced when enteral feeding volumes were increased to maintain the daily aimed volume (in ml)/kg/day. Initially 1.0 g of fortification powder was added to 100 mL of HM in both groups, which was increased to a maximum of 2.8 g BC/100 mL HM and 4.0 g CF/100 mL HM within 3–4 of days to meet the maximum added protein of 1.4 g (according to the recommendation of 3.5–4.0 g protein/kg/day [29,30]) in both groups. Fortification was administered according to detailed instructions in a standard operating procedure (SOP) (only in Danish) for clinical personnel. Enteral feeding was initially provided as 12 meals pr day. The BC-product contains intact protein while CF contains partly hydrolyzed protein. Detailed contents of macro- and micro-nutrients and individual AA levels from the two products are provided in Table 1 for the maximum amount of fortifier added to 100 mL of human milk.

One unit in Eastern Denmark used targeted fortification [31], based on the protein level in MOM. Remaining units used individualized fortification procedures, based on local guidelines using weekly growth and, in some units, combined with BUN values [26,32]. Supplementation with multi-vitamins, vitamin D, iron and phosphorus were given according to a detailed SOP (only in Danish) that corresponded to international guidelines [5]. The intervention continued until the infants reached PMA 34+6 weeks or was stopped earlier if the infant was moved to a non-participating unit, were fed a preterm formula prior to week 34+6, participated in an early discharge program prior to 34+6 weeks, or suffered from diseases that led to discontinuation of fortification. If partial breastfeeding was achieved before end of intervention, the fortifier was added to the HM for tube feeding. If the infants needed fortification after PMA 34+6 weeks (as judged by their growth rates and clinical condition), infants in both groups continued with the conventional fortification product as long as needed according to local guidelines. We realized during the trial period that randomization was uneven between Eastern and Western Denmark. Nutrition strategies (use of DHM and MOM) also differed more than expected between Eastern and Western Denmark, and probiotics were only provided to infants in 4 (Eastern Denmark) of 8 units.

2.5. Outcome measures

Primary outcome was growth. Secondary outcomes were morbidities including infections (LOS), NEC (Bell's classification system [33] with or without surgery), clinical biochemistry and AA plasma profile. Adverse events were defined as any such event occurring in the infant from start of intervention until final discharge. These included clinical signs and symptoms, or irregular results of routine blood samples that did not necessarily have any known causal relationship with the intervention.

Table 1
Composition of macronutrients and amino acids in CF and BC.

	CF product[a]	BC product[a]
	Per 4.0 g	Per 2.8 g
Energy, kcal	17.0	13.0
Macronutrients:		
Carbohydrate, g	1.3	0.48
Fat, g	0.7	0.64
Protein, g	1.4	1.40
- of which immunoglobulin G, g	–	0.62
- of which casein, g	–	0.44
Micronutrients:		
Calcium, mg	76	25.8
Phosphorus, mg	44	22.7
Zinc, mg	0.96	0.20
Sodium, mg	36.7	7.9
Iron, mg	1.8	<0.3
Vitamin D-3, μg	3.5	<0.01
Vitamin A, μg	333	27.8
Vitamin E, mg	3.8	0.05
Vitamin C, mg	19	0
Amino acids:		
Alanine, mg	79	52
Arginine, mg	34	53
Aspartate, mg	169	109
Citrulline, mg	0[b]	0[b]
Glutamate, mg	225	208
Glycine, mg	22	33
Histidine, mg	28	33
Isoleucine, mg	79	60
Leucine, mg	186	123
Lysine, mg	170	106
Methionine, mg	34	28
Ornithine, mg	2[b]	1[b]
Phenylalanine, mg	55	62
Proline, mg	64	108
Serine, mg	65	99
Taurine, mg	2[b]	1[b]
Threonine, mg	76	78
Tyrosine, mg	46	66
Valine, mg	78	99
Osmolarity, mOsm/L[c]	409	334

[a] Nutrient levels provided by the manufacturers, amino acid levels analyzed separately after product hydrolysis and as part of intact protein.
[b] The amino acid was not present in product protein.
[c] Osmolarity measured within 24 h of adding fortifier to donor human milk (osmolality 295 mOsm/L).

2.6. Data collection

Clinical data collected from electronic medical records included anthropometry at birth and weekly (weight in gram; crown-heel length in cm; head-circumference (HC, occipital-frontal in cm using a measuring tape) until end of intervention, and finally at discharge. Reasons for preterm birth (e.g. maternal complications with abruptio placenta, chorioamnionitis, rupture of membranes, preeclampsia), early onset sepsis (EOS) (antibiotic treatment initiated within 48 h from birth and for 5 days or more), LOS (at least 5 days of antibiotic treatment, with or without increased C-reactive protein (CRP) levels, or a positive bacterial culture in blood or cerebral spinal fluid). Medication given to the infant during hospitalization, type and time periods for parenteral/enteral nutrition, clinical biochemistry including CRP, white blood cell count and results from bacterial culture in cases of suspected sepsis. Plasma AA

profile, hemoglobin, phosphate, calcium, pH, BUN and sodium levels were recorded at baseline and after two weeks of fortification. During the study period, nurses and/or parents filled out a paper case report form (CRF) after each meal during the intervention period. Data collected from CRFs included feeding volumes, type of milk (MOM and/or DHM), amount and type of fortifier (grams of BC or CF). All data were entered into the online database, REDCap.

Blood and stool samples were collected up to two days before the first fortified meal and approximately on day 7 and 14 after start of fortification. Blood samples were collected about 1.5–2 h after the last enteral feeding and prior to the next meal. A maximum of 500 μL EDTA-stabilized blood was collected at each time point and was immediately cooled and centrifuged (2500×*g*, 4 °C, 10 min) within the first hour, and plasma kept frozen (−60 to −80 °C), together with stool samples (stored for later analyses). Plasma AA levels were determined by reverse-phase HPLC [34]. Other analyses performed on fecal and blood samples will be reported separately.

2.7. Statistical analyses

Baseline characteristics are presented as means and corresponding standard deviations (SD), medians and corresponding min and max values or counts (n) and percentages and Student's t-test or Wilcoxon rank sum test was used to compare the distribution of continuous variables between groups and chi-square or Fisher's exact test for categorical variables (e.g. all variables in the table for characteristics). Log rank test was used for time-to-event data. All numerical data was checked for normal distribution and logarithmic transformation was performed if needed.

Growth data (weight, length and HC) was transformed into Z scores, separately for each sex [35]. Nested linear mixed effect regression models (including a random intercept for each participating infant and nesting for siblings) were used for longitudinal growth data measured at weekly time points from birth until end of intervention, and from birth until discharge while linear regression models were used for data analyzed at specific time points. A logistic regression model was used to investigate incidences of NEC, LOS, bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP) and intraventricular hemorrhage (IVH) and a Cox proportional hazards model was used for time-to-event data.

Main analyses were adjusted for predefined fixed effects, including fortification group, GA subgroups (GA at birth 26+0 to 28+6 vs. 29+0 to 30+6) and presence of SGA (yes/no). For all analyses of SGA infants, only fortification group and GA subgroups were used for adjustment.

Growth data were analyzed using both intention-to-treat (ITT) and per-protocol (PP) infant cohorts. Infants were excluded from the PP analyses if they were given fortification for less than two weeks. ITT data is presented, unless otherwise indicated. Moreover, the SGA infants were analyzed separately. Sensitivity analyses were made based on the ITT data, adjusting for Eastern versus Western Denmark, as a fixed effect in the statistical models. This sub analysis was performed because we realized during the trial period that randomization was uneven between Eastern and Western Denmark, nutrition strategies differed more than expected, and only infants in Eastern Denmark received probiotics.

Statistical significance was defined as p values < 0.05. All analyses were performed using the statistical software R (version 4.0.0, R Foundation for Statistical Computing, Vienna, Austria) and Stata version 16 (StataCorp, Lakeway Drive, TX 77845, USA). Graphical illustrations were produced in GraphPad Prism (version 8.3.0, GraphPad Software, La Jolla California, USA).

3. Results

3.1. Recruitment

The initial cohort consisted of 590 infants. Of these, 137 did not meet the inclusion criteria. Parents declined participation for 85 infants, and 126 infants did not participate for other reasons (e.g., parents did not read nor speak Danish or English or the mother was critically ill). A total of 242 infants were randomized (Fig. 1). Ten infants were later excluded due to major congenital anomalies with Downs syndrome (n = 1), abdominal vessel defect (a possible variation of a vitellini cord with no blood flow to part of the gut) (n = 1), never received fortification due to bloody stools prior to intervention (n = 2), intracardial thrombosis with severe oedema and death (n = 1), death prior to intervention (n = 2), withdrawal by parental request (n = 3). In total, 232 infants remained in the ITT analyses with 219 in the PP analyses.

3.2. Baseline characteristics

There were no significant differences in baseline characteristics at birth or within the first days of life between the groups regarding e.g. antenatal steroids, GA, sex, weight, length, HC, SGA or Apgar score at 5 min. The proportion of multiple births was similar between groups, however only the CF group contained triplets (three sets). A total number of 162/230 (70%) infants received PN. No significant difference in number of infants receiving PN or days with PN (until day 8 in BC vs. day 9 in CF infants). There was no difference between groups regarding antibiotic treatment at birth (total 30% of infants treated) or in EOS or LOS diagnosis prior to intervention (total 11% diagnosed with EOS and 10% with LOS prior to intervention). The number of infants receiving ventilator treatment was also similar between groups (Table 2).

All infants had reached full enteral feeding (160 mL/kg/day) on postnatal day 8–9. In both groups, MOM was introduced as enteral feed from day 1 after birth, but the proportion of meals with MOM at start of fortification was lower among infants later to be fortified with BC vs. CF infants (76 vs. 86%, $p < 0.001$, Table 2).

3.3. Growth variables

The infants regained their BW at postnatal day 11 in both groups (Table 3). The mean weight Z score at start of intervention was similar across groups (−2.1 to −2.2 SDS) while it tended to be marginally lower in BC infants at the end of intervention (−1.5 vs. −1.2 SDS, $p = 0.07$), but not at final discharge (−1.3 vs. −1.2 SDS, $p = 0.3$, Table 3, Fig. 2). In other terms, the mean weight delta Z score from birth until end of intervention did not differ between BC and CF infants (−0.3 vs. −0.1 SDS, $p = 0.2$) or from birth until discharge (−0.1 in both groups), showing that the majority of infants in both groups achieved catch-up growth during hospitalization. The weight Z scores was analyzed as repeated measures over time from birth until end of intervention, and from birth until discharge and did not differ between groups ($p = 0.10$ and 0.13, respectively). Likewise, there were no differences in Z scores on length or HC.

SGA infants reached catch-up growth during the intervention period with a weight delta Z score from birth until end of intervention of 0.1 vs. 0.3 SDS, respectively (non significant between groups, Table 3, Fig. 2). These weight delta Z scores increased further until discharge, reaching 0.6 vs. 0.5 SDS, with no differences between BC and CF SGA infants. Further, there was no significant differences between groups on length and HC Z scores from birth until end of intervention, or until discharge in SGA infants, when

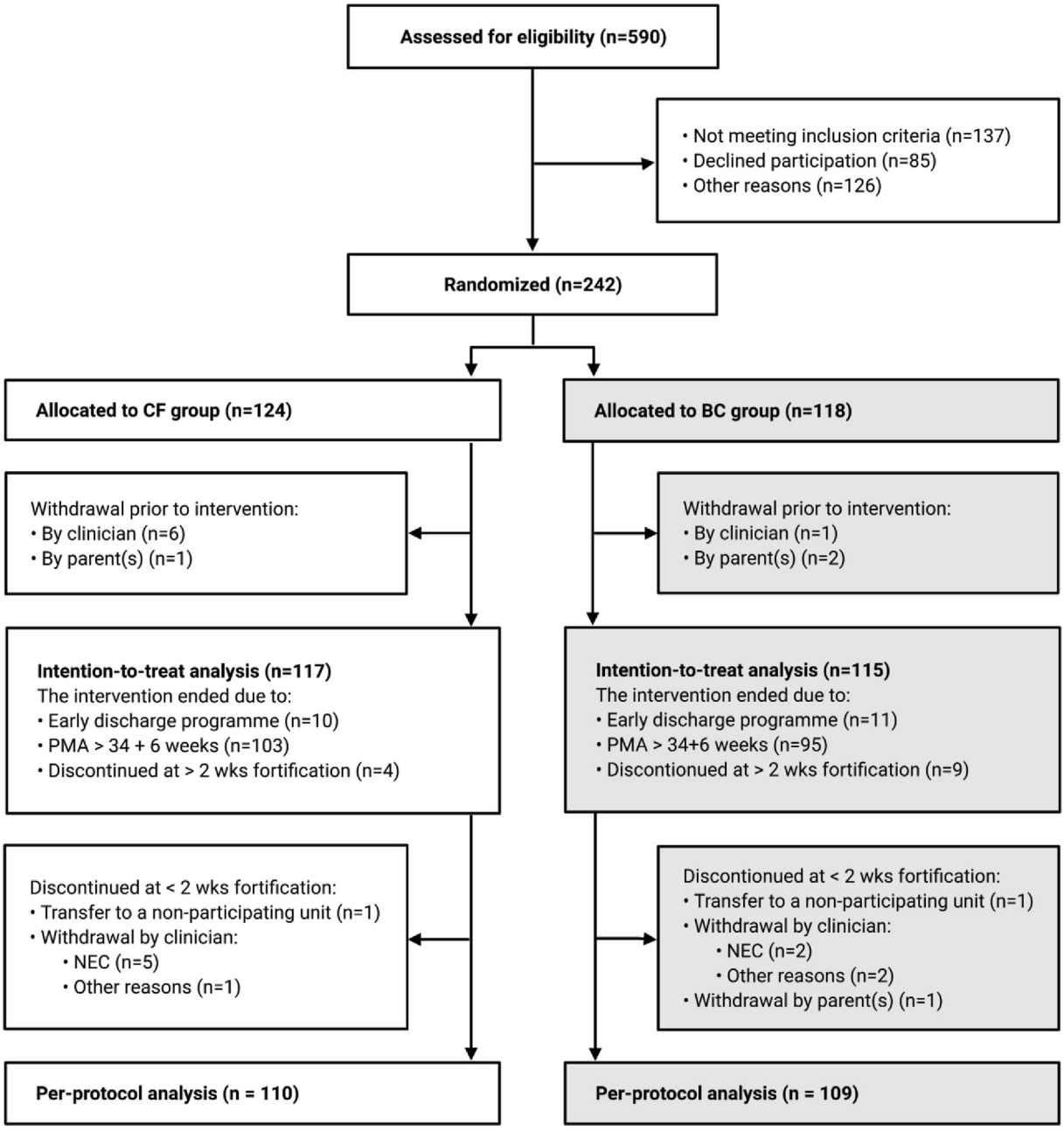

Fig. 1. Participant flow chart.

calculated as repeated measures over time (ITT with and without sensitivity analyses and PP, Table 3). Anthropometric data, especially on length, can be missing due to infants not measured (forgotten by staff or unstable infant).

3.4. Incidence of NEC, LOS and other morbidities

Three BC infants were diagnosed with NEC of which two had surgery, and five CF infants were diagnosed with NEC of which none had surgery (Table 3). The proportion of infants given antibiotics at any time during hospitalization (including less than 5 days, and before the intervention started) was 58% (133/229), with significant difference between fortification groups (BC 65% vs 51%, $p = 0.03$). During the intervention, there was no significant difference in the incidence of LOS between groups, but the proportion was highest in BC compared to CF infants (20 vs. 12%, $p = 0.08$). Infants with the lowest compared to the highest GA had an increased risk of LOS (OR 4.7, $p = 0.001$, 95% CI 1.9 to 11.4, data not shown). Incidences of BPD, ROP and IVH were similar between groups (Table 3).

3.5. Enteral nutrition intakes

The intervention with nutrient fortification started on postnatal day 8 [3–17] (median (min–max)) in the BC group and on day 9 [4–26] (median (min–max)) in the CF group ($p = 0.09$, Table 4), at a time when HM feeding volumes had reached 150–160 mL/kg/day (80–180 mL/kg/day) with only 87 of 232 (38%) infants starting fortification before 140 mL/kg/day (43 in BC and 44 CF group). We do not have the exact number of infants with delayed start of fortification due to BUN values $\geq$ 5 mmol/L or other reasons. While

Table 2
Baseline characteristics for included infants.

Characteristics	CF group		BC group		*p*-value
	N		*N*		
GA at birth, wk + d, median (min–max)	117	28+5 (26+0 – 30+6)	115	28+6 (26+0 – 30+6)	0.51
GA groups, 26+0 to 28+6 / 29+0 to 30+6, n/n	117	64/53	115	61/54	0.80
SGA, n (%)	117	26 (22)	115	28 (24)	0.70
Girls/boys, n/n	117	53/64	115	46/69	0.50
Birth weight:					
All infants, g, mean ± SD	117	1164 ± 323	115	1170 ± 333	0.89
All infants, Z score, mean ± SD	117	−1.1 ± 1.2	115	−1.2 ± 1.2	0.22
All girls, g, mean ± SD	53	1165 ± 306	46	1077 ± 285	0.14
All boys, g, mean ± SD	64	1162 ± 338	69	1232 ± 349	0.25
SGA infants, Z score, median (min–max)	26	−2.64 (−4.37 to −2.11)	28	−2.91 (−4.43 to −2.01)	0.29
Birth length:					
All infants, cm, mean ± SD	117	37.4 ± 3.4	115	37.5 ± 3.7	0.85
All infants, Z score, mean ± SD	117	−1.4 ± 1.8	115	−1.4 ± 2.0	0.95
Girls, cm, mean ± SD	53	37.6 ± 3.3	46	36.9 ± 3.6	0.37
Boys, cm, mean ± SD	64	37.3 ± 3.4	69	37.9 ± 3.8	0.34
Birth HC:					
All infants, cm, mean ± SD	117	26.3 ± 2.1	115	26.5 ± 2.2	0.61
All infants, Z score, mean ± SD	117	−0.8 ± 1.0	115	−0.7 ± 1.0	0.54
All girls, cm, mean ± SD	53	26.4 ± 2.1	46	25.8 ± 2.1	0.18
All boys, cm, mean ± SD	64	26.2 ± 2.0	69	26.9 ± 2.2	0.07
Single/multiple birth(s), n/n	117	78/39	115	81/34	0.63
Antenatal steroids (min. 1 ds), n (%)	117	110 (94)	115	112 (97)	0.35
Complications during birth:					
Preeclampsia, n (%)	113	23 (20)	113	26 (23)	0.81
Preterm premature rupture of membranes, n (%)	117	25 (21)	115	25 (22)	0.95
Chorioamnionitis, n (%)	114	3 (3)	115	5 (4)	0.29
Placental abruption, n (%)	115	7 (6)	113	13 (12)	0.15
C-section, n (%)	117	87 (74)	115	80 (70)	0.51
Apgar score <7, 5m, n (%)	110	8 (7)	108	13 (12)	0.23
Nutrition:					
Parenteral nutrition, n (%)	116	85 (73)	114	77 (68)	0.34
Parenteral nutrition, d, mean ± SD	116	9 ± 4	114	8 ± 2.4	0.47
Time to full enteral feeding (160 mL/kg/day), DOL, median (min–max)	115	9 (4–26)	113	8 (4–17)	0.60
DHM given the first time, DOL, median (min–max)	116	0 (0–2)	111	0 (0–3)	0.01
MOM given the first time, DOL, median (min–max)	107	1 (0–7)	106	1 (0–11)	0.11
Only MOM, DOL, median (min–max)	84	4 (0–19)	79	4 (0–42)	0.25
Only MOM at start of intervention (% meals)	100	86	102	76	<0.001
Antibiotics:	116		113		
At birth, n (%)		29 (25)		39 (35)	0.15
EOS ≤48h after birth, n (%)		12 (10)		13 (12)	0.83
LOS pre-intervention, n (%)		15 (13)		9 (8)	0.28
Ventilator treatment: n (%)	115	30 (26)	114	27 (23)	0.76

fortified, the BC infants received greater amounts of HM than CF infants (week 2: 167 vs. 159 mL/kg/day and repeated measures over time, both $p < 0.001$, Table 4). Normal range of HM feeding volumes in Danish NICU's is 150–200 mL/kg/day (solely enteral feeding). The daily amount of protein from only fortification added to HM in week 2 was not higher ($p = 0.10$) in the BC group, but significantly higher when calculating total protein from fortification, MOM (estimated 1.7 g (week 1) and 1.5 g (week 2) protein/100 mL) [36] and/or DHM (estimated 1.1 g protein/100 mL), resulting in higher total protein intake for BC infants in week 2 ($p = 0.006$, Table 4). There was a tendency to a lower proportion of MOM feeding in the BC infants at end of intervention (73 vs. 85% MOM, $p = 0.06$) and at discharge ($p < 0.05$, Table 4). The infants stopped nutrient fortification at PMA 36+2 and 37+0 weeks, respectively (BC vs. CF, $p = 0.03$). BC group was discharged at PMA 40+1 vs. CF-group at PMA 39+1 weeks, respectively, $p = 0.01$. No difference was detected for BUN values after 2 weeks of fortification (Table 5).

3.6. Clinical biochemistry and plasma AA values

Clinical biochemistry measured before the first fortified meal was similar between groups, except for ionized calcium (BC: 1.29 vs. CF: 1.32 mmol/L, $p = 0.04$). After two weeks of fortification, BC infants showed reduced sodium (135.6 vs. 137.1 mmol/L), base excess (0.59 vs. 2.82 mmol/L) and pH values (7.33 vs. 7.35, all $p < 0.05$, Table 5). Lactate and pCO_2 levels were similar between groups (data not shown).

After two weeks of fortification, mean levels of many AAs were elevated in 96 BC vs. 98 CF infants (Supplementary Fig. 1 "Amino acids"), with the most pronounced increases observed for tyrosine and valine (+35%, $p < 0.001$). Across all measured AAs, levels were 11% higher in BC vs. CF infants ($p < 0.001$). Combined, both essential AAs (EAAs: His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val) and branched-chain AAs (BCAAs: Val, Leu, Ile) were increased in BC infants (+20%, $p < 0.001$), together with reduced Gly:Val ratio (−20%, $p < 0.001$, a marker of protein deficiency [37]). The BCAA per aromatic AA (Phe, Tyr, Trp) proportion was unchanged (a measure of liver maturation [38]) but ornithine levels were increased in BC (+19%, $p < 0.05$, a urea cycle metabolite). Levels of citrulline (a marker gut enterocyte metabolism) and kynurenine (a tryptophan metabolite) were unchanged. Lys is the only AA with lowered level in plasma of BC infants and Lys content was also 30% lower in the BC versus CF protein. The essential BCAAs, Leu and Ile were reduced in BC versus CF protein without effects on plasma AAs, contrasting Val that showed +30% increases in both BC product and BC infant plasma. The number of infants reaching the low ranges for one or

Table 3
Clinical outcomes and morbidities until end of intervention and discharge.

Clinical outcomes	Intention-to-treat analyses						Per-protocol analyses				
	CF group		BC group		p-value	Sensitivity p value	CF group		BC group		p value
	N		N				N		N		
Days to regain birth weight, mean ± SD	115	11 ± 4	111	11 ± 4	0.14	0.21	109	11 ± 4	104	11 ± 4	0.06
Anthropometry at start of intervention (wk0):											
PMA, wk+d, mean ± SD (days)	117	29+6 ± 1.3	115	29+6 ± 1.3	0.94	0.96	110	29+6 ± 1.3	108	29+6 ± 1.3	0.97
Weight Z score, mean ± SD	117	−2.1 ± 1.0	114	−2.2 ± 0.9	0.41	0.39	110	−2.1 ± 1.0	107	−2.2 ± 0.9	0.67
Length Z score, mean ± SD	103	−1.9 ± 1.8	93	−2.0 ± 1.8	0.71	0.74	97	−1.9 ± 1.8	88	−1.9 ± 1.8	0.78
HC Z score, mean ± SD	107	−1.5 ± 0.9	101	−1.4 ± 0.9	0.68	0.65	101	−1.5 ± 0.9	95	−1.4 ± 0.9	0.55
Weight z score among SGA, mean ± SD	26	−3.4 ± 0.5	28	−3.5 ± 0.6	0.91	0.64	24	−3.4 ± 0.5	25	−3.5 ± 0.6	0.78
Anthropometry at the end of intervention:											
PMA, wk+d, mean ± SD (days)	117	34+6 ± 0.4	115	34+5 ± 0.6	0.19	0.21	110	34+6 ± 0.3	108	34+6 ± 0.3	0.20
Weight Z score, mean ± SD	117	−1.2 ± 1.1	112	−1.5 ± 1.1	0.07	0.09	110	−1.2 ± 1.1	106	−1.4 ± 1.1	0.09
Length Z score, mean ± SD	108	−1.6 ± 1.6	104	−2.0 ± 1.9	0.25	0.33	103	−1.6 ± 1.6	99	−1.9 ± 1.8	0.50
HC Z score, mean ± SD	108	−0.6 ± 0.8	102	−0.7 ± 1.1	0.99	0.88	103	−0.6 ± 0.8	96	−0.7 ± 1.1	0.57
Weight Z score among SGA, mean ± SD	26	−2.5 ± 0.7	28	−2.8 ± 0.7	0.23	0.25	24	−2.4 ± 0.7	25	−2.8 ± 0.7	0.18
Anthropometry at final discharge:											
PMA, wk+d, mean ± SD (days)	117	39+1 ± 2.1	113	40+2 ± 3.5	0.004	0.005	110	39+0 ± 2.2	108	40+0 ± 3.3	0.01
Weight Z score, mean ± SD	117	−1.2 ± 1.1	113	−1.3 ± 1.0	0.28	0.31	110	−1.1 ± 1.0	108	−1.2 ± 1.0	0.47
Length Z score, mean ± SD	114	−1.4 ± 1.5	104	−1.5 ± 1.7	0.84	0.83	107	−1.3 ± 1.5	100	−1.5 ± 1.6	0.86
HC Z score, mean ± SD	116	0.1 ± 0.9	102	0.2 ± 1.0	0.68	0.73	109	−0.1 ± 0.9	98	−0.2 ± 0.9	0.86
Weight Z score among SGA, mean ± SD	26	−2.3 ± 0.8	27	−2.3 ± 0.7	0.72	0.99	24	−2.3 ± 0.8	25	−2.3 ± 0.7	0.87
Difference (Δ) in Z score from birth until end of intervention:											
Δ weight Z score, mean ± SD	117	−0.1 ± 0.8	112	−0.3 ± 0.7	0.17	0.24	110	−0.1 ± 0.7	106	−0.3 ± 0.6	0.15
Δ length Z score, mean ± SD	94	−0.2 ± 1.4	93	−0.5 ± 1.3	0.18	0.21	89	−0.2 ± 1.3	89	−0.4 ± 1.3	0.28
Δ head circumference, Z score, mean ± SD	96	0.1 ± 0.8	90	0.0 ± 0.8	0.63	0.77	91	0.1 ± 0.8	85	0.0 ± 0.8	0.86
Δ weight Z score among SGA, mean ± SD	26	0.3 ± 0.6	28	0.1 ± 0.6	0.13	0.34	24	0.4 ± 0.5	25	0.1 ± 0.6	0.09
Difference (Δ) in Z score from birth until final discharge:											
Δ weight Z score, mean ± SD	117	−0.1 ± 0.9	113	−0.1 ± 0.9	0.65	0.73	110	−0.1 ± 0.9	108	−0.1 ± 0.8	0.79
Δ length Z score, mean ± SD	101	0.1 ± 1.4	91	−0.1 ± 2.0	0.22	0.19	94	0.1 ± 1.4	89	0.0 ± 1.9	0.25
Δ HC Z score, mean ± SD	104	0.7 ± 0.9	89	0.5 ± 0.9	0.29	0.33	97	0.7 ± 0.9	86	0.5 ± 0.9	0.33
Δ weight Z score among SGA, mean ± SD	26	0.5 ± 0.7	27	0.6 ± 0.6	0.72	0.68	24	0.5 ± 0.7	25	0.6 ± 0.5	0.88
LOS (AB ≥ 5 d after start intervention), n (%)	116	14 (12)	113	23 (20)	0.08	0.07	–	–	–	–	–
NEC ≥2, n (%)	117	5 (4)	115	3 (3)	0.72	–	–	–	–	–	–
Surgery, n (%)	5	0 (0)	3	2 (1)	–	–	–	–	–	–	–
BPD, n (%)	111	17 (15)	114	24[a] (21)	0.55	–	–	–	–	–	–
Moderate, FiO2 <30%, n (%)	17	15 (88)	23	16 (70)	0.39	–	–	–	–	–	–
Severe, FiO2 >30%, n (%)	17	2 (12)	23	7 (30)			–	–		–	
ROP, n (%)	112	8[a] (7)	108	12 (11)	0.58	–	–	–	–	–	–
Level 1, n (%)	7	1 (14)	12	0 (0)	0.58	–	–	–	–	–	–
Level 2, n (%)	7	5 (71)	12	11 (92)			–	–	–	–	
Level 3, n (%)	7	1 (14)	12	1 (8)			–	–	–	–	
IVH, n (%)	115	15 (13)	114	22 (19)	0.22	–	–	–	–	–	–
Grade 1, n (%)	15	8 (53)	22	14 (63)	0.80	–	–	–	–	–	–
Grade 2, n (%)	15	2 (13)	22	4 (18)			–	–	–	–	
Grade 3, n (%)	15	1 (6)	22	0 (0)			–	–	–	–	
Grade 4, n (%)	15	4 (27)	22	4 (18)			–	–	–	–	

[a] Missing data on severity from one infant.

more AAs did not differ (BC: 5, CF: 2, $p > 0.05$). Neither did the number of infants with at least one EAA being below reported reference values for unfortified preterm infants [39] differ between groups (BC: 24 vs. CF: 33; $p > 0.05$).

3.7. Adverse events

Two SGA infants (BW Z scores −3.4 and −4.3, respectively) were diagnosed with metabolic bone disease (MBD) based on high parathyroid hormone (PTH) levels measured during and after intervention (20 and 34 pmol/L, respectively). Fortification with BC did not affect hemoglobin levels (7.1 vs 6.8 mmol/L, $p = 0.5$) or number of infants receiving blood transfusion (31 vs. 34%, $p = 0.92$). Details are available in (*Supplementary Adverse events FortiColos*).

3.8. Sub analysis for Eastern and Western Denmark

Parameters reported separately for units in Eastern vs. Western Denmark in (*Supplementary Eastern and Western* Table 1). The proportion of infants (randomly) recruited to the BC group tended to be higher in Eastern Denmark (56 vs. 44%, $p = 0.11$) with no differences in GA, BW, BW Z score, SGA or age at start of fortification.

4. Discussion

In our clinical trial we show that BC fortification of HM induced similar growth, clinical and biochemical outcomes as a conventional bovine-milk-based fortifier, with or without being SGA at birth. The number of infants diagnosed with NEC, LOS, IVH, ROP or BPD did not differ between groups. For clinical biochemistry, BC fortification moderately reduced blood pH, base excess and increased of many AA levels, although mean values remained within normal reference values for preterm infants. Our results are based on comparison with a chosen control fortifier (FM85, Nestlé) and results might differ when using other commercial bovine-milk- or human-milk-based fortifiers.

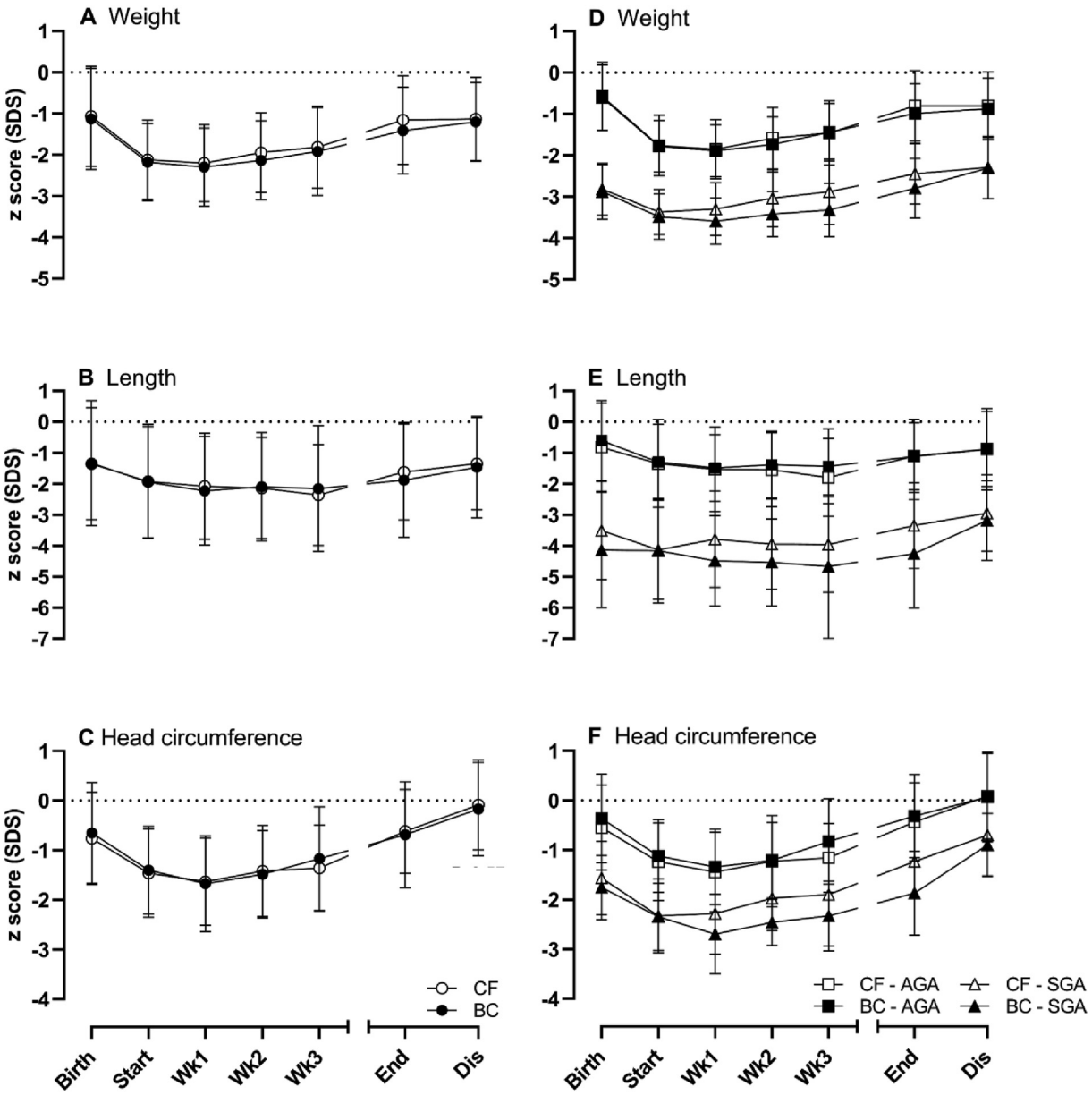

Fig. 2. Growth during intervention and until discharge across included infants and for the subgroup of small for gestational age (SGA) infants. A–C: Mean Z scores (SDS) for body weight, length and head circumference in very preterm infants receiving human milk fortified with bovine colostrum or a conventional fortifier from birth until end of intervention (End) and to discharge (Dis). D–F: Mean Z scores for infants born appropriate for gestational age (AGA, defined as birth weight > −2 SD) or SGA. Start of intervention (Start) was on postnatal day 3–26. Number of observations, see Tables 2 and 3.

Our rationale for testing BC, as a new nutrient fortifier for very preterm infants was related to its high contents of protein and milk bioactive components (including immunoglobulin G, IgG), and its possible benefits to growth, NEC and LOS, based on a series of preterm pig studies [20–23,40–46]. Our study was not powered to detect a difference in morbidities including NEC and LOS, and the incidence of NEC was very low (8/232, 3.4%, with only two patients requiring surgery). We cannot explain the tendency towards more BC infants treated with antibiotics, but overall, relatively few infants were treated (at birth 30%, EOS 11% and LOS 24%) compared with other studies [47,48].

4.1. Bovine colostrum

In preterm infants, parts of BC protein may pass the gut undigested, leading to less BC protein being metabolically available but also allowing intact BC proteins like immunoglobulins to remain bioactive and modulate mucosal immunity [14]. Negligible amounts of luminal BC immunoglobulins are absorbed intact by preterm infants [24,25] but such bioactive proteins may have systemic effects by reducing luminal gut mucosal permeability and inflammation, in turn affecting systemic immunity. These mechanisms may explain that BC supplementation to formula-fed preterm infants induced systemic immune effects (more T regulatory cells) in preterm infants in an RCT from Egypt [49]. The apparent gut and immune benefits of BC in the Egyptian and our previous studies (with BC fed just after birth to replace DHM) cannot be compared directly to the results of the present trial. Using BC as a fortifier may have limited gut and immune benefits, but further evidence will be obtained from ongoing analyses on gut microbiota, hormones and blood immune markers.

Table 4
Enteral nutrition intake during intervention, until end of intervention and at discharge.

Enteral nutrition	Intention-to-treat analyses					
						Sensitivity
	CF group		BC group		*p* value	*p* value
	N		N			
Enteral volume of human milk given during intervention (mL/kg/day):						
Start of fortification (mL/kg/day), median (min–max)	117	150 (100–180)	115	160 (80–180)	0.51	0.39
Week 1, mL/kg/day, median (min–max)	99	158 (111–189)	97	162 (135–204)	<0.001	<0.001
Week 2, mL/kg/day, median (min–max)	90	159 (0–188)	86	167 (82–203)	<0.001	0.003
End of intervention, mL/kg/day, median (min–max)	76	157 (78–180)	66	160 (127–207)	0.001	0.002
Overall (all measurements until end of intervention)					<0.001	<0.001
Start of fortification, DOL, median (min–max)	117	9 (4–26)	115	8 (3–17)	0.09	0.049
Protein provided from fortifier added to human milk, g:					0.08	0.02
Week 1, g, median (min–max)	107	1.0 (0.0–1.5)	107	1.0 (0.0–2.0)	0.38	0.27
Week 2, g, median (min–max)	104	1.0 (0.0–1.6)	105	1.0 (0.0–1.5)	0.10	0.02
End of intervention, g, median (min–max)	96	1.0 (0.0–1.4)	98	1.0 (0.2–2.0)	0.07	0.04
Overall (all measurements until end of intervention)					0.08	0.02
Protein from human milk and fortifier						
Week 1, g/kg, median (min–max)	82	4.6 (3.0–6.7)	88	4.5 (2.4–7.3)	0.78	0.64
Week 2, g/kg, median (min–max)	76	3.7 (1.6–5.0)	74	4.1 (1.8–5.6)	0.006	0.002
Infants still in need of fortification at end of intervention, n (%)	116	93 (82)	114	87 (74)	0.15	0.45
Infants still in need of fortification at final discharge, n (%)	114	21 (19)	112	16 (14)	0.30	0.56
PMA when fortification ended, wk+d, mean ± SD	105	37+0 ± 2.6	104	36+2 ± 2.7	0.03	0.02
Only MOM given at end of intervention, n (%)	117	100 (85)	115	84 (73)	0.02	0.06
Breastfeeding at discharge, n (%)	117	78 (67)	112	56 (50)	0.01	0.02

DOL, day of life; Human milk, donor human milk and/or mother's own milk; PMA, postmenstrual age.

Table 5
Clinical biochemistry before and two weeks after start of fortification.

Clinical biochemistry	Week	CF group		BC group		*p* value
		N		N		
Base excess(P), mmol/L, mean ± SD	0	95	−1.43 ± 3.02	98	−1.13 ± 2.86	0.84
	2	80	2.82 ± 2.74	79	0.59 ± 2.43	0.046
Blood urea nitrogen(P), mmol/L, mean ± SD	0	93	4.37 ± 2.15	97	4.01 ± 2.28	0.65
	2	76	2.50 ± 1.36	77	2.74 ± 1.31	0.27
Calcium(P), mmol/L, mean ± SD	0	97	1.32 ± 0.10	96	1.29 ± 0.10	0.04
	2	81	1.35 ± 0.06	78	1.35 ± 0.06	0.78
Hemoglobin(B), mmol/L, mean ± SD	0	92	9.26 ± 1.54	98	9.38 ± 1.65	0.70
	2	82	6.92 ± 1.05	83	7.08 ± 1.35	0.54
pH(P), mean ± SD	0	96	7.32 ± 0.04	98	7.32 ± 0.04	0.84
	2	81	7.35 ± 0.04	79	7.33 ± 0.05	0.03
Phosphate(P), mmol/L, mean ± SD	0	89	1.82 ± 0.36	75	1.80 ± 0.35	0.58
	2	76	2.01 ± 0.30	75	2.00 ± 0.31	0.60
Potassium(P), mmol/L, mean ± SD	0	97	4.88 ± 0.94	97	4.72 ± 0.58	0.24
	2	76	4.49 ± 0.49	77	4.51 ± 0.55	0.89
Sodium(P), mmol/L, mean ± SD	0	97	137.5 ± 5.4	98	138.2 ± 4.6	0.28
	2	81	137.1 ± 2.6	80	135.6 ± 3.1	<0.001

P; plasma and B; blood.

4.2. Feasibility and safety

The intact, powdered BC product contained more whole protein (both caseins and whey proteins) but less sodium, phosphate, calcium, iron, zinc and vitamin D (all necessary for growth, organ and bone development) than the CF fortifier containing partly hydrolyzed whey protein plus added minerals and vitamins. Among BC infants, supplementary phosphorous was required to reach recommended intakes and blood biochemistry values within the normal range. Ten infants were supplemented with extra calcium, 2 in the CF group and 8 in the BC group. The number of infants with anemia or in need of extra blood transfusions did not differ between groups. Reduced sodium levels in BC-fortified infants (although within normal reference values) may reflect lower sodium content in BC vs. CF. Use of intact BC as a fortifier may require supplementation with several micronutrients. We are looking into more details on this topic.

4.3. Fortification

Nutrient fortification is recommended to start at 50–100 mL/kg/day enteral feeding, even in settings where feeding advancement rates are relatively fast [31,50]. In our study, fortification was initiated at higher feeding volumes (above 100–140 mL/kg/day, at one week of age or later), probably partly related to our per protocol pre-fortification maximum BUN level of 5 mmol/L. In the first weeks, BUN values may be a poor measure of protein metabolism due to immature kidney function [51] and may not relate closely to protein intake until later [52]. In our study, both standard and individualized protein fortification were practiced among participating units [32], the latter securing that fortification is adjusted to protein levels in MOM and DHM. At all units, growth rate was a main parameter to guide nutritional intake, including amount of fortification and feeding volume. The trend to higher enteral volume and protein intake in BC infants may be explained both by

lower digestibility of BC-protein (see above) and a slightly higher proportion of BC infants being fed DHM, with subsequent need for feeding volume adjustment to achieve target weight gains.

4.4. Amino acids

Increased AA levels in BC infants may reflect a less efficient use of intact protein from BC for tissue accretion, relative to hydrolyzed CF protein, and this may have contributed to the decreased blood pH and base excess, consistent with previous studies on protein quality and quantity for preterm infants [52–55]. Basal levels of AAs (especially EAAs) increase in response to protein fortification [37] but correlations with diet protein composition, growth rates or clinical outcomes are highly variable [37–39]. Until a certain threshold, blood EAA levels (especially BCAAs) are assumed to correlate positively with growth rates and to be stimulated by protein supply [56], but reference values for AAs in very preterm infants are not known. In our study, the number of infants having levels of one or more EAAs below a chosen reference value for unfortified preterm infants [39] did not differ between groups. While extreme levels of plasma AAs would indicate potential deficiency or toxicity, values within the normal physiological range remain difficult to use to assess optimal quality of protein supplements for very preterm infants [57]. Especially hypertyrosinemia is considered a marker for excessive/unbalanced protein supply to preterm infants due to limited (hepatic) breakdown of Tyr in the first weeks after preterm birth [37,39,58]. Considering that Tyr levels in plasma were elevated 40% in BC infants, together with 40% greater Tyr contents in BC versus CF protein, supports that elevations of this EAA in plasma was mainly of dietary origin and probably partly related to casein in BC [57]. Elevated levels of some non-EAAs in the BC infants could arise from the combined effects of increased dietary intake and other factors.

4.5. Strength and limitations

The use of different fortification products could not be blinded to clinical personnel, and units were allowed to follow own standards on advancement in fortification. We realized during the trial period, that units in Eastern and Western Denmark varied slightly in their feeding practice, and by randomization, relatively more infants were randomized to BC in Eastern Denmark, potentially contributing to a lower breastfeeding rate at discharge in BC-fortified infants.

5. Conclusion

MOM is the best nutrition for preterm infants, but extra nutrients are needed, including non-nutrient milk bioactivity, especially in highly sensitive preterm infants. It is critical, that we further define the optimal origin and optimal processing of fortifiers added to human milk to allow preterm infants to grow and develop optimally without compromising or exceeding their nutrient metabolic capacity. Our results on growth rates and enteral nutrition intake demonstrates it is feasible and safe to use BC as a fortifier. Yet, our clinical biochemistry data and plasma AA values indicate that adjustment in protein composition and further micronutrient supplementation to the BC product may be necessary. However, any nutrient adjustment must always be balanced against the potential damaging effects of industrial processing to milk bioactive factors supporting the immature gut and metabolism of preterm infants.

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Statement of authors' contribution

Designed the overall research plan; AM Ahnfeldt, PT Sangild, L Aunsholt, SS Kappel, G Zachariassen. Conducted research; L Aunsholt, BM Hansen, B Hoest, V Jóhannsdóttir, SS Kappel, A Klamer, BK Moeller, AL Skovgaard, LD Vibede, AM Ahnfeldt, G Zachariassen. Did the amino acid analyses; G van Hall. Analyzed data and performed statistical analysis; S Möller, AM Ahnfeldt, SS Kappel, G Zachariassen. Wrote the paper; AM Ahnfeldt, PT Sangild, G Zachariassen.

All authors have read and approved the final manuscript.

Sharing data

Data described in the manuscript, code book and analytic code will be made available upon request pending application and approval.

Conflict of interest

All authors have no conflicts of interest or financial relationships relevant to this article to disclose. University of Copenhagen holds a patent on the use of bovine colostrum for human infants (PCT/DK2013/050,184) in collaboration with Biofiber Damino that is given a license option. Per T Sangild is listed as sole inventor but has declined any share of potential revenue arising from commercial exploitation of the patent.

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Appendix A. Supplementary data

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