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Contribution to Human Milk use in Neonatology

Contribution à l'utilisation du lait maternel en néonatologie

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Contribution to Human Milk use in Neonatology

Summary

In preterm infants, Human milk (HM) feeding is associated with significant benefits on health and development. The mother's own milk (OMM) is recommended as the first nutritional choice (1). When OMM is unavailable, the use of donor milk (DM) should be the second alternative. However, HM use in neonatal intensive care units may raise some concerns. The purpose of this work is to evaluate them and to find appropriate solutions in order to promote the HM use for preterm infants.

HM despite anti-infective properties is not sterile, and additional contaminations may occur during handling and processing for use in neonatology. We confirmed that expressed HM may harbor skin flora, as well as less frequent pathogenic bacteria with potential infectious risk for vulnerable preterm infants (chapter 2) (2). It therefore requires careful monitoring and eventual processing to eradicate pathogens while preserving its immune function. Holder Pasteurization is the method currently used in HM banks and should also be considered for OMM in the most immature preterm infants although, unfortunately, it alters some HM components, especially among those with immune function. It was suggested that raw OMM is superior to pasteurized OMM in protective effects against infections and other morbidities, but clinical evidence is still lacking. We also demonstrated that colostrum is less contaminated than mature milk and therefore could be used raw. The results of our study on bacterial contamination of HM and the literature review of the postnatal cytomegalovirus (CMV) infections from OMM of CMV positive mothers suggest that pasteurization of OMM remains worthwhile in very preterm infants (<32 weeks GA) fed OMM heavily contaminated with pathogenic bacteria as well as in extremely preterm infants (<28 weeks GA) of CMV positive mothers .

Nutritional requirements of preterm infants are high, explaining the risks of nutritional deficits and extrauterine growth restriction. Exclusive HM feeding with both OMM and DM cannot meet their nutritional needs without being fortified. However, despite standard fortification, growth of HM fed preterm infants remains suboptimal and lower than those of fed preterm formula. These differences could be related to large variations in the macronutrients content of expressed HM, that is frequently lower than their assumed concentration. Additional explanations include lower HM metabolizable protein and energy availability for new tissue synthesis and negative impact of pasteurization. Therefore, optimization of HM fortification is required. Both improving the quality of the fortifiers, including by increasing the protein content, and individualization of the fortification have been suggested. In a controlled, multicenter, double-blind study, we demonstrated an improved weight gain during the study period (+1.18 g/kg/d; p=0.013) in infants fed HM supplemented with a new HM fortifier providing similar energy but higher protein intakes (4.5 vs 3.8 g/kg/d) (chapter 3) (3). However, in this study, nutrients intakes were not measured and were probably overestimated in both groups. This study suggests that nutritional requirements of preterm infants fed human milk could be higher that of preterm infants fed formula and that separate nutritional recommendations should be published for preterm fed HM.

Devices using infrared technology allow rapid analysis of macronutrients concentrations but require a careful validation before their use for HM. We evaluated several infrared analyzers (Milkoscan® minor and three generation of Miris®) and demonstrated that after individual adjustments, infrared analyzers provide precise and accurate determination of protein and lipid concentration (chapter 4) (4,5). However, as validation techniques are time consuming and request several chemical analyses not

available in most NICUs, their use needs to be reserved for research pending the availability of dedicated validation kits.

Using the Milkoscan®, we confirmed the high variability of HM contents and the appeal of individualized HM fortification to optimize the fortified OMM or DM nutritional compositions up to the nutritional needs of preterm infants (chapter 5) (6,7). In clinical settings, applying our individualized fortification protocol allowed us to provide daily remarkably similar controlled nutritional intakes to a group of 101 VLBW infants fed fortified OMM or DM (chapter 6) (8). Therefore, independently from protein and energy intakes, it was possible for the first time to demonstrate that fortified(OMM promoted growth of premature infants with increased weight gain velocity of 1.6 g/kg/d (p=0.002) and length gain of 0,18 cm/week (p=0.02), relative to fortified DM. This result could be partially explained by the pasteurization of DM as the weight gain differences between raw and pasteurized OMM were in the same range. This suggests that pasteurization impaired the bioavailability of nutrient intakes. According to our results, we speculate that energy requirements could be higher in preterm infants fed pasteurized DM or OMM versus those fed raw OMM. In view of these results, we also suggested that nutritional recommendations need to take into consideration the types of HM: OMM or DM, raw or pasteurized.

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Contribution à l'utilisation du lait maternel en néonatologie

Résumé

Le lait maternel (LM) présente de nombreux avantages pour la santé et le développement du nouveau-né prématuré. Le lait maternel de la propre mère constitue ainsi le premier et meilleur choix d'alimentation. Si le lait maternel de la propre mère n'est pas disponible, le lait humain de don représente la meilleure alternative (1). Cependant, l'utilisation du lait maternel dans les unités néonatales peut soulever certaines questions. Le sujet de ce travail est d'évaluer ces problèmes et de tenter de trouver des solutions appropriées afin de promouvoir l'usage du lait maternel pour l'alimentation du nouveau-né prématuré.

Le lait maternel, malgré ses propriétés anti-infectieuses, n'est pas stérile et peut être contaminé durant les manipulations nécessaires à son usage en néonatologie. Nos études bactériologiques des laits tirés amenés par les mères en néonatologie confirment la présence de bactéries de la flore cutanée mais aussi de bactéries pathogènes avec un risque potentiel d'infection chez le grand prématuré (chapitre 2) (2). Par conséquent, il parait prudent de réaliser une surveillance bactériologique attentive ainsi qu'un traitement des laits contaminés, éliminant virus ou bactéries pathogènes tout en préservant au maximum les propriétés immunologiques du LM. La pasteurisation de Holder, malgré ses effets délétères sur certains composants, surtout immunitaires du LM est la méthode actuellement utilisée dans les banques de lait et peut être considérée pour traiter le LM de la propre mère des enfants prématurés très immatures et vulnérables. Le LM de la propre mère cru est généralement considéré comme supérieur au LM pasteurisé dans son rôle de protection contre les infections et autres morbidités mais des données cliniques probantes manquent. Nous avons également démontré que le colostrum est moins contaminé que le lait mature et pourrait être utilisé cru. Notre étude sur la contamination bactérienne du LM et la revue de littérature concernant le risque d'infection postnatale au cytomégalovirus (CMV) via le LM de mères séropositives pour le CMV suggère que la pasteurisation demeure préférable chez les prématurés extrêmes de moins de 28 semaines à la naissance, nés de mères séropositives pour le CMV ainsi qu'en cas de contamination significative du LM avec des bactéries pathogènes chez ceux nés avant 32 semaines.

Les besoins nutritionnels de l'enfant prématuré sont très élevés avec un risque de déficit nutritionnel et de retard de croissance extra-utérin. L'alimentation exclusive au LM de la propre mère ou au lait de don ne peut satisfaire ces besoins nutritionnels. Le LM doit donc être fortifié. Cependant, malgré la fortification, la croissance des enfants prématurés alimentés au LM fortifié reste inférieure à celle de ceux alimentés avec une formule destinée aux prématurés. Ces différences pourraient être expliquées par la grande variabilité nutritionnelle du LM tiré avec un contenu nutritionnel réel plus faible que présumé mais aussi par la moindre disponibilité en protéines et énergie métabolisables du LM pour la synthèse de nouveaux tissus, auxquelles s'ajoute l'impact négatif d'une éventuelle pasteurisation. Une optimisation de la fortification du LM est donc requise. L'amélioration de la qualité des fortifiants en augmentant le contenu en protéines et l'individualisation de la fortification ont été des pistes suggérées. Dans une étude multicentrique contrôlée en double-aveugle, nous avons montré une amélioration du gain pondéral (+1,18 g/kg/j; p=0,013) chez les enfants alimentés au LM enrichi avec un nouveau fortifiant apportant plus de protéines (4,5 vs 3,8 g/kg/j) pour un apport similaire en énergie (chapitre 3) (3). Cependant, dans cette étude, les apports nutritionnels étaient non directement mesurés et probablement surestimés dans les deux groupes. Cette étude suggère des besoins

nutritionnels plus élevés chez les enfants prématurés alimentés au LM fortifié par rapport à ceux alimentés avec une formule ainsi que la nécessité d'élaborer et de publier des recommandations nutritionnelles spécifiques pour l'enfant alimenté au LM fortifié.

Des appareils utilisant la technologie infra-rouge permettent une détermination rapide de la composition en macronutriments du lait de vache mais nécessitent une validation minutieuse avant utilisation pour l'analyse du LM. Nous avons évalué plusieurs analyseurs (le Milkoscan® minor et trois générations de Miris®) et démontré, qu'après ajustement individuel, ces analyseurs à infra-rouge permettent une détermination exacte et précise de la concentration en lipides et en protéines du LM (chapitre 4) (4,5). Cependant, la validation est laborieuse et demande des analyses chimiques de référence non disponibles dans la majorité des services de néonatologie. Leur usage devrait donc être réservé à la recherche tant que des kits de validation adéquatement certifiés ne sont pas disponibles.

En utilisant le Milkoscan®, nous avons confirmé la grande variabilité de composition du LM et démontré l'intérêt d'une fortification individualisée du LM, de la propre mère ou de don, pour en optimiser sa composition nutritionnelle et permettre ainsi de rencontrer les besoins nutritionnels élevés des enfants prématurés (chapitre 5) (6-7).

En appliquant notre protocole de fortification individualisée en clinique, nous avons pu fournir des apports nutritionnels contrôlés et similaires dans un groupe de 101 enfants grands prématurés alimentés au LM fortifié de la propre mère ou de don (chapitre 6) (8). Pour la première fois, nous avons pu démontrer que, de façon indépendante des apports en macronutriments, le LM de la propre mère favorisait la croissance par comparaison au lait de don avec un bénéfice de gain pondéral de 1,6 g/kg/j ($p=0,002$) et statural de 0,18 cm/sem ($p= 0,02$). Cette différence pourrait être partiellement expliquée par la pasteurisation du lait de don car nous avons aussi observé une différence de gain pondéral similaire entre le LM de la propre mère cru et pasteurisé, suggérant que la pasteurisation altérait la biodisponibilité des apports nutritionnels du LM.

Tenant compte de nos résultats, nous spéculons que les besoins en énergie pourraient être plus élevés chez les enfants alimentés au LM pasteurisé par rapport à ceux alimentés au LM de la propre mère cru. Nous suggérons également que les recommandations nutritionnelles prennent en considération le type de LM utilisé : LM de la propre mère ou de don, cru ou pasteurisé.

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Abbreviations

ADJ: adjustable fortification

BPD: bronchopulmonary dysplasia

BUN: blood urea nitrogen

CMV: cytomegalovirus

CNS: coagulase negative staphylococcus

CFU: colony-forming unit

DM: donor milk

E: energy

ELBW: extremely low birth weight

EPT: extremely preterm

EUGR: extra-uterine growth retardation

FHM: fortified human milk

FM: fat mass

GA: gestational age

HC: head circumference

HM: human milk

HMA: human milk analyzer

HMF: human milk fortifier

IHMF: individualized human milk fortification

IRA: infrared analyzer

ITT: intent to treat

LBM: lean body mass

LOS: late onset sepsis

MCT: medium chain triglycerides

NEC: necrotizing enterocolitis

NICU: neonatal intensive care

OC: oral colostrum

OMM: own mother's milk

PER: protein energy ratio

PTF: preterm formula

PUFA: polyunsaturated fatty acid

RCT: randomized controlled trial

ROP: retinopathy of prematurity

SD: standard deviation

SF: standard fortification

TF: targeted fortification

VLBW: very low birth weight

VPT: very preterm

Chapter 1- Introduction

1. Background and issues

1.1. Prematurity: incidence and challenges

The global burden of preterm birth includes the morbidity and mortality of babies born before 37 completed weeks of gestation. Prematurity is the most frequent worldwide cause of neonatal mortality, and now also becomes the leading cause of childhood mortality through age five years leading to approximatively 1 000 000 deaths in 2015 (1). Extremely preterm (EPT) birth is defined by the World Health Organization (WHO) as birth before 28 weeks of gestation, birth occurring between 28 and less than 32 weeks are very preterm (VPT) and those between 32 to 36 $^{6/7}$ weeks are moderate to late preterm. Morbidity and mortality increase exponentially with decreasing gestational ages. Incidence of preterm deliveries is around 11% worldwide, leading to the birth of 15 million babies annually (1). Furthermore, compared to full term infants, surviving preterm infants have higher risk of long-term morbidities, including neurodevelopmental disabilities and increased chronic diseases in adulthood (2, 3)(Table 1.1). In addition, prematurity is associated with psychological, economic, physical burdens and morbidities affecting infants, mothers, families and communities (4). The incidence of prematurity (2011-2015) was 8.5 % in Wallonia and Brussels and 8.9% in Province of Liège (5).

Table 1.1. Potential long-term consequences in adulthood of preterm birth

System-specific outcomes	Adverse consequences in adults born preterm
Neurological and cognitive	Increased risk of cerebral palsy, Impaired psychomotor development Reduced IQ and intellectual disability Neurosensory deficits
Cardiovascular	Increased blood pressure Impaired vascular growth
Metabolic	Increased intra-abdominal fat tissue Low insulin sensitivity Abnormal lipid profiles
Pulmonary	Higher risk of asthma Pulmonary function abnormalities
Renal	Reduced renal function
Bone	Lower bone mineral density
Social and mental health	Increased anxiety and depression Higher risk of mental disorders Difficulties in relationships Lower level of education Lower income levels

Modified from (2, 3)

The past 50 years have seen major advances in obstetric and neonatal clinical intensive care for preterm infants. Significant progresses have been made in respiratory care, thermoregulation, cardiopulmonary monitoring, nutrition and infection's treatment. Associated to improvement in diagnosis and imaging technics, they have resulted in a dramatic increase in survival rate without

sequalae up to adulthood. The International Network for Evaluating Outcomes of Neonates reported survival ranging from 78% to 93% in 24 weeks to 29 weeks preterm infants (6).

1.2. Impact of perinatal nutrition and growth on long term health and neurodevelopmental outcomes

Introduction of early nutritional support was a major contribution to the neonatal care of preterm infants, and was associated with a dramatic reduction in mortality and morbidity. In the early 20th century starvation during 24 to 48 hours was generally recommended for VLBW infants, considering both the immaturity of the gastro-intestinal tract and the risk of early enteral nutrition. Given their extremely low metabolizable stores of energy and protein, most of the ELBW infants died of malnutrition during the first few days of life (Figure 1.1) (7). The introduction of glucose perfusion and early introduction of human milk feedings improved survival rates but were still followed by relatively severe growth restriction.

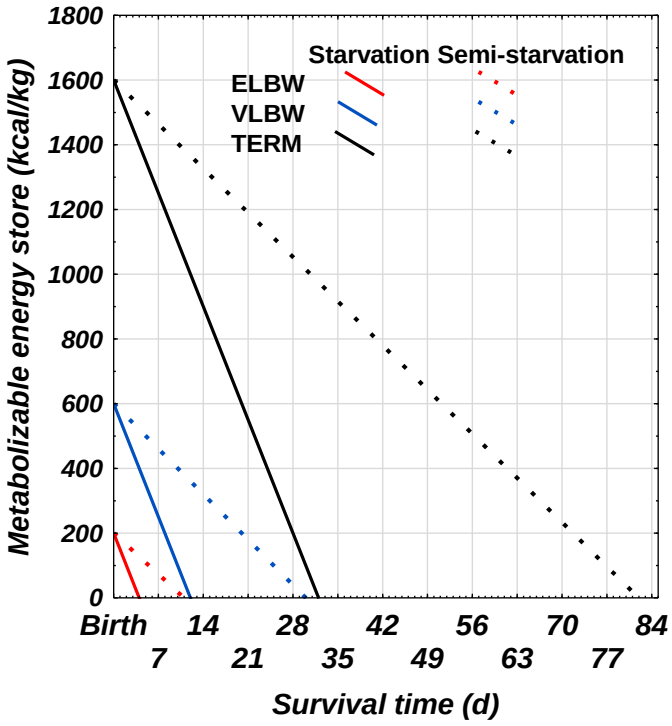


Figure 1.1. Duration of survival expected in starvation and semi-starvation (75 ml/kg perfusion glucose 10%)(7).

The introduction of parenteral nutrition at the end of the of the 1960's, and higher nutritional support at the turn of the millennium were correlated with rapid improvements in mortality and morbidity (8).

It has become increasingly recognized that nutrition during the fetal and neonatal periods may have an important impact upon health and diseases throughout adult life, a concept now known as the "developmental origins of health and disease" hypothesis (DOHAD).

The theory indicating that early-life experiences result in long-lasting alterations in health was first proposed by David Barker (9). Epidemiologic retrospective and observational studies suggested an association between in-utero growth restriction and later development of metabolic syndrome, cardiovascular diseases and mortality independently of cofounding factors such socio-economic status

(9-14). In 1992, Barker and Hales (15) proposed the “thrifty hypothesis” which states that inadequate nutrition in utero may cause metabolic adaptations that allow the organism to better survive in limited nutritional resources. While useful in limited resources, organisms so programmed might be disadvantaged when placed in an environment where food is readily available.

The concept of “Programming” introduced by Alan Lucas (16) implies the existence of a specific critical period in early life during which alterations in nutrient supply and several others environmental stimuli may result in permanent physiologic changes and adaptations, not only for the individual’s lifetime but even for subsequent generations (17). Programming is the consequence of the plasticity of the cells and tissues during development. The timing of exposure is critical. Greatest sensitivity occurs during the periods of most rapid growth and maturation. In some cell types, the adaptive capacity remains present throughout life. However, most tissues display plasticity only during the critical periods of embryonic and fetal development, during which nutrition or environmental perturbations can trigger physiological adaptations to ensure survival and may then have a permanent impact on health and disease (10).

Numerous experimental controlled animal studies have shown that nutrition in early life can influence, in adulthood, many outcomes such as hypertension, hyperlipidemia, obesity, type-2 diabetes, atherosclerosis, behavior, learning and longevity (10, 16).
In humans, retrospective and observational studies suggested a relationship between adult diseases and events in early life, though causal effect is difficult to prove from observational associations (10). However the results of randomized trials of early nutrition with long-term follow-up are emerging and suggest nutritional programming (16).

As increasingly more immature preterm infants with several morbidities survive, providing an optimal nutrition for adequate growth in the NICU is particularly challenging. Indeed, many of these babies experience cumulative nutritional deficits and extrauterine growth delay during the first weeks of life (18-21).

It has been suggested that the neonatal period corresponds to a critical window when early diet and nutrition affect brain development and will have an impact on later cognition (16, 19, 22-24). Recent data indicate that adequate early growth may be beneficial for neurocognitive function (22). However, given the delay to obtain long term follow-up, it remains unclear whether early postnatal adequate growth leads to health benefits up to adulthood compared to premature infants suffering from extra-uterine growth retardation (EUGR), with or without early catch-up growth.

Unfortunately, the literature exploring the relationship between nutritional support provided to preterm infants in the NICU and growth and neurodevelopmental outcomes is poorly strengthened by double blind randomized control trials (DBRCT) demonstrating clear causes and effect relationships. In fact, most reports describe observational studies or historical control studies where the impacts of nutritional practice changes are reported (25, 26). Optimizing nutrition in the neonatal period seems nevertheless to be a way to reduce the adverse health effects of preterm birth.

1.3. Nutritional requirements and growth of premature infants

Nutritional requirements are defined as the amount of nutrients needed to support normal health, metabolism, growth and development. Nutrient requirements of preterm infants have been

determined by two methods, the factorial method and the empirical method (27). The factorial assessed requirements from accretion rates of nutrients derived from the analysis of fetal body composition at different stages of gestation. The empirical method involved the manipulation of nutrient intakes and observation of growth and metabolic responses such as protein, energy and/or mineral retentions.

Because preterm infants are born during the period of maximal fetal growth, their nutritional requirements are particularly high. Currents Nutrients Recommendations (27-31) depend largely on expert opinion, due to a lack of evidence and are based on the estimated average requirement of a population group according to birth weight, with low consideration given to gestational age and type of (human) milk use. Thus, The validity of these recommendations is a matter of ongoing debate. (32). International consensus guidelines for enteral nutrition of the preterm infants formulated by several expert groups are gathered in table 1.2. However, individual requirements vary according to each infant's clinical conditions and characteristics at birth and in the course of his or her NICU stay.

Table 1.2. Current recommendations for enteral nutrition for preterm infants.

Nutrients	**ELBW or <1000 g**			**VLBW or 1000-1500 g (1800 g EPSGHAN)**		
	Tsang 2005	**EPSGHAN 2010**	**Koletzko 2014**	**Tsang 2005**	**EPSGHAN 2010**	**Koletzko 2014**
Energy (kcal/kg/d)	130-150	110-135	110-130	110-130	110-135	110-130
Protein (g/kg/d)	3.8-4.4	4-4.5	3.5-4.5	3.4-4.2	3.5-4	3.5-4.5
Protein /100kcal	2.5-3.4	3.6-4.1	3.2-4.1	2.6-3.8	3.2-3.6	3.2-4.1
Lipids, g	6.2-8.4	4.8-6.6	4.8-6.6	5.3-7.2	4.8-6.6	4.8-6.6
Carbohydrate, g	9-20	11.6-13.2	11.6-13.2	7.17	11.6-13.2	11.6-13.2
Calcium, mg	100-220	120-140	120-200	100-220	120-140	120-200
Phosphorus, mg	60-140	60-90	60-140	60-140	60-90	60-140

Adapted from (28, 30, 31)

The ideal goal of premature infant nutrition would be to maintain a postnatal growth trajectory similar to that of the same gestational age fetus in utero. However, whether this is an appropriate goal, how growth should be monitored, and what is the ideal pattern of growth remain controversial (33). Such a growth velocity is difficult to achieve during the first weeks in preterm infants. ELBW infants, specifically those with several morbidities, are at risk of cumulative nutritional deficits and postnatal growth restriction, which may be associated with poorer neurocognitive outcome (16, 19, 22-24). The current assessment of growth is predominantly based on weight, length and head circumference with insufficient attention given to the quality of growth in term in lean body mass, fat mass or bone density (32, 34). Clinical studies in humans and in animal models demonstrate that both the dietary composition and the growth trajectory affect long term outcomes (35).

1.4. Breastfeeding and prematurity

Mother's own milk (OMM) is the gold standard for nutrition for newborn infants and should always be recommended (36). World Health Organization (WHO) recommends exclusive breastfeeding until 6 months of age and continued breastfeeding until 2 years of age (37). Breastfeeding and human milk (HM) are also recommended for preterm infants and strong efforts should be made to promote lactation (36, 38). Overall, breastfeeding rates remain low in premature infants with large variations between regions (39-41) (Figure 1.2). Breastfeeding in the neonatal intensive care unit (NICU) represents a highly challenging issue due to the separation of the mother from her infant, the difficulties encountered by the mothers in initiating and maintaining milk production and finally in the transition from tube to oral feeding in recovering immature infants (42, 43). Support in initiating milk expression early in the hospital course will help to increase the likelihood of achieving full breast milk feeding up to discharge (42, 43).

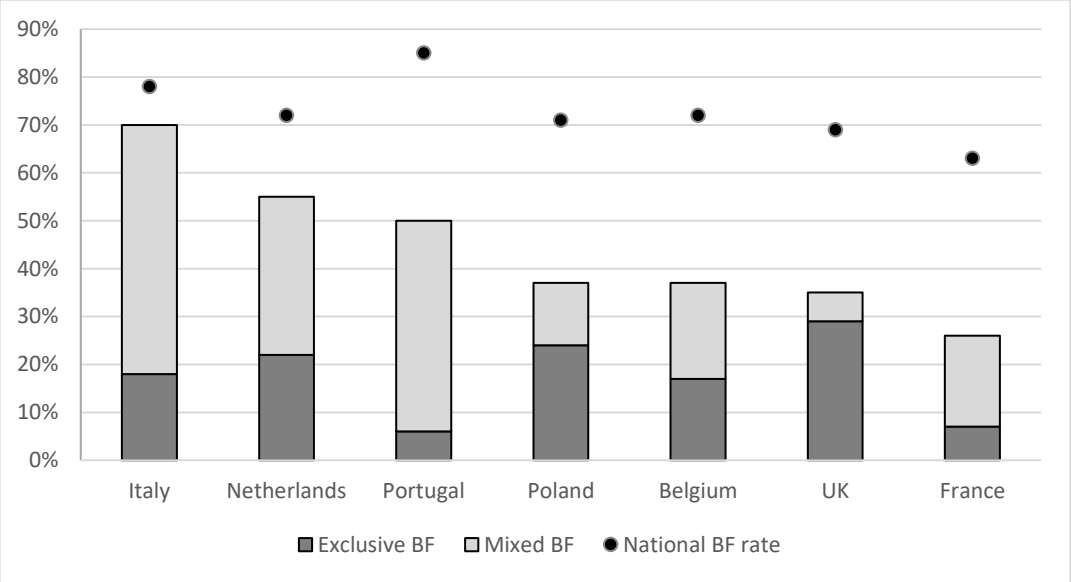


Figure 1.2. Rates of exclusive and mixed breast feeding at discharge in very preterm infants in each study region and overall national breastfeeding rates: Belgium/Flanders; France/Ile-de-France; Italy/Lazio; Netherlands/Central-East; Poland/Wielkopolska-Lubuskie; Portugal/North; UK United Kingdom/Trent (adapted from Bonet et al) (40)

2. State of knowledge

2.1. Human milk benefits and limitations

Evidence indicates that human milk is the gold standard in nutrition not only for healthy newborns but also for preterm infants (36, 44). Its specific composition in nutrients with optimal bioavailability, hormones, enzymes, anti-infective, trophic and growth factors, immune and stem cells, oligosaccharides, probiotics and a myriad of others bioactive proteins makes human milk unique and particularly adapted to infant's growth and development (45).

HM feeding is associated with significant benefits on health and development, especially in preterm infants (36, 46). HM improves feeding tolerance (47, 48), reduces necrotizing enterocolitis (NEC) and sepsis incidences (46, 48-54), possibly bronchopulmonary dysplasia (55) and severe retinopathy of prematurity (46, 56). It improves neurodevelopmental outcomes (57-60) and reduces long-term

cardiovascular and metabolic diseases (61, 62). Furthermore, breastfeeding is an opportunity to involve mothers in infant care during hospitalization and encourage mother-infant bonding.

HM, preferably own mother's milk (OMM), is highly recommended for feeding preterm infants but on its own does not provide adequate nutritional support for optimal growth and development. Thus, the use of exclusive OMM feeding is related to a significant risk of cumulative nutritional deficits and postnatal growth restriction not only during the first weeks of life but also up to the time of theoretical term or discharge (18, 63-65).

The many anti-infective properties of HM help protect babies against infections (36). Breast and HM are not sterile and represent a complex ecosystem with a large diversity of bacteria reflecting mother's biotope (66). The mother's microbiome plays an important role in the development of baby's gut colonization, with potential consequences on immunity building, growth and future infant's health (67). However, HM may also contain potentially pathogenic bacteria and viruses (68, 69) that may be transmitted to immature infants with decreased immunity and induce contaminations, infections and moderate to severe morbidities. In addition, as VLBW infants are not able to breastfed directly, HM expression, collection, transport and storage are necessary, increasing the risk of contamination and sepsis as suggested by several case- reports (70-72).

Donor milk (DM) is systematically screened and pasteurized. It is discarded in case of high contamination (73, 74). The need of bacterial screening of OMM before raw administration is controversial. When it is performed, the same bacteriological criteria as those used for DM are generally applied (68, 75). The OMM pasteurization guidelines are also controversial and based on precautionary principles (75, 76). In 2016, The Superior Health Council of Belgium provides recommendations on the use of raw own mother's milk for preterm infants (≤ 28 weeks and/or < 1000 g) in Neonatal Intensive Care with strict bacterial criteria for raw OMM use and for OMM pasteurization (75, 76). Pasteurization destroys both pathogenic bacteria and beneficial HM microbiota, and also alters cellular and some immunological HM properties but many anti-infectious properties remain preserved (77). Therefore, while theoretical arguments suggest that raw OMM is superior in protective effects against infections compared to pasteurized OMM, clinical evidence lacks to support either approach (78, 79). Unfortunately, the type and proportion of HM used (raw OMM, pasteurized OMM or pasteurized DM) are only exceptionally recorded in studies. The morbidities associated with postnatally acquired cytomegalovirus (CMV) infection are of great concern for preterm Infants (80). More than 90% of seropositive mothers for CMV reactivate and excrete the virus in OMM after birth (81). Postnatal CMV infection remains generally mild or asymptomatic but a serious illness is observed in 4% of preterm infants of seropositive mothers (82) and in up to 40% of EPT infants <26 weeks (80). The effect of postnatal CMV infection on long-term neurodevelopmental outcomes is unclear (83-86). Holder pasteurization is currently the recommended method to inactivate CMV (81). Alternatively, freezing mother's milk at -20°C for a certain period of time has been shown to reduce the viral concentration but it is not effective in complete elimination of the virus (87).

Another limitation on OMM use is related to the potentially limited availability or inadequate supply of OMM. Unfortunately, mothers of preterm infants are less likely to initiate milk expression, sustain lactation and to provide full OMM, suggesting that DM is necessary to provide an exclusive HM diet to VLBW infants during their first weeks of life (88).

2.2. Donor milk

Mother's own milk (OMM) should always be recommended as the first choice of nutrition for newborn infants (36). When OMM is unavailable or insufficient, the use of donor milk (DM) should be the first alternative for feeding VLBW infants (36, 88-91). Therefore, the use of DM is increasing, and the number of HM banks is growing worldwide (73, 92-94). DM is collected and distributed following standards similar to blood donation (88) and undergoes processing to reduce bacterial and viral contaminants (pasteurization) that influences its bioactive properties with potentially fewer benefits than raw milk (95, 96). However, pasteurized DM maintains documented advantages compared to preterm formula, mainly in reducing NEC incidence (46, 48, 50).

2.3. Processing and pasteurization's effects

Pasteurization and, to a lesser extent, storage and processing result in the loss of some biological and nutritional properties of HM. Holder pasteurization destroys the beneficial microbiota, living white blood cells, IgM and lipase activity, decreases the concentration and activity of immunoglobulins IgA, IgG, lactoferrin, lysozyme, some cytokines [interleukin (IL)-10, IL-1b, tumor necrosis factor-a], some growth factors [insulin-like growth factor 1 (IGF1), IGF2, insulin and adiponectin] and vitamins (C and folate)(77, 89). Other nutritional and biological components, such as oligosaccharides, long-chain polyunsaturated fatty acids, lactose, vitamin A, D, E, B2, some cytokines (IL-2, IL-4, IL-5, IL-12, IL-13) and growth factors (epidermal growth factor and transforming growth factor-b1) are preserved (77, 89). Therefore pasteurized HM, despite partial destruction of its immune components, maintains some bactericidal activity, albeit significantly reduced compared with raw milk (97). Clear evidence of deleterious clinical effects of pasteurized OMM versus raw OMM has not been demonstrated.

2.4. Cumulative nutritional deficit, postnatal growth and HM fortification

2.4.1. Need of HM fortification to improve postnatal growth

HM macronutrients content is insufficient to cover the high nutritional needs required for postnatal growth and development of VLBW infants. Preterm infants and particularly extremely preterm infants are at risk of cumulative nutritional deficits and postnatal growth restriction (18, 63-65, 98, 99) which have been associated with altered neurological outcomes (19, 22, 26, 100, 101). Preterm infants have higher protein, energy, mineral and electrolytes requirements (28, 30, 31). The main challenge is to meet their high and variable nutrients requirements during the whole NICU hospitalization period to prevent postnatal growth retardation and specific deficiency diseases such calcium and phosphorus deficiency leading to osteopenia. Exclusive HM diet, even from infant's OMM or banked DM cannot meet nutritional recommendations for ELBW infants. Fortification of OMM and DM is therefore recommended for all preterm infants to improve nutrients accretion and in-hospital growth (90, 102)

2.4.2. Use of fortified HM improve postnatal growth

Human milk fortification aims to provide nutritional intakes at the levels recommended by guidelines mainly designed for preterm formula (PTF), according to gestational age and clinical conditions, during the first weeks of life (Table 2.1). Feeding fortified HM improves in- hospital growth (103) and bone mineralization (104) and should be associated with favorable neurodevelopmental outcomes (105) although evidence for a long term impact on growth and developmental outcomes is limited (103). However, while energy and protein intakes are similar to those of PTF, HM fortification does not lead to comparable growth. (106-108).

2.4.3. Why does extra uterine growth restriction persist despite HM fortification?

Metabolic balances studies showed (109-112) that energy and nitrogen absorption, retention and utilisation is higher with PTF than with fortified HM.

In addition, in most of the growth studies, energy and protein intakes were not measured but based on a theorical composition of DM and OMM. Many studies suggest that the macronutrient composition of HM is highly variable, especially in protein and fat (113, 114).The use of theoretical reference values may induce an over- or mostly an under-estimation of energy and protein contents of the fortified HM (113, 115) especially after the first month of lactation when the OMM protein concentration decreases (114, 116). Moreover, handling, processing and tube feeding could also reduce the fat content of expressed HM (75, 117).

As a result, studies showed that increases in protein and energy concentration of the fortified HM improve postnatal growth (103, 118).

2.4.4. Current Human milk fortifiers

Several commercial HM fortifiers products are available for preterm (102). The fortifiers currently available in Europe are bovine protein-based and contain varying amounts of protein, energy, vitamin, minerals, elements and electrolytes. There were generally designed to reach an energy content of 80 kcal/dL with a protein concentration around 1,8 g to 2,8 g/dL, to meet nutritional guidelines. A new generation of fortifiers with higher protein content has been designed and was shown to improve short term weight gain (31, 115). More recently, a HM based liquid fortifier obtained by concentrating donor milk (54) and a HM derived cream supplement (119) have been launched on the US market and are increasingly being used.

2.4.5. Methods of HM fortification: Standard or individualized HM fortification

The common strategy for HM fortification assumes an average HM composition and a multicomponent fortifier is added in a fixed dosage (Standard fortification). Nevertheless, the use of standard fortified HM failed to achieve adequate postnatal growth similar to that observed with preterm formula (106-108).

Considering that HM energy and protein contents are unpredictable and likely overestimated when based on theoretical reference values, new strategies for fortification have been suggested in order to reach an optimal growth. “Adjustable fortification” (ADJ) and “Targeted fortification” (TF) are 2 methods of individualized fortification (102). In ADJ method, fortification and protein intakes are adjusted on the basis of individual metabolic response, considering blood urea nitrogen (BUN) levels as a marker of protein metabolism (120, 121). This method is easy to apply, does not require daily milk analysis and improved in-hospital growth compared to standard fortification (120). On the other hand, it still has some limitations; It did not correct the potential energy deficit and did not improve sufficiently the protein and growth deficits. BUN is also poorly correlated to protein intakes during the first month of life but mostly reflects the renal immaturity of preterm infants (122, 123). By contrast, targeted individualized fortification analyzes HM macronutrients content and allows fortification to reach the targeted nutriments intakes appropriate to postconceptional age. This method was first suggested by Polberger et al (124). Studies of HM individualized targeted fortification represent one of the main topics of this research.

2.4.6. Influence of HM types on growth

Various studies showed growth deficits with DM compared to OMM (125-129) and formula (49). In a recent meta-analysis, infants randomized to receive DM had slower growth than infants receiving formula; however, only 5 of 12 trials analyzed fortified DM (49). Studies of the impact of fortified DM vs fortified OMM on growth of VLBW infants are scarce. A few studies (two observational and one retrospective) suggested growth deficits with fortified DM compared to fortified OMM (125, 126, 129) whereas, others retrospective studies did not observe any growth difference (127, 130, 131). A recent randomized trial could not demonstrate a significant growth difference between nutrient-enriched DM compared to preterm formula as a supplement to OMM (50). However, in these studies the macronutrient composition of both DM and OMM was not assessed. A possible explanation for slower growth with DM is its lower fat due to processing steps and lower protein content, as most often DM is given by mothers who delivered term infants and collected milk at a later stage of lactation.

3. Objectives and study plan

In preterm infants, HM is associated with significant benefits on health and development. The mother's own milk (OMM) is always recommended as the first nutritional choice (36). When OMM is unavailable, the use of donor milk (DM) rather than formula could be the second alternative (91). However, breastfeeding rates remain low in preterm infants (41) and HM use could be problematic in neonatal intensive care in term of growth restriction and infectious risk. The purpose of this work is to evaluate some of the problems currently reported by NICU's with HM feeding and to find appropriate solutions in order to promote the HM use for VLBW infants.

3.1. Bacteriologic composition of expressed human milk

HM has many active anti-infective properties, but HM is not sterile and may be also contaminated during manipulations with potential infectious risk. The objective of our study was to evaluate the bacterial contamination of human milk provided to our NICU in order to evaluate the potential need of OMM pasteurization in VLBW infants.

3.2. Influence of new fortifier with higher protein content on growth of preterm infants

It was suggested that protein and protein: energy ratio determine the rate and composition of growth (34, 106, 132). Feeding fortified HM improves growth (103), mineralization and is associated with more favorable outcomes but incidence of postnatal growth restriction is still high, especially when DM is used. The nutritional content of some available HM fortifiers may be inadequate to ensure adequate growth. In a controlled, multicenter, double-blind study, we assessed growth and nutritional markers of preterm infants fed HM supplemented with a new HM fortifier providing a higher protein: energy or a control HM fortifier (115).

3.3. Evaluation of a rapid infrared technology for determining nutritional composition of expressed human milk.

HM composition is highly variable in protein and lipid concentration. This variability may lead to insufficient dietary intakes and growth of premature infants. The objective of our studies was to evaluate the interest of a rapid method to determine HM composition for clinical use in NICU. Analyzers based on infrared technology are currently available in the dairy industry but are originally developed for cow milk analysis and require additional calibration for HM use. We determined macronutrients composition of HM using infrared analyzers and compared results to chemical reference analyses performed in our laboratory. Two mid infra-red milk analyzers were evaluated: the

Milkoscan minor ® and the Miris ® (3 devices). Equations of calibration were specifically established and validated for each device. The accuracy and precision's stability over time were also examined for the Milkoscan minor®.

3.4. Nutritional composition of expressed human milk and Interest of human milk individualized fortification on nutritional intakes

Following the calibration and validation of the Milkoscan minor®, we evaluated its use in clinical setting.

We assessed the variability of the macronutrient's composition of different HM types: OMM, colostrum and donor milk pools provided to our milk bank.

In a pilot study, we evaluated the impact of a new protocol of HM individualized targeted fortification on nutritional intakes and growth of a cohort of premature infants compared to a historical cohort fed with standardized fortified HM and preterm formula.

After implementation of an individualized fortification procedure in our NICU, we assessed the influence of an individualized HM fortification on nutritional intakes and their variability in preterm infants and compared it to theoretical intakes that would have resulted from standard fortification.

3.5. Influence of human milk type on growth of premature infants

The last part of this work evaluated an individualized HM fortification on nutritional intakes and growth in VLBW infants fed donor milk (DM) versus own mother's milk (OMM). We hypothesized that individualized fortification, by standardizing protein and energy intakes, would allow to evaluate the influence of the type of HM (raw own mother's milk, pasteurized own mother's milk and pasteurized donor milk) on growth regardless of protein and energy intakes. We postulated that that raw OMM promoting nutrient's bioavailability could induce a higher neonatal growth than pasteurized DM.

Chapter 2- Bacteriologic composition of Human milk

Vervoort A, Delsat L, Pieltain C, de Halleux V, Rigo J. Evaluation de la qualité bactériologique du lait maternel dans un service de néonatologie (NIC). Revue médicale de Liège. 2007;62(3):159-165.

Simon L, Kessen C, Rigo J, de Halleux V. Bacteriologic quality of colostrum, comparison with mature milk: Thèse pour diplôme de docteur en Médecine et Pédiatrie, University of Nantes, France; 2012.

2.1. Introduction

While breastfeeding is recommended and particularly beneficial for preterm newborns (36, 46, 58), the use of raw expressed own mother milk remains a matter of debate due to its potential role in infection**.** Breast milk is a non-sterile complex ecosystem, which reflects the mother's microbiome and may contain a combination of non-pathogenic germs and potentially pathogenic bacteria (133). Non-pathogenic germs usually present include *coagulase-negative Staphylococci*, *alpha hemolytic Streptococci*, *Serratia* and *Corynebacteria*, part of the normal skin flora, as well as *Lactobacillus* and *Bifidobacteria* (134). Breast milk microbiota plays a probiotic role in infant's gut providing anti-infective properties, particularly against *Staphylococcus aureus* (135), and virulent strains of *Coagulase negative Staphylococci* (136) and may also contribute to the maturation of immune system (137).

However, HM microbiota of mothers of preterm infants shown lower species diversity of bacteria with higher counts of virulent strains of *Coagulase negative Staphylococci* (138). Breast milk may also contain pathogens such as *group B Streptococcus* (135, 139), *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus* (140). Transmission and infections trough breastmilk of such pathogens have been documented (71, 72, 139, 141-143). Whereas unexpressed HM likely contains bacteria, further potential sources of bacterial contamination occur in the process of expressing and storing milk. Expressed HM is more frequently contaminated than unexpressed OMM (144). Secondarily contamination during handling (extraction, storage, repackaging) may be a source of infection in preterm newborns (70). Therefore, to minimize bacterial contamination and risk of transmission to immunodeficient VLBW infants, expressed OMM for preterm infants is frequently pasteurized, as DM. Some of the HM beneficial properties are unfortunately lost during pasteurization such as the cellular immune components or reduced such as the immunologic proteins (IgA, IgG, lactoferrin, lysozyme…) (75, 77, 89). Colostrum is richer in immunoglobulin A, growth factors and anti-inflammatory cytokines (45, 145) and plays an essential role, through its anti-infectious, anti-inflammatory and gastrointestinal tract maturation activities (146). Colostrum due its particular composition could be more altered by pasteurization (147). Raw colostrum presents many interesting properties for preterm infants. However, data on its bacteriological contamination and its infectious risks are scarce.

Our first objective was to study bacterial contamination of expressed mother's milk brought to our NICU after a limited time (18-24 hours) of incubation in order to safely use raw own mother's milk in VLBW Infants. Our second aim was to compare colostrum and mature milk bacterial contamination of own mother's milks.

2.2. Material and method

2.2.1. The microbiological composition of expressed human milk

The microbiological composition of expressed HM was evaluated on each sample of breast milk brought by the mothers to the NICU of the University of Liège from november,1, 2003 to January 31, 2005. 1 µl of each sample was cultured on a Tryptone Soya Agar medium with sheep blood and immediately placed in an incubator at 37°C. After 18-24 hours of incubation colony counts were assessed and bacteria were identified by the bacteriologist. Results of HM were classified as "clean" (commensal bacteria $<10^5$ colony-forming unit (CFU) per mL and no pathogen), "contaminated" (commensal bacteria $\geq 10^5$ CFU per mL) and were pasteurized or "inappropriate" (presence of a pathogen) and were discarded. Milks classified as "contaminated" were all pasteurized in a climatic chamber (Dry Pasteurization, CLIMATS, France) before administration. Holder Pasteurization consists of a rise in temperature up to 62,5°C for 30 minutes. Seasonal influence and maternal profiles over time were also studied.

2.2.2. Microbiological composition of colostrum compared to mature milk

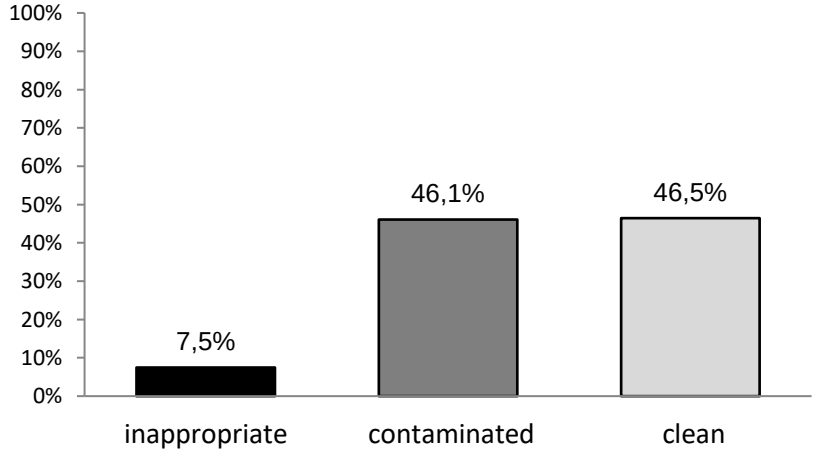
Bacteriological cultures of colostrum samples expressed during the first 4 days of life by mothers of infants in the NICU were collected and compared to bacteriological cultures of their mature milk expressed between day 28 and day 31 between January 2010 and April 2011 (148). Milks were classified slightly differently than before as "clean" (commensal bacteria $<10^5$ CFU per mL and no pathogen), "contaminated" (commensal bacteria $\geq 10^5$ CFU per mL and/or pathogen $< 10^4$ CFU per mL) and "inappropriate" (pathogen $\geq 10^4$ CFU per mL or the presence of Bacillus Cereus). In the NICU, milks classified as "clean" were administered raw within 72 hours to infants <32 weeks gestational age (GA) if the mother was seronegative for the cytomegalovirus (CMV), and to infants ≥ 30 weeks regardless of the mother's CMV status. Milks classified as "contaminated" were all pasteurized in a climatic chamber (Dry Pasteurization, CLIMATS, France) before use. "Inappropriate" milks were discarded.

Statistical analyzes were performed by comparing percentage with the chi squared test using Statistica software version 10 (StatSoft). A $p<0.05$ was considered statistically significant.

2.3. Results

2.3.1. The microbiological composition of expressed human milk

During the study period, bacteriological results of 5842 samples from 176 mothers were included (Figure 2.1). 46,5% of the HM samples were considered as "clean" and used raw to feed premature infants. 46,1% were considered as "contaminated" and pasteurized before utilization. 7,5% were



"inappropriate" (contaminated with potentially pathogenic bacteria) and were discarded. 26% of pathogenic bacteria found in discarded milk samples were gram-positive bacteria, mainly *Staphylococcus aureus*, 72% Gram-negative bacteria and 2% a combination of both. *Escherichia coli* was the gram-negative bacteria most commonly found. According to seasons, HM samples were more often contaminated during the spring and the summer and the incidence of pasteurization tended to increase (52% vs 37%, p<0,0001). Maternal profiles were established longitudinally. 116 mothers (66%) brought OMM without pathogen contamination. Among the 60 mothers with at least one sample had pathogen contamination, 27% had contamination occurring only during a few days, but 73% had more than 50% of their OMM discarded.

Figure 2.1. Percentage of milks classified as "clean", "contaminated" or "inappropriate" in mother's milk samples brought to NICU (N = 5842).

2.3.2. Microbiological composition of colostrum compared to mature milk

Between January 2010 and May 2011, 644 colostrum and 314 mature milk samples were collected from 292 mothers of 318 hospitalized newborns. Out of all the samples, the absence of contamination was significantly higher for colostrum than for mature milk, the number of milk samples considered "clean" represented 547/644 (84.9%) vs. 232/314 (73.9%) (p <0.001), respectively (Figure 2.2). The percentage of milk considered "contaminated" was less for colostrum (78/644 (12.1%) vs. 71/314 (22.6%), p <0.001) (Figure 2.2). The percentage of milk considered "inappropriate" was similar in both groups (19/644 (2.9%) vs. 11/314 (3.5%), p=0.65) (148). The bacteriological classification of colostrum was not significantly influenced by gestational age (data not shown).

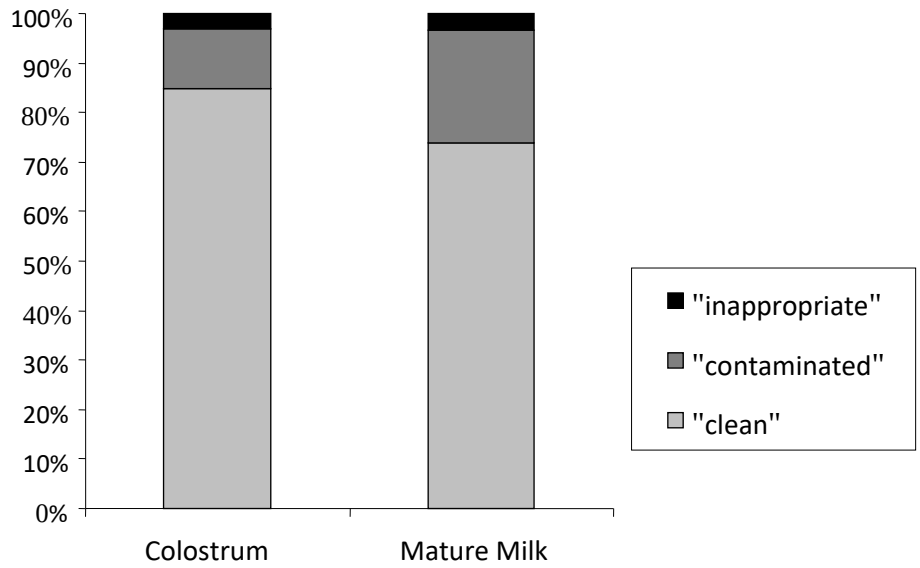


Figure 2.2. Percentage of milks classified as "clean", "contaminated" or "inappropriate" in colostrums and mature milks. N = 644 colostrum and 314 mature milks.

The identification of pathogenic bacteria differs between the two types of milk (Figure 2.3). 61% of pathogenic contaminated colostrum samples were related to gram-positive bacteria and 39% to gram-negative bacteria. No colostrum sample contained several pathogenic bacteria. Among the mature milk samples, 88% contained

one Gram-negative bacteria, 7% a combination of gram-positive and negative bacteria and only 5% contained only one Gram-positive bacteria ($p < 0.001$).

In colostrum, *Staphylococcus aureus* was the bacteria most commonly found (58.3%), followed by *Escherichia coli* (11.1%), then *Klebsiella pneumoniae* and *Enterobacter aerogenes* (5.6% each), group B *Streptococcus* and *Klebsiella oxytoca* (2.8% each). In mature milk, the bacteria most often highlighted were *Escherichia coli* (61.3%), *Klebsiella oxytoca* (17.7%), *Enterococcus* and *Enterobacter cloacae* (6.4% each), *Staphylococcus aureus* (4.8 %) and *Acinetobacter junii* (3.2%) (Figure 2.4).

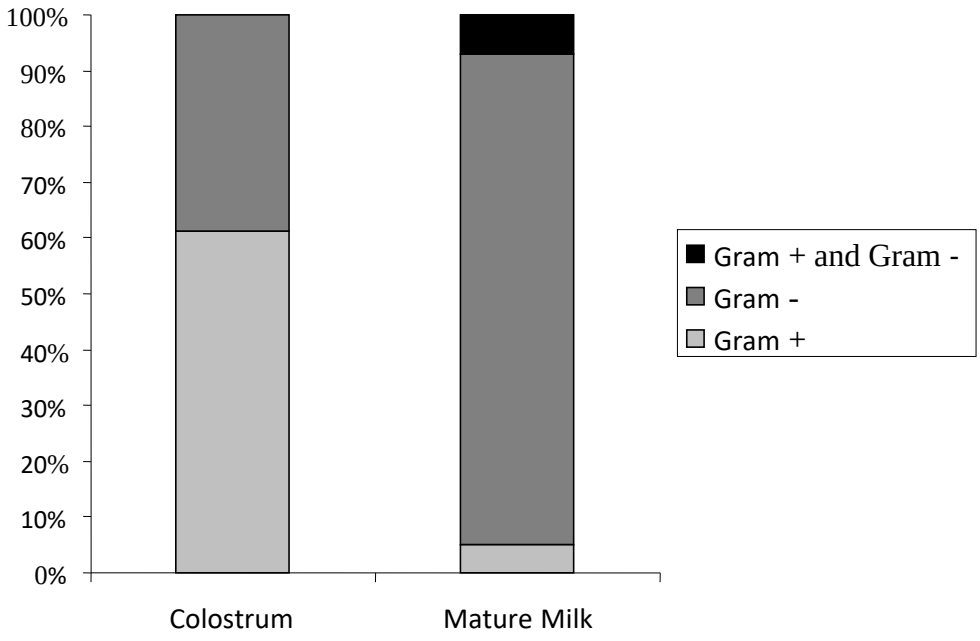


Figure 2.3. Distribution in percentage of pathogen bacteria identified in colostrums and mature milks, according to the Gram stain. N = 41 colostrums and N = 61 mature milks with a pathogen bacteria

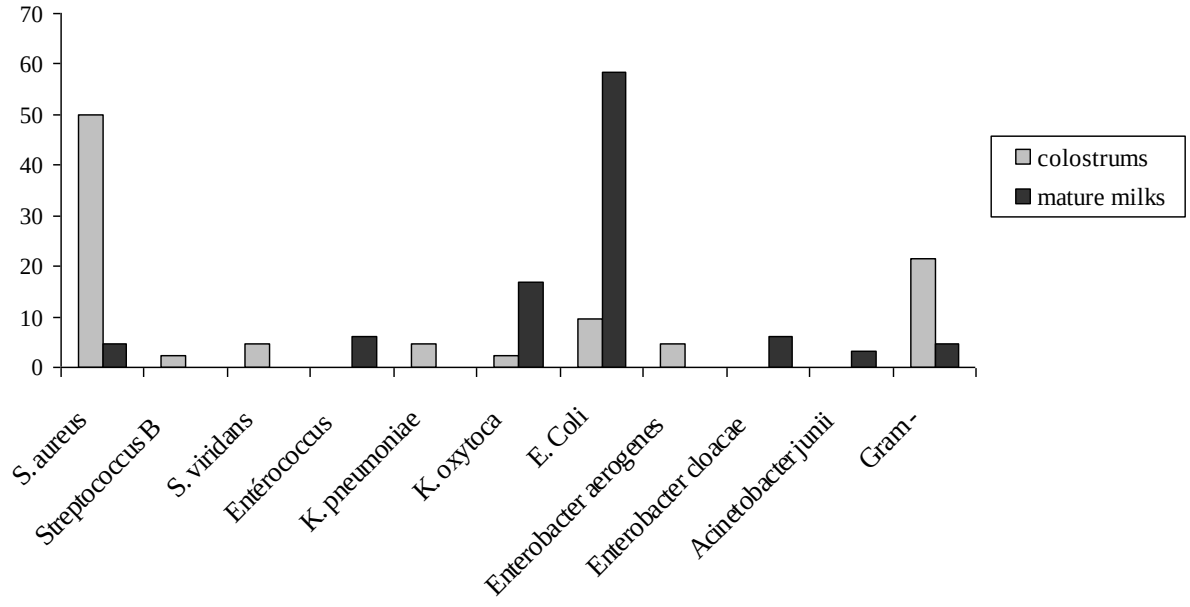


Figure 2.4. Bacterial identification as percentage of "contaminated" and "inappropriate" milks, outside commensal flora. N = 41 colostrums and 61 mature milks with a pathogen bacteria

2.4. Discussion

The first part of our study confirms that HM is not sterile and contains non-pathogenic and sometimes potentially pathogenic bacteria. Several studies based on in vitro culturing methods have shown that most of HM samples collected from mothers in NICU would be colonized with significant normal skin flora, predominately *Coagulase negative Staphylococcus* and that 10-40% of HM samples were contaminated with pathogenic germs (66, 69, 140, 149).

In our study, 66% of the mothers always brought OMM samples without pathogenic germ throughout the NICU stay. Our study also suggests that heavy contamination of OMM in our neonatal unit accounts for about 25% of the mothers with a discard rate of more than 50% of their samples. An influence of the seasons was also highlighted on the bacteriological contamination of the OMM. These results suggests that a careful survey of OMM contamination and the constitution of an educational program are necessary to improve the use of OMM in VLBW infants.

Although bacteria are expected in raw HM, there is no international consensus for acceptable threshold level of bacteria prior feeding vulnerable premature infants. Published guidelines issued from HM banks exist concerning safe contamination thresholds prior and after pasteurization for donor milk (73) but should not be necessary applied to OMM. Raw OMM is still used in many countries without bacteriologic testing. Although there is a well-known bactericidal effect of raw mother's milk (97), premature infants receiving raw OMM were exposed to many bacteria. OMM bacterial contamination does not typically result in infant's infection (69). However, there are several case reports suggesting that HM may be a source of infection (71, 72, 139, 141-143) with particularly significant consequences in the most vulnerable preterm infants with gastro-intestinal immaturity, reduced gastric acidity and increased risk of digestive translocation. Therefore, on the principle of precaution, some authors have suggested a bacterial monitoring of OMM, discarding HM with high pathogenic germ counts and pasteurizing in case of lower HM contamination (75, 76), arguing that pasteurization does not reduce the protective effect of OMM on sepsis and NEC in preterm infants (48-50, 78, 150).

At the time of the first study, we applied similar technic and criteria than those applied in the HM bank; I µl of sample incubated during 18-24 h in order to obtain a result before 48h allowing to provide the raw OMM before 72h. OMM with $<10^5$ saprophyte bacteria were provided raw, OMM with $\geq 10^5$ were pasteurized and all samples with pathogenic germ were discarded. Thus, half of OMM were pasteurized because of saprophyte contamination, losing some HM properties (cellular immune components, IgA, IgG, lactoferrin, lysozyme…). In addition, since the time of the study, it has been suggested that gut contamination by HM *Coagulase negative Staphylococci* (CNS) is promoted by the load of CNS *mec*-A negative gene of the HM and plays a probiotic role in reducing the risk of late onset sepsis (LOS) mainly resulting from NICU environmental contamination with methicillin resistant strains CNS *mec*-A positive (136). Therefore, the need for pasteurization of the heavy CNS colonized HM is questionable and requires further evaluations. Similarly, during the study, any sample with pathogenic bacteria were discarded, even in case of low contamination ($<10^4$ CFU/mL), to avoid potential toxin

production. This position is also questionable in regard to the efficacity of pasteurization and the potential benefits of OMM in VLBW infants. Cacho et al have shown that each mother has unique OMM microbiota and suggested to inoculate DM, with small proportions of OMM (10-30%) in order to restore the live potentially beneficial OMM microbiota in pasteurized DM (151).
In the light of our study and the literature, our bacterial threshold have gradually evolved (table 2.1). Only pathogenic bacteria are considered for pasteurization or elimination. Saprophytic microorganisms like CNS are regarded as a part of the personalized OMM microbiome and not requiring treatment.
In 2016, The Superior Health Council of Belgium provides recommendations on the use of raw own mother's milk for preterm infants (≤ 28 weeks and/or < 1000 g) in Neonatal Intensive Care (75, 76), but bacterial criteria for raw milk use (≤ 10^4 CFU/mL total aerobic flora) and for pasteurization (≤ 10^5 CFU/mL total aerobic flora) were stricter than we used in 2010 (< 10^5 CFU/mL total commensal bacteria for raw use an >10^5 CFU/mL total commensal bacteria for pasteurization) and in use since 2013 (no limit for commensal bacteria for raw OMM) with the consequence of having to pasteurize OMM previously used as raw and to eliminate OMM that we would have pasteurized (table 2.1).

Table 2.1 : Evolution of bacteriologic criteria for OMM use in CHR in 2005, 2010 and 2013 compared to guidelines of CHS 2016

	CFU/mL in OMM			
OMM	**CHR 2005**	**CHR 2010**	**CHR 2013**	**CHS 2016**
Raw	< 10^5 commensal bacteria and no pathogenic bacteria	<10^5 commensal bacteria and no pathogenic bacteria	No limit for commensal bacteria and no pathogenic bacteria	**≤10^4 commensal bacteria** and ≤10^2pathogenic bacteria
Pasteurized	≥10^5 commensal bacteria and no pathogenic bacteria	≥10^5 commensal bacteria and/or <10^4 pathogenic bacteria	<10^4 pathogenic bacteria	**≥10^4commensal bacteria** and/or <10^4 pathogenic bacteria
Discarded	Any pathogenic bacteria	≥10^4 pathogenic bacteria	≥10^4 pathogenic bacteria	**≥10^5commensal bacteria or** ≥10^4 pathogenic bacteria

CHR= Centre Hospitalier Régional de Liège; CHS= The Superior Health Council of Belgium**;**
CFU = colony forming unit/mL. The grey cells show the changes in CHR criteria. Bold type indicates more stringent CHS criteria compared to CHR criteria 2010 and 2016.

Our study also suggests that bacterial contamination could be the result of further potential source of contamination occurring in the process of expressing and storing HM (149). Ensuring the microbiological safety of expressed HM requires avoiding any microbiological contamination while preserving the immune components (75). The clean collection of expressed HM is important to avoid external contamination (152). Collecting HM in NICU was shown to reduce the risk of microbial

contamination compared to home collection (75). Guidelines for collection and handling of HM must be provided to mothers and followed both ,at hospital and at home (75, 152, 153).

The second part of our study suggests that the bacteriological quality of colostrum is significantly higher compared to mature milks. The pathogens predominantly identified in colostrum are gram-positive bacteria, especially *Staphylococcus aureus*, by contrast to the gram-negative bacteria predominance in mature milk, especially *Escherichia coli*. It has been suggested that oropharyngeal colostrum is a continuation of the exposure of the foetal oropharynx to growth and protective bio factors of the amniotic fluid during foetal life. Early oropharyngeal administration of raw colostrum (OC) is safe and could be preferentially given as raw in premature newborns for its anti-infectious and immune properties (146) and its role to promote gut maturation (154, 155). Colostrum secreted 1 to 4-5 days after birth, is richer in immunoglobulins (IgG, IgA, IgM) and lactoferrin than mature milk, and is the fluid of which secretory IgA is the highest in all exocrine fluids (156). Limited available data suggested that OC could potentially reduce the risk of LOS, NEC, death, feeding intolerance and other complications of prematurity (157). Recently, a systematic review and meta-analysis was performed on RCT evaluating OC in VLBW infants (158). In all, eight RCTs involving 682 patients (OC group: 332; non-OC group: 350) were included in the meta-analysis. The results suggested that OC was associated with a significantly reduced incidence of ventilator associated pneumonia, [odds ratio (OR) = 0.39, 95% CI: 0.17–0.88, n=365; P = 0.02],reduced time to full enteral feeding days (mean difference = −2.66 days, 95% CI: −4.51 to −0.80, n=662; P = 0.005),as well as a potential significant reduction of NEC (OR = 0.51, 95% CI: 0.26–0.99, n=677; P = 0.05) and of proven LOS (OR = 0.64, 95% CI: 0.40–1.01, n=585;P = 0.06).This review suggested also that OC tends to reduce mortality rate (OR = 0.60, 95% CI: 0.34–1.08,n=493; P = 0.09).

2.5. Conclusion

HM is a non-sterile complex ecosystem and expressed colostrum is less contaminated than mature OMM. Potential bacterial contamination occurs in the process of expressing and storing HM. The risk of bacterial contamination of OMM seems to be permanent throughout the NICU stay. Ensuring the microbiological safety of expressed OMM requires avoiding any microbiological contamination while preserving the immune components. Guidelines for collection and handling of OMM must be provided to child mothers. A bacteriological HM testing and HM pasteurization should be considered for this vulnerable population of preterm infants.

Although bacteria are expected in raw HM, there is no consensus for acceptable thresholds of bacteria prior feeding vulnerable premature infants and controversy persists about significance of HM bacteria and the risk-benefit balance of raw OMM and pasteurized OMM, between the multiple advantages of raw OMM and the possible risk of infection transmission via raw OMM, especially in fragile preterm infants. We suggest to perform a bacterial screening in VPT infants <32 weeks GA and pasteurization of OMM contaminated only with pathogens ($<10^4$ CFU/mL) (table 2.1). Saprophytic microorganisms like CNS are regarded as a part of the personalized OMM microbiome and not requiring treatment. However, colostrum is less contaminated and could be given as early as possible via oropharyngeal administration preferentially as raw in premature newborns for its anti-infectious and immune properties and potential beneficial impact on complications associated with prematurity.

A better understanding of the link between HM microbiome and health benefits and the potential factors influencing this relationship open new perspectives of future research.

Chapter 3 - Influence of new fortifier with higher protein content on growth of preterm infants: a RCT

Rigo J, Hascoët JM, Billeaud C, et al including de Halleux V. Growth and Nutritional Biomarkers of Preterm Infants Fed a New Powdered Human Milk Fortifier: A Randomized Trial. J Pediatr Gastroenterol Nutr. 2017;65(4):e83-e93.

3.1. Introduction

Even if both qualities of the fortifiers and methods of HM fortification have improved over times, nutrient fortification remains suboptimal. Incidence of postnatal growth restriction is more frequently reported in VLBW infants fed fortified HM rather than preterm formulas (49, 106-108, 127). These differences could be related to an over-estimation of HM composition and to its variability (113). Therefore, it has been suggested that currently available multicomponent HM fortifiers are not adequately designed for use in VLBW infants. A new powdered HM fortifier has been developed with higher protein: energy ratio (PER) (1.4 g of protein provided as partially hydrolyzed whey/100 mL HM), use of non-protein energy from lipids (0.7 g medium-chain triglycerides and docohexanoic acid/100mL HM) and carbohydrate, and higher electrolytes and vitamins levels in line with EPSGHAN and expert group recommendations (28, 31). The aim of the study was to assess growth and nutritional biomarkers of preterm infants fed HM supplemented with a new fortifier (nHMF) or a control HM fortifier (cHMF). The study 's primary objective was to demonstrate that weight gain (g/d) during the 3 weeks study period of infants fed nHMF would be both non inferior (lower limit of 95% confidence interval [CI] of mean difference > –1 g/ day) and superior (lower limit of 95% CI of mean difference > 0 g/ day) to that of infants fed cHMF. The secondary objectives of the study were to evaluate the other growth parameters: weight gain (g/kg/d), length gain (cm/week), head circumference (HC) gain (cm/week), z-score gain for those three parameters, feeding tolerance, adverse events, times to full fortification and to full enteral feeding, as well as markers of protein-energy, electrolytes, bone metabolic status.

3.2. Material and method

In this controlled, multicenter, double blind study, a sample of preterm infants ≤ 32 weeks or ≤ 1500 g and tolerating ≥ 100ml/kg/d were randomized to receive a new HMF (n=77) with higher protein (1.4 g), fat content (0.7 g) and 1,3 g of carbohydrate or a control HMF (n=76) with 1 g of protein, no lipid and 3.3 g of carbohydrate for a minimum of 21 days. The HM fortifiers were both cow's milk based and provided similar energy supplementation.

Infant's weight was measured daily, lengths and HC were measured weekly. Weight-for-age, length-for-age, HC-for-age z scores were calculated according to Fenton growth charts (159). Weight gain velocity (g/kg/day) was calculated using the average of the start and end weights as the denominator. Markers of protein-energy, electrolytes and bone metabolic status were also collected in blood (serum creatinine, prealbumin, BUN, hemoglobin, hematocrit, electrolytes, calcium, phosphorus, alkaline phosphatase) and urine (urinary urea, creatinine, electrolytes, calcium and phosphorus) samples.

Weight gain was analyzed in the intent-to-treat (ITT) and per-protocol populations by analysis of covariance (ANCOVA) adjusting for postmenstrual age, sex, weight and center.

Secondary endpoints were analysed in the ITT population only. For noninferiority and superiority tests, 1-sided P values are provided and should be compared to a reference value of 0.025. For other tests, 2-sided P values are provided and should be compared to a reference value of 0.05. Additional information about the statistical analysis is available in the published article attached as Appendix 3.

3.3.1. Population

A total of 274 infants were screened, with 153 were enrolled and randomized to either the nHMF (n=77) or cHMF (n=76) (figure 3.1). Baseline demographic and anthropometry data of infants were similar in both groups except for parental smoking (table 3.1). The majority (84% and 87% by volume in nHMF and cHMF, respectively) of milk provided to infants across all study sites was pasteurized. Donor milk was always pasteurized and accounted for 49% and 51% of the fortified HM volume provided during the study in the nHMF and cHMF groups, respectively. There was no significant difference in the mean volume of fortified milk intake between treatment groups (152.7±13.0 and 152.6±17.2 ml/kg/day in nHMF and cHMF, respectively). Protein intake estimated using standard values for preterm HM composition per 100mL (160) was significantly greater in the nHMF group compared to cHMF (4.48±0.38 vs. 3.81±0.43 g/kg/day, respectively; $p<0.001$) due to the higher protein content of the nHMF. Estimated energy intake was not significantly different between groups (125 kcal/kg/day in both groups).

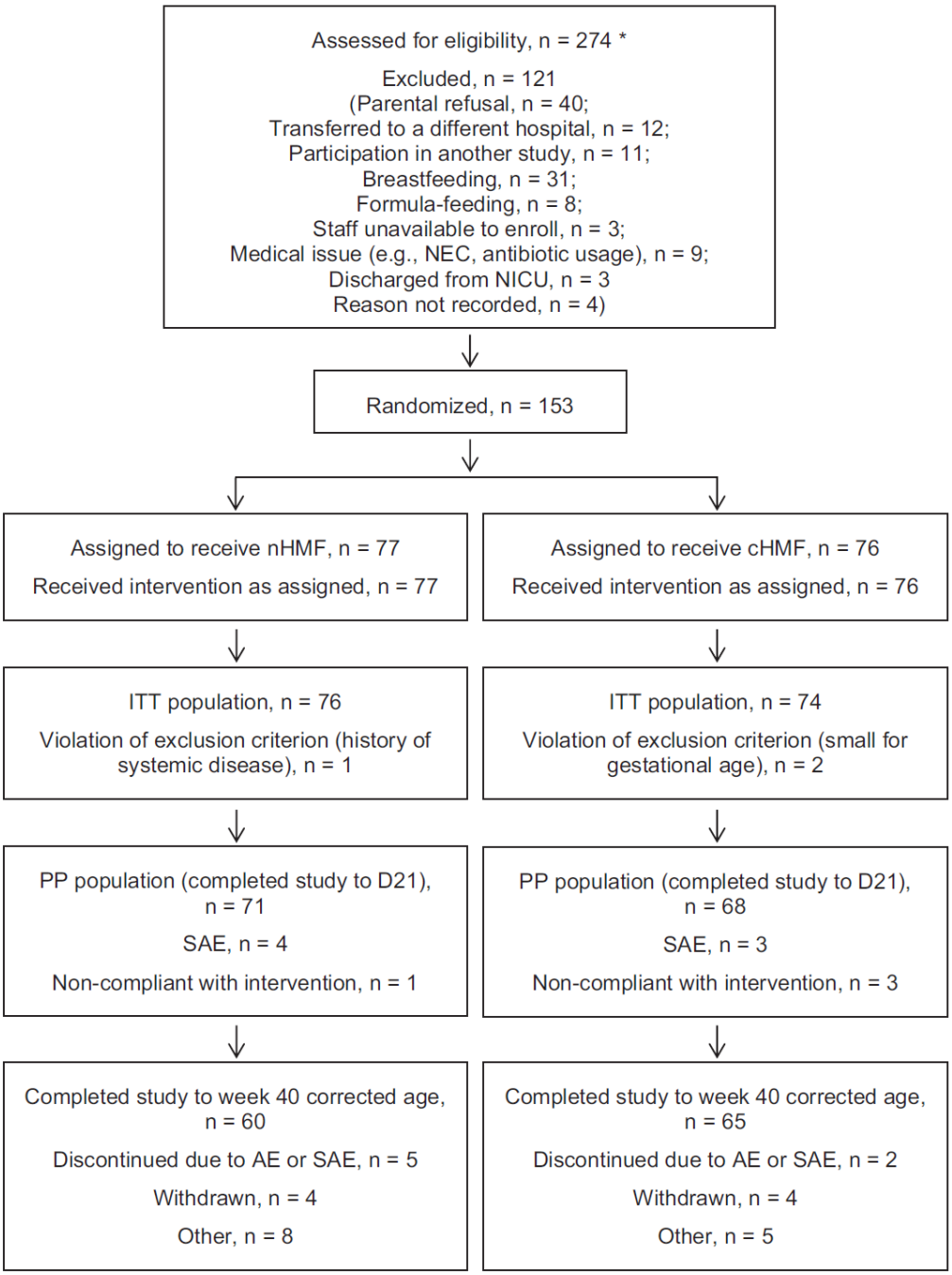


Figure 3.1. Flow of study participants

nHMF = new human milk fortifier; cHMF = control human milk fortifier; ITT = intent-to-treat; PP = per-protocol; D21 = study day 21; SAE = serious adverse event; AE = adverse event.* Although screening procedures were standardized across sites, some variability in pre-screening procedures did occur.

Table 1.1.Demographic and baselines characteristics of infants and parents

	nHMF (n = 76)	cHMF (n = 74)	p-value
Infant characteristics			
Boys	38 (50)	35 (47)	0.747
Vaginal delivery	24 (32)	20 (27)	0.593
Twin	18 (24)	16 (22)	
Birth weight, g	1147 ± 258	1156 ± 289	0.829
< 1000 g			
n (%)	24 (32)	26 (35)	
Birth weight, g	850.5 ± 118.9	847.3 ± 105.1	0.921
≥ 1000 g			
Birth weight, g	1283.6 ± 175.4	1323.9 ± 206.2	0.296
Birth length, cm	37.1 ± 2.7	37.1 ± 3.1	0.945
Birth head circumference, cm	26.5 ± 2.7	26.7 ± 2.5	0.650
Gestational age at birth, weeks	28.8 ± 2.1	28.7 ± 1.8	0.730
Postnatal age at study time points, days *			
Day 1	16 (13, 20)	17 (13, 23)	
Day 21	36 (33, 40)	37 (33, 43)	
Week 40 corrected age	76 (66, 91)	76 (67, 83)	
Apgar score			
1 min	5.8 ± 2.5	5.8 ± 2.3	0.995
5 min	8.0 ± 1.8	7.7 ± 1.9	0.250
Parent characteristics			
Mother smoker during pregnancy	6 (9)	18 (29)	0.006
Father smoker	3 (5)	12 (21)	0.013
Mother drank alcohol during pregnancy	0 (0)	4 (6)	0.054
Mother's age, y	31.1 ± 5.1	30.8 ± 5.5	0.739
Mother's BMI before pregnancy, kg/m^2 *	23.2 (20.6, 27.2)	21.3 (19.7, 26.1)	0.278
Mother's weight gain during pregnancy, kg	11.2 ± 6.8	9.2 ± 5.2	0.094

*nHMF = new human milk fortifier; cHMF = control human milk fortifier; FSI1 = fortification strength increase day 1; BMI = body mass index. Data are presented as n (%) for categorical variables and mean ± SD for continuous variables except where noted. * Data are presented as median (Q1,Q3).*

3.3.2. Growth

In the overall ITT population, adjusted weight gain from D1 to D21 was 2.3 g/day higher in the nHMF group, with the 95% CI ranging from 0.4 to 4.2 g/day, demonstrating the non-inferiority (p<0.001) and the superiority (p=0.01) of the nHMF. Weight gain from D1 to D21 remained significantly higher in the nHMF group when expressed in g/kg/day (Table 3.2). Weight-for-age *z* scores (Figure 3.2) remained stable from D1 to D21 in the nHMF group, in contrast to the cHMF group where they decreased (p=0.007 vs. D1). At D21, the weight-for-age *z* score was significantly higher in the nHMF group compared to cHMF (+ 0.12 [95% CI: 0.03 to 0.22]). Length and HC gains during the study period were similar between groups (Table 3.2). However, Length-for-age *z* scores from D1 to D21 (Figure 3.2) decreased significantly in the cHMF group (p=0.041). In addition, at W40CA (week 40 gestational age), adjusted HC-for-age *z* score was significantly higher in the nHMF group compared to cHMF (+ 0.41 [95% CI: 0.14 to 0.68]).

3.3.3. Protein-Energy Status

Blood urea nitrogen (BUN) decreased progressively in the cHMF group (p=0.004 for D21 vs D1) while it increased in the nHMF group (p<0.001 for D10/11 vs D1) and remained stable up to D21. Urinary urea excretion (corrected for creatinine excretion) at D1 was similar in the two groups. Urea excretion remained at the same level in the cHMF group but increased sharply with the use of the nHMF. At D21 urea excretion was significantly higher in the nHMF group than in cHMF (+108.7% [95% CI: +66.0% to +162.5%]).

Table 3.2. Anthropometric gains from D1 to D21

	Treatment group				
	n	**nHMF**	**n**	**cHMF**	***P****
Weight gain, g/kg/day	64	18.3 ± 3.7	67	16.8 ± 3.7	0.013 †
Length gain, cm/week	55	1.23 ± 0.62	65	1.18 ± 0.49	0.842
HC gain, cm/week	57	1.04 ± 0.32	65	0.96 ± 0.26	0.125

D1 = study day 1 (day 1 of full-strength fortification); D21 = study day 21; nHMF = new human milk fortifier; cHMF = control human milk fortifier; HC = head circumference. Data are presented as unadjusted mean ± standard deviation.

* One-sided superiority P-value based on ANCOVA model adjusted for postmenstrual age and relevant anthropometric measure at D1, sex, and center.

† Adjusted difference in weight gain (nHMF – cHMF): mean difference = 1.18 g/kg/day; 95% CI = 0.14, 2.21.

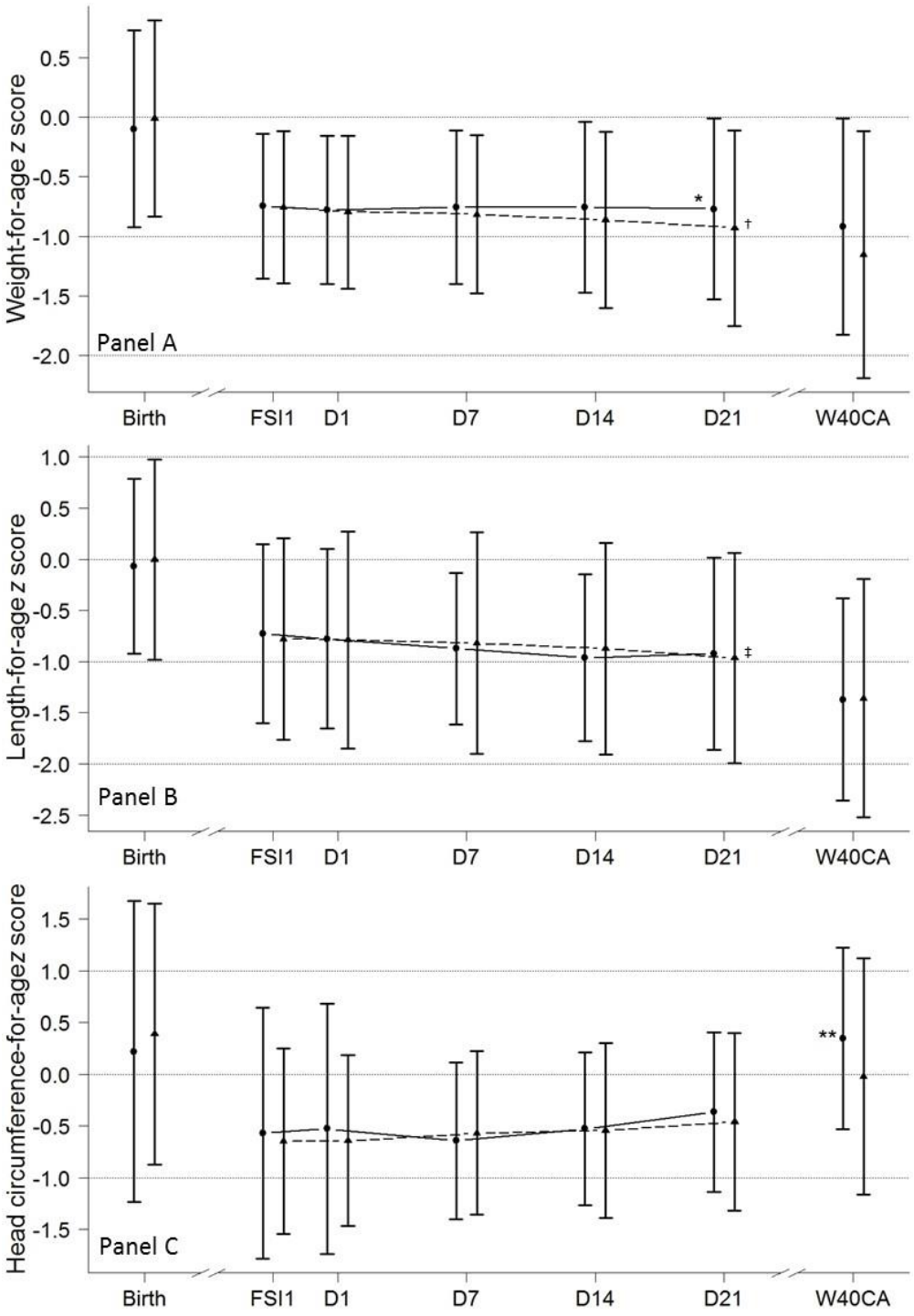


Figure 3.2. Mean ± SD weight-for-age (panel A), length-for-age (panel B), and head circumference-for-age (panel C) z scores for the overall ITT population.

*Circle symbols / solid line = nHMF; Triangle symbols / dashed line = cHMF; SD = standard deviation; ITT = intent-to-treat; FSI1 = fortification strength increase day 1; W40CA = week 40 corrected age; z scores calculated using Fenton preterm growth chart (159). * p=0.013 vs. cHMF (by ANCOVA, adjusting for value at D1, sex, and center); † p=0.007 vs. day 1 (by t-test); ‡ p=0.041 vs. day 1 (by t-test); ** p=0.003 vs. cHMF (by ANCOVA, adjusting for value at D1, sex, and center).*

3.4. Discussion

In this multicentric study, we showed that in VLBW infants fed fortified HM at isocaloric intakes, an increase of 0,65 g of protein/kg/d induces an adjusted weight gain benefit of 2.3 g/d (p=0.01) corresponding to 1.18 g/kg/d (95% CI = 0.14, 2.21; p=0.013) during the study period. In addition, weight and length z-scores remained stable during the study period (-0.06 SD, p=0.39 and -0.17 SD, p=0.10) with the new HMF but decreased significantly (-0.18 SD, p=0.007 and -0.20 SD, p=0.04) in the group receiving the control HMF. Therefore, the additional protein intake promotes growth and reduces postnatal growth restriction in VLBW infants.

The value of protein supplementation in HM has been suggested by several metabolic balance studies performed in our laboratory. These studies indicated that increasing the protein content of HM improves the weight gain and reduces the relative fat mass deposition (161-163).

Computing the results of metabolic and energy balance studies performed in 286 preterm infants with a mean BW of 1354 g for a GA of 30.5 weeks fed unfortified or fortified HM and preterm formulas, we revealed that the major determinants of weight gain were protein intake and protein energy ratio of the diet (PER) whereas protein intake was the only significant determinant of lean body mass (LBM) gain. By contrast, fat mass (FM) gain was positively related to energy intake and negatively related to the PER of the diet. These observations suggest that in the present study, the additional protein supply provided by the nHMF could improve not only the weight gain but also the weight gain composition, promoting LBM and reducing FM deposition (34).

Our results are consistent with those of a recent meta-analysis of 5 studies (comprising 352 infants with GA ≤34 weeks) concluding that infants receiving higher protein fortifiers had significantly greater weight (mean difference of 1.77 g/kg/d, length (0.21 cm/week, and HC (0.19 cm/week) (118). However, such benefits were not always observed in all recent studies. Maas and al (164) showed in a randomized trial that an increase in protein intake by 0.6 g/kg/d to a mean intake of 4.3 g/kg/d did not further enhance growth of very preterm infants, who achieved near fetal growth rates. Reid et al (165) in a small RCT neither found differences in growth rate for weight, length and HC between a standard HMF (1 g/100mL) and a high protein HMF (1.8 g/100mL) providing an increased protein intake of 0.7 g/kg/d. In this study, both groups also achieved growth rates approaching intra-uterine growth.

In all those studies, as well as in our multicentric trial, and in contrast to our previous metabolic balances research, composition of the HM provided to the preterm infants was not actually measured but estimated according to theoretical reference values. In our study, the composition of the preterm OMM during the early phase of lactation was taken as reference although with large use of DM and pasteurized OMM, energy and protein intakes really administrated may have been overestimated. In addition, the carbohydrate to non-protein energy ratio was lower in the nHMF, leaving the possibility that the additional metabolizable energy supplementation provided by the nHMF was lower than that provided by the cHMF. This could therefore induce differences in the total metabolizable energy intake between the two HMF. Additionally, increases in BUN and urinary urea excretion indicated an increase in urea production in the nHMF group. Both findings led us to speculated that the protein utilization in the new HMF group may have not been optimal due to a relative deficiency in the metabolizable energy intake. Therefore, we suggested that an increase in energy supply in the nHMF could improve protein utilization and potentially growth of the VLBW infants.

Current available nutritional guidelines for premature infants<32 weeks gestation at birth recommend an enteral protein intake in the range of 3.5-4.5 g/kg/d (31) without distinction of the type of enteral feed (OMM, DM or preterm formula). Achieving these intakes using unfortified HM is clearly impossible due to the low protein content of HM. In order to meet recommended intakes, the use of multicomponent fortifiers is required. Nevertheless, most of the available HM fortifiers (adding 0.8-1.1 g/100mL) do not allow to reach such protein intakes, given the variability of HM composition and its progressive decrease in protein content during lactation (113). Therefore, we suggest that specific recommendations should be formulated for infants fed fortified HM promoting new concepts of fortification that address the variability of protein and energy contents in HM (DM or OMM), their bioavailability, and the additional factors that might help to optimize nutrition for preterm infants.

3.5. Conclusion

A new HM fortifier made with partially hydrolyzed whey protein, the use of non-protein energy from lipids and a higher protein: energy ratio is well tolerated. It improved weight gain of preterm infants compared to control fortifier but may benefit from further additional energy supplementation.

Chapter 4 – Quantification of HM macronutrients composition by infrared method: calibration and validation of an infrared analyzer using reference's methods.

Buffin R, Decullier E, De Halleux V, et al. Assessment of human milk composition using mid-infrared analyzers requires calibration adjustment. J Perinatol. 2017;37(5):552-557.

de Halleux V, Buffin R, Picaud J-C, Studzinski F, Rigo J. Is Milkoscan® a rapid infrared analyzer, after a specific calibration, accurate and precise enough for human milk fortification? . Congress of joint European Neonatal Societies (jENS 2015), Budapest. Journal of Pediatric and Neonatal Individualized Medicine 2015;4(2):e040210; 2015.

4.1. Introduction and objectives

Analysis of milk by mid-infrared (IR) technology is based on the principle that different functional groups absorb mid-IR energy at different wavelengths (Figure 4.1).

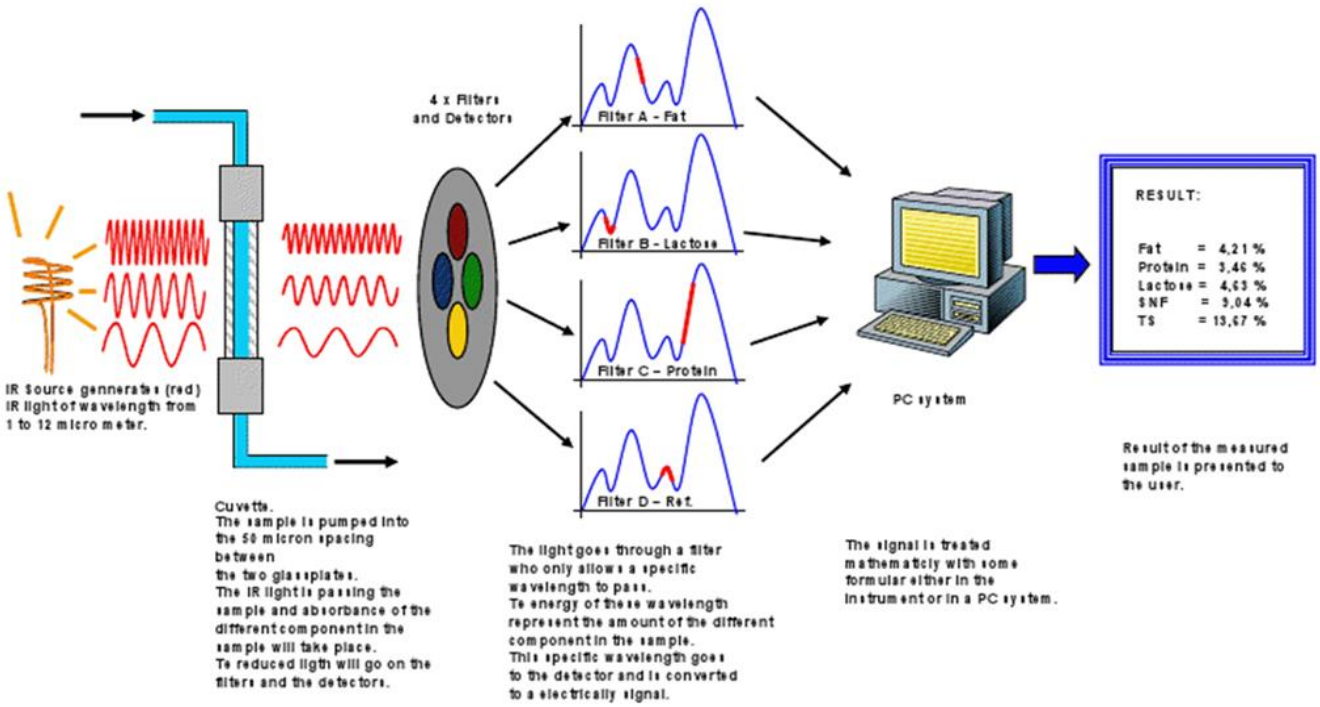


Figure 4.1. Analysis method based on filters (FOSS IR – Technology)

It requires 1-2mL (Miris®) to max 10 mL HM (Milkoscan®) to provide data on protein, fat, and carbohydrate contents in 90 seconds. Milk analysis by IR is an indirect method, so instruments must be calibrated using milk samples with reference values established by reference methods. Moreover, the instrument was originally developed for cow milk analysis in the dairy industry, and requires additional calibration for HM use (166-168). Our objective was to establish calibration's equations for lipid and protein measurement for 2 infrared analyzers (IRA) currently available (Milkoscan® Minor and Miris® HMA) and then validate our algorithms using new HM samples for each device.

4.2. Material and method

HM analyses with a mid-infrared analyzer were performed at the Human Milk Bank of Liège (Milkoscan® Minor; Foss, Hilleroed, Denmark) and at the HM bank of Lyon (Miris® HMA; Miris AB, Uppsala, Sweden) (113, 169). HM was warmed to 37°C and homogenized using an ultrasonic homogenizer (Sonicator®, Uppsala, Sweden) before analyze.

In 2005, the individual equations of correction for lipid, protein and carbohydrate were established for the IRA Milkoscan® Minor at the HM bank of Liège, by the plotting of measurements readouts obtained by the IR analyzer against those generated tough reference chemical methods for nitrogen (nitrogen analyzer EP Analyzer EP 428; Leco France) and fat ("Soxhlet" Soxtec Aventi 2055; Foss) performed in the laboratory of Liège (170). We did not have a reference method for measuring total carbohydrates in the laboratory. The raw carbohydrate values obtained by IRA (n=12) were stable and similar (mean of 6,7±0,3 g/dL) to those found in the literature (114). We decided to keep the value obtained by the Milkoscan and calibrate it in relation to the supplementation and dilution of a HM sample. The inverse linear function of the resulting regression equation was used as a correction algorithm and subsequently applied to future read outs of the IRA Milkoscan (170). In 2011, results of HM samples from HM bank were analyzed in our laboratory, for comparison to chemical analysis to check the validation of Milkoscan. In 2015, we performed 70 HM analysis using reference methods to evaluate the accuracy of lipid and protein concentration assessed by 3 generations of IRA Miris used at HM bank of Lyon (Croix Rousse University Hospital) (169) and at that time we also re-validate accuracy and precision of calibration equations for protein and fat, in use on our IRA Milkoscan and evaluate possible temporal changes (171). Protein nitrogen equivalent (g/100 mL) was calculated as nitrogen in g *6.25 (172, 173). Our aim was to calibrate each IR instruments to provide the total protein concentration including non-protein nitrogen of HM similar to the data obtained in metabolic balance studies.

Regression analysis was performed to assess relationship between IRA and reference chemical analysis. Inverse relationships were used to obtain the correction equations and these equations were then applied to the raw HMA values to calculate the corrected HMA values. Pearson correlation was analyzed between the two methods and relationship strength was represented coefficient of determination R^2. A $p < 0.05$ was considered as significant.
Comparisons were performed using the Bland-Altman statistical method (174). The Bland–Altman plots were drawn with reference method as the *x*-axis and the difference between reference method and HMA as the *y*-axis. The mean and SD of the difference between the corrected HMA values and the reference values represented the accuracy and precision of HMA. All statistical analyses were performed by using Tibco Statistica software version 13 (TIBCO, Palo Alto, CA, USA)

4.3. Results

Correlation between IR measurements and reference methods for fat and protein are illustrated figures 4.2 for Milkoscan and 4.4 for Miris 1,2 and 3. Correlation between Carbohydrate IR values obtained by Milkoscan and theoretical values are presented in figure 4.3.

Raw values obtained by both IRA Milkoscan®minor and Miris®1,2 and 3 in comparison to chemical analysis were not accurate for fat and protein determination. Milkoscan underestimated substantially the protein and fat concentration of HM and slightly the carbohydrate concentration. While the IRA Miris 1,2,3 also underestimated the HM protein concentration, they overestimated the fat concentration.

Correction equations for fat and protein were therefore different for each device and each generation of IRA Miris®. After introduction of the correction equations, the agreements between the calibrated IRA and the reference methods were high. The fat and protein levels measured by HMA were significantly correlated to those determined using reference methods ($P < 0.001$) with relationships reported as R^2. For fat, protein, R^2 were as follows: Milkoscan 0.97 and 0.93; Miris1 0.96 and 0.87; Miris2 0.96 and 0.89; Miris3 0.95 and 0.88. R^2 for Carbohydrate with Milkoscan was 0.99.

Scatters plots of differences between IRA measurements and reference methods are presented in figure 4.5 for fat and figure 4.6 for protein.

After individual calibration and validation of each instrument, both analyzers provided similar, accurate and precise results for determination of protein (Milkoscan ±0.13 g /dL or ±9.4 % vs Miris ±0.12 g or ± 8.1 %; p=0.10) and fat. The Milkoscan offers higher degree of precision for fat determination compared to Miris (±0.16 g /dL or ±4.9 % vs ±0.31 g or ± 9.3 %; p<0.01). In addition, our data with re-validation analysis for the Milkoscan performed in 2011 and 2015 suggested that the accuracy and the precision of the Milkoscan remained stable according to time (171).

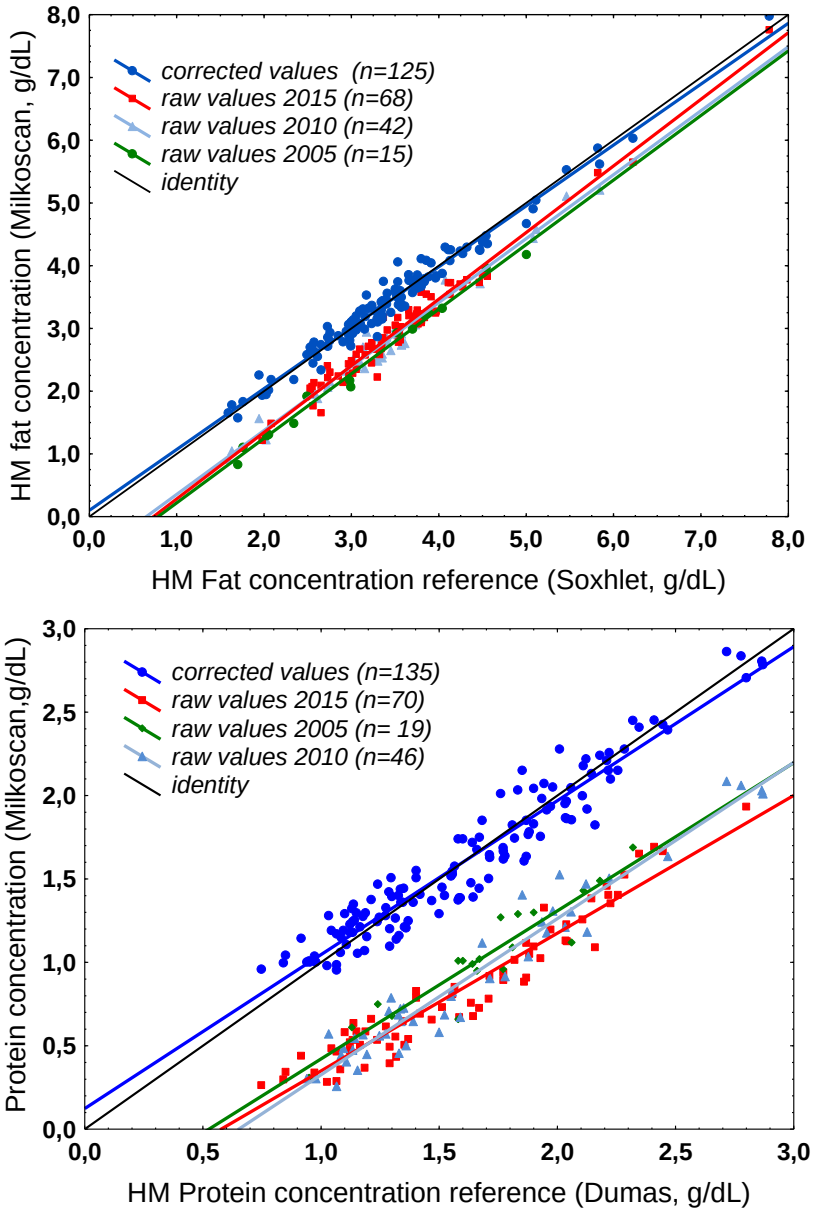


Figure 4.2. Correlation between HM fat and protein concentration measured by mid-infrared analyser Milkoscan and reference method before and after integration of correction equations

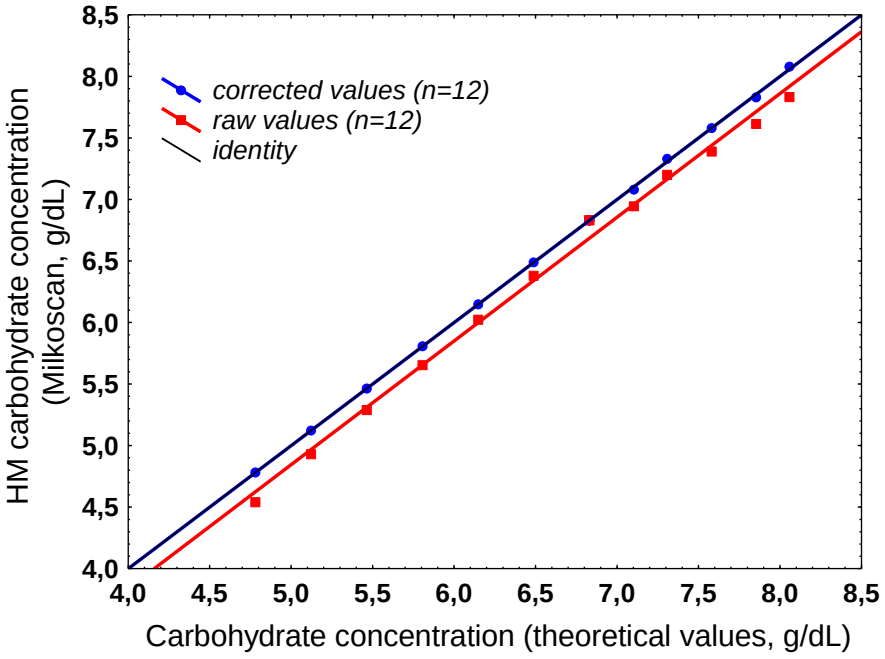


Figure 4.3. Correlation between carbohydrate concentration measured by mid-infrared analyzer (Milkoscan) and theorical values before and after integration of correction equation

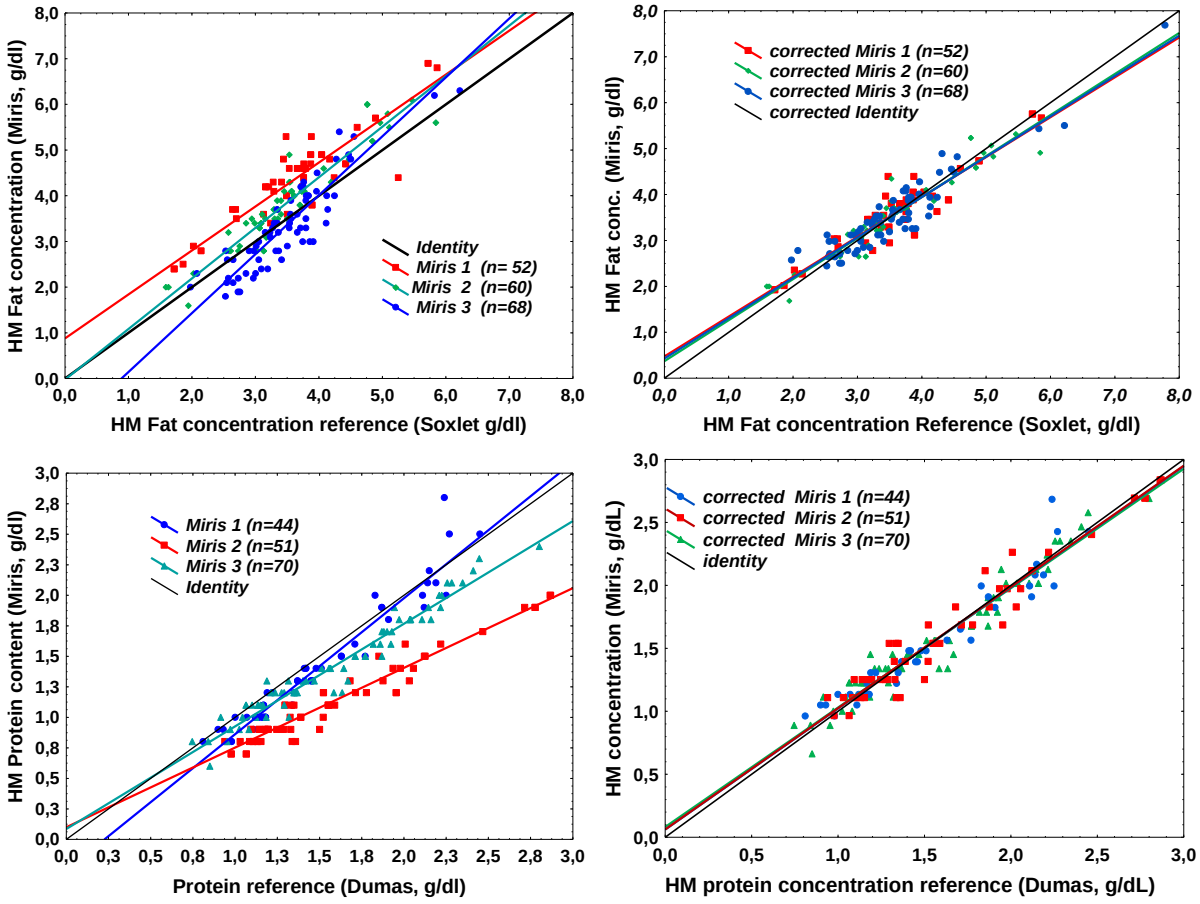


Figure 4.4. Correlation between HM fat and protein concentration measured by mid-infrared analyzers Miris 1,2,3 and reference method before and after integration of correction equations

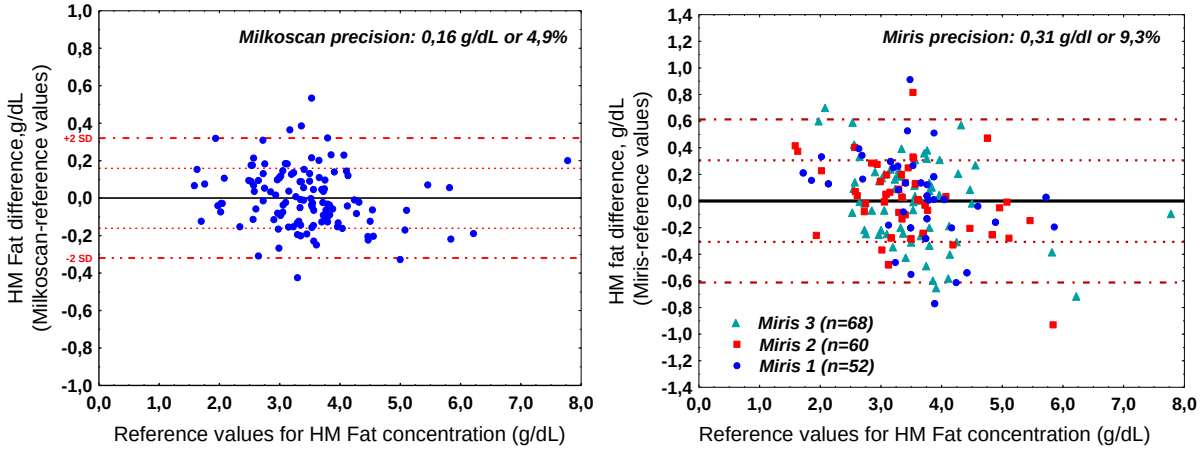


Figure 4.5. Precision of mid-infrared analyzers (Milkoscan, n=125 and Miris 1-2-3, n= 180) compared to the reference method (Soxhlet) in HM fat concentration by Bland-Altman test.

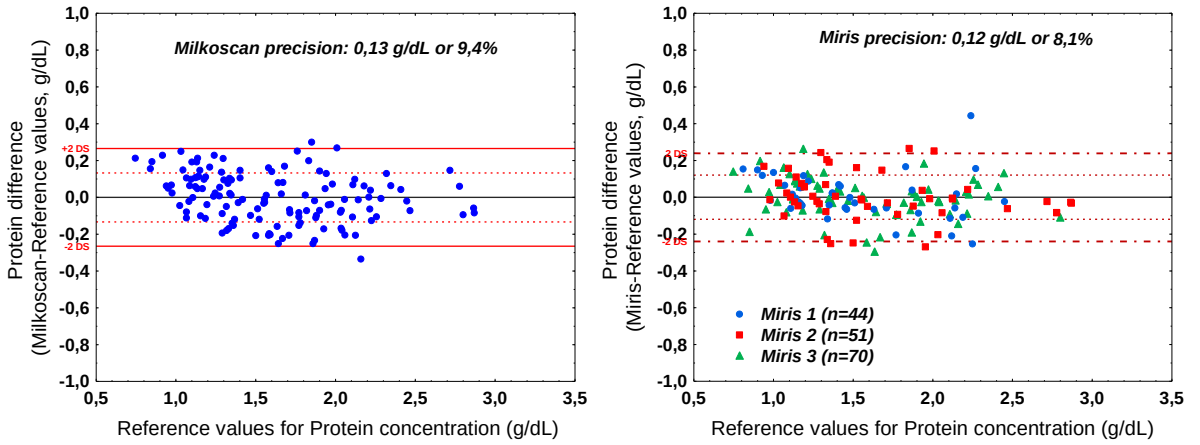


Figure 4.6. Precision of mid-infrared analyzers (Milkoscan, n=135 and Miris 1-2-3, n=165) compared to the reference method (Dumas) in HM protein concentration by Bland-Altman test.

4.4. Discussion

In our collaborative study with the HM bank of Lyon, we showed that raw values obtained by IRA Milkoscan® minor and Miris®1.2 and 3 were not accurate for fat and protein determination and that correction equations were specific for each IRA and each generation of devices (Miris®).

Milkoscan underestimated the fat and protein concentration of HM but after correction, the predicted values were highly similar to the chemical data with a precision of 4.9% and 9.4% respectively. Similarly, evaluating three different generations of Miris, we observed that the 3 Miris also underestimated differently the protein content of HM and required separate adjustments. However, after those adjustments, all three generations of Miris provided accurate evaluation with a combined precision of 8.1%. The fat content appeared overestimated but after calibration the accuracy and precision were improved with a precision reaching ± 9.3%.

For carbohydrate measurement, values obtained with our Milkoscan were stable and closed to those reported in the literature (6.7-7 g/100mL) (114). We decided to calibrate our device by using dilution and lactose supplementation. While this method affecting HM matrix is less accurate, it should be sufficient for clinical use as HM variability for carbohydrate is lower and less clinically relevant. Values reported by IRA Miris in the study of Perrin et al were 20-50% higher than reference lactose values (168). This finding is explained by oligosaccharides being present in significant numbers in HM without the IR technology being able to differentiate them from lactose.
After that correction factors specifically calculated were integrated in each device, Milkoscan and Miris became sufficiently accurate and precise for clinical use and individualized targeted fortification (113, 169). Several publications evaluated various IRA and all highlighted the need to perform chemical controls before implementation in clinical practice or research (166-168). Unfortunately, IR analyzers were marketed and often use in clinical practice without any calibration, validation and quality assurance (166). Results obtained by HMA can thus be charged with significant errors in protein and fat concentrations. Under or over-estimation of HM macronutrients concentration will affect daily individualized fortification prescriptions and lead to differences in growth rates, restriction or overgrowth (167). It is therefore important to introduce standards for good clinical laboratory practice (GCLP) when using HM analyzers to avoid introducing measurement errors. However, performing chemical analysis for HM calibration of IRA is not easy and remain time consuming. It is also important to cover the full range of macronutrients expected in the expressed HM for the analyzer's calibration and to minimize the errors that are produced from matrix measurement (166, 175).

Our study with data of revalidation (2011 and 2015) suggested that the accuracy and precision of corrected Milkoscan® remained stable according to time. While Perrin et al reported statistically significant temporal changes in some components, they concluded that these minimal changes were not likely clinically relevant (168). In contrast, Fusch et al suggest that the long-term stability of the devices vary and therefore advocate periodic revalidation (166). Recently, in 2018, Miris introduced a calibration control kit designed for HM analysis using standardized solutions with known concentrations of fat, protein and carbohydrates in the line of the GLCP concept. However, there are currently no published data on the validation of this kit (Kwan 2019).

The reproducibility of Milkoscan and Miris, was satisfactory with a coefficient of variation < 3 % for all the parameters (169). The Milkoscan is easier to use in practice, requires less manipulations and benefits from good after-sales service because of its frequent use in the cow's milk industry in Belgium. On other hands, the volume necessary for analysis is smaller for Miris (1-2 mL) than Milkoscan (10 mL). This is an important factor in clinical practice because mother's milk is often scarce and each drop of mother's milk counts. Fortunately, the new generation of Milkoscan devices need a smaller amount of milk. However, in a study from Kwan et al the sample's volume had an impact on measurement quality. They showed for the Miris that larger sample volume of 4-5 mL resulted in less random variation compared to smaller volumes of 1.5 mL (167). If there is enough milk, duplicate or triplicate analyses are recommended to control for errors.

4.5. Conclusion

Infrared analyzers allow rapid determination of HM macronutrients content using small HM volumes. However, differences between raw values obtained by IR analyzers and those by references methods are significant. Therefore, each IRA device requires an individual calibration and validation with

chemical references before implementation in clinical and research settings. After introduction of specific and individual equations of correction in their software, our results suggest that IRA Milkoscan® and Miris® provide accurate and precise protein and fat concentrations, allowing their use in clinical practice to provide the basis for individual HM fortification. However, whether such a calibration can be applied to all the devices of the same series of a single company remains to be evaluated.

Chapter 5- Variability in composition of expressed HM and benefits of Individualized compared to standard HM fortification on nutritional intakes

de Halleux V, Rigo J. Variability in human milk composition: benefit of individualized fortification in very-low-birth-weight infants. The American Journal of Clinical Nutrition. 2013;98(2):529S-535S.

de Halleux V, Close A, Stalport S, Studzinski F, Habibi F, Rigo J. Intérêt de la supplémentation du lait maternel « à la carte «. Archives de Pédiatrie. 2007;14, Supplement 1(0):S5-S10

5.1. Introduction

Optimal protein, fat, and carbohydrate concentrations in HM are crucial to the healthy growth of neonate and premature infants (36). Specifically, for VLBW infants, macronutrient content in HM is insufficient and HM needs to be fortified to meet nutritional requirements (90, 103). However, macronutrient content of HM is highly variable, particularly for fat and protein contents (114). The nutrient composition varies between mothers, within the same mother, according to length of gestation, through the lactation period, during the same day, and even during feeds (116, 176). The current practices, to fortify HM with standard amounts of fat, protein and carbohydrates assume an uniform HM composition, and may, therefore, be inadequate to overcome this variability in macronutrient composition and to meet the nutritional needs of rapidly growing preterm infants (177).

Our objectives were:

1. To assess the variability in HM composition of different HM types: from an infant's own mother's milk (OMM) or pooled donor milk (DM).
2. To evaluate the advantages of individual fortification on nutritional intakes over standard fortification.

5.2. Material and methods

5.2.1. Variability in composition of expressed human milk

By using a calibrated mid-infrared analyzer (Milkoscan Minor), the macronutrient composition of 428 HM samples used for individualized OMM fortification were obtained between June 13, 2007 and January 5, 2012. In addition, data from 138 HM pools from one single donor (each pool consisted of 5 L HM from one mother), 224 pools from multiple donors (each pool consisted of 5 L from multiple-donor mothers), and 14 pools of colostrum milk (<8 d lactation, multiple donors) were also obtained between march, 1, 2006 and August, 31, 2011 at our milk bank of the NICU at the University of Liège, Belgium. All donor HM has been frozen and pasteurized by the Holder method (62.5C for 30 min) and warmed by thawing to 37°C before analysis. The energy content was calculated by using the Atwater factors: 4 kcal/g for protein and carbohydrate, and 9 kcal/g for fat.

Data are reported as mean and SD. The variability in nutritional concentrations of each milk group was calculated as the mean value of the absolute difference between each individual value and the mean according to the following formula: variability (%) = mean[|χ(1 to n)-mean|x100/mean].
Macronutrient compositions and variabilities in OMM and DM pools from a single donor, multiple donors, and colostrum pools were compared by using 1-factor ANOVA with Bonferroni correction for multiple comparisons.

5.2.2. Effects on nutritional intakes of individualized compared to standard human milk fortification

After a pilot study showing potential benefits of individualized HM fortification procedure on nutritional intakes and growth, this individualized HM fortification protocol has been implemented for clinical use for VLBW infants since 2007 (178). A sample of the daily pool was taken. Macronutrient HM concentration was determined using an infrared analyzer (Milkoscan minor®, Foss) validated for HM use. Data of protein and fat content were gathered in an Excel® table to calculate the supplementation required to reach current nutritional recommendations (30, 31). The individualized HM fortification protocol was designed in 2 steps. Firstly, the fat content of HM was adjusted up to 4 g/dL, if necessary, by using medium-chain triglycerides (MCT; Liquigen Danone Nederland), a stabilized 1:1 mixture of MCTs and water (0.5 g/ mL). Secondly, protein content was adjusted by using a complete powdered HM fortifier (Enfamil Human Milk Fortifier; Mead Johnson) to provide 4.3 g protein/kg/d according to prescribed daily volumes of feeding. The nutritional composition of HM, the MCT and fortifier supplementations, the prescribed volume, and the infant's body weight at the day of prescription were collected at the milk bank between June 13, 2007 and January 5, 2012 and allowed calculating the nutritional intakes per kilogram of body weight per day (mean ± SD). In addition, the theoretical nutritional intakes per kilogram of body weight per day corresponding to a standard HM procedure (4 packets complete HM fortifier/dL, adding 1.1 g protein, 1 g lipids, and 14 kcal energy; Enfamil Human Milk Fortifier) were also estimated.

Nutritional intakes and variability (variability (%) = mean [|χ(1 to n) – mean|x100/mean]) resulting from individualized and standard fortifications are reported as mean with standard deviation and were compared by using paired Student's t test. All statistical analyses were performed by using Statistica software version 10 (StatSoft).

5.3. Results

5.3.1. Variability in composition of expressed human milk

Of all daily 804 OMM and HM pool samples, 56% (n=453) were below the usually assumed 1.5 g/dL of protein whereas 79 % (n=638) were below 4 g/dL of lipids and 67% (n=535) below 67 kcal/dL energy.

Significantly higher protein contents and lower fat, carbohydrate and energy contents were observed in the colostrum pools (donor milk from <8 days of lactation) compared to the other groups. In OMM, mean protein, fat and energy contents were significantly higher than in single and multiple donor milk pools. In addition, the protein content of single donor milk pools was significantly lower than those of multiple donor milk pools (Table 5.1 and Figure 5.1). Variability of protein, fat and energy contents was high in the various groups (Table 5.2 and Figure 5.1). Variability of protein content was higher between single donor pools and lower in colostrum pools than in the other two groups. Variability of fat content was higher in OMM than in all other groups but the difference was not significant compared to colostrum pool (p=0.08).

Protein values of preterm mother's milk are higher in the early postnatal period and decrease during lactation. However, a high variability remains between and within mothers (Figure 5.2)

Table 5.1. Protein, fat, carbohydrate, and energy concentrations of own mother's milk, single- and multiple-donor milk pools, and colostrum pools[1]

	Own mother's milk	**Single-donor milk pool**	**Multiple-donor milk pool**	**Colostrum pool**
n	428[2]	138	224	14[3]
Protein (g/dL)	1.52 ± 0.28[a]	1.34 ± 0.37[b]	1.46 ± 0.24[c]	2.00 ± 0.09[d]
Fat (g/dL)	3.79 ± 0.73[a]	3.45 ± 0.60[b]	3.39 ± 0.448[b]	2.92 ± 0.35[c]
Carbohydrate (g/dL)	6.76 ± 0.27[a]	6.93 ± 0.38[b]	6.81 ± 0.20[a]	6.51 ± 0.14[c]
Energy (kcal/dL)	67.3 ± 6.5[a]	64.1 ± 5.9[b]	63.6 ± 4.5[b]	60.3 ± 3.5[b]

[1] All values are means ± SDs. Values not sharing a common superscript letter are significantly different, $P < 0.05$ (1-factor ANOVA with Bonferroni correction for multiple comparisons).
[2] Own mother's milks: 28 ± 10 days of lactation.
[3] Colostrum pool: donor milk <8 d.

Table 5.2. Variability in protein, fat, and energy contents of own mother's milk, single- and multiple-donor milk pools, and colostrum pools[1] in %

	Percentage of variability[2]			
	Own mother's milk	Single-donor milk pool	Multiple-donor milk pool	Colostrum pool
n	428	138	224	14
Protein	14.7 ± 10.6[a]	19.3 ± 19.4[b]	13.5 ± 9.9[a]	3.8 ± 2.4[c]
Fat	14.5 ± 12.7[a]	10.3 ± 8.4[b]	10.6 ± 9.4[b]	9.7 ± 6.5[a, b]
Energy	7.3 ± 6.26[a]	6.9 ± 6.0[a]	5.3 ± 4.7[b]	4.4 ± 3.6[a, b]

[1] All values are means ± SDs. Values not sharing a common superscript letter are significantly different, $P < 0.05$ (1-factor ANOVA with Bonferroni correction for multiple comparisons).

[2] Variability (%) = mean [|χ (1 to n) - mean|x100/mean].

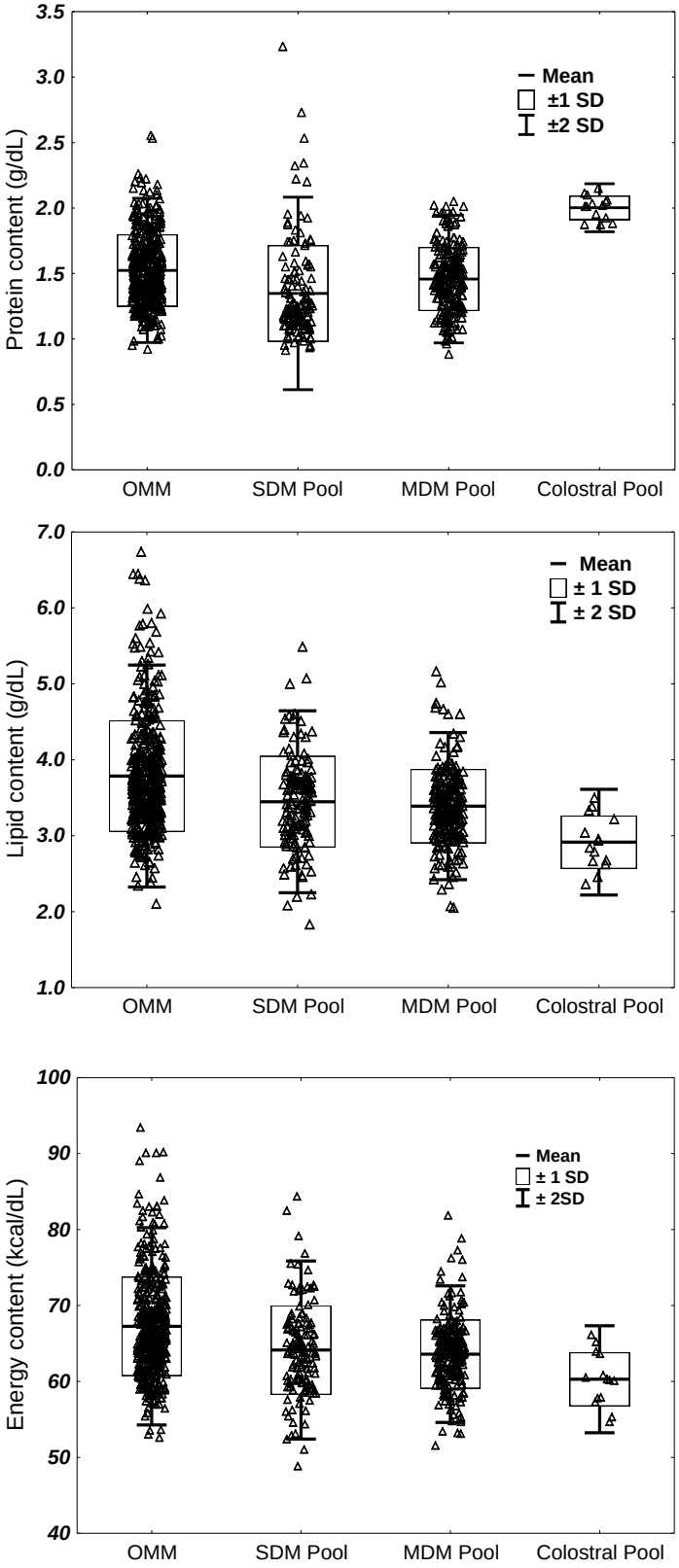


Figure 5.1. Protein, fat and energy contents of own mother's milks (OMM), single donor milk (SDM) and multiple donor milk (MDM) pools, and colostrum pools

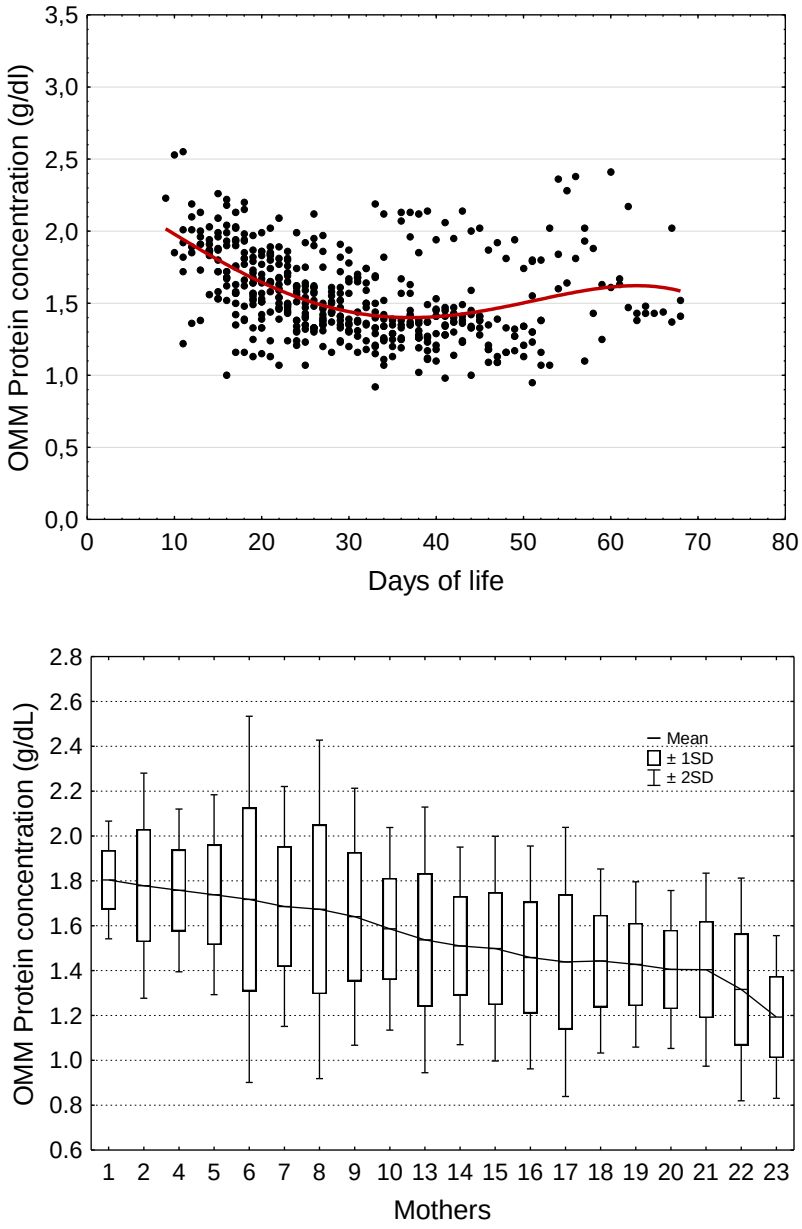


Figure 5.2. Protein concentration of OMM (n=428) according to postnatal age (first graph) and Variability of Protein concentration between and within the mothers (second graph)

5.3.2. Effects on nutritional intakes of individualized versus standard human milk fortification.

428 daily OMM individualized fortifications were performed in 24 preterm infants (mean ± SD birth weight = 1140 ± 230 g; gestational age = 28.6 ± 1.6 week) over >3 weeks. MCT supplementation was necessary in 64% (272 of 428) of daily OMM pools and HM fortifier was necessary in 99.5% (426 of 428) of daily OMM pools. The nutritional content of OMM after MCT supplementation and HM fortification is shown in Table 5.3. By comparison to theoretical values that would have resulted from standard fortification, protein intakes and the protein/energy ratio of individualized fortifications were significantly lower, whereas the fat and the energy contents were significantly higher. The variability in nutritional intakes and protein: energy ratio was significantly lower using individualized compared with standard fortification (Table 5.4 and Figure 5.3).

Table 5.3. Composition of OMM before and after individualized fortification with MCTs and HMF[1]

	OMM	OMM+MCTs[2]	OMM+MCTs+HMF[3]
Protein (g/dL)	1.52 ± 0.28	1.52 ± 0.27	2.51 ± 0.14
Fat (g/dL)	3.79 ± 0.73	4.20 ± 0.45	5.09 ± 0.48
Carbohydrate (g/dL)	6.76 ± 0.27	6.76 ± 0.27	7.11 ± 0.28
Energy (kcal/dL)	67.26 ± 6.49	70.13 ± 4.52	82.66 ± 4.42
Protein energy ratio	2.27 ± 0.37	2.17 ± 0.35	3.04 ± 0.19

[1] HMF, human milk fortifier; MCT, medium-chain triglyceride; OMM, own mother's milk. All values are means ± SDs; n = 428

[2] Fat concentration of human milk was adjusted up to 4 g/dL, when necessary, by adding MCTs.

[3] Protein content was adjusted by using HMF to provide 4.3 g protein· kg^{-1}· d^{-1}according to daily volume of feeding.

Table 5.4. Comparison of individualized fortification intakes and percentage of variability with theoretical values obtained after standard fortification[1]

	Individualized fortification	Standard fortification
Intake		
Protein ($g.kg^{-1}.d^{-1}$)	4.25 ± 0.13*	4.45 ± 0.51
Fat ($g.kg^{-1}.d^{-1}$)	8.6 ± 0.9*	8.1 ± 1.3
Energy ($kcal.kg^{-1}.d^{-1}$)	140 ± 9*	138 ± 13
Protein: energy ratio	3.04 ± 0.19*	3.24 ± 0.32
Variability (%)		
Protein	2.0 ± 2.3*	9.2 ± 6.8
Fat	6.6 ± 7.4*	12.1 ± 10.3
Energy	4.8 ± 4.5*	7.3 ± 6.1
Protein: energy ratio	4.5 ± 4.3*	7.6 ± 6.5

[1]All values are means ± SDs; *n* = 428. Intakes and variability resulting from individualized and standard fortifications were compared by using paired Student's *t* test. *$P < 0.05$ when compared with standard fortification.

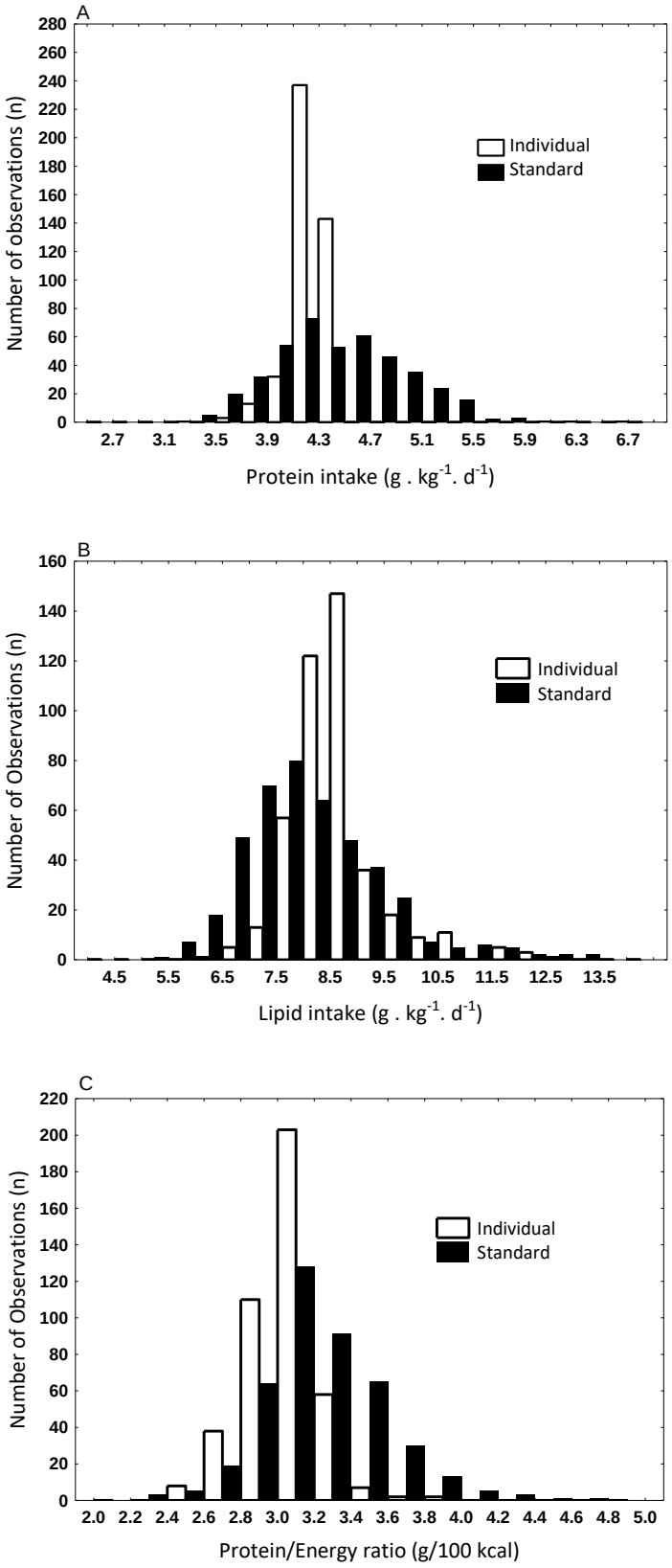


Figure 5.3.: Protein (a) and lipid (B) intakes and protein energy ratio (C) according to individualized or standardized human milk fortification (n=428)

5.4. Discussion

In the present study, we confirmed that the macronutrients and energy compositions of OMM and banked donor HM used for nutrition of preterm infants in the NICU are highly variable and unpredictable, as reported by several studies (114, 166, 179, 180). Protein, lipid and energy concentrations are often lower than values commonly used as reference for HM composition in clinical practice, especially for DM (Figure 5.4). This overestimation of intakes leads to a high rate of protein and energy deficit (179, 181) which could affect postnatal infant's growth.

Temporal changes in HM are well described in the first postnatal weeks, as HM moves from colostrum to transitional and then to mature milk (45). During this transition, protein declines while fat and lactose concentrations increase (114, 182). Protein content of preterm mothers' milk is generally higher than term milk in the early postnatal period then decreases rapidly over time during lactation (116). A meta-analysis of Gidrewicz et al suggested that most of the difference between preterm and term milk were within 0.2 g/dL after postnatal week 3 and that by 3 months of lactation's age, preterm milk may have the same protein content as term milk (114). They also showed that the variability of the protein content was higher during the first days of lactation and in preterm milk. In contrast, we found high variability of protein remaining over time both for individual mothers and between mothers (Figure 5.1).

The high variability of HM macronutrients composition between individuals and even each milk samples, in particular for fat, was established previously (114, 179, 180). This suggests that average values often assumed for HM composition are likely not representative of an individual HM composition (181). Evidence suggests that multiple external factors can affect the fat composition of HM, including incomplete milk expression, manipulations and processing of expressed HM. Reduced energy and fat content could also be due to nutrients loss during processing of DM (container changes, freeze-thaw cycles, and pasteurization) (77, 183).

Our study confirmed the lower concentrations of DM in protein and fat (Table 5.1). Lower protein content could be explained by the fact that mothers donating their milk were in later stages of lactation of delivered term infants. However, at our milk bank, many of donors were themselves the mothers of preterm infants. This could explain these relatively higher mean protein concentrations found in DM compared to values reported in other milk banks (184, 185). The strategy of pooling milk from a variety of batch of donors is likely to lead to a relatively more stable nutrient composition (Table 5.1).

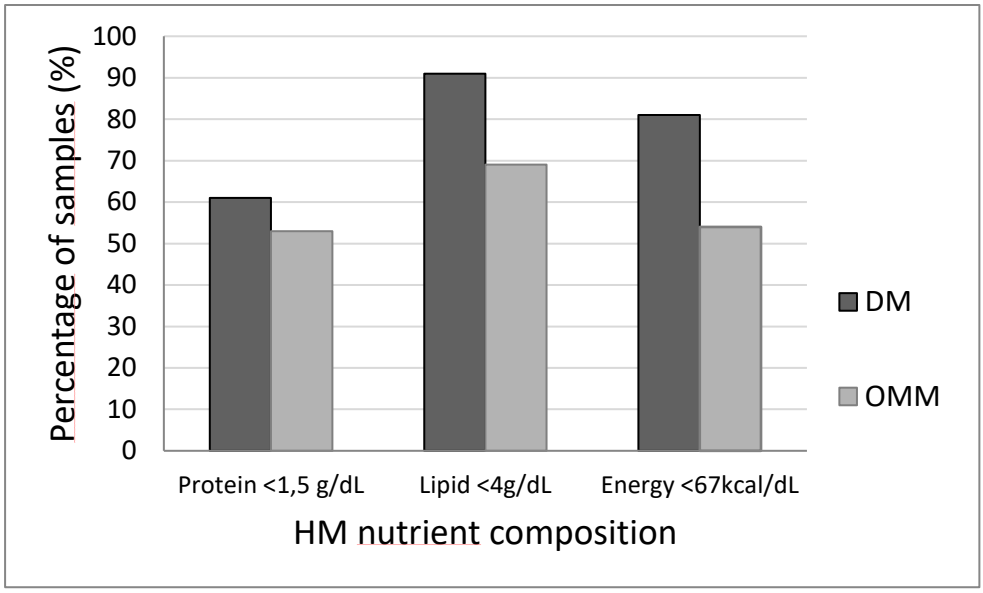


Figure 5.4. Sample's proportion (%) of donor milk (DM) and own mother's milk (OMM) in the milk bank with lower protein, lipid and energy concentration than values of HM composition commonly used in clinical setting (n=804; 428 OMM; 376 DM)

Standard fortification, adding a fixed amount of a fortifier as recommended by the manufacturer, is the most commonly used method to fortify mother's milk. This method was not designed to address the variability in HM nutritional contents and often failed to meet the nutritional recommendations for preterm infants (107, 181, 186-189). Deficit in protein intakes is recognized as limiting factor for growth of HM fed preterm infants (186, 188). Using metabolic balances and indirect calorimetry, we previously showed that protein intake and protein energy ratio were the major determinants of weight gain in VLBW infants (34). Some authors proposed to increase fortifier strength or added extra proteins (115, 164, 165, 190, 191) and reported varying results on growth suggesting a potential ceiling effect of proteins on growth. A recent meta-analysis (118) concluded that HMF with higher protein content can improve preterm infant growth compared with standard HMF but additional randomized controlled trials are needed to clarify the long-term effects of HMF containing higher levels of proteins on infant growth and outcomes and to finally define the optimal dose of enteral protein supply.

However, such an increase in protein fortification does not compensate the HM variability and there is a risk of energy deficiency as well as protein overload with its potential long-term adverse effects (192). Considering the high variability of protein and energy content of expressed HM and the significant differences with an assumed theoretical composition, new strategies of individualized fortification were suggested to improve nutritional intakes and growth in preterm infants. Arslanoglu et al (120, 121) adjusted the protein supplementation on the basis of the values of blood urea nitrogen (BUN) considered to be a marker of protein adequacy in preterm infants (193). This BUN method, which was developed to avoid inadequate and excessive protein intake, is easy to apply and does not require daily milk analyses. However, it has been shown that BUN is not correlated to protein intakes during the first weeks of life but reflects the renal immaturity of ELBW and VLBW infants (122, 194). Therefore, the use of BUN as a threshold level to adjust protein intake could be inadequate, leading to a cumulative protein deficit.

In the present study, we confirmed the high daily variability in the nutritional value of HM within a large number of samples of standardized fortified OMM, and that this variability could be reduced using our original daily individualized fortification. With standard fortification, protein deficiency or overload, and energy deficiency were frequently observed (Figure 5.3., A and B). By contrast, after individualized fortification, the range of protein intake decreased from 3.3–6.6 to 3.6–4.5 g. kg^{-1} d^{-1} and that of the protein: energy ratio from 2.4–4.7 to 2.4–3.8 g/100 kcal (Figure 5.3, A and C). In addition, with individualized fortification, the mean use of fortifier was significantly lower (3.6 compared with 4.0 packets/dL), decreasing the osmolality of the fortified HM and the risk of gastric intolerance.

This daily HM Individualized fortification allows to provide appropriate daily nutritional intakes in the upper range of recent nutritional recommendations (31). As a result of the lower energy and protein bioavailabilities of HM (34, 110, 112, 161), an energy intake of 140 kcal. kg^{-1} d^{-1} and a protein intake of 4.2 g. kg^{-1} d^{-1} were estimated to be necessary to ensure an adequate growth. These values are slightly higher than those recommended by the ESPGHAN Committee on Nutrition in 2010 (31).These recommendations are more related to preterm infants fed formula than to those fed fortified HM, and we suggest that specific recommendations for the use of HM are necessary. These new recommendations will need to consider the lower metabolizable energy and protein content of fortified HM (34), the HM type (OMM or DM) (127), the effect of processing, possible pasteurization, and the additional nutritional losses suggested during continuous feeding (117, 195).

The currently available multicomponent HM fortifiers are not adequately designed for use in VLBW infants. In the present study, the relative fat deficit of expressed HM provided to the NICU was corrected with a medium chain triglyceride emulsion. However, the fatty acid profile of the fortified HM remains inadequate for preterm infants, especially in terms of long-chain PUFA content. Therefore, newer fortifiers providing high protein and energy intakes with adequate long-chain PUFA content, but without inducing a gastrointestinal osmotic load >360–400 mOsm/kg H_2O, need to be developed in order to improve the nutritional supply with minimal side effects for the preterm infants.

Although individualized fortification is time consuming, expensive and requires additional equipment and well-trained staff, the use of infrared technology to determine the macronutrient composition of HM is likely to expand its availability in NICUs. It could have practical application in HM banks for DM composition or to develop specific HM pools with higher protein and/or energy content.

5.5. Conclusion

The macronutrient concentrations of expressed OMM and DM are highly variable and unpredictable, especially for protein, fat and energy. In our study, protein and energy content of DM was also significantly lower than that of OMM. HM standard fortification often does not meet the high nutritional requirements of preterm infants and doesn't address HM variability, possibly resulting in under or over-nutrition. Individualized fortification based on daily HM infrared analysis improves and regulates the protein and energy intakes.

Chapter 6- Growth of preterm infants fed individualized fortified human milk with different types of human milk

de Halleux V, Pieltain C, Senterre T, et al. Growth Benefits of Own Mother's Milk in Preterm Infants Fed Daily Individualized Fortified Human Milk. Nutrients. 2019;11(4):772

6.1. Introduction

Own mother's milk (OMM) provides many health benefits and is preferred for feeding preterm infants (36, 46). However, many preterm infant's mothers are unable to express a sufficient volume of milk. Where available, donor milk (DM) is provided rather than formulas (89-91) contributing to decrease in NEC rates (46, 49). Studies evaluating growth in VLBW infants fed fortified DM versus fortified OMM are generally not controlled for nutritional intakes and report controversial results. In this way, some studies reported growth deficits with fortified DM compared to fortified OMM (125, 126, 129, 196, 197). Others studies did not find that negative impact of DM on growth (50, 127, 130, 131). The aim of the present study was to evaluate the influence of HM type (raw OMM, pasteurized OMM and DM) on growth in premature infants fed exclusive HM and receiving controlled nutritional intakes using daily individualized HM fortification.

6.2. Material and method

This is a single center prospective and non-interventional study conducted in the NICU of the University of Liège, evaluating growth of preterm infants fed HM with individualized targeted fortification previously described (IHMF). From January 1, 2007 to December 31, 2014, data on HM use, HM composition and fortification in preterm infants < 32 weeks GA were collected daily in our NICU human milk bank. To evaluate the respective influences of OMM and DM, growth and nutritional intakes (mean ± SD), during the study period, were compared in preterm infants fed predominantly OMM (≥75%) or predominantly DM (≥75%). Evolution of growth were assessed in each group, OMM (≥75%) and DM (≥75%) during and after the study period.

In addition, effects of HM types (raw OMM, pasteurized OMM and DM) on growth during the study period were evaluated on the whole population (n=101) including a third group receiving a mixed HM diet ranging from 26 to 74% of OMM. This population was also evaluated according to the main HM type received, DM > 50% (DM), DM ≤ 50%, pasteurized > raw OMM (POMM), and DM ≤ 50% and raw > pasteurized OMM (ROMM).

Infant's weight (to the nearest 1 g) was measured daily by nurses using a calibrated electronic scale. Length and head circumference (HC) were assessed weekly (both to the nearest 0.1 cm), length using a length board and HC using a non-stretch measuring tape. Weight gain velocity (grams per kilogram per day) was calculated during the IHMF period using the 2-point average method (198).

$$\textbf{Weight gain} = \frac{\mathbf{1000} * (\mathbf{W2} - \mathbf{W1})}{\frac{\mathbf{W1} + \mathbf{W2}}{\mathbf{2}} * (\mathbf{d2} - \mathbf{d1})}$$

where W = weight in grams; d = day; 1 = beginning of the time interval; and 2 = end of the time interval.

Weight for age, Length for age and Head Circumference for age Z scores were calculated using Fenton reference growth calculators according to corrected GA (159).

Normally distributed data were reported as a mean with standard deviation and groups were compared by using Student's t-tests or one-way analysis of variance (ANOVA) with Bonferroni's correction for post hoc pairwise comparisons. Non-normally distributed data were presented as a median with a range, and groups were compared by Kruskal-Wallis ANOVA tests. Categorical data were presented as numbers and percentages and groups were compared by Chi-squared tests. Data of evolution of growth in each group, were compared using paired Student's t-Test. A p-value of <0.05 was considered as significant.

Stepwise multivariate analysis was performed to evaluate the respective influences of significant univariate variables and type of HM (raw OMM, pasteurized OMM, and DM) on growth parameters during the study period. The relation was presented by Pearson correlation coefficient (r or r^2). A $p < 0.05$ was considered as significant.

All statistical analyses were performed by using Tibco Statistica software version 13 (TIBCO, Palo Alto, CA, USA).

6.3. Results

6.3.1. Study population and clinical variables

Out of 101 preterm infants of less than 32 weeks (BW 975 ± 255 g for a GA of 27.8 ± 1.9 weeks), IHMF was initiated at 19 ± 8 days of life during 26 ± 8 days. Thirty-seven infants were fed ≥75% of intake with OMM, 33 infants were fed ≥75% of intake with DM, and 31 with a mixed HM diet (26%–74% OMM). Clinical characteristics of infants according to HM diet are detailed in table 6.1. Demographic parameters at birth were similar in the three groups with the exception of HC being significantly lower in the DM group compared to those fed the mixed HM diet, but not with those fed with OMM. Neonatal morbidities at study baseline were also similar in the three groups with a trend to a higher incidence of late onset sepsis in the DM group ($p = 0.062$). However, no other significant difference in morbidities that could influence growth was reported between the three groups during and after the study period.

6.3.2. Influence of OMM versus DM on nutritional intakes and growth

Contributions of raw OMM, raw pasteurized OMM and pasteurized DM composition in each HM group are gathered in table 6.2. OMM accounted respectively to mean 95.4 % in in OMM ≥75% and 2.2 % in group ≥75 % DM. Native lipid concentration was significantly higher in the OMM group. However, after individualized fortification, similar protein and energy intakes were provided (Table 6.2).

Weight (p = 0.002) and length gain (p = 0.020), but not HC gain (p = 0.120), were significantly higher in infants receiving predominantly OMM compared to those fed predominantly DM during the IHMF period (Table 6.3). Similarly, Z-scores evolutions for weight (p < 0.0001), length (p = 0.004), and HC (p = 0.013) were all significantly higher in infants receiving mostly OMM than in those fed mostly DM during the IHMF period (Figure 6.1, Table 6.3).

At the end of the IHMF period, Z-scores in DM group decreased significantly for weight (p=0.0003) and length (p<0001) but increased for HC (p=0,04). By contrast in OMM group, Z-scores showed a minimal increase for weight (SD=013; p=0.08), a significant decrease for length (p=0,0004) and a significant increase for HC (p<0.0001) (table 6.3). After the study period, Z-scores for weight (p=0.94) and length (p=0.4) remain stable in the DM group whereas Z-scores for HC showed a significant catch-up growth (p=0.0001). By contrast, after the study period, a reduced growth rate was observed in the OMM group, with a significant decrease in Z-scores for weight (p<0.0001) and length (p=0.003) but not for HC (p=0.33) (fig 6.1).

Table 6.1. Clinical characteristics of infants according to human milk diet

	≥75%OMM n = 37	**26-74%OMM n = 31**	**≥75%DM n = 33**	**All subjects n=101**	**p=**
Male sex, n (%)	18 (49)	15 (48)	17 (52)	50 (50)	0.96
Gestational age, weeks, m ±SD	27.7 ± 2.1	28.2 ±1.9	27.5 ±1.8	27.8 ±1.9	0.26
Birth Weight, g, m ±SD	983 ± 244	1042 ± 312	901 ± 185	975 ± 255	0.08
Birth Weight <1000 g, n (%)	20 (54)	16 (52)	24 (73)	60 (59)	0.16
Mean Weight z score, m ±SD	-0.19 ± 0.99	-0.37± 0.89	-0.48± 0.82	-0.34± 0.91	0.47
Birth Length, cm, m ±SD	35.0 ± 3.3	35.8 ± 3.9	34.6 ± 2.9	35.1 ± 3.4	0.34
Birth HC, cm, m ±SD	24.9± 1.9	25.8± 2.3	24.5± 1.7	25.0± 2.0	**0.02**
Vaginal Delivery, n (%)	16 (43)	9 (29)	7 (21)	32 (32)	0.13
Twin, n (%)	8 (22)	12 (39)	6 (18)	26 (26)	0.13
Apgar Score 1 min, m ±SD	6.5 ± 2.2	6.1 ± 2.2	6.1 ± 2.0	6.2 ± 2.1	0.60
Apgar Score 5 min, m ±SD	7.9 ± 1.5	7.8 ± 1.5	7.9 ± 1.1	7.9 ± 1.4	0.92
Antenatal steroids, n (%)	35 (95)	27 (87)	29 (88)	91 (90)	0.30
Study duration, m ±SD	27 ± 8	27 ± 8	24 ± 6	26 ± 8	0.14
Postnatal age at study d1,weeks, m ±SD	30,5 ± 1,5	30,8 ± 1,6	30,5 ± 1,5	30,6 ± 1,5	0.64
Postnatal age at study end, weeks	34.2 ± 1.4	34.7± 1.8	33.9 ± 1.5	34.3± 1.6	0.12

Data are presented as n (%) for categorical variables and mean (m) ± standard deviation (SD) for continuous variables; p<0.05 based on ANOVA for continuous variable and chi square for categorical variables.

Table 6.2. Human milk composition and nutritional intakes during study in the two groups.

	≥75% OMM *n* = 37	**≥75% DM *n* = 33**	***p*-value**
Human Milk Category (%)			
Raw OMM	31.3 ± 33.6	0.5 ± 3.0	<0.001
Pasteurized OMM	64.1 ± 33.1	1.7 ± 4.7	<0.001
Pasteurized DM	4.6 ± 7.8	97.8 ± 5.4	<0.001
Human Milk Composition (Infrared) before fortification			
Protein, g/dL	1.44 ± 0.22	1.35 ± 0.14	0.056
Lipid, g/dL	3.87 ± 0.59	3.61 ± 0.23	0.022
Carbohydrates, g/dL	6.84 ± 0.22	6.86 ± 0.19	0.695
Nutritional Intakes (Units/kg/day)			
Volume, mL	167 ± 10	166 ± 8	0.536
Energy, kcal	143 ± 8	141 ± 6	0.148
Protein, g	4.17 ± 0.15	4.15 ± 0.14	0.512

Data are presented as mean ± SD; $p < 0.05$ based on *t*-tests

Table 6.3. Growth rate and Z-score gain in preterm infants fed individualized fortified with predominantly own mother's milk (OMM) or donor milk (DM.)

	OMM ≥ 75% *n* = 37	**DM ≥ 75% *n* = 33**	**Delta OMM vs. DM**	***p* =**
Weight gain, g/kg/day	19.8 ± 2.0	18.2 ± 2.2	+1.6	0.002
Length gain, cm/week	1.17 ± 0.26	0.99 ± 0.36	+0.18	0.020
Head circumference, cm/week	1.13 ± 0.22	1.04 ± 0.27	+0.09	0.120
Weight Z-score gain, g/kg/d	0.13 ± 0.35	−0.26 ± 0.41	+0.39	<0.001
Length Z-score gain, cm/week	−0.25 ± 0.41	−0.59 ± 0.52	+0.33	0.004
HC Z-score gain, cm/week	0.59 ± 0.50	0.24 ± 0.65	+0.35	0.013

Data are presented as mean ± standard deviation; $p < 0,05$ based on *t*-tests

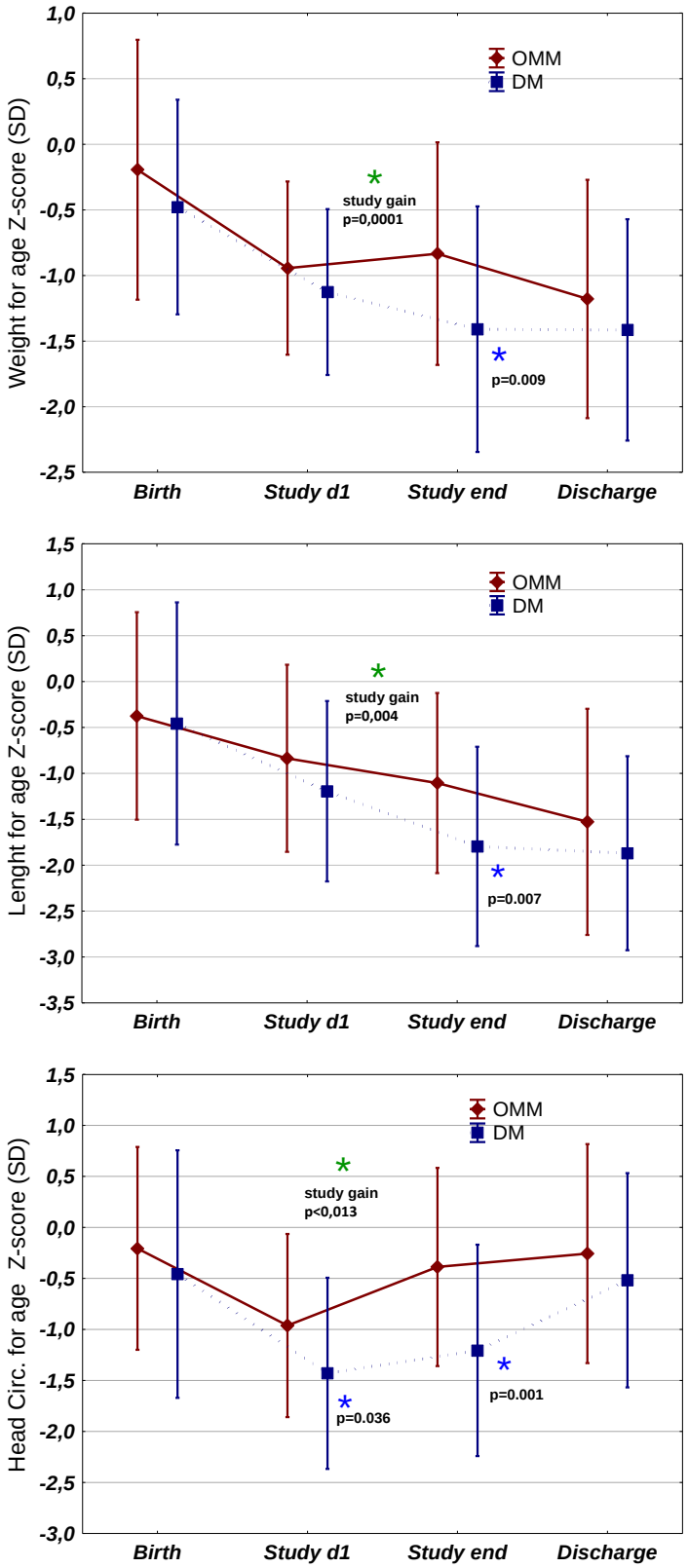


Figure 6.1. Weight, Length and HC for age Z-scores according to Fenton from birth to discharge in infants receiving predominantly OMM (n=37) compared to those predominantly DM (n=33).

**p<0,05 for Z-score gain (OMM vs DM) during study (d1-end)* **p<0,05 for age Z-scores (OMM vs DM)*

6.3.3. Effects of types of human milk (raw OMM, pasteurized OMM, pasteurized DM) on nutritional intakes and growth

The whole population was evaluated according to the main HM type received during the study period, DM > 50% (DM), DM ≤ 50%, pasteurized > raw OMM (POMM), and DM ≤ 50% and raw > pasteurized OMM (ROMM) to evaluate the influence of OMM pasteurization on growth velocity during the study period. As shown in Table 6.4. DM accounted to 88.5% in the DM group (*n* = 45), pasteurized OMM to 70.3% in the POMM group (*n* = 41), and raw OMM to 69.1% in the ROMM group (*n* = 15). Energy and protein intakes during the study period were similar in the three groups.

Both weight gain and weight Z-score evolution in the DM group were significantly lower than in the other two groups (p=0.035 and p<0.001 DM vs POMM; p<0.001 and 0.003 DM vs ROMM). In addition, weight gain, but not weight Z-score gain, was significantly higher in the ROMM versus POMM group (p<0.001 and p=0.546). Length and HC gains were similar in the three groups. Nevertheless, the length and HC Z-score evolutions were significantly improved in the ROMM group compared to the DM group (p=0.013 and p=0.016) (Table 6.3).

Table 6.4. Growth rate and nutritional intakes according to the main human milk type received during the study period

Human Milk Type Volume Intake (%)	**DM 88.5 ± 16.9**	**POMM 70.3 ± 22.6**	**Delta vs. DM**	***p* vs. DM**	**ROMM 69.1 ± 19.9**	**Delta vs. DM**	***p* vs. DM**	**Delta vs. POMM**	***p* vs. POMM**
n	**45**	**41**			**15**				
Energy, kcal/kg/day	141.3 ± 6.3	142.4 ± 7.3	-	0.432	143.7 ± 6.2		0.210	-	0.552
Protein, g/kg/d	4.15 ± 0.14	4.19 ± 0.13	-	0.211	4.18 ± 0.15		0.494	-	0.855
Weight gain, g/kg/d	18.2 ± 1.9	19.1 ± 1.8	**+0.87**	0.035	21.1 ± 1.6	**+2.83**	**<0.001**	**+1.96**	**<0.001**
Length gain, cm/week	1.04 ± 0.36	1.13 ± 0.33	+0.10	0.193	1.17 ± 0.28	+0.14	0.194	+0.04	0.697
HC gain, cm/week	1.04 ± 0.24	1.10 ± 0.20	+0.05	0.258	1.10 ± 0.24	+0.06	0.409	+0.01	0.937
Weight Z-score gain	–0.23 ± 0.39	0.09 ± 0.31	**+0.31**	**<0.001**	0.15 ± 0.44	**+0.38**	**0.003**	+0.06	0.546
Length Z-score gain	–0.53 ± 0.52	–0.36 ± 0.45	+0.17	0.116	–0.14 ± 0.50	**+0.39**	**0.013**	+0.22	0.114
HC Z-score gain	0.28 ± 0.59	0.51 ± 0.56	+0.23	0.068	0.70 ± 0.41	**+0.41**	**0.016**	+0.18	0.252

DM = donor milk; POMM = pasteurized own mother's milk; ROMM = raw own mother's milk. Data are presented as mean ± standard deviation; $p < 0.05$ based on *t*-tests.

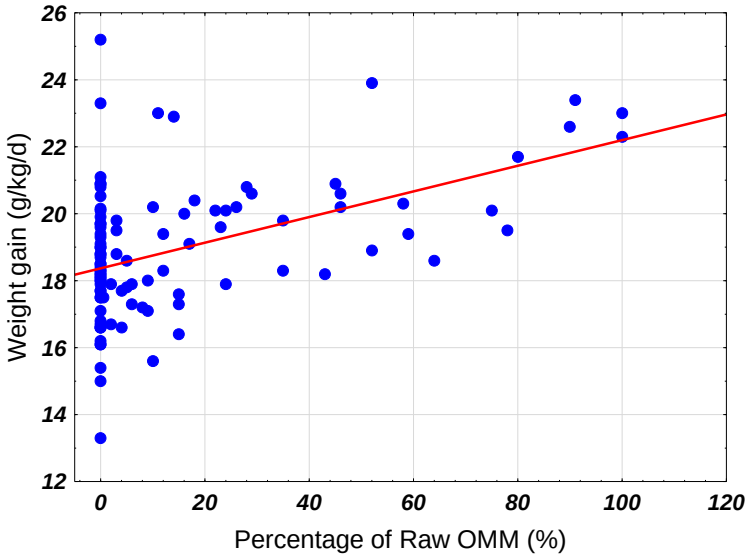


Figure 6.2. Weight gains according to proportion of raw OMM

6.3.4 Univariate and Multivariate analysis on the whole population

Univariate linear regressions on the whole population demonstrated that birthweight, GA, postnatal age at study 1, as well as protein and energy intakes did not influence weight and length gain during the study period. Weight gain during IHMF period was significantly influenced by two factors: percentage of raw OMM (r^2 = 0.227, p < 0.00001) and additionally study duration (r^2 = 0.1015, p = 0.00097). For length, the percentage of total OMM (r^2 = 0.04, p = 0.046) was the only factor significantly influencing length gain.

Stepwise multivariate analysis demonstrated that weight gain was positively related to the proportion of raw OMM, proportion of pasteurized OMM, and postnatal age at the first day of study, but negatively related to study duration and birthweight. Those factors explained 22.7%, 3.7%, 3.1%, 9.8%, and 3.0% of the weight gain, respectively. It was also estimated that the weight for age Z-score difference during IHMF was correlated to raw OMM proportion, gestational age, and birth weight, contributing, respectively, to 18.0%, 12.1%, and 10.7% of the Z-score difference.
For length gain, only two parameters remained significant: the proportion of total OMM (raw + pasteurized) and postnatal age at baseline, explaining, respectively, 4.0% and 4.4% of the length gain. Similarly, length for age Z-score difference was related to the proportion of total OMM (raw + pasteurized) and study duration, contributing, respectively, to 6.5% and 5.4% of the difference.

6.4. Discussion

This study is the first to provide daily controlled nutritional intakes in preterm infants fed HM with individualized fortification after daily determination of HM composition by a validated infrared method (113, 178). Because of IHMF, protein and energy intakes were similar with very low variability (table 6.2) in the two groups, thus allowing comparisons of growth and metabolic tolerance in VLBW infants fed exclusively fortified OMM (95.4% ± 7.8%) or DM (97.8% ± 5.4%). This study found that weight gain velocities during IHMF were on average 1.6 g/kg/d higher in infants fed OMM than in those fed DM (p=0.002), with an additional benefit on length gain of around 0.18 cm/week on average (p=0.020), suggesting a growth specific effect of OMM in preterm infants. In addition, the use of predominant

OMM (≥75%) instead of predominant DM (≥75%) significantly improved weight (p<0001), length (p=0.004), and HC (p=0.013) Z-score changes during the study period (Table 6.3 and Figure 6.1).

As shown in table 6.2, around two thirds of OMM were provided after Holder pasteurization and not as raw OMM. This is mostly explained by the strategy applied to decrease the risk of infectious transmission with raw milk. The distinct patterns of the raw OMM intakes in our whole population allowed us to evaluate the respective roles of raw OMM versus pasteurized OMM or (pasteurized) DM on growth velocity in the preterm infants. We found that ROMM and POMM both have a positive effect on weight gain, contributing to an increase of +2.8 g/kg/day (p<0.001) and +0.9 g/kg/day (p=0.035), respectively, compared to DM. This suggests that the major positive effect of OMM could be the result of its use as a raw product, with a mean weight gain difference of 2.0 g/kg/day compared to pasteurized OMM (p<0.001) (Table 6.4).

A positive relationship was observed (figure 6.2.) between the weight gain and the proportion of raw OMM intake. Our study also indicates that the use of raw OMM induces a significant positive effect on weight (p = 0.003), length (p = 0.013), and HC (p = 0.016) Z-score gains during the study period compared to DM. The benefits of POMM on DM was limited on weight gain (p=0.035) and weight Z-score gain whereas benefits on length and HC Z-scores (P=0.068) were not significant (p=0.0116 and P=0.068), contrasting with the benefits observed with ROMM. Therefore, the limited beneficial effect of POMM versus DM remains to be confirmed in larger populations. This study demonstrates a significant positive impact of both OMM and raw OMM on growth in preterm infants fed HM. This effect seems independent of nitrogen and energy content as this was controlled by the IHMF in this study. Nutritional and growth benefits of fortified OMM versus fortified DM are still debated and studies report controversial results regarding growth and Z-score changes in preterm infants. Thus, in two observational and one retrospective study, a weight gain benefit was reported in preterm infants fed fortified OMM. In 2011, Montjaux et al. (125) suggested that weight gain was directly proportional to the amount of fresh raw OMM compared to pasteurized fortified DM (n = 48). More recently, Madore et al. (129) showed a significantly higher weight gain in preterm infants fed predominantly fortified OMM compared to those fed predominantly fortified DM during the first month of life (n = 56). Brownell et al (126), using OMM as a reference, also reported a significant decrease in mean weight and head velocity during a hospital stay for every 10% increase of the total feeding volume provided as DM (n = 314). By contrast, two retrospective studies did not detect any significant difference in weight gain between premature infants receiving either exclusively OMM or DM as a sole diet (n = 92) (130) or in those fed predominantly (>50%) fortified OMM or fortified DM (n = 299)(131). In addition, a third retrospective study found no significant difference in weight Z-score change by HM diet (n = 88) (>75% donor vs >75% OMM; p = 0.28) (127). In contrast to our study, none of those studies precisely controlled the protein and energy intakes, and the rate of pasteurization, if any, in the OMM groups was rarely specified.

The effect of pasteurization on growth velocity was recently evaluated as a secondary outcome in a randomized study of more than 300 premature infants receiving fortified OMM either raw or pasteurized. In that study, a similar growth was observed between the two groups (78). Lloyd et al in a retrospective clinical audit reported a higher weight growth velocity and head circumference at 34 weeks PMA in the OMM group (n=43) compared DM group(n=53) but found no difference at discharge and at 12 month corrected age (196). They suggested that, after 34 weeks, the majority of preterm infants receiving DM were transitioned to preterm formula. Small deficit in growth over the period in

which the preterm infants received DM was transient and catch-up growth was evident at discharge. We found a similar effect in our study (figure 6.1). Z-scores in DM group decreased significantly at the end of the study period for weight (p=0.0003) and length (p<0001) but increased for HC (p=0,04). After the study period and in accordance with our protocol, donor milk was progressively replaced by a preterm formula among infants over 32 weeks of post-menstrual age and over 1500 g. During this period no or minimal changes in Z-scores for weight (p=0.94) and length (p=0.4) were noted with a catch-up growth for HC (p=0.0001). By contrast, preterm infants in OMM group continue to receive standard fortified OMM but started breastfeeding and experienced a slower growth than during the IHMF period with a significant decrease in Z-scores for weight (p<0,0001) and length (p=0.003) after the study period.

In preterm infants fed fortified HM, postnatal growth restriction was frequently reported as well as loss of Z-score during the full HM fortification period (107, 127). Repetitively, recommendations from various expert committees suggest that nutritional requirements are similar in VLBW infants fed fortified HM or preterm formula (PTF) (28, 31). Until now, no specific guidelines have been proposed for fortified HM fed preterm infants. However, it is recognized that at similar controlled protein and energy intakes, growth velocity is significantly lower in preterm infants fed fortified HM than in those fed PTF (106). Metabolic and energy balance studies show that such a difference could be the result of lower metabolized protein and energy contents of fortified HM compared to PTF (34). The mean difference in nitrogen utilization (retention/intake) as well as the mean difference in energy absorption rates measured by bomb calorimetry were both about 10% less with fortified HM (111). This difference could be partially due to the use of pasteurization. Andersson et al found 17% higher fat absorption with raw as compared to pasteurized milk (199). In addition, as shown more recently, the use of standard reference values for OMM and DM may induce an overestimation of the protein and energy content of fortified HM (113, 181).While preterm OMM, with its higher protein content, could improve growth compared to DM, it remains insufficient to support adequate growth, especially after the first month of lactation when the protein concentration decreases (114). Our previous study found that the macronutrient and energy content of OMM was highly variable and unpredictable but also that protein and energy content of DM was also significantly lower than that of OMM (113). Of all the daily OMM and DM samples (*n* = 2630) measured in the present study, 67% were <1.5 g protein/dL and 62% were <67 kcal energy/dL, values commonly considered as reference values for HM composition to estimate nutrient intakes in clinical practice.

Considering the variability of HM macronutrient contents and the lower bioavailability of HM, in the present study, we targeted higher mean protein and energy intakes than those generally recommended (28, 29, 31). Thus, during the study period, preterm infants received controlled mean intakes of 143 kcal/kg/day and 4.2 g/kg/day of proteins between 30.5 and 34 weeks of post-menstrual age, resulting in mean positive weight and HC Z-scores changes of 0.13 and 0.59, respectively, but a limited negative mean length Z-score change of 0.25 in preterm infants fed ≥75% OMM. By contrast, negative Z-score changes for weight (-0.26 on average) and length (-0.59 on average) were observed in the group receiving ≥75% DM (Table 6.2 and figure 6.1). These results suggest that such intakes are close to the minimal requirements for preterm infants fed fortified OMM in such a range of post-menstrual age, but could still be limited in those fed fortified pasteurized DM. Hoban et al suggested routine protein supplementation of all DM in addition to standard fortification, regardless of infant's growth. Their feeding protocol avoided a negative impact of DM on growth (200). Target-pooling is

another strategy to increase nutritional content by combining milk of multiple donors according to energy and protein values (201). Another specific technique is the addition of skimmed fat from lower-calorie DM to higher-calorie DM in order to achieve a minimum of 67 kcal/100mL. However, target-pooling DM still does not meet recommended protein intake and VLBW infants fed calorically target-pooled DM continued to demonstrate negative changes in Z-scores, especially for length (202). This suggests that nutrients loss likely occurred during preparation and handling (183, 202). Developing consistent feeding preparation techniques to improve homogenization, minimize fat loss, and optimize nutrient delivery is an important focus for further research and improvement for quality of care.

In addition, knowing that postnatal growth quality differs to that of fetal growth by an increase in fat deposition, the discrepancies between weight and length Z-scores benefits could be the result of a relative deficit in the lean body mass accretion rate during the study period in preterm infants fed pasteurized DM. Therefore, our study also suggests that protein and energy requirements of preterm infants fed fortified HM are higher than those currently recommended (28, 29, 31) and that specific nutritional guidelines for HM fed preterm infants need to be designed, promoting the use of OMM, and considering the limitations of its use as raw OMM in VLBW infants. Improving HM fortification by IHMF using infrared technology and extra protein and energy supplementation may be one of the strategies to optimize the nutritional composition of HM in order to meet the nutritional needs of preterm infants, especially when DM is used. IHMF decreases the variability linked to HM content and safely optimizes protein and energy intakes (113, 166, 203, 204). Premature infants fed with low macronutrient content HM benefit most from IHMF, with improved growth outcomes (204).

6.5. Conclusion

Our study demonstrated that growth velocity within reference ranges can be attained in preterm infants receiving a daily controlled intake of fortified OMM providing high protein and energy intakes (4.2 g of proteins and 143 Kcal/kg/d). It also revealed that fortified raw OMM use is associated with growth benefits compared to DM, irrespective of protein and energy contents. This suggests that pasteurization impaired significantly the bioavailability of those protein and energy intakes. The interest of an increase in protein and/or energy intakes in preterm infants receiving fortified pasteurized HM could be postulated in view of those results but needs to be demonstrated in a further randomized control study. In addition, our study also suggests that specific nutritional recommendations need to be designed for preterm infants fed OMM and DM.

Chapter 7- General discussion

Associated with significant health and developmental benefits, own mother milk (OMM) is the food of choice for preterm infants (36, 46). Providing fresh mother's own milk should be part of a global strategy that includes strong and specific breastfeeding support for mothers (205) and healthcare organization optimizing collection, storage and handling of OMM. However, some mothers who deliver prematurely struggle to produce breast milk and when OMM is limited or unavailable, donor milk (DM) should be the first alternative (36, 88, 89). Thus, breastfeeding support should be associated with a DM program, which has been shown to decrease NEC risk (49, 50) and to increase consumption of OMM during NICU stay and at discharge (206, 207).

Currents HM practices differ widely between neonatal units worldwide (208, 209). Process for safe handling of OMM in healthcare facilities has not received the same attention as the use of infant's formula or DM. Specific regulations are developed for composition, preparation, storage and handling of formula (210). There are also guidelines published for HM bank organization and donor milk use (73). By contrast, guidelines for own mother milk use in NICU are scare and given its specificity, it would be desirable not to apply the same criteria to both types of human milk (OMM and DM) (75, 76). Thus, the need for pasteurization of OMM remains a largely controversial issue considering the relative deleterious effect of pasteurization on several components of HM.

The use of HM in preterm infants has some limitations. HM has evolved to provide optimal nutritional support for term infants but its composition remains insufficient to cover the high nutritional needs and to support adequate growth of preterm infants. OMM macronutrient content is slightly higher than that of DM, generally provided by mothers of term infant. Therefore, both OMM and DM need to be fortified and HM fortifiers have been designed to reach the nutritional requirements of VLBW infants. Unfortunately, metabolic balance studies in VLBW infants have shown that macronutrient bioavailability of HM nitrogen and energy content is be lower than that of preterm formula (109-112). Nutritional requirements could therefore be higher in VLBW infants fed fortified HM (FHM). Indeed, it has been suggested that VLBW infants fed FHM are at higher risk of postnatal growth restriction than those fed formula (18, 63-65, 98, 99). Hence, is it possible to limit or even to abolish postnatal growth restriction in VLBW infants fed fortified OMM or DM?

The purpose of this thesis was to study various controversial aspects of the HM feeding in premature infants, and to suggest potential solutions contributing to promote HM use in the NICU (neonatology).

Should OMM be pasteurized like DM? What are the risks and benefits associated with use of raw versus pasteurized OMM in preterm infants?

Mother's milk is not sterile and can transmit commensal and potential pathogenic microorganisms derived from the mother or the NICU- environment, occasionally causing late onset sepsis (69-72) with particularly significant consequences in the most vulnerable preterm infants with gastro-intestinal immaturity, reduced gastric acidity and increased risk of digestive translocation.

Most microorganisms found in HM come directly from the mother. Newer techniques of microbial identification not relying on culture, such as quantitative polymerase chain reaction (PCR) and PCR

based on the amplification of the gene coding for bacterial 16S ribosomal RNA, identified a greater bacterial diversity in HM than previously assumed. Accordingly, an individual and personalized microbiome also exists in HM, as described for other microbial communities in other sites (66, 67). Several factors influencing HM microbial composition have been described and include length of gestation, mode of delivery, lactational stage, use of antibiotics, environmental settings, geographical and lifestyles differences (67). These data suggest the presence of personalized bacteria in the milk of each mother, probably not contaminating but optimized for her own child (151). The exact mechanisms (skin contamination and salivary backwash versus migration by "entero-mammary pathway") by which the bacteria reach the mammary gland have been the subject of much debate (66, 67, 137). The role of OMM bacteria appears to have implications on infant's gut microbiome (211-213) and short-term outcomes with protection against NEC and infections but also on long term infant's health in programming the neonatal immune system (66, 67, 212). New microbiological research is highlighting the importance of non-sterile HM complex ecosystem. Soeorg et al evaluated coagulase negative staphylococcus (CNS) and did not find late onset sepsis-causing strains from OMM prior sepsis onset, suggesting that risk of infection caused by these bacteria colonizing OMM is low. In contrast, they suggest that gut colonization with less virulent CNS from OMM may even protect against late onset sepsis (136). Cacho et al in a recent study showed that by using small amount of OMM (10-30%) to inoculate pasteurized DM, it was possible to restore the live microbiota in pasteurized DM, potentially improving its bioactivity (151).

Out of the microorganism directly transferred from the mother, OMM collected, transferred to NICU, and conditioned to feed the preterm infant could also be contaminated by several pathogenic bacteria associated with LOS as shown in several case report publications (70-72). However, it appears that exposition of premature infants to raw OMM does not typically result in infant's infection (69).

A recent systematic review and meta-analysis of HM feeding and morbidity in VLBW infants (46) confirmed that both NEC and LOS were significantly reduced in premature infants fed exclusive HM versus exclusive PTF and also reported those benefits in those fed partially HM versus exclusive PTF. This reduction appears to be positively related to the percentage of HM intakes. However, in most of those studies, OMM and DM were not reported separately and the rate of raw OMM was not detailed. In the meta-analysis, the authors also evaluated the effect of pasteurisation on NEC and report similar rates in 1 RCT (n=303; OR 0.64-3.30) (78) and 5 observational studies (n= 1894; OR 0.68-2.43) regardless of pasteurization of OMM or DM. Similarly, no significant difference was reported in the incidence of LOS, BPD or retinopathy [in the same 1 RCT (n=303; OR 0.43-1.18) and 5 observational studies (n= 1875; OR 0.86-1.27)]. Only one RCT (78) aimed to compare raw versus pasteurized OMM. Its design was however inadequate, in that some PTF was provided in the two groups when OMM was not available, and that raw OMM was discarded (and replaced by PTF) when growing any gram-negative organisms, *Staphylococcus aureus or Enterococci*.

Thus, the potential deleterious effects of pathogen bacterial contamination in VLBW infants appear relatively marginal and are mainly supported by case report publications as up to now, no RCT reported the incidence of LOS according to feeding regimen (strictly raw [contaminated or not] or pasteurized OMM). Although it is a well-known that some antibacterial HM properties are lost (cellular immune components) or reduced (IgA, IgG, lactoferrin, lysozyme...) (75, 77, 89) during pasteurization process, it was suggested that the bactericidal effect of raw mother's milk remains preserved after pasteurization (46, 97). Nevertheless, mainly on the base of the precautionary principle, expressed

OMM is still often pasteurized like donor milk (68). In a large survey in Germany, Switzerland and Austria, 43 % of 152 level II or III neonatal units routinely screened for bacterial colonization, and pasteurized or discarded OMM if bacterial counts exceeded pre-defined thresholds. However, the practice for bacterial count and reduction varied considerably between the units. The Superior Health Council of Belgium provided controversial recommendations in 2016 on the use of raw own mother's milk for EPT and ELBW infants in NICU (75, 76). The bacterial thresholds guidelines for raw OMM use and for pasteurization were stricter than we used with the risk of reducing raw OMM consumption and having to dispose of more OMM (see chapter 2).

In the light of our study on HM bacterial contamination and the literature including the importance of OMM microbiome, our guidelines are evolved. Actually, in our neonatal unit, we use bacteriological screening for premature infants born before 32 weeks; daily for less than 28 weeks and 3 times a week from 28 to 32 weeks of postmenstrual age. Only pathogenic bacteria are considered for pasteurization($< 10^4$ CFU/mL). or elimination ($\geq 10^4$ CFU/mL). Saprophytic microorganisms like CNS are regarded as a part of the personalized OMM microbiome and not requiring treatment. Colostrum (OMM expressed before day 5) with its higher immune factors and lower contamination risk is not screened and is administrated as raw, orally and early as possible. To date, we have not observed any deleterious consequences attributable to the softening of our criteria.

In addition to bacterial risk of contamination and LOS, CMV transmission can also occur via breast milk. Between 66 and 97% of seropositive mothers shed CMV into their milk (214).Breast milk is therefore an important mode of CMV transmission to newborn infants (215).

The morbidities associated with postnatally cytomegalovirus infection are a great concern for preterm Infants (80, 86, 216). Most CMV seropositive mothers (up to more than 90%) will excrete the virus in OMM after birth in the range of 8-119 days (214) with the highest level of HM excretion between 4 to 8 weeks postpartum (217). Postnatal CMV infection remains generally mild or asymptomatic but a serious illness may be observed in 4% of preterm infants of seropositive mothers (82) and up to 35% of infants born before 25 weeks of gestation (80) (Table 7.1). The risk factors for severe CMV infections are birth before a gestational age of 26 weeks, severe comorbidities and transmission before 8 weeks of life or before 32 weeks of postmenstrual age (82, 85). The impact of early-life postnatal CMV infection on long-term neurodevelopmental outcomes of VLBW infants remains unclear. The few available studies reported conflicting results, thus highlighting several unresolved questions about potential long-term neurodevelopmental sequelae after acquired CMV infection by premature infants during the neonatal period (81, 85, 86, 218). In addition, emerging concepts arise regarding other complications of prematurity that could be likely impacted by CMV infection. Studies suggested a role for CMV infection as a risk factor for bronchopulmonary dysplasia (BPD) (86, 219) and NEC (220). CMV infection was also associated with longer duration of hospitalization and had a negative impact on infant's growth (86). With the accumulating evidence that postnatal CMV infection causes short-term and probably long-term health risks to the VLBW infant, some systematic efforts toward prevention seem warranted. Processing breastmilk from CMV-seropositive mothers may be necessary until preterm infants reach a certain postmenstrual age after which the risk of symptomatic disease and long-term sequelae decreases. HM of CMV seropositive mothers is endowed with anti-CMV activity and its potency is higher in the colostrum (221) therefore reducing the risk of transmitting infectious viruses to the infant during the first days of life. Freezing-Thawing breast milk does not eliminate the risk of CMV infection (82, 222). Pasteurization is currently the recommended method to prevent HM

CMV transmission (79, 81). Short-term pasteurization (62°C for 5 seconds seems to minimize the impact on the immune components of HM, while still retaining the ability to inactivate virus (223, 224). Recently, other novel less deleterious technics (Ultraviolet- C irradiation and Microwave irradiation) are being explored, and promising in eradicating CMV from breast milk (216).Thus given the high rate of CMV positive mothers, the evaluation of the risk benefit ratio of raw versus pasteurized OMM on short and long- term health is still of importance and need more investigations. More research is also required to better describe risk factors for severe postnatal CMV infection, long-term neurodevelopmental outcomes, new strategies of prevention such as routine screening of breastmilk and postnatal infection as well as potential benefits or toxicity of antiviral therapy for infants with asymptomatic infection.

Table 7. 1. Transmission of cytomegalovirus via breast milk to the prematurely born infant: review of 18 studies (adapted from Lanzieri et al 2013, Kurath et 2010 and Josephson et al 2014)(82, 214, 222)

	Infants fed BM from CMV + mothers	**Infants with BM-acquired CMV infection**		**Symptomatic CMV infection**		**Severe CMV disease**	
	n	n	%	n	%	n	%
Raw	299	65	22	29	10	9	3
Frozen-Thawed	212	26	12	13	6	13	4
Raw+frozen+pasteurised or unspecified	596	50	8	9	2	7	1

Our guidelines on OMM and CMV have progressively evolved according to progresses in understanding risk of HM related CMV infection. Initially, OMM was systematically pasteurized for all infants born before 32 weeks GA until 34 weeks of postmenstrual age. In a second period, OMM from CMV seropositive mothers was systematically pasteurized after 5 days of life (colostrum given as raw) for preterm infants born before 30 weeks of gestational age up to postmenstrual age of 34 weeks and more recently for those born before 28 weeks up to postmenstrual age of 32 weeks.

Is human milk suitable for nutrition of preterm infants? Why do growth rates of preterm infants fed fortified HM tend to be lower than that of those fed adapted formula?

Human milk at usual feeding volumes does not on itself provide adequate nutrition for optimal growth in preterm infants. Indeed, native HM macronutrients content is insufficient to cover the high nutritional needs of those babies. Growth velocity during the fetal life is about twice higher than that of term newborn infants during the first month of life. It represents a gain of around 17 g/kg/d in weight and 1,3 cm/week in length compared to around 9 g/kg/d and 0.9 cm/week in the term infants. Thus protein, energy, mineral and electrolytes requirements are higher in preterm infants (28, 29, 31). VLBW and particularly ELBW infants are at risk of cumulative nutritional deficits and postnatal growth restriction (18, 63-65, 98, 99) which has been associated with altered neurological outcomes (19, 22, 26, 100). Fortification of OMM and DM is therefore recommended for all preterm infants to improve nutrients supplies and in-hospital growth (29, 102, 103).

Human milk fortification aims to increase the nutritional intakes up to the levels required for fetus-like growth taking in to account the fetal weight gain composition, according to gestational age and clinical conditions, during the first weeks of life (102). Growth of preterm infants is usually monitored using intrauterine growth trajectories from birth weight percentile or SD charts derived from cross-sectional studies (159).However, during immediate postnatal period, the trajectory for extrauterine growth may deviate from the birth reference mostly due to physiologic loss of extracellular fluid during postnatal adaptation to extrauterine conditions rather than because of nutritional deficit (225, 226). To which new physiological growth trajectory preterm infants should adjust to after immediate postnatal adaptation is still unknown. The final goal is to achieve growth rate that optimize later health outcomes and more attention should be given to body composition and growth quality rather than quantity. Birth weight charts derived from cross-sectional studies (159) do not reflect the normal postnatal adaptation, but can still be used to assess whether preterm infants grow at rates at similar to intrauterine rates (225). In the study of Rochow, healthy preterm infants transitioned to growth trajectories parallel to Fenton chart percentiles but 0.8 Z-score below their birth reference (225). However, the growth offset is frequently compensated at the term time when the growth velocity of preterm infants is faster than that of their healthy term newborn counterpart. Another recent study compared different approaches to create individualized postnatal growth trajectories with appropriate body composition for monitoring growth (226). Their approach using of a day-specific fetal median growth velocities starting at day 21 seems promising .

To adapt the HM composition to the nutritional requirements of preterm infants, various HM fortifiers based on cow's milk proteins have been designed. However, after fortification, growth of preterm infants fed fortified HM (FHM), remains lower than that observed in preterm infants fed PTF (106) even if both provide similar protein and energy intakes. This result from nutritional guidelines for preterm infants which do not fully consider the nutritional specificities of FHM versus PTF. Older studies using metabolic balances and calorimetry clearly demonstrated that the bioavailability of nutrient's content of FHM differs to that of formula. Net nitrogen utilization is significantly lower in preterm fed FHM (59.7±7.7%) than in those fed PTF (71.5±6.5%) as a consequence of differences in both nitrogen absorption and nitrogen efficiency rates (retention to absorbed). Energy absorption is also lower with FHM (82 ± 5%) than with PTF (92 ± 3 %) (109). Accordingly, those studies suggest that with similar protein and energy intakes, growth rates could be different for preterm infants fed FHM or PTF. In addition, by contrast to PTF, the macronutrient composition of expressed HM is highly variable and related to several factors such as gestational age, postnatal age, as well as mode and time of expression (early milk versus late milk) (113, 114, 227). For preterm infants not breastfed, the additional manipulations of HM necessary for conservation, transport, pasteurization, fortification, and administration could also alter the nutritional composition and therefore the nutritional value of HM (183, 195, 227)

Up to now, energy value of preterm diet, PTF or HM, are still often estimated using the Atwater factors derived from adult data on digestibility and bioavailability of the nutrients (4 kcal/g for protein and carbohydrate and 9 kcal g for fat). However, all those values were found to be overestimated when applied to preterm infants (Table 7.2).

Table 7.2. Energy derived from Protein, Fat and Carbohydrates according to milk (HM or PTF) in preterm infants compared to Atwater Factors (109-112)

Per g	Protein			Fat			Carbohydrate		
	Atwater Adult	Preterm		Atwater Adult	Preterm		Atwater Adult	Preterm	
		PTF	HM		PTF	HM		PTF	HM
Gross Energy, kcal	5.4	5.4	5.4	9.3	9.3	9.3	4.0	4.0	4.0
Digestible Energy, kcal	5.1	4.8	4.5	9.0	7.9	7.2	4.0	3.8	3.7
(%)	(95)	(90)	(83)	(96)	(85)	(77)	(99)	(95)	(93)
Metabolizable E, kcal	4.0	3.7	3.2	9.0	7.9	7.2	4.0	3.8	3.7
(%)	(78)	(77)	(72)	(100)	(85)	(77)	(100)	(100)	(109)
% of Gross Energy	74%	72%	60%	96%	85%	77%	99%	95%	93%

All those factors could explain why postnatal growth in preterm infants fed FHM could be lower than that of those fed PTF. It reminds us of the need for specific nutritional recommendations for fortified OMM or DM. The differences between OMM and DM also suggests that the use of standardized composition to evaluate the nutritional intakes in clinical practice remains inadequate.

In practice, in preterm infants fed HM or FHM, nutritional intakes cannot be easily controlled by the volume and preterm infants could be at risk of relative protein or energy overload or deficiency (113).

Therefore, this analysis strongly supports the need of specific nutritional recommendations for preterm fed fortified OMM and DM.

Are Infrared cow milk analyzers appropriate in clinical practice to determinate the macronutrient composition of human milk (OMM or DM)?

In the dairy industry, cow milk composition is rapidly determined using infrared technology based on the principle that different functional groups absorb IR energy at different wavelengths. This technology has been adapted to HM more recently.

Our studies evaluating the infrared analyzer (IRA) Milkoscan minor® accuracy in comparison to chemical analysis, have shown that the IRA Milkoscan® underestimated dramatically the protein content of HM but that after adjustment of the software, the predicted values were highly correlated to the chemical data with a precision of 0.13 g/dL or 9.4 % (Figure 7.1).

Similarly, evaluating three different generations of IRA Miris® instruments in collaboration with the University of Lyon (Prof JC Picaud), we observed that the 3 generations of Miris® also underestimated differently the protein content of HM and need separate adjustment. However, after adjustment, all the tree generations of Miris provided adequate evaluation of the protein content with a mean combined precision of 0.12/dL or 8.2% (Figure 7.1).

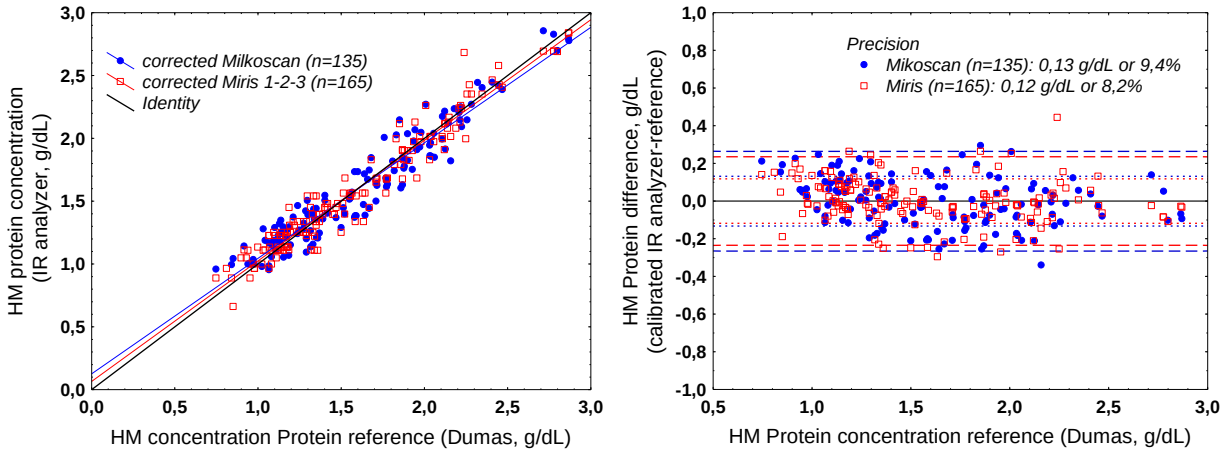


Figure 7.1. Validation of infrared analyzers (Milkoscan minor and Miris 1-2-3) for protein determination

For the Fat content of HM, the IRA Milkoscan® under-estimated the fat content by around 20%. Again, after software adjustment, the precision was improved with a standard deviation of ±0.16 g/dL or ± 4.9 %. By contrast, using the IRA Miris®, the Fat content appeared overestimated. Calibration improved accuracy with a precision reaching ± 0.31 g/dL or ± 9.3% (Figure 7.2).

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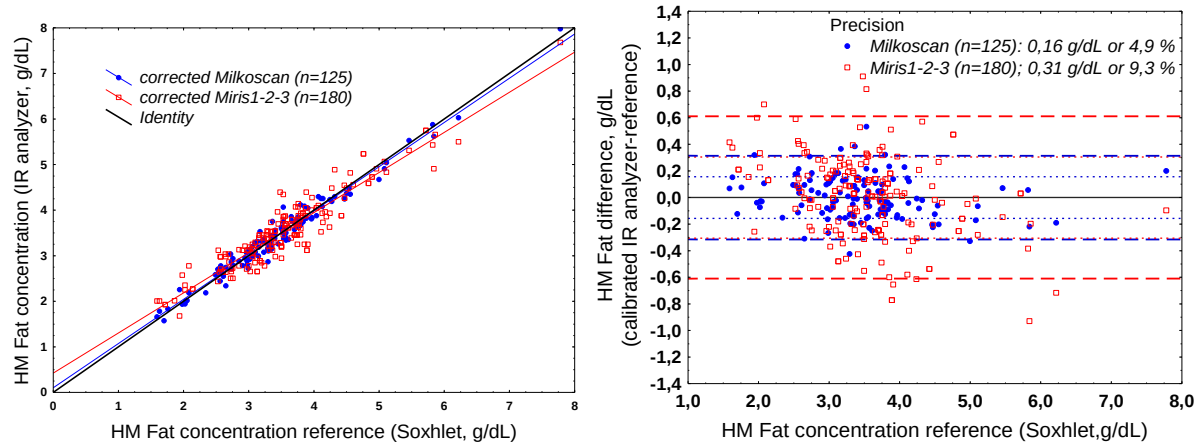

Figure 7.2. Validation of infrared analyzers (Milkoscan minor and Miris 1-2-3) for fat determination

As validations of IRA Milkoscan were performed at different periods, our study also suggests that the accuracy remains stable according to time and that its use could be of interest in clinical practice.
Thus, our studies suggest adjustments are needed to each generation or all individual IRA (Milkoscan® or Miris®) to provide accurate protein and fat measurements allowing their use in clinical practice. Unfortunately, up to recently, IRA were put on the market and use in clinical practice without any chemical additional validation. This led to increasing confusion surrounding the nutritional value of HM.

Currently, several publications have evaluated various IRA devices: the MIRIS (Uppsala, Sweden), the MilkoScan (Foss, Hillerod, Denmark), the Unity Scientific SpectraStar (Brookfield, CT, United States), the Calais Human Milk Analyzer (Cleveland, OH, United States), the near-infrared reflectance analysis (NIRA) (Fenir 8820; Esetek Instruments, Marino, Rome, Italy), the Delta LactoScope (Drachten, the

Netherlands), the AcuDairy Mid-Range Infrared instrument from Analytical Technologies (Westfield, NY, United States), and the York Dairy Analyzer from Metron Instruments (Bedford Heights, OH, United States) (227). These can be grouped into two main types of IRA according to the spectral range used: the mid-IR and the near-IR human milk analyzers. It was suggested that standards for good clinical laboratory practice (GCLP) are important when using HM IRA to avoid additional random errors (167). Thus, All IRA operators must be trained to reduce manipulations errors, such as inadequate cleaning of the test chamber, sampling errors, defect of homogenization or suboptimal temperature of the sample. This is important as human milk is a complex environment, and therefore relatively unstable, and all those processing steps could interfere with IRA (227). In an international multicentre study, Kwan et al (167) developed a calibration sets of pooled human milk controlled by chemical analysis to assess accuracy and precision of different IRA, their need for individual calibration curves and their long-term stability. This study compared ten Miris® and 5 Unity SpectraStar ® devices use in various centres in USA, Canada and European Union (167). They reported wide variation in accuracy and precision between devices, as well as in long term stability. They found that the accuracy of fat and protein measurements could be improved by establishing individual correction algorithms, and that once validated the long-term results were quite robust. However, they consider that a ring trial in milk analyzers is feasible and seems to be useful to control the potential systematic errors of the instruments. Thus, in line with our observations, this study suggests that whatever the manufacturer, each IRA needs individual calibration and control for long term stability before its clinical use in the NICU or HM bank.

As chemical analyses to obtain a controlled calibration sets of pooled human milk require specific tools, experience staff, and are time consuming, the Miris company introduced a calibration control kit for HM analysis, in line with the GCLP concept. However, there are currently no published data on the interest and the precision of this non- human milk calibration kits.

In conclusion, our study suggests that IRA could be useful to analyze macronutrients in HM with acceptable accuracy and precision after recalibrating fat and protein levels of field samples. However, as such a calibration should be performed to all devices whatever the series or the company, their use should be restricted to facilities where calibration processes are available.

What is the optimal fortification for human milk fed to preterm infants? It is possible to improve growth and reduce postnatal growth restriction in ELBW and VLBW infants fed fortified OMM or DM?

Feeding fortified HM helps to support adequate in-hospital growth (103) and bone mineralization (104). It also seems to be associated with favorable neurodevelopmental outcomes (105) although evidence for long term effects on growth and developmental outcomes is limited (103). Previously it was suggested that protein intakes and protein energy ratio were the main determinant of postnatal growth in VLBW infants (34) and that protein and energy requirements could be higher in preterm infants fed FHM compared to PTF.

Therefore, in an international DBRCT (Chapter 3), we evaluated the effect of the protein content of HMF on growth by randomizing VLBW infants fed FHM to intakes of either 4.5 or 3.8 g of protein /kg*d, while providing similar energy intakes. A significant adjusted weight gain difference of 1.18 g/kg/d (95% CI = 0.14, 2.21; p=0.013) in infants fed more protein was observed during the study period while body length and HC gains remained similar between the 2 groups. Unfortunately, if this study confirms the positive effect of protein and protein energy intakes on weight gain, it still has some limitations.

Thus, the energy and protein intakes were evaluated on the base of a theoretical preterm OMM content and were not actually measured. In addition, a large part of HM was pasteurized donor milk, where the protein and energy intakes were likely overestimated. Therefore, we suggested that protein utilization could be suboptimal. Indeed, urea production tended to be higher in the high protein HMF group, suggesting a decreased protein utilization rate. This difference could be the result of a relative lower energy bioavailability of HM (DM and expressed OMM) as shown in our HM composition study (113). In addition, while the energy intakes appear very similar between the 2 groups, their sources are different. The introduction of a part of energy as fat in the nHMF induces a reduction in the carbohydrate/non-protein ratio, and could by itself decrease the potential growth rate velocity within the nHMF group as suggested by Kashyap 2001 (132). Thus, the metabolizable energy supplies of the new HMF could be limiting for protein utilization and growth in those VLBW infants.

The significance of protein intakes was also demonstrated in studies evaluating, in preterm infants, growth velocity during the HM fortification period (115, 118, 228, 229) as summarized in figure 7.3. Higher enteral protein intakes (4-4,5 g/kg/d) also are suggested to have a positive effect on head circumference growth in preterm infants (228), an important clinical indicator of brain growth associated with better neurodevelopmental outcomes (230-234). Early postnatal lipid and energy intakes seem to be also important for brain growth and white matter maturation (235, 236). A recent systematic review performed by Katherine Ottolino investigated nutritional effects on postnatal brain development in healthy term and prematurely born infants by advanced MRI tools. She confirmed the importance of early postnatal growth and nutrition, with a balanced protein, fat, and caloric content for early brain development (236).

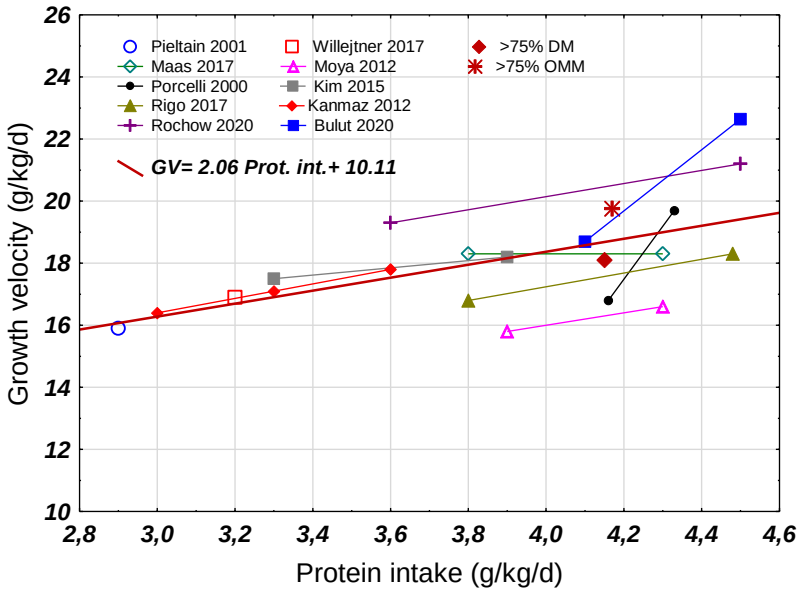

Figure 7.3. Effect of protein intakes on growth velocity (106, 115, 164, 191, 204, 237-242)

In the early postnatal period, protein content of preterm mothers' milk is generally higher than in their term counterpart but it decreases rapidly over time during lactation (116). HM composition may be

also affected by different steps in processing (container changes, freeze-thaw cycles, and pasteurization…) (77, 183).

Knowledge of preterm and term human milk compositions is generally based on meta-analyze or systematic reviews of studies analyzing the composition of HM collected in optimal clinical and laboratory conditions (114). Thus, those studies were generally limited to 24 hours collections. By contrast, our focus in the NICU (113) was to obtain the HM composition of HM expressed in various real-world conditions in the hospital or at home and brought to the NICU or the Human Milk Bank. With such conditions, the mean composition of HM determined using IRA Milkoscan remained in the range of the published reference values. However, our study confirms that the high variability of the HM composition and that mean values do not give, contrary to PTF, accurate estimates of its actual composition in view to provide adequate HM fortification

Standard fortification (SF) adds a fixed amount of multinutrient fortifier in HM as determined by the manufacturer, assuming a fixed protein and energy concentration. This is currently the most used fortification strategy in NICUs, but this practice could still prove inadequate in nutrients supplies, particularly in protein and for satisfactory growth (106, 107, 243).

Considering the variability and unpredictability of HM energy and protein contents, new strategies of individualized fortification have been suggested to improve growth. “Adjustable fortification” (ADJ) and “Targeted fortification” (TF) are 2 methods of individualized fortification (102).

Adjustable fortification (ADJ) was designed to avoid relative protein excess and deficit. ADJ fortification starts as standard fortification with a multi-nutrient fortifier. Protein intakes are then adjusted according to each infant’s metabolic response, guided by blood urea levels considered as a marker of adequate protein intake (120, 121). This method is easy to apply, does not require daily milk analysis or special equipment and improves in-hospital growth (120, 121, 244-247) and neurodevelopment outcomes (246, 248) compared to standard fortification. However some cumulative deficit in protein and growth persist (120). In adjustable fortification, the need for extra proteins is adjusted weekly based on serum urea concentration, urinary urea excretion and growth velocity (249, 250). However, it has been shown that serum urea concentration is not correlated to protein intakes during the first weeks of life, but rather reflects the renal immaturity of preterm infants (122, 238). In addition, the most adequate range of urea for VLBW infants during the early weeks of life remains controversial. Thus, in the study of Bulut comparing target and adjustable fortification, BUN values decreased regardless of protein intakes and showed no consistent relationship with increased protein intakes (238).

In our center, Senterre et al (18, 251) found that in preterm infants below 1250 g for a GA< 30 weeks, blood urea concentration increased during the first early days of life up to a mean value of 12.5 mmol/L at day 4, and thereafter decreased progressively down to around 6.5 mmol/L at the end of the second weeks of life and reached a stable plateau around 2.5 mmol/L at the end of the fifth weeks of life (figure 7.4.). During the first month of life, multivariate analysis showed that creatinine, GA, protein intake and day of life were the main factors contributing respectively to +68%, -20%, +12% and -9% to serum urea concentration. Thereafter, the contribution of protein intake increased progressively to become the main determinant with creatinine after the first month of life. Consequently, targeting the protein intake to a serum urea level during the first month of life could induce a delay to reach an adequate protein intake and an optimal growth. By contrast after the first month of life, as shown in

the study of Picaud et al in 2016, the use of a threshold BUN of 3 mmol/L (18 mg/dL of urea) to provide protein supplementation in VLBW infants allowed to significantly improve all growth parameters during the study period (249). However, the disadvantage of such of adjusted fortification could be that the energy intakes could be inappropriate, inducing an excessive protein/energy ratio.

VLBW <1250 g n=102
PN 1005±157 g, GA 28.5 ± 1.9 weeks

Postnatal age	n	Mean ± SD	P10-P90
1-7 days	367	11.1 ± 4.6	5.8-17.0
8-14 days	181	8,7 ± 6.4	3.3-16.2
15 days	55	6.7 ± 5.3	2.2-11.9
week 3	73	5.2 ± 3.3	1.8-10.0
week 4	63	3.9 ± 2.8	1.7-7.5
weeks 5-6	108	3. 1± 1.9	1.3-4.7
weeks 7-8	69	2. 9± 1.4	1.2-4.5
weeks 9-10	40	2. 5± 1.4	1.0-4.3
weeks >10	28	2.9 ± 1.2	1.5-3.8

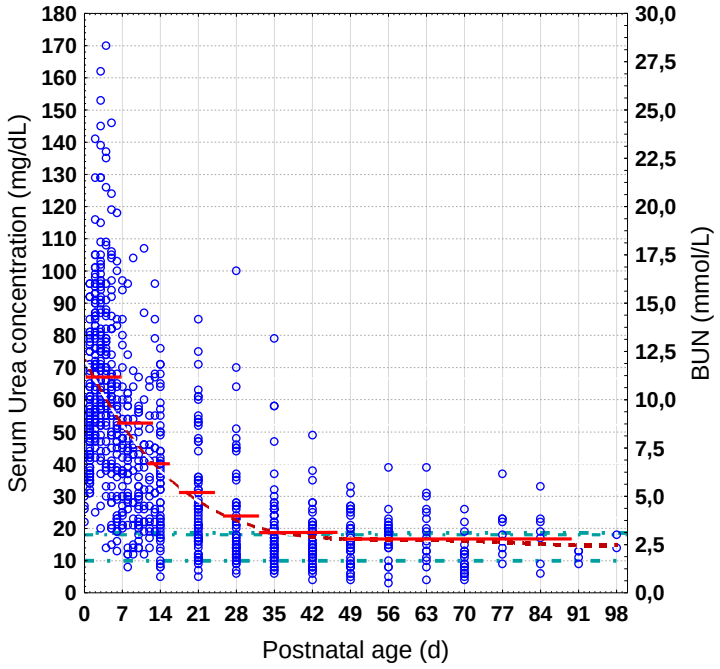

Figure 7.4. Evolution of serum urea concentration and BUN according to postnatal age

Targeted individualized fortification (TF**)** starts with analyzing HM macronutrients composition and then adapt fortification to target recommended intakes related to postconceptional age. TF is usually based on measurement of macronutrients concentration by infrared spectrophotometry as discussed in Chapter 4 and before in the general discussion.

Various strategies of individualized target fortification are currently used and reported (178, 203, 250, 252-256). Some suggest using HMA once or twice a week to guide daily fortification. Other including us advocate daily HMA, given the high variability of HM. Various multicomponent HMF containing intact or hydrolyzed cow milk protein, energy as carbohydrate and fat or exclusively as carbohydrate plus electrolytes and trace elements might be use for fortification. More recently an exclusive HM fortifier based on a concentrate pasteurized DM was put on the market. To perform individualized HM fortification and provide both protein and energy requirements, the combined use of 2 fortifiers is frequently required: a multicomponent fortifier and a protein or an energy enrichment. The energy adjustment is frequently provided as fat using MCT, LCFA or a HM Cream to reach the target energy intake (119). Target threshold for protein and fat ranged between 3.5 to 4,5 /kg/d for protein and 110 to 145 kcal/kg/d for energy (30, 31).

Several studies suggest that TF improve growth in VLBW infants. In a recent double blind RCT (n=101), Rochow et al reported that TF compared to SF resulted in higher macronutrients intakes and improved growth outcomes in preterm infants <30 weeks (204). The impact of TF on growth was most pronounced in a subgroup of infants receiving HM with lower protein content (204). A trend toward improvement in neurodevelopment outcomes in the TF group was also suggested (257) but still need

to be confirmed. Higher free fat mass deposition at term compared to SF was also reported (204, 256). Recently, in a prospective randomized controlled study, Kadioglu and al (244) compared the effects of adjustable, target and standard fortification on early growth of VLBW <32 weeks. Both ADJ and TF of HM significantly improved weight gain compared to SF (24, 25,5 vs 12 g/kg/j; p<00,1). Daily protein and energy intakes were similar in ADJ and TF group (protein 4,5 and 4,3 g/kg/d; energy 133 and 131 kg/d/d) but significantly higher to than those in SF group (protein 3,6 g/kg/d and energy 128 kcal/kg/d). Significant improvements in weight, length ad HC percentiles were observed in ADJ and TF group compared to SF. A recent meta-analyze (247) found that individualized (either targeted or adjustable) fortification of HM feeds VLBW infants increases growth velocities of weight, length, and head circumference during the intervention compared with SF. However, this meta-analysis also highlights some limitations as the included studies are mainly restricted to a short period of time and don't provide evidence of significant benefits on in-hospital and/or post-discharge clinical outcomes. That the studied regimens were not persistent to discharge is a possible explanation (247). On the contrary, another recent prospective randomized study by Bulut et al (238) demonstrated that TF method for feeding VLBW infants had a significantly increased positive effects on short-term growth in weight (23,1 vs 18,7 g/kg/d; p=0,014) and HC gains (9,8 vs 8,4 mm/week; p=0,048) compared to ADJ fortification. Mean protein intakes were also higher in the TF group 4,5 g/g/kg/d vs 4,01 g/kg/d (p=0.001) suggesting that the level of BUN used for to adapt ADJ fortification may have been insufficient (250). By contrast to ADJ fortification, TF tends to adapt both protein and energy intakes in the range of the recommendations and decreases the FHM protein/Energy ratio variability (113, 204). However, TF may be time consuming and requires specialized staff and measuring equipment. Despite these developments, consensus on the optimal individualized fortification method is lacking. Further randomized controlled trials are needed to determine the optimum components and method for individualized fortification and to assess effects on body composition and long-term outcomes.

In our studies, we suggest an original protocol of TF. HM macronutrient's concentration was determined daily by IRA to allow TF performed in two steps: first, fat was adjusted up to 4.0 g /dl with MCT and then a multicomponent HM fortifier was added to provide 4.3 g /kg/d of protein according to the daily volume prescribed. The protein and energy intakes during the study period were thus very stable. In our first experimental study (chapter 5), nutritional intakes resulting from our TF strategy were compared to potential intakes resulting from SF. We showed that the TF decreases significantly (p<0.05) the variability of the protein and energy intakes compared to SF (from 9.2 to 2.0% for protein, from 12.1 to 6.6% for fat, from 7.3 to 4.8% for energy (113, 178). In 2007, this fortification strategy was introduced in our NICU for feeding micropremies and allowed for improving the mean weight gain, reaching 18-20 g/kg/d (237).

As summarized in table 7.3, this strategy of individualized TF applied in a prospective cohort of 101 VLBW infants provided daily stable nutritional intakes with low variability, allowing an optimal growth (113, 178, 237).

Table 7.3. Weight gain and nutritional intakes according to HM type received during the study period

Human Milk Type **Volume Intake (%)**	**DM** **88.5 ± 16.9**	**POMM** **70.3 ± 22.6**	**ROMM** **69.1 ± 19.9**	**Total**	
n	45	41	15	101	Variability (%)
Weight gain, g/kg/d	18.2 ± 1.9	19.1 ± 1.8	21.1 ± 1.6	19.0 ± 2.0	8,3
Volume, ml/kg/d	168.9 ± 8.4	168.4 ± 10.5	166.7 ± 5.6	167,5 ± 9,0	3,99
Energy, kcal/kg/d	141.3 ± 6.3	142.4 ± 7.3	143.7 ± 6.2	142,20 ± 6,42	3,33
Protein, g/kg/d	4.15 ± 0.14	4.19 ± 0.13	4.18 ± 0.15	4,17 ± 0,14	2,67
Fat, g/kg/d	8.36 ± 0.61	8.59 ± 0.62	8.73 ± 0.55	8,51 ± 0,61	4,72

In conclusion, our data shown that individualized daily target fortification allows to provide stable nutrient supplies in VLBW infants leading to an optimal growth in the range of the recommendations and reduces the incidence of postnatal growth restriction in VLBW infants.

Are donor milk, pasteurized OMM and raw OMM equivalent for nutrition of preterm infants and to ensure an adequate growth?

In a clinical setting, our individualized fortification protocol provided similar controlled nutritional intakes in VLBW infants fed fortified HM. We postulated that this would allow to highlight the influence of others factors on growth than protein and energy intakes. Effectively, in our recent prospective study evaluating growth in 101 VLBW infants receiving target fortified OMM or DM, we reported that nutritional intakes were very similar in the different groups of HM types as shown in table 7.3 with a variability of less than 5%. It was therefore possible to assess effects of the type of HM fed to those infants (mostly DM, Pasteurized OMM or Raw OMM).

As for the first time, in this population, weight and length gains were independent of nutritional intakes, significant differences could be detected between the types of HM. OMM promoted growth of premature infants compared to DM with higher weight gain velocity of 1.6 g/kg/d; p=0.002 and length gain of 0.18 cm/week; p= 0.02 (see chapter 6). Weight gain was also significantly higher in ROMM compared to POMM group with a mean weight gain difference of 2.0 g/kg/day (Table 7.4). The benefits of pasteurized OMM on DM was limited on weight gain, contrasting with the benefits on all parameters of growth observed with raw OMM. It suggests that the major positive effects of OMM could be the result of its use as a raw product. This lower weight velocity in preterm infants fed DM and POMM seems to be related to the pasteurization destroying some hormones (258), growth factors and mainly the bile-salt stimulated lipase, reducing long chain fatty acid absorption and metabolizable energy (199). The significant difference (p≤0.001) detected between serum urea concentration in the ROMM group (13.6±3.9 mg/dl) versus the two other groups (17.8±4.1 in DM and 19.1±4.0 g/dl in POMM) as well that the significant negative relationship observed (Figure 7.5.) between blood urea concentration and energy intake (p=003) support this hypothesis.

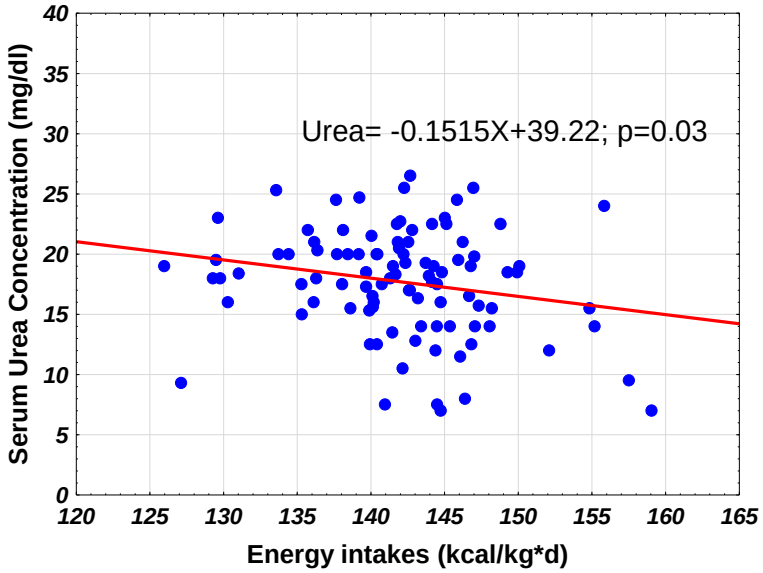

Figure 7.5. Relationship between blood urea concentration and energy intake

The optimal composition of HM fortifiers is still debated. A large range of products are available for fortifying HM and differ by the origin of milk used (bovine or human), by nutrient composition and by conditioning (powder or liquid) (102). Bovine based multi-nutrient fortifiers contain protein, energy as lipid or/and carbohydrates, minerals, traces elements, vitamins and electrolytes and are the most used in Europe. The addition of lipids as energy to replace partially or completely carbohydrates in fortifiers has allowed a reduction in osmolality (259) potentially improving digestive tolerance. In addition, lipid is a source of essential fatty acids which have been shown to improve essential fatty acid status in preterm infants (260) and seem to be essential to brain development (236). New fortifiers with higher protein content have been shown to improve weight gain (115, 229). However, bioavailability of fat is less than that of carbohydrate in VLBW infants and this difference needs to be compensated by energy supplementation.

Recently, a human milk-based fortifier obtained by concentrating heat-treated DM with added vitamins and minerals (Prolacta Bioscience, USA), was recently proposed considering that feeding bovine protein may negatively impact gut permeability, influence gut epithelial cell cytotoxicity and promote dysbiotic gastro-intestinal colonization (261). It has been suggested that exclusively HM diet could reduce feeding intolerance, NEC and ROP (54, 262-265). However, there are still concerns about the efficacity, safety and ethical issues of these products (102, 266, 267). Low-level evidence from the only blinded randomized trial (OptiMoM study) suggests that in exclusively human milk-fed preterm infants use of human milk-derived fortifiers (n=64) in comparison with bovine milk-derived fortifier (n=63) did not reduce feeding intolerance, mortality or morbidity (NEC, LOS, BDP, severe brain injury) or did not improve growth (261) and 18-mo neurodevelopment outcomes (268). The only difference between groups was a lower incidence of ROP for infants in exclusive HM diet (261). A recent systematic review and meta-analysis found a very-low-quality evidence that HM based fortifier use decreased risk of NEC stage 2 and surgical NEC in preterm infants, but resulted in slower weight gain (269). However, the beneficial effects of HM based fortifier on NEC in this review were no longer significant in sensitivity analyses excluding studies with high risk of bias (269). In addition, the NEC incidence reported in the Prolacta studies are similar to that observed in European countries using cow milk based HMF (88). The use of a liquid form of the HM based fortifiers replaces a significant fraction

of OMM (20 to 50 % of feed volume) and reduces significantly the infant's exposure to OMM, potentially impacting its benefits on infant's outcomes (267). Another concern is its extremely high financial cost that must be weighed into consideration of its potential effectiveness. The volume and origin of DM used to manufacture these products could be also an ethical concern (102, 267). The volume of DM needed to produce the concentrate might find a better use as a replacement of preterm formula. Well-designed and adequately powered randomized controlled trials, free from the influence of industry are needed to evaluate benefit-risk ratios on short- and long-term outcomes of full HM diet (269).

In conclusion, our study highlights that the use of OMM improve growth in VLBW infants and suggests that the quality of growth could be enhanced by the use of raw OMM, promoting length gain and lean body mass deposition. This benefit requires further evaluation. We also speculate that nutritional requirements could be slightly higher in VLBW infants fed fortified pasteurized OMM or DM and that a limited increase in energy intake may improve growth and protein utilisation.

Conclusion and perspectives

Human milk is the gold standard for nutrition of term and preterm infants. The function of maternal milk goes well beyond nutritional support to the infant given its roles in immune function and promotion of organ development. Human milk feeding emerged as a potential strategy to enhance vulnerable preterm infant's outcomes. Therefore, great attention should be given to methods to sustain mother's milk supply. Initiation of lactation should start early to have OMM available as soon as possible, and the presence of the mothers at the bedside of their children should be encouraged. When OMM is unavailable, DM is the recommended second-best nutritional choice.

Human milk despite anti-infective properties is not sterile and additional contaminations may occur during handling and processing for use in neonatology, leading to a potential infectious risk in vulnerable preterm infants. To decrease this risk, HM requires careful monitoring and approaches to prevent viral or bacterial contamination that also preserve the immune function. Holder Pasteurization is the method currently used in HM banks and should be considered although, unfortunately, it alters some HM components, especially immune, and impairs nutrients bioavailability. While theoretical arguments and in-vitro studies suggest that raw OMM may be superior to pasteurized OMM in protective effects against infections and other morbidities, clinical evidence is still lacking. Controversies also arise on the risk-benefits of pasteurization in regard of postnatal CMV infection, especially at the lower gestational ages, and in regard of acceptable bacteria types and thresholds. Belgian guidelines on the use of raw own mother's milk for EPT and ELBW infants in NICU were developed in 2016 but are controversy and need to be adapted. In the light of our study on HM bacterial contamination and the literature review on postnatal CMV contamination from OMM of CMV positive mother and infection risk of vulnerable preterm infants, we consider that pasteurization remains preferable in EPT infants <28 weeks GA of CMV positive mothers and for OMM contaminated with pathogen in VPT infants <32 weeks GA. Colostrum is less contaminated than mature milk and is also richer in immune components, and therefore could be used raw. Greater understanding of the link between HM microbiome and health benefits of preterm infants opens new perspectives of future research. Future well designed studies are needed to clarify the potential beneficial of raw mother's milk use. Novel methods need to be explored to ensure bacteriologic and virologic quality of HM while preserving all its beneficial properties and to restore the live microbiota in pasteurized DM, potentially improving its bioactivity.

Nutritional requirements of preterm infants are high leading to a risk of nutritional deficits and extrauterine growth restriction. On the other hand, an improved growth seems to be associated with better neurodevelopment. Exclusive HM feeding with both OMM and DM cannot meet nutritional recommendations for preterm infants and HM needs to be fortified. However, despite standard fortification, growth of HM fed preterm infants remains suboptimal and lower than that of those fed preterm formula. These differences could be related to large variations in the macronutrients content of expressed HM, that is frequently lower than the theorical assumed concentration. Additional explanations include different individual nutritional requirements, lower metabolizable protein and energy availability for new tissue synthesis and a negative impact of pasteurization. Therefore, optimization of HM fortification is required. Individualization of fortification and improving quality of the fortifiers have been suggested. Devices using infrared technology allow rapid analyses of macronutrients concentrations but require a careful, individual and time-consuming validation before

use in clinical setting. Using an infrared analyzer (Milkoscan®), we confirmed the high variability of HM content and the interest of individualized HM fortification to optimize HM nutritional composition in order to meet nutritional requirements of VPT infants, especially when DM is used. In a clinical setting, our daily individualized fortification strategy allowed us to obtain similar controlled nutritional intakes in a group of VLBW infants fed fortified OMM or DM. Therefore, independently from protein and energy intakes, it was possible for the first time to demonstrates that OMM promoted growth of premature infants compared to DM. This difference could be partially explained by use of raw OMM suggesting that pasteurization impaired the bioavailability of the protein and especially the energy intakes. The interest of an increase in protein or in energy intakes in preterm infants fed pasteurized HM could be postulated but needs to be demonstrated in further studies. We also suggest that specific nutritional recommendations need to be designed for preterm infants fed HM rather than preterm formula, taking into consideration the human milk types: OMM or DM, raw or pasteurized. Developing consistent feeding preparation techniques to improve homogenization, minimize fat loss, and maximize nutrient delivery is another relevant focus for further research and quality improvement. Optimization of the quality of fortifiers is also important in the future: amount and quality of protein, carbohydrates or lipids as source of energy, PUFA content. We showed that a new fortifier with higher protein content improved short term weight gain. Recent HM based fortifiers still lack evidence to support their use, and raise safety, ethical, and financial issues. More RCTs comparing efficacity of individualized to standardized fortification or different ways to achieve individualized fortification are required to determine the optimal method of fortification. Relevant endpoints such as morbidity, growth with body composition as well as longer-term effects on health including neurocognitive outcomes in large cohorts are also needed to determine the optimal nutrition for vulnerable preemies and adapt our guidelines and clinical practices in the future.

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Annexes

1. de Halleux V, Pieltain C, Senterre T, Rigo J. Use of donor milk in the neonatal intensive care unit. Semin Fetal Neonatal Med. 2017 ;22(1):23-29.

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Review

Use of donor milk in the neonatal intensive care unit

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SUMMARY

Own mother's milk is the first choice in feeding preterm infants and provides multiple short- and long-term benefits. When it is unavailable, donor human milk is recommended as the first alternative. Donor milk undergoes processing (i.e. pasteurization) to reduce bacteriological and viral contaminants but influences its bioactive properties with potentially fewer benefits than raw milk. However, there is no clinical evidence of health benefit of raw compared to pasteurized human milk, and donor milk maintains documented advantages compared to formula. Nutrient content of donor and own mother's milk fails to meet the requirements of preterm infants. Adequate fortification is necessary to provide optimal growth. There are significant challenges in providing donor milk for premature infants; therefore, specific clinical guidelines for human milk banks and donor milk use in the neonatal intensive care unit should be applied and research should focus on innovative solutions to process human milk while preserving its immunological and nutritional components. In addition, milk banks are not the only instrument to collect, process and store donor milk but represent an excellent tool for breastfeeding promotion.

1. Introduction

Human milk (HM) is the gold standard to provide nutritional support for all healthy and sick newborn infants including the very low birth weight (VLBW) infant (<1500 g) [1]. It contains nutrients necessary for infant's growth but also numerous bioactive factors contributing to beneficial effects on gastrointestinal maturation [2], host defence, infection [3–6], cardiovascular risks [7], metabolic disease [7] neurodevelopmental outcome [8,9] as well as in infant's and mother's psychological well-being. Several studies in preterm infants have reported short- and long-term benefits of HM compared with preterm formula [4,8–10]. Due to the specific mother and infant dyad, own mother's milk (OMM) should always be the first choice in preterm infants [1,11]. Unfortunately, mothers of preterm infants are less likely to initiate milk expression, sustain lactation and to provide full OMM soon after birth, suggesting that donor milk (DM) and HM banks are necessary to provide an exclusive HM diet in VLBW infants during their first weeks of life [1,12]. Therefore, the use of DM is increasing in the NICU and the number of HM banks is growing worldwide [13–15]. DM is collected and distributed following standards similar to blood donation and is generally pasteurized [15–17]. As with OMM, DM needs to be fortified to provide the high nutritional requirements, to reduce cumulative nutritional deficits and promote optimal growth in VLBW infants. Although storage, processing and pasteurization could reduce the nutritional value of DM and alter some of the immune components found in HM [18], beneficial health outcomes are also reported in preterm infants fed with DM compared with those fed formula [19]. However, it is unclear whether the use of pasteurized OMM or of DM confers the same clinical health benefits as does raw OMM.

2. Clinical benefits of donor milk

2.1. Necrotizing enterocolitis

Donor milk is widely used to prevent necrotizing enterocolitis (NEC) for vulnerable premature infants when OMM is unavailable [1]. Both older and more recent studies suggest that DM is as efficacious in preventing NEC in preterm infants [14,20,21]. Many observational studies suggest that the incidence of NEC is HM dose-dependent in premature infants [10,22]. A recent meta-analysis of data from six trials found a statistically significantly higher incidence of NEC (twice the risk) and feeding intolerance (Risk Ratio: 4.92) in the formula-fed group compared to HM groups. It has been

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estimated that one extra case of NEC will occur in every 25 preterm infants who receive formula. This beneficial effect exists even when DM is given as supplement to OMM rather than as a sole diet and also when DM is fortified [19]. However, the specific effect of HM fortification on the incidence of NEC is still controversial. In a randomized control trial (RCT), Lucas et al. showed a small but not significant increase in NEC in preterm infants fed fortified HM (5.8%) compared to unfortified HM (2.2%) [23]. From that study, it has been speculated that a bovine protein diet may be associated with higher intestinal inflammation and permeability and that the use of bovine-derived HMF may be inadequate to protect infants against NEC. Thus, in two recent RCTs, an exclusive HM diet exempt from bovine-based formula (DM or OMM fortified with DM fortifier) has been reported to significantly reduce the incidence of NEC compared with an exclusive bovine based formula (3% versus 21%, p=0.04) [21] or a bovine-derived fortifier (6% versus 15.9%, p=0.02) [24]. However, in these prospective randomized trials the bovine-based cohorts had higher NEC rates (16% and 21%) than in many units using bovine fortifier and formula (3% and 6%) [25]. In our country between 2010 and 2015, the national rate of NEC in 8402 preterm infants at <32 weeks or <1500 g, fed HM supplemented by bovine-derived fortifier or fed preterm formula, is 4.4% (NICAUDIT, Belgian network), suggesting that the results of these trials should be interpreted with caution.

Similarly, it has also been suggested in one RCT that pasteurization by itself does not increase significantly the incidence of NEC $\geq$ Bell's stage 2 in preterm infants $\leq$32 weeks and $\leq$1500 g fed OMM (13/151, 8% raw OMM versus 9/152, 5% in pasteurized OMM; $P = 0.39$) [26]. Similarly, in California NICUs it has been suggested that the increased availability of DM over time has been associated with a significant reduction in NEC incidence [14]. More recently, it has been suggested that the introduction of preterm formula or DM as OMM supplementation during the first 10 days of life does not increase significantly the incidence of NEC in VLBW infants (8.9% versus 9.3%; $P = 0.95$) but that the provision of OMM >50% of the intake tends to improve the event-free survival rate in both groups [27].

These studies suggest that DM could be as effective as OMM in reducing the incidence of NEC but that the use of bovine-based fortifier or formula could be a major risk factor for NEC in VLBW infants, and that further studies are still required to determine whether raw OMM, pasteurized OMM or DM offers any advantage against NEC.

2.2. Infection

Human milk is not sterile and represents a complex ecosystem with a large diversity of bacteria reflecting mother's biotope [28]. HM is known to be colonized by non-pathogenic bacterial flora with a majority of bifidobacteria, promoting development of infant's healthy gut microbiota. These bacteria could protect the infants against infections and contribute, among other functions, to the maturation of the immune system. However, HM may also contain potentially pathogenic bacteria species [29,30]. The expression, collection, storage and transport of HM may introduce pathogenic contamination, increasing the risk of sepsis to these vulnerable premature infants, as suggested by several case-reports in the literature [31–33]. The need for bacterial screening of OMM before raw administration is controversial but when performed there is a general consensus to discard or pasteurize contaminated OMM [26,30]. Several studies demonstrate that HM reduces the sepsis risk in premature infants with a dose–response relationship [4,6,8]. They also suggest that OMM provision from the first few days of life plays a major role in this phenomenon [5].

Many studies do not record the type and proportion of HM used: pasteurized DM, pasteurized OMM or raw OMM. By contrast, DM is widely pasteurized to ensure safety [15–17]. Pasteurization alters cellular and some immunological properties of HM but many bioactive components and anti-infectious properties are preserved [34,35], maintaining health advantages over formula. Therefore, there are theoretical arguments suggesting that fresh OMM is superior in protective effects against late-onset sepsis (LOS) versus pasteurized OMM but no clinical evidence has been demonstrated. Recently, Cossey et al.'s RCT reported no significant difference in the rate of LOS between infants fed raw (22/151; 15%) versus pasteurized OMM (31/152; 20%; $P = 0.23$) [26]. In this study, bi-weekly bacteriological evaluations were performed in order to discard or pasteurize contaminated OMM. Similarly, Stock et al. did not find significant differences in the rate of LOS between unpasteurized and raw milk [36].

Therefore, these recent studies failed to demonstrate a significant superiority of raw OMM over pasteurized OMM on LOS, suggesting persistent protective effects [26,36]. By contrast, the clinical superiority of fresh OMM over DM to prevent LOS in preterm infants is still debated, with a recent study suggesting that the provision of fresh OMM for >50% of the diet reduces the incidence of LOS in VLBW infants [27].

Recently, there have been concerns about short- and long-term morbidities associated with postnatally acquired cytomegalovirus (CMV) infection in very preterm infants. Postnatal CMV infection related to fresh HM in preterm infants remains generally mild or asymptomatic, but a serious illness “sepsis-like syndrome” may be observed in 4% of preterm infants of seropositive mothers [37]. By contrast, the incidence can reach up to 40% in extremely low birth weight (ELBW) infants <26 weeks of gestational age [38]. The effect of postnatal CMV infection on long-term neurodevelopmental outcomes is unclear. Limited studies suggest that cognitive and motor function could be affected in contaminated infants compared with uninfected controls [39,40]. By contrast to the freezing process, the use of pasteurized OMM or of DM prevents completely the risk of postnatal transmission of CMV via breast milk [36].

2.3. Feeding tolerance and donor milk's influence on feeding practices

The trophic effects of HM are attributed to multiple HM components stimulating the maturation of the premature gut [2]. Clinically, it improves feeding tolerance and reduces delay to full enteral feeding. Available data from older studies support the hypothesis that DM improves feeding tolerance [12,19]. In a recent study, preterm infants fed exclusive DM-fortified diet required fewer median days of parenteral nutrition [27 (14–39) days] compared with those fed preterm formula [36 (28–77) days] ($P = 0.04$). However, the time to establish full enteral feeding was not significantly different [21].

An international survey evaluating differences in feeding practices found that most NICUs with access to DM started enteral feeding earlier and advanced more rapidly. Units without access to DM frequently delayed the introduction of enteral feeds until OMM was available [41].

2.4. Other long-term benefits

2.4.1. Neurodevelopment

The survival rate for early preterm infants is improving but with high risk of neurological impairments. More attention is being focused on the quality of survival through optimal nutrition management. Several studies suggested that the use of HM compared with preterm formula during the early weeks of life of VLBW infants was associated with better neurodevelopment outcome with a dose-dependent relationship despite a slower early growth rate (breastfeeding paradox) [8,42,43]. These studies suggest that HM

may have an independent, positive dose-effect on the psychomotor development of preterm infants. HM via multiple bioactive components provides optimal substrates [long-chain polyunsaturated fatty acids (LC-PUFA), oligosaccharides] for brain development and protects infants from morbidities associated with early preterm birth (NEC, infections), considered as risk factors for adverse neurocognitive outcome. However, these studies should be interpreted with caution due to the presence of many confounding factors and lack of detailed information about the HM diet (OMM or DM, OMM completed with DM, pasteurized or unpasteurized OMM). Moreover, no beneficial effect on neurocognitive outcome has been demonstrated in the only available RCT comparing non-fortified DM and formula despite higher growth in infants fed preterm formula [12].

There are several ongoing, blinded randomized trials to investigate the neurodevelopmental outcomes and other morbidities of very preterm infants fed DM compared with those fed formula (as supplement to insufficient OMM or as the sole diet) in the era of routine fortification [18,44].

2.4.2. Bronchopulmonary dysplasia

A reduction in the incidence of bronchopulmonary dysplasia has been observed in one RCT [45]. Further studies are needed to confirm this observation.

2.4.3. Long-term cardiovascular and metabolic diseases

Donor milk in early life may have beneficial effects on cardiovascular risk factors measured during adolescence; the significance of these findings for the development of cardiovascular diseases is uncertain [12]. A limitation of these findings is that the comparison was made between preterm formula and unfortified DM. It is important to consider whether positive effects would persist with use of fortified DM and early faster growth.

2.4.4. Allergy

The neonatal period is a critical window for immunological tolerance. HM contains many immune-modulating factors and could probably play a protective role against the development of allergy in preterm infants. The only available RCT does not show protective effects of DM against allergy later in life even when a protective effect against eczema in preterm infants at high risk of allergy is reported [12].

2.4.5. Breastfeeding rate of VLBW infants

Having a DM bank feeding practice in the NICU does not reduce OMM proportion in the infant's diet but significantly decreases the formula exposure [13,46]. The available evidence does not support the hypothesis that the introduction of DM has an adverse effect on breastfeeding rates in VLBW [12,47]. An Italian survey showed that exclusive breastfeeding at discharge was significantly higher in NICUs with an HM bank when compared to NICUs without (29.6% vs 16%, $P = 0.007$) [48]. In a recent study examining the impact of DM use in California NICUs, Kantorowska found that the availability of a donor HM bank in a hospital was associated with a mean increase of 10% in the breastfeeding rate at NICU discharge [14].

3. Concerns and problems of donor milk

3.1. Growth and nutritional composition of donor milk

Preterm infants and especially ELBW (<1000 g) infants are at risk of cumulative nutritional deficits and postnatal growth restriction during the first weeks of life up to the time of discharge or theoretical term [49,50]. It has been suggested that the neonatal period corresponds to a critical window when under-nutrition affects brain development [51]. Preterm infants have higher protein, energy, minerals and electrolytes requirements than term infants. Exclusive HM, even from OMM, cannot meet nutritional recommendations for ELBW infants [11,52]. Protein content of preterm mother's milk is generally higher in the early postnatal period and decreases during lactation. This problem may be amplified with banked DM which is most often provided by mothers of term infants who are in their later stages in lactation. Therefore, various HM fortifiers were developed to increase protein, energy, minerals, electrolytes, traces elements, and vitamin supplies [53,54]. Nevertheless, the use of fortified HM failed to obtain postnatal growth in the range of fetal growth or similar to that observed in infants fed adapted preterm formulas [24,55]. These differences could be related to the large variation in the macronutrient contents of expressed HM, especially in terms of energy, fat and protein [56,57]. A recent study performed in our NICU milk bank showed that protein, fat and energy contents of DHM were significantly lower than those of OMM (Table 1). Variability of DHM contents was also high, ranging from 0.9 to 3.2 g/dL for protein, from 1.8 to 5.5 g/dL for fat, and from 48 to 85 kcal/dL for energy [56]. Furthermore, out of all DM samples, 63% were <1.5 g/dL of protein whereas 90% were <4 g/dL of lipids and 81% were <67 kcal/dL energy, all values frequently considered as reference values for HM used in the NICU (Fig. 1).

In addition, growth differences between fortified HM and preterm formula-fed VLBW infants receiving an apparent similar energy and protein intake could also be related to a lower metabolizable protein and energy available for new tissue synthesis [55,57]. Metabolic balance studies [57,58] showed that nitrogen absorption as well as nitrogen utilization were lower in preterm infants fed fortified HM than in those fed preterm formulas. In all, the mean difference in nitrogen utilization (retention/intake) accounted for 11.8% and could be related to absorption of the non-nutritional proteins (lactoferrin, IgA) as well as to non-protein nitrogen utilization (urea) in HM. Net absorption of energy as measured by bomb calorimetry was reported lower (78.3%) in infants fed HM than in those fed formula (88.4%) resulting in a higher fecal energy loss [57,58]. This difference could be partially due to the use of pasteurized HM [59]. Pasteurization leads to inactivation of the bile salt-stimulated lipase of HM as well as possible changes in the milk fat globule structure [59].

Moreover, incomplete milk expression, manipulations of HM during expression, storage, transport, and processing are all additional factors influencing the high variability of expressed HM composition, especially reducing the fat content. In a recent prospective trial evaluating HM cream supplement on growth, 85% of the preterm infants fed DHM required the extra cream supplement because of energy density <20 kcal/oz (70 kcal/dL) [60]. In addition, VLBW premature infants are frequently continuously fed by gastric tube, inducing fat adherence to tubing and a substantial loss of phosphorus, calcium, and other nutrients bound to fat [61]. Fat lost may account for up to 25–34% and has been reported both in OMM and DM with or without fortification.

Table 1
Protein, fat, and energy concentrations of own mother's milk (OMM) and donor milk (DM).

	OMM[a] ($n = 428$)	DM[b] ($n = 362$)	P
Protein (g/dL)[c]	1.52 ± 0.28	1.42 ± 0.30	<0.0001
Fat (g/dL)	3.79 ± 0.73	3.41 ± 0.53	<0.0001
Energy (g/dL)	67.26 ± 6.49	63.80 ± 5.06	<0.0001

Values are expressed as mean ± SD.
[a] Own mother milks 28 ± 10 days of lactation.
[b] A proportion of DM is provided by preterm delivery mothers.
[c] Protein is measured as total nitrogen.

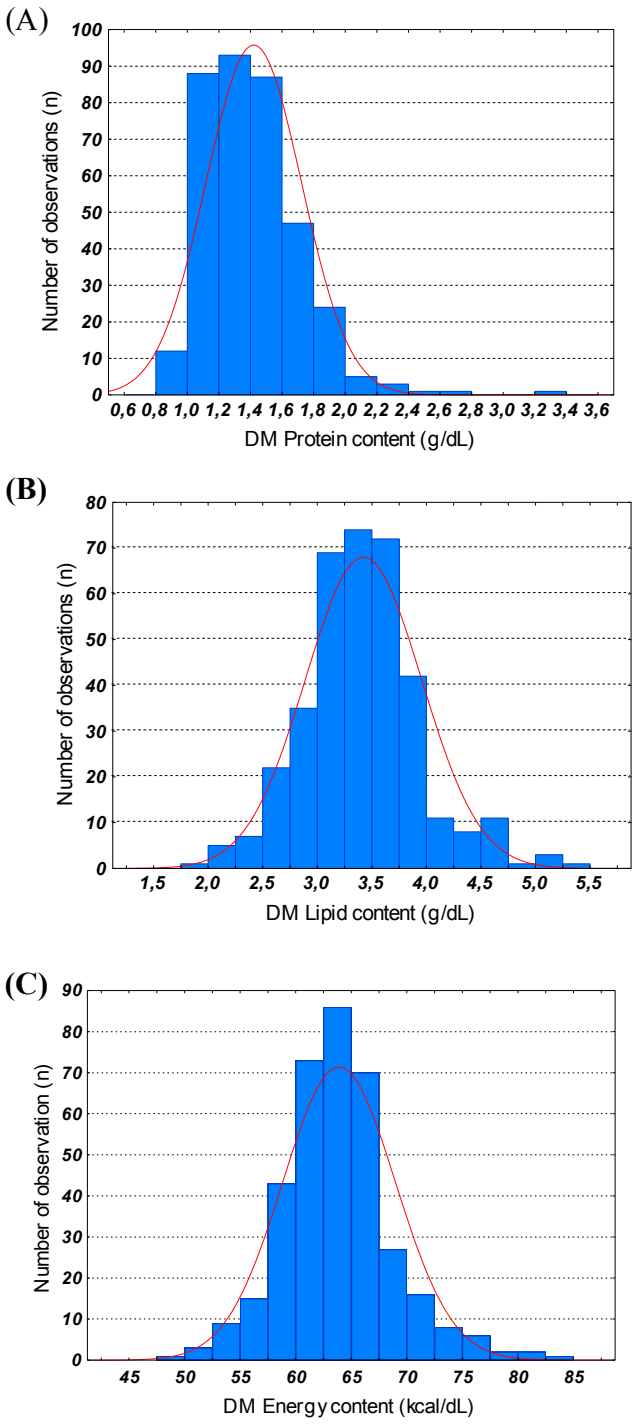

Figure 1. Variability of protein (A), fat (B), and energy (C) contents of donor milk (DM) ($n = 362$).

Standard fortification, adding a fixed amount of fortifier as recommended by the manufacturer, is the most widely used method to fortify HM. This method was not associated with a reduction in the variability of the HM macronutrient contents and often failed to meet the adequate nutritional supply for preterm infants [56]. Considering that true energy and protein contents are unpredictable and differ significantly from that calculated using a fixed composition for OMM or banked DM, new modes of fortification have been suggested.

In case of insufficient growth, some authors propose to increase fortifier strength or arbitrarily add extra protein, glucose or fat. We recently performed an RCT using a new, isocaloric HMF with a higher protein:energy ratio during a 21 d study interval in clinically stable preterm infants ($n = 153$). Infants in the intervention group had a significantly higher weight gain compared with the control HMF group. The adjusted beneficial effect amounted to 2.28 g/d (CI: 0.38–4.18; $P = 0.010$) compared with 1.18 g/kg*d (CI: 0.14–2.21) ($P = 0.013$) [62]. However, such an increase in protein fortification does not compensate for the variability of native HM composition and the risk of energy deficiency as well as protein overload with its potential long-term adverse effects [56]. Hair et al. provided an exclusive HM diet (OMM ± DM) with the use of a donor milk-derived fortifier (Prolacta®, Prolacta Bioscience, Inc., Los Angeles, CA, USA). Protein and energy intakes ranged from 130 kcal/kg/day with 3.6 g/kg/day of protein up to 150 kcal/kg/day and 5.25 g protein/kg/day when growth was <15 g/kg/day. The authors reported a high mean weight gain of 24.8 g/kg/day, exceeding targeted growth standards. In this study, HM composition was based on a fixed value. According to the variability of OMM and DM composition, overfeeding and protein/energy imbalance could be present and inappropriate to achieve a normal body composition [63].

Two new fortification strategies (adjustable and individualized fortification) were suggested to improve nutritional intakes and growth in preterm infants. Arslanoglu et al. adjusted the fortifier supply according to the values of blood urea nitrogen (BUN), considered as a marker of metabolic response for protein adequacy in preterm infants [64]. This BUN method, which was developed to avoid inadequate and excessive protein intake, is easy to apply and does not require daily milk analysis. However, it has been shown that BUN is not correlated to protein intakes during the first weeks of life but reflects the renal immaturity of preterm infants [65]. Therefore, the use of BUN as a threshold did not allow the provision of adequate nutrition and growth during the early weeks of life. Thus in the study of Arslanoglu, protein intake increased progressively from 2.9 to 3.4 g/kg*d during the three weeks of study (in mean from 2.5 to 5.5 weeks of life) leading to a cumulative protein deficit of around 7 g/kg during the study period.

Individualized fortification analyzes HM composition and provides fortification to achieve target recommended intakes related to postconceptional age. Polberger et al. have proposed analyzing, once or twice a week, the macronutrient content of 24 h OMM collections so as to adapt the fortification in the range of nutritional needs [66]. In 2007, we suggested that daily individualized HM fortification could provide nutritional supplies in the range of the nutritional recommendations and improve growth in VLBW infants. Infrared protein and fat determinations are performed daily for OMM and DM in our NICU milk bank. Fat content is first adjusted to 4 g/dL using a medium chain triglyceride solution, whereas protein intake is adjusted to provide 4.2 g/kg*d according to the daily volume order. This procedure of fortification was routinely introduced for feeding micropremies in our NICU, improving the mean weight gain up to 19–20 g/kg*d [67].

It has also been shown that targeted fortification of HM based on a daily measurement of macronutrient contents reduces the HM nutritional variability, provides nutritional intakes in the range of recent nutritional recommendations, and leads to adequate individual growth [56,68]. Although individualized fortification is time consuming, expensive and requires additional equipment and well-trained staff, the use of infrared technology to determine macronutrient composition of HM is likely to expand its availability in the NICUs and milk bank. Infrared analyzers could have practical applications in HM banks for DM composition to select specific HM pools with higher protein and/or energy content and allowing optimized fortification. Commercial infrared milk analyzers, originally developed for use in the dairy industry, are available but need to be validated before utilization for clinical HM analysis. Indeed there are

some differences in matrix composition between human and cow milk (oligosaccharides, fatty acid profiles, etc.) and these could interfere with the accuracy and precision of the results. Ideally, an independent calibration algorithm resulting from chemical analysis comparison should be generated for each infrared analyzer [69].

The currently available multicomponent fortifiers are not adequately designed for their use in VLBW infants. They are generally designed to obtain an energy content of 80 kcal/dL and a protein content around 3.1–3.5 g/100 kcal to mimic the nutritional recommendations mainly designed for preterm formula [11]. Due to the relative protein and fat deficit of expressed HM provided by HM banks to the NICU, as well as the difference in protein and energy bioavailability of fortified HM compared to preterm formula, VLBW infants fed fortified HM failed to reach an optimal growth and required extra protein and a lipid supplement. In Europe, fat supplementation is generally provided as a medium chain triglyceride emulsion. However, the fatty acid profile of the fortified HM remains inadequate for preterm infants, especially in terms of LC-PUFA content. An HM-derived cream supplement is now available in the USA, providing 2.57 kcal/mL, mainly as HM fat [60]. The use of an exclusive HM fortifier is attractive as suggested by recent studies [21,24,60] but these pasteurized DM-based liquid fortifiers replace a large proportion of OMM, potentially more beneficial for VLBW infants. In addition, exclusive HM fortifier use is very expensive and only available in USA.

Therefore, newer fortifiers providing high protein and energy intakes with balanced fatty acid and LC-PUFA content, need to be developed to improve the nutritional supply with minimal side effects for the preterm infants. From our recent data, we suggested that an intake of 140 kcal/g*d of energy and 4.2 g/kg*d of protein are necessary to ensure adequate growth [56,60]. These values are slightly higher than those recently recommended by the ESPGHAN [11] or expert committee (WRND) [70]. These recommendations are more related to preterm infants fed formula than to those fed fortified HM, and recent studies suggest that specific recommendations for the use of HM are necessary. These new recommendations need to consider the lower metabolizable energy and protein content of the fortified HM, the effect of pasteurization and the additional nutritional losses suggested during continuous feeding [61].

3.2. Safety

A first challenge of DM is to provide a safe feeding regimen to VLBW infants. For this reason, DM milk should be obtained from established HM banks that follow specific guidelines [15–17]. Donors should be screened by lifestyle questionnaire (alcohol, nicotine, drugs, etc.) and tested serologically for human immunodeficiency virus, hepatitis B and C, syphilis and human T-lymphotropic virus in some countries, in a similar way as for blood donation. DM samples should be checked microbiologically before and after processing. As a safeguard against the transmission of virus and pathogens, the DM must be pasteurized. Currently, Holder pasteurization (process at 62.5°C for 30 min) inactivates most of the viral and bacterial contaminants, is highly effective at minimizing the risk of disease transmission via HM and is recommended by the guidelines of most HM banks [36]. However, HM banks in Norway and Japan have a long tradition of using raw milk, preserving all its bioactive properties but requiring a strict control and screening of donors, especially for CMV infection and bacteria [31,71].

3.3. Effects of the pasteurization process

Indeed pasteurization and, to a lesser extent, storage and processing, result in the loss of some biological and nutritional properties of HM. Holder pasteurization destroys the beneficial microbiota, living white blood cells, IgM and lipase activity, decreases the concentration and activity of immunoglobulins IgA, IgG, lactoferrin, lysozyme, some cytokines [interleukin (IL)-10, IL-1β, tumor necrosis factor-α], some growth factors [insulin-like growth factor 1 (IGF1), IGF2, insulin and adiponectin] and vitamins (C and folate) [12,34]. Other nutritional and biological components, such as oligosaccharides, long-chain polyunsaturated fatty acids, lactose, vitamin A, D, E, B2, some cytokines (IL-2, IL-4, IL-5, IL-12, IL-13) and growth factors (epidermal growth factor and transforming growth factor-β1) are preserved. Therefore pasteurized HM, despite partial destruction of immune components, maintains some bactericidal activity, albeit significantly reduced compared with raw milk [35]. This in-vitro finding might suggest that preterm infants fed pasteurized HM may be more susceptible to clinical bacterial infections and other morbidities than those fed raw milk. However, recent studies did not confirm this hypothesis [26,36].

3.4. Costs

Expense is the most widely reported reason for not providing DM [72]. In 2013 in USA, the average cost of providing DM to preterm infants ranged from $27 to $590 for infants who received no OMM [73]. However, provision of DM to preterm vulnerable infants translates to substantial cost-saving in the NICU due to reduction in NEC and other potential long-term morbidities [6,72,74]. It is less clear whether an exclusive HM diet, including HM-derived fortifier rather than bovine-derived, is similarly cost-effective. The balance of short- and long-term costs and savings needs to be estimated through economic evaluation [18]

4. Criteria for donor milk use

Trends of increasing use of donor HM banks in NICU are increasing: 59% of respondents from level 3 and 4 NICUs in the USA are providing DM in the survey by Hagadorn et al. [72]. The criteria used to initiate DM varied but included: insufficient OMM supply or as a temporary substitute for formula feeding in high-risk preterm infants <1500 g (ranging from 1000 to 1800 g) and/or 32 weeks (ranging from 28 to 34 weeks) or severe intrauterine growth restriction, feeding after proven NEC and post gastrointestinal surgery and, sometimes, in cases of congenital heart disease with potential low gut perfusion. DM is generally discontinued after 33–34 weeks when mothers do not intend to continue breastfeeding. Most units using DM had specified guidelines (79%) for use and required signed parental consent (86%) [44,72].

5. Future research and development

Longer clinical impacts of pasteurized DM feeding of preterm infants need to be established. Several ongoing randomized trials in VLBW infants may answer important questions [18,44]. These studies are investigating the cognitive outcomes of very preterm infants fed DM compared to those fed formula (as supplement to OMM or as the sole diet) in the era of current clinical NICU practice, especially fortification. More than 1100 newborns will be included in the three studies combined, allowing secondary investigation of outcomes of other neonatal morbidities (mortality, NEC, LOS, chronic lung disease, retinopathy) and growth associated with DM. Further large controlled, masked and randomized studies are required to determine the NEC rates when HM is supplemented with HM fortifier compared to HM supplemented with bovine-derived fortifier but lacking formula.

Future research should also focus on development of alternative methods to process HM, preserving its nutritional and bioactive properties while inactivating potential pathogens with a high level

of safety. New pasteurization methods, including ultraviolet irradiation, ultrasonication and high-short-time pasteurization are under investigation [34].

6. Conclusion

Preterm infants are a vulnerable population and nutrition is a major element of care which may contribute to improved growth, and short- and long-term outcomes including neurodevelopment. Fortified OMM is the optimal way to feed VLBW infants. However, when OMM is unavailable or in short supply, fortified human DM bank is recommended as an alternative [1,11,12]. DM offers significant health benefits over formula, especially a reduction in NEC and an improvement in feeding tolerance. Growth may be lower with the use of DM because of its lower nutrient content but an adequate, individualized fortification plan can resolve this problem and achieve appropriate growth. Pasteurization of DM is usually recommended to ensure safety from infectious agents. Pasteurization and additional processing result in a loss of some nutrients and immune functions; however, many bioactive components, absent in formula, remain. Future research should focus on innovative solutions to process HM while preserving its nutritional and bioactive properties with a high level of safety.

In addition to DM availability, considered as one of many strategies to achieve better nutritional outcomes, increased efforts are needed to improve the provision of OMM to preterm infants in the NICU and at discharge, and to evaluate the impact of these combined efforts to reduce the rate of health morbidities in fragile preterm infants. HM banks may also play an important role in promotion of lactation.

Practice points

- Despite pasteurization, DM maintains documented advantages compared to formula.
- Nutrient content of DM is generally less than that of preterm OMM. That difference needs to be compensated with fortification.
- Early HM fortification ($\leq$50 mL/kg*d) for both DM and OMM is necessary to reduce protein and energy cumulative deficits and postnatal growth restriction during the early weeks of life in VLBW infants.
- Individualized fortification reduces the HM nutritional variability, provides nutritional intakes in the range of recommendations, and leads to adequate growth.
- Guidelines for the use of DM have been well established by HM bank organizations. By contrast, guidelines for the use of OMM in the NICU are lacking.
- Due to the variability of HM composition, and the differences in nutrient bioavailability between HM and preterm formulas, specific nutritional recommendations for VLBW infants fed OMM and/or DM need to be designed by scientific expert committees.
- Further research is needed to evaluate the clinical impacts of OMM pasteurization as well as the potential advantages of the use of OMM versus DM in VLBW infants. In addition, further studies are needed to determine, in VLBW infants, the effects on morbidities of HM supplementation with donor HM fortifiers versus specific bovine-derived fortifiers with the exclusion of preterm formula use.

Conflict of interest statement

None declared.

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EVALUATION DE LA QUALITÉ BACTÉRIOLOGIQUE DU LAIT MATERNEL DANS UN SERVICE DE NÉONATOLOGIE

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RÉSUMÉ : De nombreuses études démontrent l'intérêt du lait de mère frais pour l'alimentation des prématurés. Ces derniers sont rarement capables de s'alimenter directement au sein de manière satisfaisante. Le lait maternel doit alors être tiré, stocké et transporté jusqu'au centre néonatal favorisant ainsi les risques de contamination. La qualité bactériologique du lait de mère a été évaluée en étudiant les résultats des analyses effectuées sur les échantillons de lait apportés au Service Universitaire de Néonatologie du CHR de la Citadelle à Liège entre le 1er novembre 2003 et le 31 janvier 2005, soit un total de 5842 prélèvements chez 176 mères. Les échantillons ont été classés en «propres» et «contaminés» en fonction de la présence exclusive de Staphylocoques coagulase négative et de leur nombre ($\leq 10^4$ et $>10^4$ colonies par ml) ou en «impropre» en cas de présence de bactéries pathogènes. Plus de 50% des laits analysés ont dû être soit pasteurisés (contaminés : 46%) soit jetés (impropres : 7%). L'incidence des laits «contaminés» augmente au cours des périodes d'été, suggérant une influence climatique. Le suivi longitudinal a permis d'établir les profils maternels. Ainsi, parmi les 60 mères dont un échantillon au moins était « impropres », 73% ont vu plus de 50% des échantillons éliminés. Pour les autres donneuses, la contamination par un germe pathogène survenait uniquement pendant quelques jours au cours de la lactation. Cette étude démontre la nécessité de créer et de financer des unités intrahospitalières de lactarium pour promouvoir, en toute sécurité, l'allaitement maternel des prématurés.

MOTS-CLÉS : *Lait maternel - Lait pasteurisé - Lactarium – Alimentation des enfants prématurés – Contaminations du lait*

EXPRESSED BREAST MILK CONTAMINATION IN A NICU IN Belgium

SUMMARY : Many studies demonstrated that human milk is the recommended source of enteral nutrition in preterm infants providing several benefits with regards to feeding tolerance, immunity and cognitive development However, neurological immaturity and associated clinical conditions prevent them from suckling effectively. Therefore, mother's milk must be expressed, stored and transported to the neonatal unit and could be contaminated. The microbiological quality of human milk was evaluated on each donation to the neonatal intensive care unit of the University of Liege, Belgium from November 1, 2003 to January 31, 2005. In all, 5842 samples from 176 mothers were included in the study. Samples were classified according to the exclusive presence of coagulase negative Staphylococcus and their number (less or more than 104 germs per ml) or to contamination with pathogens. More than 50% of analyzed milks had to be pasteurized (46%;>104 coagulase negative Staphylococcus per ml) or to be discarded (7% pathogen contamination). The incidence of pasteurisation tends to increase during the summer, suggesting a seasonal influence. Maternal profiles were established longitudinally. Among the 60 mothers whose at least one sample had pathogen contamination, 27% had a contamination occurring only during a few days, but 73% had more than 50% of their samples discarded. This study suggest the need to promote the use and the financial support of intrahospital human milk bank units to support the safe use of raw and pasteurised human milk in preterm infants.

KEYWORDS : *Expressed breast milk - Pasteurised human milk - Milk bank premature - Infant feeding - Bacterial contamination*

INTRODUCTION

Tant pour le nouveau-né à terme que pour le prématuré, le lait maternel offre de nombreux avantages sur le plan nutritionnel, anti-infectieux et développementaux et ce jusqu'à l'âge adulte. Le lait maternel n'est pas qu'un simple ensemble de nutriments, il contient des hormones, des facteurs de croissance, des cytokines, des cellules immunocompétentes, etc., qui lui confère de nombreuses propriétés biologiques. Sa composition varie en fonction du terme de la grossesse, de l'âge postnatal de l'enfant et du moment de la tétée (1). L'allaitement maternel, même partiel, contribue significativement au développement des nouveau-nés prématurés (2).

Puisque les mamans vivent séparées de leur nourrisson hospitalisé au centre néonatal et que ceux-ci ne sont pas toujours capables d'aller au sein, le lait doit être tiré. Si l'apport de lait par la mère n'est pas suffisant, on peut recourir au lait d'une donneuse. Ainsi, le don de lait est organisé dans nos maternités auprès des mères de nouveau-nés à terme ayant un excédent de lait. Lors de sa récolte, de sa conservation ou de ses manipulations ultérieures, le lait risque d'être contaminé. Il est dès lors pris en charge par des unités de lactarium intrahospitalier qui vont assurer le contrôle de sa qualité et organiser sa distribution sur prescription médicale (1).

Pour permettre son utilisation frais et ainsi conserver au maximum ses propriétés anti-infectieuses et nutritionnelles, le lait maternel doit être collecté le plus aseptiquement possible. En effet, le lait de femme n'est pas stérile (3). Lors de la tétée, il se contamine au contact des canaux galactophores externes, du mamelon, de l'aréole et de la peau du sein par des cellules cutanées et, donc aussi, par des germes cutanés (4). Les germes habituellement présents dans le lait sont des Staphylocoques coagulase négatives (c'est-à-dire les Staphylocoques épidermidis

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et Staphylococcus saprophyticus, etc.), Streptocoques alpha-hémolytiques, des Micrococques et des bacilles diphtéroïdes, tous faisant partie de la flore cutanée normale et également des Lactobacillus et des Entérocoques (5-8).

Le lait tiré peut être une source d'infections chez le nouveau-né, comme l'indiquent plusieurs cas rapportés dans la littérature (9-14). Ainsi, une épidémie d'infections nosocomiales à Pseudomonas aeruginosa a été rapportée récemment dans un centre de néonatologie. La source de contamination mise en évidence, était un appareil utilisé au lactarium pour la décongélation des échantillons de lait (15).

Le but de notre étude est d'évaluer l'épidémiologie bactériologique du lait de femme apporté au centre néonatal de la Citadelle à Liège pendant la période du 1er novembre 2003 au 31 janvier 2005. Avec ces données, nous avons tenté de déterminer l'influence de certains facteurs comme la saison ou le lieu de récolte (hôpital versus domicile) ainsi que l'évolution de la qualité des laits au cours de la lactation.

MATÉRIEL ET MÉTHODE

Au Centre Hospitalier Régional de la Citadelle à Liège, tous les échantillons de lait maternel amenés au centre néonatal font l'objet d'un ensemencement et d'une mise en culture sur un milieu de Tryptone Soya Agar au sang de mouton (à 37°C) pendant une durée de 18 à 24 h.

Notre étude reprend les résultats des échantillons collectés pendant la période du 1er novembre 2003 au 31 janvier 2005. Seules les mères qui ont donné leur lait sur des périodes égales ou supérieures à sept jours ont été sélectionnées.

Les échantillons ont été classés en trois catégories en fonction de la présence exclusive de Staphylocoques coagulase négative inférieure ou égale à 104 colonies par ml (laits «propres»), supérieure à 104 colonies par ml (laits «contaminés») ou de la présence de bactéries pathogènes (laits «impropres»). Les germes pathogènes à l'origine de la contamination ont été identifiés.

Ces résultats ont également été étudiés en fonction du temps pour évaluer l'influence des saisons et mettre en évidence d'éventuelles tendances épidémiques. Les profils maternels ont été recherchés. Pour ce faire, les mères ont été classées en deux groupes, celles qui ont toujours donné du lait propre à la consommation (avec ou sans pasteurisation) et celles qui ont donné au moins une fois un échantillon de lait contaminé devant être jeté. Dans le premier groupe, les donneuses ont été réparties selon le pourcentage d'échantillons de lait nécessitant une pasteurisation (laits «contaminés»). Dans le deuxième groupe, elles ont été réparties en fonction du pourcentage d'échantillons de lait jetés (laits «impropres»).

Enfin, les échantillons des mères ont été suivis longitudinalement pour observer l'évolution de la qualité des échantillons de lait au cours du don et tenter de mettre en évidence une influence de l'environnement maternel (maternité *versus* domicile).

RÉSULTATS

Pendant la période étudiée, 6.227 échantillons de lait ont été collectés et 5.842 résultats ont été retenus chez les 176 mères qui ont donné leur lait au service néonatal pendant plus de sept jours.

Sur l'ensemble, 7% des échantillons de lait étaient «impropres», contaminés par des germes pathogènes et ont été automatiquement jetés. 46% des échantillons était «contaminés» contenant plus de 104 Staphylocoques coagulase négative par ml de lait, ils ont été pasteurisés, et 47% pouvaient être considérés comme «propres» et administrés directement aux nourrissons sans pasteurisation. Ainsi, pour 93% des échantillons, l'ensemencement ne montrait la croissance que de Staphylocoques coagulase négative.

Les échantillons de laits «impropres» étaient à 72% contaminés par des bacilles à coloration de Gram négative contenant par ordre de fréquence décroissante : Escherichia Coli (15,7%), Acinetobacter iwoffii (9%), Klebsiella pneumoniae (6,7%), Proteus mirabilis (4,4%), Acinetobacter baumannii (3,9%), Morganella morganii (3,5%), Enterobacter cloacae (2,1%), Pseudomonas stutzeri (0,7%). 26% des laits «impropres» étaient contaminés, quant à eux, par des germes à coloration de Gram positive, principalement le Staphylocoque aureus, et le Bacillus spp plus rarement. Par ailleurs, les bacilles à coloration de Gram négative et positive étaient associés dans 2% des échantillons.

Au cours de notre étude, la fréquence des laits «contaminés» a augmenté au cours des mois d'été, suggérant une influence climatique (Fig. 1). Ainsi, d'octobre à mars, 37%, soit 1.268 échantillons sur 3.437, étaient contaminés tandis que 52%, soit 1.363 échantillons sur 2.627, étaient contaminés entre avril et septembre. Cette augmentation n'apparaît pas significative en ce qui concerne les germes pathogènes; les laits «impropres» représentant respectivement 7

et 6% des échantillons au cours de ces même périodes.

Par contre, l'apparition des germes pathogènes semble survenir de manière épidémique comme illustré dans la figure 2. L'Escherichia coli dans les laits était surtout présent en novembre 2003 et l'Entérocoque en juillet 2004. Le dernier pic épidémique était dû au Klebsiella pneumoniae, en janvier 2005. Toutefois, l'analyse plus fine de ces résultats montre que les échantillons formant les « pics épidémiques » sont donnés par une seule mère en ce qui concerne le Klebsiella pneumoniae, par deux donneuses pour le pic du Staphylocoque aureus, de l'Entérocoque ainsi que celui de l'Escherichia coli et par trois donneuses pour l'Acinetobacter iwoffii. En dehors de ce dernier germe, on ne peut pas réellement parler d'épidémie.En ce qui concerne le profil des donneuses, 116 mères, soit 66% de la population, ont toujours apporté des échantillons de lait sans germe pathogène qui ont pu être administrés à leur enfant (avec ou sans traitement thermique). Comme illustré dans la figure 3a, pour la majorité de ces donneuses (65%), moins de 50% des échantillons ont dû être pasteurisés et pour 9% aucun lait n'a été pasteurisé.

A l'opposé, parmi les 60 autres mères représentant 34% de la population qui ont apporté au moins un échantillon de lait impropre à la consommation, on constate que la contamination était relativement importante. En effet, pour la majorité de ces donneuses (73%), plus de 50% des échantillons ont été jetés et seul 5% des donneuses ont eu moins de 20% de laits considérés comme «impropres» (Fig. 3b). Les laits «non jetés» ont pu être donnés aux nourrissons soit directement soit après pasteurisation.

Le suivi longitudinal des échantillons des mères ne permet pas de mettre en évidence une influence systématique de l'environnement maternel (maternité versus domicile) sur la qualité bactériologique des laits apportés : la contamination étant prédominante tantôt au cours des premiers jours tantôt en fin d'allaitement. Par contre, on peut observer chez certaines mères, l'émergence de contaminations occasionnelles par un germe pathogène pendant quelques jours au cours de l'allaitement (Fig. 4).

DISCUSSION

Cette étude met en évidence que la contamination du lait maternel, apporté au Centre Néonatal, par des germes pathogènes est relativement rare puisqu'elle ne concerne que 7% des échantillons recueillis durant les quinze mois de ce travail. Il existe peu d'études publiées récentes qui quantifient la présence de bactéries dans le lait maternel. Notre étude suggère que la contamination par des germes pathogènes se concentre sur une fraction modérée de la population (34%) mais que, pour les trois-quarts de celle-ci, cette contamination par des germes pathogènes est importante puisqu'elle représente plus de 50% de leurs échantillons. Le risque de contamination du lait par un germe pathogène reste permanent tout au long de la période d'allaitement. Ainsi, on peut observer brutalement l'émergence de germes pathogènes pendant quelques jours chez une mère dite «propre», sans facteur prédisposant mis en évidence. Tout au long de la période étudiée, nous n'avons pas observé des contaminations du lait maternel de type épidémique.

A l'heure actuelle, en Belgique, il n'existe aucune réglementation quant à l'utilisation du lait maternel dans les services de néonatologie et quant à l'organisation des lactariums. Dans notre service, nous avons choisi d'éliminer systématiquement les échantillons de lait contenant des germes pathogènes sans recourir à la pasteurisation. Ainsi, nous avons jeté les laits maternels contenant des Staphylococcus aureus ou des bacilles à coloration de Gram négative. Cette façon de procéder évite de donner aux nouveau-nés des laits pouvant contenir des entérotoxines staphylococciques thermorésistantes ou des endotoxines produites par les bacilles à coloration de Gram négative (16).

Les doses infectantes par voie orale chez le nourrisson ne sont pas connues, mais il est très peu probable qu'un enfant nourri directement au sein ingère un nombre suffisant de germes pathogènes pour développer une infection. Toutefois, des cas de septicémies néonatales tardives à Streptocoque hémolytique du groupe B associée à une alimentation au sein ont été rapportées (12, 13, 17).

Par contre, un défaut d'hygiène personnel, des manipulations non optimales et/ou une conservation inadéquate du lait peuvent permettre une contamination externe et/ou une multiplication des bactéries potentiellement pathogènes et ainsi faciliter le développement d'une infection systémique (3, 9, 18). En effet, il a été montré qu'à la suite d'une ingestion de lait contenant des bactéries à coloration de Gram négative, la moitié des enfants exposés se colonisait au niveau du tractus gastro-intestinal (source potentielle d'infection invasive) (6). Une revue de la littérature montre que la contamination par des germes pathogènes du lait maternel apporté dans les centres de néonatologie est courante et qu'elle peut entraîner le développement de septicémies ou d'entérocolites ulcéro-nécrosantes parfois

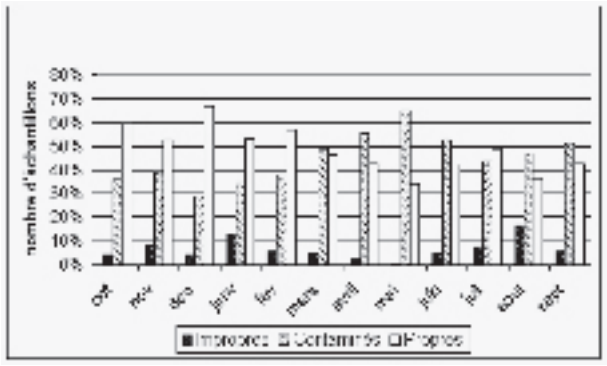

Figure 1 : Répartition mensuelle des différentes catégories de laits

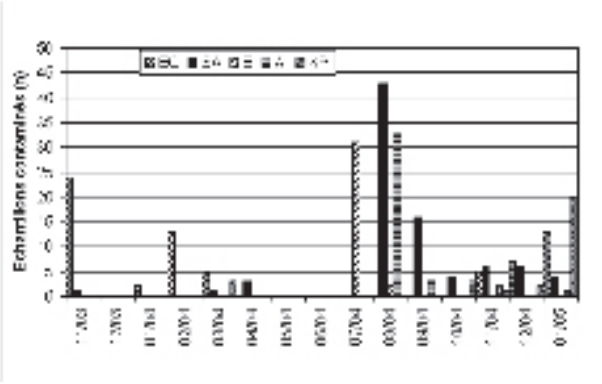

Figure 2 : Distribution des contaminations par des germes pathogènes en fonction du temps. SA : Staphylococcus aureus; EC : Escherichia coli; AI : Acinetobacter lwoffii; KP : Klebsiella pneumoniae; E : Entérocoque

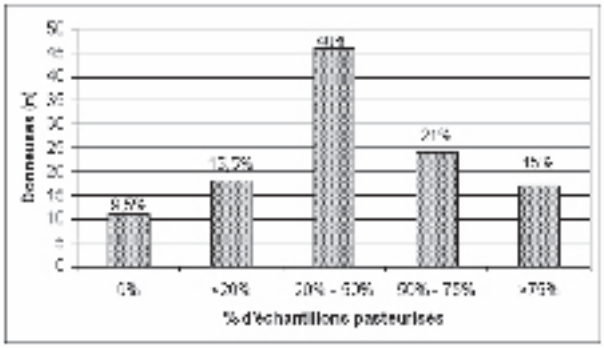

Figure 3a: Répartition des mères dont aucun lait n'a été jeté en fonction du pourcentage d'échantillons pasteurisés.

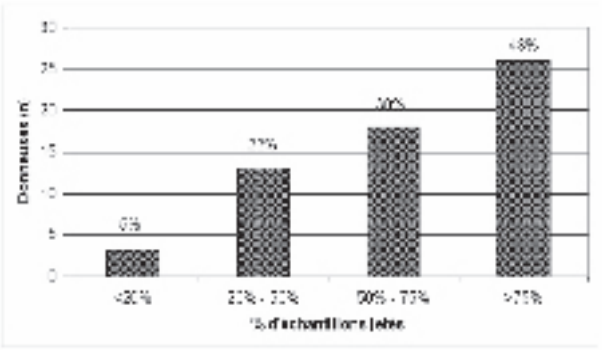

Figure 3b : Répartition des 60 mères dont au moins un lait a été jeté en fonction du pourcentage d'échantillons jetés.

fatales (11). L'incidence de ces complications est très probablement sous-estimée du fait de la difficulté d'établir un lien causal entre l'infection et la contamination du lait dans des services qui favorisent l'utilisation du lait de mère cru et où l'analyse bactériologique du lait maternel n'est pas systématique.

En dehors des germes pathogènes évoqués plus avant, le lait tiré contient des germes commensaux cutanés comme les Staphylocoques coagulase négative ou le Streptocoque du groupe A (8). Cette contamination est directement influencée par les conditions climatiques. En effet, dans notre étude, les laits «contaminés» prédominent au cours des mois d'été. En conséquence, les précautions d'hygiène et le respect de la chaîne du froid doivent être renforcés pendant les périodes chaudes. En effet, il a été bien démontré qu'une bonne conservation des échantillons au réfrigérateur à 4° pendant 24 heures n'altérait pas la qualité bactériologique du lait de mère (8).

Notre position au centre néonatal du CHR de Liège était avant cette étude de pasteuriser tous les échantillons de lait contenant plus de 104 Staphylocoques coagulase négative et de donner frais les échantillons ayant une quantité inférieure ou égale à 104 germes et recueillis dans les 48 heures. A la suite de cette étude, considérant que les prématurés de très faible poids à la naissance et les enfants hospitalisés au centre néonatal présentant une immaturité immunologique sont particulièrement à risque de développer des infections, nous pasteurisons systématiquement tous les échantillons destinés aux enfants < 1,250 g. Cette attitude est loin de faire l'unanimité parmi les différentes unités de néonatologie. Ainsi, certaines unités néonatales donnent le lait frais de la propre mère recueilli dans les 72 heures et sans contrôle bactériologique. Doit-on, dès lors, considérer que les Staphylocoques coagulase négative n'ont pas de potentialité infectante chez le prématuré? Cette question reste débattue. Dans les services de néonatologie, la majorité des infections nosocomiales sont dues aux Staphylocoques coagulase négative mais la constatation d'une contamination du lait maternel par le même germe ne permet pas d'établir avec certitude un lien de causalité. En effet, il

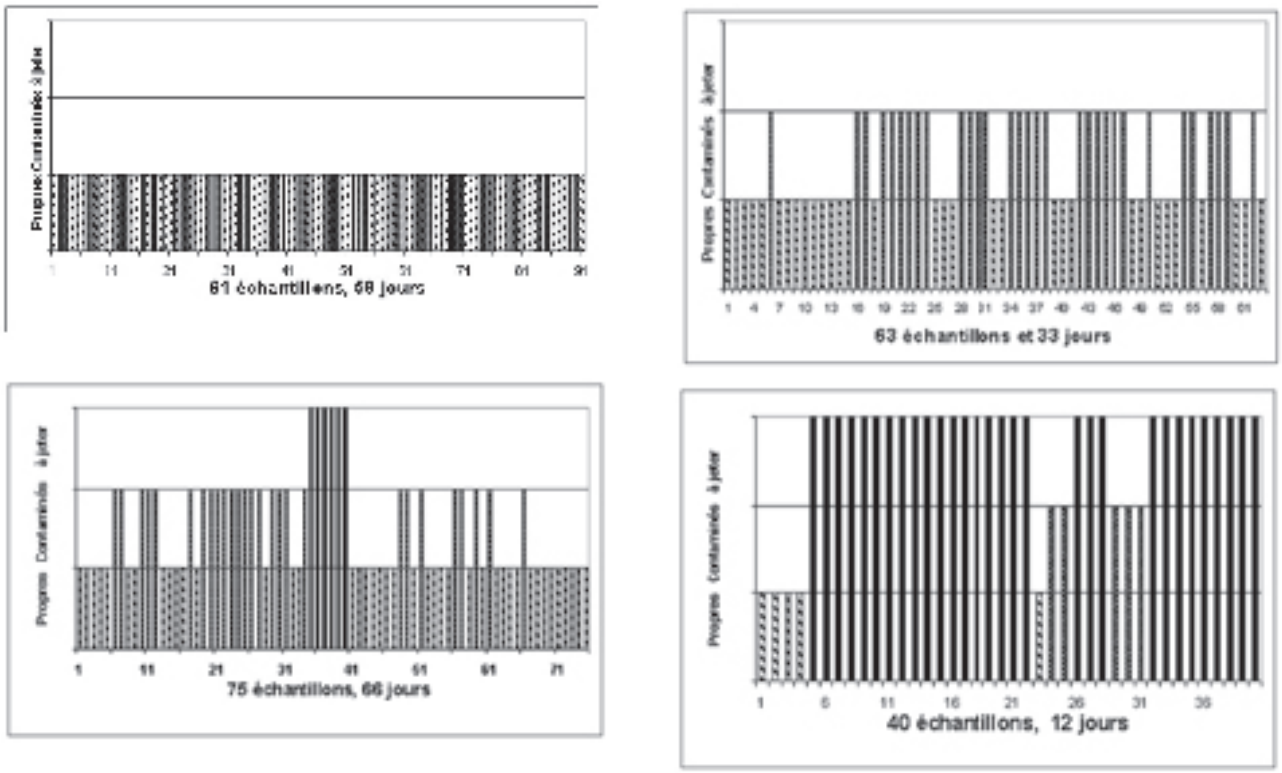

Figure 4: Exemple de suivi longitudinal de quatres donneuses

est difficile de déterminer chez un nouveau-né prématuré si une septicémie à Staphylocoques trouve son origine dans le lait maternel, car les sources de contaminations sont multiples (cathéters veineux, artériel, intubation,…). Toutefois, ce lien a été suspecté à plusieurs reprises (11, 19). Il a également été démontré que la colonisation du tube digestif par le Staphylocoque coagulase négative, était plus fréquente chez les prématurés alimentés avec du lait de mère frais que chez ceux recevant du lait pour prématuré (20) et, aussi, que le lait maternel pouvait être le réservoir de Staphylocoques multirésistants et porteurs de facteurs de virulence importants (21).

Par contre, des études récentes (22, 23) suggèrent que dans une population d'enfants de très petit poids à la naissance, l'instauration d'une alimentation entérale complète précoce par du lait de mère permet de réduire significativement l'incidence des infections nosocomiales, de l'entérocolite nécrosante et de la mortalité néonatale. Toutefois, dans ces études, le lait de mère frais n'était administré qu'après un contrôle bactériologique et, lorsque la contamination bactérienne était inférieure à 104 colonies par ml. On est en droit, dès lors, de se demander si la discordance avec les études précédentes n'est pas qu'apparente et relative à une différence de degré de contamination.

Dans notre étude, le degré de contamination des laits par le Staphylocoque coagulase négative est relativement important puisqu'en dehors des laits contaminés par des germes pathogènes (7%), près de 50% des échantillons restants contenaient plus de 104 colonies par ml. Cette valeur est largement supérieure à celle rapportée récemment par Lindemann et al. (24) dans une étude évaluant la qualité du lait de 69 mères donneuses en Norvège. Sur cette population, 8% des échantillons testés contenaient des germes pathogènes et seulement 2,5% du total ont dû être pasteurisés parce qu'ils contenaient des Staphylocoques coagulase négative à un taux supérieur à 104 colonies par ml. Cette étude réalisée sur une population de mères dont le niveau d'éducation était élevé et dans un pays à haut niveau d'hygiène suggère qu'une réduction du taux de contamination pourrait être obtenue dans notre population en sensibilisant les mères aux mesures d'hygiène ainsi qu'à la nécessité de respecter rigoureusement la chaîne du froid entre le prélèvement et l'arrivée du lait au centre néonatal.

Les bactéries ne sont pas les seuls microorganismes qui peuvent contaminer le lait maternel, des virus sont également présents. La contamination virale est d'origine maternelle; il est donc recommandé de connaître le statut sérologique de la mère, en particulier, pour le cytomégalovi-

rus, l'hépatite B, l'hépatite C, le VIH et le HTLV (5, 8). Seuls, le VIH et le HTLV constituent une contre-indication à l'allaitement au sein. La congélation du lait pendant au moins 72 h réduirait significativement le titre du cytomégalovirus dans le lait sans en supprimer complètement le risque de transmission (25). Par contre, la pasteurisation du lait permettrait d'éliminer la présence du cytomégalovirus (1) ainsi que d'autres virus (hépatites B, hépatites C, VIH, HTLV).

L'objectif des lactariums est de promouvoir l'allaitement maternel chez les prématurés en tentant de favoriser l'utilisation du lait cru de la propre mère et, éventuellement, de donneuses en raison de ses propriétés nutritionnelles et immunologiques supérieures. Mais également, parce que les nombreuses bactéries commensales et les bactéries lactiques qui font partie intégrante de la composition du lait, lui confèrent une activité probiotique ainsi que des propriétés anti-infectieuses en particulier contre le Staphylocoque aureus (7, 26).

Toutefois, dans notre service la distribution de lait cru ne représente que 47 % du lait récolté, car nous avons opté pour une pasteurisation systématique de tous les laits des mères donneuses et de tous les laits de la propre mère lorsqu'ils étaient destinés à des prématurés de moins de 1.250g ou lorsqu'ils contenaient plus de 104 germes Staphylocoques coagulase négative par ml.

Depuis la fermeture du dernier lactarium, celui de Liège, en décembre 2001, les services de néonatologie ont dû s'organiser indépendamment tant pour favoriser la collecte et la distribution du lait de la propre mère, que pour développer une unité restreinte de lactarium permettant la collecte, le dépistage, le traitement et la distribution du lait de la propre mère, mais également celui des mères donneuses. Devant l'absence de réglementation européenne ou nationale, voire même de *consensus* entre les néonatologues, chaque service a édité ses propres règles de fonctionnement en tenant plus ou moins compte de réglementation ou "guidelines" éditées ou publiées dans d'autre pays.

Les lactariums ont un rôle important pour le contrôle de la qualité des échantillons de lait, mais aussi dans l'éducation des mères à propos des mesures d'hygiène qui entourent le prélèvement du lait et, plus particulièrement, le tirage du lait, de l'entretien du matériel nécessaire (tire lait, biberons…) ainsi que sur les règles à respecter lors de la manipulation des échantillons et leur transport jusqu'au centre néonatal. Il est tout aussi important que les lactariums eux-mêmes rédigent les procédures et contrôlent l'application de ces formations. C'est à eux également que revient la tâche de promouvoir le don de lait en informant les jeunes mères en maternité du rôle du lait maternel dans l'alimentation du prématuré.

CONCLUSION

Notre étude nous a permis de montrer que la contamination des laits de mère apportés au service de néonatologie par le Staphylocoque coagulase négative à un taux supérieur à 104 germes par ml était importante dans notre population (46% des échantillons), vraisemblablement en raison des conditions socio- économiques et d'hygiène. Elle a montré également que la contamination par des germes pathogènes n'était pas si rare (7% des échantillons), ce qui pourrait favoriser le développement d'infection nosocomiale chez le prématuré comme en témoignent les liens entre contamination du lait et infection sévère démontrés dans de nombreuses publications.

Ainsi, ces résultats indiquent l'importance du contrôle bactériologique du lait maternel et de la pasteurisation pour permettre en toute sécurité l'alimentation des prématurés au lait de femme. Ceci nécessite le financement et l'équipement d'unités intrahospitalières de lactarium. Pour favoriser l'utilisation du lait de mère frais, il convient de promouvoir l'éducation et de mettre sur pied des programmes de suivi personnalisé des mères des enfants hospitalisés dans un service de néonatologie.

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OPEN

Growth and Nutritional Biomarkers of Preterm Infants Fed a New Powdered Human Milk Fortifier: A Randomized Trial

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ABSTRACT

Objectives: The aim of this study was to assess growth and nutritional biomarkers of preterm infants fed human milk (HM) supplemented with a new powdered HM fortifier (nHMF) or a control HM fortifier (cHMF). The nHMF provides similar energy content, 16% more protein (partially hydrolyzed whey), and higher micronutrient levels than the cHMF, along with medium-chain triglycerides and docosahexaenoic acid.

Methods: In this controlled, multicenter, double-blind study, a sample of preterm infants ≤32 weeks or ≤1500 g were randomized to receive nHMF (n = 77) or cHMF (n = 76) for a minimum of 21 days. Weight gain was evaluated for noninferiority (margin = −1 g/day) and superiority (margin = 0 g/day). Nutritional status and gut inflammation were assessed by blood, urine, and fecal biochemistries. Adverse events were monitored.

Results: Adjusted mean weight gain (analysis of covariance) was 2.3 g/day greater in nHMF versus cHMF; the lower limit of the 95% CI (0.4 g/day) exceeded both noninferiority ($P < 0.001$) and superiority margins ($P = 0.01$). Weight gain rate (unadjusted) was 18.3 (nHMF) and 16.8 $g \cdot kg^{-1} \cdot day^{-1}$ (cHMF) between study days 1 and 21 (D1–D21). Length and head circumference (HC) gains between D1 and D21 were not different. Adjusted weight-for-age *z* score at D21 and HC-for-age *z* score at week 40 corrected age were greater in nHMF versus cHMF ($P = 0.013$, $P = 0.003$ respectively). nHMF had higher serum blood urea nitrogen, pre-albumin, alkaline phosphatase, and calcium (all within normal ranges; all $P \leq 0.019$) at D21 versus cHMF. Both HMFs were well tolerated with similar incidence of gastrointestinal adverse events.

Conclusions: nHMF providing more protein and fat compared to a control fortifier is safe, well-tolerated, and improves the weight gain of preterm infants.

Key Words: growth, human milk, low birth weight

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What Is Known

- Due in part to variability in human milk composition, incidence of postnatal growth restriction is more frequently reported in very-low-birth-weight infants fed fortified human milk compared to those fed preterm formulas.
- The optimal composition of human milk fortifier and nutritional recommendations for preterm infants fed fortified human milk are still debated.

What Is New

- A new human milk fortifier containing partially hydrolyzed protein, fat, and carbohydrate provides a higher protein:energy ratio while achieving lower osmolality versus a current fortifier.
- In preterm infants, the new fortifier improves weight gain and reduces postnatal growth restriction compared to the current fortifier.

Feeding of human milk (HM) rather than preterm formulas provides many benefits to preterm infants (eg, accelerated gut maturation (1); protection against infections (2), sepsis (3), necrotizing enterocolitis (2), and retinopathy of prematurity (4); possible

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protective effect on neurodevelopment (5)) that are mediated by protective biomolecules and trophic factors in HM. HM, however, provides inadequate protein and micronutrients to support the rapid growth and bone mineralization of preterm infants. These deficits are particularly acute in the smallest infants (birthweight <1500 g) who have the highest protein and mineral needs (6). Fortification of mother's own milk or banked HM is therefore recommended for all preterm infants with birthweight <1800 g to improve nutrient accretion and in-hospital growth (7,8).

Feeding fortified HM helps support adequate growth and bone mineralization (9), and is associated with favorable neurodevelopmental outcomes (10), although evidence for improved outcomes other than in-hospital growth is limited (11). The nutritional content, however, of some currently available fortifiers may be inadequate for many preterm infants. Incidence of postnatal growth restriction is more frequently reported in very-low-birth-weight infants fed fortified HM compared to those fed preterm formulas (12,13). In addition, the nutritional profile of HM from mothers of premature infants varies greatly (14) and may differ from published reference compositional data, which may lead to less-than-recommended intakes of protein and energy (15,16). These nutritional inadequacies may worsen with use of donor HM, which is often from mothers of term infants >1-month postpartum (17).

A new powdered HM fortifier has been developed with a higher protein:energy ratio (protein provided as partially hydrolyzed whey), non-protein energy from lipids and carbohydrate, and higher electrolyte and vitamin levels (enriching HM in line with ESPGHAN (18) and expert group (19) recommendations) versus a control fortifier. When mixed with HM containing 1.5 g protein/100 mL (2–4 week milk) (20–22), it provides 3.6 g protein/100 kcal (within the ESPGHAN-recommended ranges (18) for protein and energy intakes for a minimal intake volume of 140 mL/kg/day in very-low-birth-weight infants up to 1.8 kg body weight), with osmolality below the recommended threshold of 450 mOsm/kg (23,24).

This study evaluated growth and nutritional biomarkers during a 21-day interval in clinically stable preterm infants receiving the new HM fortifier (nHMF) compared to infants fed a control fortifier (cHMF). The primary objective was to assess weight gain velocity (grams per day); evaluations of other growth parameters (including weight gain velocity in gram per kilograms per day) and intervals (eg, to 40 weeks corrected age [W40CA]), feeding tolerance, adverse events, time to full fortification/full enteral feeding, and markers of protein-energy, electrolytes, bone metabolic status, gut inflammation, and maturity of gastrointestinal (GI) function were also conducted as secondary outcomes. We hypothesized that weight gain of infants fed nHMF would be both noninferior (lower limit of 95% confidence interval [CI] of mean difference >-1 g/day) and superior (lower limit of 95% CI of mean difference >0 g/day) to that of infants fed cHMF.

METHODS

Study design and participants

This was a controlled, double-blind, randomized, parallel-group study conducted in neonatal intensive care units (NICUs) at 11 metropolitan hospitals in France, Belgium, Germany, Switzerland, and Italy. NICU size ranged from 25 to 45 beds. Clinically stable male and female preterm infants with gestational age ≤32 weeks or birthweight ≤1500 g and born to mothers who had agreed to provide expressed or donor breastmilk for the entire 21-day study duration were enrolled in the study from April 2011 to March 2014. Infants were excluded if they had a history of or current systemic, metabolic, or chromosomic disease, any congenital anomalies of the GI tract, were small for gestational age (defined in this study as bodyweight ≤5th percentile (25)), or were receiving steroids or preterm formula during the study period. For multiple births, the first sibling was randomized and other siblings were allocated to the same group. The study was reviewed and approved by an institutional review board/independent Ethics Committee at each study site. Each subject's parent/legal representative provided written informed consent before participating in the study.

Infants tolerating $\geq 100\,\text{mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ of HM for >24 hours were randomized to receive either nHMF or cHMF for a minimum of 21 days; infants continued to receive their allocated study fortifier (or were transitioned to a routine/standard fortifier) until NICU discharge or medical decision to stop fortification, and fortification was stopped after discharge. The fortifiers were both cow's milk-based and provided similar energy supplementation (17 kcal/100 mL of HM). For every 100 mL of HM, nHMF provided 1.4 g partially hydrolyzed whey protein, 0.7 g lipids (primarily medium chain triglycerides and docosahexaenoic acid), 1.3 g carbohydrate (maltodextrin), with a blend of micronutrients. cHMF (FM85 Human Milk Fortifier, Nestlé, Switzerland) provided 1.0 g extensively hydrolyzed whey protein, no lipids, 3.3 g carbohydrate (lactose and maltodextrin), with a blend of micronutrients. nHMF contained higher concentrations of some vitamins and electrolytes compared to cHMF, but both contained similar levels of minerals, including calcium (as calcium glycerophosphate and calcium phosphate) and phosphorus. Table 1 presents the estimated composition and osmolality of preterm HM (22) fortified with each fortifier. Fortifiers were fed beginning at half-strength (Fortification Strength Increase day 1; FSI1), then advanced per hospital practice, with full-strength fortification occurring once infants could maintain intakes of 150 to $180\,\text{mL}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (ie, full enteral feeds; study day 1 [D1]). A study plan schematic is presented in Figure 1.

Study Procedures

Growth

Infant nude weight (to the nearest 1 g) was measured daily by trained nursery personnel using a calibrated electronic scale (Baby Scale 717, Seca, Semur-en-Auxois, France). Recumbent length and head circumference (HC; both to the nearest 0.1 cm) were measured at FSI1, D1, and weekly thereafter. At least 2 trained examiners measured recumbent length using a length board (Mobile Measuring Board 417, Seca, Semur-en-Auxois, France) while maintaining proper body alignment and full body extension with feet flexed. HC was measured using a nonelastic measuring tape (Measuring Tape 212 or 218, Seca, Semur-en-Auxois, France) placed over the largest circumference of the skull (above the supraorbital ridges while covering the most prominent part of the frontal bulge anteriorly). The same calibrated equipment was used for anthropometric measures for each infant at all sites. Weight-for-age, length-for-age, and HC-for-age z scores were calculated using Fenton (25). Weight gain velocity (grams per kilograms per day) was calculated using the average of the start and end weights as the denominator.

Markers of Protein-energy, Electrolyte, and Bone Metabolic Status

Blood and urine samples were collected at D1, D10/11, and D21 and analyzed for serum creatinine and prealbumin, blood urea nitrogen (BUN), urinary urea, hemoglobin, hematocrit, electrolyte status, and bone metabolic status. All blood and urine parameters were analyzed as part of routine clinical assessments at each NICU. Since 24-hour urine collections were not performed in this study owing to logistical infeasibility, urinary markers were corrected for 24-hour creatinine excretion (26) assuming a standard urinary excretion in preterm infants of $10\,\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (27).

TABLE 1. Calculated* nutrient composition of fortified preterm human milk

	Preterm HM + nHMF			Preterm HM + cHMF			
	4 g fortifier alone	4 g fortifier per 100 kcal milk	4 g fortifier per 100 mL milk	5 g fortifier alone	5 g fortifier per 100 kcal milk	5 g fortifier per 100 mL milk	Recommended intake range (per 100 kcal)†
Nutrient							
Energy, kcal	17.4	100	84.6	17.4	100	84.5	
Protein, g	1.42	3.6	3.04	1.0	3.10	2.62	3.2–4.1
Protein source		Partially hydrolyzed whey			Extensively hydrolyzed whey		
Fat, g	0.72	5.00	4.23	0.02	4.16	3.52	4.4–6
MCT, g	0.50	0.59	0.50	0	0	0	
DHA, mg	6.3	19.3	16.3	0	11.8	10.0	(16.4–) 50–55
Carbohydrate, g	1.30	10.17	8.60	3.30	12.53	10.60	10.5–12
Carbohydrate source		Maltodextrin			Lactose and maltodextrin		
Calcium, mg	76	119	101	75	118	100	109–182
Phosphorus, mg	44	69	58	45	70	59	55–127
Magnesium, mg	4.0	8.6	7.3	2.4	6.7	5.7	7.3–13.6
Sodium, mg	36.7	76.5	64.7	20.0	56.8	48.0	63–105
Potassium, mg	48.4	116.4	98.4	42.0	108.8	92.0	71–177
Chloride, mg	32.1	106.6	90.1	17.0	88.7	75.0	95–161
Iron, mg	1.80	2.23	1.89	1.30	1.64	1.39	1.8–2.7
Zinc, mg	0.94	1.55	1.31	0.80	1.38	1.17	1.3–2.3
Manganese, μg	8.08	9.98	8.44	5.00	6.34	5.36	0.9–13.6
Copper, mg	0.05	0.11	0.09	0.04	0.09	0.08	0.09–0.21
Iodine, μg	16.9	36.6	30.9	15.0	34.3	29.0	9–50
Selenium, μg	3.7	7.2	6.1	1.5	4.6	3.9	4.5–9
Vitamin A, IU	1183	1754	1483	500	946	800	1217–3333
Vitamin D, IU	150	187	158	100	128	108	100–350
Vitamin E, IU	4.4	5.6	4.7	2.2	3.0	2.5	2.2–11.1
Vitamin K, μg	8.0	9.8	8.3	4.0	5.1	4.3	4–25
Thiamin, mg	0.15	0.19	0.16	0.05	0.07	0.06	0.13–0.27
Riboflavin, mg	0.20	0.27	0.23	0.10	0.15	0.13	0.18–0.36
Vitamin B_6, mg	0.13	0.16	0.14	0.05	0.07	0.06	0.05–0.27
Vitamin B_{12}, μg	0.20	0.26	0.22	0.10	0.14	0.12	0.09–0.73
Niacin, mg	1.50	2.02	1.71	0.80	1.19	1.01	0.9–5
Folic acid, μg	40.0	51.0	43.1	40.0	51.0	43.1	32–91
Pantothenic acid, mg	0.70	1.10	0.93	0.40	0.74	0.63	0.45–1.9
Biotin, μg	3.50	4.78	4.04	3.00	4.19	3.54	1.5–15
Vitamin C, mg	20.0	28.9	24.4	10.0	17.0	14.4	18–50
Osmolality‡, mOsm/kg			390			441	

cHMF = control human milk fortifier; DHA = docosahexaenoic acid; HM = human milk; nHMF = new human milk fortifier; MCT = medium chain triglycerides.

*Calculated based on preterm human milk composition from Tsang et al, 2005 (22).

†Recommended nutrient intakes for fully enterally fed preterm very low birth weight infants (19).

‡Measured immediately after fortification at room temperature (25°C).

Feeding Tolerance and Adverse Events

Feeding tolerance was evaluated by trained nursery staff who recorded daily milk intake (milliliters), stool pattern (defecation frequency and stool consistency [5 = hard, 4 = formed, 3 = soft, 2 = liquid, or 1 = watery]), presence of abdominal distention, and incidence of spitting-up (defined as return of a small amount of swallowed food, usually a mouthful, and usually occurring during or shortly after feeding) and vomiting (defined as return of a larger amount of food with more complete emptying of the stomach, and usually occurring sometime after feeding). In addition, frequency, type, and attribution to fortifier intake of adverse events (AEs; including clinical and laboratory) were evaluated using physician-reported information recorded using standardized forms from enrollment to W40CA. AEs were categorized by the reporting investigator as ''serious'' in accordance with International Conference on Harmonization criteria (28) and as ''related to the intervention'' based on detailed, standardized criteria provided in the protocol.

Statistical Analysis

Sample size was based on a previous study (29), which investigated growth and zinc status in preterm infants fed fortified HM. In the present trial, a group-sequential design was chosen (Wang and Tsiatis) (30) with 1 interim analysis. To detect a noninferior weight gain in infants fed with nHMF versus cHMF from D1 to D21 (noninferiority margin −1 g/day, expected weight gain difference 2 g/day, standard deviation 4.73 g/day, type I error 5%, power 80%) (29), 192 subjects (males and females combined)

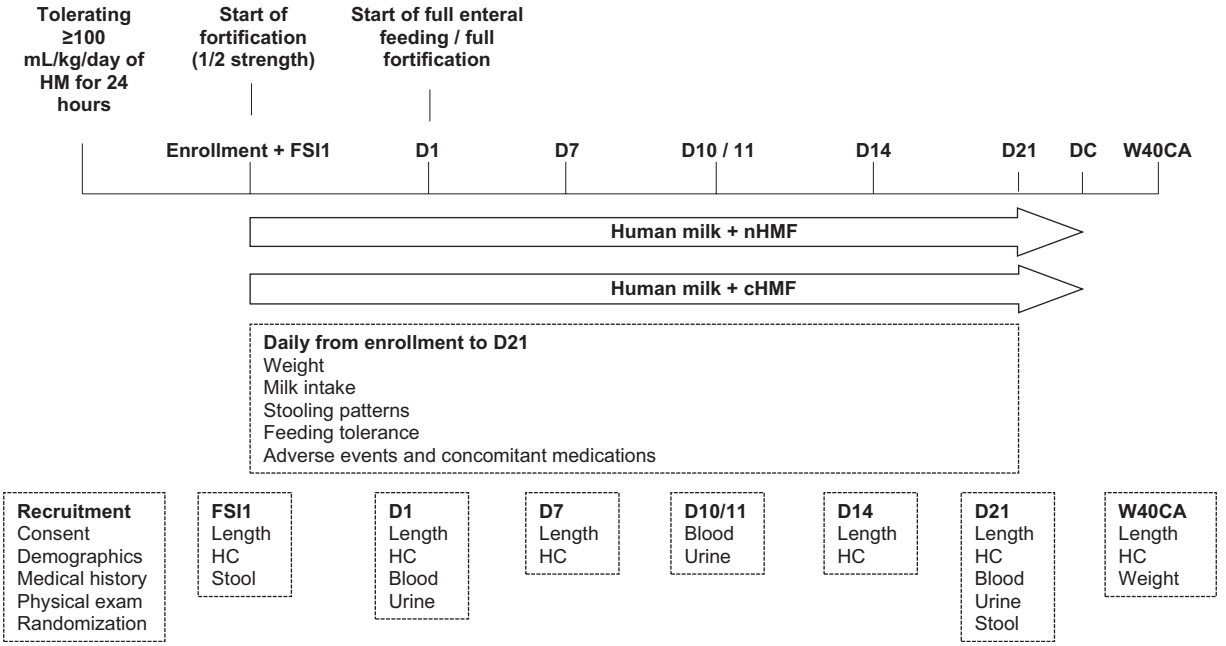

FIGURE 1. Study design. cHMF = control human milk fortifier; D1 = study day 1; D7 = study day 7; D10/11 = study day 10/11; D14 = study day 14; D21 = study day 21; DC = discharge (note that infants continued to receive their allocated study fortifier [or were transitioned to a routine/standard fortifier] until neonatal unit discharge or medical decision to stop fortification if length of stay was >21 days, and fortification was stopped after discharge) ; FSI1 = fortification strength increase day 1; HC = head circumference; HM = human milk; nHMF = new human milk fortifier; W40CA = week 40 corrected age.

were needed. A computer-generated list of random numbers was used to allocate group assignments. Minimization algorithm with allocation ratio 1:1 and second best probability of 15% was used. Stratification factors were center, sex, and birthweight (100g intervals). Group coding was used with 2 nonspeaking codes per group; fortifier packaging was coded accordingly but otherwise identical in appearance. Infants were enrolled and assigned to their intervention by the study investigators or trained delegates. All study personnel (both site- and sponsor-based) and participants (infants' families) were blind to group assignment. Noninferiority was demonstrated if the lower limit of the 2-sided 95% CI of the difference in weight gain from D1 to D21 was larger than the noninferiority margin. Superiority was evaluated if noninferiority was demonstrated. Weight gain was analyzed in the intent-to-treat (ITT) and per-protocol populations by analysis of covariance (ANCOVA) adjusting for D1 postmenstrual age and weight, sex, and center (random effect). Sensitivity analyses were conducted using ANCOVA models that adjusted for covariates that were determined post hoc to be significantly different between groups and which may have confounded the primary results (eg, mother smoking status). Secondary endpoints were analyzed in the ITT population only. For noninferiority and superiority tests, 1-sided *P* values are provided and should be compared to a reference value of 0.025. For other tests, 2-sided *P* values are provided and should be compared to a reference value of 0.05. 95% CIs provide estimates for feeding effects on all endpoints. Based on prespecified guidelines in the independent Data Monitoring Committee's (DMC) charter, a single interim analysis was conducted when 134 subjects had completed their D21 visit. The interim analysis was planned to occur when the first 100 infants completed at least 21 days of full fortification; however, the analysis was conducted using data from 134 infants owing to unforeseen delays in conducting the analysis (eg, performing statistical programming, data cleaning, and query resolution) while recruitment continued. The type 1 error rate was adjusted to account for the analysis being conducted at ~70% enrollment rather than the planned 52%. The DMC consisted of independent experts (2 clinicians, 1 biostatistician) who reviewed growth, formula intake, and key biochemical data as well as AEs. The purpose of the interim analysis was to examine unblinded growth velocity results and determine whether the trial could be stopped early for success or futility, or whether the targeted sample size required adjustment (the interim statistical analysis plan was finalized before unblinding, and the analysis was unblinded only to the DMC to facilitate ethical decision-making) (31). On April 2, 2014, the DMC recommended to stop the trial, as noninferiority and superiority in regard to the primary outcome had been demonstrated. The sponsor was notified of this decision on April 3, 2014, and the final study population included infants enrolled through March 31, 2014.

RESULTS

A total of 274 infants were screened, with 153 enrolled and randomized to either nHMF (n = 77) or cHMF (n = 76) (Fig. 2). Demographic and baseline anthropometry data are summarized in Table 2. There was no evidence of imbalance between the 2 groups with respect to infant characteristics. A significantly lower percentage of mothers and fathers of infants in the nHMF group, however, smoked during pregnancy. Number of twins was similar in each group.

The majority (84% and 87% by volume in nHMF and cHMF, respectively) of milk provided to infants was pasteurized. Donor milk was always pasteurized and accounted for 49% and 51% of the fortified HM volume provided in the nHMF and cHMF groups, respectively. There was no significant difference in mean volume of fortified milk intake between groups (152.7 ± 13.0 and $152.6 \pm 17.2\,\mathrm{mL \cdot kg^{-1} \cdot day^{-1}}$ in nHMF and cHMF, respectively).

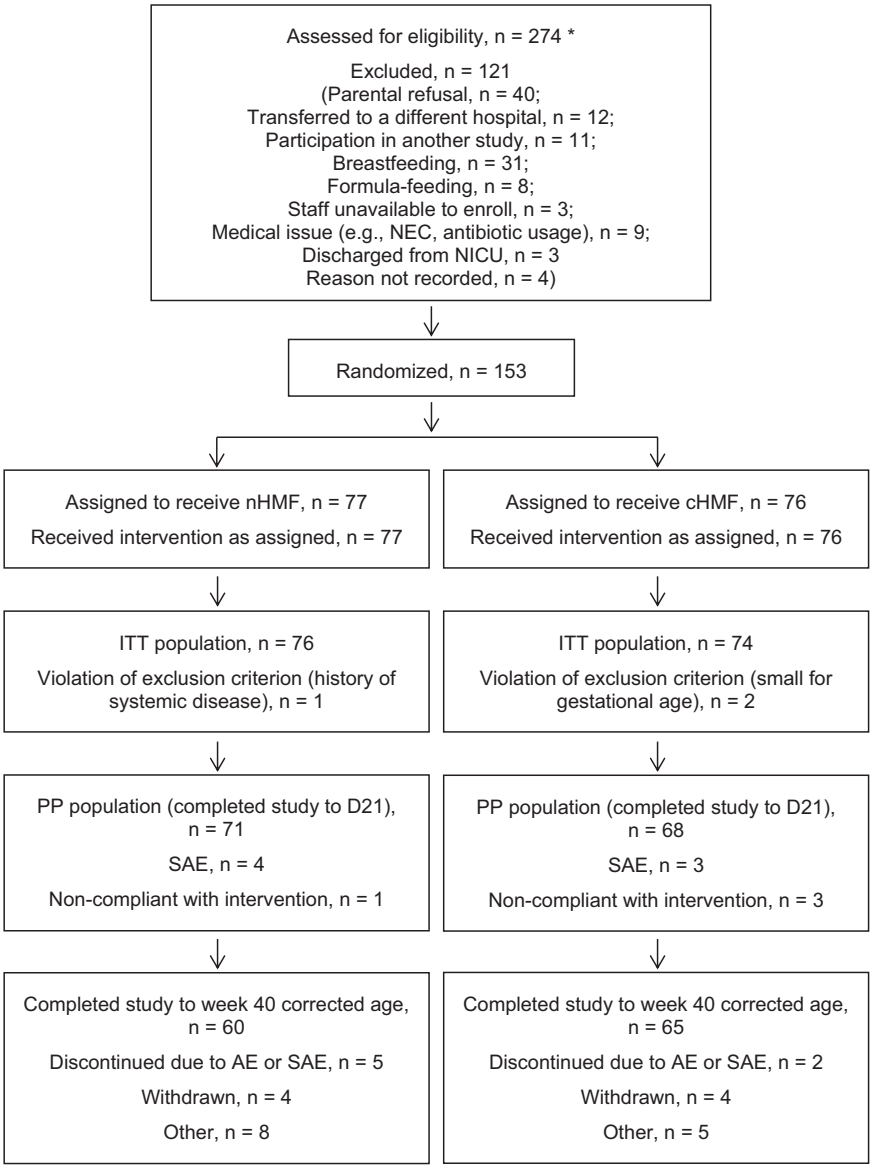

FIGURE 2. Flow of study participants. AE = adverse event; cHMF = control human milk fortifier; D21 = study day 21; ITT = intent-to-treat; NEC = necrotizing enterocolitis; nHMF = new human milk fortifier; NICU = neonatal intensive care unit; PP = per-protocol; SAE = serious adverse event. *Although screening procedures were standardized across sites, some variability in prescreening procedures did occur. Based on the typical clinical characteristics of infants who were admitted to each NICU during the study interval, the total number of infants who would have been theoretically considered eligible for the study was higher than the number shown here.

Protein intake estimated using standard values for preterm HM composition per 100 mL (22) was significantly greater in nHMF compared to cHMF (4.48 ± 0.38 vs 3.81 ± 0.43 g · kg^{-1} · day^{-1}, respectively; $P < 0.001$) because of higher protein content of the nHMF. Estimated energy intake was not significantly different between groups (125 kcal · kg^{-1} · day^{-1} in both groups). There was no significant difference in number of days between FSI1 and D1, but adjusted time between birth and D1 was significantly shorter in nHMF (16.8 ± 5.4 vs 18.7 ± 8.8 days; −8.5% [95% CI: −15.0%, −1.0%]).

Growth

In the ITT population, adjusted weight gain from D1 to D21 was 2.3 g/day higher in nHMF, with the 95% CI ranging from 0.4 to 4.2 g/day, demonstrating noninferiority ($P < 0.001$) and superiority ($P = 0.01$) of nHMF. Per-protocol results were similar. Weight gain from D1 to D21 remained significantly higher in nHMF when expressed in grams per kilogram per day (Table 3). Weight-for-age z scores (Fig. 3) remained stable from FSI1 to D21 in nHMF, but continued to decrease in cHMF ($P = 0.007$ vs D1). At D21, weight-for-age z score was significantly higher in nHMF compared to cHMF (0.12 [95% CI: 0.03, 0.22]). Length and HC gains during the D1 to D21 period were not significantly different between groups (Table 3), with comparable results observed from analyses of unadjusted means (Table 4). Length-for-age z scores at D21 (Fig. 3) were significantly lower than D1 values in cHMF ($P = 0.041$). Additionally, at W40CA, adjusted HC-for-age z scores were significantly higher in nHMF compared to cHMF (0.41 [95%

TABLE 2. Demographic and baseline characteristics of infants and parents

	nHMF (n = 76)	cHMF (n = 74)
Infant characteristics		
Sex		
Boys	38 (50)	35 (47)
Delivery type		
Vaginal	24 (32)	20 (27)
Twin	18 (24)	16 (22)
Birth weight, g	1147 ± 258	1156 ± 289
Birth weight by birth weight category		
<1000 g		
n (%)	24 (32)	26 (35)
Birth weight, g	850.5 ± 118.9	847.3 ± 105.1
≥1000 g		
Birth weight, g	1283.6 ± 175.4	1323.9 ± 206.2
Birth length, cm	37.1 ± 2.7	37.1 ± 3.1
Birth head circumference, cm	26.5 ± 2.7	26.7 ± 2.5
Gestational age at birth, weeks	28.8 ± 2.1	28.7 ± 1.8
Postnatal age at study time points, days*		
FSI1	13 (11, 18)	14 (10, 20)
Day 1	16 (13, 20)	17 (13, 23)
Day 21	36 (33, 40)	37 (33, 43)
Week 40 corrected age	76 (66, 91)	76 (67, 83)
Apgar score		
1 min	5.8 ± 2.5	5.8 ± 2.3
5 min	8.0 ± 1.8	7.7 ± 1.9
Parent characteristics		
Smoking status		
Mother smoker during pregnancy	6 (9)	18 (29)
Father smoker	3 (5)	12 (21)
Mother drank alcohol during pregnancy	0 (0)	4 (6)
Mother's age, y	31.1 ± 5.1	30.8 ± 5.5
Mother's BMI before pregnancy, kg/m^2*	23.2 (20.6, 27.2)	21.3 (19.7, 26.1)
Mother's weight gain during pregnancy, kg	11.2 ± 6.8	9.2 ± 5.2

BMI = body mass index; cHMF = control human milk fortifier; FSI1 = fortification strength increase day 1; nHMF = new human milk fortifier . Data are presented as n (%) for categorical variables and mean ± SD for continuous variables except where noted.

*Data are presented as median (Q1, Q3).

CI: 0.14, 0.68]). Mean weight, length, and HC at D1, D21, and W40CA are summarized in Table 5.

Protein-Energy Status

BUN decreased progressively in cHMF ($P = 0.004$ for D21 vs D1), whereas it increased in nHMF ($P < 0.001$ for D10/11 vs D1 [data not shown]) and remained stable up to D21 (Table 6). Prealbumin levels were similar at D1 and increased in both groups during the study (Table 6). The increase from D1 to D21, however, was only significant in nHMF ($P = 0.004$). At D21, adjusted mean prealbumin in nHMF was significantly higher (+11.8% [95%CI: +2.3%, +22.2%]) than in cHMF. Urinary urea excretion (corrected for creatinine excretion) at D1 was similar in the 2 groups (Table 6). Urea excretion remained steady in cHMF but increased sharply in nHMF ($P < 0.001$ for D10/11 vs D1 [data not shown]), after which it remained stable (to D21). At D21, urea excretion was significantly higher in nHMF versus cHMF (+108.7% [95% CI: +66.0%, +162.5%]).

TABLE 3. Anthropometric gains from D1 to D21

	Treatment group				
	n	nHMF	n	cHMF	P*
Weight gain, g · kg^{-1} · day^{-1}	64	18.3 ± 3.7	67	16.8 ± 3.7	0.013†
Length gain, cm/wk	55	1.23 ± 0.62	65	1.18 ± 0.49	0.842
HC gain, cm/wk	57	1.04 ± 0.32	65	0.96 ± 0.26	0.125

cHMF = control human milk fortifier; D1 = study day 1 (first day of full-strength fortification); D21 = study day 21; HC = head circumference; nHMF = new human milk fortifier. Data are presented as unadjusted mean ± SD.

*One-sided superiority P value based on analysis of covariance model adjusted for postmenstrual age and relevant anthropometric measure at D1, sex, and center.

†Adjusted difference in weight gain (nHMF–cHMF): mean difference = 1.18 g · kg^{-1} · day^{-1}; 95% CI = 0.14, 2.21.

Bone Metabolic Status

Serum calcium concentrations were generally stable during the study (Table 6), with mean values for both groups within the normal range (32). Nevertheless, adjusted mean serum calcium concentration in nHMF was minimally but significantly higher than in cHMF at D21 (+1.9% [95% CI: +0.3%, +3.5%]). Serum phosphorus increased slightly in the 2 groups (Table 6). At D1, relative hypophosphatemia (<1.55 mmol/L) was observed in 13 infants in both groups; this was corrected in 11 infants by D10/11 and 12 infants by D21. At D1, serum alkaline phosphatase was not significantly different in nHMF versus cHMF ($P = 0.208$). Thereafter, serum alkaline phosphatase decreased significantly in both groups (D21 vs D1: $P = 0.005$ for nHMF, $P < 0.001$ for cHMF), with mean values significantly higher in nHMF versus cHMF at D10/11 (+8.6% [95% CI: +1.0%, +16.8%]; data not shown) and D21 (+12.1% [95% CI: +2.8%, +22.3%]) (Table 6). Declines from baseline were significantly greater in cHMF versus nHMF at D10/11 ($P < 0.001$; data not shown) and D21 ($P = 0.035$). At D1, spot urinary excretions of calcium and phosphorus corrected for urinary creatinine excretion were similar in the 2 groups (Table 6). Calcium excretion tended to increase slowly during the study in both groups, with mean concentration significantly lower in nHMF compared to cHMF at D21 ($P = 0.011$). Phosphorus excretion increased in both groups, resulting in a decreased median urinary calcium:phosphorus molar ratio in both groups (Table 6).

Electrolytes

Serum electrolyte concentrations were stable during the study and similar in both groups (Table 6). Urinary sodium and potassium concentrations were significantly higher (sodium: +31.1% [95% CI: +1.7%, +68.9%], potassium: +22.5% [95% CI: +1.0%, +48.6%]) in nHMF compared to cHMF at D21 (Table 7).

Stool Characteristics and Feeding Tolerance

Stool frequency from D1 to D21 was not significantly different in nHMF and cHMF (3.9 ± 1.05 vs 3.6 ± 0.93 stools/day; 0.29 [95% CI: −0.05, 0.63]). Stool consistency was slightly more ''formed'' in nHMF compared to cHMF during this interval (3.1 ± 0.26 vs 3.0 ± 0.27; 0.12 [95% CI: 0.02, 0.21]). Most infants (>90%) had stool consistency scores of ''soft.'' There were no significant differences between groups in frequencies of

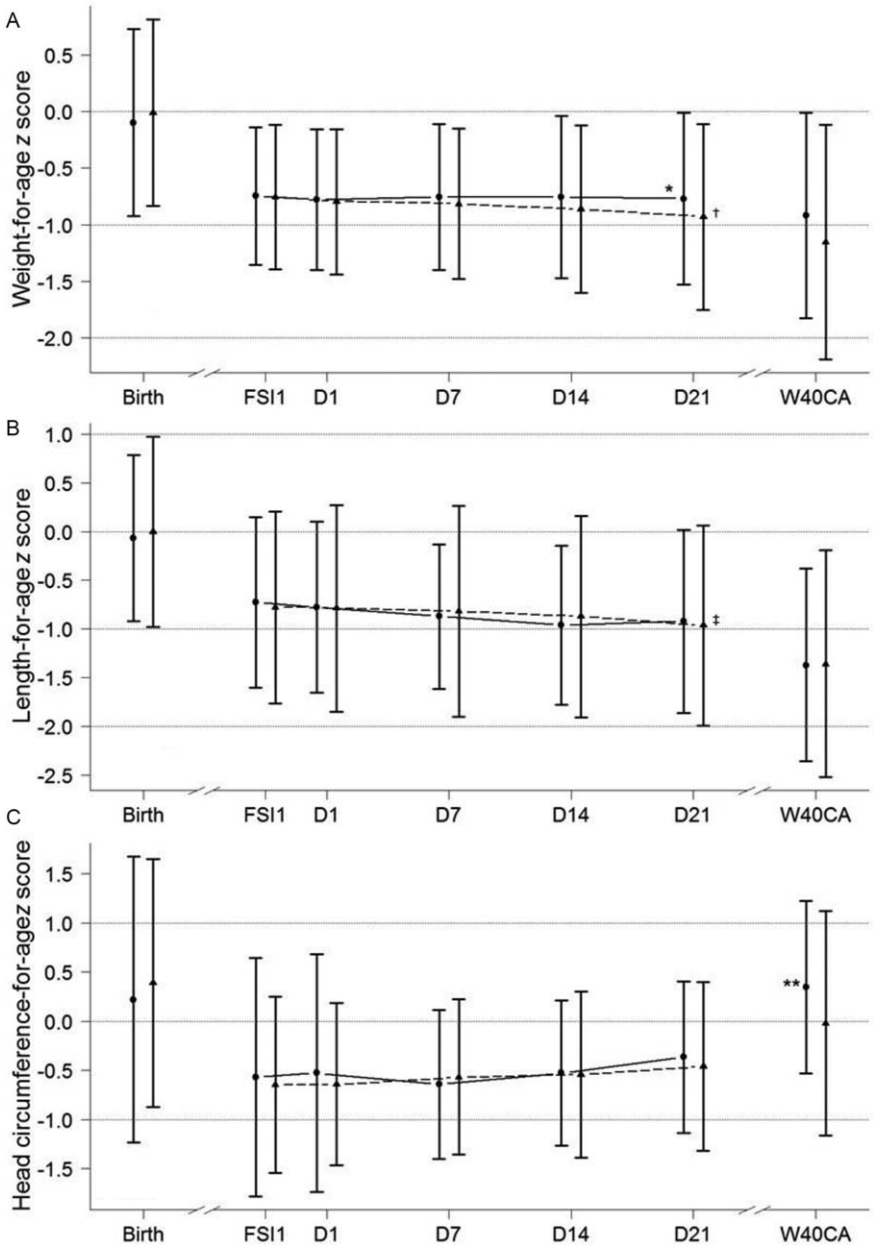

FIGURE 3. Mean ± SD weight-for-age (panel A), length-for-age (panel B), and head circumference-for-age (panel C) *z* scores for the overall ITT population. Circle symbols/solid line represents nHMF. Triangle symbols/dashed line represents cHMF. FSI1 = fortification strength increase day 1; ITT = intent-to-treat; SD = standard deviation; W40CA = week 40 corrected age; *z* scores calculated using Fenton preterm growth chart (25). $^{*}P = 0.013$ vs cHMF (by analysis of covariance, adjusting for value at D1, sex, and center); $^{\dagger}P = 0.007$ vs day 1 (by *t* test); $^{\ddagger}P = 0.041$ vs day 1 (by *t* test); $^{**}P = 0.003$ vs cHMF (by analysis of covariance, adjusting for value at D1, sex, and center).

spitting-up, vomiting, or abdominal distention. There also were no group differences in incidence of AEs indicative of feeding intolerance (all $P \geq 0.25$).

Adverse Events

The overall incidence of AEs was significantly larger in nHMF (103 events in 56 infants, including 26 events categorized as GI disorders, 18 as infections or infestations, and 5 as metabolism and nutrition disorders) compared to cHMF (78 events in 41 infants, including 21 events categorized as GI disorders, 18 as infections or infestations, and 1 as metabolism and nutrition disorder; odds ratio: 2.26 [95% CI: 1.10, 4.47]). Other AEs that occurred more frequently in nHMF included several that were classified by study investigators as unlikely to be related to consumption of milk fortifiers (eg, cardiac disorders [16 events in nHMF vs 5 in cHMF], eye disorders [10 events in nHMF vs 3 in cHMF]). The number of AEs considered related to study product intake as determined by physician report was low (3 events in nHMF [2 events of hyponatremia, 1 of vomiting] and 0 events in cHMF). No significant difference was demonstrated in overall incidence of serious AEs between the 2 groups (7 events in 7 infants [including 2 events of necrotizing

TABLE 4. Body length and head circumference gains between study days 1 and 21, by infant sex and by birth weight category

	Unadjusted length gain, cm/wk*					Unadjusted head circumference gain, cm/wk*				
	nHMF		cHMF			nHMF		cHMF		
	n	Mean ± SD	n	Mean ± SD	$P^{\dagger}$	n	Mean ± SD	n	Mean ± SD	$P^{\dagger}$
Overall	55	1.23 ± 0.62	65	1.18 ± 0.49	0.842	57	1.04 ± 0.32	65	0.96 ± 0.26	0.126
Boys	27	1.40 ± 0.65	28	1.18 ± 0.49	0.364	28	1.12 ± 0.28	28	0.99 ± 0.22	0.062
Girls	28	1.08 ± 0.56	37	1.17 ± 0.50	0.510	29	0.97 ± 0.35	37	0.93 ± 0.29	0.598
<1000 g	19	1.07 ± 0.52	21	1.27 ± 0.52	0.563	19	1.04 ± 0.34	21	0.94 ± 0.28	0.223
≥1000 g	36	1.32 ± 0.66	44	1.13 ± 0.48	0.499	38	1.05 ± 0.32	44	0.96 ± 0.26	0.270

cHMF = control human milk fortifier; nHMF = new human milk fortifier.
*Data are presented as unadjusted mean ± SD.
†Superiority *P* value for gain differences adjusted for postmenstrual age and the relevant anthropometric measure at D1, sex, and center by analysis of covariance.

TABLE 5. Weight, length, and head circumference at selected study time points

	nHMF			cHMF		
Variable	n	Mean	SD	n	Mean	SD
Weight, g						
D1	72	1346	271	74	1347	270
D21	64	1884	336	67	1863	328
W40CA	60	3076	519	63	2897	416
Length, cm						
D1	67	38.7	2.5	74	38.7	2.8
D21	58	41.8	2.4	65	42.0	2.7
W40CA	60	47.6	2.6	62	47.3	2.5
Head circumference, cm						
D1	68	27.7	2.5	73	27.6	1.9
D21	59	30.2	2.2	66	30.3	2.0
W40CA	59	35.3	1.4	64	34.6	1.5

cHMF = control human milk fortifier; D1 = study day 1; D21 = study day 21; nHMF = new human milk fortifier; SD = standard deviation; W40CA = week 40 corrected age.

TABLE 6. Markers of protein-energy status, electrolytes, and bone metabolic status at study days 1 and 21

	nHMF				cHMF				
Variable	n	Median	IQR	Geometric mean	n	Median	IQR	Geometric mean	P^{*}
Serum creatinine, μmol/L									
D1	69	44.0	36.2–48.0	41.5	70	44.1	38.0–51.8	43.5	0.303
D21	63	28.0	23.5–32.0	26.7	65	30.0	25.0–35.0	29.5	0.001
BUN, mmol/L									
D1	70	3.10	1.70–4.56	2.89	71	2.50	1.65–4.67	2.73	0.585
D21	63	3.90	3.05–4.65	3.89	64	2.15	1.50–2.63	2.15	<0.001
Serum prealbumin, mg/L									
D1	51	100	80–120	96.8	46	90	80–100	87.8	0.073
D21	46	116	91.3–140	113.8	41	100	90–120	98.1	0.015
Urinary urea†, mmol/10 mg creatinine									
D1	47	2.7	2.0–4.7	2.8	53	2.5	1.9–3.3	2.5	0.302
D21	42	5.8	4.6–6.8	5.1	40	2.8	2.0–3.3	2.7	<0.001
Serum calcium, mmol/L									
D1	50	2.44	2.31–2.53	2.41	54	2.47	2.38–2.56	2.44	0.445
D21	50	2.47	2.40–2.54	2.46	48	2.43	2.34–2.53	2.43	0.019
Serum phosphorus, mmol/L									
D1	68	1.99	1.85–2.22	1.96	71	1.94	1.76–2.25	1.94	0.816
D21	62	2.10	1.93–2.23	2.05	64	2.12	1.93–2.26	2.08	0.681
Alkaline phosphatase, U/L									
D1	67	353.0	298.5–459.5	377.9	63	333.0	250.0–438.5	343.8	0.208
D21	62	320.5	273.3–405.5	337.5	62	270.5	233.0–354.3	297.5	0.010
Urinary calcium †, mmol/10 mg creatinine									
D1	60	0.11	0.07–0.19	0.12	69	0.14	0.09–0.20	0.12	0.985
D21	55	0.14	0.09–0.23	0.15	54	0.21	0.13–0.32	0.19	0.011
Urinary phosphorus†, mmol/10 mg creatinine									
D1	59	0.41	0.12–0.66	0.22	65	0.34	0.14–0.65	0.23	0.867
D21	52	0.68	0.44–1.10	0.53	52	0.71	0.40–0.92	0.58	0.896
Urinary calcium:phosphorus molar ratio									
D1	59	0.39	0.15–0.90	0.50	64	0.41	0.16–1.34	0.47	0.824
D21	53	0.22	0.12–0.48	0.28	53	0.31	0.19–0.60	0.34	0.054
Serum sodium, mmol/L									
D1	71	138.0	137.0–140.0	138.6	72	138.6	136.6–140.0	138.5	0.891
D21	65	138.0	136.4–140.0	138.0	64	138.0	137.0–139.9	138.3	0.449
Serum potassium, mmol/L									
D1	71	4.73	4.30–5.32	4.83	72	4.77	4.40–5.10	4.78	0.685
D21	64	4.74	4.29–5.10	4.72	64	4.51	4.14–4.88	4.54	0.091
Serum chloride, mmol/L									
D1	71	106.0	104.0–109.0	106.1	72	105.0	102.8–108.0	105.2	0.148
D21	63	105.0	103.0–107.0	104.6	62	105.0	104.0–107.0	105.3	0.111

BUN = blood urea nitrogen; cHMF = control human milk fortifier; D1 = study day 1; D21 = study day 21; IQR = interquartile range; nHMF = new human milk fortifier.
*D1 geometric mean values were log-transformed and analyzed using *t* test; D21 geometric mean values were log-transformed and analyzed using analysis of covariance (adjusting for the relevant biochemical parameter at D1, sex, and center).
†Corrected for urinary creatinine excretion of 10 mg/kg body weight/day.

TABLE 7. Markers of kidney function, blood count, and urinary electrolyte status at study days 1 and 21

	nHMF				cHMF				
Variable	n	Median	IQR	Geometric mean	n	Median	IQR	Geometric mean	P^*
Urinary creatinine, μmol/L									
D1	63	1300.0	785.5–1685.5	1224.7	69	1105.0	900.0–1500.0	1182.3	
D21	57	1030.0	660.0–1609.0	1000.3	55	854.0	618.0–1273.0	900.8	0.447
Serum hemoglobin, mmol/L									
D1	68	2.08	1.84–2.29	2.14	72	2.02	1.84–2.26	2.18	
D21	63	1.71	1.56–1.91	1.83	66	1.69	1.50–1.98	1.76	0.936
Serum hematocrit, %									
D1	68	0.40	0.35–0.43	0.39	72	0.39	0.35–0.43	0.38	
D21	63	0.32	0.29–0.38	0.33	66	0.33	0.28–0.38	0.33	0.805
Urinary sodium, mmol/L									
D1	66	37.0	23.3–57.3	37.5	69	32.0	19.4–54.0	31.2	
D21	59	34.0	21.1–48.0	33.3	56	23.0	14.3–36.4	24.0	0.037
Urinary potassium, mmol/L									
D1	66	25.9	13.6–37.0	23.6	69	21.8	15.0–32.2	20.0	
D21	59	30.0	16.9–45.0	27.6	57	22.9	16.9–30.4	22.8	0.040
Urinary chloride, mmol/L									
D1	60	37.0	26.3–60.0	40.2	67	33.0	20.5–55.0	34.2	
D21	54	31.0	17.8–43.8	30.7	55	26.0	18.0–39.5	27.8	0.558

cHMF = control human milk fortifier; D1 = study day 1; D21 = study day 21; IQR = interquartile range; nHMF = new human milk fortifier .

*D21 geometric mean values were log-transformed and analyzed using analysis of covariance (adjusting for the relevant biochemical measure at D1, sex, and center).

enterocolitis, 0 events of bronchopulmonary dysplasia, 0 events of sepsis, 0 events of retinopathy] in nHMF and 12 events in 11 subjects [including 4 events of necrotizing enterocolitis, 1 event of bronchopulmonary dysplasia, 0 events of sepsis, 0 events of retinopathy] in cHMF; odds ratio: 0.54 [95% CI: 0.17, 1.58]).

DISCUSSION

This study demonstrated that weight gain from D1 of full fortification until D21 in preterm infants fed HM fortified with a new fortifier designed to add 1.4 g partially hydrolyzed protein and 0.7 g fat to 100 mL of HM was significantly greater than weight gain in infants fed HM fortified with an isocaloric control fortifier designed to add 1.0 g extensively hydrolyzed protein and no fat. The mean difference was 2.3 g/day or 1.2 $g \cdot kg^{-1} \cdot day^{-1}$, consistent with our hypothesized difference of 2 g/day, and which indicates the superiority of the new fortifier compared to the control with regard to weight gain. In addition, the weight gain benefit tended to persist until discharge, with a significantly higher adjusted weight gain difference in the nHMF group compared to cHMF from FSI1 to W40CA (2.01 g/day; $P = 0.009$). In the nHMF group, weight-for-age z scores were stable from FSI1 to D21 and average weight gain exceeded 18 $g \cdot kg^{-1} \cdot day^{-1}$, matching recommended rates of postnatal weight gain to mimic intrauterine growth (33,34). Consistent with the increased protein content of the new fortifier, the nHMF group had significantly higher serum prealbumin concentrations, suggesting an increase in nitrogen retention compared to cHMF. The lack of difference, however, in length gain during the study may be in part the result of the relatively limited period of protein supplementation (only 21 days) or because mean length gains in both groups were already quite high (ie, ≥1.1 cm/week), whereas the significantly higher HC-for-age z score at W40CA in the nHMF group may be because of the increased protein and lipid content of the new fortifier. In contrast, the absence of a significant difference at earlier timepoints could be attributable to the relatively high variability of HC gain (31% and 27% for nHMF and cHMF, respectively, from D1 to D21) induced by the natural dolichocephalic evolution of the skull that occurs in preterm infants (35). Feeding tolerance and stool patterns were similar in each group, and AEs related to feeding were low and not significantly different between groups, consistent with fortified HM osmolality values slightly lower in nHMF versus cHMF and below the recommended cutoff (23,24) in both groups.

Although there was no evidence of imbalance between the 2 fortifier groups with respect to infant baseline characteristics, significant differences in maternal weight gain, smoking, and alcohol usage during pregnancy were observed. As these may be confounding factors in the analysis of weight gain, post hoc ANCOVAs including these parameters were performed. The post hoc results were essentially the same as the main results, indicating that differences in maternal baseline characteristics did not confound the results. Additionally, to determine the possible impact of including clustered data from twins in the analyses, a sensitivity analysis on weight gain (grams per day) from D1 to D21 accounting for the correlated multiple-birth data was performed. Again, these results were similar to those of the main analysis (weight gain 3.2 g/day higher in nHMF [95% CI: 0.5, 5.9 g/day]).

Our results are consistent with those of previous studies (36–42). A recent meta-analysis of 5 studies (comprising 352 infants with birthweight ≤1750 g and gestational age ≤34 weeks) compared growth of infants fed HM fortified with either lower-protein or higher-protein fortifier (43). Infants receiving higher-protein fortifier had significantly greater weight (mean difference 1.77 g/kg/day), length (0.21 cm/week), and HC gains (0.19 cm/week) compared to those receiving lower-protein fortifier (43). Miller et al (39) used a higher-protein fortifier similar in protein content to the one used in the present study, and reported a higher bodyweight at study end among infants in the higher-protein HMF group (mean difference 220 g), but no significant differences in length or HC. In contrast, Moya et al (40) observed a significantly higher achieved weight, length, and HC in the experimental group compared to controls, but their fortifier had a slightly higher protein content

(3.2 g/100 mL) versus the one used in the present study (3.04 g/100 mL), plus the intervention lasted 28 rather than 21 days.

Energy and protein content of HM samples were not analyzed in this study but estimated according to Tsang et al (22). Variability of protein, fat, and energy content of HM fed to preterm infants in the NICU is high (15,21). In addition, fat content may be reduced during processing of HM from expression to administration (44), which could be exacerbated with the use of continuous tube feeding (45). In our study, percentage of intake from mother's own milk, donor milk, and pasteurized HM was assessed. Pasteurized donor milk accounted for 51% of the fortified HM provided during the study, whereas 56% of mother's own milk was also pasteurized. Considering that protein content of donor HM is lower than that of mother's own milk (46) and that all the required processing steps (eg, collection, transfer, refrigeration, pasteurization, tube feeding) may significantly decrease fat and energy content (47), the characteristics of the HM used in the present study suggests that protein and energy content could be overestimated when based on a theoretical composition of preterm HM.

In the present study, the mean increase in protein supplementation provided by nHMF compared to cHMF was $0.65\,g \cdot kg^{-1} \cdot day^{-1}$ or $7.4\,mmol \cdot kg^{-1} \cdot day^{-1}$ of nitrogen, from which approximately $6.14\,mmol \cdot kg^{-1} \cdot day^{-1}$ of nitrogen (83%) is absorbed (based on data from balance studies) (48). During the study, urea production increased significantly in the nHMF group leading to an increase in BUN of 1.7 mmol/L at D21 and in urea excretion of $2.3\,mmol \cdot kg^{-1} \cdot day^{-1}$ (2.3 mmol/10 mg creatinine). These data suggest that the nitrogen balance was improved to ~3.8 mmol nitrogen (52% of nitrogen intake) in preterm infants fed nHMF compared to control. This relatively limited protein utilization could result from reduced energy bioavailability of HM, and an increase in energy supply could improve protein utilization in preterm infants fed fortified HM. These data also suggest that specific nutritional recommendations should be formulated for infants fed fortified HM. Nevertheless, the increase in nitrogen retention ($\sim 3.8\,mmol \cdot kg^{-1} \cdot day^{-1}$) appears to be higher than the nitrogen content of the higher weight gain observed with the nHMF (12% of the $1.5\,g \cdot kg^{-1} \cdot day^{-1}$ corresponding to $2\,mmol \cdot kg^{-1} \cdot day^{-1}$ of nitrogen), suggesting an increase in lean body mass accretion and a moderate reduction in fat mass gain as previously demonstrated in preterm infants fed protein-fortified HM (49).

Indices of bone metabolism were satisfactory in both groups, with a significant decrease in serum alkaline phosphatase observed in both groups and 98% of the infants having normal serum phosphorus concentrations at D21. Adequate postnatal bone mineralization is difficult to obtain in preterm infants owing to the interruption of mineral transplacental transfer (50). Although elevated alkaline phosphatase activity may be associated with reduced bone mineralization when mineral intake is deficient (51), the decrease in enzyme levels observed in the presence of normal serum phosphorus values, as well as the low urinary calcium and moderate urinary phosphorus excretion observed in both groups in this study, suggest that intakes were adequate to promote bone mineralization and limit postnatal osteopenia. Mean serum creatinine concentration decreased significantly in both groups suggesting a similar maturation of renal function during this period. Urinary electrolyte concentrations were higher in nHMF versus cHMF at D21, likely in parallel with the higher electrolyte content of nHMF.

A lack of HM composition data (allowing estimation of nutritional balance) is a limitation of our study, although standardized accurate techniques are still not available in the NICU. Additionally, the composition of the faster weight gain can only be estimated as lean body mass and/or bone mineralization were not determined. As a result, nutrient absorption and metabolism can only be estimated from serum and urinary metabolite concentrations. Lastly, the results need to be confirmed in a broader population of preterm infants commonly admitted to the NICU including SGA infants and partially breast-fed infants, as these infants were excluded by design. Strengths of this study include the size and multiple sites (11 pediatric hospitals in 4 European countries), which enhances external validity.

In conclusion, these results indicate that the new HM fortifier, made with partially hydrolyzed whey protein and a higher protein:energy ratio is safe, well-tolerated, and improves weight gain of preterm infants compared to control fortifier. Providing some energy as fat and replacing extensively hydrolyzed with partially hydrolyzed protein in the new HM fortifier allows a reduction in osmolality <400 mOsm/kg immediately after fortification. Protein intakes from HM supplemented with the new fortifier are within the range of the most recent nutritional recommendations for preterm infants.

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ORIGINAL ARTICLE

Assessment of human milk composition using mid-infrared analyzers requires calibration adjustment

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OBJECTIVE: Nutrient composition of human milk (HM) is highly variable. Targeted HM fortification has been proposed to address these variations and reduce the cumulative nutritional deficit in preterm infants. Near-infrared analysis is used to measure the protein and fat content in HM; however, the reliability of this technique has not been evaluated. The objective of this study is to evaluate the reproducibility and accuracy of two generations of HM analyzers (HMA1 and HMA2) in estimating protein and lipid contents.

STUDY DESIGN: Reproducibility was assessed by analyzing in duplicate 146 and 128 HM samples with HMA1 and HMA2 (*Miris*), respectively. To evaluate the accuracy, lipid and protein concentrations were assessed in 31 and 39 samples using HMA1 or HMA2, respectively. Values were compared with measurements obtained using reference methods and correction equations were calculated. After applying the correction equations on 12 HM samples, the performance of the two devices were compared and the equation was validated according to the reference methods.

RESULTS: The coefficients of variation for protein and lipid assessments were below 3% for both HMA1 and HMA2. Protein concentrations were significantly underestimated by HMA2 (-0.53 ± 0.23 g dl^{-1}). Lipid content was significantly overestimated by both devices, but the error was greater with HMA1 (0.76 ± 0.48 g dl^{-1}) than with HMA2 (0.36 ± 0.33 g dl^{-1}). Correction equations were specific for each generation of HMA. Finally, after correction, both instruments provided similar and accurate results.

CONCLUSION: HMAs require calibration adjustment before their use in clinical practice, to avoid inappropriate HM fortification.

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INTRODUCTION

In preterm infants, human milk (HM) has several health benefits, including reduction of necrotizing enterocolitis or late-onset sepsis and improved neurodevelopmental outcomes.[1–4] However, protein and lipid contents of expressed HM are highly variable and fortification is needed to support optimal postnatal growth in preterm infants.[4–11] Assessment of HM composition is required to perform individualized targeted fortification.[12–14]

Available infrared HM analyzers (HMAs) allow the rapid determination of protein and lipid concentrations using a small volume of HM.[15–21] However, dairy industry consider mid-infrared analyzers as secondary testing instruments requiring calibration by chemical reference (CR) methods.[22] Among the available, *Miris* HMA (Miris, Uppsala, Sweden) is a compact, easy-to-use and relatively inexpensive device. It is widely used, because it requires only 1 ml of HM, which is a concern for new mothers producing limited amounts of milk. In 2009, Menjo *et al.*[18] used a first-generation *Miris* HMA and reported that protein and lipid contents differed significantly from those obtained with CR. Casadio *et al.*[19] found that HMAs overestimated the lipid and protein content of HM but used a recalibrated software for both diluted and undiluted milk samples, in order to measure broader range of HM. Similarly, Silvestre *et al.*[20] found that *Miris* HMA provided reproducible results but overestimated the lipid content, while underestimating the protein content, therefore requiring the application of correction equations. In 2015, Fusch *et al.*[23] achieved good precision and accuracy after major adjustments.

We aimed to evaluate the reproducibility, accuracy and precision of protein and lipid assessment of HM by comparing first- and second-generation HMA devices (HMA1 2008 and HMA2 2011 software XMA SW Ver. 18.06.2010, Miris AB, Uppsala, Sweden).

MATERIALS AND METHODS

Collection of HM samples

This study was performed at the regional HM bank at the Croix Rousse University Hospital (Lyon, France) from 2008 to 2011. All samples (30 to 180 ml) of pasteurized HM were bacteriologically contaminated and unfit for consumption by preterm infants.[24] The samples were issued from mothers who delivered term or preterm, to obtain a wide range of protein and lipid contents, representative of HM composition. Some samples were obtained from a pool of several donors' milk. According to French regulations no ethics approval was required, as measurements were performed before destruction on unusable milk. Informed consent for the use of their milk for research purposes was obtained from the donors before the study.

HMA sample analysis

We used the 'processed milk' mode on the *Miris* HMA (Miris AB, Uppsala, Sweden); Figure 1. Samples were thawed by warming the milk to 40 °C and then homogenized using an ultrasonic probe (20 KHz s^{-1} per ml milk; Sonics Vibracell, CIAB, Sollentuna, Sweden; 1 s ml^{-1}). Only the lipid and

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protein contents of the milk samples were analyzed, because lactose levels are relatively stable.[9–12]

Experiment 1. Reproducibility of HMA1 and HMA2 for protein and lipid assessment. To determine the reproducibility of each instrument, samples of frozen HM were evaluated in duplicate during two periods, in 2008 and in 2011. The coefficient of variation of each instrument (%) was calculated as the mean and s.d. of the difference between the two determinations expressed in % of the mean value (s.d. of difference × 100/mean).[25]

Experiment 2. Accuracy of HMA1 and HMA2 versus CR for protein and lipid assessment, and calculation of the correction equation. Seventy samples selected, because they were considered representative of HM composition, were refrozen to − 20 °C after HMA measurements in Lyon and then sent to the Nutritional Laboratory at the University of Liege for chemical analysis. Total nitrogen was determined by the method of Dumas using a nitrogen analyzer (Analyzer EP 428, Leco, Garges les Gonesse, France) and total lipid content using the Soxhlet method (Soxtec Aventi 2055; Foss, Hillerød, Denmark), as described previously.[11] Samples were homogenized before assessment. The conversion factor used to calculate the protein equivalence was nitrogen (*g*) × 6.25.[26] The results obtained with each device were then compared with the CR.

Experiment 3: Validation of the correction equations and comparison of values obtained with both devices using the same samples. A subset of HM samples was assessed with HMA1 and the chemical analysis performed in 2008 and re-assessed 3 years later with HMA2 and chemical analysis. This re-evaluation was performed, because HMA1 broke down in 2010 and was replaced by HMA2. To overcome the effects of time and freezing on protein and lipid contents, the values obtained using HMA1 and HMA2 were compared with each other and also with the values obtained using the reference chemical methods at each time point.

The correction equations for HMA1 and HMA2 calculated in Experiment 2 were applied on the protein and lipid HMA values obtained from this subset of samples. Then, the corrected protein and lipid HMA1 and HMA2 values were compared with each other and with the CR values.

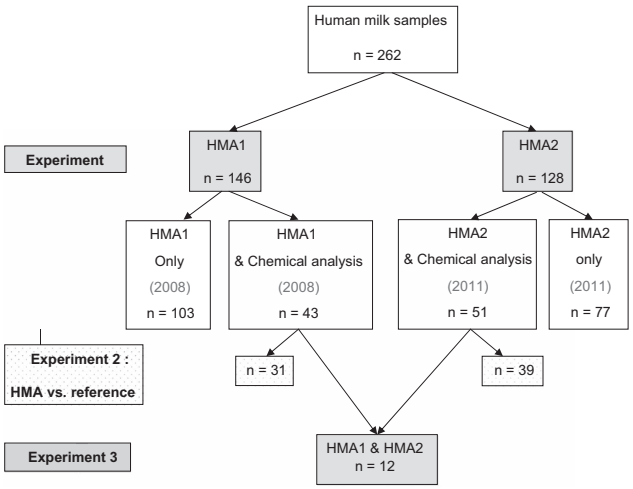

Figure 1. Study protocol. Human milk (HM) mid-infrared analyzer (HMA) 1 and HMA2 are first- and second-generation HMAs, respectively. Chemical analysis is reference biological methods (Dumas method for protein content and Soxhlet method for lipids).

Statistical analyses

The protein and lipid values and the coefficients of variation are reported as means ± s.d. Passing–Bablok regression analysis was performed to estimate the relationship between the HMA and CR.[27,28] Inverse relationships were used to obtain the correction equations and these equations were then applied to the HMA values, to calculate the corrected HMA values. The corrected HMA values were plotted against the CR according to the recommendations of Bland and Altman.[29] The means and s.d. of the differences between the corrected HMA values and the CR represented the accuracy and precision of the measurement, respectively. Wilcoxon signed-rank tests were performed to compare the results obtained for the same samples with the two instruments. Mann–Whitney tests were performed to compare the reproducibility of the two instruments. Statistical analysis was performed with the MedCalc software and SAS version 9.2 (SAS France, Brie Comte Robert, France). Differences with *P*-values < 0.05 were considered statistically significant.

RESULTS

Mid-infrared protein and lipid measurements were performed for 262 samples of HM (146 samples for HMA1 and 128 for HMA2). Among the 70 HM samples selected to assess the accuracy, 31 were used for HMA1 and 39 for HMA2 evaluation. A subset of 12 samples was assessed using both HMA1 (first period) and HMA2 (second period), and by CR at each period (Figure 1).

Experiment 1: reproducibility of HMAs

The coefficients of variation for the assessment of proteins and lipids using HMA1 (146 samples) and HMA2 (128 samples) were both below 3% for all parameters and were similar between the two devices (Table 1).

Experiment 2: accuracy of protein and lipid assessment

The accuracy of measurements by HMA1 was evaluated in 31 HM samples for protein and 28 of 31 HM samples for lipids.

For protein. The agreement between the HMA1 values and the CR was high. Mean difference between the HMA1 and CR was -0.02 ± 0.18 g dl^{-1}, suggesting that HMA1 underestimated the protein content, especially for the lowest values (Figure 2a). The conversion equation was calculated as follows: HMA1-PROT corrected (g dl^{-1}) = 0.135+(0.95 × HMA1-PROT (g dl^{-1})). HMA2 also underestimated the protein content of HM (39 samples). The mean difference between the HMA2 and CR was -0.53 ± 0.23 g dl^{-1} (Figure 2b). The conversion equation was calculated as follows: HMA2-PROT corrected (g dl^{-1}) = − 0.08 +(1.47 × HMA2-PROT (g dl^{-1})).

Table 1. Parameters measured in HM samples by first-generation (HMA1, $n = 146$) and second-generation (HMA2, $n = 128$) mid-infrared HMAs and CVs of these duplicated measurements

	Lipids (g per 100 ml)	*Protein (g per 100 ml)*	*Lactose (g per 100 ml)*	*Solids (g per 100 ml)*	*Energy (kcal per 100 ml)*
HMA1 ($n = 146$)	3.8 ± 1.5	1.3 ± 0.6	7.1 ± 0.5	12.6 ± 1.7	68.9 ± 14.0
HMA1 − CV (%)	1.06 ± 0.01	1.42 ± 0.05	1.30 ± 0.02	1.08 ± 0.02	1.08 ± 0.02
HMA2 ($n = 128$)	3.6 ± 1.0	1.2 ± 0.5	6.9 ± 0.4	14.0 ± 2.3	65.5 ± 10.2
HMA2 − CV (%)	1.18 ± 0.02	2.86 ± 0.07	0.62 ± 0.01	1.04 ± 0.02	1.13 ± 0.02

Abbreviations: CV, the coefficients of variation; HM, human milk; HMA, human milk analyzers. Values are expressed as mean ± s.d.

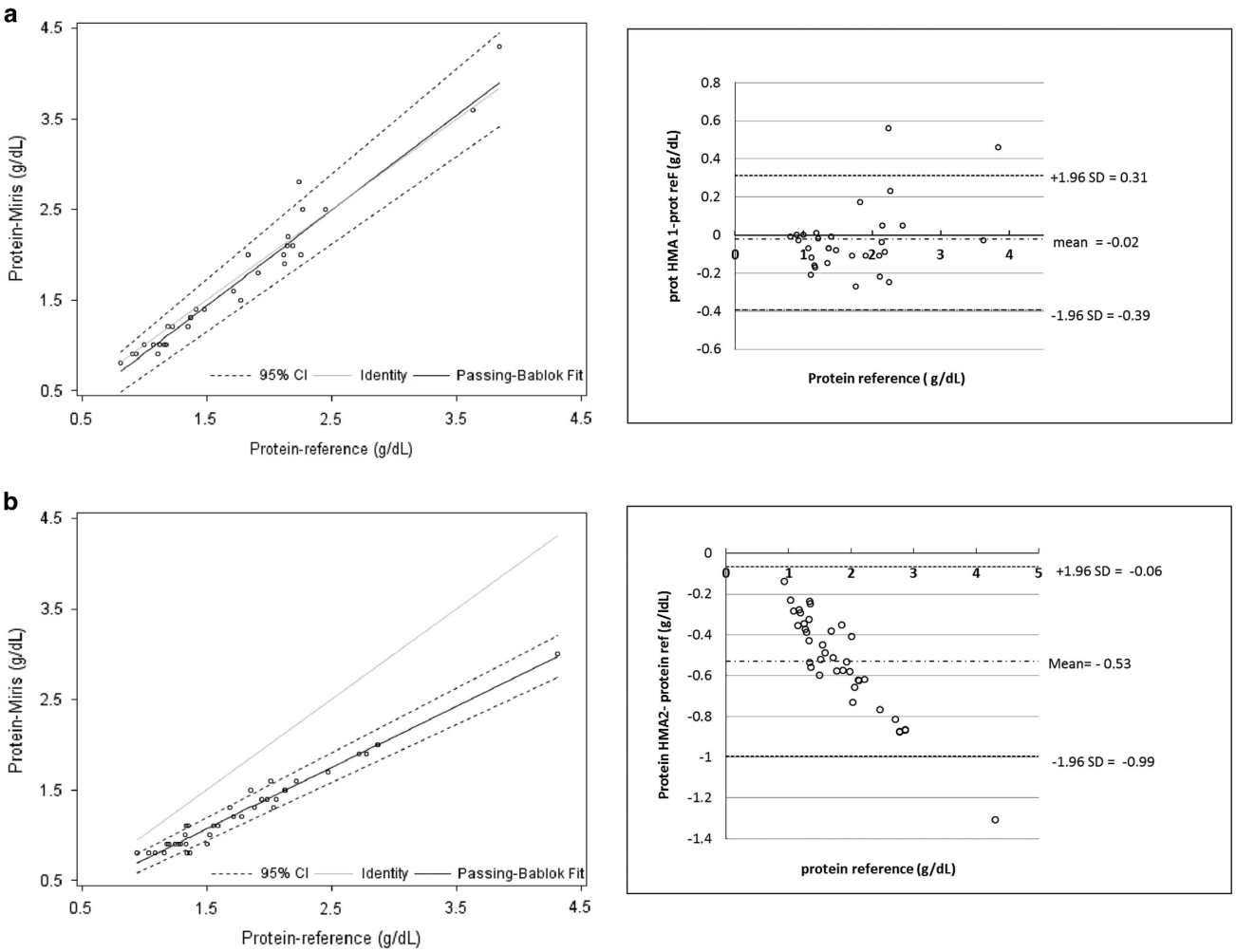

Figure 2. Relationship between protein concentrations measured using first-generation (HMA1) or second-generation (HMA2) *Miris* human milk (HM) mid-infrared analyzers (HMA) and a reference biochemical method (Kjeldhal). Relationships were assessed using Passing–Bablock linear regression analysis and the error is represented by Bland and Altman graphics (HMA1 (**a**) and HMA2 (**b**).

For lipids. The HMA1 device significantly overestimated the lipid concentrations in HM; the mean difference between the HMA1 and CR was 0.76 ± 0.48 g dl^{-1} (Figure 3a). The conversion equation was: HMA1-LIPID corrected (g dl^{-1}) = − 0.51+(0.93 × HMA1-LIPID (g dl^{-1})). Similar results were obtained for HMA2 (37 of 39 milk samples), although the overestimation of lipid content was not as dramatic as for HMA1; the mean difference between the HMA2 and CR was 0.36 ± 0.33 g dl^{-1}. The conversion equation was as follows: HMA2-LIPID corrected (g dl^{-1}) = − 0.03+(0.9 × HMA2-LIPID (g dl^{-1}); Figure 3b).

Experiment 3: validation of correction equations and comparison of both devices using the same samples.

Testing of correction equations. The assessment of the 12 samples by HMA1, HMA2 and CR were compared. Protein and lipids values, as assessed by the CR, ranged from 0.98 to 1.95 g dl^{-1} and 2.14 to 4.89 g dl^{-1}, respectively. After correction according to the equations mentioned above, the lipid values for HMA1 ($P = 0.33$ for lipids), and the protein and lipid values for HMA2 ($P = 0.7$ for protein and $P = 0.09$ for lipids) were not significantly different from the CR However, a significant difference was noted between the HMA1 protein corrected values and CR ($P = 0.012$).

Comparison of both devices using the same samples. Analyses of the subset of 12 samples using the CR methods, 3 years apart, demonstrated a significant decrease in lipid content ($P = 0.02$), whereas protein content was similar ($P = 0.14$; Table 2). A similar result was obtained only when the corrected HMA1 and HMA2 values were compared (Table 2); otherwise, HMA1 and HMA2 demonstrated a significant difference in both the protein ($P = 0.0002$) and lipid ($P = 0.0015$) contents.

DISCUSSION

Our results suggest that the assessment of protein and lipid content in HM using *Miris* mid-infrared analyzers is significantly influenced by the device used. However, when correction factors specifically calculated for each device were employed, the *Miris* HMAs were sufficiently accurate for the targeted fortification of HM.

The reproducibility of mid-infrared assessment was satisfactory, yielding coefficients of variation of $< 3\%$ for all parameters. These results were consistent with previous reports for *Miris* HMAs,[19,20] as well as the Milkoscan 104 system,[15] suggesting that the *Miris* HMA is comparable to other similar instruments in terms of reproducibility. The volume of milk required is comparable or

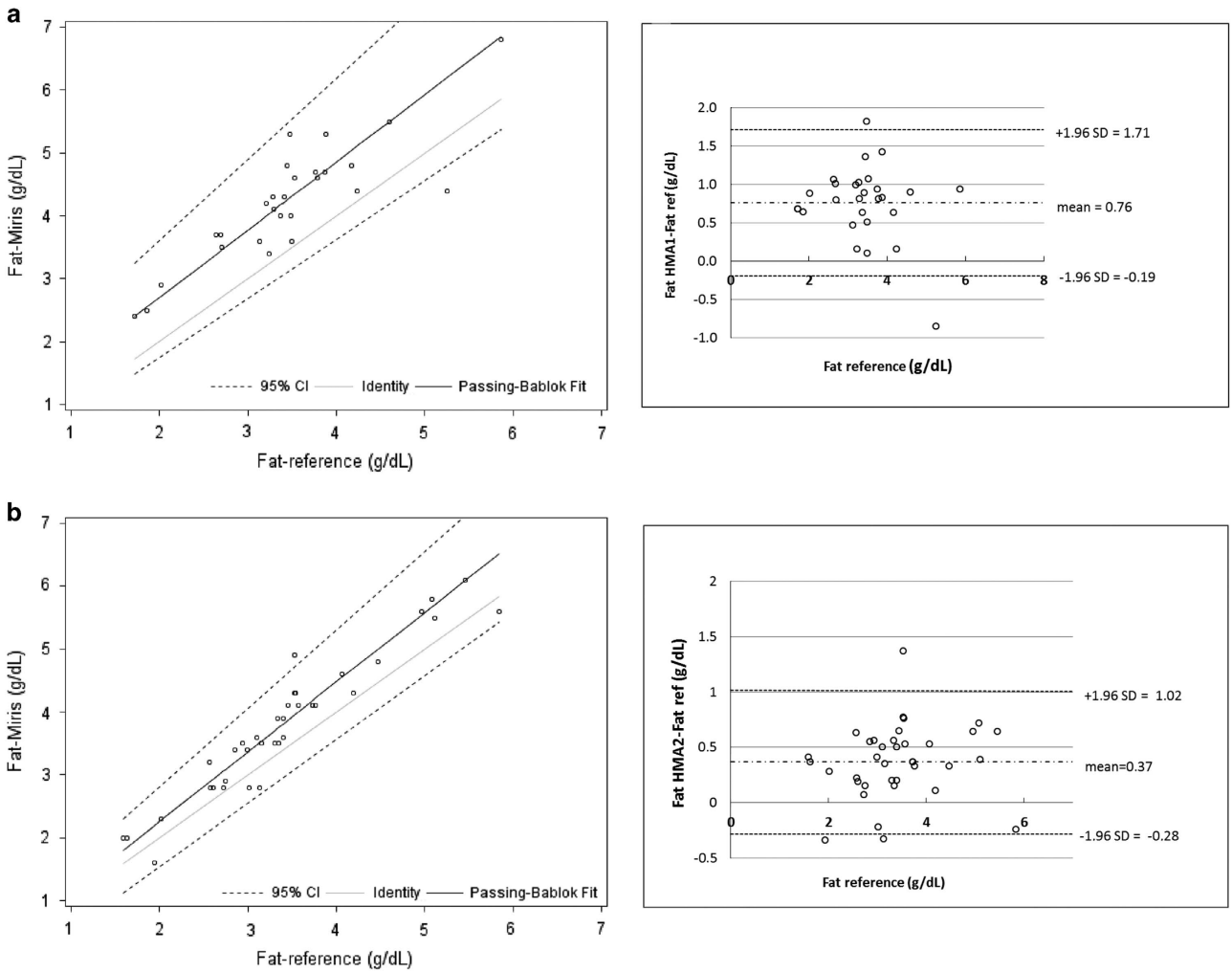

Figure 3. Relationships between fat concentrations measured using first-generation (HMA1) or second-generation (HMA2) *Miris* human milk mid-infrared analyzers (HMAs) and the reference biochemical method (Soxhlet). Relationships were assessed using Passing–Bablock linear regression analysis and the error is represented by Bland and Altman graphics (HMA1 (**a**) and HMA2 (**b**).

Table 2. Comparison on nutrient content assessment by HMAs (HMA1 versus HMA2)

Nutrient content of HM	*Period 1 (HMA1)*	*Period 2 (HMA2)*	P-*values*
Protein			
Biochemical method (total nitrogen), g dl^{-1}	1.33 ± 0.25	1.29 ± 0.28	0.14
HMA, g dl^{-1}	1.20 ± 0.29	0.94 ± 0.17	0.0002
Corrected HMA, g dl^{-1}	1.28 ± 0.28	1.31 ± 0.26	0.288
Lipids			
Biochemical method (Soxhlet), g dl^{-1}	3.90 ± 0.88	3.59 ± 0.6	0.0264
HMA, g dl^{-1}	4.59 ± 0.97	3.88 ± 0.9	0.0015
Corrected HMA, g dl^{-1}	3.77 ± 0.94	3.47 ± 0.81	0.0317

Abbreviations: HM, human milk; HMA, human milk analyzer. Corrected values and biochemical reference values in 12 identical human milk samples analyzed in 2008 (period 1) and in 2011 (period 2). Mean (s.d.) protein and lipid concentrations were measured with the first-generation device (HMA1) during period 1 and with the second-generation device (HMA2) during period 2. HMA results are presented as initial values (before correction) and corrected values (after using the correction equation calculated in the present study).

lower than that for other instruments such as Spectrastar[21] (1 ml), Milkoscan[11] (10 ml) and Fenir 8820 (Corvaglial *et al.*[16]; 5 ml).

We could have overestimated the protein content in HM, as we did not deducted non-protein nitrogen (free amino acids, ammonia and urea) from total nitrogen measured by chemical analysis. Non-protein nitrogen constitutes ~25% of total nitrogen in HM, much more than in most species (~5%).[30] However, the percent of urea nitrogen utilization is a function of the nitrogen requirement, which is high in very low birth weight infants. Using the method of metabolic balances, we previously observed that urea nitrogen excretion is higher in preterm infants fed fortified HM than in those fed a preterm formula.[31] The increase in urea nitrogen excretion accounted for around 50% of the nitrogen urea content of HM, suggesting that a significant part of the nitrogen urea content of HM is used for protein accretion. Therefore, subtracting the non-protein nitrogen to total nitrogen could significantly underestimate the protein equivalent nitrogen really.

In our study, HMA1 less underestimated the protein content, but greatly overestimated the fat content when compared with HMA2. The HMA1 instrument used in the present study was similar to the device used by Casadio *et al.*[19] Contrary to our results, Casadio *et al.*[19] reported an overestimation of protein content and for fat an overestimation that was far less remarkable than ours. We used the standard factory software dedicated to undiluted HM samples when Casadio *et al.*[19] used a device

specifically calibrated for both normal and diluted samples of HM. The findings of Casadio *et al.*[19] clearly suggest a negative correlation between the HMA-reference differences and the CR, indicating that a correction factor needs to be applied in order to achieve accurate results.

Silvestre *et al.*[32] also reported an underestimation of protein content. The protein content was markedly lower than that reported in our study and in the literature.[32] Moreover, their HMA also overestimated the fat content and they concluded that the instrument was not sufficiently accurate for clinical use without rigorous and systematic calibration.[20] Our results confirmed these findings, suggesting that Miris HMA2 requires calibration adjustment when used to measure samples for individual fortification because of the risk of protein overload associated to a relative deficiency in fat and energy supplies.

In a recent study, Fusch *et al.*[23] also reported underestimation of protein content, similar to our results with HMA2. Concerning lipids, the overestimation was less remarkable than that in the current study.[23] Although the two studies did not use the same reference method, this finding suggests that each machine, even if from the same manufacturer needs to be calibrated independently.

Therefore, our results confirmed that all mid-infrared analyzers require calibration by CR methods.[22] We calculated the correction factors for the two first generation *Miris* analyzers used in our unit by correcting the slope and intercept with the appropriate correction equation. This equation for protein assessment using HMA1 should be used with caution, because it was calculated for a relatively small number of samples. In the case of lipid assessment by HMA1, and protein and lipid assessment by HMA2, after applying this correction, we observed that the accuracy of both instruments was sufficiently high to avoid under- or over-fortification. However, our results indicated that both devices provide different results for the same sample when used without correction. Michaelsen *et al.*[15] and Fusch *et al.*[23] also reported that a correction factor is required for the Milkoscan 104 analyzer and SpectraStar, respectively. These data were further confirmed by De Halleux *et al.*[11] who found that the HMA measurement errors for Milkoscan Minor were 6.7% for nitrogen and 4.3% for fat, after correction. In our study, we evaluated two generations of *Miris* HMA on the same HM samples, to confirm the specificity of the correction equation and to compare their accuracy. After calibration adjustment, HMA1 was not sufficiently accurate but HMA2 provided good accuracy for protein and fat measurements. HMA1 is an older instrument and has been replaced progressively by new generation devices; however, it is still used by some medical teams.

Furthermore, our results suggest that the generation of each device should be clearly identified by the manufacturer; the identification number should be provided and this information should be presented in further articles. For Miris instruments, users should refer to the appearance of the machine and to the way the results are presented. The first-generation device has four control buttons and generates a single protein value; the second-generation device has six control buttons and presents a single protein value. The third-generation device, which became available recently, has six control buttons, but presents both 'crude' and 'true' protein values (the 'true' protein value is 80% the 'crude' protein value, as calculated by HMA3).

Our study has some limitations. First, we analyzed a defined range of protein and lipid contents, and did not include extreme values, because we were only analyzing undiluted HM samples. We choose to use undiluted milk, because dilution may introduce potential methodological errors. For the same reason, we did not manipulate the HM samples to prepare ranges of macronutrient content, as in recent studies,[23,33] and we processed the HM samples as recommended by the manufacturer, to ensure that the practices employed during routine conditions of use were followed.

A second limitation is that we calculated the correction equation using a limited number of samples. However, when we applied this equation on new samples, we observed no difference between the CR and the corrected HMA values for lipids in the case of HMA1 and for proteins and lipids in the case of HMA2, suggesting that this equation is reliable. For protein calculations with HMA1, the equation could be improved by using more samples.

Another limitation was the fact that 3 years had elapsed between the HMA and chemical analyses; the composition of HM could have changed during this period. The measurements for the reference method were performed 3 years apart and no variations were observed in the protein values. However, a remarkable difference was observed between the initial protein values of HMA1 and HMA2, highlighting the difference between the two devices. For lipids, a small variation of 8% was observed between the values and the same variation was also observed in the corrected values, consistent with the findings of Garcıa-Lara *et al.*[34] However, this does not explain the difference in lipid content assessed by HMA, which was 15% lower in the case of HMA2 than in HMA1.

In conclusion, our study demonstrated that HMA1 and HMA2 show differences in assessment, and that the crude values measured by first- and second-generation *Miris* HMAs were not appropriate for individualized protein and/or energy fortification without calibration adjustment by CR methods. Further investigations are needed to evaluate the intervariability of nutrient assessment by instruments of the same generation. Clear identification of each device's generation by the manufacturer is essential for the application of the appropriate correction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Is Milkoscan® a rapid infrared analyzer, after a specific calibration, accurate and precise enough for human milk fortification?

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Introduction

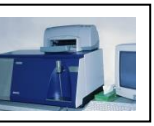
Human milk (HM) is the feeding of choice for preterm infants. However HM's macronutrient content is insufficient to cover their high nutritional needs, postnatal growth and development. Expressed HM is highly variable especially for protein and fat suggesting the need of individual fortification. Mid-infrared analyzer, originally developed for cow's milk analysis has been suggested as a rapid and simple method to analyze HM optimizing individual fortification in clinical routine (de Halleux 2013).

Objective

The aim of the study was to revalidate with chemical methods for protein and fat, our calibration equations in use on our mid-infrared analyzer.

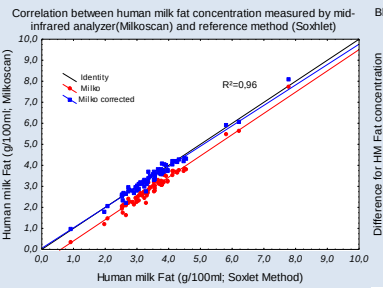
Material and methods

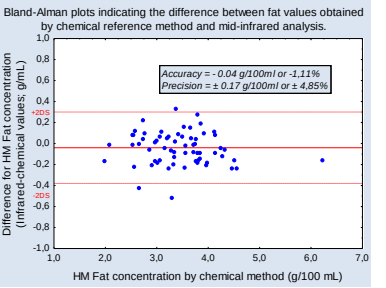
70 HM samples provided by mother of preterm infants to our NICU were evaluated with a mid-infrared analyzer (Milkoscan minor®,57 Foss) and the results of protein and fat contents were compared to chemical method providing total nitrogen (nitrogen analyzer EP 61 analyzer EP 428 Leco France) and fat ("Soxhlet") contents determined in our laboratory.Comparisons were performed using the Bland and Altman statistical method.



Results I: Fat

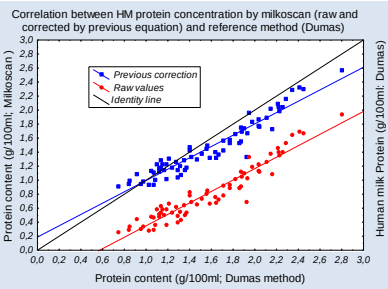
For fat, the agreement between the calibrated Milkoscan® and the "Soxhlet" method was high with a slope of 0.970 ± 0.016 and a correlation coefficient of R^2=0.96.

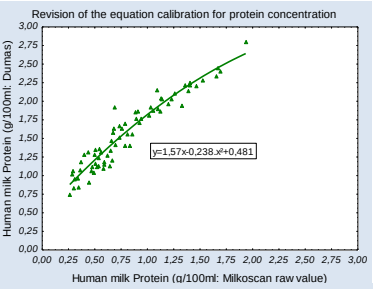


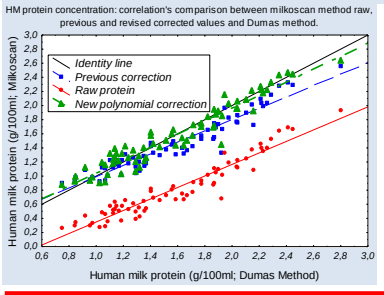


Results II: Protein

Protein equivalent* provided by previously calibrated Milkoscan® underestimated the values achieved by chemical method with a slope of 0.81±0.03 and a correlation coefficient of (R^2=0.91). Mean error of estimation was -0,11±0,15 g/dl. Precision was higher for nitrogen value ≤ 1, 5 g /dl (n=34) with an mean error of -0,02±0,12 g than for higher concentration > 1,5 g/dl (n= 36) with a mean error was -0,2 ±0,11 g.



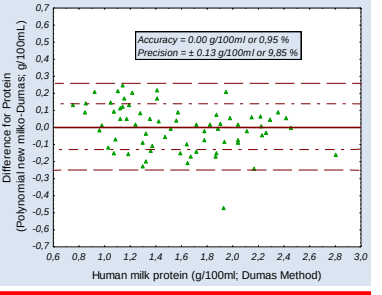




Results III: Protein concentration corrected by revised polynomial equation

After revision of the calibration's equation according to the present data, Protein nitrogen equivalent measured by the Milkoscan® was improved with a mean error of estimation < 1% ± 10% g/dl.

*Protein nitrogen equivalent (g/100 mL) was calculated as nitrogen in g *6.25



Conclusion:

Human milk calibrated Milkoscan® provides accurate protein and fat concentrations with a higher precision for fat than for protein allowing its use in clinical practice to provide individual HM fortification. In addition, our data suggest that the accuracy and precision remain stable for several months.

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Variability in human milk composition: benefit of individualized fortification in very-low-birth-weight infants[1–3]

Virginie de Halleux and Jacques Rigo

ABSTRACT

Background: Preterm infants fed fortified human milk (HM) grow more slowly than those fed preterm formulas. These differences could be related to the variability in the macronutrient composition of expressed HM, resulting in inadequate nutrient intake in relation to the estimated needs of the preterm infants.

Objectives: The aim of this article was to show the variability in HM composition from an infant's own mother's milk (OMM) or pooled HM from the milk bank. The second objective was to evaluate the advantages of individual fortification on nutritional intakes over standard fortification.

Design: The macronutrient composition of 428 OMM, 138 HM pools from single donors, 224 pools from multiple donors, and 14 pools from colostral milk was determined by using a mid-infrared analyzer. Individualized fortification was performed after analysis of the milk samples in 2 steps: adjustment of fat content up to 4 g/dL, followed by the addition of an HM fortifier to provide 4.3 $g \cdot kg^{-1} \cdot d^{-1}$ according to the daily prescribed volume of feeding. Nutritional intakes resulting from the individualized fortification were compared with calculated intakes resulting from standard fortification (HM fortifier: 4 packets/dL).

Results: The variability in contents of fat, protein, and energy was high for all types of HM samples. Compared with standard fortification, individual fortification significantly reduced the variability in nutritional intakes, allowing the maintenance of protein intake and the protein:energy ratio in the range of the nutritional recommendations.

Conclusions: The variability in expressed HM with respect to its protein and energy content is high. This variability persists after standard fortification, possibly resulting in under- or overnutrition. Because both over- and undernutrition confer risks in later development, individualized fortification optimizes protein and energy intake. *Am J Clin Nutr* doi: 10.3945/ajcn.112.042689.

INTRODUCTION

Human milk (HM)[4] is regarded as the gold standard in the provision of nutritional needs for all healthy and sick newborn infants during the first months of life (1). It contains nutrients necessary for growth and development but also numerous bioactive factors contributing to beneficial effects on host defense, gastrointestinal maturation (2, 3), infection rate (4–7), neurodevelopmental outcome (8–10), cardiovascular and metabolic disease (11, 12), and the infant's and mother's psychological well-being.

In preterm infants, there is a general agreement that the use of exclusive HM has short- and long-term beneficial effects on health and neurodevelopmental outcomes (1). However, preterm infants and particularly extremely-low-birth-weight (ELBW) infants are at risk of cumulative nutritional deficits and postnatal growth restriction during the first weeks of life up to the time of discharge or theoretical term (13, 14). It has been suggested that the neonatal period corresponds to a critical window when undernutrition does affect brain development (15–17). Preterm infants have higher protein, energy, mineral, and electrolyte requirements than term infants. Exclusive HM, even from an infant's own mother's milk (OMM) or banked HM cannot meet nutritional recommendations for ELBW infants (18, 19). Despite the benefits of HM fortification (20), growth in preterm infants fed fortified HM differs qualitatively and quantitatively from the optimal fetal growth and is also slower than that of preterm infants fed adapted preterm formulas (21–23). These differences could be related to the large variation in the nutritional value of expressed OMM or banked HM, particularly in terms of fat and protein contents (24–26). We recently suggested that the use of individualized HM fortification improves nutritional support and growth in very-low-birth-weight (VLBW) infants (27). As a result, since 2006, this procedure of fortification has been used for feeding VLBW in our neonatal intensive care unit (NICU).

The aim of the present study was to evaluate the variability in HM composition of both OMM and bank HM pools provided daily to our NICU. The secondary objective was to evaluate the influence of an individualized HM fortification procedure on nutritional intakes in preterm infants compared with standard fortification.

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[4] Abbreviations used: BUN, blood urea nitrogen; ELBW, extremely low birth weight; HM, human milk; MCT, medium-chain triglyceride; NICU, neonatal intensive care unit; OMM, own mother's milk; VLBW, very low birth weight.

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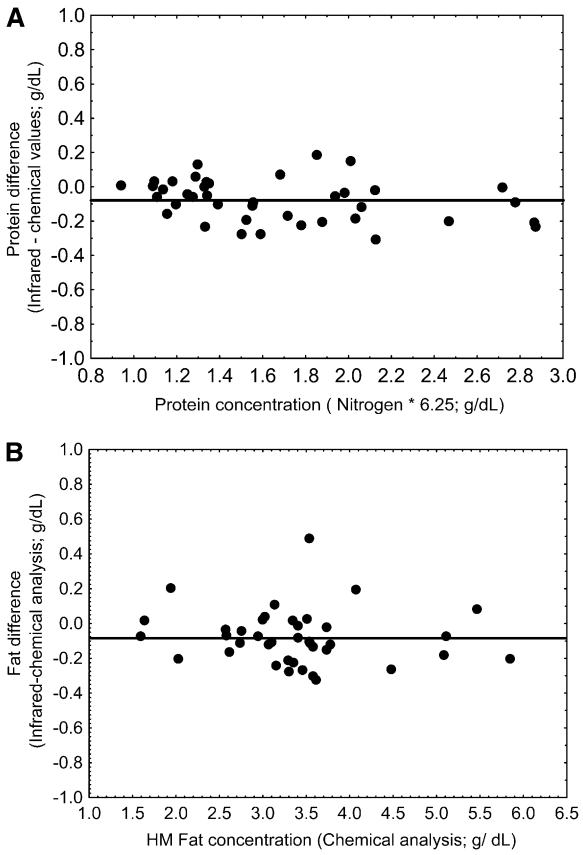

FIGURE 1. Accuracy of protein (A) and fat (B) determination in human milk (n = 40) with the use of infrared technology compared with chemical analysis as the gold standard using Bland-Altman plots (29). HM, human milk.

SUBJECTS AND METHODS

Validation of an infrared HM analyzer

HM analyses were performed with a mid-infrared analyzer (Milkoscan Minor; Foss) (27, 28). The instrument, originally developed for cow milk analysis in the dairy industry, requires additional calibration for HM use. It needs ~10 mL HM to provide data on protein, fat, and carbohydrate contents in 90 s. Results of 40 HM samples from our HM bank were analyzed in our laboratory, for comparison to chemical analysis for nitrogen (nitrogen analyzer EP Analyzer EP 428; Leco France) and fat ("Soxhhlet" Soxtec Aventi 2055; Foss).

Variability in daily composition of OMM and of pools of HM from the milk bank

By using a mid-infrared analyzer (Milkoscan Minor), the macronutrient composition of 428 OMM samples used for individualized OMM fortification were obtained. In addition, data from HM pools from one single donor (5 L HM from one mother), pools from multiple donors (5 L from multiple-donor mothers), and pools of colostral milk (<8 d lactation, multiple donors) were also obtained at the milk bank of the NICU at the University of Liège, Belgium. HM was expressed at the hospital or at home, by manual expression or by using an electric pump, and transported under aseptic HACCP (Hazard Analysis Critical Control Point) conditions in accordance with written instructions to the mothers regarding mechanical expression, milk collection, storage, and transport. OMM provided by the mother was kept at 4°C and used within 72 h. A bacteriologic count was performed on the day of receipt to allow its use as raw milk or as requiring pasteurization or elimination. Milk samples of cytomegalovirus-positive mothers were also pasteurized. To allow individualized fortification, a sample of 10 mL was taken from the daily pool and analyzed before fortification. The surplus milk could be kept in the refrigerator to be used within 72 h of extraction or frozen for later use. All donor HM had been frozen and pasteurized by the Holder method (62.5°C for 30 min) and warmed by thawing to 37°C before analysis. The energy content was calculated by using the Atwater factors: 4 kcal/g for protein and carbohydrate and 9 kcal/g for fat.

Nutritional intakes resulting from individualized and standard HM fortification procedures

The individualized HM fortification protocol was designed in 2 steps to meet the current nutritional recommendations for premature growing infants (18, 19). This protocol has been routinely in use in the NICU for VLBW infants since 2006. First, the fat content of HM was adjusted up to 4 g/dL when necessary by using medium-chain triglycerides (MCTs; Liquigen Danone Nederland), a stabilized 1:1 mixture of MCTs and water (0.5 g/mL). Second, protein content was adjusted by using a complete powdered HM fortifier (Enfamil Human Milk Fortifier; Mead Johnson) to provide 4.3 g protein $\cdot$ kg^{-1} $\cdot$ d^{-1} according to the daily prescribed volume of feeding. The nutritional composition of OMM, the MCT and the fortifier supplementation, the prescribed volume, and the infant's body weight at the day of prescription were collected at the milk bank for calculating the

TABLE 1
Protein, fat, carbohydrate, and energy concentrations of own mother's milk, single- and multiple-donor milk pools, and colostral pools[1]

	Own mother's milk (n = 428)[2]	Single-donor milk pool (n = 138)	Multiple-donor milk pool (n = 224)	Colostral pool (n = 14)[3]
Protein (g/dL)	1.52 ± 0.28[a]	1.34 ± 0.37[b]	1.46 ± 0.24[c]	2.00 ± 0.09[d]
Fat (g/100 mL)	3.79 ± 0.73[a]	3.45 ± 0.60[b]	3.39 ± 0.48[b]	2.92 ± 0.35[c]
Carbohydrate (g/dL)	6.76 ± 0.27[a]	6.93 ± 0.38[b]	6.81 ± 0.20[a]	6.51 ± 0.14[c]
Energy (kcal/dL)	67.3 ± 6.5[a]	64.1 ± 5.9[b]	63.6 ± 4.5[b]	60.3 ± 3.5[b]

[1] All values are means ± SDs. Values not sharing a common superscript letter are significantly different, $P < 0.05$ (1-factor ANOVA with Bonferroni correction for multiple comparisons).
[2] Own mother's milk: 28 ± 10 d of lactation.
[3] Colostral pool: donor milk <8 d.

nutritional intakes per kilogram of body weight per day. In addition, the theoretical nutritional intakes per kilogram of body weight per day corresponding to a standard HM procedure (4 packets complete HM fortifier/dL, providing 1.1 g protein, 1 g lipids, and 14 kcal energy; Enfamil Human Milk Fortifier) were also estimated.

Statistical analysis

The difference between infrared analyzer and chemical analysis for nitrogen and fat concentrations were evaluated by regression analysis and Bland-Altman plots (29) by using chemical analysis as the gold standard.

Macronutrient composition and variability in OMM and HM pools from a single donor, multiple donors, and colostral pools were compared by using 1-factor ANOVA with Bonferroni correction for multiple comparisons.

The variability in the nutritional content of the different milk groups and the nutritional intakes resulting from individualized or standard fortification were calculated as the mean value of the absolute difference between all individual values and the mean according to the following formula:

$$\text{Variability}(\%) = \text{mean}[|x(1 \text{ to } n) - \text{mean}| \times 100/\text{mean}] \quad (1)$$

Nutritional intakes and variability resulting from individualized and standard fortifications were compared by using paired Student's t test. All statistical analyses were performed by using Statistica software version 10 (StatSoft).

RESULTS

Validation of an infrared HM analyzer

Validation of the infrared HM analyzer was determined on 40 HM samples. A highly significant positive linear correlation was found between chemical reference values and infrared analysis ($P < 0.001$; $r = 0.97$ and 0.99 for protein and fat, respectively). Both regression lines did not differ significantly from the identity line. With the use of chemical analysis as the gold standard, Bland-Altman plots (29) showed that the precision for nitrogen and fat estimation using infrared analysis corresponded to 6.7% and 4.3%, respectively, of the reference values (**Figure 1**).

Variability in daily composition of OMM and in HM pools from the milk bank

Mean (±SD) values for protein, fat, carbohydrate, and energy content of OMM ($n = 428$), single-donor HM pools ($n = 138$), multiple-donor HM pools ($n = 224$), and colostral pools ($n = 14$) are shown in **Table 1**. Significantly higher protein content and lower fat, carbohydrate, and energy contents were observed in the colostral pools (donor milk from 1 to 7 d of lactation) than in all the other groups. In OMM, mean protein, fat, and energy contents were significantly higher than in single- and multiple-donor milk pools. In addition, the protein content of single-donor milk pools was significantly lower compared with multiple-donor milk pools. Overall, of the 804 samples, 80% ($n = 640$) had a fat content <4 g/dL, whereas 51% ($n = 413$) had an energy content <65 kcal/dL. The protein content was <1.2 g/dL in 17% of samples ($n = 141$), between 1.2 and 1.6 g/dL in 50% of samples ($n = 402$), and >1.6 g/dL in 30% of samples ($n = 243$) (**Figure 2**).

The variability in protein, fat, and energy contents was high in the various groups (**Table 2** and shown in Figure S1 under "Supplemental data" in the online issue). The variability in protein content was higher in single-donor pools and lower in colostral pools than in all other groups. The variability in fat content was higher in OMM than in all other groups, but the

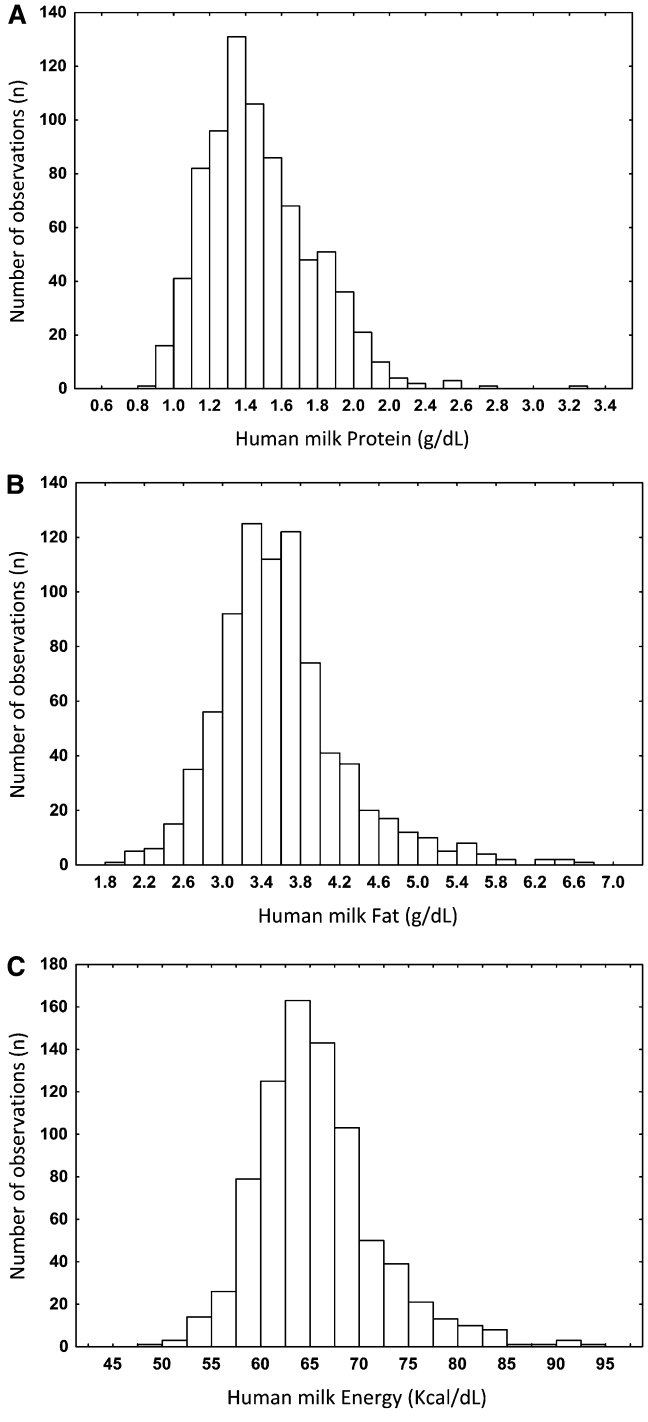

FIGURE 2. Variability in protein, fat, and energy concentrations of own mother's milk and human milk pools ($n = 804$).

TABLE 2
Variability in protein, fat, and energy contents of own mother's milk, single- and multiple-donor milk pools, and colostral pools[1]

	Percentage of variability[2]			
	Own mother's milk (n = 428)	Single-donor milk pool (n = 138)	Multiple-donor milk pool (n = 224)	Colostral pool (n = 14)
Protein	14.7 ± 10.6[a]	19.3 ± 19.4[b]	13.5 ± 9.9[a]	3.8 ± 2.4[c]
Fat	14.5 ± 12.7[a]	10.3 ± 8.4[b]	10.6 ± 9.4[b]	9.7 ± 6.5[a,b]
Energy	7.3 ± 6.26[a]	6.9 ± 6.0[a]	5.3 ± 4.7[b]	4.4 ± 3.6[a,b]

[1] All values are means ± SDs. Values not sharing a common superscript letter are significantly different, $P < 0.05$ (1-factor ANOVA with Bonferroni correction for multiple comparisons).
[2] Variability(%) = mean[|x(1 to n) − mean| × 100/mean].

difference was not significant compared with the colostral pool (P = 0.08).

Nutritional intakes and variability resulting from individualized and standard HM fortification procedures

Between June 2006 and December 2011, 428 daily OMM individualized fortifications were performed in 24 VLBW preterm infants (mean ± SD birth weight = 1140 ± 230 g; gestational age = 28.6 ± 1.6 wk) over >3 wk. MCT supplementation was necessary in 64% (272 of 428) of daily OMM pools and HM fortifier was necessary in 99.5% (426 of 428) of daily OMM pools. The nutritional content of OMM after MCT supplementation and HM fortification is shown in **Table 3**. By comparison to standard fortification, protein intakes and the protein:energy ratio of individualized fortification were significantly lower, whereas the fat and the energy contents were significantly higher, with individualized fortification. The variability in nutritional intakes and protein:energy ratio were significantly lower using individualized compared with standard fortification. Thus, the variability in protein intake after individual fortification was reduced by 21% of the variability after standard fortification (**Table 4** and **Figure 3**).

DISCUSSION

Several studies have shown an association between short- and long-term health, as well as neurodevelopmental outcomes, and cumulative intakes of HM during the early weeks of life in VLBW infants (20, 30). However, the use of HM as a sole source of nutrients is insufficient to cover the high nutritional requirements of growing preterm infants. OMM, with its higher protein content, improves growth compared with banked HM (31, 32), but remains suboptimal to support growth, especially lean body mass gain after the second or third week of lactation. Despite various HM fortifiers developed to increase protein, energy, minerals, electrolytes, trace elements, and vitamin supplies (20, 33), the use of fortified HM has failed to obtain postnatal growth in the range of fetal growth or that observed in infants fed preterm formulas (21–23).

In the present study, we showed that the macronutrient and energy composition of OMM and banked donor HM used for nutrition in preterm infants in the NICU are highly variable, leading to a high rate of protein and energy deficits compared with reference values.

As shown in Figure 1, protein, fat, and energy contents ranged from 0.8 to 2.4 g/dL for protein, from 1.8 to 6.6 g/dL for fat, and from 47 to 85 kcal/dL for energy. Furthermore, as shown in Figure 2, of all daily OMM and HM pool samples, 56% were <1.5 g protein/dL, whereas 79% were <4 g lipids/dL, and 67% were <67 kcal energy/dL (values frequently considered as reference values for preterm milk composition). These results differ from the recent reference values reported by Bauer and Gerss (34) who evaluated nutritional composition of OMM collected longitudinally from mothers of ELBW infants. In this study, they suggested that in OMM between 28 and 32 wk the protein content could be as high as 2.3–1.9 g/dL, whereas the fat and the energy content accounted for 4.4 g/dL and 77 kcal/dL, respectively.

Protein values of preterm mother's milk are generally higher in the early postnatal period and decrease during lactation. However, a high variability remains between and within mothers (34). The present study confirms these 2 observations as shown in Figures S2 and S3 under "Supplemental data" in the online issue. Incomplete milk expression and manipulations of HM during expression, storage, transport, and processing are all additional factors influencing the high variability in expressed HM composition. Indeed, in clinical practice, it is not possible for mothers of preterm infants to follow the strict guidelines and methodology as proposed in a prospective study on HM composition (34). The fat content is highly related to manipulation and processing between expression and delivery to the preterm infants. As a result, the true energy and protein contents are unpredictable and differ significantly from those calculated by using a fixed composition for OMM or banked HM.

TABLE 3
Composition of OMM before and after individualized fortification with MCTs and HMF[1]

	OMM	OMM + MCTs[2]	OMM + MCTs + HMF[3]
Protein (g/dL)	1.52 ± 0.28	1.52 ± 0.27	2.51 ± 0.14
Fat (g/dL)	3.79 ± 0.73	4.20 ± 0.45	5.09 ± 0.48
Carbohydrate (g/dL)	6.76 ± 0.27	6.76 ± 0.27	7.11 ± 0.28
Energy (kcal/dL)	67.26 ± 6.49	70.13 ± 4.52	82.66 ± 4.42
Protein:energy ratio	2.27 ± 0.37	2.17 ± 0.35	3.04 ± 0.19

[1] All values are means ± SDs; n = 428. HMF, human milk fortifier; MCT, medium-chain triglyceride; OMM, own mother's milk.
[2] Fat concentration of human milk was adjusted up to 4 g/dL when necessary by adding MCTs.
[3] Protein content was adjusted by using a complete HMF to provide 4.3 g protein · kg^{-1} · d^{-1} according to daily volume of feeding.

TABLE 4
Comparison of individualized fortification intakes and percentage of variability with theoretical values obtained after standard fortification[1]

	Individualized fortification	Standard fortification
Intake		
Protein ($g \cdot kg^{-1} \cdot d^{-1}$)	4.25 ± 0.13*	4.45 ± 0.51
Fat ($g \cdot kg^{-1} \cdot d^{-1}$)	8.6 ± 0.9*	8.1 ± 1.3
Energy ($kcal \cdot kg^{-1} \cdot d^{-1}$)	140 ± 9*	138 ± 13
Protein:energy ratio	3.04 ± 0.19*	3.24 ± 0.32
Variability (%)		
Protein	2.0 ± 2.3*	9.2 ± 6.8
Fat	6.6 ± 7.4*	12.1 ± 10.3
Energy	4.8 ± 4.5*	7.3 ± 6.1
Protein:energy ratio	4.5 ± 4.3*	7.6 ± 6.5

[1] All values are means ± SDs; $n = 428$. Intakes and variability resulting from individualized and standard fortifications were compared by using paired Student's t test. *$P < 0.05$ when compared with standard fortification.

Growth differences between fortified HM and preterm formula-fed VLBW infants receiving an apparently similar energy and protein intake could also be related to a lower content of metabolizable protein and energy available for new tissue synthesis. Metabolic balance studies (35, 36) showed that nitrogen absorption as well as nitrogen utilization were lower in preterm infants fed fortified HM than in those fed preterm formulas. In all, the mean difference in nitrogen utilization accounted for 5.5% and could be related to nonnutritional proteins (lactoferrin, IgA) or nonprotein nitrogen content (urea) in HM. Net absorption of fat-derived energy was also frequently lower (78.3%) in infants fed HM than in those fed formula (88.4%), resulting in a higher fecal loss of energy. This difference could be increased by the use of pasteurized HM (37). Pasteurization of HM for high-risk preterm infants is frequently applied in milk banks and in neonatal units to reduce bacterial contamination and the risk of cytomegalovirus infection (38, 39). Pasteurization leads to inactivation of the bile salt–stimulated lipase of HM (40) as well as possible changes in the milk fat globule structure (41).

Standard fortification, adding a fixed amount of a fortifier as recommended by the manufacturer, is the most commonly used method to fortify mother's milk. This method was not associated with a reduction in the variability in HM nutritional contents and often failed to meet the nutritional recommendations for preterm infants (42, 43). A more suitable fortification regimen was suggested to improve nutritional intakes and growth in preterm infants. Arslanoglu et al (44) adjusted the fortifier supply according to the values of blood urea nitrogen (BUN) considered to be a marker of protein adequacy in preterm infants. This BUN method, which was developed to avoid inadequate and excessive protein intake, is easy to apply and does not require daily milk analyses. However, it has been shown that BUN is not correlated to protein intakes during the first weeks of life but reflects the renal immaturity of ELBW and VLBW infants (45, 46). Therefore, the use of BUN as a threshold level to adjust protein intake is inadequate. Polberger et al (47, 48) have proposed analyzing, once or twice a week, the macronutrient content of 24-h OMM collections so as to adapt the fortification in the range of nutritional needs. Recently, Miller et al (49) suggested that an increase in the protein fortification from 1 g/dL to 1.4 g/dL produces a minimal benefit on growth in preterm infants. They found no significant increase in daily weight gain but a significant reduction in incidence of growth restriction in the higher protein group. However, such an increase in protein fortification does not compensate for the variability in HM composition. The risk of energy deficiency as well as of protein overload remains, with its potential long-term adverse effects. In 2007 we suggested that daily individualized HM fortification could provide nutritional supplies in the range of the nutritional recommendations and improve growth in VLBW infants (27).

In the present study, we confirm the high daily variability in the nutritional value of HM within a large number of samples of OMM, and that this variability could be reduced by daily individualized fortification. With standard fortification, protein deficiency or overload, and energy deficiency were frequently observed (Figure 3, A and B). By contrast, after individualized fortification, the range of protein intake decreased from 3.3–6.6 to 3.6–4.5 $g \cdot kg^{-1} \cdot d^{-1}$ and that of the protein:energy ratio from 2.4–4.7 to 2.4–3.8 g/100 kcal (Figure 3, A and C). With this technique, we showed that appropriate nutritional intakes could be provided daily in the upper range of recent ESPGHAN (European Society of Paediatric Gastroenterology, Hepatology, and Nutrition) recommendations (19). In addition, with individualized fortification, the mean use of fortifier was significantly lower (3.6 compared with 4.0 packets/dL), decreasing the osmolality of the fortified HM and the risk of gastric intolerance.

The currently available multicomponent HM fortifiers are not adequately designed for use in VLBW infants. In the present study, the relative fat deficit of expressed HM provided to the NICU was corrected with an MCT emulsion. However, the fatty acid profile of the fortified HM remains inadequate for preterm infants, especially in terms of long-chain PUFA content. Therefore, newer fortifiers providing high protein and energy intakes with adequate long-chain PUFA content, but without inducing a gastrointestinal osmotic load >360–400 mOsm/kg H_2O, need to be developed to improve the nutritional supply with minimal side effects for the preterm infants.

Although individualized fortification is time consuming and expensive and requires additional equipment and well-trained staff, the use of infrared technology to determine the macronutrient composition of HM is likely to expand its availability in NICUs. It could have practical application in HM banks for donor milk composition or to develop specific HM pools with higher protein and/or energy content.

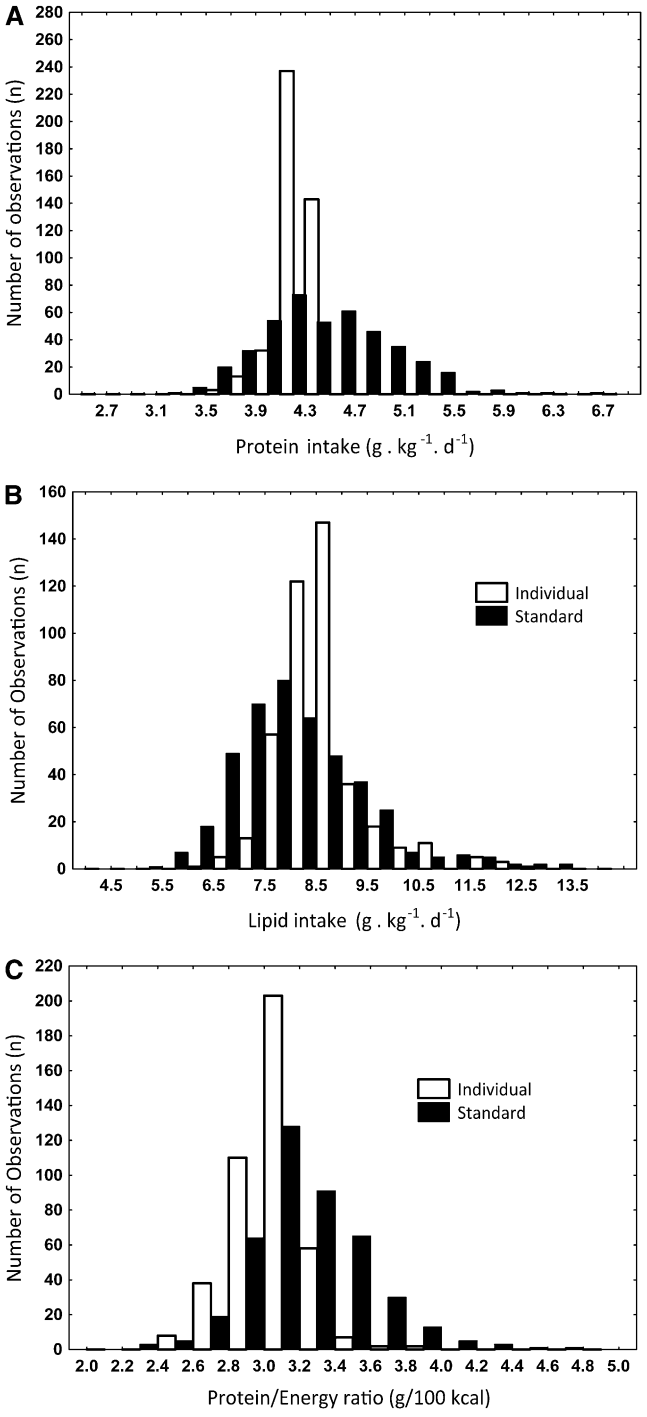

FIGURE 3. Protein (A) and lipid (B) intakes and protein:energy ratio (C) according to individualized or standard human milk fortification (n = 428).

As a result of the lower energy and protein bioavailability of HM, an energy intake of 140 kcal · kg^{-1} · d^{-1} and a protein intake of 4.2 g · kg^{-1} · d^{-1} were estimated to be necessary to ensure an adequate growth. These values are slightly higher than those recently recommended by the ESPGHAN Committee on Nutrition in 2010 (19). These recommendations are more related to preterm infants fed formula than to those fed fortified HM, and recent studies suggest that specific recommendations for the use of HM are necessary. These new recommendations need to consider the lower metabolizable energy and protein content of fortified HM, the effect of pasteurization, and the additional nutritional losses suggested during continuous feeding (27, 50).

In conclusion, the macronutrient content of expressed preterm OMM and donor HM pools is widely variable, especially for protein, fat, and energy. Standard fortification, as recommended by the manufacturer, does not meet the high nutritional requirements of immature infants, thereby creating conditions for under- or overnutritional risks. Individualized fortification based on daily HM analysis improves and regulates the protein and energy intakes in preterm infants but requires equipment and a well-trained staff. Further studies are necessary to improve the fortifier formulation to meet individual needs and new recommendations, and studies particularly dedicated to ELBW and VLBW infants fed HM need to be developed.

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The authors' responsibilities were as follows—VdH: was the principal investigator in the study and contributed to the conception and design of the study and acquisition, analysis, interpretation of data; drafted the manuscript; revised the manuscript for important intellectual content; and had final approval of the draft that was submitted for publication; and JR: contributed significantly to the conception and design of the study and analysis and interpretation of data, participated in drafting the manuscript and providing in-depth revision for important intellectual content, and had final approval of the draft that was submitted for publication. Neither of the authors had a conflict of interest to declare.

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Intérêt de la supplémentation du lait maternel « à la carte »

Advantages of individualized fortification of human milk for preterm infants

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Résumé

Malgré l'utilisation de lait maternel fortifié pour l'alimentation des grands prématurés, leur croissance reste généralement inférieure à celle des prématurés alimentés avec une formule de lait adapté. Une variabilité de composition du lait maternel tiré, fréquemment plus pauvres en protéines et en énergie que sa composition théorique, peut être à l'origine d'une telle différence. Dès lors, une analyse rapide de la composition initiale du lait de mère devrait permettre d'ajuster la composition du régime de manière individualisée et adaptée aux besoins du prématuré. Dans ce but, une méthode rapide d'analyse de la composition du lait par spectroscopie infrarouge (Milkoscan®) a été validée pour le lait maternel. Nous avons ensuite étudié la variation de composition en protéines, lipides et énergie des échantillons de lait apportés dans notre unité et comparé les apports nutritionnels selon deux méthodes de fortification, standardisée et « à la carte ». Avec la fortification standardisée, la variabilité de composition en protéines et lipides du lait maternel persiste entrainant un risque de carence ou de surcharge en protéines ainsi qu'un risque de déficit énergétique. En revanche, la fortification « à la carte » permet de stabiliser l'apport protéique avec un apport moyen de fortifiant moindre, réduisant le risque d'hyperosmolarité. De même, l'adaptation de la composition du lait maternel en graisse permet d'obtenir un apport énergétique plus élevé conforme aux besoins. Évalués chez 10 prématurés de très faible poids à la naissance, cette fortification « à la carte » favorise la croissance et permet d'obtenir un gain pondéral moyen de 21 g/kg/j.

Abstract

Despite the benefits of human milk fortification, nutrients of human milk are not sufficient to cover the greater needs of very low birth weight and to ensure a growth similar to that of premature infants fed with preterm formula. These differences could be related to the variation in the macronutrient composition of expressed breast milk with lower protein and energy content. Unfortunately there is unusually no information on macronutrients composition prior human milk fortification. With such data, it would be possible to individualize the fortification. In order to use adjustable fortification of human milk, we have assessed a rapid and simple method using full spectrum infrared laser technology (Milkoscan®) to analyze human milk composition. We describe the variation in concentration of protein, lipid and energy in the human milk received in our neonatal unit. Then we evaluate the benefit of adjustable fortification of human milk compared with standard fortification. After standard fortification the variability of protein and lipid remains with a risk of protein deficiency or excess and a risk of energy deficiency. After adjustable human milk fortification based on human milk analysis using Milkoscan®, we observe a more stable protein content and a lower amount of added fortifier decreasing the risk of hyperosmolarity. Furthermore, the energy content is higher following of the fat human milk adjusted content. Up to now, our preliminary results suggest that individualized fortification of human milk improves growth rate in preterm infants (21 g/kg/d) to a level close to formula fed infants.

Mots clés : Croissance ; Fortification ; Lait maternel ; Prématurés ; Nutrition

En raison de ses nombreux avantages, l'alimentation au lait de mère est recommandée pour tous les enfants y compris les prématurés [1]. Les bénéfices du lait de mère sur la

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fonction gastro-intestinale, l'immunité, le développement neurologique et relationnel sont bien connus [1-3]. Afin de préserver au maximum ses multiples propriétés, l'utilisation du lait maternel cru doit être favorisée [3]. Une étude récente, portant sur des anciens prématurés âgés de 18 mois, a montré que le quotient de développement serait fonction de la quantité de lait maternel reçue [4].

Cependant, l'utilisation du lait de mère dans l'alimentation du prématuré a ses limites. Les mères d'enfants prématurés ont souvent une production de lait maternel limitée et l'accès au lait de lactarium n'est pas toujours possible. L'excrétion de virus potentiellement transmissibles au prématuré [5] et le risque de contamination bactériologique au cours des différentes manipulations peuvent rendre le lait impropre à la consommation ou nécessiter une pasteurisation [6]. Le lait maternel, de par sa composition, ne permet pas de couvrir les besoins nutritionnels élevés des prématurés et de leur assurer une croissance optimale [7].

Il en résulte dès lors, que pour les prématurés alimentés au lait de mère, il est nécessaire d'enrichir le lait maternel tant en protéines qu'en énergie, minéraux, sodium, oligoéléments et vitamines [8]. Différentes méthodes de fortification ont été proposées dans la littérature. La supplémentation est classiquement utilisée de façon standardisée, en ajoutant une quantité fixe de fortifiant sans tenir compte de la composition nutritionnelle initiale variable du lait de la propre mère, de celle des laits de lactarium utilisés en complément, ainsi que des réductions de valeurs nutritionnelles (pertes de graisses et d'énergie) pouvant survenir au cours des manipulations.

Les études réalisées avec ces types de fortification ont montré que la croissance des enfants prématurés nourris au lait de mère enrichi était inférieure à celle des enfants nourris avec une formule de lait adaptée [9-11]. La réalisation de bilans métaboliques chez ces prématurés a montré que cette différence pouvait en partie s'expliquer par des apports moindres en protéines et en énergie mais aussi par une moindre biodisponibilité des protéines et de l'énergie fournies [11].

Dès lors, d'autres types de fortifications ont été développés [12-14]. Certains proposent d'adapter la supplémentation du lait maternel à la tolérance métabolique du prématuré en se basant sur le dosage de l'urée plasmatique. Cette méthode présente l'avantage de pouvoir être facilement réalisée. Cependant l'urée ne reflète pas uniquement la charge protéique du prématuré et peut être influencée par des situations tels que l'insuffisance rénale, la déshydratation ce qui retarde l'augmentation progressive des apports protéiques. Dès lors, une autre forme de fortification est actuellement développée : la supplémentation « à la carte » qui tente d'adapter la composition du lait maternel aux besoins du prématuré et à la composition initiale du lait de mère [12,14]. Dans notre service, au cours de ces derniers mois, nous avons tenté d'évaluer cette forme de supplémentation « à la carte » en développant tout d'abord une méthode rapide d'analyse de la composition du lait de mère, et en comparant par la suite les apports nutritionnels ainsi que la croissance des prématurés selon le mode de fortification standardisée et « à la carte ».

1. Fortifiants de LM disponibles

La fortification du lait de mère au moyen de fortifiants complexes contenant à la fois des protéines, de l'énergie des minéraux, des électrolytes, des oligoéléments et des vitamines est devenue le standard de la majorité des unités néonatales (Tableau 1). La composition de ces fortifiants a évolué au cours des années. Au départ, ils étaient constitués principalement de protéines entières ou hydrolysées et d'énergie sous forme de dextrines.

Différentes études ont montré, que l'adjonction de ces fortifiants, modifiait les propriétés du lait maternel. Ainsi en raison de leur teneur osmotique, mais aussi de l'activité persistante de l'amylase du lait de mère même après pasteurisation, l'osmolarité du lait maternel fortifié est significativement augmentée [15]. L'expérience clinique montre que cette augmentation d'osmolarité peut altérer la tolérance digestive du prématuré et mener à des modifications de prescription et dès lors d'apports nutritionnels. Néanmoins, une méta-analyse comparant les laits de mère enrichis ou non n'a pas permis de mettre en évidence de différence significative concernant la tolérance digestive chez le prématuré [8]. En revanche, la fortification semble constituer un facteur de risque pour l'entérocolite nécrosante chez les grands prématurés [16]. Ce risque reste cependant inférieur à celui des enfants nourris au lait artificiel [3]. L'effet de la fortification sur les propriétés immunologiques du lait de mère semble peu importante [17].

Plusieurs études ont évalué l'impact de la fortification du lait maternel sur la croissance, la balance métabolique et la qualité de la prise pondérale [8-11]. L'ajout d'un fortifiant améliore la croissance staturo-pondérale et celle du périmètre crânien. Néanmoins, la croissance obtenue après fortification du lait de mère reste inférieure à la croissance obtenue avec les formules pour prématurés.

Tableau 1
Composition de différents fortifiants exprimée pour 1 g de protéine

Pour 1 g de protéine	BMF (Numico) poudre	FM-85 (Nestlé) poudre	EHMF3 (Mead Johnson) sachet 0,7 g
Protéine (g)	1	1	1
Graisse (g)	0	0,0	0,91
Glucides (g)	3,75	3,3	0,2
Na (mg)	12,5	20	15
K (mg)	10	42	26
Ca (mg)	81	75	82
Mg (mg)	7,5	2,4	0,9
P (mg)	56	45	45,0
Cl (mg)	8.7	17	12
Energie (kcal)	18,8	17,4	12,7
Osmol.(mOsm)	85	96	40,0

Plus récemment, de nouveaux fortifiants contenant des graisses en lieu et place de dextrine comme substrat énergétique ont été développés particulièrement aux États-Unis. Ces fortifiants ont l'avantage d'augmenter l'apport énergétique sans augmenter l'osmolarité et ne semblent pas perturber la stabilité du lait maternel frais qui conserve son activité lipasique. Quelques études ont permis de montrer récemment que l'utilisation de ces fortifiants contenant des graisses permettait d'améliorer significativement la croissance des prématurés [18,19].

2. Intérêt d'une supplémentation « à la carte » par rapport à une supplémentation standard

2.1. Analyse de la composition du lait de mère

L'évaluation d'une supplémentation « à la carte » nécessite de disposer d'une méthode rapide et fiable d'analyse de la composition nutritionnelle du lait de mère. Au préalable, la composition du lait maternel était réalisée dans notre laboratoire à l'occasion de la réalisation de bilan métabolique [20]. Ces méthodes classiques requièrent des échantillons de lait volumineux et sont relativement longues et fastidieuses à réaliser. Elles sont mal adaptées à une utilisation clinique quotidienne.

2.2. Intérêt du Milkoscan®

Dans l'industrie laitière, il existe une méthode simple de mesure de la composition en protéines, glucides et lipides du lait de vache qui peut être réalisée sur un faible échantillon (10 ml). Il s'agit d'un appareil d'analyse par spectroscopie proche infrarouge dont le principe repose sur l'absorption du rayonnement proche infrarouge par la matière organique [21]. Cette méthode peut être utilisée également pour le dosage des nutriments du lait de mère [12,22] mais nécessite une calibration préalable de l'appareil. La première partie de notre travail a consisté à établir les équations de corrections permettant de déterminer les teneurs en azote total, en graisses et en hydrate de carbone du lait de mère (Close A, mémoire de fin d'étude de Licence en sciences biomédicales, ULg, 2005). Dans la suite, ces équations ont été validées afin de déterminer la précision de la mesure par le Milkoscan®. Ainsi, nous avons pu montrer que la précision de la mesure était de 3,6 % pour les protéines, 2,7 % pour les lipides et 1,8 % pour les glucides. Ces valeurs sont tout à fait satisfaisantes et nous pouvons dès lors considérer que l'utilisation de cet appareil peut être recommandée pour l'analyse en routine du lait de mère au sein d'un lactarium ou d'une biberonnerie. Une analyse complète de la composition du lait de mère peut ainsi être réalisée sur un échantillon de 10 ml en moins de deux minutes.

2.3. Variabilité de la composition en protéines et en énergie du lait de mère utilisé pour l'alimentation du prématuré dans un centre néonatal

La composition du lait maternel a fait l'objet de nombreuses études [3,7,22-25]. Le lait de mère ayant donné naissance à un prématuré a, au cours de la phase colostrale et jusqu'à la quatrième semaine après la naissance, une teneur moyenne en protéines et en électrolytes plus élevée que le lait de mère d'enfant à terme [7]. Ceci le rend plus apte à couvrir les besoins nutritionnels des prématurés. Cette composition particulière n'est pas réellement une adaptation physiologique à la prématurité mais résulte plutôt d'une immaturité, d'une interruption inopinée de la préparation de la glande mammaire à la lactation. Toutefois, cette teneur en protéines diminue avec la durée de la lactation tant chez le prématuré que chez l'enfant à terme [23,24]. Si la teneur en protéines des différents échantillons apportés par une même mère est relativement stable, la comparaison des échantillons apportés par différentes mères de prématuré montre une variabilité relativement importante.

La composition en graisses moyenne semble assez stable avoisinant les 4 g par 100 ml tant chez le prématuré que chez l'enfant à terme [7], mais présente une grande variabilité tant pour des échantillons recueillis auprès d'une même mère que pour ceux provenant de mères différentes. Cette plus grande variabilité semble la résultante de l'évolution de la teneur en graisse du lait maternel au cours de la tétée d'une part, mais aussi d'une perte de graisses pouvant survenir lors des manipulations de tirage ou de transvasements souvent nécessaires avant son utilisation pour l'alimentation du prématuré.

À l'aide du Milkoscan®, nous avons mesuré les teneurs en protéines, lipides et glucides des échantillons de lait de mère d'enfants nés prématurés apportés au centre néonatal ainsi que celles des laits de pool du lactarium, provenant essentiellement de mères d'enfants nés à terme et utilisés également à titre de complément pour l'alimentation des prématurés dans notre service (Fig. 1) (Stalport S., mémoire de fin d'études en diététique, 2006). Cette étude confirme la grande variabilité des teneurs en protéines et surtout en lipides des échantillons de laits utilisés dans notre service. En comparant les teneurs en énergie, on peut constater que la valeur énergétique d'un lait de mère de prématuré est plus élevé mais plus variable que celle d'un pool de lait de lactarium. Nos données confirment donc bien les données retrouvées dans la littérature concernant la grande variabilité de composition du LM dans les services de néonatologie et la nécessité de le supplémenter pour satisfaire les besoins des prématurés et leur assurer une croissance optimale.

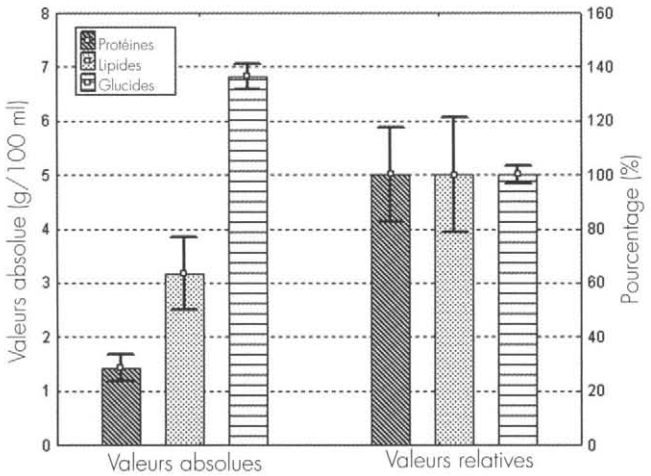

Fig. 1. Variabilité du lait maternel tiré.

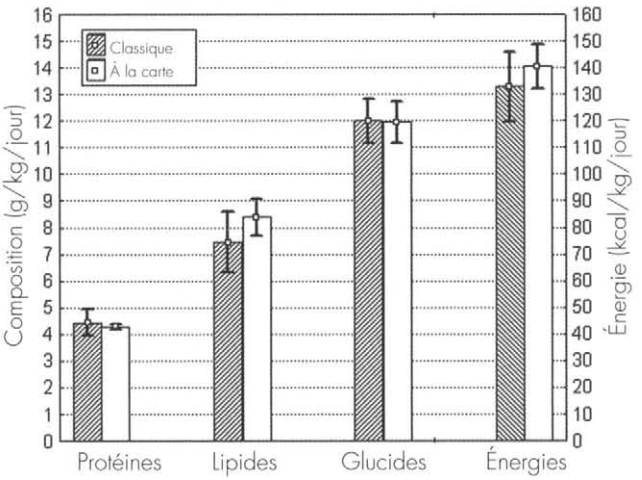

Fig. 2. Comparaison des apports nutritionnels après supplémentation « à la carte » ou classique.

2.4. Avantage d'une fortification « à la carte »

À la suite de ces travaux, nous avons voulu évaluer l'intérêt d'une fortification « à la carte » par rapport à celle d'une fortification classique. Par fortification classique, nous entendons l'addition au volume de lait maternel prescrit à l'enfant de 4 % de fortifiant du lait maternel, à savoir l'Enfamil Human Milk fortifier® (EHMF) et par fortification « à la carte », tout d'abord la normalisation de la teneur en graisses du lait maternel (ajustée à 4 g par 100 ml au moyen de triglycérides à chaînes moyennes, Liquigen®) suivi de l'addition de fortifiant dont la concentration est ajustée en fonction du volume prescrit pour obtenir un apport protéique de l'ordre de 4,3 g par kg et par jour. Nous avons utilisé le Milkoscan® d'une part, pour analyser la composition nutritionnelle du lait maternel de départ et d'autre part, pour analyser la composition du lait de mère après fortification « à la carte ». Pour la fortification classique, considérant que la composition du fortifiant est stable, la composition du régime a été calculée théoriquement en additionnant les teneurs mesurées dans le lait maternel à celles rajoutées par l'addition de 4 % de fortifiant. Cinquante-quatre régimes ont été analysés (Fig. 2). La supplémentation classique ne tient pas compte de la composition variable du lait de mère. Après fortification, la variabilité en protéines et en lipides du lait persiste, associée avec un risque de déficit ou de surcharge en protéines, et un risque de carence en énergie combiné avec un rapport protéino-énergétique élevé.

Lors d'une supplémentation « à la carte », on observe une diminution significative de la variabilité de la composition du lait de mère en protéines permettant ainsi un apport optimal et stable en protéines. En outre, l'apport énergétique est en moyenne plus élevé grâce à l'enrichissement en lipides. Ceci stabilise d'une part l'apport protéino-énergétique et augmente l'apport énergétique fourni aux prématurés. De plus, la quantité de fortifiant EHMF nécessaire à la fortification est moindre (± 3,5 %), diminuant ainsi les risques d'hyperosmolarité.

2.5. Évaluation de la fortification « à la carte » sur la croissance

La supplémentation du lait maternel « à la carte » permet d'adapter la composition du régime de manière individuelle en fonction des besoins spécifiques du prématuré. Chez le prématuré, cette supplémentation devrait permettre théoriquement d'obtenir une meilleure croissance staturo-pondérale, tout en gardant les avantages de l'alimentation au lait maternel. Dans notre service, cette étude de croissance est menée en deux étapes successives. Dans un premier temps, nous avons mené une étude-pilote en étudiant la composition du régime et la croissance de 10 prématurés d'un âge gestationnel moyen de 28,4 ± 0,7 semaines avec un poids de naissance moyen de 1195 ± 225 g alimentés au lait de mère supplémenté « à la carte ». Ce régime est débuté lorsque les enfants sont totalement alimentés et après leur avoir laissé un temps d'adaptation métabolique de 2-3 jours. Durant une période de 10 ± 2 jours, ces enfants prématurés ont reçus 174 ± 8 ml/kg/j. Avant et après fortification, la composition moyenne en nutriments du lait maternel est passée de 1,47 ± 0,33 à 2,39 ± 0,12 g pour les protéines, de 2,9 ± 0,26 g à 4,84 ± 0,28 g pour les lipides et de 6,86 ± 0,1 g à 7,2 ± 0,17 g pour les glucides, ce qui correspond à l'ajout de 2,17 ± 0,46 ml de Liquigen® et 3,38 ± 1,15 sachets de EHMF par 100 ml de lait. L'apport journalier en protéines et en énergie respectivement de 4,09 ± 0,35 g et de 140 ± 7,64 g répond aux nouvelles recommandations nutritionnelles des prématurés [26]. Un dosage d'urée plasmatique moyen de 17,8 ± 6 mg/dl et l'absence d'acidose reflètent une bonne tolérance métabolique du régime « à la carte ». En comparant à nos données antérieures nos résultats préliminaires, ceux-ci suggèrent que la croissance des prématurés nourris exclusivement au lait de mère enrichi « à la carte » (21 ± 1,8 g/kg/j) est supérieure à celle de ceux alimentés au lait de mère enrichi de façon classique (15,7 ± 1,3 g/kg/j) et pourrait être au moins équivalente à celle obtenue chez des prématurés en alimentation artificielle (19,6 ± 3,1 g/kg/j) (Fig. 3). Ces

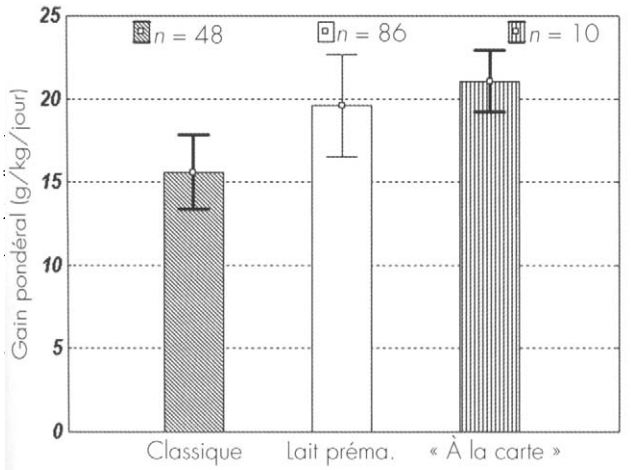

Fig. 3. Comparaison de croissance pondérale de prématurés en fonction du régime.

résultats favorables nous encouragent à poursuivre notre étude et à évaluer non seulement la croissance, mais également la composition du gain pondéral en termes de masse grasse et de masse maigre par la réalisation d'examens d'absorptiométrie successifs.

3. Conclusion

En raison de ses nombreuses propriétés particulières, le lait maternel constitue l'aliment de choix pour l'enfant prématuré. Sa composition en nutriments étant cependant insuffisante pour couvrir les besoins nutritionnels élevés des prématurés et leur assurer une croissance optimale, il est nécessaire d'enrichir le lait de mère avec des fortifiants. Généralement, la fortification ne tient pas compte de la composition initiale fort variable du lait de mère, notamment en protéines, lipides et donc en énergie. En outre, les pertes en graisses surviennent au cours des différentes manipulations. Nous proposons une méthode de supplémentation du lait maternel « à la carte » qui, à l'aide du Milkoscan® une technique de mesure rapide de la teneur en nutriments du lait, tient compte de l'analyse de composition initiale du lait de mère amené au centre néonatal et permet d'adapter de manière individualisée la composition du lait aux besoins spécifiques du prématuré. Ce mode de supplémentation présente comme avantages de diminuer la variabilité de composition du régime, d'optimaliser ainsi les apports en protéines, en énergie tout en diminuant les risques d'hyperosmolarité.

Cette méthode de fortification semble favoriser la croissance, se rapprochant de celle obtenue avec les laits adaptés pour prématurés, tout en conservant les nombreux avantages du lait maternel. Toutefois, des études ultérieures complémentaires sur une plus grande population d'enfants prématurés sont nécessaires pour confirmer ces résultats ainsi que pour préciser la composition qualitative du gain pondéral en termes de masse maigre et de masse grasse. Il serait également intéressant d'étudier l'application de cette méthode au sein d'une unité néonatale en termes de coût, de personnel et de temps.

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Growth Benefits of Own Mother's Milk in Preterm Infants Fed Daily Individualized Fortified Human Milk

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Abstract: The influence of types of human milk (HM)—raw own mother's milk (OMM), pasteurized OMM, and donor milk (DM)—was evaluated for growth in premature infants fed exclusively HM with controlled nutritional intakes using daily individualized HM fortification (IHMF). Growth and nutritional intakes were prospectively collected in preterm infants (<32 weeks) fed IHMF and compared in infants fed predominantly (≥75%) OMM and DM. The influence of HM types (raw OMM, pasteurized OMM, and DM) on growth were also evaluated in the whole population. One-hundred and one preterm infants (birth weight 970 ± 255 g, gestational age 27.8 ± 1.9 weeks) were included. Energy (143 ± 8 vs. 141 ± 6 kcal/kg/day; $p = 0.15$) and protein intakes (4.17 ± 0.15 vs. 4.15 ± 0.14 g/kg/day; $p = 0.51$) were similar in both groups. Infants receiving predominantly OMM ($n = 37$), gained significantly more weight (19.8 ± 2.0 vs. 18.2 ± 2.2 g/kg/day; $p = 0.002$) and length (1.17 ± 0.26 vs. 0.99 ± 0.36 cm/week; $p = 0.020$) than those fed predominantly DM ($n = 33$). Stepwise multivariate analysis ($n = 101$) suggests that raw OMM was the major determinant of growth, contributing 22.7% of weight gain. Length gain was also related to OMM (raw + pasteurized) intakes, explaining 4.0% of length gain. In conclusion, at daily controlled similar protein and energy intakes, OMM had significant beneficial effects on weight and length versus DM in VLBW infants. This difference could be partially explained by the use of raw OMM.

Keywords: preterm; growth; human milk; donor milk; own mother's milk; fortification

1. Introduction

In premature infants, human milk (HM) is associated with significant benefits on health and development. The mother's own milk (OMM) is therefore always recommended as the first nutritional choice. When OMM is unavailable, the use of donor milk (DM) rather than formula could be the first alternative for very low birth weight (VLBW) infants of less than 32 weeks [1–3].

Preterm infants have high nutritional requirements, [4–6] and exclusive HM, even from infant's OMM or banked DM, will not provide intakes that reach current nutritional recommendations. Fortification is therefore recommended to improve post-natal growth [7,8]. Nevertheless, the use of fortified HM could still fail to obtain qualitative and quantitative postnatal growth in the range of fetal growth [9–11]. That remains a concern as postnatal nutritional deficit and growth restriction during the neonatal period could be linked to altered long term health and neurodevelopment outcomes [12–14] in spite of the beneficial advantages associated with the early HM use [15–17].

Worldwide, OMM and DM use in neonatal intensive care units (NICUs) increased over the last decade, but without practical and clear nutritional recommendations [18]. Recent studies, using infrared method, demonstrated the wide variability of protein and energy contents of either DM or OMM, suggesting that the use of theoretical composition values could induce nutritional deficiency or overload [19]. Studies of the impact of individualized HM fortification versus targeted or standard fortification on growth of VLBW infants are scarce [20–23]. In addition, nutritional interests of fortified raw OMM versus pasteurized OMM or DM are still controversial [10,24–27]. A few studies have showed lower growth rates in infants receiving fortified DM compared to fortified OMM [25,26,28]. One frequently suggested explanation was the lower protein and fat content of DM frequently provided by mothers who delivered term infants or were in later stages of lactation [29]. Another explanation could be a reduction of the nutrient contents or bioavailability with the processing of DM [27,30].

The primary objective of the present study is to evaluate growth in VLBW infants fed individualized fortified HM with predominant OMM (≥75%) or predominant DM (≥75%). We hypothesized that, using individualized fortification providing controlled similar protein and energy intakes, the use of OMM could improve growth during the early weeks of life. The secondary objective is to determine the influence of raw versus pasteurized OMM, hypothesizing that pasteurization could impair nutrients' bioavailability and therefore reduce the neonatal growth rate during the study period.

2. Materials and Methods

2.1. Study Population and Study Design

This is a single center prospective and non-interventional study conducted in the NICU of the University of Liège, Belgium evaluating growth in preterm infants fed HM with individualized fortification (IHMF). From January 1, 2007 to December 31, 2014, data on HM use, HM composition, and fortification in preterm infants born <32 weeks gestation (GA) were collected daily in our NICU human milk bank. From those datasets, preterm infants receiving IHMF as previously reported [19,20] were included in the present study. Infants with chromosomal or congenital anomalies impacting growth and those receiving IHMF for less than 14 days where excluded.

To evaluate the respective influences of OMM and DM, growth and nutritional intakes (mean ± standard deviation (SD), during the study period, were compared in preterm infants fed predominantly OMM (≥75%) or predominantly DM (≥75%). In addition, the effects of HM types on growth during the study period were evaluated on the whole population, including a third group receiving a mixed HM diet ranging from 26% to 74% of OMM. Under existing Belgian law at the time of the study, the collection of anonymized data concerning clinical routine practices does not require approved of the Ethical Committee. However, the parents were informed and provided consent for donor milk use as necessary, as well as HM analysis and individualized fortification.

2.2. Nutritional Practices

Global nutritional management was previously reported [31]. According to our protocol, all VLBW infants received parenteral nutrition on the first day of life with a balanced standardized parenteral solution, designed to provide preterm infants a mean intake of 37–38 kcal/kg/day and 2.4–2.5 g/kg/day of protein on the first day of life followed by a rapid increase to a target intake of 3.8 kcal/kg/day of protein and 120 kcal/kg/day by 5 to 8 days of life [31]. Insulin therapy was only used in case of hyperglycemia (>10 mmol/L) during parenteral nutrition. Enteral nutrition (10–20 mL/kg/day) was initiated within the first hours of life with maternal colostrum or unfortified DM and progressively increased by 10 to 20 mL/kg/day until 160 to 180 mL/kg/day according to tolerance. Mothers were encouraged to breastfeed and received support from dedicated nurses in the unit. HM was expressed at the hospital or at home, by manual expression or by using an electric pump, and transported under aseptic HACCP (Hazard Analysis Critical Control Point) conditions, and mothers were provided with written instructions regarding mechanical expression, milk collection,

storage, and transport. OMM provided by the mother was kept at 4 °C and used within 72 h. DM was obtained from our own NICU HM Bank. Milk donors were unpaid volunteers. Informed consent for the use of their milk for feeding preterm infants or for research purposes was obtained Most of these donors had delivered preterm. DM from the early stage of lactation (first week) was separately pooled, processed, labeled, and used during the first days of life in extremely preterm infants in the absence or as a supplement of OMM. DM was always Holder pasteurized (62.5 °C for 30 min) in batches of 5 L. OMM was used as previously described. OMM of cytomegalovirus positive mothers of infants of less than 32 weeks GA at birth was pasteurized until postconceptional age of 34 weeks. A bacteriologic count of OMM was performed after 24 h of incubation, allowing heavy contaminated OMM to be discarded or to use it directly as raw milk or pasteurized milk in case of light contamination [32,33]. Supplemental parenteral nutrition was withdrawn when enteral intakes reached 100 to 120 mL/kg/day. Standard HM fortification was introduced at 25% (addition of 0.275 g of protein and 3.5 kcal in 100 mL of HM) of full fortification once preterm infants tolerated a minimum of 50 mL/kg/d enterally and was gradually increased to full fortification (addition of 1.1 g of protein and 14 kcal in 100 mL of HM). IHMF was considered when a minimum of 140 to 150 mL/kg/day was provided. As IHMF requires extra workload for the HM Bank, its prescription was left to the attending neonatologist.

2.3. Individualized HM Fortification (IHMF)

Fortified HM was prepared daily in the HM Bank. To allow individualized fortification, a sample of 10 mL of HM was taken from the daily pool. Macronutrient HM concentration was determined using a mid-infrared analyzer (Milkoscan minor®, Foss, Hillerød, Denmark) previously validated for HM [19]. The Milkoscan analyzer was calibrated to provide the total protein concentration of HM similar to the total nitrogen content, including non-protein nitrogen, measured by a chemical method. HM was warmed to 37 °C and homogenized using an ultrasonic homogenizer (Sonicator®, Uppsala, Sweden) before analysis. Data of protein and fat contents were gathered in an excel table to calculate the needs of supplementation according to recommendations [5]. IHMF was performed in two steps: (1) Adjustment of fat content up to 4 g/dL by adding medium-chain triglycerides (MCTs; Liquigen® Danone, The Netherlands), (2) addition of a multicomponent powdered HM fortifier (Enfamil Human Milk Fortifier powder; Mead-Johnson or Nutrilon B.M.F.; Nutricia) to finally provide 4.3 g/kg/day of protein according to the daily volume order.

2.4. Data Collection and Growth Assessment

Day 1 of the study was defined as the first day of IHMF. Weight, HM type (raw OMM, pasteurized OMM, and pasteurized DM), macronutrient composition of HM, MCTs, and fortifier addition and volume intakes were prospectively collected daily during all the IHMF period and used to calculate the nutritional intakes. The energy content was calculated using the Atwater factors: 4 kcal/g for protein and carbohydrate and 9 kcal/g for fat.

Other clinical and demographic data were collected from the medical charts of infants, and this included prenatal complications, delivery information, and neonatal outcomes in the NICU until discharge or transfer to another hospital.

Infants weight (to the nearest 1 g) was measured daily by nurses using a calibrated electronic scale. Length and head circumference (HC) were assessed weekly (both to the nearest 0.1 cm), length using a length board and HC using a non-stretch measuring tape. Weight gain velocity (grams per kilogram per day) was calculated during the IHMF period using the 2-point average method [34].

$$\text{Weight gain} = \frac{1000 * (W2 - W1)}{\frac{W1+W2}{2} * (d2 - d1)}$$

where W = weight in grams; d = day; 1 = beginning of the time interval; and 2 = end of the time interval.

Weight for age, length for age, and head circumference for age Z scores were calculated using Fenton reference growth charts according to corrected GA [35].

2.5. Statistical Analysis

Normally distributed data are reported as a mean with standard deviation and groups are compared by using *t*-tests or one-way analysis of variance (ANOVA) with Bonferroni's correction for post hoc pairwise comparisons. Non-normally distributed data are presented as a median with a range, and groups were compared by Kruskall-Wallis ANOVA tests. Categorical data are presented as numbers and percentages and groups were compared by Chi-squared tests. A p-value of <0.05 was considered as significant.

Stepwise multivariate analysis was performed to evaluate the respective influences of significant univariate variables and type of HM (raw OMM, pasteurized OMM, and DM) on growth parameters during the study period. The relation was presented by Pearson correlation coefficient (r or r^2). A $p < 0.05$ was considered as significant.

All statistical analyses were performed by using Tibco Statistica software version 13 (TIBCO, Palo Alto, CA, USA).

3. Results

3.1. Study Population

Between January 1, 2007 and December 31, 2014, 726 infants with gestational age of less than 32 weeks were admitted to the University of Liège NICU by birth or transfer, of which 665 were discharged alive. The total number of infants that received IHMF during NICU hospitalization was 204. Eighty-two were excluded as they received IHMF of less than 14 days, 12 for chromosomal or congenital anomalies impacting growth, and 9 for incomplete data, leaving 101 subjects included in the study.

3.2. Clinical Variables

Out of 101 preterm infants (BW 975 ± 255 g for a GA of 27.8 ± 1.9 weeks), IHMF was initiated at 19 ± 8 days of life during 26 ± 8 days. Thirty-seven infants were fed ≥75% of intake with OMM, 33 infants were fed ≥75% of intake with DM, and 31 with a mixed HM diet with (26%–74% OMM). Demographic and clinical characteristics according to the three HM diets (≥75% OMM versus ≥75% DM versus 26%–74% OMM) are detailed in Table 1. Demographic parameters at birth were similar in the three groups with the exception of HC being significantly lower in the DM group compared to those fed the mixed HM diet.

Neonatal morbidities at study baseline were also similar in the three groups (Table S1) with a trend to a higher incidence of late onset sepsis in the DM group ($p = 0.062$). However, no other significant difference in morbidities that could influence growth was reported between the three groups during and after the study period (Table S2). Necrotizing enterocolitis was observed in three infants, two in the DM group after the study period, (two days after the introduction of preterm formula and the day before suggested discharge in a preterm infant fed formula for several weeks), and the last one in the intermediate group, during the study period, the day after a transfusion. Two infants in the DM category, one in OMM and three in the intermediate group presented clinical infection during or after the study: Five respiratory infections and one urinary tract infection. Insulin treatment rate was similar in all the groups and was only used in case of hyperglycemia during parenteral nutrition. No infants received insulin during the study period.

Table 1. Infants clinical characteristics according to human milk diet.

m ± SD	≥75% OMM *n* = 37	26%–74% OMM *n* = 31	≥75% DM *n* = 33	All Subjects *n* = 101	*p*
Male sex, n (%)	18 (49)	15 (48)	17 (52)	50 (50)	0.96
Gestational age, weeks,	27.7 ± 2.1	28.2 ± 1.9	27.5 ± 1.8	27.8 ±1.9	0.26
Birth Weight, g,	983 ± 244	1042 ± 312	901 ± 185	975 ± 255	0.08
Birth Weight < 1000 g, n (%)	20 (54)	16 (52)	24 (73)	60 (59)	0.16
Mean Weight z score,	−0.19 ± 0.99	−0.37 ± 0.89	−0.48 ± 0.82	−0.34 ± 0.91	0.47
Birth Length, cm,	35.0 ± 3.3	35.8 ± 3.9	34.6 ± 2.9	35.1 ± 3.4	0.34
Birth HC, cm,	24.9 ± 1.9	25.8 ± 2.3	24.5 ± 1.7	25.0 ± 2.0	0.02
Vaginal Delivery, n (%)	16 (43)	9 (29)	7 (21)	32 (32)	0.13
Twin, n (%)	8 (22)	12 (39)	6 (18)	26 (26)	0.13
Apgar Score 1 min,	6.5 ± 2.2	6.1 ± 2.2	6.1 ± 2.0	6.2 ± 2.1	0.60
Apgar Score 5 min,	7.9 ± 1.5	7.8 ± 1.5	7.9 ± 1.1	7.9 ± 1.4	0.92
Antenatal steroids, n (%)	35 (95)	27 (87)	29 (88)	91 (90)	0.30
Study duration,	27 ± 8	27 ± 8	24 ± 6	26 ± 8	0.14
GA age at study day 1, weeks,	30.5 ± 1,5	30.8 ± 1,6	30.5 ± 1,5	30,6 ± 1,5	0.64
GA age at study end, weeks,	34.2 ± 1.4	34.7 ± 1.8	33.9 ± 1.5	34.3 ± 1.6	0.12

OMM = own mother's milk; DM = donor milk; GA = gestational age; data are presented as n (%) for categorical variables and mean (m) ± standard deviation (SD) for continuous variables; $p < 0.05$ based on ANOVA for continuous variable and chi square for categorical variables.

3.3. Influence of OMM Versus DM

According to the primary objective of the study, nutritional intakes and growth during IHMF were compared in VLBW infants fed predominantly OMM and DM.

3.3.1. Human Milk Composition and Nutritional Intakes

The contributions of the HM categories in the two groups are gathered in Table 2. OMM accounted for, respectively, 95.4% and 2.2% of the HM intakes during the IHMF study. Lipid content was significantly higher in the OMM than in the DM group. Nevertheless, in both groups, fortified HM provided similar mean energy and protein intakes with low variability, accounting for, respectively, less than 5.6% and 3.6 % for energy and 3.6% and 3.4% for protein.

Table 2. Human milk composition and nutritional intakes during study in the two groups.

	≥75% OMM *n* = 37	≥75% DM *n* = 33	*p*-Value
Human Milk Category (%)			
Raw OMM	31.3 ± 33.6	0.5 ± 3.0	<0.001
Pasteurized OMM	64.1 ± 33.1	1.7 ± 4.7	<0.001
Pasteurized DM	4.6 ± 7.8	97.8 ± 5.4	<0.001
Human Milk Composition (Infrared)			
Protein, g/dL	1.44 ± 0.22	1.35 ± 0.14	0.056
Lipid, g/dL	3.87 ± 0.59	3.61 ± 0.23	0.022
Carbohydrates, g/dL	6.84 ± 0.22	6.86 ± 0.19	0.695
Nutritional Intakes (Units/kg/day)			
Volume, mL	167 ± 10	166 ± 8	0.536
Energy, kcal	143 ± 8	141 ± 6	0.148
Protein, g	4.17 ± 0.15	4.15 ± 0.14	0.512

Data are presented as mean ± SD; $p < 0.05$ based on *t*-test.

3.3.2. Growth

As shown in Table 3, weight ($p = 0.002$) and length gain ($p = 0.020$), but not HC gain ($p = 0.120$), were significantly higher in infants receiving predominantly OMM compared to those fed predominantly DM during the IHMF period. Similarly, Z-scores gains for weight ($p < 0.0001$), length ($p = 0.004$),

and HC ($p = 0.013$) were all significantly higher in infants receiving mostly OMM than in those fed mostly DM during the IHMF period.

Table 3. Growth rate and Z-score gain in preterm infants fed individualized fortified with predominantly own mother's milk (OMM) or donor milk (DM.)

	OMM ≥ 75% ***n* = 37**	**DM ≥ 75%** ***n* = 33**	**Delta OMM vs. DM**	***p***
Weight gain, g/kg/day	19.8 ± 2.0	18.2 ± 2.2	+1.6	0.002
Length gain, cm/week	1.17 ± 0.26	0.99 ± 0.36	+0.18	0.020
Head circumference, cm/week	1.13 ± 0.22	1.04 ± 0.27	+0.09	0.120
Weight Z-score gain, g/kg/d	0.13 ± 0.35	−0.26 ± 0.41	+0.39	<0.001
Length Z-score gain, cm/week	−0.25 ± 0.41	−0.59 ± 0.52	+0.33	0.004
HC Z-score gain, cm/week	0.59 ± 0.50	−0.24 ± 0.65	+0.35	0.013

Data are presented as mean ± standard deviation; $p < 0,05$ based on *t*-test.

3.4. Effects of Type of Human Milk (Raw OMM, Pasteurized OMM, and Pasteurized DM)

3.4.1. Human Milk Composition and Nutritional Intakes

In line with the secondary objective of the study, the whole population was evaluated according to the main HM type received during the study period, DM > 50% (DM), DM ≤ 50%, pasteurized > raw OMM (POMM), and DM ≤ 50% and raw > pasteurized OMM (ROMM) to evaluate the influence of OMM pasteurization on growth velocity during the study period. As shown in Table 4, DM accounted to 88.5% in the DM group ($n = 45$), pasteurized OMM to 70.3% in the POMM group ($n = 41$), and raw OMM to 69.1% in the ROMM group ($n = 15$). Energy and protein intakes during the study period were similar in the three groups.

Table 4. Growth rate and nutritional intakes according to the main human milk type received during the study period.

Human Milk Type Volume Intake (%)	**DM 88.5 ± 16.9**	**POMM 70.3 ± 22.6**	**Delta vs. DM**	***p* vs. DM**	**ROMM 69.1 ± 19.9**	**Delta vs. DM**	***p* vs. DM**	**Delta vs. POMM**	***p* vs. POMM**
n	45	41			15				
Energy, kcal/kg/day	141.3 ± 6.3	142.4 ± 7.3	-	0.432	143.7 ± 6.2		0.210	-	0.552
Protein, g/kg/d	4.15 ± 0.14	4.19 ± 0.13	-	0.211	4.18 ± 0.15		0.494	-	0.855
Weight gain, g/kg/d	18.2 ± 1.9	19.1 ± 1.8	+0.87	0.035	21.1 ± 1.6	+2.83	<0.001	+1.96	<0.001
Length gain, cm/week	1.04 ± 0.36	1.13 ± 0.33	+0.10	0.193	1.17 ± 0.28	+0.14	0.194	+0.04	0.697
HC gain, cm/week	1.04 ± 0.24	1.10 ± 0.20	+0.05	0.258	1.10 ± 0.24	+0.06	0.409	+0.01	0.937
Weight Z-score gain	–0.23 ± 0.39	0.09 ± 0.31	+0.31	<0.001	0.15 ± 0.44	+0.38	0.003	+0.06	0.546
Length Z-score gain	–0.53 ± 0.52	–0.36 ± 0.45	+0.17	0.116	–0.14 ± 0.50	+0.39	0.013	+0.22	0.114
HC Z-score gain	0.28 ± 0.59	0.51 ± 0.56	+0.23	0.068	0.70 ± 0.41	+0.41	0.016	+0.18	0.252

DM = donor milk; POMM = pasteurized own mother's milk; ROMM = raw own mother's milk. Data are presented as mean ± standard deviation; $p < 0.05$ based on *t*-test.

3.4.2. Growth

Both weight gain and weight Z-score gain in the DM group were significantly lower than in the other two groups. In addition, weight gain, but not weight Z-score gain, was significantly higher in the ROMM versus POMM group. Length and HC gains were similar in the three groups. Nevertheless, the length and HC Z-score gains were significantly improved in the ROMM group compared to the DM group.

3.5. Univariate and Multivariate Analysis on the Whole Population

3.5.1. Univariate Analysis

Univariate linear regression analysis on the whole population, showed that birthweight, gestational age, postnatal age at study day 1, as well as protein and energy intakes did not significantly influence weight and length gain during the study period.

Weight gain during the IHMF period was significantly influenced by two univariate factors; study duration (r = 0.31, $p = 0.0014$) and percentage of raw OMM (r = 0.47, $p < 0.00001$). For length, the percentage of total OMM (r = 0.20, $p = 0.046$) was the only factor significantly influencing length gain.

3.5.2. Multivariate Analysis

Weight Gain and Weight for Age Z-score Difference

Stepwise multivariate analysis demonstrated that weight gain (g/kg/day) was positively related to the proportion of raw OMM, proportion of pasteurized OMM, and postnatal age at the first day of study, but negatively related to study duration and birthweight. Those factors explain 22.7%, 3.7%, 3.1%, 9.8%, and 3.0% of the weight gain, respectively. It was also estimated that the weight for age Z-score difference during IHMF was related to the raw OMM proportion, gestational age, and birth weight, contributing, respectively, to 18.0%, 12.1%, and 10.7% of the Z-score difference.

Length Gain and Length for Age Z-score Difference

For length gain, only two parameters were significant; the proportion of total OMM (raw + pasteurized) and postnatal age at baseline, explaining, respectively, 4.0% and 4.4% of the length gain. Similarly, length for age Z-score difference was related to the proportion of total OMM (raw + pasteurized) and study duration, contributing, respectively, to 6.5% and 5.4% of the difference.

4. Discussion

This study is the first providing daily controlled nutritional intakes in preterm infants fed HM with individualized fortification after daily determination of HM composition by a validated infrared method [19,20]. Because of IHMF, protein and energy intakes were similar with very low variability (Table 2) in the two groups, it adequately allows for comparisons of growth and metabolic tolerance in VLBW infants fed exclusively fortified OMM (95.4% ± 7.8%) or DM (97.8% ± 5.4%). This study found that weight gain velocity during IHMF was on average 1.6 g/kg/d higher in infants fed OMM than in those fed DM, with an additional benefit on length gain of around 0.18 cm/week on average, suggesting a growth specific effect of OMM in preterm infants. In addition, the use of predominant OMM (≥75%) instead of predominant DM (≥75%) significantly improved weight, length, and HC Z-score changes during the study period (Table 3).

As shown in Table 3, around two thirds of OMM was provided after Holder pasteurization and not as raw OMM. This is mostly explained by the strategy applied to reduce the risk of infectious transmission with raw milk. According to our previous study [32,33], up to 20%–50% of the OMM samples were contaminated and were either pasteurized or discarded. In addition, to avoid CMV contamination or infection [30], OMM of CMV seropositive mothers of VLBW infants was also systematically pasteurized. The variability of the raw OMM intakes in our whole population allowed us to evaluate the respective role of raw OMM versus pasteurized OMM or DM on growth velocity in the preterm infants. We found that ROMM and POMM both have a positive effect on weight gain, contributing to an increase of +2.8 g/kg/day and +0.9 g/kg/day, respectively, compared to DM. It suggests that the major positive effect of OMM could be the result of its use as a raw product, with a mean weight gain difference of 2.0 g/kg/day compared to pasteurized OMM (Table 4). Our study also suggests that the use of raw OMM also induces a significant positive effect on weight ($p = 0.003$), length ($p = 0.013$), and HC ($p = 0.016$) Z-score gains during the study period compared to DM. The benefits

of POMM on DM was limited on weight gain and weight Z-score gain whereas benefits on length and HC Z-scores were not significant with p values of 0.2 and 0.07, respectively, contrasting with the benefits observed with ROMM. Therefore, our study suggests that the limited beneficial effect of POMM versus DM remains to be confirmed in additional studies.

The optimal reference growth chart to evaluate postnatal growth velocity in preterm infants is still debated as discussed recently by an international expert group [34]. From this review, it was recommended to use the average 2 points or the exponential 2 points methods to evaluate the growth velocity. Both formulas provide similar results that are highly correlated with a slightly higher value for the exponential method as shown in the Figure 1. In our study, we chose to use the average 2 points method for comparison to our previous studies [9,20,31,36].

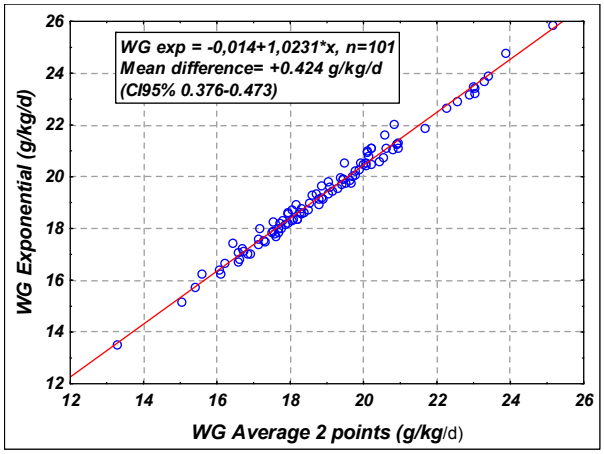

Figure 1. Evaluation of growth velocity by the exponential and the average 2 points methods.

In addition, this review and others [34,37] suggest that it is time to report growth studies in a standardized fashion. The standardized growth report is also debated and several growth charts have been proposed to evaluate postnatal Z-scores in VLBW infants. Recently, it was suggested that the Fenton revised growth charts of 2013 could be outdated by the recent INTERGROWTH-21st Postnatal Follow-up Study of preterm infants [38]

We agree that both the Fenton growth chart and the INTERGROWTH-21st have some limitations. The Fenton growth charts are built with a meta-analysis of cross-sectional fetal charts without take into account that postnatal growth composition differs to that of fetal growth composition. By contrast, the INTERGROWTH-21st chart is longitudinal, not cross-sectional and non-fetal. However, this contains some limitations, including the small number of very preterm infants included in the database, as well as the lack of "gold standard nutrition" [39,40]. Indeed, the description of the feeding regimen in the INTERGROWTH study is limited. It is specified that the main feeding regimen of the included preterm infants was human milk and that the use of HMF was only added to expressed HM until a baby's weight reached 1800–2000 g and not up to discharge. The daily protein and energy intakes were not adequately controlled during the postnatal period, suggesting that some cumulative protein and energy deficits could induce relative postnatal growth restriction in the evaluated population of preterm infants. The authors of the INTERGROWTH-21 group [39] recommend the INTERGROWTH-21st Preterm Postnatal Growth Standards for monitoring the growth of more than 90% of preterm infants who are born at ≥32 weeks and recognize that the construction of charts for very preterm infants (<32 weeks' gestation) is problematic. We consider that our population, including 30% of preterm infants with a GA < 27 weeks and 100% at <32 weeks at birth, but also 80% still <32 weeks at baseline, is not in the optimal range of the INTERGROWTH reference. Therefore, our results were compared to the combined references growth chart of the fetus and the term infants as proposed by Fenton et al. in 2013 [35]. However, data of preterm infants >27 weeks GA were also compared to the INTERGROWTH-21st reference in Figures S1 and S2.

This study demonstrates a significant positive impact of both OMM and raw OMM on growth in preterm infants fed HM. This effect seems independent of nitrogen, lipid, and carbohydrate content as this was controlled by the IHMF in this study. Nutritional and growth benefits of fortified OMM versus fortified DM is still debated and studies report controversial results regarding growth and Z-score changes in preterm infants. Thus, in two observational and one retrospective study, a weight gain benefit was reported in preterm infants fed fortified OMM. In 2011, Montjaux et al. [25] suggested that weight gain was directly proportional to the amount of fresh raw OMM compared to pasteurized fortified DM (n = 48). More recently, Madore et al. [26] showed a significantly higher weight gain in preterm infants fed predominantly fortified OMM compared to those fed predominantly fortified DM during the first month of life (n = 56). Brownell et al. [28], using OMM as a reference, also reported a significant decrease in mean weight and head velocity during a hospital stay for every 10% increase of the total feeding volume provided as DM (n = 314). By contrast, two retrospective studies did not observe any significant difference in weight gain between premature infants receiving either exclusively OMM or DM as a sole diet (n = 92) [41] or in those fed predominantly (>50%) fortified OMM or fortified DM (n = 299) [42]. In addition, a third retrospective study found no significant difference in weight Z-score change by HM diet (n = 88) (>75% donor vs. >75% OMM; p = 0.28) [10]. In contrast to our study, none of those studies precisely determined and controlled the protein and energy intakes, and the rate of pasteurization, if any, in the OMM groups was not specified. Still, the effect of pasteurization on growth velocity was recently evaluated as a secondary outcome in a randomized study of more than 300 premature infants receiving fortified OMM either raw or pasteurized. In that study, a similar growth was observed between the two groups [43].

In preterm infants fed fortified HM, postnatal growth restriction was frequently reported as well as loss of Z-score during the full HM fortification period [10,44]. Repetitively, recommendations from various expert committees suggest that nutritional requirements are similar in VLBW infants fed fortified HM or preterm formula (PTF) [4,6]. Until now, no specific guidelines have been proposed for fortified HM fed preterm infants. However, it is recognized that at similar controlled protein and energy intakes, growth velocity is significantly lower in preterm infants fed fortified HM than in those fed PTF [9]. Metabolic and energy balance studies show that such a difference could be the result of lower metabolized protein and energy contents of fortified HM compared to PTF [36]. The mean difference in nitrogen utilization (retention/intake) as well as the mean difference in energy absorption rates measured by bomb calorimetry were both about 10% less with fortified HM [45]. This difference could be partially due to the use of pasteurization. In addition, as shown more recently, the use of standard reference values for OMM and DM may induce an overestimation of the protein and energy content of fortified HM [19,46]. While preterm OMM with its higher protein content could improve growth compared to DM, it remains insufficient to support adequate growth, especially after the first month of lactation when the OMM protein concentration decreases [29]. A previous study performed in our NICU found that the macronutrient and energy content of OMM was highly variable and unpredictable. Protein and energy content of DM was also significantly lower than that of OMM [19]. Of all the daily OMM and DM samples (n = 2630) measured in the present study, 67% were <1.5 g protein/dL and 62% were <67 kcal energy/dL, values commonly considered as reference values for HM composition to estimate nutrient intakes in clinical practice.

By using metabolic balance studies and indirect calorimetry, we previously showed that protein intake and the protein energy ratio were major determinants of weight gain in VLBW infants [36]. In a recent multicentric study [46], we showed that theoretical intakes of 4.46 g/kg/day of protein and 125 kcal/kg/day (not confirmed by HM content analysis) led to a stable weight Z-score during the study period in VLBW infants receiving new HMF while the weight Z-score decreased significantly in the control HMF group theoretically receiving 3.81 g/kg/day of protein and 125 kcal/kg/day. Trends in the same direction were observed for length Z-score changes. In that study, the protein intakes were not measured, but estimated according to a preterm HM reference [47] and were therefore probably overestimated in regard to the large use of DM and pasteurized OMM [46]. Based on blood urea

nitrogen and urinary urea excretion, we speculated that protein utilization in the new HMF might not have been optimal due to a relative deficiency in metabolized energy intake [46].

Considering the variability of HM macronutrient contents and the lower bioavailability of HM, in the present study, we targeted higher mean protein and energy intakes than those generally recommended [4,6,48]. Thus, during the study period, preterm infants received controlled mean intakes of 143 kcal/kg/day and 4.2 g/kg/day of proteins between 30.5 and 34 weeks' post-menstrual age, resulting in mean positive weight and HC Z-scores changes of 0.13 and 0.59, respectively, but a limited negative mean length Z-score change of 0.25 in preterm infants fed ≥75% OMM. By contrast, negative Z-score changes for weight (0.26 on average) and length (0.59 on average) were observed in the group receiving ≥75% DM (Table 3). These results suggest that such intakes are close to the minimal requirements necessary for preterm infants fed fortified OMM in such a range of post-menstrual age, but could still be limited in those fed fortified pasteurized DM. In addition, knowing that postnatal growth quality differs to that of fetal growth by an increase in fat deposition, the discrepancies between weight and length Z-scores benefits could be the result of a relative deficit in the lean body mass accretion rate during the study period. Therefore, our study also suggests that protein and energy requirements of preterm infants fed fortified HM are higher than that currently recommended [4,6,48] and that specific nutritional guidelines for HM fed preterm infants need to be designed, promoting the use of OMM, but considering the limitations of its use as raw OMM in VLBW infants.

Improving HM fortification by IHMF through the use of infrared technology and extra protein and energy supplementation may be one of the strategies to optimize the nutritional composition of HM to meet the nutritional needs of preterm infants, especially when DM is used. It was demonstrated that IHMF decreases the variability linked to HM content and safely optimizes protein and energy intake [19,21,49,50]. Premature infants fed with low macronutrient content HM benefit the most from IHMF, with improved growth outcomes. However, infrared devices, originally developed for use in the dairy industry, must be calibrated and validated for HM analysis before clinical use by following good laboratory and clinical practice, and appropriate sample preparation must be done otherwise their use can affect the growth outcomes of preterm infants [19,50,51].

5. Conclusions

Our study is one of the first studies showing that a daily controlled high protein and energy intakes (4.2 g of protein and 143 Kcal/kg/day) of fortified raw OMM is associated with important growth benefits in preterm infants. It also suggests that pasteurized OMM provides a limited, but significant growth benefit compared to DM, suggesting that pasteurization significantly impaired the bioavailability of protein and energy intake. The increase in protein and/or energy intakes in preterm infants receiving fortified pasteurized HM could be postulated in view of these results, but needs to be demonstrated in further studies. In addition, our study also suggests that specific and different nutritional recommendations need to be designed for preterm infants fed OMM and DM.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/4/772/s1, Table S1: Initial clinical outcomes before daily individualized HM fortification (IHMF); Table S2: Post baseline clinical outcomes. Figure S1. Weight Z-score and Weight Z-score change according to Fenton and Intergrowth during the study in all preterm infants included in the study with a GA > 27 weeks; Figure S2. Weight for age Z-score at day1 and at the end of the study period, and Z-score gain during the study in infants fed mostly donor (n = 45) versus raw OMM (n = 15). Comparison of FENTON and INTERGROWTH's references.

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