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GAVIN PUBLISHERS

Advances in Pediatrics and Neonatal Care

Cayer MP and Brouard D. Adv Pediatr Neonatol care: APNC-120.

DOI: 10.29011/APNC-120.100020

Review Article

Microbiological Safety of Banked Human Milk: Current Status and Future Directions

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Citation: Cayer MP, Brouard D (2021) Microbiological Safety of Banked Human Milk: Current Status and Future Directions. Adv Pediatr Neonatol care: APNC-120. DOI: 10.29011/APNC-120.100020

Received Date: 15 February, 2021; **Accepted Date:** 19 February, 2021; **Published Date:** 25 February, 2021

Abstract

Recent studies have clearly shown the numerous benefits of a human milk diet over a diet of bovine milk-based products for neonates and premature newborn children. More specifically, short- and long-term positive effects of human milk on growth and development of newborns have recently been demonstrated. Access to human milk can be invaluable for extremely premature or ill infants hospitalized in neonatal intensive care units (NICU), and especially for those who do not have access to their mother's own milk (MOM), in which case donor human milk (DHM) represents the best alternative. DHM is supplied to the NICU by human milk banks (HMB) which must test, control and ensure microbiological safety, which can be accomplished by thermal processing and screening for harmful contaminants at different times during the milk preparation process. In close collaboration with standards development organizations, HMBs have set up validated methods to meet requirements associated with their national regulatory guidelines. A lack of consistency in established global recommendations has been noted by several groups who have highlighted differences in HMB operations, and more worryingly, in screening methods performed before (in-process) and after thermal processing (end-process). The Holder pasteurization and the plate count method (PCM) are both recognized as gold standards in the field of human milk processing. However, new technologies currently emerging from the food and cosmetic industries could be adapted for performing DHM screening in HMB facilities. The main objective of this review is to outline inconsistencies in DHM microbial assessment methods, which contributes to the observed variability in human milk banking practices. Finally, we discuss a microbiological screening strategy that could be implemented within most DHM preparation processes to improve production efficiency and end-product safety.

Keywords: Donor human milk; Human milk bank; Bacteriology testing; Donor screening; Quality control

Introduction

The numerous benefits of human milk for neonates, especially for extremely preterm infants, are widely recognized; therefore, the World Health Organization (WHO), UNICEF, and professional associations recommend that children initiate breastfeeding immediately after birth and should be exclusively breastfed for the first six months of life [1-3]. In addition to its nutritional content linked to lipid, protein and carbohydrate components, human milk provides antimicrobial and growth factors as well as many others bioactive components [4,5]. Recent scientific advances, thanks in part to the more widespread use of "omics" approaches, have made it possible to deepen the state of knowledge associated with the complex composition of human milk and its impact on newborn health and growth [6,7]. Its natural and evolving composition is known to be responsible for reductions in mortality, incidence of nosocomial infections, and sepsis in premature babies fed with breast milk compared to infant formula [8,9]. Unfortunately, many preterm infants do not have access to their mother's milk for various reasons, including health conditions, suckling inability or delayed milk production. In these cases, the use of donor human milk (DHM) represents the best alternative, owing to the fact that strong immunological benefits have been rigorously studied and are associated with significant reductions in the incidence of infectious complications and necrotizing enterocolitis (NEC) in preterm and low birth weight infants [10,11]. Even though bacteria found in human milk contributes to the establishment of the infant's gut microbiota [12], the weakened state and incomplete maturation of the immune response in premature infants [13] requires DHM to be processed before being administrated. Most often, DHM is prepared, processed and managed by human milk banks (HMB), which operate by following internal procedures

similar to those found in blood banks. Indeed, HMB must follow validated operating procedures for donor screening, bacterial testing, processing, storage and distribution. Their primary aim is to ensure that DHM is microbiologically safe, by eliminating potentially harmful microorganisms while minimizing the impacts of processing on milk quality and its bioactive properties.

Microflora of Donor Human Milk

Fresh human milk is known for shaping the gut microbiota in early life [14] and its microbiological complexity mainly originates from breast tissue, maternal skin and gut flora [15]. Culture-based methods have been used to define the DHM microbiota diversity in the past and have paved the way for a deeper characterization using emerging analytical approaches like 16S rRNA gene amplicon next-generation sequencing and cultivation/matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry [16,17]. Culture studies published by human milk banks and independent researchers have confirmed the predominance of *Staphylococcus* spp. and *Streptococcus* spp. [18]. The presence of other bacteria belonging to *Corynebacterium, Bifidobacterium, Lactobacillus*, and *Acinetobacter* genera has also been reported as being part of the expected microbiota [15,19] . Specific and potentially pathogenic bacteria such as *Staphylococcus aureus*, Group B *Streptococcus*, *Escherichia coli*, *Pseudomonas* spp., *Klebsiella* spp., *Enterococcus* spp., *Enterobacter* spp., *Bacillus* spp. and *Moraxella* spp., have also been reported [20,21]. Raw human milk usually contains between 2 and 3 $\log_{10}$ colony forming units/mL (CFU/mL) [19,22], but bacterial overgrowth can be observed in some cases where one or two dominant species gain the upper hand and can increase bacterial concentration up to 10^6 CFU/mL [18]. Breastfeeding problems, such as cracked or chapped nipples and breast infections, have also been reported in a significant proportion of donors. Indeed, the conclusions of a recent survey indicate that these symptoms were experienced by more than 20% of participants [23]. The microbial concentration in mastitis milk samples has been assessed, and the mean total bacterial count was calculated at 4 $\log_{10}$ CFU/mL, in which *Staphylococcus epidermidis* was the most prevalent species (92%) and the presence of *Staphylococcus aureus*, a pathogenic bacterium, was detected in 30% of the samples [24]. Dewitte et al. reported an overall 11% rate of non-compliant milk pre-pasteurization, and 55% of these were attributable to the presence of *S. aureus* [25].

Exogenous Microbial Contamination of Donor Human Milk

One of the most effective preventing methods leading to the delivery of pathogen-free milk is to perform collection in the most aseptic conditions possible. Several HMB have recently come up with detailed procedures aimed at improving collection practices and milk safety, including recommendations on hygienic measures for the expression, cleaning, storage and transport of human milk [26-28]. Mothers with increased bacterial content in their breast milk, especially with high *Enterobacteriaceae* counts [29], are frequently associated with confirmed divergence from guidance [30]. Ineffective cleaning or decontamination of breast pump systems, as well as inadequate maintenance of collection or storage equipment and non-compliance with recommended milk storage conditions, are considered primary sources of increased bacterial counts and higher risks of detecting the presence of harmful bacteria [30,31]. This underlines the important role of HMB in ensuring that their milk donor population can access proper training on best hygienic practices and effectively adhere to recommended guidelines promoting DHM microbiological safety [32]. Ubiquitous environmental bacteria can also be introduced during milk collection procedures or HMB operations via aerial contamination [33]. However, despite frequent observations of *Bacillus* species in contaminated DHM [34,35], it would be perilous to associate their inoculation specifically at the time of collection [36,37]. The most likely contaminating sources, probably as spores, are air, direct food contact, linen and disposable items [33]. It has been shown that *B. cereus* spores found in raw milk are the major source of *B. cereus* in pasteurized DHM [38]. Adjidé et al. has reported on a 27% rate of discarded pasteurized batches, of which 63% and 38% were attributed to *Bacillus* spp. and *Bacillus cereus* as the predominant species detected, respectively [35].

Current and Emerging Methods for Milk Safety

Current international DHM processing practices suggest to perform a low-temperature (62.5°C), long-time (30 min) pasteurization (LTLT), also known as Holder pasteurization (HoP), to inactivate non spore-forming and pathogenic bacteria [39-41]. Unfortunately, HoP is also known for having moderate impacts on the nutritional milk quality and for decreasing the amount of biologically active components when compared to fresh milk [3,42-44]. In addition, a few sporulating bacteria and viruses can survive HoP, such as *Bacillus* spores and hepatitis B virus [45-47]. Inspired by the specific needs of the dairy and cosmetic industries, new processing methods have emerged and translated to milk applications and adapted to DHM [48,49]. Some of these methods claim to be less time-consuming, more cost-effective, or claim greater nutritional value recovery post processing compared to HoP. Two extensive reviews discuss emerging DHM processing technologies and their respective impacts on human milk [41,50]. High pressure processing [51], ultraviolet-C irradiation [52] and the optimization of thermal processes [53], such as high-temperature short-time treatment (HTST) [54], figure among the most recently developed and studied DHM processing strategies. As some of them show better preservation of the DHM nutritional content and immunological properties, there is still a lack of evidence supported by compelling published data on their respective impacts on bacterial spores, viruses or fungi

[41,55,56]. Commercial sterilization, called retort pasteurization or shelf-stable process, has been preferred to HoP by commercial HMB despite the fact that the technology is no longer used in the dairy industry. Retort sterilization, similar to canning, uses high temperature (115°C to 145°C) under pressure for several minutes to sterilize DHM. The process eliminates heat-stable toxins and spores that cannot be completely eradicated by HoP, while offering an improved microbiological risk profile. However, recent studies have demonstrated a significant loss in end-product bioactive proteins for shelf-stable processes compared to HoP [57,58]. Further research on the development and evaluation of emerging processing techniques are still needed, as few of the available ones have been thoroughly evaluated for their respective impacts on DHM microbial and bioactive content, including HoP, in the context and experimental conditions of HMB operations [50]. Indeed, the somewhat relatively high cost for HoP replacement and the implementation and validation of a new technology has to be justified with demonstrated and clinically relevant gains expected from an improved nutritional content retention and an increased microbiocidal action. The incomplete elimination of bacterial spores, or alterations in the nutritional and immunological potency resulting from the application of these technologies, provides an explanation as to why none of them have been reported to be formally implemented in HMB. In the meantime, as indicated by professional organizations, HoP still remains the reference method for DHM processing to meet the needs of the medically fragile infant [3,56].

The Importance of Microbial DHM Monitoring

Microbial monitoring is one of the most effective tools for ensuring DHM safety within HMB operations. There is still no global consensus for microbiological test protocols or as to when exactly they need to be performed during the DHM preparation process [50]. The European Milk Bank Association (EMBA) has recently highlighted existing discrepancies between international guidelines and suggested standardized recommendations for microbiological screening based on collective expert opinions [59]. The published best practices state that:

- Before pasteurization
  - All pools of milk must be tested prior to pasteurization.
  - Acceptance criteria: 10^5 CFU/ml or less of non-pathogenic organisms and no pathogens for each pool of milk tested prior to pasteurization. Discard all samples of milk from a pool that does not meet this standard.
- After pasteurization
  - Each batch of milk must be tested after pasteurization.
  - Discard the batch if there is any microbial growth detected in a random sample taken after pasteurization.

In Canada and the United States, the Human Milk Bank Association of North America (HMBANA) has formulated guidelines to frame HMB operations, and pre-pasteurization testing is not formally required nor specified [39]. This situation influences North American HMB practices regarding pre-pasteurization testing, which relies upon internal guidelines most often established by local medical authorities. For smaller HMB, the cost-effective decision not to perform pre-pasteurization culture screening is frequently made [33]. HMB performing both pre- and post-pasteurization have shown higher DHM discard rates, up to 29% [20,60]. In these instances, high contamination rates before pasteurization were responsible for the elevated discard rates [33,53]. Even though higher rejection rates are expected from an early screening strategy, the end result is a reduced rate of non-compliant milk batches after post-pasteurization testing (< 1%) [53]. Moreover, the presence of pathogens in DHM before thermal processing is concerning, given the ability of certain strains to produce heat-stable enterotoxins that can survive pasteurization [61]. Recent studies have demonstrated that *S. aureus* bacterial counts must be as high as $>10^6$ CFU/ml in milk for toxins to become detectable [62], and the minimal dose of enterotoxins that can lead to complications in preterm infants remains unknown [63]. The strategy of detecting staphylococcal enterotoxins (SE) post-treatment has been considered; however, of the known 27 SE, only the five classical SEs (SEA-SEE) found in food matrices can be detected by enzyme immunoassays [64]. There are a few reports of infections due to the food poisoning agent *Bacillus cereus* in neonates, but to date, no studies have been able to establish a direct association with DHM consumption [65]. Despite the lack of undisputable evidence for the involvement of DHM in cases of sepsis in preterm infants, *Bacillus cereus* is recognized for its pathogenic potential, its sporulation and cleaning resistance capabilities, which is why this microorganism is closely monitored by HMB. Another important parameter to consider regarding milk safety is to validate and control the HoP process. There is no consensus on how quality control should be performed for commercially available pasteurizers. Data from the literature have shown important discrepancies and a lack of standardization in HoP processes [66]. Most studies evaluating HoP performance in human milk used *in vitro* methodologies based on simulated experiments [50]. Temperature profile variabilities can be observed and considered non-compliances when compared to the 62.5°C target maintained for 30 minutes followed by rapid cooling. Buffin et al. have proposed criteria for qualification of human milk pasteurizers, and have highlighted the importance of regular quality controls performed by the HMB on each pasteurizer to optimize milk quality [66].

Common Practices of the Microbiological DHM Screening Process

Most HMB performing pre-pasteurization microbiological screening test a sample from the first donation. If any contamination is found, milk is discarded and the subsequent donations must be rechecked until the milk donor demonstrates appropriate hygienic practices. Once mothers are approved, suitable milk is frozen at -20°C as quickly as possible and kept in freezer until expiration, which varies from one country to another (between 3 and 12 months from the earliest pumping date of milk within the pool). Before pool preparation, milk collections are gradually thawed at 1-4°C. The time spent in the refrigerator may vary among HMB, and can last up to three days [33,67]. Standards or guidelines do not specify a recommended time frame, but generally a maximum of 24 hours is suggested [68,69]. Previous studies have concluded that fresh human milk that is refrigerated for several days remains safe and retains a good proportion of its nutritional value, but significant changes in pH, total protein and fatty acids, as well as a decrease in bacterial colony counts, were observed [70,71]. Some HMB pool milk collections from multiple donors which produces a more uniform product in terms of nutrient content, but in return complicates the trace back process aimed at identifying the original source of contamination. Donations from the same milk donor can also be mixed to generate an individual donor pool, from which a sample can be collected to screen for microbiological content. If the microbiological criteria are not met, a retroactive intervention can be carried out on recommended hygiene practices to be followed by the milk donor. Furthermore, a collective pool can be prepared by combining individual donor pools into clean containers prior to pasteurization. Milk from the collective pool can be sampled to perform bacteriological culture procedures before and after pasteurization. Any bacterial growth is deemed unacceptable for heat-processed, unfrozen DHM, so if there is any growth in culture tests, the collective pool is rejected. Final bacteriological analysis results are obtained after collective pool processing is completed. The collective pool can remain at 4°C for up to 72 hours before being frozen [39].

Plate Count Method for Screening DHM Contaminants

Conventional plate count culture techniques are widely considered as the gold standard for DHM microbiological testing. Innovative techniques are trying to break into the market of quality control in the food industry; among these, molecular biology methods are the most widely studied [72,73], but are still not implemented in milk bank operations. The main problem arises from the fact that drawing conclusions based on the viability of detected microorganisms remains challenging. An HMB international guidance for DHM testing by culture methods has not been clearly defined, or lacks stringency. A summary of HMBANA's recommended method for post-pasteurization analysis states [74]:

- Milk samples subjected to testing must be chosen randomly, adequately mixed and collected aseptically.
- 100 µl of milk is tested by either a spread plate or pour plate method.
- After incubation, CFU are counted and the plate count result is reported as CFU/100 µl.

Most HMB cultivate raw or pasteurized DHM on solid media to perform visual enumeration of aerobic flora and pathogens, usually on plate count agar (PCA) or blood agar [20]. Media containing specific nutrients that select facultative anaerobic populations can be used for pre-pasteurization testing (MacConkey agar for *Enterobacteriaceae* spp. or Chapman and Baird-Parker agar for *Staphylococcus* spp.) [21,34]. However, culturing bacteria for DHM quality control applications is a time-consuming process requiring expertise and highly qualified personnel. Blood and Chapman agars were originally designed as isolation media. When used for enumeration purposes, there may be uncertainty in the accuracy of the observed counts. Consequently, an additional identification step may become necessary, which has the effect of extending the analysis time up to 96 hours [75]. Most HMB use a qualitative method to estimate the burden of *S. aureus*, while growth of other bacterial species on the same medium can impact accuracy of the final count. An interesting study has presented data on a validated process associated with a quality approach that leads to final results for *S. aureus* counts; however, this strategy still requires an end-process confirmatory testing step [76]. The same cultivation method concerns arise for *Bacillus* sp. and its associated challenge to clearly distinguish it from concomitants on agar plates. Potentially pathogenic *Bacillus* sp. are known to generate beta-hemolysis on blood agar; accordingly, several HMB use this criterion as a first identification test. Highly selective media (PEMBA, MYPA, BBC agar) are available for enumerating *B. cereus* in food matrices likely to give rise to high background competing microflora, however they are relatively expensive [77]. Protocols including DHM dilutions prior to inoculation should be considered for improving bacterial determination, and also because the presence of antimicrobial molecules, such as lysozyme and lactoferrin, can interfere with measurements. Sample dilution to a threshold where antimicrobial molecules are no longer active should be performed whenever possible. Rigourd et al. proposed an 18-hour long incubation of pasteurized milk at 37°C as a preliminary enrichment step to increase the detection limit of *B. cereus*, as they have shown an increased rejection rate from 6.3% to 12.6% following implementation [78]. In the same study, the authors have highlighted the need to define more or less stringent recommendations on pre-pasteurization discard criteria, and to what extent bacterial

screening test cut-offs must be performed [79]. Table I summarizes the sparse information found from culture-based protocols, which again partly explains the observed variations in bacterial content assessment from different HMB practices.

Revisited Process of Microbiological Screening for HMB

One of the reasons why DHM microbiological assessment procedures are not easy to standardize is its reliance on the use of PCM. Plate counts and subsequent confirmatory bacterial identification tests involve several tedious steps and partial subjectivity, which collectively introduce some variability in the microbial content determination process. Our group has recently presented data on the potential of the Tempo® technology (bioMérieux, Marcy L'Étoile, France) for DHM bacterial screening applications within HMB operations [75]. Tempo® is an automated system based on the most probable number (MPN) quantification method, equipped with filling and reading units that is designed to detect microorganisms in complex sample matrices. Analyses are performed on single-use test cards comprising 48 wells spanning three dilutions. The Tempo® device is currently used for quality control applications in food and cosmetic industries which have to demonstrate that their product meets specific microbiological criteria [80,81]. Our comparative study aimed at demonstrating that the analytical performance of the Tempo® system for DHM bacteriological testing was equivalent to the reference PCM. Moreover, it was shown that results could be obtained within a 24-hour turnaround time from sample inoculation, with no need for supplementary testing. The semi-automated culture-based method could be implemented within HMB operations as an in-process monitoring technology to optimize end-product quality and safety. Figure 1 shows a typical contaminant screening process followed by HMB, and how the Tempo® technology could allow in-process monitoring and consequent reductions in milk losses. In this scenario, each bottle or donor pool obtained from raw milk is screened before pasteurization for the four most relevant quality indicators (total aerobic flora, *Enterobacteriaceae*, *S. aureus* and *Bacillus cereus* group). Raw milk is then refrigerated until quantitative bacterial results are obtained, within a 24-hour period. A collective pool can be prepared from strictly uncontaminated donations, since contaminated milk bottles can be discarded prior to pasteurization. The results are a more efficient screening strategy, a significant reduction in the analysis time, an improved and simpler traceability and finally, an easier milk process management and a contamination risk reduction in the end-product. In order to reduce the 24-hour window of complete treatment, rapid thawing of the DHM bottles in a water bath at ≤ 7.2°C could be considered. Further research and optimization are still needed to demonstrate feasibility, especially if one would consider using this technology with pasteurized DHM as release criterion testing.

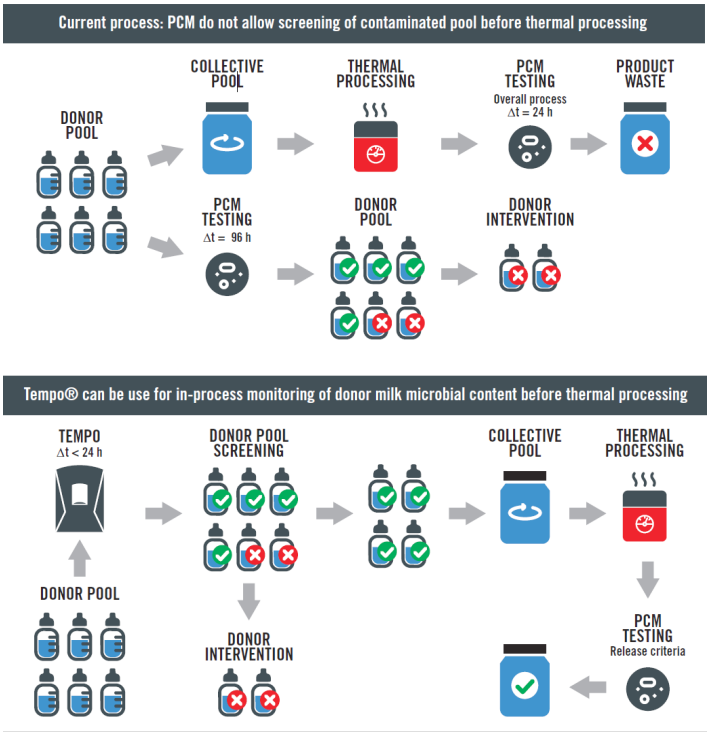

Figure 1: Human milk microbiological screening process before and after thermal processing using **(A)** the plate count method and **(B)** the proposed process involving the automated Tempo® device to achieve complete bacterial conformity testing within a 24 hours turnaround.

HMB location (reference)	Factors influencing bacteriological analysis	Culture conditions
Perth, Australia (Almutawif et al., 2017)	Volume of DHM tested BP	100 µl (single)
	Dilutions used for enumeration BP	100
	Volume of DHM tested AP	200 µl
	Enumeration medium for TAF	5% horse blood agar
	Selective medium used for pathogen detection	CLED (Cystine-lactose-electrolyte deficient)
	Plate incubation time, temperature, and percent CO_2	18-24 h; 35 °C in 5% CO_2
	Type of confirmatory testing	Gram staining, colony morphology and biochemical tests
Amiens, France (Mullié et al., 2018)	Volume of DHM tested BP	1 µl (in duplicate)
	Dilutions used for enumeration BP	10^0, 10^{-1}, 10^{-2} (tryptone-salt broth)
	Volume of DHM tested AP	500 µl
	Enumeration medium for TAF	Columbia 5% sheep blood agar
	Selective medium used for pathogen detection	Chapman (CPS)
	Plate incubation time and temperature	48 h; 36 ± 2°C
	Type of confirmatory testing	Agglutination test (CPS); MALDI-TOF mass spectrometry (non-compliant TAF)
Corbeil-Essonnes, France (Lecointe et al., 2016)	Dilutions used for TAF enumeration BP	10^0; 10^{-3}; 10^{-4}
	Enumeration medium for TAF	Blood agar
	Selective medium used for pathogen detection	Chapman (CPS)
	Plate incubation time and temperature	48 h; 37°C
	Type of confirmatory testing	Agglutination and biochemical tests (CPS)
Australian Red Cross Lifeblood, Australia (Clifford et al., 2020)	Detection Method	Spread or pour plate
	Enumeration medium for TAF	Plate count agar
	Selective medium used for pathogen detection	Baird Parker agar (CPS); RAPID Enterobacteriaceae Agar (EB)
	Plate incubation time and temperature	72 ± 2 h; 30 ± 1°C (TAF) / 48 ± 2 h; 37 ± 1°C (CPS) / 24 ± 2 h; 37 ± 1°C (EB)

BP: before pasteurization; AP: after pasteurization; DHM: donor human milk; TAF: total aerobic flora; CPS: coagulase-positive *Staphylococcus*; EB: *Enterobacteriaceae*

Table I: HMB practices for donor human milk microbiological testing based on plate count methods.

Conclusion

Further research focusing on the analytical performance of emerging technologies for processing and bacterial monitoring of human milk is needed. Rigorous comparative studies carried out within HMB environments, with continuous follow-up monitoring, will be required to convince regulatory agencies governing human milk production that they can be implemented for HMB quality control and release criteria testing. Clear demonstrations of efficacy could convince automated testing technology manufacturers to consider human milk in their application development process. Ultimately these technologies be recommended by official guidelines.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

MPC wrote the first draft of the manuscript and approved the final draft. DB contributed to the redaction process, critically reviewed and edited the manuscript, and approved the final draft.

Acknowledgments

The authors would like to thank Marie-Joëlle de Grandmont and Jean-François Leblanc for critically reading and reviewing the manuscript, and Stanko Josimov for the Figure 1 design.

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