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Human Milk Oligosaccharide Associated with Infant Nutritional Status and Macroscopic Stool Examination among Stunted Infants in Malang Indonesia

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Abstract

This study aims to investigate the concentration of 2'-FL HMO and its relationship with infant health status, then examine their correlation to macroscopic stool examination. A case-control study was conducted among 103 mother-infant pairs in 3 primary health care in Malang City, Indonesia. HPLC analyzed HMO quantification and fecal assessment by gross macroscopic stool examination. The findings showed that 49 infants had stunted nutrition status, and 54 had not stunted nutritional status. Among the group of stunted infants came from mothers with secretor-positive status (40.81%), while all infants with not stunted nutritional status came from mothers with secretor-positive status (100%). However, the status of secretor mothers to nutritional status was not significantly related (p>0.05). Levels of 2'-FL HMO in breast milk in stunted infants had a lower average than in non-stunted infants (1.21 mg/L vs. 1.40 mg/L). After analysis with the Mann-Whitney Test, 2'-FL HMO levels had a significant relationship with the baby's nutritional status, the yellow color in infant stool, and mucus in large amounts of stool (p>0.05). 2'-FL HMO has a significant role in the nutritional status of infants. Further analysis is needed to validate the macroscopic assessment of stool to detect inflammation and indigestion in stunted infants.

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Human Milk Oligosaccharide;
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Introduction

Stunting is a malnutrition problem faced by the world, including Indonesia.[1] According to the statistical evidence provided by the Ministry of Health of the Republic of Indonesia, 7.6 million children (accounting for 37% of the total population of children) are suffering from stunting. In Indonesia, the problem of stunting is further compounded by

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the scarcity of sufficient diagnostic instruments that could effectively direct therapeutic interventions and mitigate the issue.[2]

Histopathology, which involves the invasive biopsy of the small intestine, is considered the benchmark for identifying and diagnosing Environmental Enteric Dysfunction (EED) associated with a dysfunctional gut barrier. Hence, it can be inferred that modified diagnostic markers with reduced invasiveness can identify gut inflammation and heightened intestinal permeability.[3, 4] To date, lactulose tests and other blood and urine are the most frequently employed diagnostic tests for identifying EED.[5, 6] However, this analysis cannot be carried out at Indonesia's household level and primary health facilities.

By assessing stool, meaningful information about digestive system diseases can be obtained. The stool can be examined macroscopically, microscopically, chemically, immunologically, and chronologically.[7] In malnourished children, stool characteristics predict impaired nutrient absorption due to infection.[8] The macroscopic characteristics of the stool are evaluated by color, consistency, and the presence of mucus.[9] Several studies assessing macroscopic stools at home by parents have shown that evaluating stool color using 'stool color cards' in newborns and stool consistency has been found beneficial in monitoring treatment efficiency in functional constipation.[7]

In addition to EED, stunting is caused by a lack of adequate nutrition consumed by infants.[10] It is widely known that breast milk is the gold standard for infant nutrition, exclusively given during the initial six months of life. Breast milk contains several bioactive components, including human Milk Oligosaccharides (HMOs), the most prominent components after fat and lactose.[11]

HMOs have importance in infant health and nutritional status. Several studies show the role of HMO in infant health through the developing gut microbiota through the mechanism of selective anti-adhesion properties to pathogenic bacteria,[12] so that beneficial bacteria can bind to the intestinal epithelium and lower intestinal pH caused by short-chain fatty acids produced by bacteria to strengthen the function of the intestinal barrier,[13] as well as modulation of the immune system by restricting lymphocyte bonds, Monocytes, and neutrophils to epithelial cells.[14]

However, limited research comparing HMOs involves countries across Asia, particularly Indonesia.[15–18] With the critical role of HMOs in the health of these infants, we hypothesize that HMOs will modulate the nutritional status of infants in Indonesia, where one of the nutritional problems faced by Indonesia is stunting, which is still high. Several interventions in research using nutritional supplementation to overcome stunting, such as multiple micronutrients (MMN), small-quantity lipid-based nutrient supplements (SQ-LNS), and individual micronutrient supplementation in Indonesia, gave inconsistent results.[1] Nutritional supplementation intervention shows a narrow understanding of stunting as a form of malnutrition by concluding feeding as a solution. Therefore, it is necessary to understand nutritional ecology, which involves nutritional status with the absorption process and metabolic of digestion and the environment.[10] This is evidenced in studies that modulate the gut microbiota of infants showing improvements in the absorption of digestive nutrients so that they can increase the growth of infants.[19]

Therefore, this study aims to measure the concentration of HMO and differentiation of maternal secretor status and mitigate its relationship with infant health status. This research is necessary because it proves that HMO contributes to the nutritional status of infants so that it can be used as a rationale for optimal exclusive breastfeeding for stunting management in Indonesia.

Materials and Methods

Study Site and Participants

This study aims to investigate the concentration of 2'-FL HMO and its relationship with infant health status, then examine their correlation to macroscopic stool examination. A case-control study was conducted among 103 mother-infant pairs in 3 primary health care in Malang City, Indonesia. HPLC analyzed HMO quantification and fecal assessment by gross macroscopic stool examination. The findings showed that 49 infants had stunted nutrition status, and 54 had non-stunted nutritional status. Among the group of stunted infants came from mothers with secretor-positive status (40.81%), while all infants with non-stunted nutritional status came from mothers with secretor-positive status (100%). However, the

status of secretor mothers to nutritional status was not significantly related (p>0.05). Levels of 2'-FL HMO in breast milk in stunted infants had a lower average than in non-stunted infants (1.21 mg/L vs. 1.40 mg/L). After analysis with the Mann-Whitney Test, 2'-FL HMO levels had a significant relationship with the infant's nutritional status, the yellow color in infant stool, and mucus in large amounts of stool (p>0.05). 2'-FL HMO has a significant role in the nutritional status of infants. Further analysis is needed to validate the macroscopic assessment of stool to detect inflammation and indigestion in stunted infants.

This study constitutes a case-control investigation executed between November 2021 and November 2022 at three primary health centers in Malang, where stunted growth was observed at a high rate. This study used sample data from 103 mother and infant pairs who had signed and agreed to the Informed Consent sheet registered at the medical faculty of Brawijaya University Number. 3969.2/ 60 / UN10.F08/ PN/ 2021. Anthropometric index data measurements conducted were that length for age with Z-score (LAZ) limit parameter < -2 Standard Deviation (SD) for stunting nutritional status. Furthermore, interviews and filling out questionnaires were conducted for maternal and neonatal data on the mother's age, mother education, parity, vaginal delivery, HMO, secretor status, and stool macroscopic examination. The control group consisted of 51 infants with no stunted nutrition status, while the case group consisted of 51 infants with stunted nutritional status, namely body length for ages and Z score (LAZ) less than -2 SD.

Breast milk Samples Collection

Breastmilk is expressed at 8-11 AM to avoid variability due to circadian rhythm. Mothers are instructed to manually express milk from the mammary glands, which is subsequently gathered in a falcon tube measuring between 10 and 15 mL. Breastmilk is divided into five 1.5 mL cryovials and stored in a -80°C freezer within twenty-four hours following its procurement and is subsequently retained until the execution of the analysis.[11]

2'-FL Standard Preparation

The commercially available 2'-fucosyllactose (2'-FL) sourced from Sigma Aldrich was prepared by weighing and dissolving 2'-FL in room temperature water (12 mg/mL). Standard solutions were made by serial dilution in the concentration range of 0.2 to 6.6 mg/mL in room-temperature water. The dispersion was filtrated using a syringe filter with a 0.45 µm pore size.

2'-FL HMO Extraction

The technique for extracting oligosaccharides has been elaborately explicated in the literature referred to Christensen 20. In preparation for analysis, it is customary to neutralize the breast milk by subjecting it to a thawing process at a temperature of 5 °C for 24 hours. The preparation of aliquots comprising a proportional ratio of breast milk and water necessitates an equilibrated proportion of 1:1. Following this, the resultant amalgamation is subjected to centrifugation at a force of 10,000 g for 30 min while maintaining a temperature of 4 °C, utilizing a centrifuge instrument of the type Thermo Fisher Scientific Heraus Multifuge X3R. The transparent liquid underwent filtration using a PTFE syringe filter with a diameter of 0.25 mm and was subsequently subjected to centrifugation at a speed of 7,500 g for 50 min at a temperature of 4°C. The filtrate is then transferred into the High-Performance Liquid Chromatography (HPLC) vial and is deemed prepared for subsequent analytical procedures.

2'-FL HMO Quantification

The quantification of 2'-FL was conducted using the Shimadzu HPLC system, which was equipped with a refractive index detector. HMO separation was achieved by utilizing an Amide column and eluting a mobile phase composed of acetonitrile, water, and triethylamine in the proportion of 785:215:5 v/v/v. The flow rate utilized in the experiment was 0.2 mL/min, and the experiment was 19 min, with the column temperature maintained at 40 °C. The filtrate is introduced to the HPLC equipment through a 2 µL HPLC syringe. Following injection, the syringe is subjected to a rinsing procedure involving a combination of water and isopropanol (900/100, v/v).

Identification of Secretor Status from Breast Milk

The determination of secretor status is carried out based on research that has been carried out elsewhere.[14] Briefly, oligosaccharide determination is carried out by measuring structures that bind to α(1,2)-Fuc through activation of the FUT2 enzyme as a carrier of the secretor gene, namely 2'-FL.

Stool Collection

Infant stool samples were collected at home from nappies immediately after defecation using a sterile spatula into a sterile stool container for about minimum 200 mg and then frozen at −20 °C until transferred to a −80 °C freezer in the laboratory until the analysis time.

Table 1: Sociodemographic profile and nutritional status of the participants

Characteristic	Stunting	Not stunted	p value
Maternal age (mean + SD)	28.48+5.26	30.59+597	0.316
Graduated from Higschool (n,%)	26 (26.53)	27 (74.07)	0.107
Parity (mean + SD)	1.78+0.79	2.05+0.95	0.667
Pervaginam delivery (n,%)	14 (14.2)	17 (44.44)	0.695
HMO (mean + SD)	1.21+0.29	1.40+0.37	NA
Positif Secretor Status (n,%)	49 (40.81)	54 (100)	0.025*
Stool macroscopic assessment:			
Yellow Color (n,%)	32 (65.30)	25 (46.29)	0.137
Loose Consistency (n,%)	4 (8.16)	2 (3.70)	0.636
Abundance Mucus (n,%)	21 (42.85)	5 (9.25)	0.560

*p-value <0.05

Statistical Analysis

Data analysis was carried out to explain the characteristics of respondents using percentages and mean values. The relationship of 2'-FL HMO with nutritional status was analyzed based on the grouping of the nutritional status of stunted and non-stunted infants. Whitney's U-Mann test was performed to compare 2'-FL of stunted and not-stunted infants. The statistical analysis utilized the SPSS v.26 software (SPSS *et al*., USA).

Table 2: Association between sociodemographic profile, HMO, nutritional status, and stool macroscopic assessment

Variables	p value
Nutritional status	0.04*
Parity	0.673
Mode of delivery	0.516
Stool macroscopic assessment:	
Color	0.011*
Consistency	0.904
Mucus	0.034*

*p-value <0.05

Results

This study analyzed 103 mothers and infant couples, of which 49 had stunted nutrition and 54 had non-stunted nutritional status. Table 1 shows, based on the nutritional status of infants, it can be seen that stunted infants are born to mothers younger than infants with no stunted nutritional status (28.48 years vs. 30.59 years), mothers with high school education (26.53% vs. 74.07%), and born with lower non-stunted childbirth (14.2% vs. 44.4%). Among the group of stunted infants came from mothers with secretor-positive status (40.81%), while all infants with non-stunted nutritional status came from mothers with secretor-positive status (100%).

However, the status of secretor mothers to nutritional status was not significantly related (p>0.05). Levels of 2'-FL HMO in breast milk in stunted infants had a lower average than in non-stunted infants (1.21 mg/L vs. 1.40 mg/L). The results of the Mann-Whitney Test indicate a statistically significant relationship between HMO levels and various indicators of infant health, including their nutritional status, presence of yellowish color in stool, and amounts of mucus in stool (p > 0.05), as presented in Table 2.

Discussion

Our study is the first to assess the concentration of 2'-FL HMO and the relationship of HMO to nutritional status in Indonesia. In this study, we observed an association of 2'-FL HMO with nutritional status in exclusively breastfed infants under six months.

Some research has shown inconsistent results about factors that can affect HMOs.[21] Factors often revealed to influence HMO include lactation stages, maternal status, and geographical conditions.[22] This study carried out the lactation stage in infants aged six months or maturing breast milk. This study's average concentration of 2'-FL HMO was 1,314 mg/L. This amount was lower than studies conducted in the USA, Canada, Europe, and China, which varied between 4-6 mg/L in breast milk.[15] This gap in HMO levels can be caused by the HMO analysis method carried out in this study using the HPLC method. The capillary electrophoresis-laser-induced fluorescence (CE-LIF) method in comparable studies conducted in China and other Asian nations resulted in notably elevated findings6, while in research using HPLC, lower HMO levels were obtained.[23]

Determination of the secretor status based on (Se) and Lewis (Le) gene codes, both of which determine the profile and relative abundance of HMOs expressed by glucosyltransferase (FUT).[24] The secretor gene (Se+) found HMO abundance α1-2-fucosylated, especially 2'-FL, whereas in non-secretors, due to lack of FUT2 enzymes, it does not contain, or contains minimal amounts of 2'-FL and other α1-2-fucosylated HMOs.[25] Maternal secretor status was determined by the absolute abundance of the 2'-FL present in every breast milk sample, employing a natural breakpoint in the dataset for accurate classification25. Based on the cut-off (1.31 mg/L), 52.42% of mothers are secretors. A similar proportion was also found in the population of breastfeeding mothers in the Philippines, only 46% of mothers' secretors.[24]

2'-FL HMO level in this study was lower than in Europe, America, and Africa, indentify as weak secretors.[21] A deficient secretor is known to generate Fucosyltransferase-II (FucT2), an enzyme responsible for synthesizing structures that carry α-1,2-linked fucose residues. However, due to alterations in the amino acid sequence, the effectiveness of this enzyme is considerably compromised. Consequently, oligosaccharides like 2'-FL in breast milk are significantly below the typical levels observed in breast milk from secretor mothers.[23]

In this study, the relationship of stunting nutritional status was not related with mother's age, mother education, parity, vaginal delivery, but significantly related with HMO, secretor status, and stool macroscopic examination. According to recent empirical investigations, a positive correlation between HMOs and early growth in infancy[14] has been established. The experimental findings suggest that 2'-FL HMOs significantly affect longitudinal development, as observed in animal model investigations.[21] According to findings presented by another author in Malawi, it was found that a positive correlation between the total quantity of HMO present in breast milk and LAZ during the crucial developmental phase of infants between 6 and 12 months.[26]

Interestingly, this study shows HMO associated with macroscopic stool examination between stunted and non-stunted infants. There was a significant association between 2'-FL HMO levels and stool color and the presence of mucus in the stool of stunted infants. The characteristics of mucus in stools were also found in 28% of stunted children and proved a correlation between gut inflammation and stunting.[26] Young infants aged 0-6 months who suckle exclusively have peanut butter paste-like stools on the brown color spectrum: brown-brown, yellow-brown, or green-brown8. Green and frothy stools may be caused by too much foremilk (low-calorie milk in breast milk) and insufficient hindmilk (higher fat and super nutritious). This could mean the mother does not breastfeed her infant long enough in each breast.[27]

This study showed that the color of the stool is predominantly yellow. This color corresponds to the non-stunted color of the infant's stool, which is brownish-yellow due to bilirubin and bile. Furthermore, the association of HMO with infant stool color shown in infants given prebiotic supplementation resulted in a meaningful stool discoloration signaling a good microbiota gut condition.[28] HMOs acting as prebiotics in infant digestion make infant stool soft to regulate the

microbiota and protect the neonatal gut against pathogens.[13,29] The presence of mucus in the stool of stunted infants in this study indicates the possibility of gut inflammation. The presence of mucus in the stool can indicate prolonged irritation of the intestinal mucosa.[27,30] This can be caused by various things, including intestinal inflammation that causes growth and development disorders in toddlers/children.[8,31] However, laboratory tests must still be conducted to determine the causative agent. One of them is with a routine stool microscopic test, the gold standard for digestive problems caused by infections.[4,6]

The limitations of this study do not calculate the total value of HMOs, so it is not biased to calculate the proportion of 2'-FL in total HMOs. Nevertheless, the 2'-FL value of this study was measured with absolute values and was associated with specifics on the nutritional status of stunted and non-stunted infants. Further studies are needed to assess total HMOs prospectively to understand the relationship of HMOs to linear infant growth clearly. This study provided primary observational data with groups of homogeneous respondents in 6-month-old infants exclusively breastfed. Moreover, apart from the chief discoveries highlighting the impact of 2'-FL HMO concentration on the nutritional status of infants, auxiliary findings have shown that 2'-FL HMO exhibits an association with the color and presence of mucus in the stool of stunted infants.

Conclusion

2'-FL HMO associated with infant gut microbiota as macroscopically evidenced by color and mucus in stunted infant stools. Further analysis is needed to assess the specificity and sensitivity of the stool macroscopic assessment to detect inflammation in stunted infants and optimize HMO's potential for improving stunting.

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Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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