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NUTRITION AND INFECTIONS

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Editorial: Nutrition and infections

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Editorial on the Research Topic
Nutrition and infections

Infection is one of the leading causes of death globally. Despite the economic development and the improvement of people's quality of life, the occurrence of infection is still very common. The individual dietary state has a significant impact on both host susceptibility to infectious illnesses and their outcome. According to available evidence, specific and aggregate dietary deficiencies can change a host's immune response and enhance infection susceptibility. Within this context, this Research Topic called for research on the relationship between nutritional factors and specific infection as well as mechanism of action. Nutritional factors are relatively controllable and easier to be implemented for intervention compared with drugs, surgery, and other treatment methods. As represented in this Research Topic, nutritional factors and their impact on various infections were evaluated.

For instance, Siziba et al. investigated the association of human milk oligosaccharides (HMOs) concentration and the risk of otitis media and lower and upper respiratory tract infections in 667 breastfed infants. Despite the potential benefits of neonatal immune enhancement from HMOs, the study suggests that neither prominent neutral individual HMOs (ranging from 2′-FL to LNDFHs) nor acidic human milk sialyllactoses or lactose are significantly associated with a reduced or increased risk of infections in infants up to 2 years of age.

To understand the potential benefit of anti-inflammatory short-chain fatty acids (SCFA) from dietary fiber, Ni et al. conducted mice experiment to investigate impact of dietary fiber on West Nile Virus (WNV) disease and associated survival and immune profile. Despite increased fecal SCFA acetate and changes in gut microbiota composition, dietary fiber did not affect clinical scores, leukocyte infiltration into the brain, or survival. The study concluded that fiber supplementation is not effective in WNV encephalitis.

Furthermore, Xu et al. investigated the discrepancy of gut microbiota among elite athletes and young adults with different physical activities. The matching study of 63 Chinese young adults found a non-linear association between physical activity and microbiota, which may be confounded by the dietary intake. In contrast, a separate study

by Li et al. found that serum albumin was associated with disability in activities of daily living, mobility, and objective physical functioning among 2,233 Chinese older adults (≥65 years) in the Chinese Longitudinal Healthy Longevity Survey. It indicates that appropriate management of poor nutritional status through lowering serum albumin levels may contribute to maintaining physical functions in older adults.

This Research Topic includes three review articles on nutrients and infections. Govers et al. provided a broad overview on the topic of nutrition and viral respiratory tract infections. This review covered which population subgroups are susceptible to respiratory infections, which immunological mechanism contribute to antiviral immunity, how nutritional components can modulate antiviral immune response and infections. In a separate review, Cai et al. conducted a comprehensive systematic review and meta-analysis to clarify the relationship between common types of vitamins and helicobacter pylori (*H. pylori*). The study found that *H. pylori* infections decrease the serum levels of several types of vitamins, eradication of *H. pylori* could rescue its adverse effects, and antioxidant vitamin supplementation may improve the *H. pylori* eradication rate.

Furthermore, a systematic review and meta-analysis on the association between vitamin D and influenza was conducted by Zhu et al. Of the nine included randomized controlled trials, the summarized results indicates that substitution of vitamin D significantly reduces the risk of influenza infections (relative risk = 0.95, 95% confidence interval: 0.73–0.98). The study confirms the preventive effect of influenza by vitamin D supplement, but also suggests further high-quality studies with better design and larger samples.

On topics in relation to nutrient intake, Lin et al. evaluated the role of oropharyngeal dysphagia in older patients on long-term enteral feeding for risk stratification of pneumonia requiring hospitalization. It is found that the risk of pneumonia requiring hospitalization was significantly lower in patients with percutaneous endoscopic gastrostomy than in those with nasogastric tube among the patients with oropharyngeal dysphagia (adjusted hazard ratio 0.26, 95% confidence interval: 0.11–0.63). Tao et al. investigated the association between stress hyperglycaemia and stroke-associated pneumonia (SAP) with a total of 2,039 patients. The study quantified the stress hyperglycaemia through dividing the blood glucose by the glycated hemoglobin to calculate the stress hyperglycaemia ratio (SHR). It is found that SHR was significantly associated with the risk of SAP in patients without diabetes, but not in patients with diabetes. Furthermore, to predict potential surgical site infections (SSI), Chen et al. developed and validated a nomogram based on geriatric nutritional risk index (GNRI) for gynaecologic oncology patients. The use of GNRI cut-off value of 101.7 could stratify patients into distinct SSI risk groups, and the use of GNRI in nomogram model could enhance its potential to predict SSI in gynaecologic oncology patients.

Nutritional factors can support immune function against infections *via* different mechanisms and at different levels as demonstrated in this *Research Topic*. It has been shown that dietary deficiencies and malnutrition could lead to infection and contribute to morbidity and mortality. Thurs, further research should focus on identifying these nutritional factors and developing preventive strategies to target diet and nutrition at population level to improve people's quality of life.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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.

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Associations of Human Milk Oligosaccharides With Otitis Media and Lower and Upper Respiratory Tract Infections up to 2 Years: The Ulm SPATZ Health Study

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Background: Human milk oligosaccharides (HMOs) support and concurrently shape the neonatal immune system through various mechanisms. Thereby, they may contribute to lower incidence of infections in infants. However, there is limited evidence on the role of individual HMOs in the risk of otitis media (OM), as well as lower and upper respiratory tract infections (LRTI and URTI, respectively) in children up to 2 years.

Objective: To investigate whether individual HMO concentrations measured at 6 weeks of lactation were associated with risk of OM, LRTI or URTI up to 2 years in breastfed infants. Associations with OM, LRTI and URTI were determined for the most prominent human milk oligosaccharides including 13 neutral, partly isomeric structures (trioses up to hexaoses), two acidic trioses, and lactose.

Design: HMO measurements and physician reported data on infections were available from human milk samples collected at 6 weeks postpartum ($n = 667$). Associations of HMOs with infections were assessed in crude and adjusted models using modified Poisson regression.

Results: Absolute concentrations (median [min, max], in g/L) of 2′-fucosyllactose (2′-FL) tended ($p = 0.04$) to be lower, while lacto-N-tetraose (LNT) was higher in the milk for infants with OM in the 1st year of life ($p = 0.0046$). In the milk of secretor mothers, LNT was significantly higher in the milk for infants with OM (RR [95% CI]: 0.98 [0.15, 2.60]) compared to infants without OM (RR [95% CI]: 0.76 [0.14, 2.90]) at 1 year ($p = 0.0019$). No statistically significant milk group differences and associations were observed for OM, LRTI, and URTI ($p > 0.0031$).

Conclusion: Our findings suggest that neither prominent neutral individual HMOs (ranging from 2′-FL to LNDFHs) nor acidic human milk sialyllactoses or lactose are

significantly associated with a reduced or increased risk of infections in infants up to 2 years of age. Further research is needed to determine whether specific HMOs could potentially reduce the incidence or alleviate the course of distinct infections in early life.

Keywords: human milk oligosaccharides (HMOs), otitis media (OM), lower respiratory tract infections (LRTI), upper respiratory tract infections (URTI), human milk groups, targeted LC-MS/MS^2

INTRODUCTION

Otitis media (OM) is a common infectious disease during infancy, which is also responsible for a huge burden of disease in both emerging and established economies (1). In addition, respiratory infections are one of the leading causes of morbidity in infants and young children (2, 3). Recent reviews (4, 5) highlighted several studies that have demonstrated a protective effect of breastfeeding on OM and respiratory infections in children up to 2 years of age. Hence, human milk is regarded important as an early life exposure for the development of a healthy immune system (6–8). Of note, human milk comprises several multifunctional components which function as chemokines, antimicrobial proteins or peptides, antioxidants, growth factors, anti-inflammatory elements, prebiotics, enzymes, probiotics, and nutrients for the infant (9, 10). However, very little is known about the nature of the relationship or interactions, if any, between these multiple components that are present in human milk, and their impact on the infant (11, 12).

Of interesting significance are human milk oligosaccharides (HMOs), important constituents of human milk that seem to have anti-microbial and anti-viral effects which could potentially contribute to lower incidence of respiratory and other infections in breastfed infants (13–17). Although the structures and quantities of HMOs differ significantly amongst women and both are dependent on maternal secretor and Lewis blood group status (18, 19), their influence on the infant's microbiome and immune maturation is well documented (14, 20–23).

However, the mechanism through which individual HMOs act as an antiviral is based on *in vitro* and *ex vivo* studies (24–27). Many viruses, bacterial pathogens or toxins need to adhere to mucosal surfaces to colonise or invade the host and cause disease (22). The biological structure of HMOs is similar to all cell surface glycan receptors and serves as decoy receptors that block the pathogens from binding to epithelial cells (20, 21). Consequently, the decoy receptor bound pathogens are then unable to attach to the cell surfaces and are excreted without causing disease. In addition, HMOs also modulate differentiation and death of intestinal cells which potentially impacts the infant's susceptibility to infections (28). As such, both neutral and acidic HMOs have been shown to exhibit anti-effective properties. For instance, some individual HMO structured have been associated with protection against diarrhoea in infants (29), postnatal HIV-transmission during breastfeeding (30), necrotizing enteritis (NEC) (31, 32) and reducing rotavirus infections (22), amongst others. As a result, HMOs play a key role in shaping the infant gut microbiome, controlling enteric infections, and protecting the newborn infant, plausibly in combination with other bioactive components in human milk, including non-immunoglobulin proteins and milk fat globule membrane.

In spite of the substantial interest in this area of research, the evidence is still scarce. We are only aware of two other studies that investigated associations of HMOs with OM and upper respiratory tract infections (URTI) (33) and acute respiratory infections (34). The outcomes among breastfed children in both studies were assessed at 6 and 24 weeks of age (33), and at 6–7 months of age (34). Still, the sample size for one study (33) was limited ($n = 49$) and human milk samples were collected only at 2 weeks (33) and 6 months (34) postpartum. We previously showed a significant relationship of time, maternal secretor and milk group status on HMOs across lactation (35). Based on this, the HMO structures present and supplied to the infant through human milk will differ significantly. Maternal secretor and milk group status should therefore be considered when investigating clinical health outcomes in infants. Yet, maternal secretor status was only accounted for in one recent study (34).

Furthermore, Stepans et al. (33) investigated one specific HMO, LNFP II (lacto-*N*-fucopentaose II), while Jorgensen et al. (34) investigated several other bioactive proteins in addition to 51 individual HMOs and structure specific groups of HMOs. In light of this, a broader understanding of the potential effects of HMOs in increasing or reducing susceptibility to infections in breastfed infants is warranted. Thus, to contribute to this area of research, we not only measured lactose and several individual HMOs (∼15) using more sensitive and robust techniques (35), but we also took into account maternal secretor status and milk groups. The aim of this study was to investigate the associations of individual HMOs measured at 6 weeks of lactation with OM, and with lower and upper respiratory tract infections (LRTI and URTI, respectively) in the first and second year of life in the context of a large birth cohort study.

MATERIALS AND METHODS

Study Design and Population

Data were obtained from the Ulm SPATZ Health Study, an ongoing birth cohort study, in which a total of 970 mothers (49% of all eligible families) and their 1,006 newborn infants were recruited shortly after their delivery, during their hospital stay at the University Medical Centre Ulm, southern Germany, between April 2012 and May 2013 (36). Of note, University Medical Centre was the only hospital in Ulm and surrounding areas, thus we have a fairly representative sample of the general population. Inadequate German language skills, outpatient childbirth, maternal age <18 years, postpartum transfer of mother or child to intensive care unit, or stillbirth were all reasons for exclusion from participating in the study. All

participants signed written informed consent prior to the study and participation in the study was completely voluntary. Ethical approval for the SPATZ study was attained from the Ethics board of Ulm University (No. 311/11).

Data Collection and Measurements

Demographic, lifestyle and birth-related data including but not limited to child sex, delivery mode, maternal age, level of education, parity, and pre-pregnancy body mass index (BMI calculated as [mass(kg)/height(m)^2]) were collected using self-administered questionnaires, electronic hospital charts, and routine screening examinations. Questionnaires documented social demographic information, living situation and lifestyle factors. Further clinical data related to child's delivery and the mother's pregnancy were obtained from routine paper documentation. Mothers were asked if the child was still getting human milk at the time of sample collection and if the mother had introduced any complementary foods or liquids in addition to human milk. When the mother stated that no further liquid, semi-solid or solid food had been given to the child up to that point, this was classified as exclusive breastfeeding. Subsequently, a more definitive category of exclusive breastfeeding was then derived, based on maternal recall of introduction of other liquids or foods, at each time point. Reported doctor's diagnoses of OM, LRTI (including pneumonia, bronchitis, pertussis, tracheobronchitis, Krupp, bronchiolitis and flu), and URTI (including rhinitis, pharyngitis, tonsillitis and epiglottitis) were assessed at 1 and 2 years of age, for the first and the second year of life, respectively, by standardised, self-administered questionnaires from the children's primary care paediatricians. Several other health outcomes were also similarly assessed concurrently using these questionnaires. Additional data was collected at 6 weeks, 6 months, and 12 months after delivery by telephone interview or self-administered questionnaires sent by post if participants were not reachable by telephone of had previously stated breastfeeding cessation. Subsequent follow-ups were done yearly, and follow-ups is still on going.

Human milk samples were collected at approximately 6 weeks [Mean (SD), 5.9 (0.7) weeks] post-delivery from willing lactating mothers who were still breastfeeding at the time of sample collection. Lactating mothers were instructed to clean the breast and manually express or pump human milk between 9 am and 12 pm, after breakfast and before lunch, but at least 1 h after the infant's last feed. Where necessary, trained study nurses helped mothers with expression of human milk. Human milk samples were stored in the refrigerator by the mothers until study nurses collected them from their homes on the same day if milk was expressed between 9 am and 12 pm (76.9%), or on the next day if milk was expressed in the evening [after 12 pm, 9%; and before 9 am, 13.8%] and delivered them refrigerated to the study centre. Following which, they were separated into aliquots and frozen within 48 h and kept until further analysis.

Analysis of HMOs

Human milk samples were stored at −80°C until analysis of HMOs in 2019 as previously described (35). Briefly, individual native HMOs were identified and quantified using targeted liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS^2) in negative ion mode. Quantification of absolute HMO concentrations was done for lactose and 16 of the most abundant HMOs comprising: 2′-fucosyllactose (2′-FL); 3-fucosyllactose (3-FL); 3′-sialyllactose (3′-SL); 4′-galactosyllactose (4′-GL); 6′-galactosyllactose (6′-GL); 3,2′-difucosyllactose (DFL); 6′-sialyllactose (6′-SL); lacto-N-tetraose (LNT); lacto-N-neotetraose (LNnT); lacto-N-fucopentaose I (LNFP I); lacto-N-fucopentaose II (LNFP II); lacto-N-fucopentaose III (LNFP III); lacto-N-fucopentaose V (LNFP V); lacto-N-difucohexaose I (LNDFH I); and the sum of lacto-N-difucohexaose II and lacto-N-neodifucohexaose II (LNDFH II + LNnDFH II, standard containing both). Milk group and secretor status in this study were determined as previously described (35), based on the presence or absence of α1,2- and α1,4-fucosylated HMOs (**Table 1**). We essentially used the concentrations of LNFP I, LNFP II, and LNDFH I as proxies for FUT2 and FUT3 activities. Thus, human milk samples which contained both α1,2- and α1,4-fucosylated HMOs, like LNDFH I, were attributed to group I. Group II milk comprised human milk samples in which LNFP I and LNDFH I were absent [i.e., below the limit of quantification (<LLOQ)]. Group III milk comprised samples in which LNFP II and LNDFH I were not present (<LLOQ) and group IV comprised samples which did not present LNFP I and LNFP II (<LLOQ). Human milk samples attributed to group I and II were then grouped as secretors, while group II and IV milk were grouped as non-secretors. For the purpose of this study, HMO concentrations measured at 6 weeks were used to determine associations with OM, LRTI and URTI in the first and second years of the infant's life, respectively. Human milk samples with a complete set of HMO data and outcomes on infections (either one, in the first or second year) were available from up to 667 mothers.

TABLE 1 | Basis on which human milk samples were classified to the different milk groups (types).

Milk type	Condition
I	If not Type II, III or IV
II	LNFP I & LNDFH I <LLOQ
III	LNFP II & LNDFH I <LLOQ
IV	LNFP I & II <LLOQ

LNFP I, lacto-N-fucopentaose-I; LNFP II, lacto-N-fucopentaose-II; LNDFH I, lacto-N-difucohexaose I; <LLOQ, below the limit of quantification.

Statistical Analysis

Data for 4′-GL were excluded because >90% of values were found to be below the lower limit of quantification (<LLOQ). For the remaining HMOs, all values <LLOQ were replaced by LLOQ / $\sqrt{(2)}$ and the values higher than the upper limit of quantification (>ULOQ) were extrapolated for 3-FL, 3′-SL, DFL, 6′-GL, 6′-SL, LNT, LNFP I, LNFP II, LNDFH I, LNDFH II + LNnDFH II (35). Absolute HMO concentrations (g/L) measured at 6 weeks of lactation stratified by OM, LRTI or UTRI in the first or second year of life are presented as mean (SD) and

TABLE 2 | Characteristics of lactating mothers and infants who had a complete set of data on human milk oligosaccharides and atopic dermatitis available in the Ulm SPATZ Health Study.

		6 weeks samples (n = 667)
Mother		*n* (%)
	Age (years)	33.05 (4.58)[a]
Age category (years)		
	<30	30 (4.5)
	30–35	467 (70.1)
	>35	169 (25.4)
BMI category 6 weeks		
	Underweight (BMI < 18.5 kg/m^2)	6 (1.0)
	Normal (18.5 ≤ BMI < 25 kg/m^2)	345 (57.9)
	Overweight (25 ≤ BMI < 30 kg/m^2)	179 (30.0)
	Obese (BMI ≥ 30·00 kg/m^2)	66 (11.1)
Parity (*n* births of foetus ≥ 24 weeks)		
	0 births	343 (51.5)
	≥1 birth	323 (48.5)
Milk group		
	I	493 (73.9)
	II	119 (17.8)
	II	47 (7.0)
	IV	8 (1.2)
Maternal allergy		
	Yes	203 (30.6)
	No	461 (69.4)
Infant		
	Female	316 (47.4)
	Male	351 (52.6)
Gestation category		
	≤ 36 weeks	183 (27.4)
	Between 36 and 41 weeks	382 (57.3)
	≥41 weeks	102 (15.3)
Delivery mode		
	Vaginal spontaneous	459 (68.9)
	Elective caesarean	66 (9.9)
	Emergency caesarean	80 (12.0)
	Vaginal assisted	61 (9.2)
Exclusive breastfeeding at 6 weeks		
	Yes	503 (75.4)
	No	164 (24.6)
Otitis media in the first year of life		
	Yes	440 (89.8)
	No	50 (10.2)
Otitis media in the second year of life		
	Yes	363 (72.0)
	No	141 (28.0)
LRTI in the first year of life		
	Yes	146 (29.6)
	No	348 (70.4)
LRTI in the second year of life		
	Yes	240 (44.6)
	No	298 (55.4)
URTI in the first year of life		
	Yes	327 (66.2)
	No	167 (33.8)
URTI in the second year of life		
	Yes	433 (80.9)
	No	102 (19.1)

[a]*Mean (SD). SD, Standard deviation; BMI, Body mass index; LRTI, Lower respiratory tract infections; URTI, Upper respiratory tract infections.*

median [min, max]. An unpaired two samples Wilcoxon sum rank test was used to evaluate differences of HMO and lactose concentrations in the milk for children with or without OM, LRTI or URTI. Modified Poisson regression analyses (37) were used to determine associations between individual HMOs and lactose with OM, LRTI or URTI in the first or second year of life, respectively in crude and in adjusted models. Risk ratios (RR) and their 95% confidence intervals (95% CI) were obtained by exponentiation of the observed coefficients, as a logarithmic link function was used to model the HMO concentrations as continuous independent variables. Models were adjusted for infant sex, maternal allergy, exclusive breastfeeding at 6 weeks of lactation, delivery mode, parity, secretor status and milk group. These covariates were selected to be included in the models based on their influence on HMOs (35, 38–40). A stratified analysis was done to investigate these associations amongst infants receiving secretor or non-secretor milk and those receiving milk attributed to milk group I or II. A Bonferroni-adjusted threshold of $\alpha = 0.05/16 = 0.0031$ was used as a level of statistical significance following correction for multiple testing. All statistical analyses were performed with R (version 3.5.1; R Foundation for Statistical Computing).

RESULTS

A total of 667 (68.8% of women participating in SPATZ) lactating women had HMO and infant data on either OM, LRTI or URTI in the first or second year of life (**Table 2**). The majority (75%) of infants were receiving human milk exclusively at 6 weeks. More than 70% of the infants had a report of at least one episode of OM in the first or second year of life. Almost a third (30%) had a positive report of LRTI in the first or second year of life. Similarly, more than half (60%) reported URTI in the first or second year of life.

Although there were some differences in absolute HMO concentrations (g/L) in the milk for infants with OM in the first or second year of life, these differences were not statistically significant (Bonferroni correction α threshold = 0.0031). However, at the conventional level of significance ($p<0.05$), 2′-FL was (Wilcoxon sum-rank test, $p = 0.04$) lower while LNT was considerably higher in the human milk for infants with

OM at 1 year ($p = 0.0046$, **Table 3**). However, after Bonferroni correction, the differences in absolute HMO concentrations (g/L) in the human milk for infants who developed OM in the first or second year of life, were not statistically significant (Bonferroni correction α threshold = 0.0031).

A similar pattern of seemingly lower 2′-FL (Median [min, max)], although not statistically significant following correction for multiple testing, was evident in the secretor milk of infants with OM (2.40 [0.36, 6.30] g/L) compared to those without OM (2.70 [0.13, 6.60] g/L) in the first year of life ($p = 0.009$, **Supplementary Table 1**). Amongst infants receiving secretor milk, absolute concentrations of LNT were significantly higher in the milk for infants with OM (0.98 [0.15, 2.60] (g/L), $p = 0.0019$) compared to infants without OM (0.76 [0.14, 2.90] g/L) in the first year of life (**Supplementary Table 1**). This statistically significant difference in LNT concentrations was not evident for OM in the second year of life (**Supplementary Table 2**).

At the conventional level of significance ($p < 0.05$), absolute concentrations of LNT were higher in group I milk of infants with OM (0.98 [0.15, 2.60] g/L) compared to their counterparts (0.77 [0.14, 2.90] g/L, $p = 0.0067$, **Supplementary Table 3**) but not in the second year of life ($p = 0.28$, **Supplementary Table 4**). There were no statistically significant differences (i.e., at the conventional and Bonferroni corrected levels of significance) of individual HMOs in the milk for infants with or without LRTI (**Supplementary Tables 5–9**). Likewise, absolute concentrations of LNFP I (0.36 [0.04, 3.00] g/L) and total HMOs (6.10 [2.41, 11.6] g/L) were lower ($p < 0.05$) in the milk of infants with URTI in the first year compared to their counterparts (0.48 [0.04, 2.70], and 6.60 [3.39, 9.80] g/L, respectively, **Supplementary Table 10**) in the second year of life. This pattern was not present in secretor milk of infants with URTI in the first year (**Supplementary Table 11**) but in the second year of life (**Supplementary Table 12**). This was also the case when human milk samples were stratified by milk group (**Supplementary Tables 13**, **14**).

Following Bonferroni correction ($\alpha = 0.0031$), there were no statistically significant associations of individual HMOs with the risk of OM in the first or second year of life (**Table 4**). However, there were some associations that were statistically significant at the conventional level (using $\alpha = 0.05$). For example, higher LNT and higher LNFP V were both associated with an increased risk of OM in the first year [RR (95% CI): 1.17 (1.02, 1.34) and RR (95% CI): 1.16 (1.01, 1.35), respectively]. Higher LNT [RR (95% CI)] in secretor milk was significantly ($p < 0.0031$) associated with a higher risk [RR (95% CI): 1.27 (1.09, 1.49), $p = 0.0025$] of OM in the first year of life in crude models (not shown). This association was not statistically significant following adjustment for infant sex, maternal allergy, delivery mode, exclusive breastfeeding, parity and milk group [RR (95% CI): 1.25 (1.06, 1.47), $p = 0.01$; **Table 5**].

Following Bonferroni correction, there were no further statistically significant associations between individual HMOs and the risk of OM, LRTI and URTI in the first or second year of life. Although, some associations were significant at conventional level ($p < 0.05$). For instance, higher 6′-GL in non-secretor milk was associated with a higher risk [RR (95% CI)] of OM in the first [1.95 (1.02, 3.73)] and second [1.61 (1.04, 2.50)] year of life (**Table 6**). Similarly, higher LNT in group I milk was associated with a higher risk [1.23 (1.04, 1.46)] of OM in the first year of life (**Table 7**). Higher 6′-GL in group II milk was also associated with a higher risk of OM in the first [1.96 (1.02, 3.74)] and second [1.60 (1.01, 2.55)] year of life (**Table 8**).

There were no other statistically significant or exploratory associations at conventional level of significance of individual HMOs with LRTI (**Supplementary Table 15**), with the exception of 3′-SL in non-secretor milk (**Supplementary Table 16**) and group II milk (**Supplementary Table 17**) which were associated with a high risk of LRTI in the second year of life, at conventional level of statistical significance ($p < 0.05$). Overall, there were no statistically significant associations between individual HMOs and URTI in the first or second year of life (**Supplementary Table 18**). However, LNFPI in secretor and group I milk was associated with a decreased risk of URTI in the first and second year of life, at conventional level of significance ($p < 0.05$, **Supplementary Tables 19**, **20**). Higher 6′-GL in non-secretor and group I milk was also associated with an increased risk of URTI in the first year of life ($p < 0.05$, **Supplementary Tables 19**, **20**). Of note, these associations were not statistically significant following Bonferroni correction (α threshold = 0.0031).

DISCUSSION

The present study including 667 breastfed children investigated associations between individual HMOs measured at 6 weeks of lactation with OM, LRTI and URTI in the first or second year of life, using data from the Ulm SPATZ Health Study. Although not statistically significant, due to the large number of associations we explored, we report a tendency of lower absolute concentrations of 2′-FL and considerably higher LNT in human milk for infants with OM in the first year of life, irrespective of maternal secretor status or milk group. This difference was statistically significant and tended towards significance amongst infants who were receiving secretor milk and milk attributed to group I, respectively. However, this and all other associations were not statistically significant following adjustment for infant sex, maternal allergy, delivery mode, exclusive breastfeeding, parity, milk group and correction for multiple testing.

The absolute concentrations of 2′-FL tended to be lower while the absolute concentrations of LNT were higher in the milk of infants with OM in the second year of life. However, there were no statistically significant associations of 2′-FL with risk of OM, LRTI or URTI in the first or second year of life, in both crude and adjusted models. Similarly, protection against diarrhoea caused by *Campylobacter jejuni* was significantly associated with 2′-FL in human milk (29). That said, while the absence of functional FUT2 activity has been associated with better resistance to some pathogens such as Norovirus genotypes and Helicobacter pylori, this reportedly comes at a cost of an increased risk of infection by other pathogens affecting the respiratory, urinary or gastrointestinal systems (40). On one hand, a profile of higher

TABLE 3 | Absolute human milk oligosaccharide concentrations (g/L) measured at 6 weeks and otitis media in infants in the first or second year of life in the Ulm SPATZ Health Study.

	OM in the first year of life			OM in the second year of life		
	OM Yes ($n = 50$)	**OM No ($n = 440$)**	***p***	**OM Yes ($n = 141$)**	**OM No ($n = 363$)**	***p***
Lactose						
Mean (SD)	66.8 (4.31)	66.6 (3.69)		66.4 (4.04)	66.7 (3.68)	
Median [min, max]	67.0 [58.0, 74.0]	67.0 [52.0, 77.0]	0.64	67.0 [56.0, 77.0]	67.0 [52.0, 74.0]	0.42
2′-FL						
Mean (SD)	1.93 (1.32)	2.31 (1.41)		2.26 (1.34)	2.29 (1.43)	
Median [min, max]	2.00 [0.13, 6.30]	2.50 [0.13, 6.60]	0.04	2.50 [0.13, 6.30]	2.50 [0.13, 6.60]	0.95
3-FL						
Mean (SD)	0.71 (0.55)	0.67 (0.54)		0.61 (0.51)	0.68 (0.55)	
Median [min, max]	0.55 [0.11, 2.10]	0.49 [0.03, 2.90]	0.65	0.42 [0.03, 2.90]	0.51 [0.03, 2.60]	0.12
3′-SL						
Mean (SD)	0.16 (0.05)	0.15 (0.04)		0.15 (0.05)	0.15 (0.04)	
Median [min, max]	0.15 [0.08, 0.27]	0.15 [0.05, 0.32]	0.64	0.15 [0.05, 0.30]	0.15 [0.07, 0.32]	0.79
6′-GL						
Mean (SD)	0.02 (0.02)	0.02 (0.01)		0.02 (0.01)	0.02 (0.01)	
Median [min, max]	0.02 [0.01, 0.15]	0.02 [0.004, 0.09]	0.67	0.02 [0.01, 0.15]	0.02 [0.004, 0.09]	0.51
DFL						
Mean (SD)	0.16 (0.15)	0.18 (0.17)		0.17 (0.14)	0.18 (0.18)	
Median [min, max]	0.15 [0.01, 0.72]	0.18 [0.01, 1.80]	0.11	0.15 [0.01, 0.72]	0.18 [0.01, 1.80]	0.56
6′-SL						
Mean (SD)	0.25 (0.09)	0.26 (0.11)		0.25 (0.10)	0.26 (0.11)	
Median [min, max]	0.24 [0.07, 0.54]	0.24 [0.06, 0.73]	0.84	0.23 [0.07, 0.54]	0.24 [0.06, 0.73]	0.33
LNT						
Mean (SD)	1.09 (0.50)	0.92 (0.49)		0.98 (0.53)	0.92 (0.48)	
Median [min, max]	0.99 [0.15, 2.60]	0.84 [0.14, 3.10]	0.0046	0.86 [0.15, 3.10]	0.84 [0.14, 2.90]	0.31
LNnT						
Mean (SD)	0.08 (0.05)	0.08 (0.06)		0.09 (0.06)	0.08 (0.06)	
Median [min, max]	0.07 [0.01, 0.26]	0.07 [0.01, 0.34]	0.50	0.07 [0.01, 0.34]	0.07 [0.01, 0.33]	0.66
LNFP I						
Mean (SD)	0.50 (0.48)	0.48 (0.50)		0.53 (0.48)	0.48 (0.46)	
Median [min, max]	0.35 [0.04, 2.20]	0.38 [0.04, 3.00]	0.97	0.42 [0.04, 2.70]	0.36 [0.04, 3.00]	0.33
LNFP V						
Mean (SD)	0.04 (0.04)	0.04 (0.04)		0.04 (0.04)	0.04 (0.04)	
Median [min, max]	0.03 [0.01, 0.17]	0.02 [0.01, 0.24]	0.08	0.02 [0.01, 0.17]	0.02 [0.01, 0.24]	0.54
LNFP III						
Mean (SD)	0.190 (0.07)	0.18 (0.07)		0.18 (0.07)	0.18 (0.07)	
Median [min, max]	0.19 [0.06, 0.43]	0.18 [0.04, 0.48]	0.31	0.17 [0.05, 0.43]	0.18 [0.04, 0.48]	0.45
LNFP II						
Mean (SD)	0.41 (0.42)	0.34 (0.40)		0.32 (0.37)	0.35 (0.41)	
Median [min, max]	0.22 [0.04, 1.60]	0.17 [0.04, 2.50]	0.23	0.15 [0.04, 1.60]	0.18 [0.04, 2.50]	0.25
LNDFH I						
Mean (SD)	0.50 (0.37)	0.53 (0.39)		0.53 (0.38)	0.52 (0.39)	
Median [min, max]	0.55 [0.02, 1.50]	0.58 [0.02, 1.90]	0.58	0.55 [0.02, 1.70]	0.58 [0.02, 1.90]	0.88
LNDFH II + LNnDFH II						
Mean (SD)	0.08 (0.12)	0.06 (0.11)		0.06 (0.10)	0.07 (0.11)	
Median [min, max]	0.02 [0.01, 0.46]	0.02 [0.01, 0.73]	0.35	0.01 [0.01, 0.46]	0.02 [0.01, 0.73]	0.28
Sum of HMOs						
Mean (SD)	6.12 (1.29)	6.23 (1.29)		6.19 (1.33)	6.24 (1.29)	
Median [min, max]	6.18 [3.70, 9.69]	6.16 [2.41, 11.6]	0.53	6.18 [2.41, 9.80]	6.17 [2.76, 11.6]	0.79

p values derived from Wilcoxon sum-rank test comparing HMO concentrations between infants with and without otitis media at 1 or 2 years. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.0031$.

OM, Otitis media; RR, Risk Ratio; CI, Confidence intervals; HMO, human milk oligosaccharides.

2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-GL, 6′-galactosyllactose; DFL, 3,2′-difucosyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, lacto-N-difucohexaose I; LNDFH II, lacto-N-difucohexaose II; LNnDFH II, lacto-N-neodifucohexaose II.

TABLE 4 | Adjusted associations between human milk oligosaccharides measured at 6 weeks of lactation with otitis media in the first or second year of life in the Ulm SPATZ Health Study.

	OM in the first year of life			OM in the second year of life		
	RR	**95% CI**	***p***	**RR**	**95% CI**	***p***
Lactose	1.00	(0.98, 1.02)	0.96	1.00	(0.99, 1.01)	0.67
2′-FL	0.87	(0.76, 1.01)	0.06	0.97	(0.91, 1.05)	0.49
3-FL	1.03	(0.90, 1.19)	0.64	0.91	(0.82, 1.02)	0.11
3′-SL	1.02	(0.93, 1.11)	0.71	1.01	(0.96, 1.07)	0.71
6′-GL	1.19	(0.92, 1.54)	0.18	1.09	(0.96, 1.25)	0.19
DFL	0.88	(0.70, 1.10)	0.25	0.93	(0.81, 1.07)	0.30
6′-SL	0.99	(0.89, 1.10)	0.89	0.95	(0.88, 1.03)	0.23
LNT	1.17	(1.02, 1.34)	0.03	1.07	(0.98, 1.18)	0.14
LNnT	0.92	(0.77, 1.09)	0.33	1.01	(0.89, 1.14)	0.90
LNFP I	1.07	(0.84, 1.37)	0.60	1.09	(0.93, 1.27)	0.30
LNFP V	1.16	(1.01, 1.35)	0.04	1.03	(0.91, 1.16)	0.66
LNFP III	1.05	(0.95, 1.16)	0.31	0.98	(0.90, 1.06)	0.55
LNFP II	1.14	(0.96, 1.36)	0.12	0.95	(0.82, 1.09)	0.47
LNDFH I	0.96	(0.82, 1.12)	0.58	1.00	(0.90, 1.11)	0.99
LNDFH II + LNnDFH II	1.17	(0.91, 1.50)	0.21	0.90	(0.74, 1.11)	0.35
Total HMOs	0.99	(0.95, 1.05)	0.79	0.99	(0.95, 1.03)	0.62

Associations determined by modified Poisson regression. Models adjusted for infant sex, maternal allergy, delivery mode, exclusive breastfeeding, parity, secretor status and milk group. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.0031$.

OM, Otitis media; RR, Risk Ratio; CI, Confidence intervals; HMO, human milk oligosaccharides.

2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-GL, 6′-galactosyllactose; DFL, 3,2′-difucosyllactose; 6′-SL; 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, lacto-N-difucohexaose I; LNDFH II, lacto-N-difucohexaose II; LNnDFH II, lacto-N-neodifucohexaose II.

TABLE 5 | Adjusted associations between human milk oligosaccharides in secretor milk measured at 6 weeks of lactation with otitis media in the first or second year of life in the Ulm SPATZ Health Study.

	OM in the first year of life			OM in the second year of life		
	RR	**95% CI**	***p***	**RR**	**95% CI**	***p***
Lactose	1.00	(0.98, 1.02)	0.77	1.00	(0.98, 1.01)	0.45
2′-FL	0.89	(0.77, 1.03)	0.12	0.98	(0.92, 1.05)	0.62
3-FL	0.98	(0.81, 1.19)	0.88	0.94	(0.83, 1.06)	0.30
3′-SL	1.02	(0.93, 1.12)	0.66	1.01	(0.95, 1.07)	0.76
6′-GL	0.98	(0.83, 1.17)	0.86	0.98	(0.88, 1.08)	0.67
DFL	0.85	(0.67, 1.06)	0.15	0.92	(0.80, 1.05)	0.21
6′-SL	1.00	(0.89, 1.13)	0.99	0.96	(0.88, 1.04)	0.33
LNT	1.25	(1.06, 1.47)	0.01	1.07	(0.96, 1.19)	0.23
LNnT	0.90	(0.74, 1.08)	0.26	0.99	(0.87, 1.12)	0.82
LNFP I	1.12	(0.88, 1.42)	0.35	1.11	(0.96, 1.29)	0.16
LNFP V	1.17	(0.94, 1.47)	0.16	1.02	(0.89, 1.17)	0.80
LNFP III	1.00	(0.88, 1.13)	0.95	0.94	(0.86, 1.03)	0.19
LNFP II	1.11	(0.83, 1.49)	0.47	0.99	(0.83, 1.18)	0.87
LNDFH I	0.91	(0.80, 1.05)	0.19	0.98	(0.90, 1.07)	0.66
LNDFH II + LNnDFH II	1.14	(0.84, 1.55)	0.39	0.98	(0.81, 1.19)	0.88
Total HMOs	0.98	(0.92, 1.06)	0.66	1.00	(0.96, 1.04)	0.90

Associations determined by modified Poisson regression. Models adjusted for infant sex, maternal allergy, delivery mode, exclusive breastfeeding, parity and milk group. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.0031$.

OM, Otitis media; RR, Risk Ratio; CI, Confidence intervals; HMO, human milk oligosaccharides.

2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-GL, 6′-galactosyllactose; DFL, 3,2′-difucosyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, lacto-N-difucohexaose I; LNDFH II, lacto-N-difucohexaose II; LNnDFH II, lacto-N-neodifucohexaose II.

TABLE 6 | Adjusted associations between human milk oligosaccharides in non-secretor milk measured at 6 weeks of lactation with otitis media in the first or second year of life in the Ulm SPATZ Health Study.

	OM in the first year of life			OM in the second year of life		
	RR	**95% CI**	***p***	**RR**	**95% CI**	***p***
Lactose	1.00	(0.96, 1.04)	0.88	1.01	(0.98, 1.03)	0.56
3-FL	0.98	(0.82, 1.18)	0.83	0.94	(0.81, 1.09)	0.42
3′-SL	0.99	(0.81, 1.20)	0.92	1.02	(0.89, 1.18)	0.78
6′-GL	1.95	(1.02, 3.73)	0.04	1.61	(1.04, 2.50)	0.03
6′-SL	0.97	(0.78, 1.20)	0.75	0.93	(0.80, 1.10)	0.40
LNT	0.98	(0.78, 1.24)	0.87	1.05	(0.87, 1.28)	0.62
LNnT	1.09	(0.78, 1.53)	0.62	1.27	(0.89, 1.81)	0.19
LNFP V	1.09	(0.91, 1.31)	0.33	1.07	(0.89, 1.29)	0.48
LNFP III	1.20	(0.99, 1.45)	0.07	1.10	(0.95, 1.26)	0.19
LNFP II	1.05	(0.87, 1.27)	0.60	1.00	(0.85, 1.18)	0.97
LNDFH II + LNnDFH II	1.09	(0.82, 1.46)	0.55	0.97	(0.77, 1.23)	0.81
Total HMOs	1.02	(0.91, 1.13)	0.78	1.00	(0.92, 1.09)	1.00

Associations determined by modified Poisson regression. Models adjusted for infant sex, maternal allergy, delivery mode, exclusive breastfeeding, parity and milk group. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.0031$.

OM, Otitis media; RR, Risk Ratio; CI, Confidence intervals; HMO, human milk oligosaccharides.

3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-GL, 6′-galactosyllactose; 6′-SL; 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH II, lacto-N-difucohexaose II; LNnDFH II, lacto-N-neodifucohexaose II.

TABLE 7 | Adjusted associations between human milk oligosaccharides in group I milk measured at 6 weeks of lactation with otitis media in the first or second year of life in the Ulm SPATZ Health Study.

	OM in the first year of life			OM in the second year of life		
	RR	**95% CI**	***p***	**RR**	**95% CI**	***p***
Lactose	1.00	(0.98, 1.02)	0.69	1.00	(0.98, 1.01)	0.48
2′-FL	0.91	(0.78, 1.05)	0.20	0.99	(0.92, 1.06)	0.76
3-FL	0.98	(0.80, 1.19)	0.83	0.94	(0.83, 1.06)	0.30
3′-SL	1.02	(0.92, 1.13)	0.68	1.00	(0.95, 1.06)	0.92
6′-GL	0.96	(0.80, 1.15)	0.66	0.98	(0.88, 1.09)	0.65
DFL	0.83	(0.66, 1.05)	0.13	0.91	(0.79, 1.05)	0.21
6′-SL	1.01	(0.89, 1.14)	0.92	0.97	(0.89, 1.06)	0.52
LNT	1.23	(1.04, 1.46)	0.02	1.06	(0.95, 1.19)	0.28
LNnT	0.90	(0.74, 1.10)	0.30	0.99	(0.87, 1.13)	0.86
LNFP I	1.11	(0.87, 1.41)	0.39	1.14	(0.97, 1.32)	0.11
LNFP V	1.17	(0.93, 1.48)	0.18	1.02	(0.88, 1.18)	0.78
LNFP III	0.95	(0.85, 1.07)	0.43	0.94	(0.85, 1.03)	0.16
LNFP II	1.11	(0.83, 1.49)	0.48	0.99	(0.82, 1.18)	0.88
LNDFH I	0.91	(0.80, 1.05)	0.19	0.98	(0.90, 1.07)	0.65
LNDFH II + LNnDFH II	1.14	(0.84, 1.56)	0.40	0.99	(0.81, 1.20)	0.89
Total HMOs	0.99	(0.92, 1.05)	0.67	1.00	(0.96, 1.04)	0.95

Associations determined by modified Poisson regression. Models adjusted for infant sex, maternal allergy, delivery mode, exclusive breastfeeding and parity. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.0031$.

OM, Otitis media; RR, Risk Ratio; CI, Confidence intervals; HMO, human milk oligosaccharides.

2′-FL, 2′-fucosyllactose; 3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-GL, 6′-galactosyllactose; DFL, 3,2′-difucosyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP I, lacto-N-fucopentaose-I; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH I, lacto-N-difucohexaose I; LNDFH II, lacto-N-difucohexaose II; LNnDFH II, lacto-N-neodifucohexaose II.

LNnT and 3′-SL in the milk of HIV-infected women compared to uninfected controls has previously been reported (30, 41). On the other hand, concentrations of LNT were significantly higher in the milk of HIV-infected mothers whose HIV-exposed uninfected children survived during breastfeeding, compared to HIV-infected mothers whose HIV-exposed children died (41). Nonetheless, we attribute the inverse pattern of 2′-FL and LNT concentrations to the fact that concentrations of LNT and LNnT

TABLE 8 | Adjusted associations between human milk oligosaccharides in group II milk measured at 6 weeks of lactation with otitis media in the first or second year of life in the Ulm SPATZ Health Study.

	OM in the first year of life			OM in the second year of life		
	RR	**Lower RR**	***P* value**	**RR**	**Lower RR**	***P* value**
Lactose	1.00	(0.96, 1.04)	0.90	1.00	(0.98, 1.03)	0.80
3-FL	0.98	(0.82, 1.18)	0.83	0.95	(0.82, 1.10)	0.46
3′-SL	0.99	(0.81, 1.21)	0.89	1.02	(0.88, 1.19)	0.74
6′-GL	1.96	(1.02, 3.74)	0.04	1.60	(1.01, 2.55)	0.05
6′-SL	0.97	(0.78, 1.20)	0.77	0.91	(0.78, 1.08)	0.27
LNT	0.98	(0.77, 1.24)	0.86	1.07	(0.87, 1.32)	0.50
LNnT	1.10	(0.78, 1.54)	0.60	1.24	(0.85, 1.82)	0.27
LNFP V	1.09	(0.91, 1.31)	0.35	1.12	(0.95, 1.33)	0.19
LNFP III	1.19	(0.99, 1.43)	0.06	1.13	(0.98, 1.31)	0.09
LNFP II	1.05	(0.87, 1.27)	0.60	1.00	(0.85, 1.18)	0.96
LNDFH II + LNnDFH II	1.09	(0.82, 1.46)	0.55	0.97	(0.77, 1.23)	0.82
Total HMOs	1.01	(0.91, 1.13)	0.79	1.01	(0.92, 1.10)	0.87

Associations determined by modified Poisson regression. Models adjusted for infant sex, maternal allergy, delivery mode, exclusive breastfeeding and parity. Bonferroni-adjusted level of statistical significance is $\alpha = 0.05/16 = 0.0031$.

OM, Otitis media; RR, Risk Ratio; CI, Confidence intervals; HMO, human milk oligosaccharides.

3-FL, 3-fucosyllactose; 3′-SL, 3′-sialyllactose; 6′-GL, 6′-galactosyllactose; 6′-SL, 6′-sialyllactose; LNT, lacto-N-tetraose; LNnT, lacto-N-neotetraose; LNFP V, lacto-N-fucopentaose-V; LNFP III, lacto-N-fucopentaose-III; LNFP II, lacto-N-fucopentaose-II; LNDFH II, lacto-N-difucohexaose II; LNnDFH II, lacto-N-neodifucohexaose II.

in human milk are core-regulated by the FUT2-dependent 2′-FL (40). In addition, although we did not find statistically significant associations, the anti-inflammatory and immunomodulatory effect of 2′-FL is well known (42–44).

Furthermore, in crude models, LNT in secretor milk was associated with a higher risk of OM in the first year of life. This association was not statistically significant following adjustment for infant sex, maternal allergy, delivery mode, exclusive breastfeeding, parity, milk group and Bonferroni correction. Likewise, another study (28) reported significantly higher levels of 2′-FL and LNT in the milk of secretor mothers whose children had symptomatic rotavirus infection. While LNFP I was higher in the milk of those who were positive for rotavirus infection compared to the rotavirus negative group of infants. Furthermore, in that same study (28), levels of 6′-SL and LNT were higher in non-secretor milk of symptomatic rotavirus group of infants, while LNFP II was significantly higher in the milk of asymptomatic rotavirus-positive group. Despite this, LNT is reportedly one of the most abundant core HMO structures present in human milk (ranging between 0.5 and 1.5 g/L in mature human milk) (19, 45). Yet, an *in vitro* study reported that LNT inhibited the growth of *Streptococcus agalactiae* (group B *Streptococcus*, GBS), a leading cause of invasive bacterial infections in newborns (46). Also, LNT has been reported to block *Entamoeba histolytica,* a major protozoan parasite in developing countries (47), from binding to epithelial cell surfaces (48). Although LNT has not yet been proposed to serve any direct antimicrobial function in infants, secretor status is useful as a stratification variable (14, 49). Thus, our results also confirm the importance of stratifying by or adjusting for maternal milk group when evaluating HMO associations with infant health outcomes.

We are only aware of two other studies that investigated similar associations with OM and respiratory problems (comprising URTI (runny nose or cold), cough, or pneumonia) (33) and acute respiratory infections (34). On one hand, Stepans et al. (33) reported associations between higher levels of LNFP II in colostrum and reduced risk of respiratory problems by 6 and 12 weeks, and non-statistically significant associations with OM. That study investigated human milk sampled at two weeks postpartum whereas we sampled human milk at 6 weeks postpartum. However, the study children (33) may have been breastfed for a longer time throughout the first year of life; while for our study the median durations of exclusive and any breastfeeding were 197 days (∼28 weeks) and 122 days (∼17 weeks), respectively (36). Still, it is possible that children in our study were breastfed for longer in the first year of life thus having a different baseline risk for infections compared to the other previous study. It should also be noted that only five infants were reported to have OM by 24 weeks, and the potential confounding of secretor status or milk group was not accounted for in that study (33).

On the other hand, Jorgensen et al. (34) showed a positive association between LNFP II in secretor milk with the incidence of acute respiratory infections in Malawian infants at age 6–7 months. However, this association was not statistically significant following correction for multiple testing using the Benjamin–Hochberg approach. Although they accounted for maternal secretor status, milk group status is also important to consider. Moreover, the secretor status of the infant, although difficult to measure, is also suggested to modify the association between HMOs and clinical health outcomes (14). Furthermore, the researchers in that study also assessed several other bioactive components in comparison

to our study. We are only aware of one other study (50) which also investigated the relationship between HMOs and other human milk immune components, simultaneously. Firstly, while both studies (34, 50) report plausible low and moderate correlations between relative abundances of HMOs, both these studies, as well as ours, do not cover 100% of the abundance of HMOs. Of note, the relative proportions or abundance of HMOs will always differ depending on the number of individual HMOs measured and quantified despite the standardised laboratory methods human milk sample collection.

Secondly, we acknowledge that several other components, which include immunological, hormonal, enzymatic, trophic, and bioactive components in human milk offer passive protection to the growing infant (51, 52). Human milk is particularly rich in maternal cells which potentially produce cytokines and have a modulatory influence on the immunological system of new borns. Among these cellular components are macrophages and leucocytes and other immunologic molecules which are present in large amounts during early lactation, particularly in colostrum compared to mature milk (53). Most of these components in human milk can interact with each other synergistically or with other additional factors related to the mucosal or systematic immune response of the infant (9). Thus, it is plausible that other bioactive compounds as well as the multiple HMOs that were not assessed in the current study have synergistic effects to decrease or increase the risk of infection. More research is needed to determine the exact nature of the correlations that exist between HMOs and other components and their potential impact on human milk composition or volume. Granted that HMOs vary greatly within and between groups of mothers, based on secretor status or milk group, as well as time of lactation, it is plausible that any associations observed in this current study, although not statistically significant, are applicable to a specific group of infants who receive human milk for a shorter duration of lactation.

Moreover, the overall beneficial effect of HMOs in reducing other infections is widely documented (14, 20–23, 54). For instance, an observational, prospective study reported reduced diarrhoea incidence caused by *Campylobacter jejuni* and *calicivirus* in infants who received human milk containing higher levels of 2′-FL and LNDFHI, respectively (29, 55). Similarly, another study (56) reported beneficial effects of fucosyl-HMOs in reducing morbidity in Gambian infants at 4 months of age. Two observational studies on African mother-infant pairs reported reduced risk of HIV transmission (30) and decrease in mortality of HIV-exposed infants (41) receiving human milk. Specific HMO structures present in secretor milk have also been associated with direct protection against specific infections like NEC in infants (57, 58). Nonetheless, although some individual HMOs are reported to be higher in the milk of infants with some infection compared to those without infection, whether or not these are true risk factors for infection remains unknown and requires further investigation. Even so, these high concentrations may be enhanced by the effect of vaccinations against infections. For instance, 2′-FL is suggested to potentially improve the effect of vaccination against influenza virus infection (27). The clinical consequence of specific HMOs, therefore, remains to be elucidated further.

A limitation of this study is its observational nature, which makes it difficult to draw conclusions without appropriate methods for assessing causal inference when using observational data. Also, the data presented here, are naturally subject to potentially unmeasured confounding factors, which include but are not limited to secretor status of the infant. Additionally, while the novel quantification method allowed us to measure absolute HMO concentrations up to hexaose structures, it is plausible that further important oligosaccharides that have not been identified in the current study as well as in previous studies may have other important biological effects. Still, we provide a much larger sample size from a large birth cohort in comparison to the other two studies (33, 34) evaluating similar associations.

In conclusion, our results do not confirm that individual HMOs are associated with either an increased or a reduced risk of OM, LRTI and URTI in infants during early life. However, we did pick up some associations which would be statistically significant at the conventional level and may thus be subject for further, exploratory or confirmatory studies. Taken together, the evidence base is still very limited, and has several issues. More studies are needed to determine the interactions and interdependencies that exist within a broader scope of human milk components. Further studies are needed to determine whether HMOs reduce the incidence or alleviate the course of specific infections in breastfed infants. However, human studies to investigate the exact metabolic pathways and implications of single HMOs in infants are limited due to a lack of HMO availability at times, and obvious ethical issues. Hence, the need to consider or select suitable surrogate animal models in which passive maternal immune transfer is avoided. These models and research studies will allow researchers to clarify the direct effect of HMOs, in more controlled experiments and their role in infections and other health outcomes.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available due to participant consent and data protection, we may not be able to share the raw data. However, the authors are open to sharing aggregate data (for instance, absolute concentrations that have been included as **Supplementary Material).** Requests to access the datasets should be directed to Jon.Genuneit@medizin.uni-leipzig.de.

ETHICS STATEMENT

This study was approved by Ethics Board of Ulm University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LPS and JGe conceived the study question and interpreted the data and wrote the manuscript. LPS conducted the statistical analyses. MM, JGo, and BB designed the LC-MS^2 method for HMOs analysis. JGo and BB validated the method for HMO analysis and analysed the HMOs in the lab. JGe and DR conceived and designed the Ulm SPATZ Health Study. JGe, DR, MM, and BS conceptualised the AMICA project for advanced compositional human milk analysis of SPATZ human milk samples. JGe and MM manage the AMICA project. All authors critically reviewed the manuscript and approved its final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.761129/full#supplementary-material

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Conflict of Interest: JGe is the project manager of unrestricted grants from Danone Nutricia Research to Ulm University and Leipzig University for research on other aspects of human milk composition in the Ulm birth cohort studies. MM, BS, JGo, and BB are employees of Danone Nutricia Research. However, the principal investigators JGe and DR along with LPS made final decisions on the interpretation and dissemination of results.

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

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Association Between Vitamin D and Influenza: Meta-Analysis and Systematic Review of Randomized Controlled Trials

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Background: Vitamin D supplementation improves the immune function of human body and can be a convenient way to prevent influenza. However, evidence on the protective effect of vitamin D supplementation on influenza from Randomized Controlled Trials (RCTs) is inconclusive.

Methods: RCTs regarding the association between vitamin D supplementation and influenza were identified by searching PubMed, Cochrane library, Embase and Chinese Biomedical Database (CBM) from inception until present (last updated on 10 November 2021). Studies that reported dosages and durations of vitamin D supplementation and number of influenza infections could be included. Heterogeneity was assessed using Cochran's Q test and I^2 statistics, the meta-analysis was conducted by using a random-effects model, the pooled effects were expressed with risk ratio (RR) with 95% confidence interval (95% CI).

Results: 10 trials including 4859 individuals were ultimately eligible after scanning. There was no evidence of a significant heterogeneity among studies ($I^2 = 27\%$, $P = 0.150$). Meta-regression analysis finding indicated that country, latitude, average age, economic level, follow-up period and average daily vitamin D intake did not cause the statistical heterogeneity. The study finding indicates that substitution with vitamin D significantly reduces the risk of influenza infections ($RR = 0.78$, 95% CI:0.64–0.95). No evidence of publication bias was observed. Omission of any single trial had little impact on the pooled risk estimates.

Conclusions: The meta-analysis produced a corroboration that vitamin D supplement has a preventive effect on influenza. Strategies for preventing influenza can be optimized by vitamin D supplementation.

Keywords: vitamin D supplementation, influenza, influenza-like illness, respiratory tract infection, meta-analysis

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INTRODUCTION

Influenza (referred to as flu) is an acute upper respiratory tract infection caused by influenza viruses. According to WHO, seasonal influenza epidemics cause an estimated 3 to 5 million severe cases and 290,000 to 650,000 deaths globally each year, indicating the substantial burden in public health worldwide with the impact of influenza (1). Although influenza vaccines are available, their efficacy is declined in mismatched seasons (2).

There are several studies concerning the effect of vitamin D supplementation on the prevention of influenza infection have been reported, but their results were inconsistent. Zhou et al. preformed an RCT in infants and found a significant difference between low-dose and high-dose vitamin D groups in the occurrence of influenza an infection ($RR = 0.56$, 95% CI: 0.42–0.77) (3). Aglipay et al. also reported a significant difference ($RR = 0.50$, 95% CI: 0.28–0.89) (4). Additionally, Urashima et al. conducted a RCT in 2010, and the result showed that vitamin D3 supplementation during the winter reduced the incidence of influenza A ($RR = 0.58$, 95% CI: 0.34–0.99) (5). In 2014, Urashima et al. preformed another RCT and reported that during the first month of follow-up, vitamin D3 supplementation was a protective factor against influenza ($RR =$ 1.11, 95% CI: 0.57–2.18), but based on the observation results at two-month follow-up, vitamin D supplementation does not play a role in preventing influenza ($RR = 0.17$, 95% CI:0.04–0.77) (6), which indicated that only short-term use of vitamin D3 dietary supplementation can temporarily decrease the incidence of influenza A during an influenza pandemic. However, Arihiro et al. have observed an insignificant difference in influenza incidence between the vitamin D and placebo group ($RR = 1.25$, 95%CI: 0.45–3.49) (7). Loeb et al. also reported ineffectiveness of vitamin D supplementation in preventing influenza (8). Studies found that vitamin D deficiencies in blood was associated with the increased incidence of respiratory tract infections and influenza (9–13). However, some opposite results were reported (14, 15).

In order to determine the overall effect of vitamin D supplementation on the risk of influenza and to identify factors that might influence the effects of this intervention on the risk of influenza, a meta-analysis and system review of RCTs was conducted in the study.

MATERIALS AND METHODS

Search Strategy

This systematic review and meta-analysis were conducted according to the Cochrane Handbook for Systematic Reviews of Interventions and the Preferred Reporting Items for Systematic Reviews and Meta-Analysis guidelines for meta-analysis (16, 17). Randomized Controlled Trials (RCTs) and clinical trials published before 10 November 2021 were collected from the electronic databases such as PubMed, Cochrane Library, Embase and CBM. Additionally, the OpenGrey database (http://www.opengrey.eu/) was also searched for any potential studies, and the International Clinical Trials Registry Platform (http://apps.who.int/trialsearch/) was scanned for ongoing trials. The references of retrieved studies and reviews were manually crosschecked.

Research terms "vitamin D or ergocalciferols or cholecalciferol" AND "influenza or pneumonia or common cold or respiratory tract infection or influenza-like illness or ILI" were used. The details of research strategy were provided in **Supplementary Table S1**. Each identified report was carefully scanned by two of the authors.

Selection Criteria

The inclusion criteria were as follows: (1) RCTs exploring the effect of vitamin D supplementation on the prevention of influenza; (2) studies reporting the dosages, modes and duration of vitamin D supplementation; (4) studies reporting the definitions of influenza patients or ILI (influenza-like illness) and the methods used to diagnose them. (5) English or Chinese articles. Reports as follow were excluded: (1) case reports, reviews, letters or comments; (2) animal or cell trials; (3) not involved in the association between vitamin D and influenza patients or ILI.

Data Collection and Quality Assessment

Two reviewers used Endnote9x software to screen literature. Data information were extracted through a standardized data extraction form. If there was any disagreement among two reviewers, the report would be sent to a third researcher and fully discussed. The Cochrane Collaboration's tool for assessing risk bias were used to assess the experimental study's quality, based on six quality criteria: sequence generation, allocation concealment, blinding; missing outcome data, selective reporting and other biases (18). Review Manager (version 5.4.1) was used here. The overall confidence in the estimate was assessed with GRADE profiler software according to the criteria published by the GRADE working group (19). The extracted information from included reports consisted of first author's name, publication year, participants, sample size, location, average age, dose design, follow-up period, season and primary outcomes.

Primary Outcomes

The primary outcome measures of the present study were the prevention of influenza infections in human including laboratory-confirmed influenza infections and ILI (body temperature greater than or equal to 38°C, accompanied by either cough or pharyngeal pain, while other laboratory diagnostic evidence is lacking).

Statistical Analysis

Study results were quantitatively combined for each outcome. For dichotomous outcomes, pooled estimates expressed as relative risks (RR) with the corresponding 95% confidence intervals (95%CIs) were calculated. Q test and I^2-statistic with its 95%CI were used to assess the heterogeneity among the effect results (20, 21), and the pooled RR was computed using a random-effects model because of the different characteristics of included studies such as diverse populations and different background diseases of subjects. If P-value of the Q test is greater than 0.05 and $I^2 \leq$ 50%, we can consider that there was no statistically heterogeneity. Forest plots were produced to show each trial's result and estimate the pooled effect sizes. Publication bias was tested by funnel plots, using Harbord tests (22). Sensitivity analysis was used to measure the impact of each individual trial on the combined effects and the robustness of results.

Statistical analysis was performed with Review Manager (version 5.4.1) and STATA 14.0 software. Meta-regression analysis was conducted to verify whether some covariates such as country, latitude (<30, 30–50 or >50°), average age (18<, 18–50

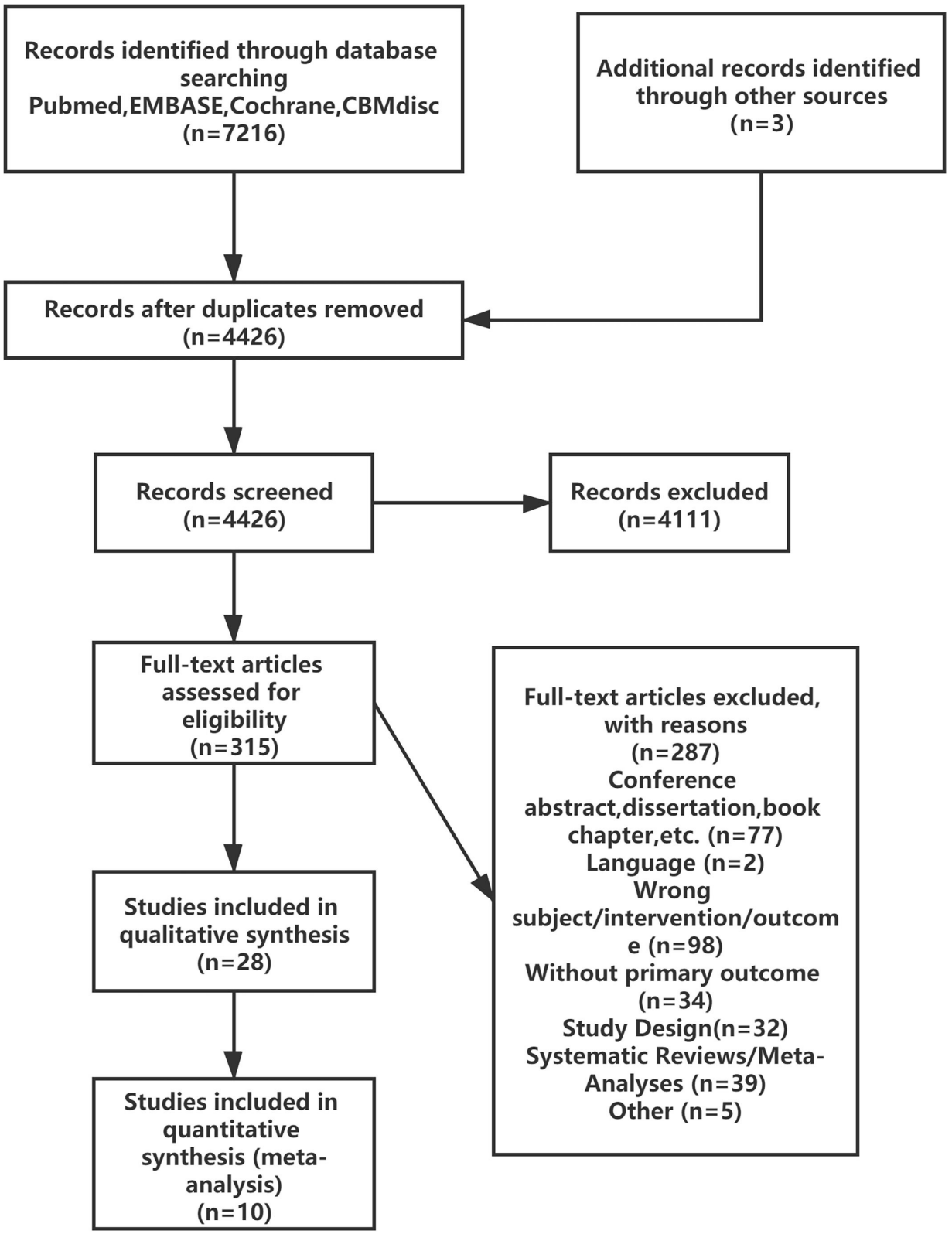

FIGURE 1 | PRISMA flow diagram of the study selection process.

TABLE 1 | Characteristics of clinical trials included in the meta-analysis.

1st author	Year	Location	Latitude	Economic level	Participants			Dosing regime		Follow–up period	
					Males/ Females	Average age (range)	Health status	Vitamin D group	Control	Duration	Season
Aglipay et al. (4)	2017	Toronto, Canada	43°N	Developed	404/296	2.7 (1–5) years	Healthy children	2000 IU/Day	400IU/Day	8 months	Winter
Arihiro et al. (7)	2019	Tokyo, Japan	35°N	Developed	136/87	44 (18–50) years	Adults with ulcerative colitis or Crohn's disease	500 IU/Day	Placebo	6 months	Winter
Loeb et al. (8)	2019	Ha Nam Province, Vietnam	21°N	Developing	621/679	8.5 (3–17) years	Healthy children and teenagers	2000 IU/Day	Placebo	12 months	All seasons
Urashima et al. (5)	2010	Tokyo, Japan	35°N	Developed	242/188	10.2 (6–15) years	Healthy children and teenagers	1200 IU/Day	Placebo	4 months	Winter
Urashima et al. (6)	2014	Tokyo, Japan	35°N	Developed	162/83	(15–18) years	Healthy teenagers	2000 IU/Day	Placebo	1 month	Winter
Zhou et al. (3)	2018	Yongkang, China	29°N	Developing	173/159	8 (3–12) months	Healthy infants	1200 IU/Day	400IU/Day	8 months	Winter
Godan et al. (25)	2021	Zagreb, Croatia	46°N	Developing	26/71	78.5 (61–96) years	Elderly patients with different kinds of chronic disease	800 IU/Day	Blank control	3 months	Winter
Rees et al. (28)	2013	Houston, Atlanta et.al	30–36°N	Developed	438/321	57.8 (45–75) years	Adults with a history of colorectal adenoma	1000 IU/Day	Placebo	5 months	Winter
Murdoch et al. (26)	2012	Christchurch, New Zealand	43°S	developed	81/241	47 years	Healthy adults	200,000 IU/monthly for 2 months; 100,000 IU/monthly	Placebo	18 months	All seasons
Jorde et al. (27)	2012				327/242	63 (32–84) years					
Jorde(1)		Troms, Norway	69°N	Developed		63 (34–82) years	1.Subjects with reduced glucose tolerance 2.Healthy adults	20000–40000 IU/week	Placebo	3 months	Winter
Jorde(2)		Vienna, Austria	48°N	Developed		61 (32–72) years	Kidney transplant recipient	6800 IU/Day	Placebo	3 months	Winter
Jorde(3)		Seattle, USA	47°N	Developed		60 (46–76) years	Subjects with Type 2 diabetes	2000 IU/Day	Placebo	3 months	Winter
Jorde(4)		Dundee, Scotland	47°N	Developed		71 (50–84) years	1.Isolated systolic hypertension 2. Adults with a past history of myocardial infarction 3.Adults with resistant hypertension	100,000 IU/quarterly	Placebo	3 months	Winter
Jorde(5)		Aarhus, Denmark	56°N	Developed		65 (33–77) years	Healthy adults	2800 IU/Day	Placebo	3 months	Winter
Jorde(6)		Leuven, Belgium	51°N	Developed		72 (56–82) years	Patients with moderate to very severe COPD	100,000 IU/monthly	Placebo	3 months	Winter

FIGURE 2 | Risk of bias summary: review authors' judgements about each risk of bias item for each included study.

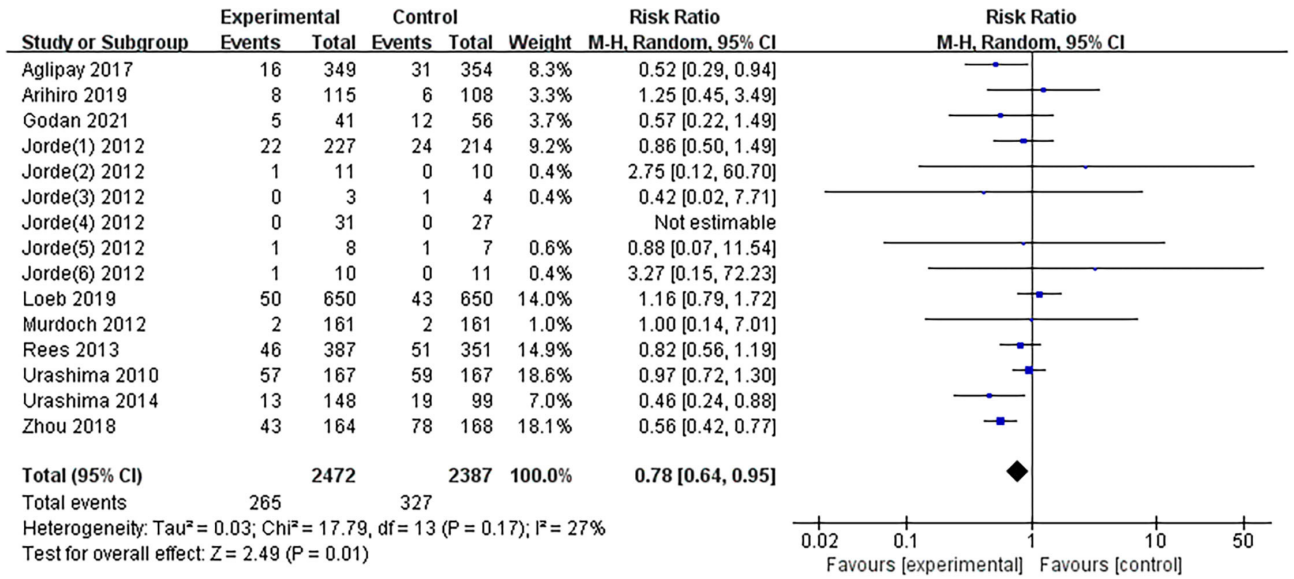

FIGURE 3 | Forest plot of comparisons of vitamin D vs. control on influenza. M-H, Mantel-Haenzsel statistics; Random, Random effects model.

TABLE 2 | Characteristics of univariate meta–regression analysis.

Covariates	Coefficient	SE	*t* value	*P* value
Country	0.988	0.109	−0.110	0.917
Latitude	1.005	0.783	0.010	0.995
Economic level	0.959	0.785	−0.050	0.960
Age	1.190	0.412	0.500	0.631
Follow–up period	1.137	0.370	0.390	0.706
Average daily vitamin D intake	1.032	0.383	0.080	0.935
Knapp–Hartung modification				0.997

or >50 years), economic level (developed or developing), average daily vitamin D intake (<2000 or ≥2000 IU/day) and follow-up period (<4, 4–8 or ≥ 8 months) would influence the association between vitamin D intake and influenza, and we could confirm the influence factor with a positive coefficient ($P \leq 0.05$). We also performed two subgroup analyses (adult vs. children and winter vs. all seasons) to explore in depth the respective influence of developmental stage and season on the effect. Some of the age data were mathematically processed (23, 24).

RESULTS

Search Results and Trial Characteristics

The literature screening process is presented in **Figure 1**; 7216 reports were identified after the databases were searched, and 28 reports were retained after reviewing the title and summary according to the exclusion criteria. Then 18 reports were excluded after full text review. A total of 10 RCTs were satisfied the criteria for inclusion and entered the meta-analysis (3–8, 25–28).

Details of the study characteristics were shown in **Table 1**. The included studies were mainly conducted in Asian, North America or Europe countries, and were all published after the influenza epidemic in 2009. All used oral cholecalciferol of these studies, six used laboratory examinations like Polymerase Chain Reaction and two used a questionnaire to diagnosis influenza, and one contained both methods. Most of the participants were healthy adults, and the number of them was almost the same for men and women (4026:4003). The risk of bias was assessed using a risk-of-bias graph (**Figure 2**). Most of the RCT exhibited good allocation concealment, reporting of blinding methods, and complete outcome data; however, one study had a high risk in blinding of participants (25), and the other had a high risk of other biases due to its procedure and diagnosis method (27). Due to the inconsistency of studies in the primary outcomes, we therefore downgraded the overall quality of evidence (**Supplementary Table S2**).

Effects of Vitamin D Supplementation on the Primary Outcomes

The results of one article (27) were divided into six parts by region. The results of the overall meta-analysis were presented in **Figure 3**. The summarized results of the included studies indicated that substitution with vitamin D significantly reduces the risk of influenza infections ($RR = 0.78$, 95% CI: 0.64–0.95). There was no evidence of a significant heterogeneity among studies ($I^2 = 27\%$, $P = 0.170$). The 95% CI for I^2 was 0–61%.

Meta-regression analysis was conducted to verify the possible influential factors, and the result showed in **Table 2** manifested that country, latitude (<30, 30–50 or >50°), average age (18<, 18–50 or >50 years), economic level (developed or developing), average daily vitamin D intake (<2000 or ≥2000 IU/day) and follow-up period (<4, 4–8 or ≥ 8 months) were not influential

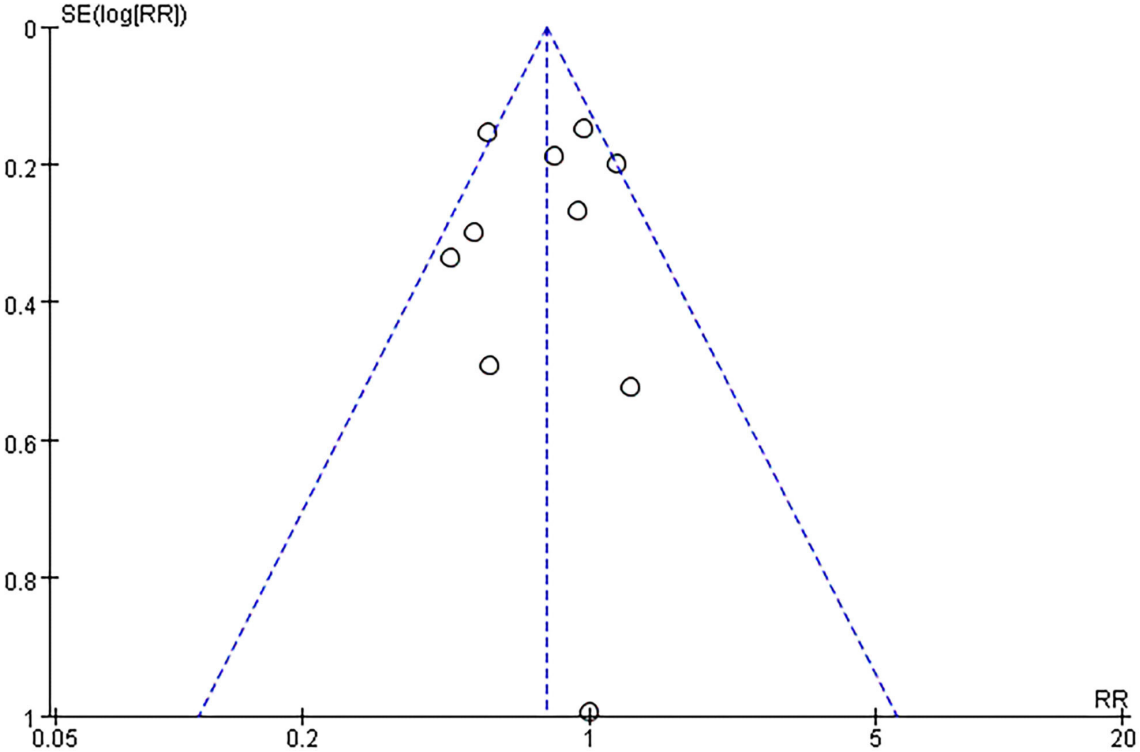

FIGURE 4 | Funnel plots with pseudo 95% confidence intervals.

factors. Therefore, all 14 estimates were incorporated into the meta-analysis.

There was no significant difference in effect between children group and adults' group as was showed in **Supplementary Figure S1** ($P = 0.470$). For season, the pooled *RR* of all seasons' group was 1.16 (95%CI: 0.79–1.70, $I^2 = 0\%$), while the pooled *RR* of winter group was 0.73 (95%CI: 0.60–0.88, $I^2 = 17\%$). Difference in heterogeneity between the two subgroups was statistically significant ($P = 0.040$), and vitamin D supplementation in winter might have a better effect of preventing influenza than in all seasons (**Supplementary Figure S2**).

Publication Bias and Sensitivity Analyses

The funnel plot, as shown in **Figure 4**, suggested we could rule out the publication bias (Harbord tests $t = -0.08$, $P = 0.937$). The meta-analysis result was robust according to the sensitivity analysis as shown in **Figure 5** and **Table 3**. Each trial had a modest influence on the overall results, and after exclusion of single studies the estimated *RR* remained within the range 0.73(95%CI: 0.59–0.90) to 0.84(95%CI: 0.69–1.04).

DISCUSSION

Several mechanisms explain the possible preventive effects of vitamin D supplementation on influenza. A recent review grouped those mechanisms into three categories: physical barrier, cellular natural immunity, and adaptive immunity (29). Cells involved in innate and adaptive immunity contain the 1-alpha-hydroxylase in their mitochondria. This series of cells including macrophages, neutrophils, dendritic cells, natural killer cells, B cells, CD4 and CD8 cells also contain surface Vitamin D Receptors (30). Calcitriol regulates local immune function by binding to Vitamin D Receptors through endocrine, paracrine, and autocrine mechanisms that influence gene transcription. Calcitriol also has a broader role in regulating secondary expression of some genes responsible for transcription of pro-inflammatory cytokines (31).

This meta-analysis of 10 RCTs indicates a protective effect of vitamin D supplementation against influenza infections with a combined risk ratio of 0.78 (95% CI: 0.64–0.95; $P = 0.010$). It is difficult to compare this study with the previous analysis because no one has done the same analysis before. Some previous meta-analyses studied the effect of vitamin D supplementation against respiratory tract infections and got inconsistent results (32–36), two of which with no restriction imposed on the origin of participants are consistent with our conclusion (32, 36), but none of them analyzed influenza as a subgroup.

Many factors such as region, age, economic level, follow-up period and average daily vitamin D intake may affect the serum vitamin D level of subjects or the mode of action of vitamin D supplementation, and then influence the outcome. Thus, we took these factors into meta-regression analysis but showed no marked impact on the conclusions.

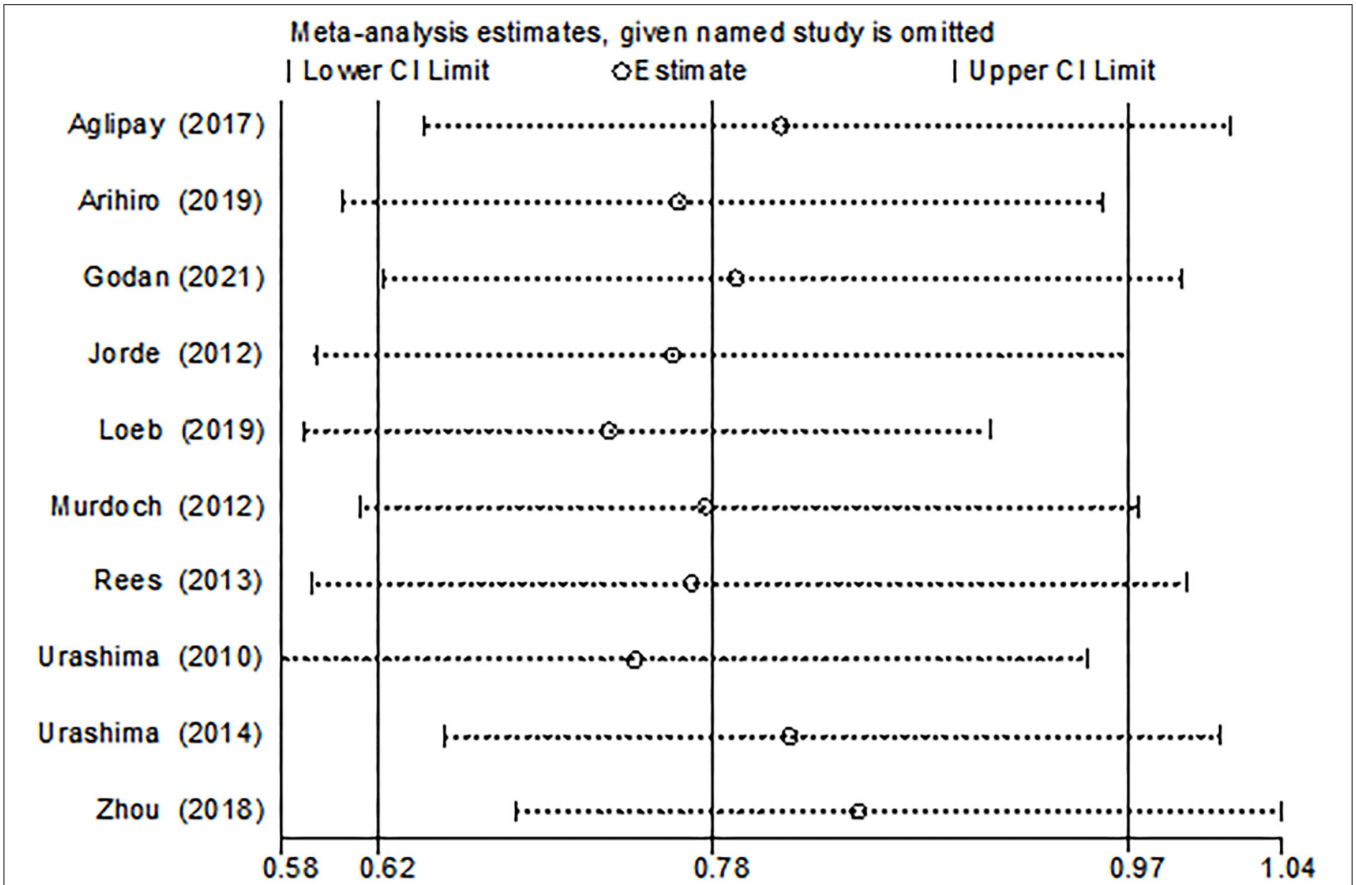

FIGURE 5 | Results of sensitivity analysis.

TABLE 3 | Sensitivity analysis results.

Study omitted	Estimate	95%CI
Aglipay et al. (4)	0.806	0.643–1.012
Arihiro et al. (7)	0.760	0.605–0.953
Godan et al. (25)	0.786	0.624–0.990
Jorde et al. (27)	0.757	0.593–0.966
Loeb et al. (8)	0.728	0.588–0.902
Murdoch et al. (6)	0.772	0.614–0.970
Rees et al. (28)	0.766	0.598–0.992
Urashima et al. (5)	0.740	0.578–0.947
Urashima et al. (6)	0.810	0.652–1.007
Zhou et al. (3)	0.842	0.685–1.035

Regions have an impact on vitamin D status and influenza infections. Different people in countries with different levels of economic development are various in vitamin D intake. Influenza mainly occurs in winter, so we mainly extracted outcome data from winter episodes (28). Winter influenza peak was due in part to the conjunction with the season when solar UVB doses, and thus 25(OH)D concentrations, are lowest in most mid- and high-latitude countries (37). Therefore, it seems reasonable that vitamin D supplementation is more effective in preventing influenza during the winter. However, Rees et al. reported null effects both in winter and all seasons (28). Most of the other included studies were conducted in winter, and only two reported the effectiveness (3, 4). The amount of ultraviolet radiation b (UVB) decreases along latitude in winter, and the UVB-related vitamin D production decreases dramatically in the areas at latitudes above 40° (38). But here, latitudes seem to have no influence on the effect of vitamin D.

Infants may lack sun exposure because they are more likely to stay at home, which causes reduced cutaneous synthesis of vitamin D3. Serum 25(OH)D concentrations tend to decline with age because aging is associated with decreased levels of 7-dehydrocholesterol, the precursor of vitamin D3 in the skin (39, 40). And both the old and infants have weak immunity and are more susceptible to influenza virus. We suspect that the preventive effect of vitamin D on influenza is more obvious in infants and the elderly. 2 studies reported the vitamin D was effective in preventing influenza in infants, which was in line with prat of our conjectures (3, 4). However, the results of meta regression and subgroup analysis (children vs. adults) showed that there were not any significant differences in effects between different age groups. In teenagers and the elderly, 5 studies included here demonstrated a null effect (5, 6, 8, 27, 28).

This may be because the subjects in these studies had higher baseline serum vitamin D levels, which compromised the effect of vitamin D supplement. Arihiro et al. reported 58.7 nmol/l (7); Loeb et al. reported 65nmol/l (8); Rees et al. reported 73.4 nmol/l (28), and Murdoch et al. reported 72.3 nmol/l (26). In a cohort study, participants with predicted baseline serum 25(OH)D $\geq$ 50 nmol/L had lower risk in influenza infection compared with 25(OH)D $\leq$ 50 nmol/L group after analyzing the combined outcome of PCR-confirmed influenza virus infection and ILI (*RR* = 0.63, 95% CI:0.52–0.76) (41). Given the higher serum vitamin D levels in our included observation subjects, as well as the lower incidence in the high vitamin D level population, differences in effects between the intervention and control groups are difficult to detect.

Vitamin D metabolites have different effects on the immune response of various respiratory viruses and regulate the secretion of cytokines (42). The cytokine secretion pattern are different between influenza A and influenza B (43). So preventive effects of vitamin D on influenza A and influenza B may also be different. Urashima et al. reported that vitamin D reduced the incidence of influenza A (*RR* = 0.58, 95% CI:0.34–0.99), but not of influenza B (*RR*= 1.39, 95% CI:0.90–2.15) (5). We estimated RR value for influenza A (*RR*= 0.36, 95% CI: 0.15–0.85) and influenza B (*RR* = 0.83, 95% CI: 0.04–18.00) respectively based on Aglipay's research outcomes by using Woolf's method, and the conclusion was consistent with Urashima's (4). But in Loeb's study, vitamin D supplement had no effect on both influenza A and B (8). This difference may be due to the experimental design and some biases, we still assume that vitamin D supplement is likely to have better effects on preventing influenza A than B.

This meta-analysis produced a corroboration that vitamin D supplement has a preventive effect on influenza, and vitamin D supplementation in winter seems to have better effect of preventing influenza than in all seasons according to subgroup analysis (winter vs. all seasons). Compared with drugs and influenza vaccines, vitamin D supplementation as a way to prevent influenza is more convenient and more acceptable due to its safety and many other benefits in maintaining bone health. Vitamin D did not have an impact on immunological effects of influenza vaccines (44, 45). So, it is recommended that people of all age can supplement vitamin D to prevent influenza and get vaccinated if necessary, during wintertime. Because of the small number of included reports and incomplete reporting of serum vitamin D levels, we did not further analyze the appropriate doses of vitamin D supplementation in different age groups. However, vitamin D supplementation may have different effects on people of various age levels, which is closely related to the physiological condition of the patients themselves. So, researchers should develop different vitamin D supplementation programs for them. Our findings can help optimize the influenza prevention strategies and provide a theoretical basis for the development of nutrition guidelines.

Our study has several strengths. First, all the enrolled studies were RCTs, which eliminates the possibility of reverse causation and minimizes selection and recall biases, while the non-RCT design has more of the above biases and confounding factors. Second, we have conducted relative comprehensive analyses, including meta-regression analysis, influence analysis and publication bias detection, to explore potential sources of heterogeneity and bias. Furthermore, no restriction was imposed on the origin of participants, which may generalize the results.

The limitation of this meta-analysis cannot be ignored: First, four articles did not report baseline serum vitamin D levels, and therefore, we could not analyze whether the baseline serum concentrations of 25(OH)D modified the effects of vitamin D supplementation. Second, two study with a relative high risk of bias was included in the analysis. Fortunately, the exclusion of these studies had only modest influence on the outcome based on the results of sensitivity analysis. Finally, the age range of the subjects in this study is very wide (3 months to 82 years). Due to the small number of studies, the three categories used in this study alone may not show a possible difference among subgroups.

CONCLUSIONS

Aggregated evidence from 10 RCTs indicates that vitamin D supplementation could be an effective for preventing influenza. This meta-analysis was conducted based on RCTs, so the strength of evidence argument is very strong. Our findings can provide a theoretical basis for the development of nutrition guidelines.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XL and ZZ conceived and designed the meta-analysis. ZZ, XZ, LG, and YZ searched the literature. ZZ and XZ extracted the data. ZZ and LC analyzed the data. ZZ contributed analysis tools and wrote the paper. XL revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.799709/full#supplementary-material

Supplementary Figure S1 | Forest plot of subgroup analysis (adults vs. children).

Supplementary Figure S2 | Forest plot of subgroup analysis (winter vs. all seasons).

Supplementary Table S1 | Search strategy and search terms.

Supplementary Table S2 | GRADE level of evidence and summary of findings of vitamin D for the prevention of influenza.

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Vitamins and *Helicobacter pylori*: An Updated Comprehensive Meta-Analysis and Systematic Review

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Background: Over recent decades, epidemiological studies have shown relationships between vitamins and *Helicobacter pylori* (*H. pylori*) infection and eradication, but the results are controversial.

Methods: A comprehensive meta-analysis and systematic review were conducted to clarify the relationships between common types of vitamins and *H. pylori*. We applied meta-regression, subgroup analysis and sensitivity analysis to obtain available evidence. Articles published from January 1991 to June 2021 in PubMed, EMBASE, and the Cochrane Library were searched.

Results: In total, we identified 48 studies. The results indicate that *H. pylori* -positive patients had lower serum vitamin B_{12} [standardized mean difference (SMD) = −0.30; 95% confidence interval (CI): −0.53 – −0.08], folate (SMD = −0.69; 95% CI: −1.34 – −0.04), vitamin C (SMD = −0.37; 95%CI: −0.57 – −0.18) and vitamin D (SMD = −0.34; 95% CI: −0.49 – −0.18) levels than *H. pylori* -negative patients. Patients in which *H. pylori* had been successfully eradicated had higher serum vitamin D levels (SMD = 1.37; 95% CI: 0.37–2.38) than in patients in which eradication had been unsuccessful. The serum vitamin B_{12} levels of *H. pylori*-positive patients improved after successful *H. pylori* eradication therapy (SMD = 1.85; 95% CI: 0.81–2.90), and antioxidant vitamin supplementation to an *H. pylori* eradication regimen improved the eradication rate (risk ratio = 1.22; 95% CI: 1.02–1.44 for per-protocol analysis; risk ratio = 1.25; 95% CI: 1.06–1.47 for intention-to-treat analysis).

Conclusions: *H. pylori* infections decrease the serum levels of several types of vitamins, eradication of *H. pylori* could rescue its adverse effects, and antioxidant vitamin supplementation may improve the *H. pylori* eradication rate.

Systematic Review Registration: identifier: CRD42021268127.

Keywords: vitamins, helicobacter pylori, meta-analysis, systematic review, relationship

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gastric gram-negative, spiral-shaped microaerophilic pathogen (1). About half of the global population is infected with *H. pylori*, and the infection rate in developing countries is higher than in developed countries (2, 3). *H. pylori* is the main risk for chronic gastritis, gastric ulcer, gastric cancer and mucosa-associated lymphoid tissue–associated lymphoma (4–6). It can damage the gastric mucosa and affect the absorption of trace elements, especially vitamins (7). Vitamin deficiency can upset the internal balance of the human body and cause a variety of diseases outside of the digestive system (8–10).

Vitamins are members of a huge family. At present, there are dozens of known vitamins that may be divided into fat-soluble and water-soluble categories. The relationships between *H. pylori* infection and various vitamins have attracted attention worldwide. Some studies found that *H. pylori* infections reduce serum vitamin levels (11, 12); several studies have revealed that after *H. pylori* eradication, the serum vitamin levels increase (13, 14). Some randomized controlled trials (RCTs) found that vitamin supplementation combined with standard anti-*H. pylori* therapy increase the *H. pylori* eradication rate (15, 16). However, the results have been inconsistent.

Meta-analyses on the relationships between vitamins and *H. pylori* have been published (17–19). These studies involved one vitamin or a certain aspect of the relationships between vitamins and *H. pylori,* or the number of included studies was limited. Yang et al. (17) found vitamin D could improve the success rate of *H. pylori* eradication. However, they only identified three relevant studies to support this conclusion. Afsar et al. (18) reported a relationship between *H. pylori* infection and micronutrient (vitamin B_{12} and folate) levels in pregnant women. Nevertheless, the effects of *H. pylori* on the population excluding pregnant women were not evaluated. Li et al. (19) assessed the effects of antioxidant vitamins supplementation on the rate of *H. pylori* eradication. However, only three supporting studies were referenced in that work. In recent years, many excellent articles have been published. To update the results and obtain more credible conclusions, we conducted this systematic review and meta-analysis to evaluate the relationships more comprehensively, thereby providing a theoretical basis for clinical practice and public health policy-making.

METHODS

Data Sources and Search Strategy

This meta-analysis was registered on PROSPERP (No. CRD42021268127) (20) and compliant with the main PRISMA statement (21). A comprehensive and systematic search was carried out for relevant studies describing relationships between vitamins and *H. pylori* in biologic and medical databases (Medline, Web of Science, Embase, Chinese Biomedical Database and Cambridge Scientific Abstracts databases). We developed a search strategy using following keywords: "vitamins," "vitamin A," "vitamin B," "vitamin C," "vitamin D," "vitamin E," "β-Carotene," "retinol," "cobalamin" "folate," "folic acid," "tocopherol," "antioxidants," "micronutritent," and "*Helicobacter pylori*" (as shown in **Supplementary Table 1**). Duplicate works were collapsed into a single entry. Additionally, we scanned the reference lists of all the relevant published studies and reviews. The two blinded reviewers (Xianlei Cai and Xueying Li) selected the studies and specified the exclusion criteria.

Inclusion and Exclusion Criteria

The inclusion criteria were as follows: (1) Observational or experimental research; (2) Comparisons of serum vitamin levels between *H. pylori* - positive and *H. pylori* - negative patients; (3) Comparisons of serum vitamin levels between successful and failed *H. pylori* eradication patients; (4) Comparisons of serum vitamin levels before and after successful *H. pylori* eradication therapy; (5) Comparisons of *H. pylori* eradication rate between antioxidant vitamin supplementation groups and controlled groups for *H. pylori* – positive patients; and (6) Original studies in English or Chinese indexed up to June 2021.

The exclusion criteria were as follows: (1) Original studies did not involve the relationships between vitamins and *H. pylori*; (3) Studies did not provide sufficient data for a meta-analysis; (4) reviews, comments, letters, and animal studies; and (5) low-quality studies [Newcastle-Ottawa scale and Cochrane collaboration's tool were used to assess the quality and bias risk for cross-sectional, cohort and random-controlled studies as described in a previous study (22)].

Data Extraction

All the data were extracted by three researchers independently (Xianlei Cai, Xueying Li and Yangli Jin) using standardized form. The characteristics of the identified relevant works were records as follows: name of first author, year of publication, country, study design (cross-sectional, case series, cohort and RCTs), age, number of subjects, gender, type of vitamins (vitamins A, B, C, D and E) and presentation of effect magnitude [mean ± standard deviation (SD), mean ± standard error of mean (SEM), median (interquartile range), median (range), odds risk (OR), risk ratio (RR), or hazard ratio (HR) with 95% confidence interval (CI)].

Statistical Analyses

Mean serum vitamin levels and the SDs were used in pooling analyses. If the original studies provided other estimates [mean ± SEM, median (interquartile range) or median (range)], we converted the data using a common method described by Hozo et al. (23). Because of the inconsistent units used in different studies, the pooled results were expressed in terms of standardized mean difference (SMD) with 95% CI. If the RCTs and cohort studies only provided 2 × 2 table data, we calculated the responding RRs. Additionally, RRs and 95% CIs were used to show the differences in the *H. pylori* eradication rates between vitamin supplementation and control groups.

A meta-regression was performed to examine the sources of heterogeneity from disparate types of vitamin supplementation (vitamin C or vitamin C plus vitamin E), and we identified influence factors having positive coefficients ($p \leq 0.05$). Q-test and I^2 were used to assess heterogeneity. If the results

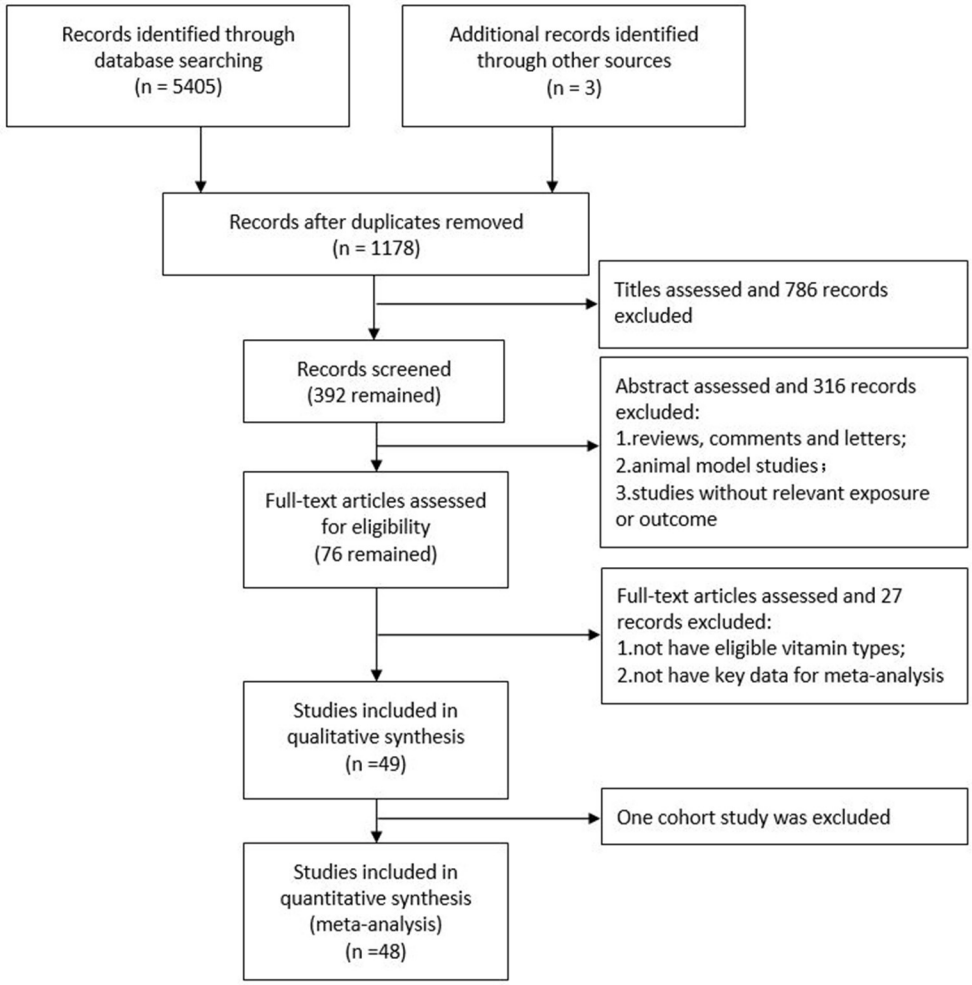

FIGURE 1 | Flow diagram of the studies search process.

showed notable heterogeneity ($p \leq 0.05$ and $I^2 > 50\%$), then pooled estimates were calculated using random-effects models (DerSimonian and Laird method) (24). Otherwise, fixed-effects models were used (Mantel-Haenszel method) (25). Subgroup analyses were performed to evaluate relationships between diverse types of vitamins and *H. pylori*. Forest and funnel plots were drawn and publication bias was tested by a weighted Egger and Begg's tests (26, 27). Sensitivity analyses were performed by omitting one estimate at a time to assess the relative influence of each work on pooled results. If the included estimates were less than four, then we did not carry out the meta-analysis and conducted systematic review instead. All the analyses were performed using STATA version 12.0 (StataCorp LP).

RESULTS

Study Characteristics

The flow-through of the study selection process is described using a modified PRISMA diagram (**Figure 1**). In total, forty-eight high quality studies (39 observational studies and 9 RCTs) with 73 independent estimates of relationships between vitamins and *H. pylori* in four fields were included in this meta-analysis and systematic review. In total, 31 studies compared serum vitamin levels between *H. pylori* - positive and - negative patients; 5 studies compared serum vitamin levels between successful and failed *H. pylori* eradication patients; 6 studies compared patient serum vitamin levels before and after successful *H. pylori* eradication therapy; and 10 studies focused on the effects of vitamin supplementation on the *H. pylori* eradication rate. Of these, three studies (28–30) considered more than one effect of *H. pylori* on vitamins. There were 173,013 participants from Turkey (12 studies), the United Kingdom (6 studies), China (4 studies), Italy (4 studies), Brazil (2 studies), Iran (2 studies), Israel (2 studies), Japan (2 studies), the USA (2 studies), and one each from Argentina, Australia, Egypt, Germany, Greece, India, Lebanon, Netherlands, Pakistan, Palestine, Poland, Saudi Arabia and Switzerland. Overall, **Tables 1–4** present the summaries of all the studies included in the meta-analysis. The original estimates reported in the articles are summarized in **Supplementary Tables 2–5**.

TABLE 1 | Basic characteristics of studies comparing serum vitamin levels between *H. pylori* + groups and *H. pylori* - negative groups.

Study	Year	Area	Design	Age of HP + groups	No. of HP + groups (male/female)	Age of HP - groups	No. of HP - groups (male/female)	Quality
Vitamin A								
Phull et al. (31)	1998	UK	Cross-sectional	Mean 46.0 y	25 (18/7)	Mean 54 y	18 (7/11)	7*
Zhang et al. (32)	2000	UK	Cross-sectional	19–89 y	41 (N/A)	19-89 y	27 (N/A)	7*
Toyonaga et al. (33)	2000	Japan	Cross-sectional	Mean 47.0 y	37 (13/24)	Mean 44.7 y	40 (14/26)	8*
Vitamin B_{12}								
Tamura et al. (34)	2002	Japan	Cross-sectional	Mean 64 y	57 (44/13)	Mean 63 y	36 (25/11)	8*
Cenerelli et al. (35)	2002	Italy	Cross-sectional	Mean 54.7 y	31 (19/12)	Mean 50.9 y	42 (23/19)	8*
Shuval-Sudai et al. (36)	2003	Israel	Cross-sectional	Mean 52.8 y	96 (N/A)	Mean 49.2 y	37 (N/A)	7*
Trimarchi et al. (37)	2004	Argentina	Cross-sectional	Mean 56.8 y	8 (3/5)	Mean 62.4 y	21 (9/12)	7*
Oijen et al. (38)	2004	Netherlands	Cross-sectional	N/A	29 (N/A)	N/A	60 (N/A)	7*
Sarari et al. (11)	2008	Palestine	Cross-sectional	Mean 43.4 y	43 (24/19)	N/A	17 (N/A)	7*
Stettin et al. (39)	2008	Germany	Cross-sectional	Mean 50.8 y	69 (27/42)	Mean 47.3 y	21 (8/13)	8*
Kakehasi et al. (40)	2009	Brazil	Cross-sectional	Mean 63.7 y	34 (0/37)	Mean 62.5 y	27 (0/27)	8*
Gerig et al. (41)	2013	Switzerland	Cross-sectional	Mean 42.3 y	85 (21/64)	Mean 40.9 y	319 (85/234)	8*
Ulasoglu et al. (42)	2019	Turkey	Cross-sectional	Mean 44.8 y	213 (N/A)	Mean 44.8 y	76 (N/A)	7*
Surmeli et al. (43)	2019	Turkey	Cross-sectional	Mean 74.7 y	43 (11/32)	Mean 78.2 y	211 (91/120)	9*
Soyocak et al. (44)	2021	Turkey	Cross-sectional	Mean 46.4 y	31 (12/19)	Mean 45.2 y	19 (8/11)	8*
Folate								
Tamura et al. (34)	2002	Japan	Cross-sectional	Mean 64 y	57 (44/13)	Mean 63 y	36 (25/11)	8*
Cenerelli et al. (35)	2002	Italy	Cross-sectional	Mean 54.7 y	31 (19/12)	Mean 50.9 y	42 (23/19)	8*
Shuval-Sudai et al. (36)	2003	Israel	Cross-sectional	Mean 52.8 y	96 (N/A)	Mean 49.2 y	37 (N/A)	7*
Stettin et al. (39)	2008	Germany	Cross-sectional	Mean 50.8 y	69 (27/42)	Mean 47.3 y	21 (8/13)	8*
Gerig et al. (41)	2013	Switzerland	Cross-sectional	Mean 42.3 y	85 (21/64)	Mean 40.9 y	319 (85/234)	8*
Ulasoglu et al. (42)	2019	Turkey	Cross-sectional	Mean 44.8 y	213 (N/A)	Mean 44.8 y	76 (N/A)	7*
Surmeli et al. (43)	2019	Turkey	Cross-sectional	Mean 74.7 y	43 (11/32)	Mean 78.2 y	211 (91/120)	9*
Soyocak et al. (44)	2021	Turkey	Cross-sectional	Mean 46.4 y	31 (12/19)	Mean 45.2 y	19 (8/11)	8*
Vitamin C								
Banerjee et al. (28)	1994	UK	Cross-sectional	N/A	19 (N/A)	N/A	10 (N/A)	7*
Rokka et al. (45)	1995	USA	Cross-sectional	Mean 43.5 y	58 (30/28)	Mean 45.5 y	30 (16/14)	8*
Webb et al. (46)	1997	Australia	Cross-sectional	N/A	666 (N/A)	N/A	737 (N/A)	7*
Phull et al. (31)	1998	UK	Cross-sectional	Mean 46 y	25 (18/7)	Mean 54 y	18 (7/11)	7*
Rokkas et al. (47)	1999	Greece	Cross-sectional	Mean 42.0 y	30 (17/13)	Mean 42.5 y	10 (6/4)	7*
Jarosz et al. (48)	2000	Poland	Cross-sectional	Mean 45.5 y/39.0 y	21 (11/10) 32 (18/14)	Mean 37.5 y/41.5 y	17 (10/7) 16 (9/7)	8*
Toyonaga et al. (33)	2000	Japan	Cross-sectional	Mean 47.0 y	37 (13/24)	Mean 44.7 y	40 (14/26)	8*
Woodward et al. (49)	2001	UK	Cross-sectional	25–74 y	765 (N/A)	25–74 y	403 (N/A)	8*
Everett et al. (50)	2001	UK	Cross-sectional	Mean 51 y	85 (47/38)	Mean 45 y	39 (25/14)	7*
Annibale et al. (29)	2003	Italy	Cross-sectional	Median 47 y	30 (6/24)	Median 37 y	13 (1/12)	7*
Capurso et al. (51)	2003	Italy	Cross-sectional	Median 44 y	32 (5/27)	Median 37 y	13 (1/12)	7*
Simon (52)	2003	USA	Cross-sectional	Mean 51 y	2189 (1072/1117)	Mean 41 y	4557 (2142/2415)	9*
Khanzode et al. (30)	2003	India	Cross-sectional	Mean 45.4 y	37 (15/22)	Mean 48.2 y	40 (22/18)	8*
Vitamin D								
Antico et al. (53)	2012	Italy	Cross-sectional	20–80 y	21 (N/A)	20–80 y	163 (N/A)	7*
Gerig et al. (41)	2013	Switzerland	Cross-sectional	Mean 42.3 y	85 (21/64)	Mean 40.9 y	319 (85/234)	8*
Han et al. (54)	2019	China	Cross-sectional	Mean 47.1 y	496 (236/260)	Mean 48.1 y	257 (127/300)	8*
Surmeli et al. (43)	2019	Turkey	Cross-sectional	Mean 74.7 y	43 (11/32)	Mean 78.2 y	211 (91/120)	9*
Assaad et al. (12)	2019	Lebanon	Cross-sectional	Mean 39.3 y	225 (88/137)	Mean 41.9 y	235 (88/137)	8*
Gao et al. (55)	2020	China	Cross-sectional	Mean 12.1 m	2113 (1202/911)	Mean 12.4 m	4783 (2865/2098)	9*
Shafrir et al. (56)	2021	Israel	Cross-sectional	Mean 41.0	75640 (38576/37064)	Mean 42.2	74843 (37421/37422)	9*

(Continued)

TABLE 1 | Continued

Study	Year	Area	Design	Age of HP + groups	No. of HP + groups (male/female)	Age of HP - groups	No. of HP - groups (male/female)	Quality
Vitamin E								
Phull et al. (31)	1998	UK	Cross-sectional	Mean 46 y	25 (18/7)	Mean 54 y	18 (7/11)	7*
Zhang et al. (32)	2000	UK	Cross-sectional	19–89 y	41 (N/A)	19–89 y	27 (N/A)	7*
Toyonaga et al. (33)	2000	Japan	Cross-sectional	Mean 47.0 y	37 (13/24)	Mean 44.7 y	40 (14/26)	8*

*UK, United Kingdom; N/A, not available; y, years; m, months; *The "star system" of the Newcastle–Ottawa scale.*

TABLE 2 | Basic characteristics of studies comparing serum vitamin levels between the successful *H. pylori* eradication groups and the failed groups.

Study	Year	Area	Design	Age of successful groups	No. of successful groups (male/female)	Age of failed groups	No. of failed groups (male/female)	Quality
Vitamin D								
Yildirim et al. (57)	2017	Turkey	Cross-sectional	N/A	170 (N/A)	N/A	50 (N/A)	7*
Shahawy et al. (58)	2018	Egypt	Cross-sectional	18–80 y	105 (N/A)	18–80 y	45 N/A)	7*
Magsi et al. (2)	2021	Pakistan	Cross-sectional	18–60 y	88 (42/46)	18–60 y	36 (18/18)	8*
Shatla et al. (59)	2021	Saudi Arabia	Cross-sectional	N/A	109 (N/A)	N/A	42 (N/A)	7*
Shafrir et al. (56)	2021	Israel	Cross-sectional	N/A	45821 (N/A)	N/A	29722 (N/A)	9*

*N/A, not available; y, years; *The "star system" of the Newcastle–Ottawa scale.*

TABLE 3 | Basic characteristics of studies comparing serum vitamin levels before and after *H. pylori* eradication therapy.

Study	Year	Area	Design	Age	No. before eradication (male/female)	No. after eradication (male/female)	Test time after eradication	Quality
Vitamin B_{12}								
Kaptan et al. (13)	2000	Turkey	Case series	Mean 59.5 y	31 (19/12)	31 (19/12)	3 or 6 m	8*
Serin et al. (60)	2002	Turkey	Case series	Mean 43 y	65 (N/A)	65 (N/A)	2–3 m	7*
Ozer et al. (61)	2005	Turkey	Case series	Mean 41 y	41 (N/A)	41 (N/A)	1 m	7*
Marino et al. (14)	2007	Brazil	Case series	Mean 72.8 y	59 (N/A)	59 (N/A)	6 m / 12 m	7*
Folate								
Kaptan et al. (13)	2000	Turkey	Case series	Mean 59.5 y	31 (19/12)	31 (19/12)	3 or 6 m	8*
Ozer et al. (61)	2005	Turkey	Case series	Mean 41 y	41 (N/A)	41 (N/A)	1 m	7*
Vitamin C								
Banerjee et al. (28)	1994	UK	Case series	N/A	11 (N/A)	11 (N/A)	1 m / 6 m	7*
Annibale et al. (29)	2003	Italy	Case series	Median 47 y	5 (N/A)	5 (N/A)	6 m	7*

*N/A, not available; y, years; m, months; *The "star system" of the Newcastle–Ottawa scale.*

Vitamin Levels Discrepancies Between *H. pylori* - Positive and - Negative Patients

The number of studies on the relationships between *H. pylori* and vitamin B_{12}, folate, vitamin C and vitamin D was sufficient for a meta-analysis (**Table 1**, **Supplementary Table 2**).

For vitamin B_{12}, 12 estimates were included in the pooled analysis. The results indicated that *H. pylori* – positive patients had lower serum vitamin B_{12} levels than *H. pylori* – negative patients (SMD = −0.30; 95% CI: −0.53 – −0.08; **Figure 2**), with heterogeneity ($P < 0.001$; $I^2 = 71.4\%$) and publication bias (Begg's test $z_c = 2.26$, $P = 0.024$; Egger's test t = 0.05; **Figure 3A**). The sensitivity analysis showed that the results were stable and reliable (**Supplementary Figure 1A**).

For folate, eight estimates were incorporated into the meta-analysis. Similarly, the results showed that *H. pylori* – positive patients had lower serum folate levels than *H. pylori*-negative patients (SMD = −0.69; 95% CI: −1.34 – −0.04; **Figure 2**), with obvious heterogeneity ($P < 0.001$; $I^2 = 95.8\%$). Publication

TABLE 4 | Basic characteristics of studies focusing on the effect of vitamin supplementation on *H. pylori* eradication rate.

Study	Year	Area	Design	Age of group 1	No. of group 1 (male/female)	Age of group 2	No. of group 2 (male/female)	Type of vitamin supplementation	Method of HP eradication
Chuang et al. (62)	2002	China	RCT	Mean 37.9 y	55 (21/34)	Mean 35.6 y	49 (19/30)	Vitamin C plus Vitamin E	Triple therapy
Everett et al. (63)	2002	UK	RCT	Mean 52 y	29 (17/12)	Mean 49 y	30 (16/14)	Vitamin C plus Vitamin E	Triple therapy
Sezikli et al. (15)	2009	Turkey	RCT	Mean 43 y	80 (24/56)	Mean 44 y	80 (28/52)	Vitamin C plus Vitamin E	Quadruple therapy
Sezikli et al. (64)	2011	Turkey	RCT	Mean 42 y	80 (25/55)	Mean 43 y	40 (15/25)	Vitamin C plus Vitamin E	Triple therapy
Sezikli et al. (65)	2012	Turkey	RCT	Mean 39.7	160 (53/107)	Mean 42.7	40 (13/27)	Vitamin C plus Vitamin E	Triple therapy
Demirci et al. (66)	2015	Turkey	RCT	Mean 40 y	100 (59/41)	Mean 41 y	100 (53/47)	Vitamin C plus Vitamin E	Triple therapy / Quadruple therapy
Kockar et al. (67)	2001	Turkey	RCT	Mean 40.0 y	30 (N/A)	Mean 38.9 y	30 (N/A)	Vitamin C / Vitamin A	Triple therapy
Chuang et al. (68)	2007	China	RCT	Mean 53.2 y	61 (N/A)	Mean 49.9 y	55 (N/A)	Vitamin C	Triple therapy
Zojaji et al. (16)	2009	Iran	RCT	Mean 43 y	150 (N/A)	Mean 45 y	162 (N/A)	Vitamin C	Triple therapy
Kaboli et al. (69)	2009	Iran	Cohort	N/A	114 (N/A)	N/A	100 (N/A)	Vitamin C	Triple therapy

Group 1: antibiotic + vitamin groups.
Group 2: antibiotic groups.
RCT, random controlled trial; N/A, not available; y, years; UK, United Kingdom.

bias was not found (Begg's test $z_c = 0.87$, $P = 0.386$; Egger's test $t = 0.254$; **Figure 3A**). The sensitivity analysis showed that the results were influenced by some positive data (**Supplementary Figure 1B**).

For vitamin C, 14 estimates were incorporated into the pooled analysis. The results revealed that *H. pylori* – positive patients had lower serum vitamin C levels than *H. pylori*-negative patients (SMD = −0.37; 95% CI: −0.57 – −0.18; **Figure 2**), with obvious heterogeneity ($P < 0.001$; $I^2 = 87.9\%$). There was no publication bias (Begg's test $z_c = 0.44$, $P = 0.661$; Egger's test $t = 0.130$; **Figure 3A**). The sensitivity analysis showed that the result was stable (**Supplementary Figure 1C**).

For vitamin D, seven estimates were included in the meta-analysis. The result also found that *H. pylori* – positive patients had lower serum vitamin D levels than *H. pylori*-negative patients (SMD = −0.34; 95% CI: −0.49 – −0.18; **Figure 2**), with heterogeneity ($P < 0.001$; $I^2 = 95.0\%$). Publication bias was not found (Begg's test $z_c = 0.60$, $P = 0.548$; Egger's test $t = 0.412$; **Figure 3A**). The sensitivity analysis revealed that the results were robust (**Supplementary Figure 1D**).

For vitamins A and E, only three studies were identified; therefore, we did not conduct a pooled analysis. Among these three studies, Phull et al. (31) indicated that there was no relationship between *H. pylori* infection and serum vitamin A and E levels, and their conclusion were similar to those of Zhang et al. (32) and Toyonaga et al. (33).

Vitamin Levels Discrepancies Between Successful and Failed *H. pylori* Eradication Patients

Overall, five studies focusing on vitamin D level discrepancies between successful and failed *H. pylori* eradication patients were included in the meta-analysis (**Table 2**, **Supplementary Table 3**). The results indicated that the patients with successful *H. pylori* eradication had higher serum vitamin D levels than the failed patients (SMD = 1.37; 95% CI: 0.37–2.38; **Figure 4**), with heterogeneity ($P < 0.001$; $I^2 = 98.4\%$). Because all five studies reported positive estimates, the funnel plot was asymmetric (**Figure 3B**). We did not assess publication bias using weighted Egger test and Begg's tests owing to the insufficient numbers of estimates. The sensitivity analysis revealed that the results were robust (**Supplementary Figure 2**).

There were no studies exploring other vitamin levels (vitamin A, B, C and E) discrepancies between successful and failed *H. pylori* eradication patients. More relevant research is recommended to clarify the relationships.

Vitamin Level Discrepancies Before and After Successful *H. pylori* Eradication Therapy

The numbers of studies for vitamins B_{12} and C were sufficient for a meta-analysis (**Table 3**, **Supplementary Table 4**). For vitamin B_{12}, five estimates were incorporated into the pooled analysis. The result indicated that after successful *H. pylori* eradication, serum vitamin B_{12} increased (SMD = 1.85; 95% CI: 0.81–2.90; **Figure 5**), with heterogeneity ($P < 0.001$; $I^2 = 96.0\%$). The funnel plot was symmetric (**Figure 3C**). The sensitivity analysis showed that the results were stable and reliable (**Supplementary Figure 3**). For vitamin C, four estimates were included in the meta-analysis. The results showed that *H. pylori* eradication did not increase the serum vitamin C level (SMD = −0.32; 95% CI: −1.56–0.91; **Figure 5**), with heterogeneity ($P = 0.002$; $I^2 = 79.7\%$). The funnel plot was symmetric (**Figure 3C**). Because the four estimates were extracted from two studies, we

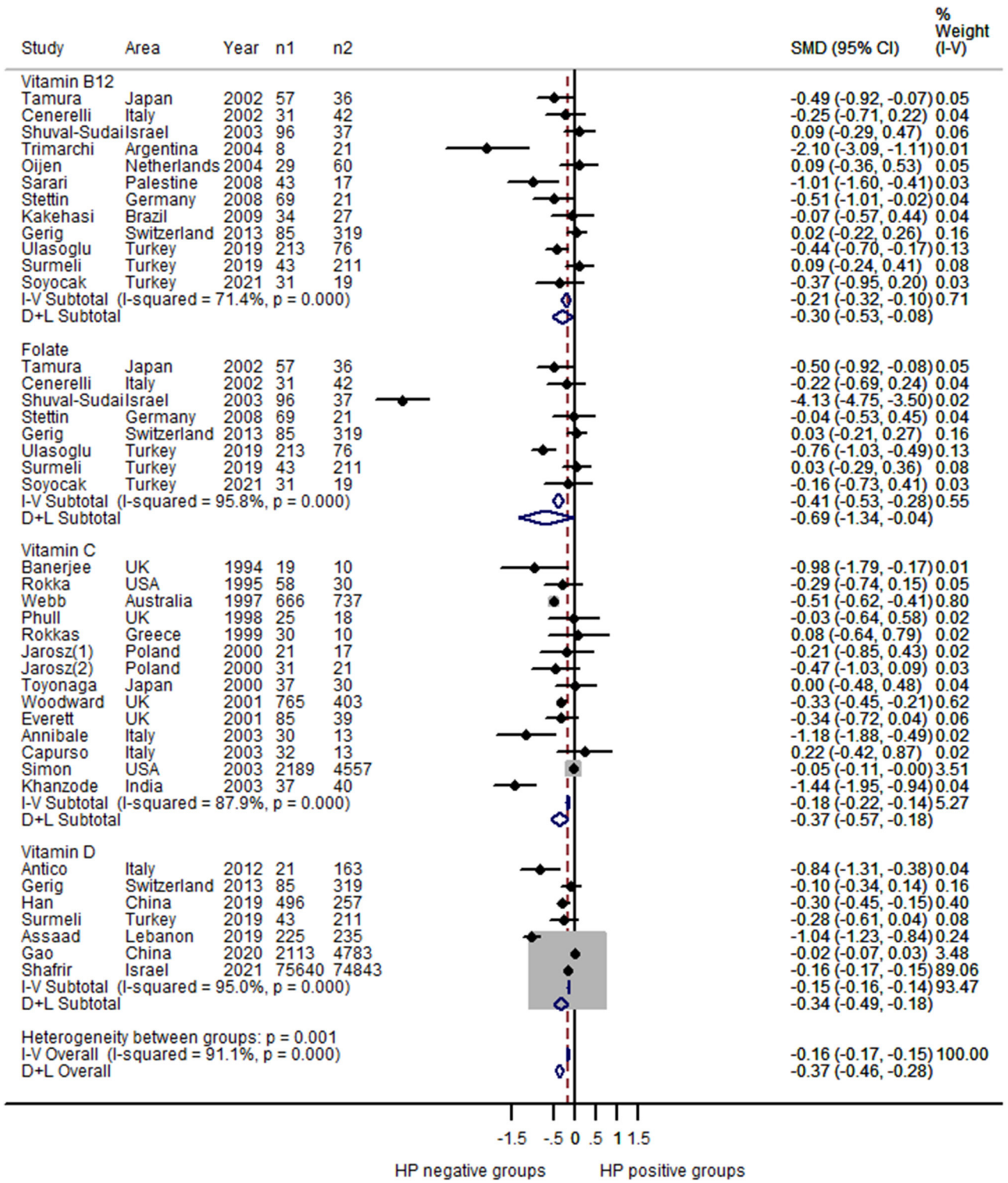

FIGURE 2 | Forest plot of the meta-analysis on comparison of serum vitamin levels between *H. pylori* - positive and *H. pylori* - negative patients (n1: number of *H. pylori – positive* patients; n2: number of *H. pylori – negative* patients); *H. pylori – positive* patients had lower serum vitamin B12 levels than *H. pylori – negative* patients (SMD = −0.30; 95% CI: −0.53 – −0.08; $P < 0.001$; $I^2 = 71.4\%$); *H. pylori – positive* patients had lower serum folate levels than *H. pylori-negative* patients (SMD = −0.69; 95% CI: −1.34 – −0.04; $P < 0.001$; $I^2 = 95.8\%$); *H. pylori – positive* patients had lower serum vitamin C levels than *H. pylori-negative* patients (SMD = −0.37; 95% CI: −0.57 – −0.18; $P < 0.001$; $I^2 = 87.9\%$); *H. pylori – positive* patients had lower serum vitamin D levels than *H. pylori-negative* patients (SMD = −0.34; 95% CI: −0.49 – −0.18; $P < 0.001$; $I^2 = 95.0\%$).

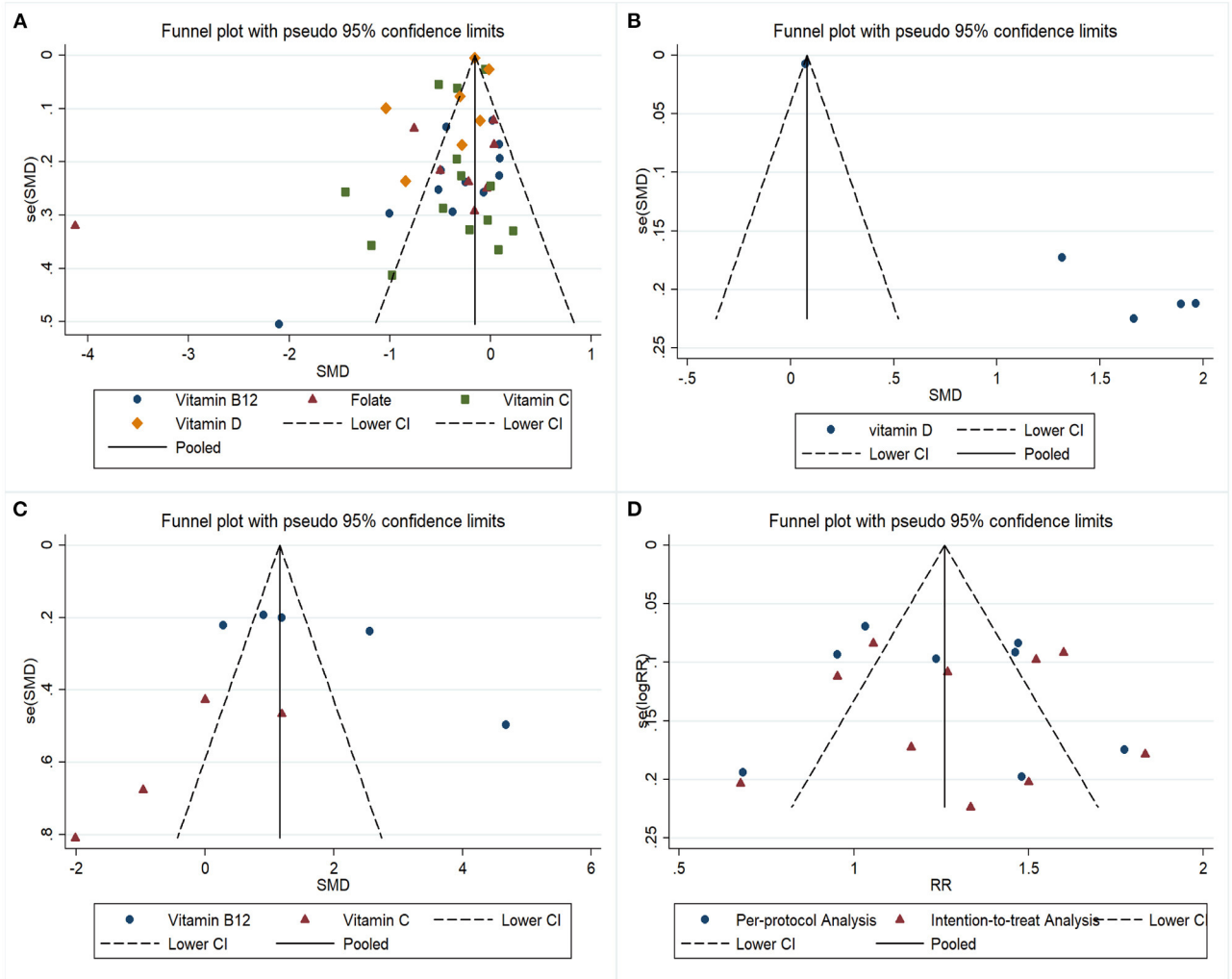

FIGURE 3 | Funnel plots of the meta-analysis on relationships between *H. pylori* and vitamins: **(A)** Comparison of serum vitamin levels between *H. pylori - positive* and *H. pylori - negative* patients; **(B)** Comparison of serum vitamin levels between *H. pylori* successful and failed eradication patients; **(C)** Comparison of serum vitamin levels before and after successful *H. pylori* eradication therapy; **(D)** Effect of vitamin supplements on the *H. pylori* eradication rate.

did not conduct a sensitivity analysis and the results should be interpreted cautiously.

For folate, we only identified two relevant studies; therefore, a pooled analysis was not performed. Both Kaptan et al. (13) et al. and Ozer et al. (61) concluded that successful *H. pylori* eradication was not associated with an increased serum folate level.

The Effects of Antioxidant Vitamin Supplementation on *H. pylori* Eradication

A total of 10 trials on the effects of antioxidant vitamin supplementation on *H. pylori* eradication were identified (**Table 4** and **Supplementary Table 4**). Among them, nine studies were RCTs, and one study was cohort research. The risks of bias were summarized in **Supplementary Table 6**. To avoid heterogeneity derived from different study designs, we did not include the cohort research (69) in the meta-analysis. We calculated the pooled results of the per-protocol analysis and intention-to-treat analysis from RCTs.

For the per-protocol analysis, eight estimates were included. A meta-regression was performed to assess potential sources of heterogeneity from eradication therapy (triple or quadruple therapy) and types of vitamin supplementation (vitamin C or vitamin C plus vitamin E). We found that eradication therapy and types of vitamin supplementation were not influencing factors ($P = 0.441$ for eradication therapy; $P = 0.707$ for types of vitamin supplementation). Therefore, all eight estimates were incorporated into the meta-analysis. The results indicated that combining antioxidant vitamin supplementation with standard therapy could increase the *H. pylori* eradication rate (RR = 1.22; 95% CI: 1.02–1.44; **Figure 6**), with heterogeneity ($P < 0.001$; $I^2 = 81.0\%$). There was no publication bias (Begg's test $z_c =$

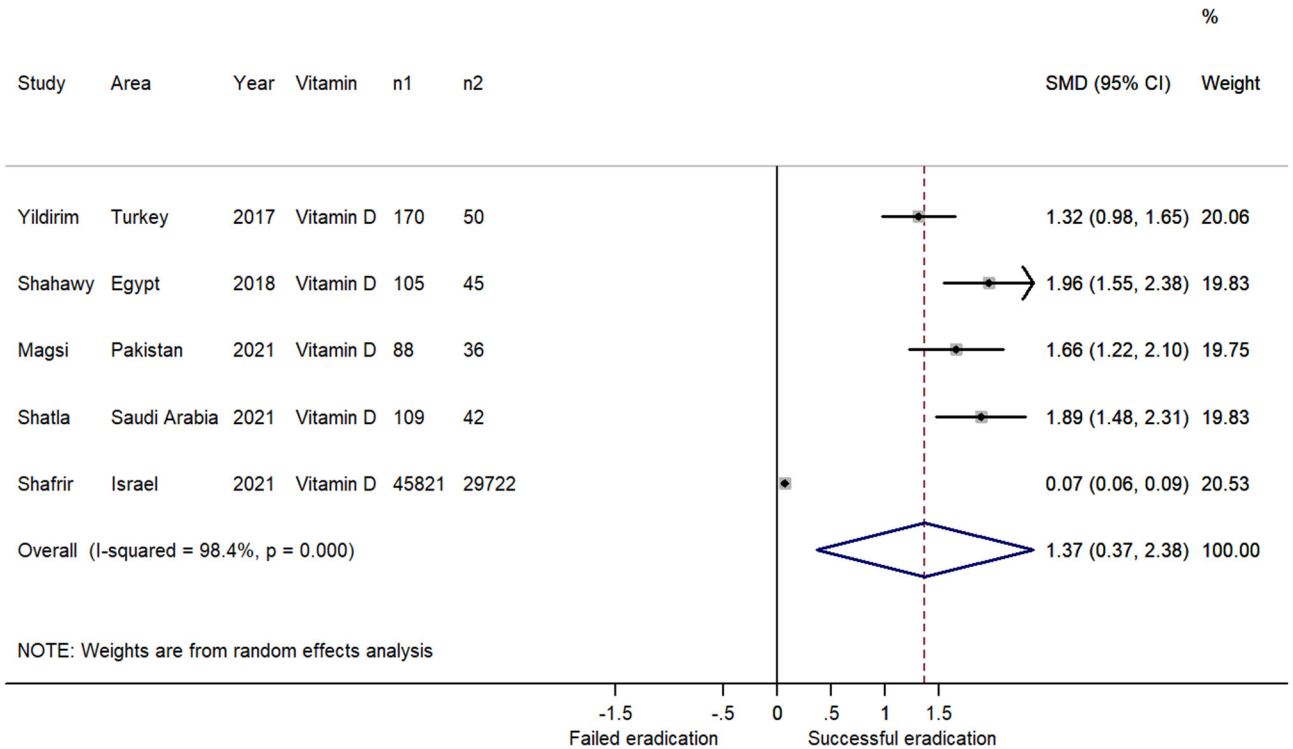

FIGURE 4 | Forest plot of the meta-analysis on the comparison of serum vitamin levels between *H. pylori* successful and failed eradication patients (n1: number of successful eradication patients; n2: number of failed eradication patients); The patients with successful *H. pylori* eradication had higher serum vitamin D levels than the failed patients (SMD = 1.37; 95% CI: 0.37 – 2.38; $P < 0.001$; $I^2 = 98.4\%$).

0.12, $P = 0.902$; Egger's test t = 0.662), and the funnel plot was symmetric (**Figure 3D**). The sensitivity analysis revealed that the results were robust (**Supplementary Figure 4**).

For the intention-to-treat analysis, 10 estimates were included. A meta-regression was conducted, and the result revealed that eradication therapy and types of vitamin supplementation were not influencing factors ($P = 0.832$ for eradication therapy; $P = 0.510$ for types of vitamin supplementation). The results of the pooled analysis showed that antioxidant vitamins supplementation increased the *H. pylori* eradication rate (RR = 1.25; 95%CI: 1.06–1.47; **Figure 6**), with heterogeneity ($P < 0.001$; $I^2 = 75.1\%$). Publication bias was not found (Begg's test $z_c = 0.18$, $P = 0.858$; Egger's test t = 0.973; **Figure 3D**). The sensitivity analysis showed that the results were stable.

Only the Kockar et al. study (67) explored the effects of vitamin A supplementation on *H. pylori* eradication. They determined that vitamin A was ineffective in *H. pylori* eradication. More relevant RCTs are recommended to clear the relationship.

DISCUSSION

The debate over the *H. pylori*-vitamin association has been persistent. The controversy mainly focuses on the following four aspects: (1) vitamin level discrepancies between *H. pylori*-positive and -negative patients; (2) vitamin level discrepancies between successful and failed *H. pylori* eradication patients; (3) vitamin level discrepancies before and after successful *H. pylori* eradication therapy; and (4) the effects of vitamin supplementation on *H. pylori* eradication. Phull et al. (31), Zhang et al. (32) and Toyonaga et al. (33) found that *H. pylori* infection was not associated with serum vitamin A and E levels. The vitamin B_{12} results from Trimarchi et al. (37), Sarari et al. (11) and Ulasoglu et al. (42) indicated that *H. pylori* had an adverse effect on serum vitamin B_{12} levels, whereas other observational studies (35, 36, 40, 44) presented a null association between *H. pylori* and vitamin B_{12}. The findings of Tamura et al. (34), Shuval-Sudai et al. (36) and Ulasoglu et al. (42) showed that *H. pylori*-negative patients had higher serum folate levels than *H. pylori*-positive patients; however, some studies (35, 39, 41, 43, 44) did not present similar results. A similar controversy exists regarding the associations between serum vitamin C or D levels and *H. pylori*. The effects of *H. pylori* eradication on serum vitamin levels have aroused extensive interest. Several studies (2, 57–59) found that patients that underwent successful *H. pylori* eradication had higher serum vitamin D levels than patients with in which eradication failed. Several studies (13, 14, 60, 61) described vitamin B_{12} level discrepancies before and after successful *H. pylori* eradication therapy; nevertheless, there were also inconsistent results. Vitamin supplementation was hypothesized to aid in *H. pylori* eradication. Some high-quality RCTs were performed to assess the assisting role of antioxidant vitamin

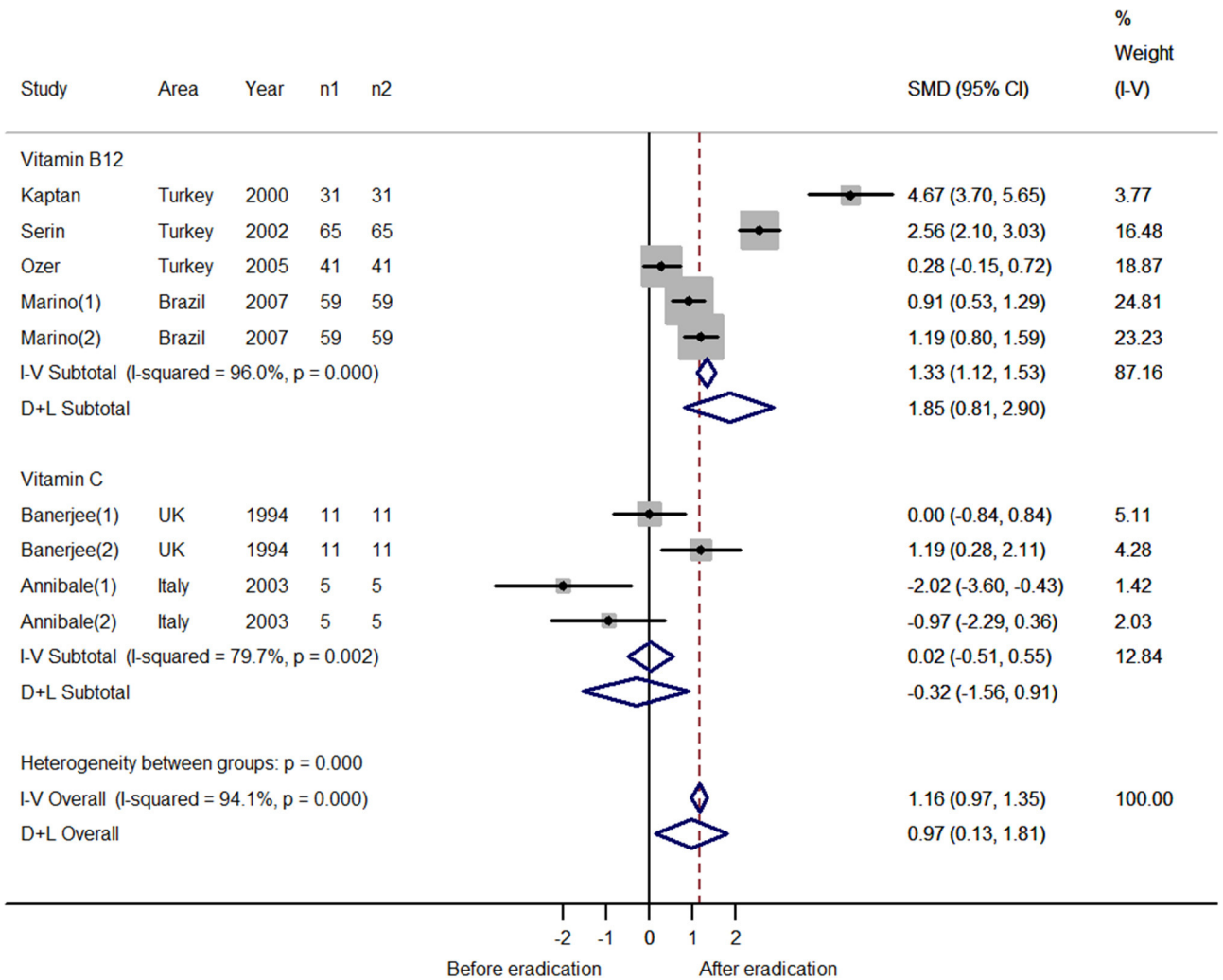

FIGURE 5 | Forest plot of the meta-analysis on the comparison of serum vitamin levels before and after successful *H. pylori* eradication therapy (n1: number of patients after eradication therapy; n2: number of patients before eradication therapy); After successful *H. pylori* eradication, serum vitamin B12 increased (SMD = 1.85; 95% CI: 0.81–2.90; $P < 0.001$; $I^2 = 96.0\%$); *H. pylori* eradication did not increase the serum vitamin C level (SMD = −0.32; 95% CI: −1.56–0.91; $P = 0.002$; $I^2 = 79.7\%$).

supplementation on *H. pylori* eradication. Sezikli et al. (65) found that supplementation with vitamin C plus vitamin E increases the *H. pylori* eradication rate; whereas Chuang et al. (68) and Zojaji et al. (16) indicated that simple vitamin C supplementation increases the *H. pylori* eradication rate. In contrast, studies from other researchers (66, 67) showed that vitamin supplementation has no effects on the *H. pylori* eradication rate. Several meta-analyses had also been published. Yang et al. (17) found that 25-hydroxyvitamin D levels in *H. pylori* - negative patients were higher than in *H. pylori* – positive patients, and patients with vitamin D deficiency had lower eradication rates of *H. pylori*. Lahner et al. (70) reported a comprehensive meta-analysis in 2012 focused on the relationships between micronutrients and *H. pylori*. This study found that *H. pylori* was associated with ascorbic acid levels in gastric juice, which were increased by the eradication treatment. At present, many relevant articles have been published, and we have the opportunity to update the results and obtain reliable conclusions.

The results of the meta-analysis suggested negative effects of *H. pylori* on serum vitamin B_{12}, folate, vitamin C and vitamin D levels. *Helicobacter pylori*-induced atrophic gastritis impairs stomach acidification and secretion functions and causes the malabsorption of nutrients. Vitamin B_{12} plays indispensable roles in promoting the development and maturation of red blood cells and in maintaining normal hematopoietic functions (71). A deficiency in an intrinsic factor caused by *H. pylori* infection would aggravate vitamin B_{12} absorption-related disorders. Folate participates in the metabolism of genetic materials and proteins, and it affects mammal reproduction (72). A folate deficiency may cause neural tube malformation, megaloblastic anemia, depression and malignant tumor formation (71, 73). A vitamin B_{12} deficiency may cause a folate metabolic disorder and

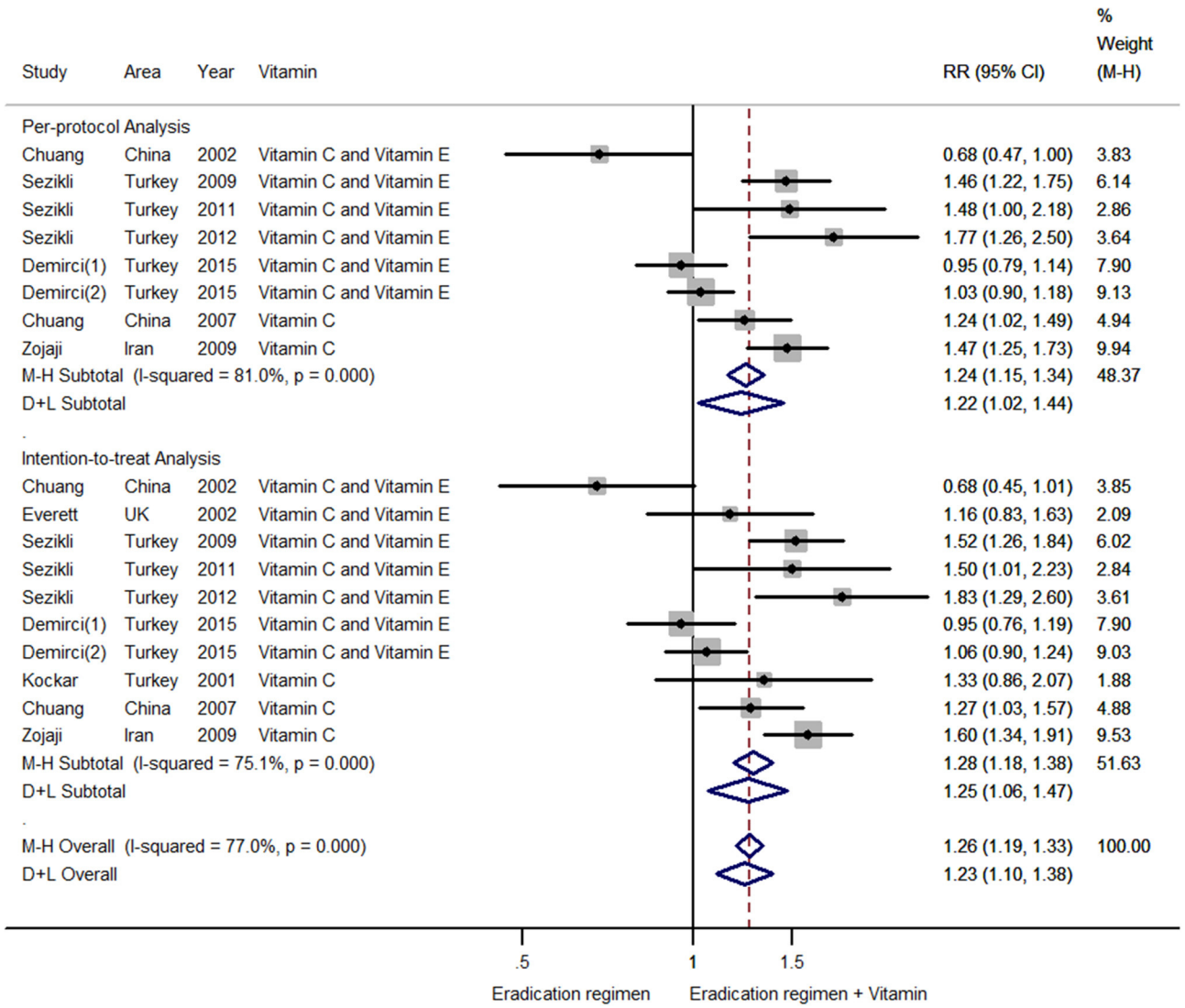

FIGURE 6 | Forest plot of the meta-analysis on the effects of vitamin supplements on *H. pylori* eradication rate (n1: number of eradication regimen plus vitamin supplement group; n2: number of eradication regimen group); For the per-protocol analysis, combining antioxidant vitamin supplementation with the standard therapy increased the *H. pylori* eradication rate (RR = 1.22; 95% CI: 1.02–1.44; $P < 0.001$; $I^2 = 81.0\%$); For the intention-to-treat analysis, antioxidant vitamins supplementation increased the *H. pylori* eradication rate (RR = 1.25; 95%CI: 1.06–1.47; $P < 0.001$; $I^2 = 75.1\%$).

aggravate the folate deficiency. Vitamin C and E, as vitamins with antioxidant effects, play roles in eradicating oxygen radicals and in maintaining the body's steady state (74, 75). Vitamin C may also promote the formation of tetrahydrofolic acid (75, 76). A shortage of vitamin C aggravates a folate deficiency. Therefore, the effects of *H. pylori* infections on human vitamin levels are not independent, and there are interactions among the various vitamins.

Our study found that patients that had undergone successful *H. pylori* eradication had higher serum vitamin D levels than patients in which *H. pylori* eradication failed. We speculated that vitamin D is a protective factor for *H.* pylori. In terms of mechanism, a combination of vitamin D and the vitamin D receptor may activate immune responses and participate in the anti-*H. pylori* process (77, 78). Yang et al. (17) also performed a related meta-analysis, but they only included three papers; consequently, the limited number of studies was not suitable for a pooled analysis. Our work included five relevant studies, including the study of Shafrir et al. (56) that covered data from more than 70,000 patients, which improved the reliability of the results. Apart from those on vitamin D, we did not find any studies that explored the relationships between other vitamins and the success of *H. pylori* eradication. Further research is needed to investigate the differences in other vitamin levels between patients with successful *H. pylori* eradication compared with those that failed.

This work revealed that serum vitamin B_{12} levels in patients after *H. pylori* eradication were significantly higher than those before *H. pylori* eradication. We reaffirmed the adverse effects of *H. pylori* on vitamin B_{12}. For patients with a vitamin

B_{12} deficiency, aggressive *H. pylori* eradication therapy and additional vitamin B_{12} supplementation are necessary. For vitamin C, this meta-analysis did not produce a statistically significant result. However, the four estimates included in this study were from only two reports. Thus, there were too few relevant studies included, and the results should be interpreted cautiously.

The pooled results of the RCTs showed that both the per-protocol analysis and intention-to-treat analysis suggested that antioxidant vitamin supplementation could improve the eradication rates of *H. pylori* of standard regimens. In the selection of standard eradication regimens, Kockar et al. (67), Chuang et al. (68) and Zojaji et al. (16) chose vitamin C for additional supplementation, whereas other studies used vitamin C combined with vitamin E for supplementation. Although both vitamin C and vitamin E have antioxidant effects, we were still concerned that inconsistent supplementation selection would introduce bias. Moreover, except the studies of Sezikli et al. (15) and Demirci et al. (66), which adopted the quadruple regimen, other studies adopted the triple regimen. The choices of eradication therapy (triple or quadruple therapy) also could introduce bias. We used a meta-regression to eliminate the possible bias caused by different kinds of vitamin supplementation and different eradication schemes from the statistical field, and then, we conducted pooled analyses to improve the reliability of the results. The meta-analysis of Li et al. (19) found that supplementation with antioxidant vitamins did not benefit the eradication rate. However, this analysis included only three studies. Although Ochoa et al. (79) also conducted a meta-analysis on a similar topic in 2018, there were errors and shortcomings in the data extraction and chart construction. Moreover, the above two articles only performed intention-to-treat analyses of the data. We believed that the per-protocol analysis should not be ignored in the RCTs. The meta-analysis of Yang-Ou et al. (80) focused on the effects of antioxidants on *H. pylori* eradication. However, this study combined antioxidant vitamins with other antioxidant (curcumin and cranberry). Our study incorporated intention-to-treat and per-protocol analyses and updated the results. Interestingly, the cohort study by Kaboli et al. (69) found that vitamin C supplementation reduces the dosage of clarithromycin. Li et al. (81) revealed that the *H. pylori* treatment and vitamin supplementation reduced the incidence of gastric cancer.

A complete and comprehensive search is necessary to find all the published data relevant to the meta-analysis. In addition to the usual English databases (Medline, Web of Science and Embase), we also searched for Chinese studies using the Chinese Biomedical Database and for conference papers using the Cambridge Scientific Abstracts databases to identify suitable studies. Although the search results did not ultimately expand the number of relevant articles, the processes remained essential. Unfortunately, it was hard for us to search manuscript with other languages, which might have resulted in the exclusion of some suitable articles. At last, we identified 48 relevant articles in this meta-analysis. We ran a better selection of studies and the number of included articles was larger than previous meta-analysis.

The heterogeneity of the selected studies cannot be ignored. The different methods of vitamin detection may result in variations in the results. The different areas, different numbers of patients enrolled in each study, some positive data and large amounts of included estimates may have increased the heterogeneity. Because the number of included studies was limited, it was hard for us to conduct subgroup analyses to reduce the influence of heterogeneity. Nevertheless, we performed meta-regression and sensitivity analyses to reduce the effects of heterogeneity on the credibility of the conclusions. The results of the meta-regression helped us eliminate the possibility that heterogeneity was a result of different eradication methods or vitamin supplementations. The sensitivity analyses helped us eliminate the influence of some positive data on the conclusions. In addition to the meta-regression and sensitivity analyses, we used random-effects models to establish relationships among the variables with high heterogeneity. Therefore, this work is appropriate to provide evidence, but the conclusions should be interpreted cautiously.

The study provides a comprehensive analysis of the interrelations between *H. pylori* and the most common vitamins from four aspects. Nevertheless, this work has some shortcomings. First, Turkish scholars have done a great deal of work on the effects of vitamin supplementation on *H. pylori* eradication. However, the contributions of researchers from other countries are limited. Different races and dietary habits may influence the results. It is difficult to conduct subgroup analyses to address this issue. Second, quadruple anti-*H. pylori* therapy is recommended currently. Some studies included in the work still used the triple approach. Moreover, the dose of vitamin supplementation is also controversial. Thus, the results should be interpreted cautiously. More RCTs with large samples focusing on the effects of vitamin supplementation on *H. pylori* eradication are needed to confirm our results and explore the appropriate dose.

CONCLUSIONS

In summary, this meta-analysis demonstrates that *H. pylori* infections can reduce the serum levels of several vitamins. The eradication of *H. pylori* rescues its adverse effects. Antioxidant vitamin supplementation may increase the rate of *H. pylori* eradication. Aggressive *H. pylori* eradication therapy is necessary, and the advantages of multivitamin supplementation for *H. pylori*-positive patients outweigh the disadvantages.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

XC, WY, and XiL conceived and designed the study. XC and XuL acquired and analyzed the data. XC, YJ, MZ, YX, YW, and CL

interpreted the data and drafted the manuscript. XiL and WY reviewed and corrected the manuscript. All authors approved the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.781333/full#supplementary-material

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Evaluation of Oropharyngeal Dysphagia in Older Patients for Risk Stratification of Pneumonia

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Objective: Nasogastric tube (NGT) and percutaneous endoscopic gastrostomy (PEG) are widely used techniques to feed older patients with oropharyngeal dysphagia. Aspiration pneumonia is the most common cause of death in these patients. This study aimed to evaluate the role of oropharyngeal dysphagia in older patients on long-term enteral feeding for risk stratification of pneumonia requiring hospitalization.

Methods: We performed modified flexible endoscopic evaluation of swallowing to evaluate oropharyngeal dysphagia in older patients and conducted prospective follow-up for pneumonia requiring hospitalization. A total of 664 oral-feeding patients and 155 tube-feeding patients were enrolled. Multivariate Cox analysis was performed to identify risk factors of pneumonia requiring hospitalization.

Results: Multivariate analyses showed that the risk of pneumonia requiring hospitalization significantly increased in older patients and in patients with neurological disorders, tube feeding, and oropharyngeal dysphagia. Subgroup analysis revealed that the risk of pneumonia requiring hospitalization was significantly lower in patients with PEG than in those with NGT among the patients with oropharyngeal dysphagia (adjusted hazard ratio 0.26, 95% confidence interval: 0.11–0.63, $P = 0.003$).

Conclusions: For older patients with oropharyngeal dysphagia requiring long-term enteral tube feeding, PEG is a better choice than NGT. Further research is needed to elucidate the role of oropharyngeal dysphagia in enteral feeding in older patients.

Keywords: older patients, oropharyngeal dysphagia, aspiration pneumonia, percutaneous endoscopic gastrostomy, nasogastric tube, tube feeding, enteral feeding

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INTRODUCTION

Population aging is a global issue, and it is estimated that the older population will reach approximately 1.5 billion in 2050 (1). Degeneration and multiple comorbidities accompany as people age, and approximately 13%–81% of older patients are affected by oropharyngeal dysphagia (1–4). This condition is common particularly in patients with neurological disorders (3, 4). Patients with oropharyngeal dysphagia may require enteral tube feeding if they cannot meet their nutritional needs orally (5). Such patients may face greater socioeconomic issues as the prevalence rates of stroke, dementia, and esophageal motility disorder increase with older age (4, 6, 7).

The two commonly used methods of enteral tube feeding are nasogastric tube (NGT) and percutaneous endoscopic gastrostomy (PEG) (8). Aspiration pneumonia is the most common cause of death in these patients with enteral tube feeding (9, 10), with estimated incidence rates of 12%–87% for NGT (11–15) and 9%–52% for PEG (12–15). At present, NGT is recommended for temporary enteral nutrition lasting less than 4 weeks, whereas PEG is recommended for cases longer than 4 weeks (16). However, in Asian counties, NGT is more frequently selected than PEG for long-term enteral feeding. Possible explanations for this choice include refusal of interventional surgical procedures by the patient's family due to considering patients has already been tortured by long-term morbidity or disability, desire to follow the custom of maintaining the "whole corpse" for burial purposes, and concerns related to complications after the PEG procedure (17–19).

The risks of aspiration pneumonia in older patients rise significantly with oropharyngeal dysphagia (20, 21). Meta-analyses have not conclusively determined whether oropharyngeal dysphagia is a risk for aspiration pneumonia in older patients on enteral feeding and whether PEG is better than NGT (15, 22). However, this may be due to the high level of statistical heterogeneity among analyzed studies. Therefore, oropharyngeal dysphagia should be evaluated and subgroup analysis regarding the risk of pneumonia in older patients on long-term enteral feeding, especially in regions in favor or NGT over PEG, should be performed.

Flexible endoscopic evaluation of swallowing (FEES) is a well-documented standard method for evaluating oropharyngeal dysphagia (23, 24). This method allows assessment of aspiration risk by direct visualization of test material accumulating in the pharyngolaryngeal region or entering the vocal cords (23–26); however, adverse events such as epistaxis, vasovagal response, and laryngospasm can develop during the procedure (27). Furthermore, this method can only be performed if the patient has adequate physical and cognitive function to cooperate with degulgitation, and thus cannot be properly applied in patients with advanced neurological diseases such as dementia, Parkinson's disease, or stroke (24–26). In contrast, a modified version of FEES that involves additional pharyngolaryngeal examination during upper gastrointestinal (UGI) endoscopy is easy and bearable to patients, and may thus help in stratifying the severity of oropharyngeal dysphagia according to the pooling of secretions in the pharyngolaryngeal region (24).

Aspiration pneumonia could develop if aspiration of potential pathogenic gastric contents or oropharyngeal secretions into the larynx or respiratory tract, which is common in older adults with dysphagia (28). As more residues in the pharyngolaryngeal region were reported in those with NGT due to NGT passes through the upper esophageal sphincter (29–32), we hypothesized that oropharyngeal dysphagia significantly increased the risk of pneumonia requiring hospitalization in older patients with NGT, and that PEG is recommended in preference to NGT for patients with oropharyngeal dysphagia requiring long-term tube feeding.

Previous studies primarily focused on the utility of FEES in evaluating the risk of aspiration pneumonia in patients with oral feeding, whereas studies examining the utility of FEES in evaluating the risks in NGT- and PEG-feeding patients remain limited. This study aimed to evaluate the role of oropharyngeal dysphagia for risk stratification of pneumonia requiring hospitalization in older patients on long-term enteral feeding.

MATERIALS AND METHODS

Patients

We performed modified FEES in older patients who received enteral feeding between January 2015 and July 2020. Additionally, we conducted a prospective follow-up of those requiring hospital admission due to pneumonia. The study was approved by the institutional review board of the Tri-Service General Hospital, No: 2-108-05-136. The participants provided written informed consent.

Study Design

Of the 2,923 patients who underwent modified FEES to evaluate oropharyngeal dysphagia (**Figure 1**), 2,031 were excluded because they were below 65 years of age, 39 were excluded because modified FEES provided a poor pharyngolaryngeal view, and 34 were excluded for missing demographic profile data. Of the remaining 819 older patients who required enteral feeding for more than 4 weeks, 664 were enrolled in the oral-feeding group and 155 in the tube-feeding group.

Demographic and Clinical Data

We recorded the demographic and clinical profiles, such as age, gender, body mass index, reasons for enteral feeding, and pneumonia requiring admission. Reasons for enteral feeding were categorized into two groups according to whether the patient had any neurological disorder, such as Alzheimer's disease, dementia, Parkinson's disease, or stroke.

Modified Flexible Endoscopic Evaluation of Swallowing

Patients fasted for at least 4 hours before UGI endoscopic examination and were placed in the left lateral decubitus position (24, 33). The endoscope tip was inserted through a mouthpiece with its axis aligned with that of the patient's esophagus. The endoscope was advanced along the palate midline, rotated slightly, and gently advanced past the uvula with anterior flexion to visualize the pyriform sinus, laryngeal vestibule, vocal cords, and upper part of the trachea (**Figure 2A**). The examination result was recorded using a digital video recorder (HVO-550MD; Sony, Tokyo, Japan) for later analysis and was reviewed frame by frame in slow motion by two endoscopists (WKC and CWY).

Evaluation of Oropharyngeal Dysphagia

When patients are placed in the left lateral decubitus position during examination, the accumulated secretions may fill the lowest area of the right side of the pyriform sinus, leak into the left side of the pyriform sinus, or flow into the laryngeal vestibule or vocal cords (24). Swallowing frequency is estimated to be about 600 times per day under normal physiological conditions (34). In most patients, no pooling or minimal pooling of

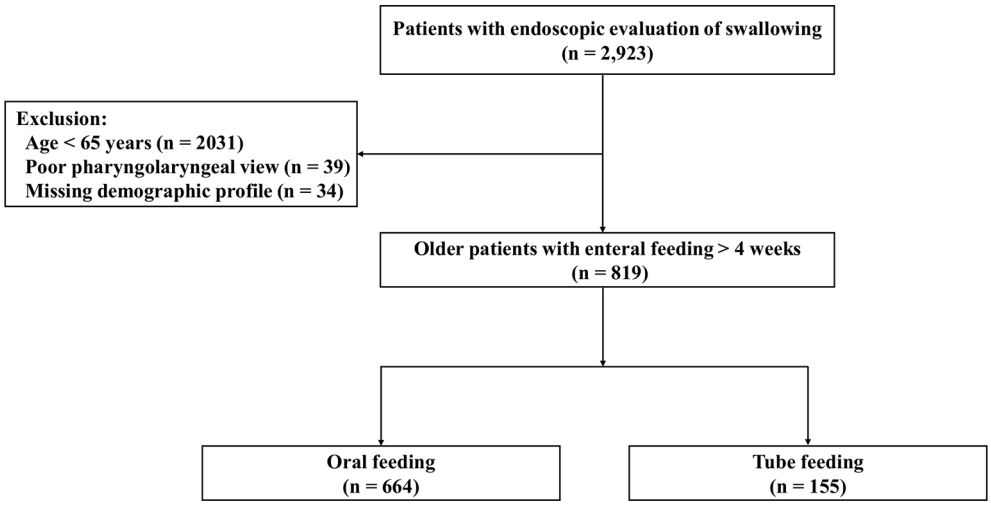

FIGURE 1 | Patient selection flow chart.

secretions in the pharyngolaryngeal region was observed under endoscopy; occasionally, thin, watery secretions or clear bubbles were observed in the pharyngolaryngeal region (24, 25). Oropharyngeal dysphagia was evaluated according to the amount and location of accumulated secretions observed endoscopically in the pharyngolaryngeal region (**Figure 2**) (24, 35). The pooling of secretions in the pyriform sinus was quantified as follows: minimal, < 25%; moderate, 25%–50%; and large, > 50% secretions filling the pyriform sinuses. Endoscopic evidence of oropharyngeal dysphagia was divided into two categories: (1) absence of oropharyngeal dysphagia (< 25% pooled secretions filling the pyriform sinus, **Figure 2A**) and (2)

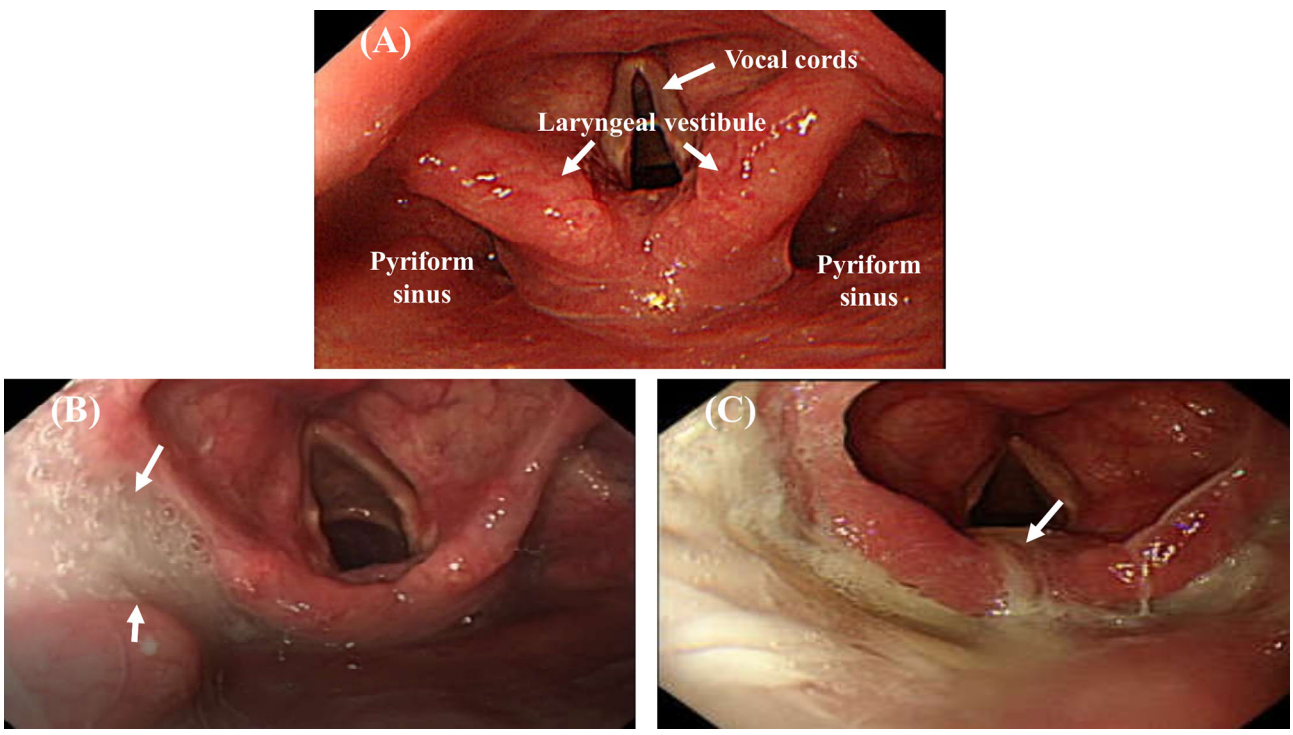

FIGURE 2 | Endoscopic views of pooled secretions (arrow) in the pharyngolaryngeal region. Absence of secretions filling the pharyngolaryngeal region **(A)**. A large amount of pooled secretions in the right side of the pyriform sinus but not entering the laryngeal vestibule **(B)**, and pooled secretions leaking into the laryngeal vestibule **(C)**.

presence of oropharyngeal dysphagia (> 25% pooled secretions filling the pyriform sinus, **Figure 2B**; or pooled secretions entering the laryngeal vestibule, **Figure 2C**) (24, 35).

Diagnosis of Pneumonia

Pneumonia was diagnosed based on radiological evidence of pulmonary consolidation, shortness of breath, body temperature above 38°C, serum white blood cell count > 10,000/mm^3, and the requirement for hospitalization within 2 years after the patient was enrolled (36).

Statistical Analysis

Statistical analyses were performed using SPSS Statistics 22.0 (IBM Inc., Armonk, NY, USA) with a two-sided significance level of 5%. Parametric continuous data were compared using the independent t-test. Categorical data were compared using the Chi-square test or Fisher's exact test. A univariate and multivariate Cox regression model was applied to determine the association between the clinical-demographic profile and developing pneumonia. The hazard ratio (HR) for pneumonia development was estimated *via* multivariate analysis using the Cox proportional hazards model after adjusting the statistically significant variables in the univariate analysis as potential confounding factors. Survival curves were plotted with the Kaplan–Meier method.

RESULTS

Patient Characteristics

A total of 819 older patients were enrolled in this study, with 664 in the oral-feeding group and 155 in the tube-feeding group. Patient characteristics are shown in **Table 1**. The mean age was 76.4 ± 7.5 years in the oral-feeding group and 78.5 ± 8.9 years in the tube-feeding group. Significant differences in age, sex, body mass index, reasons for enteral feeding, and oropharyngeal dysphagia were observed between the two groups.

Risk Factors for Pneumonia Requiring Hospitalization

Multivariate analyses showed that the risk of pneumonia requiring hospitalization was significantly increased in patients with older ages (with each year of age increase, adjusted HR 1.03, 95% CI 1.01–1.06, $p = 0.004$), neurological disorders (adjusted HR 2.39, 95% CI: 1.49–3.84, $P < 0.001$), tube feeding (adjusted HR 2.57, 95% CI: 1.61–4.12, $P < 0.001$), and oropharyngeal dysphagia (adjusted HR 1.59, 95% CI: 1.02–2.47, $P = 0.041$) (**Table 2**).

Subgroup Analysis: Presence Versus Absence of Oropharyngeal Dysphagia

Subgroup analysis was conducted for patients on enteral feeding based on whether the patients had oropharyngeal dysphagia (**Figure 3A**). Presence of oropharyngeal dysphagia significantly increased the risk of pneumonia in patients with enteral feeding (adjusted HR 1.59, 95% CI: 1.02–2.47, $P = 0.041$). However, it did not increase the risk in patients with oral feeding (adjusted HR 1.63, 95% CI: 0.68–3.88, $P = 0.274$) and in patients with tube feeding (adjusted HR 1.54, 95% CI: 0.91–2.62, $P = 0.109$).

Subgroup Analysis: PEG Versus NGT

The risk of pneumonia showed no significant difference between PEG and NGT in all tube-feeding patients (adjusted HR 0.58, 95% CI: 0.32–1.04, $P = 0.070$). We conducted subgroup analysis for all enteral feeding patients with tube feeding based on the presence of NGT or PEG long-term feeding (**Figure 3B**). The risk of pneumonia was not significantly different between PEG and NGT (adjusted HR 1.57, 95% CI: 0.71–3.45, $P = 0.261$) in patients with absence of oropharyngeal dysphagia; however, the risk of pneumonia was significantly lower in PEG than NGT in patients with presence of oropharyngeal dysphagia (adjusted HR 0.26, 95% CI: 0.11–0.63, $P = 0.003$).

Cumulative Proportion of Pneumonia Requiring Admission

Kaplan–Meier analysis indicated that the cumulative proportion of pneumonia was significantly increased in patients with tube feeding compared with oral feeding ($P < 0.001$) (**Figure 4A**). When subgroup analysis patients had absence of oropharyngeal dysphagia, the cumulative proportion of pneumonia was not significantly different between PEG and NGT ($P = 0.425$) (**Figure 4B**). However, when subgroup analysis patients had presence of oropharyngeal dysphagia, the cumulative proportion

TABLE 1 | Patients characteristics.

Variable	Oral feeding (n = 664)	Tube feeding (n = 155)	*P* Value
Age (years)	76.4 ± 7.5	78.5 ± 8.9	0.006
Sex			0.001
Female	349 (52.6%)	58 (37.4%)	
Male	315 (47.4%)	97 (62.6%)	
Body mass index (kg/m^2)	23.6 ± 3.8	21.9 ± 3.6	<0.001
Reasons for enteral feeding, no. (%)			<0.001
Neurological disorders	135 (20.3%)	118 (76.1%)	
Non-neurological disorders	529 (79.7%)	37 (23.9%)	
Oropharyngeal dysphagia, no. (%)			<0.001
Absence	613 (92.3%)	96 (61.9%)	
Presence	51 (7.7%)	59 (38.1%)	

TABLE 2 | Multivariable analysis of the factors associated with pneumonia.

Variable	With pneumonia (n = 113)	Without pneumonia (n = 706)	Crude HR (95% CI)	*P* value	Adjusted HR (95% CI)	*P* value
Age (years)	79.8 ± 8.7	76.3 ± 7.6	1.06 (1.03–1.08)	<0.001	1.03 (1.01–1.06)	0.004
Sex, no. (%)						
Female	45 (39.8%)	362 (51.3%)	Reference		Reference	
Male	68 (60.2%)	344 (48.7%)	1.57 (1.08–2.29)	0.018	1.22 (0.83–1.79)	0.302
Body mass index (kg/m^2)	22.5 ± 3.9	23.4 ± 3.8	0.95 (0.90–0.99)	0.03	0.99 (0.94–1.05)	0.842
Reasons for enteral feeding, no. (%)						
Non-neurological disorders	38 (33.6%)	528 (74.8%)	Reference		Reference	
Neurological disorders	75 (66.4%)	178 (25.2%)	4.92 (3.33–7.27)	<0.001	2.39 (1.49–3.84)	<0.001
Enteral feeding, no. (%)						
Oral feeding	53 (46.9%)	611 (86.5%)	Reference		Reference	
Tube feeding	60 (53.1%)	95 (13.5%)	5.53 (3.82–8.01)	<0.001	2.57 (1.61–4.12)	<0.001
Oropharyngeal dysphagia, no. (%)						
Absence	77 (68.1%)	632 (89.5%)	Reference		Reference	
Presence	36 (31.9%)	74 (10.5%)	3.61 (2.43–5.37)	<0.001	1.59 (1.02–2.47)	0.041

HR, hazard ratio; CI, confidence interval.

of pneumonia was significantly decreased in patients with PEG compared with NGT (P = 0.001) (**Figure 4C**).

DISCUSSION

This was a prospective study of older patients requiring long-term enteral tube feeding in a tertiary care center. We found that (1) multivariate analyses showed that older ages, neurological disorders, tube feeding, and oropharyngeal dysphagia were associated with an increased risk of pneumonia requiring hospitalization; (2) oropharyngeal dysphagia significantly increased the risk of pneumonia in patients on long-term enteral feeding; (3) pneumonia significantly decreased in patients with PEG compared to those with NGT among the patients with oropharyngeal dysphagia. The implication of this study is that the evaluation of oropharyngeal dysphagia is crucial for older patients on long-term enteral feeding. In older patients

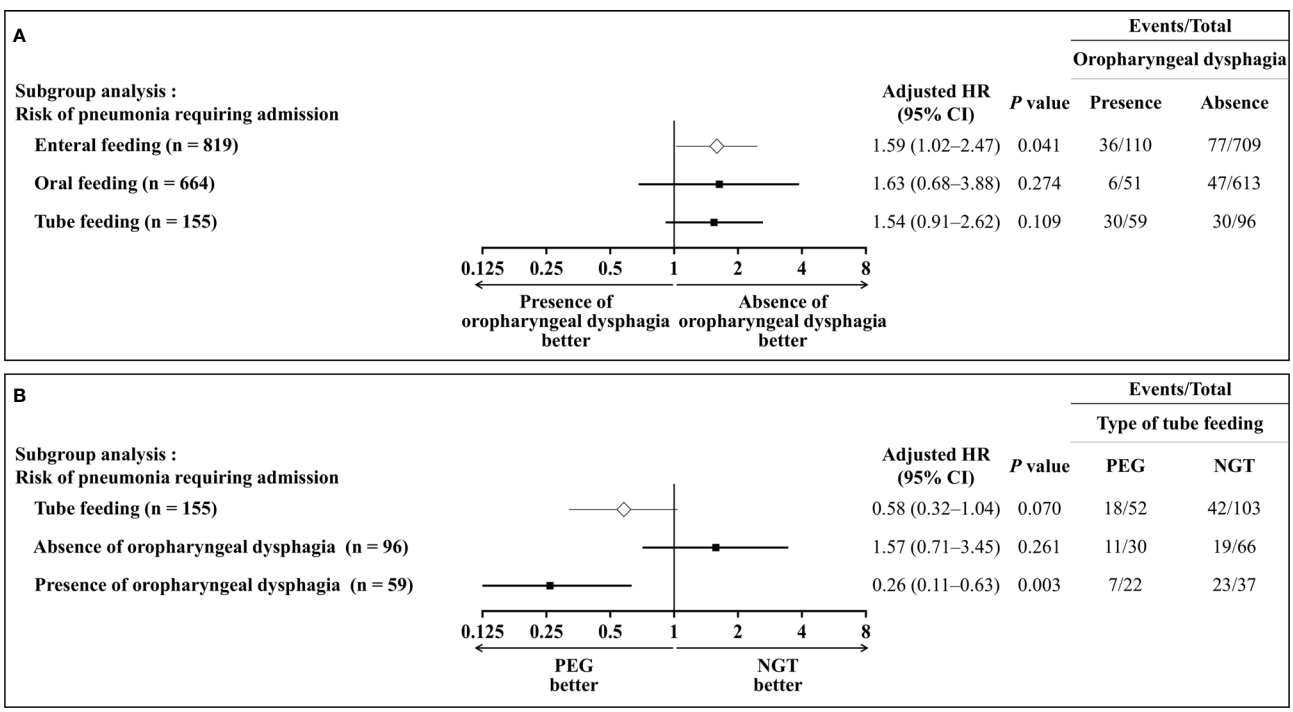

FIGURE 3 | Subgroup analysis of risk of pneumonia requiring admission in older patients with enteral feeding. **(A)** Based on whether the patients had oropharyngeal dysphagia, the risk of pneumonia requiring hospitalization increased significantly in overall enteral feeding patients, but not in oral-feeding and tube-feeding patients. **(B)** Based on the type of tube feeding, PEG did not decrease the risk of pneumonia requiring hospitalization in the overall tube-feeding patients and patients without oropharyngeal dysphagia, but the risk was reduced significantly in patients with oropharyngeal dysphagia.

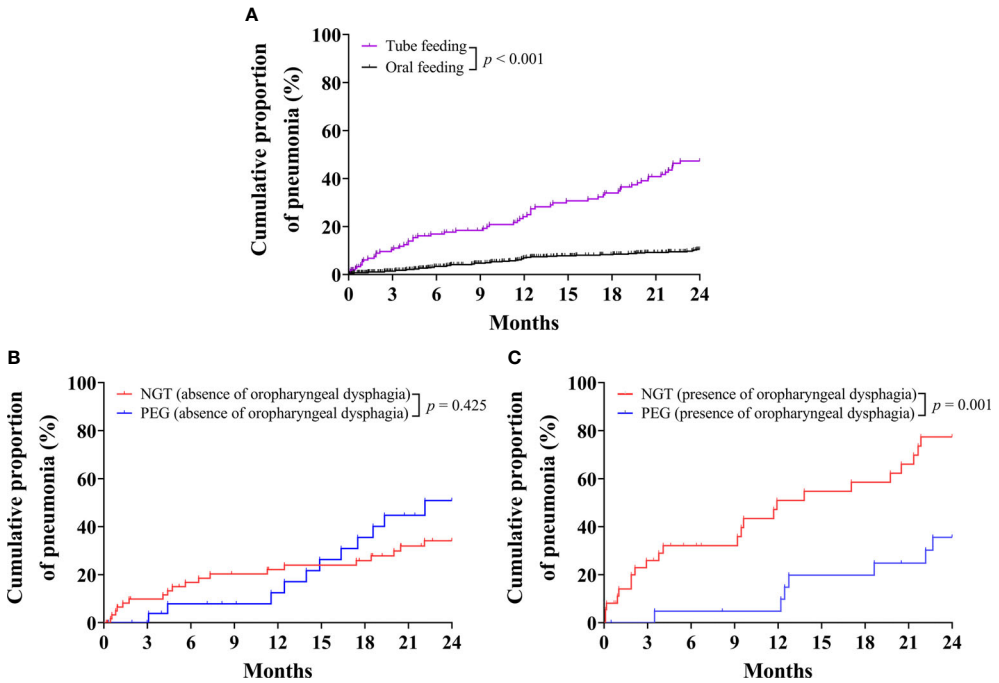

FIGURE 4 | Kaplan–Meier curves for the cumulative proportion of pneumonia requiring hospital admission in older patients. **(A)** Cumulative proportion of pneumonia in older patients for tube-feeding and oral-feeding group. **(B)** Cumulative proportion of pneumonia in older patients without oropharyngeal dysphagia for nasogastric tube (NGT) and percutaneous endoscopic gastrostomy (PEG) groups. **(C)** Cumulative proportion of pneumonia in older patients with oropharyngeal dysphagia for NGT and PEG groups.

with oropharyngeal dysphagia, PEG is a better choice than NGT for long-term enteral tube feeding.

Evaluation of Oropharyngeal Dysphagia

Detailed history-taking, physical examination, and FEES are important for evaluating oropharyngeal dysphagia (37), and evaluation of oropharyngeal dysphagia is recommended in patients requiring long-term enteral feeding (33, 38, 39). Digital video recording systems can aid in overcoming the limitations of conventional UGI endoscopy for pharyngolaryngeal examination due to limited photographic recording within a short observation period (24, 40). Furthermore, the recorded video can facilitate the evaluation of oropharyngeal dysphagia by recording protective cough reflex, vocal cord adduction, and movements for clearing secretions during the process (24, 40). Although a swallowing test was not conducted as a part of the traditional methodology of FESS due to the patient characteristics (23–26), we were able to stratify the groups with higher pneumonia risk using modified FESS with UGI endoscopy through the direct visualization of the pharyngolaryngeal region.

Older Patients With Oropharyngeal Dysphagia

Deterioration in neural control and structural alteration of the pharyngeal region can lead to swallowing difficulties (41), which can gradually worsen with increasing age of people (3, 4, 6, 7). In the current study, the multivariate analyses revealed that the risk of pneumonia requiring hospitalization was significantly higher in older patients (with each year of age increase, adjusted HR 1.03, 95% CI 1.01–1.06, P = 0.004) and in patients with neurological disorders (adjusted HR 2.39, 95% CI: 1.49–3.84, $P < 0.001$). Oropharyngeal dysphagia is associated with increased risk of aspiration pneumonia or death (42, 43). The risk of pneumonia requiring hospitalization also increased significantly in patients with oropharyngeal dysphagia (adjusted HR 1.59, 95% CI: 1.02–2.47, P = 0.041) in the multivariate analyses in the current study. If oropharyngeal dysphagia is suspected in older patients, referral to a physician or speech therapist is warranted (44). A multidisciplinary team should develop a management plan to monitor, assess, and prevent aspiration pneumonia in patients receiving long-term enteral feeding who are at risk of oropharyngeal aspiration (5, 45). The development of malnutrition might be related to reduced swallowing function due to oropharyngeal dysphagia (20, 46), and further deterioration of laryngeal muscle might lead to a vicious cycle (20, 46). Effective swallowing function and salivary flow are crucial for the clearance of most oropharyngeal pathogens in healthy individuals (47), whereas reduced mechanical clearance might be associated with oropharyngeal residue or bacterial colonization (46, 47). Aspiration pneumonia could develop due to pulmonary aspiration induced by impaired swallowing function, particularly if the aspirated material contains an abundant bacterial load or in the presence of impaired mechanical or immune defense mechanism (48).

Tube Feeding in Older Patients With Oropharyngeal Dysphagia

Tube feeding is associated with a higher risk of aspiration pneumonia compared to oral feeding (32, 49), and our study showed similar results (adjusted HR 2.57, 95% CI: 1.61–4.12, $P < 0.001$) under multivariate analysis. Although PEG has not shown more favorable outcomes compared to NGT in meta-analyses (15, 22), subgroup analysis in this study demonstrates that the risk of pneumonia was significantly reduced in PEG compared to NGT in older patients with oropharyngeal dysphagia (adjusted HR 0.26, 95% CI: 0.11–0.63, $P = 0.003$), but not in patients without oropharyngeal dysphagia (adjusted HR 1.57, 95% CI: 0.71–3.45, $P = 0.261$). Bacterial colonization and gastroesophageal reflux might increase due to interference with protective cough reflexes in patients with NGT (29–32). The NGT passes through the upper esophageal sphincter, and studies have reported more residues in the pharyngolaryngeal region (29–32), and aspiration of pathogenic bacterial colonization from oropharyngeal secretions could lead to aspiration pneumonia (48). Therefore, in older patients requiring long-term enteral tube feeding with oropharyngeal dysphagia, PEG is a better choice than NGT.

Management of Oropharyngeal Dysphagia in Older Patients

The management of oropharyngeal dysphagia should include familial support and a multidisciplinary team (45). Strategies for reducing the rate of aspiration pneumonia in patients with oropharyngeal dysphagia include (1) maintaining a semi-recumbent position and turning the patient's head to one side with the chin down during feeding to reduce the risk of gastric aspiration (50); (2) maintenance of oral hygiene by proper tooth brushing, mechanical oral cleaning, and oral rinsing with chlorhexidine (50–52); (3) dental examination to remove debris, plaque or treat tooth decay (50–52); (4) suctioning of subglottic and oropharyngeal secretions (33, 53); (5) avoidance of oversedation, antipsychotics, and cough suppressants that weaken the cough reflex (50); (6) regular swallowing rehabilitation (54); (7) feeding with PEG rather than NGT in patients requiring long-term tube feeding (24, 33).

Limitations

Several limitations should be considered when interpreting our results. First, the study was a prospective single-center non-randomized design, and due to the limited sample size, the statistical power is limited. Second, although direct visualization of the pharyngolaryngeal region was feasible with FEES, we could not assess the contents or amounts aspirated during follow-up and determine their relationship to the development of pneumonia. In addition, swallowing trial was not conducted since a significant proportion of enrolled patients had advanced neurological diseases who could not follow instructions during the examination and penetration-aspiration scale was not assessed (55–57). Third, information on body weight variation, feeding intolerance, and gastric residual volume was not available; thus, we could not adjust for these possible confounders (58). However, to the best of our knowledge, the present study is the first prospective study designed to evaluate the optimal choice of long-term tube feeding in older patients with oropharyngeal dysphagia.

CONCLUSIONS

Despite the limitations, this study shows that oropharyngeal dysphagia significantly increased the risk of pneumonia requiring hospitalization in older patients with NGT. PEG is recommended in preference to NGT for patients with oropharyngeal dysphagia requiring long-term tube feeding. Further study will be needed to elucidate the role of oropharyngeal dysphagia in enteral feeding in older patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because legal restrictions imposed by the government of Taiwan in relation to the "Personal Information Protection Act". Requests to access the datasets should be directed to Tri-Service General Hospital.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by institutional review board of the Tri-Service General Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

T-HL and W-KC contributed to conception and design of the study. T-HL organized the database, performed statistical analysis, and wrote the first draft of the manuscript. C-WY and W-KC wrote sections of the manuscript. All authors contributed to manuscript revision, reading, and approval of the submitted version.

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Higher Stress Hyperglycemia Ratio Is Associated With a Higher Risk of Stroke-Associated Pneumonia

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Objective: Stroke-associated pneumonia (SAP) is a frequent complication in stroke patients. This present study aimed to investigate the association between stress hyperglycemia and SAP.

Methods: Patients were screened between February 2013 and August 2020 from the First Affiliated Hospital of Wenzhou Medical University. We divided the blood glucose of the patients at admission by the glycated hemoglobin to calculate the stress hyperglycemia ratio (SHR). Binary logistic regression analysis was used to identify the association between SAP and SHR, with the confounders being controlled. Further, subgroup analyses were separately performed for stroke patients with and without diabetes.

Results: A total of 2,039 patients were finally recruited, of which 533 (26.14%) were diagnosed with SAP. SHR were divided into four quartiles in the logistic regression analysis, the highest SHR quartile (SHR $\geq$ 1.15) indicated a higher risk of SAP (OR = 1.57; 95% CI = 1.13–2.19, $p = 0.01$) in total patients. In patients without diabetes, the third quantile (SHR = 0.96–1.14) and the highest quantile (SHR $\geq$ 1.15) were both related to a higher risk of SAP (both $p < 0.05$). However, we did not find such an association in diabetic patients.

Conclusion: SHR was significantly associated with the risk of SAP in patients without diabetes. Adequate attention should be paid to the patients with high SHR levels at admission, especially those without diabetes.

Keywords: stroke, pneumonia, glucose, stress hyperglycemia, diabetes

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INTRODUCTION

Stroke-associated pneumonia (SAP) is a frequent and serious complication in stroke patients (1). Accumulating studies have observed that SAP was associated with a higher risk of mortality, a longer length of hospitalization, and poorer functional outcomes in stroke survivors independently (2–4). Given the clinical importance of SAP, early identification of SAP is important to improve the prognosis of stroke.

As far as we know, nearly half of patients suffering from stroke may develop stress hyperglycemia (5, 6), which is defined as temporarily increased glycemia in the emergency situation (7). A previous study showed that stress hyperglycemia was associated with a high risk of mortality and recurrence after stroke (6). It should be noted that hyperglycemia was also linked to an increased risk of infection *in vitro* (8) and after surgical procedures (9), which is due to the excessive release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) (10, 11). These elevated cytokines would further interfere with the insulin signaling pathway, reduce insulin production in peripheral tissues, and further increase blood glucose, resulting in a vicious cycle (12). Furthermore, the aforementioned pro-inflammatory molecules were significant contributors to SAP as well (13–16), and the stroke-induced immunosuppression and infection will promote and accelerate the occurrence and development of SAP (17). Thus, stroke patients with a stress hyperglycemia-induced high inflammatory state may be associated with a high risk of SAP, and it was worth exploring the relationship between stress hyperglycemia and SAP.

Many studies adopted the stress hyperglycemia ratio (SHR) to indicate stress hyperglycemia (18–21), which is defined as the ratio of glycated hemoglobin (HbA1c) to blood glucose (21, 22). Moreover, SHR was considered as an independent influencing factor for hemorrhagic transformation in stroke patients (23). In addition, increasing evidence demonstrated that SHR was useful in assessing neurological deterioration and prognosis in patients with ischemic stroke (24), and was related to all-cause death in ischemic stroke patients (25). Moreover, SHR for acute ischemic stroke after intravenous thrombolysis was predictive for worse outcomes (26, 27).

Up to now, the relationship between SHR and SAP was poorly explored. This research aimed to explore the role of SHR in SAP, and we hypothesized that higher SHR may indicate a higher risk of SAP in patients with stroke.

MATERIALS AND METHODS

Study Design and Setting

This study was a retrospective analysis of the patients admitted to the Department of Neurology at the First Affiliated Hospital of Wenzhou Medical University between February 2013 and August 2020. This study was approved by the local Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. Because this was a retrospective study, written informed consent was waived, and all data were kept anonymous.

Participants

All the patients were screened from the First Affiliated Hospital of Wenzhou Medical University. The inclusion criterias were: (1) age $\geq$ 18 years old; (2) onset of stroke within 7 days; (3) acute stroke (including acute ischaemic stroke and intracerebral hemorrhage) verified by a radiological examination [either magnetic resonance imaging (MRI) or cranial computed tomography (CT)]. The exclusion criterias were as follows: (1) preexisting pneumonia before stroke; (2) fever or active infection within 2 weeks before admission; (3) diagnosis of transient ischaemic attack (TIA); (4) data incomplete. The diagnosis of SAP was determined by two well-trained and experienced neurological physicians during the 1st week of hospitalization after the onset of stroke, according to the modified Centers for Disease Control and Prevention criteria for hospital-acquired pneumonia, combining the clinical, laboratory, and radiological examinations (28, 29).

Data Collection

Data were collected from the electronic medical record system. Demographic variables included age, body mass index (BMI, kg/m^2), and gender. The medical histories were gathered, including hypertension, coronary heart disease, diabetes mellitus, kidney disease, tuberculosis, chronic hepatitis, and atrial fibrillation. Besides, other clinical characteristics, including cigarette smoking, alcohol consumption, blood pressure, subtypes of stroke, and swallowing function, were recorded as well. The patients' swallowing functions were assessed by the specialists, and thus the diet was determined, including general diet, semi-liquid diet, paste meal, and nasal feeding. The diet was adopted as an indicator of the swallowing function in this study. Furthermore, the National Institutes of Health Stroke Scale (NIHSS) score was also collected, which is a well-validated and reliable scale for evaluating the severity of stroke (30).

Blood samples were carried out after overnight fasting within 24 h of admission to the hospital. We conducted the blood parameters including low-density lipoprotein cholesterol (LDL-C), triglyceride, leukocyte count, serum creatinine (Cr), glucose levels, and HbA1c. Finally, we calculated the stress hyperglycemia ratio by dividing the fasting plasma glucose at admission with HbA1c.

Study Size

As was shown in **Supplementary Figure S1**, 2616 patients were screened and 2,039 patients with acute stroke were finally included in this study, of which 533 participants were confirmed as SAP. Meanwhile, 577 patients did not meet the entry criteria and were excluded, of which 220 subjects had a fever or active infection within 2 weeks before admission, 80 had a transient ischemic attack, 67 had pneumonia before the stroke, and 210 had incomplete data.

Statistical Methods

Continuous variables were displayed as mean ± standard deviation (SD) or median (interquartile range, IQR). A Student's *t*-test (normal distribution) or Mann–Whitney *U*-test (non-normal distribution) was used to compare the continuous variables between the SAP and non-SAP groups. The Chi-square test or Fisher's exact test was used to analyze categorical variables, which were shown as frequencies (percentage). The sample mean approach was conducted to replace missing values. To identify the relationship between SHR and SAP, binary logistic regression analysis was applied with the confounding variables being controlled (including atrial fibrillation, NIHSS score, swallowing function, creatinine, and leukocyte count). SHR was stratified into quartiles ($\leq$0.84, 0.85–0.95, 0.96–1.14,

TABLE 1 | Comparison of characteristics between non-SAP and SAP groups in the total patients.

Variables	Total (n = 2,039)	non-SAP (n = 1,506)	SAP (n = 533)	$\chi 2/t/U$	P
Age, Mean ± SD	70.27 ± 11.60	70.18 ± 11.44	70.51 ± 12.08	−0.559	0.576
Gender (male), *n* (%)	1,272 (62.38)	926 (61.49)	346 (64.92)	1.828	0.176
BMI, Mean ± SD	23.85 ± 2.52	23.80 ± 2.53	23.99 ± 2.50	−1.519	0.129
Hypertension, *n* (%)	1,446 (70.92)	1,082 (71.85)	364 (68.29)	2.241	0.134
Heart disease, *n* (%)	280 (13.73)	195 (12.95)	85 (15.95)	2.742	0.098
Diabetes, *n* (%)	612 (30.01)	444 (29.48)	168 (31.52)	0.684	0.408
Kidney disease, *n* (%)	48 (2.35)	33 (2.19)	15 (2.81)	0.421	0.516
Tuberculosis, *n* (%)	21 (1.03)	18 (1.20)	3 (0.56)	0.986	0.321
Liver disease, *n* (%)	38 (1.86)	29 (1.93)	9 (1.69)	0.026	0.872
Atrial fibrillation, *n* (%)	369 (18.10)	225 (14.94)	144 (27.02)	37.926	<0.001***
Smoking, *n* (%)	699 (34.28)	511 (33.93)	188 (35.27)	0.258	0.612
Drinking, *n* (%)	617 (30.26)	452 (30.01)	165 (30.96)	0.124	0.724
SBP (mmHg), Mean ± SD	157.17 ± 23.93	157.21 ± 23.94	157.03 ± 23.93	0.152	0.879
DBP (mmHg), Mean ± SD	86.78 ± 22.67	86.75 ± 24.77	86.87 ± 15.27	−0.134	0.893
Stroke type (Hemorrhage), *n* (%)	720 (35.31)	519 (34.46)	201 (37.71)	1.68	0.195
Swallowing function, *n* (%)				379.432	<0.001***
General diet	462 (22.66)	419 (27.82)	43 (8.07)		
Semi-liquid diet	873 (42.82)	745 (49.47)	128 (24.02)		
Paste meal	87 (4.27)	59 (3.92)	28 (5.25)		
Nasal feeding	617 (30.26)	283 (18.79)	334 (62.66)		
NIHSS, Median (IQR)	5.00 (2.00, 10.00)	4.00 (2.00, 8.00)	9.00 (5.00, 15.00)	230,768	<0.001***
Triglyceride, Mean ± SD	1.56 ± 1.01	1.57 ± 0.93	1.51 ± 1.19	1.153	0.249
LDL_C, Mean ± SD	2.81 ± 0.93	2.82 ± 0.92	2.78 ± 0.95	0.751	0.453
Leukocyte count (10^9/L), Mean ± SD	7.98 ± 2.70	7.54 ± 2.36	9.23 ± 3.18	−11.176	<0.001***
Creatinine (μmol/L), Mean ± SD	77.31 ± 37.89	75.56 ± 34.41	82.23 ± 46.00	−3.058	0.002**
Glucose levels (mmol/L), Mean ± SD	6.57 ± 2.74	6.25 ± 2.46	7.48 ± 3.24	−8.006	<0.001***
HbA1c (%), Mean ± SD	6.32 ± 1.35	6.31 ± 1.34	6.35 ± 1.40	−0.585	0.558
Glucose/HbA1c ratio, Mean ± SD	1.03 ± 0.32	0.98 ± 0.27	1.17 ± 0.40	−10.27	<0.001***

***P < 0.01; ***P < 0.001. SAP, stroke-associated pneumonia; BMI, body mass index; NIHSS, National Institutes of Health Stroke Scale; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation; IQR, interquartile range; Fisher, Fisher exact test.*

≥1.15) in the regression model. Odds ratios (OR) with the corresponding 95% confidence intervals (CIs) were adopted to show the association between the risk factors and SAP. To further explore how diabetes modulated the relationship between clinical presentations and SAP, we conducted binary logistic regression on diabetic and non-diabetic patients. A forest plot was drawn to show the results of the logistic regression analysis.

The significance level was set at $P < 0.05$ (two-tailed). All statistical analyses were performed using SPSS version 21.0 and R (version 4.0.3).

RESULTS

Differences in Clinical Presentations Between SAP and Non-SAP Patients

Differences in clinical characteristics between SAP and non-SAP patients were listed in **Table 1**. Among the total patients, 533 (26.14%) had SAP. These two groups demonstrated no statistical differences in age, gender, BMI, smoking, drinking, hypertension, heart disease, diabetes, kidney disease, tuberculosis, and liver disease (all $P > 0.05$). SHR, leukocyte count, glucose, and creatinine levels were higher in the SAP group than in the non-SAP group (all $P < 0.01$). Significant differences were also found between the groups in the percentage of atrial fibrillation, NIHSS scores, and swallowing function (all $P < 0.001$). Besides, there were no differences between the included and excluded patients in SHR and glucose levels, except for some secondary variables (see **Table 2** for details).

Impact of Diabetes on the Relationship Between Clinical Presentations and SAP

Table 3 showed the subgroup analysis of patients stratified by diabetic status. Both the diabetic and non-diabetic patients showed significant differences (SAP vs. non-SAP) in the atrial fibrillation history, NIHSS score, swallowing function, leukocyte count, glucose levels, and SHR (all $P < 0.001$). Additional differences (SAP vs. non-SAP) were found in the non-diabetic patients, including hypertension history, HbA1c, and creatinine (all $P < 0.05$).

TABLE 2 | Comparison of characteristics between included and excluded patients.

Variables	Total ($n = 2,616$)	Excluded ($n = 577$)	Included ($n = 2,039$)	Statistic	p
Age, Mean ± SD	69.59 ± 11.75	67.22 ± 11.95	70.27 ± 11.60	−5.446	< 0.001***
Gender (male), *n* (%)	1,588 (60.7)	316 (54.77)	1,272 (62.38)	10.623	0.001**
BMI, Mean ± SD	25.28 ± 18.8	30.34 ± 39.35	23.85 ± 2.52	3.958	< 0.001***
Hypertension, *n* (%)	1,880 (71.87)	434 (75.22)	1,446 (70.92)	3.902	0.048*
Heart disease, *n* (%)	376 (14.37)	96 (16.64)	280 (13.73)	2.853	0.091
Diabetes, *n* (%)	770 (29.43)	158 (27.38)	612 (30.01)	1.376	0.241
Kidney disease, *n* (%)	64 (2.45)	16 (2.77)	48 (2.35)	0.178	0.673
Tuberculosis, *n* (%)	27 (1.03)	6 (1.04)	21 (1.03)	0	1
Liver disease, *n* (%)	50 (1.91)	12 (2.08)	38 (1.86)	0.026	0.871
Atrial fibrillation, *n* (%)	451 (17.24)	82 (14.21)	369 (18.10)	4.491	0.034*
Smoking, *n* (%)	871 (33.3)	172 (29.81)	699 (34.28)	3.851	0.05
Drinking, *n* (%)	775 (29.63)	158 (27.38)	617 (30.26)	1.65	0.199
SBP (mmHg), Mean ± SD	157.23 ± 24.55	157.44 ± 26.63	157.17 ± 23.93	0.225	0.822
DBP (mmHg), Mean ± SD	86.67 ± 21.15	86.28 ± 14.54	86.78 ± 22.67	−0.639	0.523
Stroke type (Hemorrhage), *n* (%)	863 (32.99)	143 (28.77)	720 (35.31)	7.61	0.006**
Swallowing function, *n* (%)				23.732	< 0.001***
General diet	647 (24.73)	185 (32.06)	462 (22.66)		
Semi-liquid diet	1,097 (41.93)	224 (38.82)	873 (42.82)		
Paste meal	115 (4.4)	28 (4.85)	87 (4.27)		
Nasal feeding	757 (28.94)	140 (24.26)	617 (30.26)		
NIHSS, Median (IQR)	5 (2, 9)	2.00 (1.00, 6.00)	5.00 (2.00, 10.00)	402,309.5	< 0.001***
Triglyceride, Mean ± SD	1.59 ± 1.02	1.70 ± 1.06	1.56 ± 1.01	2.913	0.004**
LDL_C, Mean ± SD	2.8 ± 0.92	2.75 ± 0.91	2.81 ± 0.93	−1.331	0.183
Leukocyte count (10^9/L), Mean ± SD	8.1 ± 2.85	8.49 ± 3.30	7.98 ± 2.70	3.411	< 0.001***
Creatinine (μmol/L), Mean ± SD	78.31 ± 46.67	81.85 ± 69.22	77.31 ± 37.89	1.515	0.13
Glucose levels (mmol/L), Mean ± SD	6.57 ± 2.69	6.57 ± 2.54	6.57 ± 2.74	−0.026	0.98
HbA1c (%), Mean ± SD	6.34 ± 1.33	6.4 ± 1.24	6.32 ± 1.35	1.386	0.166
Glucose/HbA1c ratio, Mean ± SD	1.03 ± 0.31	1.03 ± 0.30	1.03 ± 0.32	0.402	0.688

**P < 0.05; **P < 0.01; ***P < 0.001. SAP, stroke-associated pneumonia; BMI, body mass index; NIHSS, National Institutes of Health Stroke Scale; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation; IQR, interquartile range; Fisher, Fisher exact test.*

Associations Between SHR and SAP

The forest plot in **Figure 1** showed that higher SHR levels (SHR $\geq$ 1.15) were associated with a higher risk of SAP both in the total patients ($P = 0.01$) and those without diabetes ($P < 0.001$), with the first quantile (SHR $\leq$ 0.84) being the reference. For patients without diabetes, the third quantile (SHR = 0.96–1.14) and the highest quantile (SHR $\geq$ 1.15) were both associated with a higher risk of SAP than the first quantile (both $P < 0.05$). However, similar results were not found in patients with diabetes (all $P > 0.05$).

We also found that the secondary variables including NIHSS score, swallowing function, leukocyte count, and creatinine were all risk factors for SAP in the total patients (all $P < 0.05$), which was shown in the forest plot. Similar results were also observed in patients without diabetes (all $P < 0.05$, data not shown). In patients with diabetes, only NIHSS score and swallowing function were risk factors for SAP (both $P < 0.05$, data not shown).

DISCUSSION

To our knowledge, the current study is the first study describing the relationship between SHR and SAP. There were two main findings of this study. Firstly, we found that higher SHR levels were significantly associated with an increased risk of SAP in patients with stroke. Secondly, this effect mainly existed in patients without diabetes.

Prior studies have reported the worse outcomes of stroke patients with comorbid stress hyperglycemia (6, 23–25). However, there was a lack of consensus on whether stress hyperglycemia deteriorated the severity of SAP. Previous studies showed that hyperglycemia was associated with community-acquired pneumonia (31, 32). In this study, we found that high SHR levels (SHR $\geq$ 1.15) were also significantly associated with SAP. The mechanism underlying the SHR and SAP was still unknown. As is known to all, hyperglycemia in the context of acute diseases is an adaptive response, which provides energy for the central nervous system, immune system, and various organs and increases the survival opportunity of the host to a certain extent (33). However, stress hyperglycemia also has some harmful effects. Specifically, glucose was identified as a pro-inflammatory mediator in animal and human studies (34). Meanwhile, hyperglycemia can promote the production of inflammatory cytokines, inflammatory processes, and insulin resistance (34). In addition, it can decrease vascular endothelial nitric oxide and promote vasoconstriction, and in turn lead to abnormal organ perfusion (34, 35). Moreover, it also disrupts the immune system, directly inhibiting the function of

TABLE 3 | Comparison of characteristics between non-SAP and SAP groups stratified by the diabetic status.

Variables	Non-diabetic group		*P*	Diabetic group		*P*
	non-SAP (*n* = 1,062)	SAP (*n* = 365)		non-SAP (*n* = 444)	SAP (*n* = 168)	
Age, Mean ± SD	69.84 ± 11.75	70.25 ± 12.80	0.592	70.99 ± 10.61	71.10 ± 10.33	0.912
Gender (male), *n* (%)	674 (63.47)	240 (65.75)	0.47	252 (56.76)	106 (63.10)	0.184
BMI, Mean ± SD	23.64 ± 2.53	23.81 ± 2.57	0.285	24.19 ± 2.50	24.40 ± 2.30	0.319
Hypertension, *n* (%)	723 (68.08)	227 (62.19)	0.046*	359 (80.86)	137 (81.55)	0.937
Heart disease, *n* (%)	129 (12.15)	56 (15.34)	0.14	66 (14.86)	29 (17.26)	0.545
Kidney disease, *n* (%)	16 (1.51)	9 (2.47)	0.33	17 (3.83)	6 (3.57)	1
Tuberculosis, *n* (%)	13 (1.22)	3 (0.82)	0.774	5 (1.13)	0 (0.00)	0.33
Liver disease, *n* (%)	22 (2.07)	7 (1.92)	1	7 (1.58)	2 (1.19)	1
Atrial fibrillation, *n* (%)	166 (15.63)	99 (27.12)	<0.001***	59 (13.29)	45 (26.79)	<0.001***
Smoking, *n* (%)	374 (35.22)	135 (36.99)	0.585	137 (30.86)	53 (31.55)	0.946
Drinking, *n* (%)	336 (31.64)	120 (32.88)	0.709	116 (26.13)	45 (26.79)	0.95
SBP (mmHg), Mean ± SD	156.41 ± 24.22	156.64 ± 24.29	0.872	159.14 ± 23.18	157.87 ± 23.18	0.545
DBP (mmHg), Mean ± SD	87.39 ± 28.16	87.33 ± 15.68	0.961	85.20 ± 13.45	85.86 ± 14.34	0.606
Stroke type (Hemorrhage), *n* (%)	410 (38.61)	157 (43.01)	0.155	109 (24.55)	44 (26.19)	0.754
Swallowing function, *n* (%)			<0.001***			<0.001***
General diet	292 (27.50)	22 (6.03)		127 (28.60)	21 (12.50)	
Semi-liquid diet	539 (50.75)	92 (25.21)		206 (46.40)	36 (21.43)	
Paste meal	42 (3.95)	23 (6.30)		17 (3.83)	5 (2.98)	
Nasal feeding	189 (17.80)	228 (62.47)		94 (21.17)	106 (63.10)	
NIHSS, Median (IQR)	4.00 (2.00, 8.00)	10.00 (5.00, 16.00)	<0.001***	4.00 (2.00, 8.00)	9.00 (4.00, 13.00)	<0.001***
Triglyceride, Mean ± SD	1.47 ± 0.85	1.36 ± 0.97	0.06	1.83 ± 1.05	1.83 ± 1.53	0.987
LDL_C, Mean ± SD	2.86 ± 0.90	2.82 ± 0.93	0.484	2.73 ± 0.95	2.71 ± 1.00	0.822
Leukocyte count (10^9/L), Mean ± SD	7.40 ± 2.24	9.27 ± 3.27	<0.001***	7.88 ± 2.60	9.12 ± 2.97	<0.001***
Creatinine (μmol/L), Mean ± SD	75.05 ± 32.43	83.06 ± 49.06	0.004**	76.80 ± 38.76	80.44 ± 38.61	0.3
Glucose levels (mmol/L), Mean ± SD	5.43 ± 1.34	6.33 ± 1.90	<0.001***	8.22 ± 3.28	9.99 ± 4.03	<0.001***
HbA1c (%), Mean ± SD	5.69 ± 0.38	5.64 ± 0.39	0.019*	7.78 ± 1.62	7.89 ± 1.56	0.427
Glucose/HbA1c ratio, Mean ± SD	0.95 ± 0.23	1.12 ± 0.33	<0.001***	1.05 ± 0.33	1.28 ± 0.50	<0.001***

**P < 0.05; **P < 0.01; ***P < 0.001. SAP, stroke-associated pneumonia; BMI, body mass index; NIHSS, National Institutes of Health Stroke Scale; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation; IQR, interquartile range; Fisher, Fisher exact test.*

immunoglobulin, T lymphocytes, and complement, and further increasing the risk of infection (34, 35). Hyperglycemia was also shown to be associated with lower bacterial clearance and higher infection-related mortality in diabetic animal models (36–38). In addition, stroke-induced immunosuppression can be observed in stroke patients, which is a systemic anti-inflammatory response (39). Studies had also found that SAP was closely related to the immunosuppression syndrome caused by stroke (40). Immunosuppression was associated with an excess of glucose, and as mentioned earlier, a hyperglycemic state increased the production of reactive oxygen species by immune cells, leading to oxidative stress and the promotion of proinflammatory cytokine cascades (34). This excessive inflammatory response might deplete the immune system, and at the same time, hyperglycemia in turn could suppress immunoglobulins, T lymphocytes, and complement (34, 40). These factors may ultimately lead to the suppression of systemic immune function, thereby predisposing patients to stroke-induced immunodepression syndrome and stroke-associated infections (40).

Furthermore, we found that the correlation between SHR and SAP was only found in the total and non-diabetic patients, but not diabetic patients. Similar results were also found in previous studies related to brain injury and stroke (24, 41). The mechanism of this issue remains obscure. One possible reason was that acute hyperglycemia could promote oxidative stress, whereas chronic hyperglycemia could induce antioxidant defenses in tissues and cells (42). Therefore, diabetes may increase antioxidant defenses, which can protect tissues from oxidative stress caused by acute hyperglycemia, and thus attenuating inflammation resulting from oxidative stress (43, 44). Future research is needed to explore the underlying mechanism of this phenomenon, which might help to further reveal the pathophysiological mechanism of diabetes development.

Moreover, we found that the secondary variables, including NIHSS score, swallowing function, and leukocyte count, were all risk factors for SAP. Growing evidence had shown that higher NIHSS scores, dysphagia, as well as elevated leukocyte count, could contribute to an increased risk of SAP (45–49). Our study further demonstrated these points.

This study has several limitations. First, this study was limited by the retrospective nature of the analysis, which relied heavily on discharge medical diagnosis coding. Second, this study was a

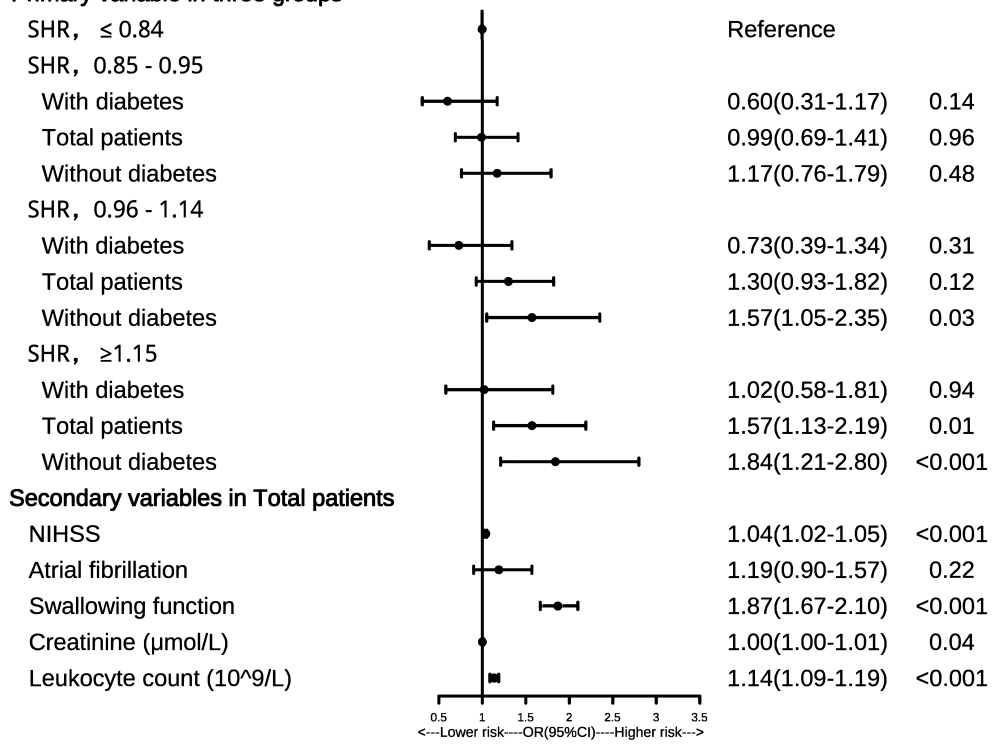

FIGURE 1 | Forest plot of odds ratios for SAP. NIHSS, National Institutes of Health Stroke Scale; SAP, stroke-associated pneumonia; OR, odds ratio; 95%CI, 95% confidence interval.

cross-sectional study and could not be done to explore causality, and patients were recruited over a long time range from 2013 to 2020, which may lead to a timely bias because the characteristics of these patients may differ in different years. Third, differences were found in some variables such as age and gender between the included and excluded patients, which may lead to selection bias to some extent. Forth, blood glucose levels are prone to fluctuation, which may have an impact on the results. Fifth, only Chinese stroke patients were included in the current study. Therefore, the findings here may not be generalized to other populations.

CONCLUSION

In conclusion, SHR was significantly associated with the risk of SAP in patients without diabetes. Adequate attention should be paid to the patients with high SHR levels at admission, especially those without diabetes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University. Written informed consent was not required for the current study in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

WR and QH: conceived and designed the study. WR, ZW, JW, XJ, and JT: made substantial contributions to data analysis and interpretation. JT, ZH, and FL: drafted the manuscript. WR, SY, QH, JT, ZH, and MW: reviewed and gave final approval of the version to be published. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Associations of Serum Albumin With Disability in Activities of Daily Living, Mobility and Objective Physical Functioning Regardless of Vitamin D: Cross-Sectional Findings From the Chinese Longitudinal Healthy Longevity Survey

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Objective: To examine the associations of serum albumin, a nutrition indicator, with disability in activities of daily living (ADL), mobility, and objective physical functioning among Chinese older adults.

Materials and Methods: Cross-sectional data of 2233 older adults (≥65 years) who participated in the 2011/2012 main survey of the Chinese Longitudinal Healthy Longevity Survey (CLHLS) and the 2012 biomarker sub-study was used. Serum albumin was measured by immunoturbidimetric assay. Physical functioning included subjectively (ADL and mobility) and objectively measured disability (standing up from a chair, picking up a book from the floor, and turning around 360°). Multivariable logistic regression models were performed.

Results: After adjusting for age and sex, compared with participants in the lowest quartile group of serum albumin, those in the highest quartile group had 45% lower odds of disability in ADL (odds ratio [OR]: 0.55; 95% confidence interval [CI]: 0.38, 0.80); 48% lower odds of disability in mobility (OR: 0.52; 95% CI: 0.38, 0.71); 46% lower odds of disability in standing up from a chair (OR: 0.54; 95% CI: 0.34, 0.85); and 37% lower odds of disability in picking up a book from the floor (OR: 0.63; 95% CI: 0.40, 0.97). We did not observe a statistically significant interaction effect between serum albumin and vitamin D on disability in physical functioning.

Conclusion: Serum albumin level was associated with physical functioning among Chinese older adults, regardless of vitamin D level. The findings indicate that appropriate management of poor nutritional status, in particular low serum albumin levels, may contribute to maintaining physical functioning in older adults.

Keywords: serum albumin, activities of daily living, mobility, objective physical functioning, disability, older adult

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INTRODUCTION

By 2050, China will have 400 million adults aged 65+ years (1). Among this older population, the prevalence of disability increases rapidly with age, posing huge burdens to the family and healthcare systems (2). Having difficulty or dependency in performing activities essential for self-care and independent living in daily life among older adults, which is termed as physical disability (3, 4). Physical disability may include basic activities of daily living (ADL) and mobility, which emphasizes different aspects of functioning. The causes of physical disability with aging are likely to be multifactorial, making disability management to be complicated. Biomarkers, usually measured in blood, urine, or other biospecimens, serve as important indicators of physiological states or conditions. Identifying biomarkers of physical disability could provide not only clues for early screening and preventive interventions but also insights into the underlying mechanisms (5–7).

Albumin, the most abundant protein in the blood, is a significant indicator of malnutrition. Recently, lower serum albumin level has been linked to a decline in physical functioning among older adults in various studies (8–13). However, these studies were limited by a lack of comprehensive assessment of physical functioning, e.g., they did not combine objective and subjective measures of physical functioning (8, 10, 12, 13), or targeting populations without representativeness (9, 11, 13). As aging-related physiological changes are linked to the function of the whole body, a relatively multi-dimensional approach to physical functioning is crucial. In addition, recent studies have revealed that a low level of vitamin D is also significantly associated with the decline in physical functioning among older adults (14–17). Malnutrition and vitamin D deficiency are very common in older adults (18, 19), and are correlated as well. As a major secondary carrier of vitamin D (20), changes in serum albumin levels are likely to cause changes in serum vitamin D levels (21). Thus, it is likely that serum albumin and vitamin D synergistically play a role in physical functioning, which, however, has been less investigated in previous studies (22).

In the context of rapid population aging in China, this study aimed to examine the associations of serum albumin with disability in ADL, mobility, and objective physical functioning among Chinese older adults. Furthermore, we aimed to evaluate the interaction between serum albumin and vitamin D on physical functioning in the same population. We used data from the 2011/2012 main survey and the 2012 biomarker sub-study of the Chinese Longitudinal Healthy Longevity Survey (CLHLS), a national survey in China.

METHODS

Study Population

CLHLS was established in 1998 as a national survey and was designed to investigate determinants of healthy longevity in Chinese older adults. Details of the methodology and the study design have been previously reported (23). In short, the CLHLS was conducted in a random sampling of 50% of cities and countries in 23 provinces across China, accounting for 85% of the total population. The CLHLS tried to interview all centenarians who agreed to participate in the study. To ensure representativeness, the CLHLS conducted a target random sampling design and performed identical interviews with approximately equal numbers of young-older adults (aged 65–79 years), octogenarians, and nonagenarians who lived in the same area as the centenarians. To fill the gap that some objective medical aspects such as biomarkers are not available in CLHLS, the researchers have added in-depth surveys in seven longevity regions where the number of centenarians is large as part of the 5th wave of CLHLS in 2009, and eight longevity regions (plus a new one based on the survey in 2009) as part of the 6th wave of CLHLS in 2012. The in-depth surveys included more comprehensive health examinations by medical personnel and collected urine and blood samples during home interviews. This in-depth surveys were called the biomarker sub-study of CLHLS, or Healthy Aging and Biomarkers Cohort Study (HABCS) (2, 24). The Duke University and the Research Ethics Committees of Peking University granted approval for the Protection of Human Subjects for the CLHLS and all participants provided informed consent.

In this study, we included participants from the 2012 biomarker sub-study and the 2011/2012 main survey of CLHLS ($N = 2439$). We excluded participants aged below 65 years ($N = 85$) or with missing data on serum albumin levels ($N = 121$), and had a sample of 2233 participants, with no missing data on covariates. Further, due to the varying numbers of missing data on each outcome, different analytic samples for each outcome were assembled (**Figure 1**).

Assessment of Serum Albumin Levels and Serum Vitamin D Levels

About 5 ml fasting venous blood samples were collected from participants by trained medical staff with heparin anticoagulant vacuum tubes. All blood samples were centrifuged at 2,500 rpm at 20°C for 10 min. The separated specimens were frozen at −20°C and transferred to Central Laboratory in Beijing, where they were stored at −80°C until biochemical analysis. Serum albumin level and vitamin D levels were measured by immunoturbidimetric assay and enzyme-linked immunosorbent assay (Immunodiagnostic Systems Limited, Bolton, UK), respectively. Further details are provided elsewhere (25, 26).

Serum albumin levels were divided into quartiles and the cutoff values were 36.8, 40.1, and 43.3 g/L. In addition, we divided serum albumin and vitamin D into a dichotomous variable. The cutoff value of serum albumin was 35 g/L, recommended to define hypoalbuminemia in literature (27). Similarly, the cutoff value of serum vitamin D was 50 nmol/L, recommended to define vitamin D deficiency (28).

Assessment of ADL Disability and Mobility Disability

In the CLHLS survey, six items of daily self-care ability were collected from each participant based on the Katz index: dressing, bathing, transferring, toileting, continence, and eating (29). Each

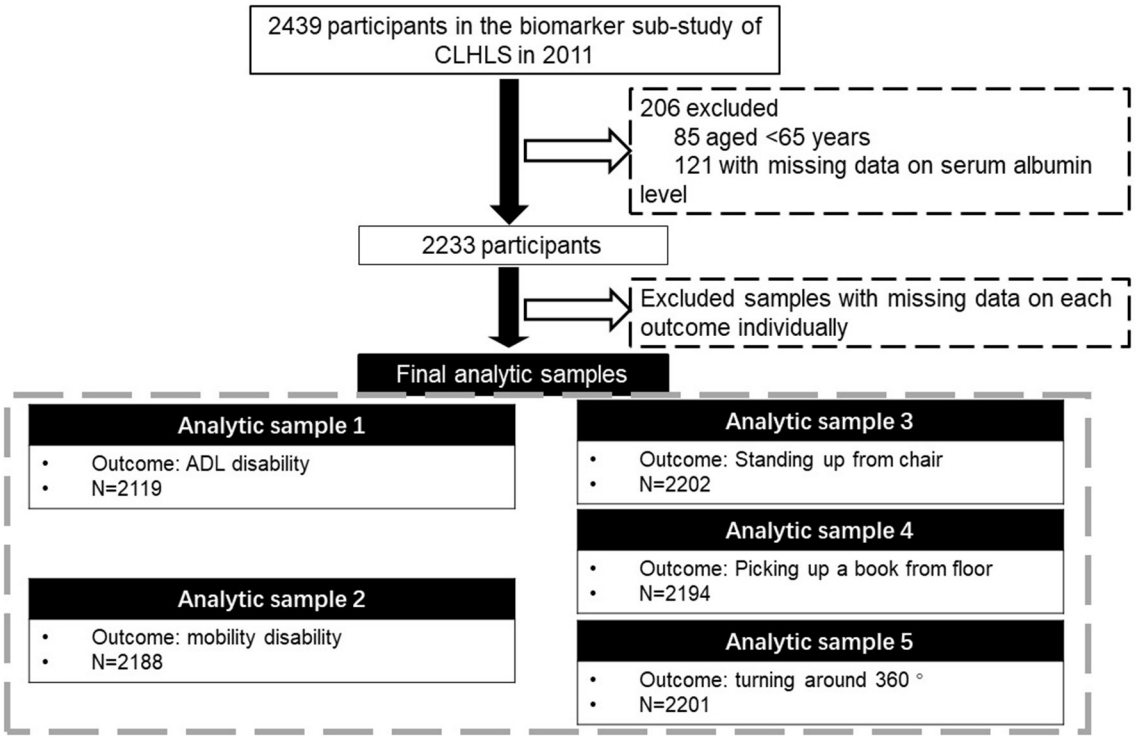

FIGURE 1 | Flowchart of assembling study participants. CLHLS, Chinese Longitudinal Healthy Longevity Survey; ADL, activities of daily living.

item was asked with three answers, i.e., "complete independence," "partially dependence," and "complete dependence" (30). As done in previous studies, ADL disability was defined as present if participants needed any assistance (i.e., "partially dependence," and "complete dependence") in performing at least one of the six self-care activities (31).

The status of mobility function was assessed by asking participants the question: "Can you walk continuously for 1 kilometer at a time by yourself?" As done in previous studies, mobility disability was defined as present if participants reflected any difficulty in this task (32).

Assessment of Disability in Objective Physical Functioning

Objective physical functioning was measured through three tasks (protected by trained technicians) in every participant's home. These tasks included picking up a book from the floor, standing up from a chair, and turning around 360°. Thus, three dichotomous indicators were used to represent the presence or absence of disability in objective physical functioning. If participants were not able to perform one task independently, they were defined as having a disability in this task (33).

Covariates

According to previous studies, we found that some population characteristics (e.g., age, sex, ethnicity, and so on) (34–39) and lifestyles (e.g., currently smoking, current consuming alcohol, and regular exercise) (40–43) may confound the associations between serum albumin and physical functioning. Thus, as done in the previous study based on CLHLS (44), the following covariates were included in our analysis: age, sex, residence (rural vs. other), ethnicity (Han vs. other), education (≥1 year of education vs. no education), economic independence (yes vs. no), lifetime primary occupation (white-collar occupation vs. others), and being in receipt of adequate medications (yes vs. no); co-residence with children (yes vs. no) and current marital status (married vs. others) were used to assess participants' social support and contact; lifestyle consisted of consuming alcohol (yes vs. no), currently smoking (yes vs. no), and regular exercise (yes vs. no). In addition, chronic diseases were included in our models for both serum albumin and physical functioning could be influenced by major chronic diseases. Five self-reported chronic diseases (diabetes mellitus, hypertension, heart disease, stroke/cardiovascular disease (CVD), and respiratory diseases) diagnosed by doctors were included. Furthermore, we additionally added mild cognitive impairment (MCI) (defined by Mini-Mental State Examination (MMSE) score below 24) in our analyses, for it may influence physical functioning (45). Body mass index (BMI) was also included. BMI was calculated as weight divided by height squared (kg/m^2). The height (in centimeter) and body weight (in kilogram) of participants were measured by interviewers during the survey (46).

Statistical Analyses

Basic characteristics of study participants were summarized in the total sample and by serum albumin quantile groups.

Continuous variables were summarized by mean ± standard deviation (SD) and categorical variables were summarized by numbers and percentage. The analysis of variance (ANOVA) or χ^2 test was used to compare basic characteristics among four quartile groups of serum albumin levels.

We used multivariable logistic regression models to examine the associations of serum albumin levels with ADL disability, mobility disability, and disability in objective physical functioning. The odds ratios (OR) and corresponding 95% confidence intervals (CI) were documented. The lowest quartile group of serum albumin levels was considered as the reference group and two models were considered. In model 1, we adjusted for age and sex. In model 2, we additionally adjusted for ethnicity, current marital status, residence, education, BMI, lifetime primary occupation, economic independence, being in receipt of adequate medication, co-residence with adult children, currently consuming alcohol, currently smoking, and regular exercise. In model 3, to determine the effect of serum vitamin D levels on associations of serum albumin with physical disability, we further adjusted for serum vitamin D levels based on model 2.

Next, we examined the association of serum albumin and disability in physical functioning by serum vitamin D status. The significance of the multiplication interaction between serum albumin and serum vitamin D levels was estimated by adding cross-product terms in models. We evaluated three indices and their 95% CI to determine if there was an additive interaction between serum albumin and vitamin D on physical functioning. The three indices included the interaction contrast ratio (ICR), attributable proportion due to interaction (AP), and the synergy index (S). If there was no statistically significant additive interaction, the 95% CI of ICR and AP included 0 and the 95% CI of S included 1 (47). In addition, restricted cubic splines (RCS) were performed to determine the Non-linear associations of serum albumin levels with ADL disability, mobility disability, and disability in objective physical functioning with four knots at the 5th, 25th, 75th, 95th of serum albumin levels distribution based on model 2.

To examine whether the associations of serum albumin with outcomes differed by age, sex, and the status of comorbidity, we repeated the main analyses by age (aged 65–79 years or ≥80 years), sex, and comorbidity with the same covariates adjusted in model 2. In addition, we conducted several additional analyses to assess the robustness of our results. First, in model 4, we repeated all analyses additionally adjusted for five chronic diseases (diabetes mellitus, hypertension, heart disease, stroke/cerebrovascular disease, and respiratory diseases) based on model 2. Second, in model 5, we repeated the main analysis further adjusting for MCI based on model 2. All analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). A $P < 0.05$ was considered to be statistically significant.

RESULTS

Basic Characteristics of Study Participants

The mean age (± SD) of study participants was 86.3 ± 12.2 years, and 55.1% ($N = 1231$) were females. Compared with participants with lower serum albumin levels (quartile 1), participants with higher serum albumin levels tended to be older, and more likely to be female, Han, residents of rural areas, to have a formal education, and to have more social contact and support (i.e., married and co-residence with children). Meanwhile, they were more likely to have a lower proportion of regular exercise, to consume alcohol, to have adequate medication, economic independence, and chronic diseases (hypertension, diabetes mellitus, and heart disease). Participants with higher serum albumin levels tended to have a higher BMI and higher serum vitamin D levels, compared with participants with lower serum albumin levels. Details about the characteristics of study participants are shown in **Table 1**.

Associations of Serum Albumin Level With Disability

The associations of serum albumin level with ADL disability, mobility disability, and disability in objective physical functioning are shown in **Table 2**. After adjusting for age and sex, we observed dose-response associations of serum albumin level with ADL disability, mobility disability, and disability in two objective tasks (i.e., standing up from a chair and picking up a book from the floor) (all P for trend <0.05). Compared with participants in the lowest quartile group of serum albumin level, those in the highest quartile group had 45% lower odds of ADL disability (OR: 0.55; 95% CI: 0.38, 0.80); 48% lower odds of mobility disability (OR: 0.52; 95% CI: 0.38, 0.71); 46% lower odds of disability in standing up from a chair (OR: 0.54; 95% CI: 0.34, 0.85); and 37% lower odds of disability in picking up a book from the floor (OR: 0.63; 95% CI: 0.40, 0.97). The findings were maintained after controlling for more covariates. For instance, the odds of ADL disability in the fully adjusted model was 0.50 (95% CI: 0.34, 0.74) for the highest group of serum albumin levels. Additionally, we observed a marginally significant association of serum albumin level with disability in turning around 360°. Compared with participants in the lowest quartile group of serum albumin levels, those in the highest quartile group had 27% lower odds of disability in turning around 360° (OR: 0.73; 95% CI: 0.51, 1.03). In model 3, after further adjusting for serum vitamin D, the statistically significant association of serum albumin with ADL disability, mobility disability, and disability in standing up from a chair were maintained. Although the associations of serum albumin with disability in picking up a book from the floor and turning around 360° did not achieve statistical significance, the potential protective effects of serum albumin on physical functioning were maintained. For instance, compared with participants in the lowest quartile group of serum albumin level, those in the highest quartile group had 34% lower odds of disability in picking up a book from the floor (OR: 0.66; 95% CI: 0.41, 1.06).

Additional Analyses

Figure 2 shows the associations of serum albumin levels and disability in physical functioning by serum vitamin D level. We did not observe the statistically significant multiplication (data not shown) or additive interaction effects (**Supplementary Table 1**) between serum albumin and vitamin D level on disability in physical functioning (all P for interaction >

TABLE 1 | Basic characteristics of study participants by serum albumin quartiles.

Characteristics	Overall (*N* = 2,233)	Serum albumin level (g/L)				*P*-value
		<36.8 g/L[a] (*N* = 554)	36.8–40.1 g/L (*N* = 571)	40.1–43.3 g/L (*N* = 544)	>43.3 g/L (*N* = 564)	
Age, mean ± SD, years,	86.3 ± 12.2	91.9 ± 10.8	88.0 ± 12.2	84.0 ± 12.1	81.3 ± 11.2	<0.001
Sex, female (%)	1,231 (55.1)	345 (62.3)	319 (55.9)	282 (51.8)	285 (50.5)	<0.001
Ethnicity, Han (%)	2,132 (95.5)	514 (92.8)	548 (96.0)	521 (95.8)	549 (97.3)	0.003
Residence, rural (%)	1,844 (82.6)	481 (86.8)	492 (86.2)	446 (82.0)	425 (75.4)	<0.001
≥1 years of education (%)	812 (36.4)	157 (28.3)	179 (31.3)	201 (36.9)	275 (48.8)	<0.001
White collar occupation (%)	46 (2.1)	9 (1.6)	7 (1.2)	12 (2.2)	18 (3.2)	0.106
Married, yes (%)	812 (36.4)	128 (23.1)	184 (32.2)	212 (39.0)	288 (51.1)	<0.001
Regular exercise, yes (%)	328 (14.7)	58 (10.5)	79 (13.8)	79 (14.5)	112 (19.9)	<0.001
Currently smoking, yes (%)	366 (16.4)	92 (16.6)	90 (15.8)	92 (16.9)	92 (16.3)	0.961
Currently consuming alcohol, yes (%)	329 (14.7)	58 (10.5)	80 (14.0)	88 (16.2)	103 (18.3)	0.002
BMI, mean ± SD, kg/m^2	21.7 ± 12.7	20.7 ± 8.2	21.7 ± 19.6	21.5 ± 8.3	22.9 ± 11.0	0.037
Adequate medication, yes (%)	2,116 (94.8)	522 (94.2)	534 (93.5)	510 (93.8)	550 (97.5)	0.008
Co-residence with children, yes (%)	1,746 (78.2)	428 (77.3)	438 (76.7)	424 (77.9)	456 (80.9)	0.337
Economic independence, yes (%)	496 (22.2)	73 (13.2)	110 (19.3)	154 (28.3)	159 (28.2)	<0.001
serum vitamin D, mean ± SD,	41.7 ± 19.4	36.9 ± 17.9	40.8 ± 18.2	43.4 ± 19.8	45.5 ± 20.7	<0.001
Hypertension, yes (%)	588 (26.3)	115 (20.8)	164 (28.7)	147 (27.0)	162 (28.7)	0.006
Diabetes mellitus, yes (%)	49 (2.2)	9 (1.6)	10 (1.8)	12 (2.2)	18 (3.2)	0.264
Heart disease, yes (%)	167 (7.5)	27 (4.9)	35 (6.1)	44 (8.1)	61 (10.8)	0.001
Stroke/CVD, yes (%)	178 (8.0)	39 (7.0)	54 (9.5)	50 (9.2)	35 (6.2)	0.118
Respiratory diseases, yes (%)	190 (8.5)	45 (8.1)	52 (9.1)	47 (8.6)	46 (8.2)	0.925
MCI, yes	825 (36.9)	316 (57.0)	236 (41.3)	148 (27.2)	125 (22.2)	<0.001

SD, standard deviation; BMI, body mass index; CVD, cardiovascular disease; MCI, mild cognitive impairment.
Values are given as No. (%) unless otherwise stated.
[a]*The cutoff values of serum albumin levels were 36.8, 40.1, and 43.4 g/L.*

0.05) in our analyses, although participants with a combination of higher serum albumin levels and serum vitamin D levels had the lowest odds of disability in physical functioning. For instance, relative to participants with a combination of lower serum albumin levels and serum vitamin D levels, those with a combination of higher serum albumin levels and serum vitamin D levels had 85% lower odds of ADL disability (OR: 0.15; 95% CI: 0.09, 0.25); 79% lower odds of disability in standing up from a chair (OR: 0.21; 95% CI: 0.12, 0.39); 84% lower odds of disability in picking up a book from the floor (OR: 0.16; 95% CI: 0.09, 0.29); and 70% lower risk of disability in turning around 360° (OR: 0.30; 95% CI: 0.19, 0.46). Similarly, the association of serum albumin level with disability in physical functioning by serum vitamin D level was maintained after further adjustment for five chronic diseases (**Supplementary Table 2**).

Supplementary Figure 1 presents the Non-linear associations of serum albumin level with ADL disability, mobility disability, and disability in objective physical functioning. We observed that with the increase of serum albumin levels, the OR of disability in physical functioning was decreasing within the serum albumin levels of roughly <52.5 g/L. For ADL disability and mobility disability, we found a linear association (*P* for nonlinear = 0.059 and 0.339, respectively) when treating serum albumin levels as continuous variables. The results of stratified analyses by age, sex, and comorbidity were shown in **Supplementary Tables 3–5**, respectively. No statistically significant interactions between age, sex, and comorbidity and serum albumin level on disability in physical functioning were found, with one exception (i.e., serum albumin and sex on disability in turning around 360°). In model 4, after further adjusting for five chronic diseases, the statistically significant associations of serum albumin levels with disability in physical functioning remained. For instance, compared with participants in the lowest quartile group of serum albumin levels, those in the highest quartile group had lower odds of disability in ADL disability, mobility disability, disability in standing up from a chair, and disability in picking up a book from the floor, with ORs of 0.51 (95% CI: 0.34, 0.76), 0.51 (95% CI: 0.37, 0.72), 0.54 (95% CI: 0.33, 0.88), 0.66 (95% CI: 0.41, 1.06), respectively. In model 5, after further adjustment for MCI based on model 2, the protective effect of higher serum albumin on physical functioning was maintained, though the associations between serum albumin levels and a few outcomes such as disability in picking up a book from the floor did not achieve the statistical significance (**Supplementary Table 6**).

DISCUSSION

In this study, we observed statistically significant associations between serum albumin level and various indicators of disability

TABLE 2 | Associations of serum albumin level with ADL disability, mobility disability, and disability in objective physical functioning.

	No. of events/No. of participants	Model 1[a]		Model 2[b]		Model 3[c]	
		OR (95% CI)	*P* for trend	OR (95% CI)	*P* for trend	OR (95% CI)	*P* for trend
ADL disability	434/2,119						
<36.8 g/L[d]	176/554	Ref.		Ref.		Ref.	
36.8–40.1 g/L	122/571	0.72 (0.53, 0.97)	0.001	0.70 (0.51, 0.96)	<0.001	0.77 (0.56, 1.07)	0.001
40.1–43.3 g/L	79/544	0.63 (0.45, 0.88)		0.60 (0.42, 0.84)		0.67 (0.47, 0.96)	
>43.3 g/L	57/564	0.55 (0.38, 0.80)		0.50 (0.34, 0.74)		0.54 (0.36, 0.80)	
Mobility disability	1,057/2,188						
<36.8 g/L	360/554	Ref.		Ref.		Ref.	
36.8–40.1 g/L	298/571	0.79 (0.59, 1.06)	<0.001	0.72 (0.53, 0.98)	<0.001	0.72 (0.53, 0.98)	<0.001
40.1–43.3 g/L	233/544	0.84 (0.62, 1.14)		0.80 (0.58, 1.11)		0.81 (0.59, 1.12)	
>43.3 g/L	166/564	0.52 (0.38, 0.71)		0.51 (0.37, 0.71)		0.51 (0.37, 0.71)	
Disability in objective physical functioning							
Standing up from a chair	213/2,202						
<36.8 g/L	97/554	Ref.		Ref.		Ref.	
36.8–40.1 g/L	47/571	0.51 (0.34, 0.74)	0.004	0.49 (0.33, 0.73)	0.003	0.53 (0.35, 0.78)	0.009
40.1–43.3 g/L	40/544	0.61 (0.41, 0.92)		0.59 (0.38, 0.89)		0.63 (0.41, 0.96)	
>43.3 g/L	29/564	0.54 (0.34, 0.85)		0.52 (0.32, 0.84)		0.54 (0.33, 0.88)	
Picking up a book from the floor	258/2,194						
<36.8 g/L	111/554	Ref.		Ref.		Ref.	
36.8–40.1 g/L	66/571	0.64 (0.45, 0.91)	0.017	0.62 (0.43, 0.89)	0.012	0.69 (0.48, 1.00)	0.057
40.1–43.3 g/L	47/544	0.67 (0.45, 0.99)		0.63 (0.42, 0.94)		0.71 (0.47, 1.08)	
>43.3 g/L	34/564	0.63 (0.40, 0.97)		0.60 (0.38, 0.95)		0.66 (0.41, 1.06)	
Turning around 360°	521/2,211						
<36.8 g/L	199/554	Ref.		Ref.		Ref.	
36.8–40.1 g/L	138/571	0.69 (0.51, 0.92)	0.053	0.65 (0.48, 0.89)	0.048	0.69 (0.51, 0.94)	0.102
40.1–43.3 g/L	101/544	0.71 (0.52, 0.98)		0.67 (0.49, 0.94)		0.72 (0.52, 1.00)	
>43.3 g/L	83/564	0.74 (0.53, 1.03)		0.73 (0.51, 1.03)		0.76 (0.53, 1.08)	

ADL, activities of daily living; OR, odds ratio; CI, confidence interval.

[a] *Model 1 adjusted for age and sex.*

[b] *Based on model 1, model 2 further adjusted for ethnicity, residence, current marital status, education, lifetime primary occupation, economic independence, being in receipt of adequate medication, co-residence with adult children, currently smoking, currently consuming alcohol, BMI, regular exercise.*

[c] *Based on model 2, model 3 further adjusted for serum vitamin D levels.*

[d] *The cutoff values of serum albumin levels were 36.8, 40.1, and 43.4 g/L.*

in physical functioning among Chinese older adults. These associations were regardless of vitamin D level since no interaction between serum albumin and vitamin D level on disability in physical functioning was observed in our study. These findings suggest that serum albumin level could be a potentially useful biomarker of disability in physical functioning among older adults.

Our findings of the associations between serum albumin level and disability in physical functioning are consistent with previous studies (8–13, 22, 48–53). Notably, one study observed that there was no association of serum albumin with the decline in functional performance in a 3 years follow-up study (54). In addition to differences in measurements of physical functioning included (chair stand, putting on and taking off a cardigan, 3-meter walk), the disparities may be attributed to the difference in characteristics of the study population. Regarding the association of a combined lower serum albumin and vitamin D level with disability in physical functioning, our results are consistent with a study performed in Japan (22). Considering the findings of previous studies that vitamin D was also associated with the decline in physical function (14, 55), we hypothesize that there would be a joint effect of a low level of serum albumin and vitamin D on the risk of disability in physical functioning. However, we did not observe a multiplication or additive interaction between serum albumin and vitamin D on physical disability, even though participants with a combination of lower serum albumin and vitamin D level had high odds of physical disability in our study. We speculate that serum albumin and vitamin D may play a different role in the process of functional decline.

The mechanisms explaining the observed association between serum albumin and disability in physical functioning are not clear. A previous study demonstrated that a lower serum albumin level was associated with poor nutritional status among older adults aged above 90 years old (56). Due to the poor nutritional status of older adults, the lack of serum proteins needed for

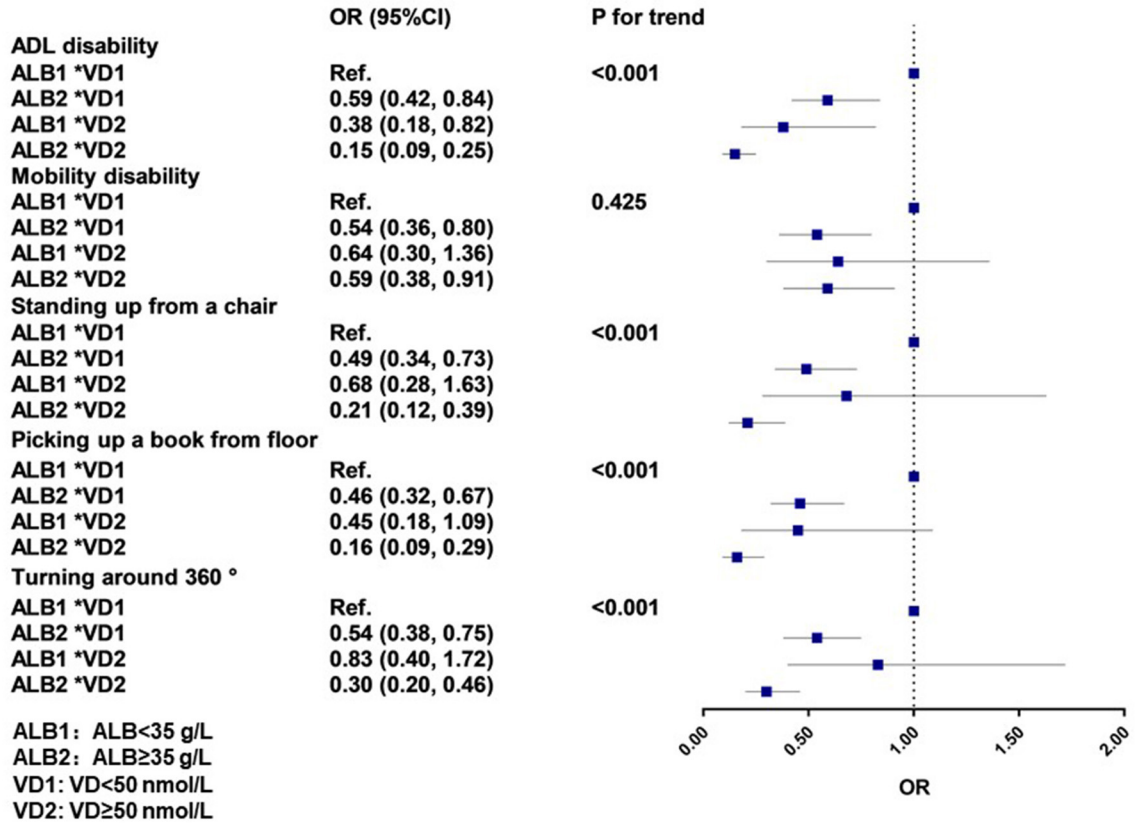

FIGURE 2 | Joint associations of serum albumin level and vitamin D with disability in physical functioning. OR, odds ratio; CI, confidence interval; ALB, albumin; VD, vitamin D; ADL, activities of daily living. The cutoff value was 35 g/L and 50 nmol/L for serum albumin and serum vitamin D level, respectively. ALB1 represents serum albumin levels of the participants were below 35 g/L (<35 g/L), ALB2 represents serum albumin levels of the participants were above 35 (≥35 g/L). VD1 represents the serum vitamin D level of the participants were below 50 (<50 nmol/L), VD2 represents the serum vitamin D level of the participants were above 50 (≥50 nmol/L). In this model, based on model 1, we further adjusted for ethnicity, residence, current marital status, education, lifetime primary occupation, economic independence, being in receipt of adequate medication, co-residence with adult children, currently smoking, currently consuming alcohol, BMI, and regular exercise.

muscle synthesis and strength maintenance might partially contribute to the decline in physical functioning. Simultaneously, a relatively low level of serum albumin *per se* reflects an unhealthy status. Previous studies demonstrated that serum albumin was associated with many diseases such as diabetes (57) and metabolic syndrome (58). Thus, we further adjusted for five main chronic diseases to control for the possible confounding effect of diseases. The results were maintained, emphasizing the potential of serum albumin as a biomarker of decline in physical functioning. In addition, researchers uncovered that serum albumin treatment reduced systemic inflammation in patients with decompensated cirrhosis (59). We speculate that a low serum albumin level reduces the ability of the body to resist inflammatory damage, a significant biological component of aging (60). Our findings indicate that appropriate management of poor nutritional status, in particular low serum albumin levels, may contribute to maintaining physical functioning in older adults. Recently, randomized controlled trials found that multimodal nutritional intervention significantly improved the physical functioning of older adults (14, 61). Although clinical trials directly targeting serum albumin have not been conducted, it is believed that such investigations would be of much value.

The main strengths of our study include the use of data from a large sample from a national survey of Chinese older adults and a relatively comprehensive assessment of physical functioning with a combination of objective and subjective measurements. In addition, the consistency of the associations between serum albumin level and these multiple indicators of physical functioning verified the robustness of our results. Nevertheless, this study has several limitations. First, an inevitable limitation is the cross-sectional design of our study, which does not allow for causal inference. Second, it is the lack of information on daily nutrition intake that may confound the findings. Third, since the level of serum albumin is dynamic, only one assessment of serum albumin level may result in some measurement errors as it may not reflect the intra-person variability and long-term average level. Forth, considering that the rural population accounts for 82.6%, our findings may not be generalizable to other cases, e.g., when having a high proportion of urban older adults. Fifth, considering that 87.1% of the participants with serum albumin levels above 35 g/L and

most of the participants in our dataset are relatively healthy, our findings may only provide instruction for relatively healthy older adults to manage nutrition status for preventing physical disability. Finally, our result may not be generalizable older adults in other countries.

In conclusion, we found that serum albumin level was associated with disability in physical functioning regardless of vitamin D level among Chinese older adults. Serum albumin level has the potential to serve as a useful biomarker of decline in physical functioning among older adults. The findings highlight the potential of preventing functional decline by managing serum albumin levels in older adults.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author Zuyun Liu, upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Duke University and the Research Ethics Committees of Peking University granted approval for the Protection of Human Subjects for the CLHLS and all participants provided informed consent. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XL: methodology, data curation, software, visualization, writing—original draft preparation, and writing—review and editing. XC: methodology, data curation, software, writing—original draft preparation, and writing—review and editing. ZY: investigation, methodology, and writing—review and editing. JZ, XS, and EH: methodology and writing—review and editing. ZL: conceptualization, methodology, data curation, software, visualization, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.809499/full#supplementary-material

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Impact of Dietary Fiber on West Nile Virus Infection

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Dietary fiber supports healthy gut bacteria and their production of short-chain fatty acids (SCFA), which promote anti-inflammatory cell development, in particular, regulatory T cells. It is thus beneficial in many diseases, including influenza infection. While disruption of the gut microbiota by antibiotic treatment aggravates West Nile Virus (WNV) disease, whether dietary fiber is beneficial is unknown. WNV is a widely-distributed neurotropic flavivirus that recruits inflammatory monocytes into the brain, causing life-threatening encephalitis. To investigate the impact of dietary fiber on WNV encephalitis, mice were fed on diets deficient or enriched with dietary fiber for two weeks prior to inoculation with WNV. To induce encephalitis, mice were inoculated intranasally with WNV and maintained on these diets. Despite increased fecal SCFA acetate and changes in gut microbiota composition, dietary fiber did not affect clinical scores, leukocyte infiltration into the brain, or survival. After the brain, highest virus loads were measured in the colon in neurons of the submucosal and myenteric plexuses. Associated with this, there was disrupted gut homeostasis, with shorter colon length and higher local inflammatory cytokine levels, which were not affected by dietary fiber. Thus, fiber supplementation is not effective in WNV encephalitis.

Keywords: dietary fiber, gut microbiota, West Nile Virus (WNV), infection, immune response, enteric neurons, cytokines

INTRODUCTION

Consumption of dietary fiber confers health benefits and correlates with decreased mortality from both infectious and non-infectious diseases (1). Dietary fiber comprises non-digestible complex carbohydrates that promote gut health through the beneficial reshaping of the gut microbiota. This occurs through the release of bacterial metabolites, particularly short-chain fatty acids (SCFA), during its fermentation in the colon (2). High-fiber feeding and SCFA reduce disease severity in models of colorectal cancer, colitis and food allergy (3, 4). The benefits of dietary fiber and SCFA extend beyond the gastrointestinal tract, influencing immune responses in the lungs in a model of allergic airway inflammation (5), in the pancreas in a model of type 1 diabetes (6) or in the brain in multiple

sclerosis (7). Dietary fiber and SCFA are also beneficial in infectious diseases with decreased mortality in a mouse model of influenza infection (8) and of *Citrobacter rodentium* infection (9).

Dietary fiber and SCFA have a broad impact on the immune system by promoting the development of anti-inflammatory regulatory T cells (10) and regulatory B cells (11), of Th1 cells (12), of memory $CD8^+$ T cells (13, 14) and by increasing B cell antibody production (15). They also affect innate immune cells by modulating the migration and activation of neutrophils (16, 17), the generation of monocytes and their differentiation into anti-inflammatory macrophages (8), the production of IL-22 by type 3 innate lymphoid cells (18) and the activity of $CD103^+$ dendritic cells (4). The mechanisms behind the immunomodulatory effects of SCFA are multifaceted. They occur *via* activation of G-protein-coupled receptors, through modulation of gene expression by inhibiting histone deacetylases, by affecting immune cell metabolic activity by fueling the tricarboxylic acid cycle, or by promoting glutaminolysis, fatty acid oxidation and gluconeogenesis (2, 19).

Antibiotic treatments disrupt both the gut microbiota and its release of SCFA (20), impairing immunity, including the IFN-γ antiviral immune response, aggravating influenza severity (21). Similarly, antibiotic treatment exacerbates disease severity in a model of West Nile Virus (WNV) infection (22), suggesting that the gut microbiota can regulate WNV, infection outcomes.

WNV is a mosquito-borne neurotropic flavivirus. Neuronal infection is associated with massive inflammatory monocyte recruitment into the central nervous system (CNS), causing life-threatening encephalitis (23–26). As dietary fiber promotes anti-inflammatory SCFA production and has been shown to be protective in a model of influenza virus infection by targeting monocytes and enhancing $CD8^+$ T cell effector function (8), we hypothesized that high-fiber feeding might also be protective in a mouse model of WNV encephalitis.

To test this hypothesis, we fed mice on diets abundant or deficient in dietary fiber, prior to infection with either 100% or 50% lethal doses (LD_{100} or LD_{50}) of WNV and investigated the impact of these diets on survival and immune profile.

MATERIALS AND METHODS

Mice and Dietary Intervention

Six-week-old female C57BL/6 mice [Animal BioResource (NSW, Australia) or Animal Resources Centre (WA, Australia)] were housed under specific pathogen-free conditions in the animal facility of the Charles Perkins Centre. Diets were purchased from Specialty Feeds (Glenn Forest, Australia) and mice were fed for 2 weeks prior to infection, either with commercially available diets deficient in dietary fiber (SF11-028), or enriched in dietary fiber (SF11-025: resistant starch gel crisp as source of fiber) as previously described (3). Experiments were performed in accordance with the animal ethics protocol 2016/976 approved by The University of Sydney Animal Ethics Committee.

WNV Infection

The original stock of WNV (lineage II Sarafend strain), acquired from The John Curtin School of Medical Research (ACT, Australia) was propagated alternately in C57BL/6 suckling mouse brains and *in vitro* in Vero cells (24). Mice anesthetized intraperitoneally with Avertin at day 0 were inoculated intranasally with 6×10^4 and 6×10^3 plaque-forming units (PFUs) of WNV to achieve 100% lethal dose (LD_{100}) and 50% lethal dose (LD_{50}) studies, respectively, as described in (27). Alternatively, mice were injected with a LD_{100} intracranially *via* the postglenoid foramen, an approach that does not penetrate the skull bones, minimizing tissue damage (28). Mice were weighed daily, and assessed for clinical symptoms, as previously described (24). Briefly, they were scored as follows: score 0: no clinical signs, 1: Weight loss < 5%, 2: Weight loss ≥ 5%, 3: Weight loss ≥ 5% with significant reduction in movement, 4: Weight loss ≥ 5% with significant reduction in movement, ruffled fur, hunched posture, and seizures and 5: Immobile, cold.

Flow Cytometry and Data Analysis

Mice were anesthetized and perfused with ice-cold PBS before collection and processing of tissues into single cell suspension as previously described (26, 29). Dead cells were excluded, based on their staining with the LIVE/DEAD™ Fixable Blue Dead Cell Stain (ThermoFisher Scientific) and anti-mouse CD16/32 (BioLegend) was used to prevent antibody non-specific binding. Cells were permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) for intracellular staining. Antibodies used for flow cytometry are listed in **Table S1**. To study cytokine expression, cells were stimulated with 50ng/ml phorbol 12-myristate 13-acetate, 500ng/ml ionomycin and 5μg/ml brefeldin A for 4 hours. Flow cytometry was run on the LSR-II analyser (Becton Dickinson, San Jose, CA, USA) using the FACSDiva software and data was analyzed with FlowJo v10.7.1. (Treestar Inc. Ashland, OR, USA) based on gating strategies presented in **Figures S2-3**.

RNA Extraction and Quantitative Real-Time PCR

Total tissue RNA was extracted using TRI Reagent (Sigma Aldrich), based on the manufacturer's protocol. cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). qPCR was conducted with the Power SYBR™ Green PCR Master Mix (ThermoFisher Scientific) on LightCycle® 480 Instrument II (Roche) with primers listed in **Table S2**.

Histology

Paraformaldehyde-fixed (4% in PBS), paraffin-embedded colon tissues were sectioned and stained with H&E and imaged using light microscopy (Zeiss Axioscope, Zeiss, Oberkochen, Germany). Colonic inflammation was scored, based on previously published guidelines (30).

Immunohistochemistry/ Immunofluorescence

For immunohistochemistry, colon tissue was fixed overnight in 2% PFA and subsequently placed in a series of solutions of progressively increasing sucrose concentration (10%, 20% and 30% sucrose in PBS), before being embedded in optimum cutting temperature compound (O.C.T.; Tissue-Tek) and frozen in hexane pre-chilled in liquid nitrogen, as previously described (26, 31). Tissue blocks were sectioned (8–9 μm), fixed in

methanol, rinsed in tris-buffered saline with 0.05% Tween 20 (TBST) and blocked with 10% FCS before being stained with primary fluorophore-conjugated antibodies targeting WNV non-structural protein 1 (NS-1) and FOX-3 in neuronal nuclei (NeuN). Tissue sections were washed twice in TBST before being counterstained with DAPI antifade (Vector Laboratories). Images were acquired on the Olympus BX51 Microscope using a DP-70 camera and Cell Sensor software.

Acetate Quantification

Fecal SCFA acetate was quantified by ^{1}H nuclear magnetic resonance spectroscopy as previously described (11). Feces were first homogenized with deuterium oxide (Sigma-Aldrich) at a concentration of 100mg/ml and centrifuged for 5 minutes at 14000 × g at 4°C. The resulting supernatant was then diluted in sodium triphosphate buffer (pH=7.0) (Sigma-Aldrich), with 0.5mM 4,4-dimethyl-4-silapentane-1-sulfonic acid as the internal standard (Sigma-Aldrich). Samples were run on Bruker 600 MHz AVANCE III Spectrometer and analyzed with the Chenomx NMR Suite v8.4 (Chenomx Inc.).

16S rRNA Gene Sequencing

DNA from fecal samples were extracted using the FastDNA Spin Kit for Feces (MP Biomedicals) following the manufacturer's protocol. The primers for 16S microbiota analysis were selected to amplify the V3-V4 region of 16S rRNA genes. Forward primer used was ACTCCTACGGGAGGCAGCAG and reverse GGACTACHVGGGTWTCTAAT. The primers contained barcodes, spacers and Illumina sequencing linkers and were designed and used as suggested by Fardosh et al. (32). The 16S rRNA gene sequencing library preparation, PCR amplification and library purification followed the Illumina recommended protocol (Illumina Inc., San Diego, CA, USA). The sequencing was performed on an Illumina MiSeq instrument using 2x300 bp paired-end sequencing. Amplicon sequence variant was generated with the dada2 package (1.16.0) using R software (4.0.2). Taxonomy was assigned using the Ribosomal Database Project classifier with species level taxonomy assignment. Alpha and beta diversity analysis was performed using the phyloseq (1.32.0), microbiome (1.10.0) and vegan (2.5-7) packages. Sequence data was deposited in the European Nucleotide Archive under accession number PRJEB50194.

Statistics

Unpaired t-test and ANOVA were used to compare two or more groups, respectively, and Mantel-Cox log-rank test was used to analyze LD_{50} study survival results. Differences were considered statistically significant when $p<0.05$.

RESULTS

High-Fiber Feeding Does Not Affect WNV-Induced Encephalitis Neuroinflammation

To determine whether beneficial reshaping of the gut microbiota could affect the severity of WNV infection, mice were fed on diets enriched in dietary fiber (high fiber, HF) or deficient in dietary fiber (zero fiber, ZF) for 2 weeks prior to infection (**Figure 1A**). These diets have previously been shown to reshape the gut microbiota composition beneficially (HF) or detrimentally (ZF) (3, 4). Mice fed on HF, as expected, had a significantly different gut microbiota composition as shown by weighted UniFrac (**Figure S1**), as well as a significantly increased production of the SCFA, acetate, in feces, compared to ZF-fed mice (**Figure 1B**), as previously published (5). These mice were inoculated intranasally with WNV LD_{100} and maintained on these diets over the course of infection. Intranasal inoculation enables reliable infection of the central nervous system (CNS), with clear separation of anti-viral responses in the CNS from systemic responses. In this model, WNV spreads from the olfactory bulb to the rest of the brain and spinal cord over the course of infection and has been described in detail by Getts et al. (26).

Both HF- and ZF-fed mice had altered gut microbiota composition after WNV infection (**Figures S1A–G**), with the bacteria from the genus *Dorea, Enterorhabdus* and *Clostidium IV* being significantly more represented in infected ZF-fed mice. Despite these differences, acetate levels remained higher in infected HF-fed mice (**Figure S1H**). Nevertheless, both groups had similar clinical scores at 7 days post infection (dpi) (**Figure 1C**), as well as undergoing comparable weight loss (**Figure 1D**). This suggests that HF diet does not protect against LD_{100} WNV infection. Consistent with this, total numbers of leukocytes in the brain and infiltration of inflammatory Ly6C^{hi} monocytes was similar in both groups, as assessed by flow cytometry, although neutrophil infiltration was significantly reduced in HF-fed mice (**Figure 1E** and **Figures S2, S3**). The aggravating effects of antibiotics have been linked to impaired WNV induced-T cell responses (22). In contrast, HF and SCFA can modulate T cell differentiation towards both Th1, Th17 and Treg, depending on the context (12). However, numbers of infiltrating CD4^{+} and CD8^{+} T cells into the brain were similar between HF and ZF groups (**Figure 1F**). Furthermore, in WNV infection, HF feeding did not affect the T cell response, with similar numbers of Th1, Th17 and Treg, as well as IFN-γ^{+} CD8^{+} T cells, both in the brain (**Figure 1F**) and its draining lymph nodes, when compared to ZF (**Figure S4**).

As beneficial immunomodulation by HF diet may be masked by overwhelming infection at the LD_{100}, we investigated the effects of dietary fiber in WNV infection at LD_{50} and assessed its impact on survival. In mice inoculated intranasally with LD_{50} as in **Figure 1A**, clinical disease scores increased to a similar extent in both dietary interventions from 5 dpi onwards (**Figure 1H**). By 10 dpi, independent of diet, the mortality of both groups was similar at 30-50% and remained stable after 16 dpi (**Figure 1G**). We confirmed that the absence of protection in the HF group was not linked to this diet in particular, as a different high-fiber diet enriched in guar gum, previously shown to improve health (3–5), also had no impact (data not shown). Taken together, these data indicate that dietary fiber does not reduce the clinical severity of WNV encephalitis.

WNV Encephalitis Is Associated With the Spread of the Virus to the Colon

Systemic infection associated with peripheral WNV inoculation has been linked to enteric neuronal infection and neuronal injury promoted by antigen-specific CD8^{+} T cells (33), resulting in

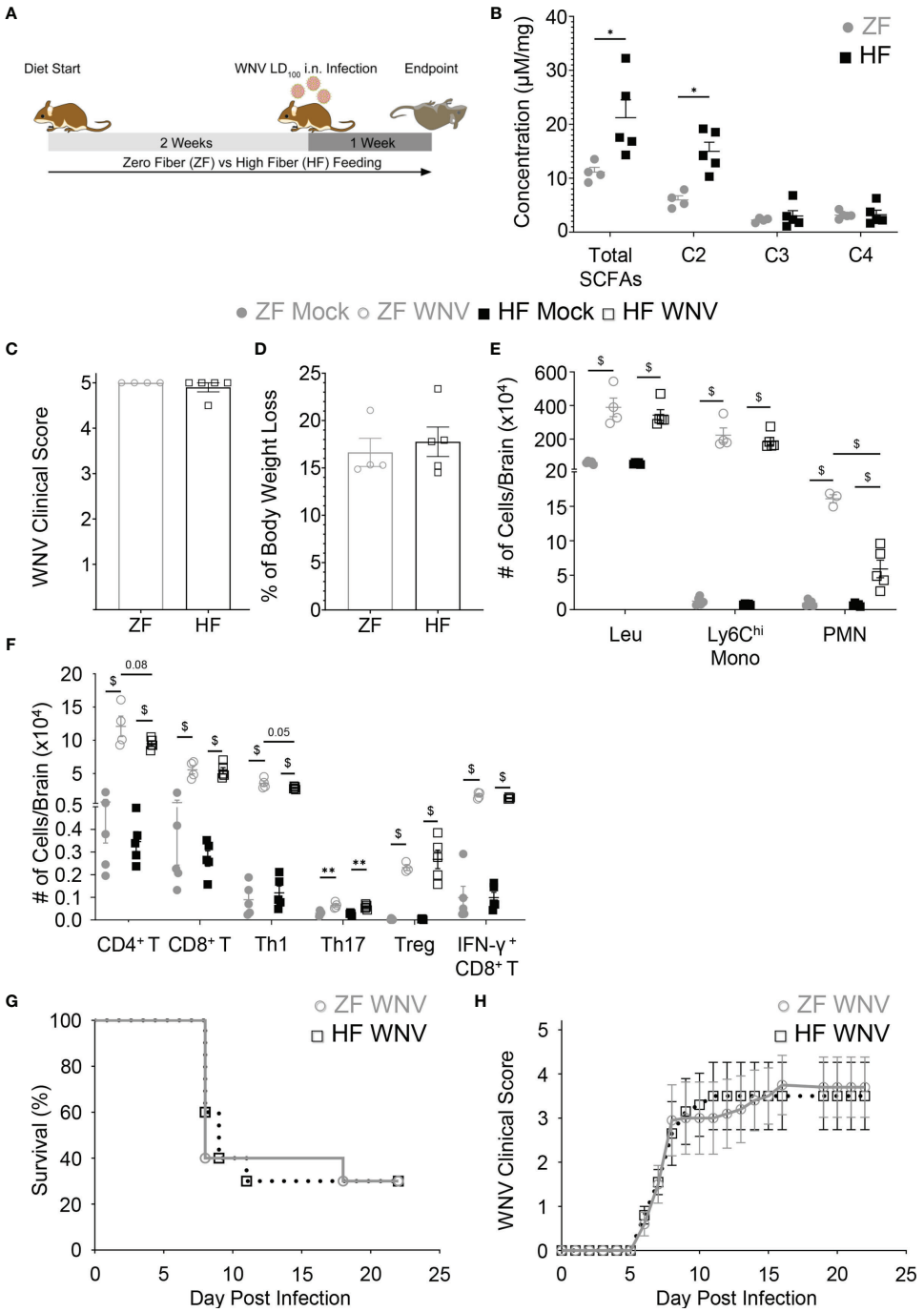

FIGURE 1 | Dietary fiber increased fecal acetate but failed to protect against WNV encephalitic neuroinflammation and improve disease survival. Mice were fed on diets either enriched (HF) or deficient (ZF) in dietary fiber for two weeks and then intranasally infected with either LD_{100} **(A–F)** or LD_{50} WNV **(G, H)**, while diets were maintained during infection. **(A)** Experimental workflow for LD_{100} WNV infection study. **(B)** The concentration of fecal total SCFA, acetate (C2), propionate (C3) and butyrate (C4) of mice fed on HF or ZF diet for two weeks was quantified by NMR spectroscopy (n = 4-5 per group). Clinical scores **(C)** and body weight loss **(D)** of ZF- or HF-diet-fed mice in WNV LD_{100} study were determined at 7 dpi (n = 4-5 per group). **(E)** Numbers of total leukocytes (Leu), Ly6C^{hi} monocytes (Ly6C^{hi} Mono), and neutrophils (PMN) in mock-infected (Mock) or 7 dpi LD_{100} WNV-infected (WNV) brain from mice fed on ZF- or HF-diets were analyzed by flow cytometry (n = 4-5 per group). **(F)** Numbers of CD4$^+$ T cells, CD8$^+$ T cells, Th1, Th17, Treg, and IFN-γ$^+$ CD8$^+$ T cells in mock-infected (Mock) or 7 dpi LD_{100} WNV-infected (WNV) brain from mice fed on ZF- or HF-diets were analyzed by flow cytometry (n = 4-5 per group). **(G)** Survival of mice intranasally infected with LD_{50} WNV fed on ZF- or HF-diets (n = 10 per group). **(H)** Average clinical scores of LD_{50}-infected mice fed on ZF- or HF-diets (n = 10 per group). Data are represented as mean ± SEM with $^*p < 0.05$; $^{**}p < 0.01$ and $^{\$}p < 0.001$ by t test or two-way ANOVA.

altered gut homeostasis, in particular, gastrointestinal motility (33). Whether CNS infection with WNV is also associated with WNV in the gastrointestinal tract is unknown. To address this question, we inoculated mice fed on normal chow diet intranasally (i.n.) with LD_{100} WNV and assessed the presence of WNV by qPCR in the brain and brain-draining cervical lymph nodes (cLN), the gastrointestinal tract (duodenum, jejunum, ileum and colon), the gut-draining mesenteric lymph nodes (mLN), the primary lymphoid organs (thymus and spleen) and peripheral organs (lung, kidney and heart). While the brain was the major site of infection, we also detected significant viral load in the colon and at lower levels in the duodenum, jejunum, ileum and their mLN, as well as the heart. Viral load in the colon was even higher in animals inoculated intracranially (i.c.) with WNV, excluding the possibility that infection was a result of systemic infection or ingestion (**Figure 2A**). We confirmed the presence of WNV by immunofluorescence in the colon, specifically in colonic neurons of the myenteric and submucosal plexuses, colocalizing with the neural marker NeuN (**Figure 2B**). We then assessed whether the presence of WNV was associated with colonic mucosal inflammation. Histologically, mice infected with WNV had significantly more mononuclear cells infiltrating the colon (**Figure 2C**), and this was associated with a significantly reduced colon length in infected mice (**Figure 2D**), as well as changes in the gut microbiota composition (**Figure S5**). Thus, CNS infection with WNV is accompanied by spread of virus in the gastrointestinal tract, particularly in colonic neurons, leading to local inflammation.

Dietary Fiber Does Not Affect Colonic Viral Load and Minimally Affects Colonic Inflammation

As dietary fiber is known to promote gut health, we then investigated whether HF affected viral load in the colon. Regardless of diet, mice fed on HF or ZF had similar colonic viral load (**Figure 3A**), showing no effect of dietary fiber. The presence of virus was associated with significantly increased mRNA expression for type 1 and type 2 IFNs, IL-6, tumor necrosis factor (TNF) and IL-10 expression in the colon in both groups (**Figures 3B–F**). While levels of mRNA for these cytokines were similar between groups, mice fed on HF had significantly decreased levels for TNF, compared to mice fed on ZF, suggesting potential local immunomodulatory effects of HF in the colon (**Figure 3E**). The similar overall levels of message for cytokines (except for TNF) correlated with similar increased mononuclear cell infiltration by histological analysis between groups (data not shown). Infected HF-fed mice also had immune phenotypic profiles in the mLN similar to infected ZF-fed mice (**Figure S6**), suggesting a similar response to infection in both groups.

The fermentation of dietary fiber affects both the colon and cecum length (3). Accordingly, the colons of mock-infected HF-fed mice were significantly longer than ZF-fed mice. However, in WNV infection by 7 dpi, colon length in HF-fed mice was significantly reduced and comparable in size to those of ZF-fed mice. Notably, the colon length in infected ZF-fed mice was similar to mock-infected ZF-fed mice (**Figure 3G**).

Together, these data show that WNV infection of the CNS is accompanied by infection of the autonomic nervous plexuses in the colon, mucosal mononuclear cellular infiltration, local elevation of immune cytokines and reduced colonic size, consistent with inflammation, which, despite reduced TNF levels, was unaffected by the fiber content in HF feeding.

DISCUSSION

In the present report, we show that dietary intervention modulating dietary fiber had no benefit in WNV encephalitic disease, notwithstanding an evident shift in the composition of the gut microbiota. Mice fed on a diet enriched or deficient in dietary fiber showed similar degrees of encephalitis, as evident by their clinical scores and mortality rates, and except for neutrophils, immune cell recruitment into the brain and draining lymph nodes was similar in both groups. We used an intranasal or intracranial inoculation model of WNV infection to limit systemic infection, detecting high levels of WNV in the brain, as expected. However, significant virus signal was also detected in the colon, principally in neurons, accompanied by a local mucosal inflammatory infiltrate. Supporting this, mRNA for both pro-and anti-inflammatory cytokines, as well as type 1 and 2 IFNs were increased locally and there was a marked reduction in colonic length, a feature of colonic inflammation. Consistent with HF treatment in other inflammatory diseases, TNF message was reduced in HF-fed mice, however, no other measured parameters were altered by high-fiber content in the diet.

Dietary fiber and SCFA have been shown to have immunomodulatory effects beneficial in lung infection (8) and in inflammatory CNS disease (7). Since detrimental changes in the gut microbiota induced by antibiotics were reported to aggravate flavivirus infection (22), we hypothesized that high-fiber diet may ameliorate disease in WNV encephalitis. However, in our experiments, neither the type of fiber, nor the amount was beneficial in WNV infection. Using two different types of diet, one supplemented with guar gum, containing the equivalent of 10-fold the recommended amount of fiber (3), we found no benefit, either in clinical scores or increased survival. Thus, increasing the consumption of dietary fiber in itself is unlikely to be an effective treatment in WNV encephalitis, as the immunomodulatory effects of dietary fiber are evidently insufficient to counteract the damaging immunopathology associated with the anti-viral response. Nevertheless, high fiber may potentiate the effects of anti-inflammatory drugs, which to our knowledge has never been investigated.

The only change we observed in the brains of HF-fed mice with WNV encephalitis was decreased neutrophilic infiltration. A similar effect of HF and acetate was reported in a mouse model of gout in which HF-fed mice had reduced neutrophilic inflammation (34). Acetate increased neutrophil apoptosis, which decreased the inflammation and promoted resolution in the knee. The role of neutrophils in WNV is unclear and may either promote virus replication or contribute to disease resolution (35). However, in our study the potential impact of

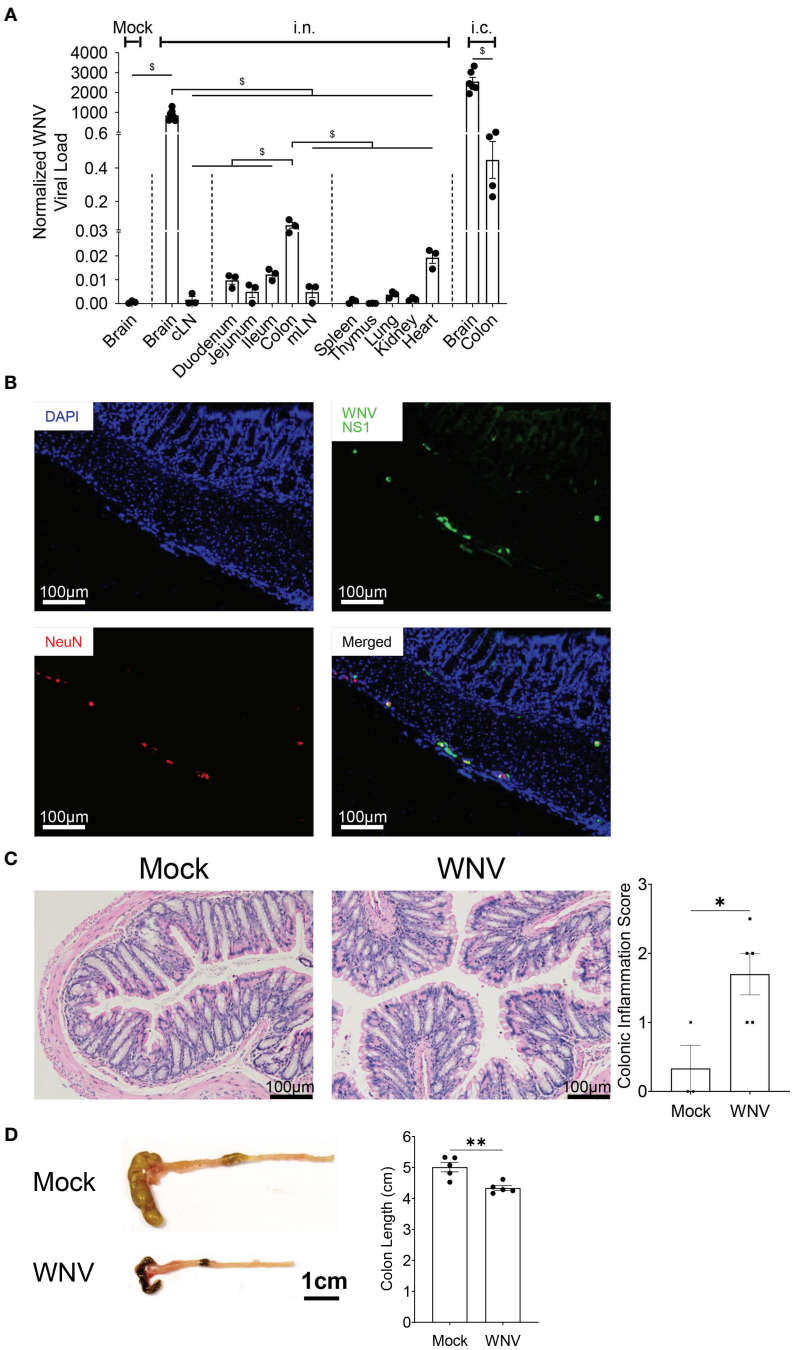

FIGURE 2 | Spreading of WNV in the colon during WNV encephalitis triggers colonic inflammation. Mice were fed on normal chow diet and were intranasally (i.n.) or intracranially (i.c.) infected with LD_{100} WNV. **(A)** WNV viral load quantified for mock-infected animals (Mock) or infected animal at 7dpi by qPCR in brain, cLN, duodenum, jejunum, ileum, colon, mLN, spleen, thymus, lung, kidney, heart, n = 3-7 per organ), as well as brain and colon from mice inoculated intracranially with LD_{100} WNV, as a control. **(B)** Representative immunofluorescence staining of WNV non-structural protein 1 (WNV NS1, green), and FOX-3 neuronal nuclei (NeuN, red) in colon, counterstained with DAPI (blue) from 7 dpi LD_{100} WNV-infected mice fed on a normal diet. Scale bar represents 100μm. **(C)** H&E-stained colonic tissue sections from mock-infected (Mock) or 7 dpi LD_{100} WNV-infected (WNV) mice fed on a normal diet evaluated for colonic inflammation. Representative histological images are shown for each group in the left panel, and quantification of colonic inflammation scoring are shown in the right panel (n = 3-5 per group). Scale bar represents 100μm. **(D)** Colon length of mock-infected (Mock) or 7 dpi LD_{100} WNV-infected (WNV) mice fed on normal diet were assessed (n = 5 per group). Scale bar represents 1cm. Data are represented as mean ± SEM with $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\$}p < 0.001$ by t test or two-way ANOVA.

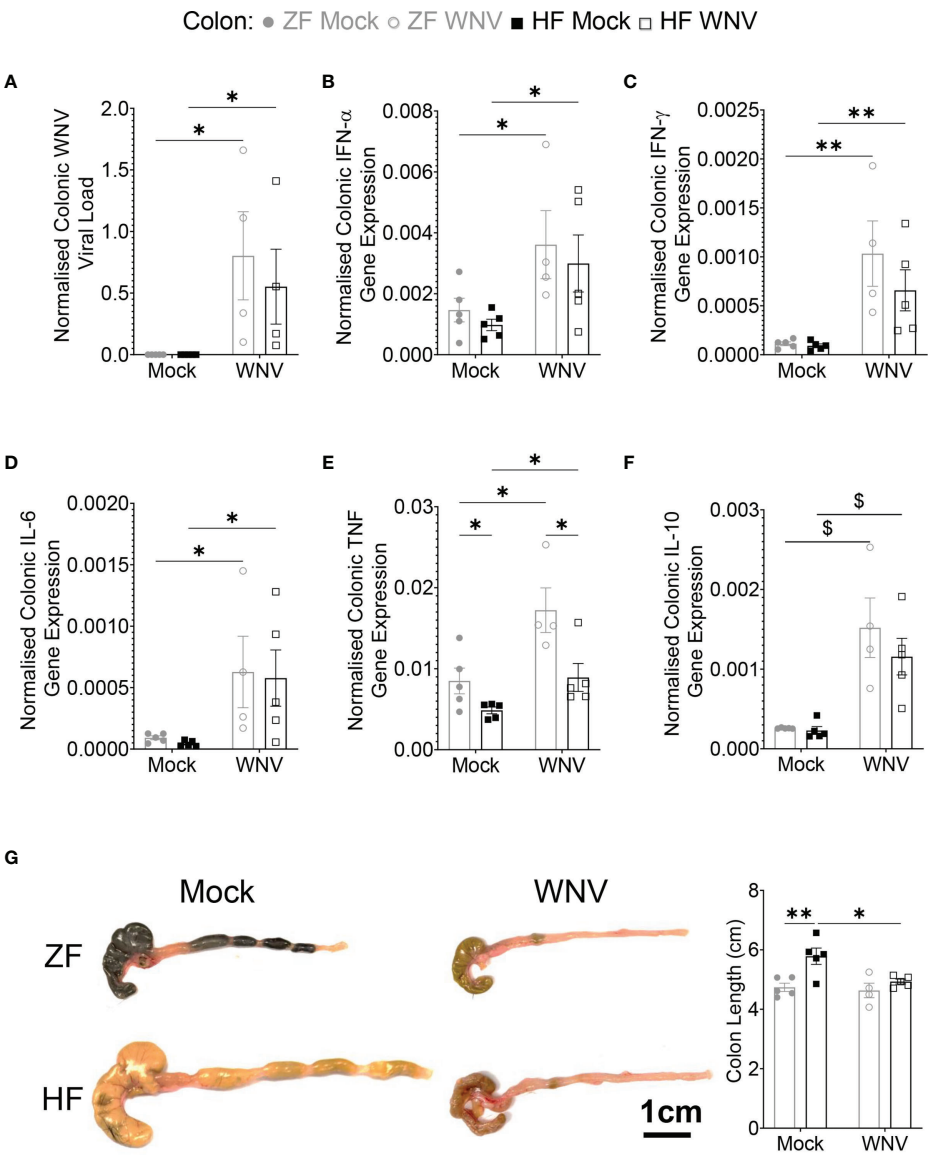

FIGURE 3 | Dietary fiber did not ameliorate WNV-induced colonic inflammation despite reducing TNF. Mice were fed on diets either enriched (HF) or deficient (ZF) in dietary fiber for two weeks and then intranasally infected with LD_{100} WNV. WNV viral load **(A)**, colonic gene expression of *Ifna* **(B)**, *Ifng* **(C)**, *Il6* **(D)**, *Tnf* **(E)**, *Il10* **(F)** was determined by qPCR (n = 4-5 per group). **(G)** Colon length of mock-infected (Mock) or 7 dpi LD_{100} WNV-infected (WNV) mice fed on ZF- or HF-diets were assessed (n = 4-5 per group). Scale bar represents 1cm. Data are represented as mean ± SEM with $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\$}p < 0.001$ by two-way ANOVA.

HF on neutrophil recruitment or apoptosis did not improve survival, suggesting a minor role of these cells, as supported by our previous studies (25). This may explain why dietary fiber may be beneficial in influenza infection (8) in which neutrophils contribute significantly to immunopathology, but not in WNV.

We used intranasal or intracranial infection to limit systemic infection. In both cases, WNV was also detectable in the colon, evidently localized to neurons of the myenteric and submucosal plexuses, as well as to a lesser extent in several other organs. The gut-brain connection has been reported in other infection models, with spread of reovirus from the intestine to the brain *via* the vagus nerve, for example (36). WNV may reach colonic neurons from the CNS *via* efferent branches of the vagus nerve. This would explain the presence of the virus in the heart, as well as other viscera supplied by the extensive parasympathetic network originating from this important cranial nerve, while the well-described interconnectivity between enteric and mucosal plexuses, would explain the presence of virus at both levels (37). The presence of WNV in the colon correlated with local mucosal inflammation characterized by increased levels of IFN-α and IFN-γ, as well as IL-6 and TNF, although the latter was reduced in HF-fed mice. While reduced TNF expression is

consistent with the immunomodulatory effects of HF reported in colitis, it is unclear why levels of IL-6 and IL-10 are not similarly affected. The presence of WNV in the gastrointestinal tract has been reported to impair gut motility (33) in a systemic infection model. It is thus of interest that direct brain infection can also be relayed to the colon, where it presumably contributes to this dysmotility by interfering with vagal stimulation, which in itself may interfere with the beneficial effect of HF feeding seen in non-infectious models of colitis.

Thus, despite its potent immunomodulation and palliative success in other inflammatory diseases, the use of high-fiber feeding is not an effective treatment in WNV infection.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The University of Sydney Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

DN and JT performed most of the experiments and analysis and wrote the manuscript. PN and GP did the flow cytometry experiments related to brains and lymph nodes and contributed to manuscript writing. AS did the immunofluorescence staining experiments and contributed to immune analysis and manuscript writing. DS helped with the 16S sequencing and analysis. NK and LM conceived, designed, and supervised the study, participated in the experiments and wrote the manuscript. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.784486/full#supplementary-material

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REVIEW
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Ingestion, Immunity, and Infection: Nutrition and Viral Respiratory Tract Infections

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Respiratory infections place a heavy burden on the health care system, particularly in the winter months. Individuals with a vulnerable immune system, such as very young children and the elderly, and those with an immune deficiency, are at increased risk of contracting a respiratory infection. Most respiratory infections are relatively mild and affect the upper respiratory tract only, but other infections can be more serious. These can lead to pneumonia and be life-threatening in vulnerable groups. Rather than focus entirely on treating the symptoms of infectious disease, optimizing immune responsiveness to the pathogens causing these infections may help steer towards a more favorable outcome. Nutrition may have a role in such prevention through different immune supporting mechanisms. Nutrition contributes to the normal functioning of the immune system, with various nutrients acting as energy sources and building blocks during the immune response. Many micronutrients (vitamins and minerals) act as regulators of molecular responses of immune cells to infection. It is well described that chronic undernutrition as well as specific micronutrient deficiencies impair many aspects of the immune response and make individuals more susceptible to infectious diseases, especially in the respiratory and gastrointestinal tracts. In addition, other dietary components such as proteins, pre-, pro- and synbiotics, and also animal- and plant-derived bioactive components can further support the immune system. Both the innate and adaptive defense systems contribute to active antiviral respiratory tract immunity. The initial response to viral airway infections is through recognition by the innate immune system of viral components leading to activation of adaptive immune cells in the form of cytotoxic T cells, the production of neutralizing antibodies and the induction of memory T and B cell responses. The aim of this review is to describe the effects of a range different dietary components on anti-infective innate as well as adaptive immune responses and to propose mechanisms by which they may interact with the immune system in the respiratory tract.

Keywords: infection, immunity, nutrition, infant, elderly, respiratory virus

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INTRODUCTION

Due to the increasing population density and the worldwide trend of urbanization, people are living closer together, thus providing a favorable environment for transmission of respiratory viruses and other infective organisms. Contacts with animals such as chickens and pigs, but also with wild animals, increase the risk of pandemic zoonotic disease outbreaks. Examples include the 2009 swine influenza pandemic and the current severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic. COVID-19, caused by SARS-CoV-2, is a reminder of the impact that respiratory viral infections can have on human health and wider society.

Respiratory infections are common in the elderly and in very young children and place a heavy burden on the health care system, especially in the winter months. In addition to the direct impact of respiratory infections, there are causal links between respiratory infections and the development and worsening of chronic respiratory diseases such as asthma in children (1–3), long COVID in children and adults (4–6), COVID-19 induced pulmonary fibrosis in adults and the elderly (7), and chronic obstructive pulmonary disease (COPD) in the elderly (8).

It is therefore highly relevant from a societal perspective, in addition to developing effective vaccines and new antiviral treatments, to understand more about possible preventive, lifestyle-related approaches, including those related to nutrition. Therefore, it is important to understand the possible role that nutrition can play in reducing the occurrence and in dampening the severity of these infections. This role has been largely neglected to date, even though there are indications that nutrition in general, and specific nutrients and other food components in particular, may improve resistance against viral infections. In this review we will describe which population subgroups are susceptible to respiratory infections, which immunological mechanisms contribute to antiviral immunity, how nutritional components can modulate antiviral immune responses and infections, and finally we will propose how these components that are ingested through the gastrointestinal tract can have effects on immunity in the respiratory tract.

IMMUNE VULNERABILITY: THE YOUNG AND OLD

Both young children (0-4 years) and the elderly (> 65 years) are more susceptible to respiratory and gastrointestinal infections than older children and young and middle aged adults (9, 10) as their immune system is less effective (11). The underlying reasons for this differ between young children and the elderly. In young children, lower immune responsiveness results from a less matured adaptive immune system, especially in the first 2-3 years of life, which cannot always respond well to external stimuli. Moreover, the type of adaptive immune response in early life is more focused on the prevention of inflammation, as a result of which Th1 immunity and pro-inflammatory responses are not yet well developed [(11, 12) and **Figure 1**]. The innate immune system, however, is already developed in young children. Pan-pathogen receptors such as toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) are present soon after birth, enabling recognition and responses by innate immune cells in the form of antiviral cytokine production after infection (11, 12). Protection of infants against infection is further provided by transplacental transport of immunoglobulins during pregnancy, and by breastfeeding after birth. In addition to immunoglobulins, breastmilk contains a wide range of immune-related components including micronutrients, oligosaccharides, antimicrobial proteins, and immunoregulatory cytokines that support immune maturation and regulation (13–15), as well as the development of the microbiota in early life [(16, 17) and **Figure 1**].

The reduced immune responsiveness/efficacy in the elderly, termed immune senescence, is characterized in particular by a less flexible adaptive immune response [(18–22) and **Figure 1**]. One contributor to immune senescence is likely to be the decreased output of immune cells from bone marrow, the site of origin of all immune cells, with increasing age. Furthermore, involution of the thymus with age decreases output of naive T cells, resulting in reduced capacity to respond to new antigens. In addition to altered numbers of immune cells in the circulation, their function is often impaired in older people. For example, neutrophils show impaired phagocytosis, respiratory burst and bacterial killing; natural killer (NK) cells have impaired cytotoxicity towards virally-infected and tumor cells; dendritic cells (DCs) have impaired responsiveness to immune signals. T cells have reduced ability to proliferate and to produce important cytokines like interleukin (IL)-2 and interferon (IFN)-γ; Cytotoxic T cell activity is reduced and antibody production by B cells is altered. Hence, older people can show a broad range of immune impairments, making them more susceptible to infectious diseases. This includes respiratory illnesses caused by viruses. Immune senescence also impairs responses to vaccination, including to the seasonal influenza vaccine (23, 24).

The reduced adaptive immune response to vaccination and to viral infection in the elderly may resemble the reduced adaptive immune response in young children, but it has a different cause. In infants and toddlers up to 2-3 years of age, T-cell responses and the induction of specific antibodies are reduced because of immaturity of the immune system; in elderly, there is a bias towards generation of memory T cells, and the ability to mount naive T cell responses to new antigens, as well as vaccines, is reduced.

Whereas even at a few weeks of age, infant already have sufficient TLR expression and responsiveness to TLR ligands like single stranded RNA (11) which induce IFN-α, the elderly have a much decreased IFN-α response to influenza viruses (25). This has been shown in particular for TLR7 and TLR8, the receptors that are important in protecting against single stranded RNA viruses, to which most respiratory viruses belong. For example, in older women, the plasmacytoid dendritic cell (pDC) response to TLR7 stimulation with R848 is notably decreased (26). In this context it is interesting to note that in COVID-19 patients with a severe clinical picture, two different TLR7 variants were found to be associated with reduced antiviral function (27).

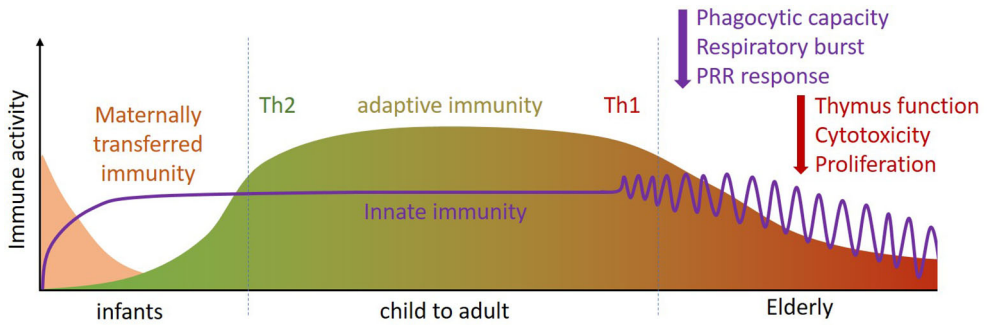

FIGURE 1 | Throughout the life course (infant, child to adult, elderly) the immune system changes in composition and activity. Innate immunity is already present at birth and quickly matures (purple line). For adaptive immune defense infants rely on placental transfer and mothers' milk for their first period (pink section). During development, the adaptive immune system phenotype changes from Th2 dominant [i.e. tolerant (green)] to Th1 dominant [i.e. inflammatory (red)]. At later age, specific functions of innate and adaptive immunity become less efficient (purple and red arrows). Th, T helper.

The immune vulnerability in the very young and the elderly thus illustrates the need for supporting antiviral immune function, for example by nutritional intervention. But let's first discuss the mechanisms of antiviral immunity.

MECHANISMS OF ANTIVIRAL IMMUNITY

The human immune system comprises an innate and adaptive component. The innate, or inborn, component is rapid (responds minutes to hours after infection), reacts to a large variety of pathogens and is important in the primary protection against infection, but is also limited in effectiveness. The adaptive immune system on the other hand is slow to respond (days to weeks), but highly specific and effective. In fact, the adaptive system is activated by its innate counterpart *via* the antigen presenting capacity of monocytes, macrophages and DCs.

Both the innate and adaptive defense systems contribute to active antiviral immunity. The initial response against viral airway infections is through sensing of viral replication in infected host cells by cells of the innate immune system *via* PRRs such as TLR7/8 and RIG-I. PRRs recognize repetitive evolutionarily conserved structures on potential pathogens, such as bacterial cell wall components (lipopolysaccharides, peptidoglycans) and sugars, and also intracellular components of pathogens such as DNA and RNA. The best studied PRRs are the TLRs, of which TLR7 and TLR8 are particularly important against single stranded RNA viruses, such as influenza virus, respiratory syncytial virus (RSV), rhinovirus and SARS-CoV-2. They recognize single stranded RNA that is not found in endosomes under normal metabolic activities of human cells. The recognition of pathogenic structures such as single stranded viral RNA by TLR7 and TLR8 results in induction of anti-viral type I and type III interferons that have a direct inhibitory effect on viral replication. In addition, these interferons (α, β and λ) cause nearby cells to heighten their anti-viral defenses, inducing an antiviral state. The innate defense system includes monocytes, myeloid and plasmacytoid DCs (mDCs and pDCs), macrophages, NK and natural killer T (NKT) cells, and neutrophils, eosinophils, and basophils. Especially the pDCs are high producers of type I interferons that play a key role in antiviral immunity (28–30) to shape the local immunological environment in order to eliminate viruses.

In addition to a first broad response and activation of the adaptive immune system, specific components of the innate immune system can also directly target respiratory pathogens. For example, NKT cells play a direct role in antiviral immunity (31–33). They have receptors that can recognize lipid when presented on major histocompatibility complex (MHC)-like molecules such as CD1c on innate immune cells. In addition, iNKT cells can inhibit the influenza A-induced immunosuppressive activity of myeloid suppressor cells (34) and iNKT activation can shorten influenza infection (35). The finding that milk contains lipids known as ligands for NKT cells (36) may indicate that the milk lipid fraction also plays a role in antiviral immunity, as well as in intestinal homeostasis as NKT cells also recognize lipids from the intestinal microbiota (37, 38). Plant lipids can also activate NKT cells (39).

Respiratory and gastrointestinal epithelial cells - which together with mucus form a first physical barrier against infection - can also contribute to antiviral immunity. Epithelial cells express TLRs and other PRRs such as RIG I and recognize viral components. In response, both intestinal epithelial and respiratory epithelial cells can produce antiviral IFNs (40–42).

The adaptive defense system consists of the B lymphocytes, which can produce antibodies against viruses, and both the CD4+ and CD8+ T lymphocytes, which can recognize protein fragments of the virus presented by MHC molecules on innate immune cells. This adaptive immune response is crucial in protecting against re-infection with the same pathogen through its long-lasting immunological memory. However, the adaptive immune response starts slowly and it takes a few days to weeks after infection before antibodies become detectable. This results from the process in which innate immune cells such as macrophages or DCs move from the site of infection to nearby specialized immunological compartments such as lymph nodes or the spleen. Monocytes, macrophages and DCs collect pathogenic

peptide fragments and present those on MHC molecules - or human leukocyte antigens (HLA) in humans - to T cells that specifically recognize the MHC-peptide complex, resulting in T cell activation and instruction of antigen-specific B cells. After activation, B cells and T cells mature and proliferate (clonal expansion) before they can either start producing antibodies or migrate to the site of infection to kill infected host cells, respectively. Maintenance of virus-specific T and B cells provides a memory giving protection against reinfection. This is the basic principle underlying pathogen-specific protection by adaptive memory induced by vaccination.

Passive immunity against virus infections can be provided by antiviral antibodies already circulating in the blood that can neutralize the virus, but there are also small molecules such as complex sugars (oligosaccharides) and oligosaccharide-containing sphingolipids that can also exert such a neutralization role (43–46).

Another way the innate immune system confers protection against infections has been revealed through vaccination with BCG (tuberculosis vaccine). Surprisingly, BCG vaccination was found to also result in better protection against other pathogens (47, 48). Although this can be partly explained by cross-reacting antibodies which can bind to more than one pathogen, it has recently been shown that the innate immune system also has a certain degree of memory. This so-called "innate immune memory" or "trained immunity" appears to result from epigenetic modifications in the DNA conferring innate immune system cells with the ability to respond more strongly to stimulation by pathogens *via* TLRs and exhibiting some degree of cross-protection (49–52). It has recently been postulated that "trained immunity" may also protect against viral respiratory infections such as COVID-19 (53), and this concept is currently being investigated in multiple studies (54).

NUTRITION AND THE IMMUNE SYSTEM

Foods and beverages provide macro-and micronutrients, as well as other bioactive components, that contribute to the normal functioning of the immune system, including supporting barrier function. The impact of several food components such as specific vitamins and minerals, and also proteins, flavonoids, and non-digestible polysaccharides, as well as probiotics and short-chain fatty acids, on the immune system has recently been comprehensively reviewed (55, 56). These effects are relevant to anti-viral immunity (57).

Food components act in a variety of ways to influence immune responsiveness against acute upper respiratory tract infections by viruses:

- Macronutrients act as fuels for energy generation by immune cells;
- Macronutrients provide substrate ("building blocks") for the biosynthesis that is involved in the immune response (e.g. amino acids for immunoglobulins, cytokines, new receptors, acute phase proteins);
- Many micronutrients are regulators of molecular and cellular aspects of the immune response (e.g. iron, zinc, vitamin A, vitamin D);
- Some nutrients are substrates for the synthesis of chemicals involved in the immune response (e.g. arginine and nitric oxide; arachidonic acid and eicosanoids);
- Some micronutrients have specific anti-infection roles (e.g. zinc, vitamin D);
- Some proteins play a direct role in pathogen clearance and immune function (e.g. breast milk proteins like IgG, lactoferrin, and lysozyme);
- Many nutrients and bioactives are involved in protection of the host from the oxidative and inflammatory stress imposed by the immune response (e.g. vitamin C, vitamin E, cysteine, zinc, copper, selenium, flavonoids);
- Many food components contribute to creating a diverse microbiota that supports the immune response (e.g. plant-derived fibers and non-digestible polysaccharides, prebiotic oligosaccharides and human milk oligosaccharides);
- Probiotic bacteria that produce metabolites like short chain fatty acids (SCFAs) and some vitamins, support barrier function and contribute to immune support;
- Some food components act as aryl hydrocarbon receptor ligands, acting to strengthen barrier function (e.g. quercetin).

These considerations suggest multiple sites of interaction of food components with the immune system. Firstly, absorbed food components can act systemically to target the different components of the immune system (e.g. in bone marrow, the thymus, the bloodstream, secondary lymphoid organs, and other organs). Secondly, multiple food components can act to influence the immune system without being absorbed systemically. For example, they could have local actions on epithelial barrier function or on the gut-associated lymphoid system, they could modulate the gut microbiota composition influencing gut microbiota-immune system cross-talk, they could be fermented by the microbiota resulting in metabolites (e.g. short chain fatty acids (SCFAs)) which can act locally on epithelial and immune cells or be absorbed and act systemically, or they could train or prime immune cells involved in surveillance of the luminal contents of the gastrointestinal tract. Although these latter actions are primarily focused on the gastrointestinal tract, because of recirculation of cells from the gut wall to the respiratory tract, effects initiated at the gut level can be enacted at the level of the airways.

MICRONUTRIENTS: SUPPORT OF NORMAL IMMUNE FUNCTION AND RELEVANCE FOR RESPIRATORY INFECTION

Multiple micronutrients (vitamins and minerals) play several roles in supporting the immune system (**Table 1**), as comprehensively reviewed elsewhere (56, 59–61). People with

low intakes or status of these micronutrients show immune impairments and increased susceptibility to infectious disease, especially respiratory and gastrointestinal (56, 60). Amongst the micronutrients, the roles of vitamins A, C and D and the minerals zinc, copper and iron are well explored, but B vitamins, vitamin E, vitamin K, selenium, magnesium and others all have roles.

Vitamin A and its metabolites (e.g. retinoic acid) are important for normal differentiation of epithelial tissue and for immune cell maturation and function (62–69). Vitamin A is key to an effective barrier function and it regulates many aspects of innate immunity including neutrophil maturation and function and NK cell activity, thus contributing to defense against viruses including respiratory viruses (**Figure 2**). With regard to acquired immunity, vitamin A controls DC and CD4+ T lymphocyte maturation and promotes Th1 type responses that are involved in antibacterial and anti-viral defenses. The vitamin A metabolite retinoic acid promotes homing of T lymphocytes to the gut-associated lymphoid tissue by upregulating surface homing molecules. In this context it is interesting to note that some gut-associated immune cells are able to synthesise retinoic acid. Retinoic acid is required for CD8+ T lymphocyte survival and proliferation and the normal functioning of B lymphocytes including antibody generation, including IgA. Thus vitamin A deficiency is associated with increased susceptibility to many infections, especially in children, including respiratory infections, diarrhea and severe measles (70, 71). Vitamin A supplementation can improve symptoms in patients with acute pneumonia (69). A meta-analysis of 15 randomized controlled trials in children with pneumonia identified that vitamin A decreased pneumonia morbidity, increased the clinical response rate, shortened clearance time of signs and shortened length of hospital stay, although it did not affect mortality (72).

Vitamin C is required for collagen biosynthesis and is vital for maintaining epithelial integrity (73, 74) (**Figure 2**). It also has roles in several aspects of immunity, including supporting leucocyte migration to sites of infection, phagocytosis and bacterial killing, scavenging reactive oxygen species (ROS), NK cell activity, T lymphocyte function and IFN-γ production (especially by CD8+ cytotoxic T lymphocytes) and antibody production (73, 74) (**Figure 2**). People that are deficient in vitamin C are susceptible to severe viral respiratory infections such as pneumonia and acute respiratory disease syndrome (ARDS) (73, 74). Trials of vitamin C supplementation report reduced risk of pneumonia, with some trials reporting decreased severity and mortality (75). A meta-analysis of trials of vitamin C and common cold infections found that it decreased the incidence in those individuals under physical stress and decreased the duration and severity (76).

Vitamin D receptors have been identified in most immune cells and, besides the occurrence of genetic polymorphisms in the vitamin D receptor gene, vitamin D has pleiotropic actions within the immune system supporting the activity of several cell types (77, 78). Furthermore, some immune cells (DCs, macrophages) can produce the active form of vitamin D suggesting it is important to immunity. Vitamin D enhances epithelial integrity and induces synthesis of antimicrobial peptides (e.g., cathelicidin) in epithelial cells and macrophages (79, 80) (**Figure 2**). Vitamin D promotes homing of immune cells to the respiratory tract (81–84). On a functional level vitamin D promotes differentiation of monocytes to macrophages and increases phagocytosis, superoxide production and bacterial killing by innate immune cells. It also promotes antigen processing by DCs although antigen presentation may be impaired. Thus, vitamin D has many actions which would support immunity against respiratory infections. Nevertheless, the effects of vitamin D on the cellular components of immunity are rather complex and paradoxical in nature as reviewed recently (85). Berry et al. described an inverse linear relationship between vitamin D levels and respiratory tract infections in a cross-sectional study of 6,789 British adults (86). In agreement with this, data from the US Third National Health and Nutrition Examination Survey which included 18,883 adults showed an independent inverse association between serum 25(OH)-vitamin D and recent upper respiratory tract infection (87). A meta-analysis of such observational studies identified that serum vitamin D is inversely associated with risk and severity of acute respiratory tract infections (88). There have been a large number of randomized controlled trials investigating the effect of vitamin D to reduce incidence or severity of respiratory tract infections and these have been subject to meta-analysis. A meta-analysis of 25 RCTs involving over 11,000 adults and children identified that vitamin D decreased the risk of acute respiratory tract infections, with greater effects being observed in those with low starting status (89).

Zinc supports the activity of many cells of the immune system, helps to control oxidative stress and inflammation and has specific anti-viral actions including inhibiting the replication of RNA viruses (90–92). Zinc-binding metallothioneins seem to play an important role in antiviral defense (93). Zinc is important in early immune cell development in the bone marrow and is important in maintaining T and B lymphocyte numbers. Zinc also supports the release of neutrophil extracellular traps that capture microbes. Zinc promotes CD4+ T lymphocyte numbers and function (e.g., IL-2 and IFN-γ production) and supports the proliferation of CD8+ cytotoxic T lymphocytes, key cells in antiviral defense (**Figure 2**). Zinc can disrupt the replication and infectivity of some respiratory viruses and prevents ARDS and lung damage in COVID-19 (94). Zinc supplementation has been reported to improve some markers of immunity especially in older people or those with low zinc intake (95). Recent systematic reviews and meta-analyses of trials with zinc report shorter duration of common cold in adults, reduced incidence and prevalence of pneumonia in children and reduced mortality when given to adults with severe pneumonia (96–98).

Iron is another micronutrient that is required by the immune system for proper functioning. Iron is important to maintain thymus function and the output of naive T lymphocytes and also to support neutrophil respiratory burst and bacterial killing, NK cell activity, T lymphocyte proliferation and production of T helper 1 cytokines (99–101) (**Figure 2**). These observations would suggest a clear case for iron deficiency increasing susceptibility to infection, which it does (102). However, many

TABLE 1 | Taken from (58). Summary of the effects of various micronutrients on different aspects of immunity.

Micro nutrient	Role in barrier function	Role in cellular aspects of innate immunity	Role in T-cell mediated immunity	Role in B-cell mediated immunity
Vitamin A	Promotes differentiation of epithelial tissue; Promotes gut homing of B- and T- cells; Promotes intestinal immunoglobulin A+ cells; Promotes epithelial integrity	Regulates number and function of NK cells; Supports phagocytic and oxidative burst activity of macrophages	Regulates development and differentiation of Th1 and Th2 cells; Promotes conversion of naive T-cells to regulatory T-cells; Regulates IL-2, IFN-g and TNF production	Supports function of B-cells; Required for immunoglobulin A production
Vitamin B6	Promotes gut homing of T-cells	Supports NK cell activity	Promotes T-cell differentiation, proliferation and function, especially Th1-cells; Regulates (promotes) IL-2 production	Supports antibody production
Vitamin B9 (Folate)	Survival factor for regulator/T-cells in the small intestine	Supports NK cell activity	Promotes proliferation of T-cells and the Th1-cell response	Supports antibody production
Vitamin B12	Important co-factor for gut microbiota	Supports NK cell activity	Promotes T-cell differentiation,, proliferation and function., especially cytotoxic T-cells; Controls ratio of T-helper to cytotoxic T-cells	Required for antibody production
Vitamin C	Promotes collagen synthesis; Promotes kerathocyte differentiation; Protects against oxidative damage; Promotes wound healing; Promotes complement	Supports function of neutrophils, monocytes and macrophages including phagocytosis; Supports NK cell activity	Promotes production, differentiation and proliferation of T-cells especially cytotoxic T-cells; Regulates IFN-g production	Promotes antibody production
Vitamin D	Promotes production of antimicrobial proteins (cathelicidin, b-defensin); Promotes gut tight junctions (*via* E-cadherin, connexion 43); Promotes homing of T cells to the skin	Promotes differentiation of monocytes to macrophages; Promotes macrophage phagocytosis and oxidative burst	Promotes antigen processing but can inhibit antigen presentation; Can inhibit T-cell proliferation, Th1-cell function and cytotoxic T-cell function; Promotes the development of regulatory T-cells; Inhibits differentiation and maturation of dendritic cells; Regulates IFN-g production	Can decrease antibody production
Vitamin E	Protects against oxidative damage	Supports NK cell activity	Promotes interaction between dendritic cells and T-cells; Promotes T-cell proliferation and function, especially Th1-cells; Regulates (promotes) IL-2 production	Supports antibody production
Zinc	Maintains integrity of the skin and mucosal membranes; Promotes complement activity	Supports monocyte and macrophage phagocytosis; Supports NK cell activity	Promotes Th1-cell response; Promotes proliferation of cytotoxic T-cells; Promotes development of regulatory T-cells; Regulates (promotes) IL-2 and IFN-g production; Reduces development of Th9 and Thl7 cells	Supports antibody production particularly immunoglobulin G
Copper		Promotes neutrophil, monocyte and macrophage phagocytosis; Supports NK cell activity	Regulates differentiation and proliferation of T-cells; Regulates (promotes) IL-2 production	
Iron	Essential for growth and differentiation of epithelial tissue	Promotes bacterial killing by neutrophils; Regulates balance of M1 and M2 macrophages; Supports NK cell activity	Regulates differentiation and proliferation of T-cells; Regulates IFN-g production	
Selenium		Supports NK cell activity	Regulates differentiation and proliferation of T-cells; Regulates (promotes) IFN-g production	Supports antibody production

IFN, interferon; IL, interleukin; NK, natural killer; Th, T-helper; TNF, tumor necrosis factor.

pathogens also have a high demand for iron and in an acute infection, host-driven iron removal inhibits the growth of pathogens (103–105). Chronic immune activation due to persistent infection, however, sequesters iron not only from infectious agents but also from erythroid progenitors, thereby causing anemia associated with chronic inflammation (106). Therefore, the relationship between iron availability, including deficiency, and susceptibility to infection remains complex (107–109). Evidence suggests that infections caused by organisms that spend part of their life-cycle intracellularly may actually be enhanced by iron, and in such situations providing iron may be harmful. Perhaps because of this harm from iron, several host immune mechanisms have developed for withholding iron from a pathogen (104, 105, 110).

Selenium has important roles in supporting the immune system in general (111, 112) and in promoting anti-viral immunity in particular (113–115). Selenium supports the activity of many cells of the immune system and helps to control oxidative stress and inflammation. Cellular entry of viruses, including SARS-CoV-2, into lung cells causes cellular

oxidative stress resulting in the formation of free radicals (ROS) and subsequent disruption of the cellular membrane and lysis because of the budding process. Selenium could protect against such stress. Extensive research in mice has shown that selenium deficiency adversely affects several components of both innate and acquired immunity, including NK cell, T and B lymphocyte function and antibody production (**Figure 2**), increases susceptibility to viral infection, permits viruses (including coxsackievirus, polio virus and influenza viruses) to mutate, and allows normally weak viruses to become more virulent (116–118). A study in South Korea showed that selenium deficiency in peripheral blood was associated with a higher mortality in COVID-19 patients (119). Selenium supplementation improves some markers of immunity especially in older people or those with low selenium intake; for example a supplementation study conducted in UK adults with marginal selenium status showed that selenium improved *ex vivo* anti-viral immune responses, promoted polio virus clearance and decreased polio virus mutation (120). It seems likely that selenium is important in reducing risk and severity of viral respiratory infections.

Taken together, it is evident that multiple micronutrients play several roles in the immune system. Individuals with a deficiency in either one of these vitamins or minerals show immune impairments and increased susceptibility to infection. These impairments can be reversed by repletion of the deficient micronutrient. Each micronutrient has its own biochemical actions that result in its effect on the immune system; although the immunologic effect (*e.g.* improved Th1 cell function) can be shared by many micronutrients, the mechanism underpinning that effect is, generally speaking, unique to each particular

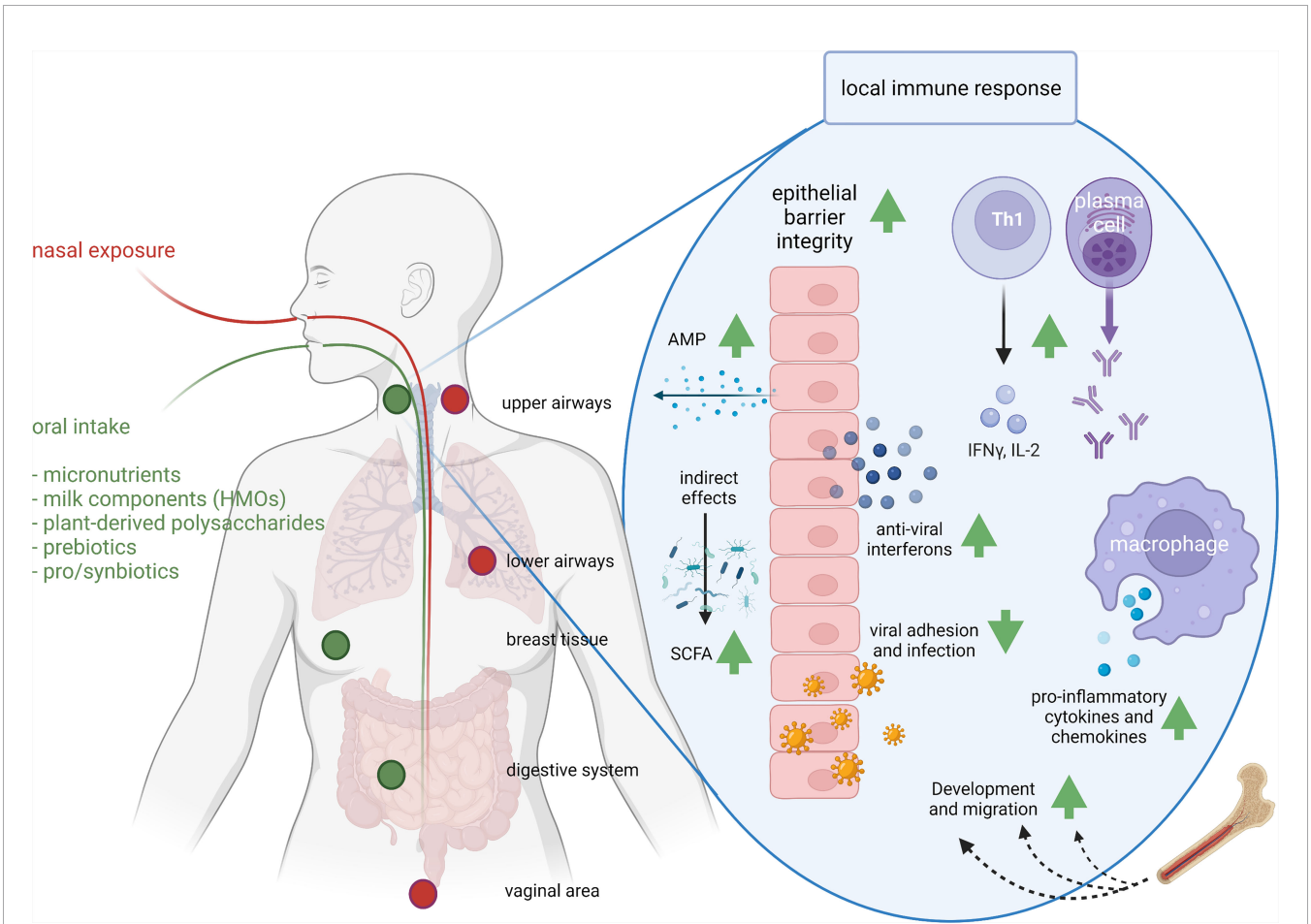

FIGURE 2 | Exposure routes and potential concerted immunomodulatory effects of food components. The route of administration determines the sites of immunomodulation. As a result of nasal exposure (the route that respiratory pathogens follow) pathogens or intranasal vaccines lead to IgA production in upper and lower airways and the vaginal area, and oral exposure leads to IgA production in the upper airways, breast and digestive system. Clinical studies have demonstrated support of respiratory immunity by many food components, whereas preclinical, *ex vivo* and *in vitro* studies identified possible mechanisms. For instance, milk-derived HMOs, pre- and probiotics modify the microbiome and SCFA production; vitamins A, C and D improve epithelial barrier integrity; vitamin D induces anti-microbial protein (AMP) secretion and acetate anti-viral interferon secretion in epithelial cells; viral adhesion and infection is reduced by zinc, bIgG, lactoferrin or HMOs; iron supports immune cell development and migration; bIgG and NDPs induce innate immune training and as a results increased cytokine and chemokine production; zinc and vitamin C support Th1 activity and IFN-γ and IL-2 release; and selenium and vitamin C support antibody production. AMP, anti-microbial peptide; HMO, human milk oligosaccharides; IFN-γ, interferon gamma; IL-2, interleukin-2; SCFA, short chain fatty acid. This figure was created with BioRender.com.

micronutrient. In other words, the immune impairments that result from deficiencies in multiple micronutrients are unlikely to be reversed by repletion of only one of those micronutrients due to limited redundancy in function. In such a scenario a multiple micronutrient mixture is likely to be more effective in supporting (impaired) immune function than any single micronutrient.

MILK COMPONENTS, IMMUNE FUNCTION, AND RESPIRATORY INFECTION

Milk production has evolved in mammals to support growth and development in early life, as well as to provide protection against infections. Therefore, there are many components in breast milk that have direct anti-infective and immune modulatory actions or are important for supporting the immune system (121–131).

Breastfeeding

The first 1000 days of a child's life are important for maturing the immune system, partly under the influence of the development of the gut microbiota. Breast milk contains many bioactive components that support the development and can regulate the immune system, as well as components that can have direct antibacterial and antiviral effects. It is therefore not surprising that breast-fed children have fewer infections than bottle-fed children (125, 132, 133). This has been reported for gastrointestinal infections, but also relates to (viral) respiratory tract infections, pneumonia and mortality (134–136).

Bovine Milk and Its Components

Even though human breastmilk contains many immune supporting components (125, 130), studies on breastfeeding cannot show exactly which components confer resistance to respiratory infections. Cow's milk contains similar components, often in comparable concentrations (131), and apparently with comparable activity on the human immune system (61, 121, 123, 131). Epidemiological studies have seen an inverse association between the consumption of raw cow's milk and asthma and respiratory infections (137–140) and have shown that it is heat-sensitive components of raw cow's milk, likely proteins or peptides, that play an important role (141, 142). In addition, it is known that, like human breast milk, raw cow's milk contains components that may have antiviral activity like IgG, lactoferrin and lactadherin (131, 143). These are not included in infant formulas in their active form due to structural alterations as a result of heating during processing. Thus, infants that are not breastfed or switch to formula at an early age are at increased risk of infection, in particular when introduced to new foods or an altered environmental exposure to microbes.

Epidemiological research has shown that the consumption of raw cow's milk by young children is associated with a lower chance of developing asthma and hay fever (137–139, 141, 142, 144–146) and respiratory infections (140). These studies show that heat-sensitive components in cow's milk - the proteins and peptides - play an important role in protecting against infections and allergies. *In vitro* research has established that milk proteins like IgG, lactoferrin, IL-10 and others can have effects on the human immune system (123, 124, 126, 127, 147–149). These components may contribute to antiviral immunity, but they are not well researched in this context. However, on a cautionary note, it should be stated that raw milk consumption also brings about risks of gastrointestinal infections and therefore consumption of raw milk is not recommended. Heating is applied to kill potential pathogens but also destroys some of the bioactive components of raw milk. Application of these components to support immune function is thus dependent on milk processing that ensures microbiological safety without disrupting their functionality.

Bovine IgG

Gamma immunoglobulin (IgG) antibodies from cow's milk can bind to respiratory pathogens such as RSV and influenza virus (148), and also to many bacterial pathogens and to allergens (128). Moreover, cows can be infected by the bovine RSV and thereby induce an anti-RSV response; the IgG antibodies produced can prevent infection of human cells with human RSV *in vitro* (127), and prevent RSV infection in an animal model (147) (**Figure 2**).

The possible role of IgG in relation to other respiratory viruses is not well known. In addition to RSV, cows encounter respiratory coronaviruses (150–152). Cows therefore have coronavirus-specific antibodies, which may also be able to bind cross-reactively to SARS-CoV-2, as can IgA against SARS-CoV-2 from breast milk of infected (153) as well as vaccinated (154) women.

Bovine IgG, like raw cow's milk, can induce trained immunity (122, 155), a concept that is discussed earlier (**Figure 2**). Through this mechanism, milk IgG antibodies may also provide protection against other pathogens. It is not yet known whether this also works against respiratory infections, but it is known that bovine IgG-induced trained immunity, as well as lactoferrin supplementation in elderly women, enhances subsequent innate immune responses *via* TLR7, a key receptor in immunity against single stranded RNA viruses (122, 155, 156).

Lactoferrin

Lactoferrin is an iron-scavenging immunomodulatory protein found in milk, but also in serum and other bodily fluids. It has anti-infective effects, including antibacterial and antiviral effects (110, 157, 158) (**Figure 2**). The antiviral properties of lactoferrin present in human breast milk and also in cow's milk make it a natural supplement with a potential to be used to help protect against respiratory viruses. Lactoferrin's benefits as a contributor to innate defense, are well documented as supported by a recent meta-analysis (158). Based on the observation that lactoferrin can inhibit infection of cells with the original SARS virus (159), there has recently been a lot of interest in whether lactoferrin can do the same for SARS-CoV-2, but data on this are not yet available (160, 161). It is also known that lactoferrin can induce IFN-λ as an antiviral cytokine in intestinal epithelial cells (40), and can partially restore the deficient response of pDCs of elderly women to TLR7 stimulation (156).

Other Milk Proteins

Cow's milk also contains other proteins that may have an effect on the immune system, such as IL-10 (mostly in colostrum), transforming growth factor (TGF)-ß, osteopontin, MFGM8 (lactadherin) and lactoperoxidase. TGF-ß, IL-10 and osteopontin are anti-inflammatory components and do not play a direct role in viral infection, but can help prevent tissue damage arising from infection. Lactoperoxidase and MFGE8 have antimicrobial and possibly antiviral effects (143, 162, 163). These components have not been studied in relation to respiratory tract infection.

Human Milk Oligosaccharides (HMOs) and Prebiotic Oligosaccharides

As mentioned above, breastfeeding is known to protect against gastrointestinal and respiratory tract infections in infants (132, 164). The concentrations of human milk oligosaccharides (HMOs) in breastmilk are inversely associated with prevalence of gastrointestinal and respiratory tract infections (165), and HMOs and prebiotic oligosaccharides have been shown to enhance immune responses to vaccination or infection (166–171). HMOs play a role in the maturation of the immune system in early life, partly because of their influence on the microbiota composition and the production of microbial metabolites such as short-chain fatty acids (SCFAs) (129, 172, 173). The composition of the intestinal microbiota has been linked to the prevalence of respiratory infections (174–177). The effect of HMOs may in part be indirect *via* an effect on Bifidobacteria (178).

Breastfeeding and early life nutrition may also influence respiratory infections through interaction with the microbiota in the nasopharynx. The composition of the microbiota in the nasopharynx of infants is associated with the occurrence of respiratory tract infections (179, 180) and asthma (181). Furthermore, in breastfed children microbial communities in the nasopharynx are significantly different from those of bottle-fed infants (182), and the early nasopharyngeal microbial composition is linked to reduced risk for respiratory infection (183). These data suggest that breastmilk, and possibly HMOs, may affect the local microbiota composition in the nasopharynx of infants as well as that of the intestinal tract (**Figures 2**, **3**).

Although most of the oligosaccharides in breastmilk are fermented by bifidobacteria and other bacteria in the intestine or are excreted, a small fraction of these HMOs can enter the circulation (184–186), as do the SCFAs that are produced after fermentation - especially acetate and to a lesser extent propionate and butyrate (186, 187). These SCFAs are also induced after the fermentation of prebiotic oligosaccharides like galactooligosaccharrides (GOS), fructooligosaccharrides (FOS) and inulin, as previously reviewed in (188).

SCFAs have been shown to protect against the development of asthma (189–191), suggesting that they might also influence immune responses to respiratory pathogens. Indeed, Trompette et al. showed in a murine influenza model that a high fiber diet and resulting increased intestinal SCFA levels led to decreased neutrophil recruitment to the airways preventing tissue damage in acute infection, as well as enhancing CD8+ T cell responses (192). Recent evidence suggests that SCFAs play a role in the control of respiratory infections through direct action both on microbiota and on host immune signaling (193). Influenza infection itself can also alter the microbiota composition (194), which leads to decreased SCFA production enabling bacterial superinfection by a reduction in the bactericidal activity of alveolar macrophages (195).

In addition to these indirect effects of HMOs *via* the microbiota, sialyllactose (SL) that is also present in bovine milk, has long been known to inhibit the binding of human influenza haemagglutinins to target cells (43, 44, 196, 197) (**Figure 2**). Likewise, HMOs that prevent bacterial adhesion to the respiratory epithelium may play a role in preventing bacterial pneumonia, as was shown for the HMO Lacto-N-Neotetraose (LNnT) in an animal model of pneumococcal pneumonia (198).

At the level of the respiratory epithelium, the HMO, 2'fucosyllactose (2'FL) could decrease the epithelial viral load and cytokine production after RSV exposure, and LNnT and 6'SL reduced viral load and cytokine production of epithelial cells after exposure to influenza virus (45), suggesting that these HMOs prevent infection of epithelial cells by these viruses directly by preventing adhesion. Interestingly HMOs can also enter the amniotic fluid (199), and may, after 'inhalation' of the fluid by the fetus, protect against respiratory infections like RSV and influenza in the first days or weeks after birth, as was also proposed for amniotic fluid antibodies by Jacobino et al. (200).

To date several studies have shown a reduction in respiratory infections by inclusion of prebiotics in infant formulas (201–205), and GOS supplementation was reported to reduce the duration of the common cold in healthy students (206). As HMOs have only recently been introduced into infant formulas, so far only a single study has addressed their effect on respiratory infections. In an infant study with formula supplemented with 2'FL and LNnT, parents reported reduced bronchitis, lower respiratory tract infection and less antibiotic use (207). In a follow-up study, infants without bronchitis or lower respiratory tract infections had increased levels of acetate and B. longum subsp. infantis in their stools, which suggests a causal relationship between HMOs, SCFAs and protection against bronchitis (178).

PLANT-DERIVED NON-DIGESTIBLE POLYSACCHARIDES, MICROBIOTA, IMMUNE FUNCTION AND RESPIRATORY INFECTION

Non-digestible polysaccharides (NDPs) constitute a large group of molecules that are found in many different foods and have been demonstrated to broadly and effectively support health and immune activity (208). NDPs include ß-glucans, pectins, resistant starch, arabinoxylans and many other types of NDPs extracted from plants (e.g. ginseng, carrot, oat), fungi (e.g. *Saccharomyces cerevisiae, Lentinula edodes*) or bacteria (e.g., *Alcaligenes faecalis*). Many *in vitro* and preclinical studies have demonstrated the immunomodulatory potency of NDPs [expertly reviewed by Ferreira and colleagues (209) and Jin and

colleagues (210)], and clinical studies confirmed some of these findings. Double-blind placebo controlled clinical studies have demonstrated that oral intake of some NDPs (e.g. β-glucans) reduces incidence and duration of upper respiratory tract infections (URTIs) in elderly subjects or athletes (211–214).

Athletes typically demonstrate enhanced frequencies of symptoms of URTIs following intensive exercise (215). Several studies were performed to investigate whether NDP administration could lower incidence of URTIs in athletes. In a first study these subjects were provided with an insoluble ß-glucan derived from the mushroom *Pleurotus ostreatus* (211). A daily intake of 200 mg ß-glucan mixed with 200 mg vitamin C was compared to the placebo intake of only 200 mg vitamin C. During three months of supplementation, subjects in the test group reported a total of 65 episodes of URTI, which was significantly less than the 117 episodes reported by subjects in the placebo group. These results might have been related to the reduced drop in phagocytic potential of PBMCs and increased frequency of NK cells in the PBMCs. In a similar setting, healthy marathon runners were provided a dairy drink as placebo or a dairy drink containing 250 mg per serving per day of insoluble or soluble ß-glucan from yeast for 91 days (213). URTI symptoms were scored according to a validated questionnaire and confirmed by a physician. The total severity of URTI symptoms over the test period was significantly lower for the subjects provided with the insoluble ß-glucan. A final study involving athletes also investigated the administration of insoluble ß-glucan from yeast (216). The ß-glucan was provided in capsules, as was the rice flour placebo, at a dose of 250 or 500 mg per day for four weeks; a significantly reduced number of subjects reported URTI symptoms after two and four weeks of β-glucan when compared to the control group.

In other study population (with older people) similar effects were observed, demonstrating that the beneficial effects are not related to this specific metabolism or physical state of athletes. Older people with an approximate age of 59 years (212) or women selected according to experiencing moderate levels of stress (217) where provided with 250 mg per day of an insoluble yeast ß-glucan for 90 days or rice flour as placebo in capsules. In the older people, the intake of ß-glucan resulted in a trend towards fewer illness episodes and fewer days of illness and in women with above average stress levels β-glucan resulted in a significant reduction in days of symptoms and fewer subjects with URTI symptoms during the test period. Similarly, ß-glucan supplementation in healthy adults reduced severity and duration of URTI symptoms (218, 219).

With ginseng extracts enriched for NDPs, it has been shown in a number of clinical trials that addition to food resulted in support of immune function (220–222) and reduced incidence and severity of viral respiratory infections (223, 224). Research has shown that so-called RG-I fibers (Rhamnogalacturonan-I, referring to specific domains of pectins) are responsible for this effect of ginseng. It has now become clear that RG-I fibers from various plant sources have a modulating effect on immune function and on the microbiota (225, 226). A recent randomized controlled trial explored a low or a higher dose of cRG-I from carrot as a dietary supplement in heathy volunteers (227). After 8 weeks, the volunteers were infected with a low dose of rhinovirus in their nasal cavity and then the immune response and the development of cold symptoms were monitored. Such studies in which the effect of a nutritional intervention on the course after a (viral) challenge is measured in humans are seen as particularly relevant by experts and regulators (228, 229). Consumption of cRG-I was found to greatly accelerate the anti-viral IFN response in infected nasal epithelium and the local innate immune response, resulting in a significantly milder clinical course with less severe and shorter cold symptoms (227).

Fluorescently labelled NDP particles, being either ß-glucan capsules or particulate ß-glucan, were demonstrated to reach the ileum, colon, liver and lung and macrophages in spleen, lymph nodes and bone marrow upon oral administration in mice (230–233). In a different study, radiolabeled NDPs were shown to reach blood, intestine, and spleen and accumulated in tumors in mice (234). These studies confirm that NDPs interact with immune cells of the intestine and that they do not only move to the colon for fermentation. Moreover, these studies show that NDPs can become available in distant peripheral tissues where they can interact with local APCs and exert their immunomodulatory effect. There is clear evidence that molecular patterns on NDPs are recognized by PRRs of the immune system like Dectin-1 while actively "sensing" the contents of the gut (235, 236). This recognition and the subsequent innate immune training or direct immune activation are manners through which NPDs could modulate peripheral macrophage activity (**Figure 2**). Human trials to investigate induction of innate immune training so far were limited to i.v. introduction of NDPs rather than oral intake. To date only a single clinical trial has been performed to investigate orally mediated innate immune training by NDPs. Unfortunately, in this study ß-glucan was barely detectable in blood and blood-derived immune cells did not demonstrate signs of innate immune training (237). Over the past decade many NDPs, including arabinoxylans, ß-glucans and pectins, have been tested for their potency to activate innate immune cells. NDP exposure to peripheral blood monocyte-derived macrophages, either anti- or proinflammatory, or pro-tumorogenic (i.e. TAMs) induced a distinct transcriptional profile (238) and a secretome rich in chemokines and increased co-stimulatory receptor expression (239, 240). Of note, studies have demonstrated that NDP characteristics of solubility, size, monosaccharide composition and side chains (226, 239, 241) were crucial to induce immunomodulation *in vitro* or *ex vivo*.

Taken together, NDPs have been demonstrated to modulate immune responsiveness beyond the GI tract and to support APCs in expressing co-stimulatory receptors and releasing pro-inflammatory cytokines through training and in addition improve recruitment of additional immune cells.

PRO AND SYNBIOTICS

The gut and airway microbiota are linked to respiratory tract health and immunity ('gut-lung axis') and modulation by

pharmacological agents and pro- and synbiotics has in some studies been shown to manage RTIs (242). One implication of the dynamic bidirectional relationship between gut microbes and the immune system is that changes in the composition and metabolic activity of the gut microbiota can alter immune function, potentially altering host susceptibility to infection including within the pulmonary system.

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (243). The most commonly used probiotics are Lactobacillus and Bifidobacterium species, followed by the genera Streptococcus, Enterococcus, Propionibacterium, Bacillus, and Escherichia coli (244, 245). In addition, some yeast species are used as probiotics, for example, *Saccharomyces boulardii* and *Saccharomyces cerevisiae* to treat gastrointestinal disorders (246, 247). Generally, probiotics do not extensively divide and therefore do not permanently colonize the gut. In the gut of children, probiotics can persist for up to 4 weeks (248, 249) as they have a relatively stable ecology, while in the gut of adults probiotics persist for only around a week after consumption (250).

Besides their well-described effects in the gastrointestinal tract and in allergic diseases, probiotics also display clinical effectiveness in respiratory tract infections and support lung immunity and inflammation in both children and adults (251). Clinical studies have shown that certain probiotic strains help to prevent bacterial and viral infections, including gastroenteritis, sepsis, and RTIs (252) although others did not find such effects (253). This dynamic bidirectional relationship between gut microbiota and the immune system is sensitive to changes in the composition and metabolic activity of the gut microbiota. This also influences the pulmonary system and alters general host immune competence and thereby susceptibility to infection.

The potential for the use of probiotics has been shown in different studies including a double-blind placebo-controlled trial, in which milk was supplemented with the probiotic *Lactobacillus reuteri*, but not *Lactobacillus casei*, which showed that acute infectious diarrhoea could be reduced in Indonesian children with a low nutritional status (254). Probiotics such as Lactobacillus and Bifidobacterium have shown direct potential in respiratory infections as illustrated in a meta-analysis in which they were able to increase the efficacy of influenza vaccines in adults (255). These probiotics enhanced the rate of seroconversion in older individuals and thereby have potential to be used as preventive treatment before seasonal influenza vaccination. In a systematic review it was shown that the use of selective probiotics and synbiotics in optimized formulations can provide prophylactic and complementary treatment benefits in patients with RTIs, including COVID-19 and influenza infections as they reduce the severity of the infection symptoms and the duration of disease (256).

Probiotics, including *Lactobacillus plantarum* and *Lactobacillus reuteri*, are mainly used to prevent infections and for recovery from gastrointestinal infections and are not yet widely used to prevent or treat URTIs, although some studies have shown them to alleviate symptoms of URTIs by strengthening the respiratory mucosa (257, 258). In recent meta-analyses of double-blind, randomized, and placebo-controlled trials it was shown that probiotics significantly reduced rates of acute URTI and the associated antibiotic use, but they did not decrease the duration of each single infection episode (259, 260). Children that were supplemented with probiotics had fewer days with URTIs and therefore had fewer days absent from day care or school compared with children receiving placebo (234, 245).

Probiotics can modulate the plasma levels of cytokines and exert differential modulation of innate and the adaptive arms of the immune response based on host (epi)genetic differences and the particular microbial strain (251, 261). Dietary supplementation with probiotics like *Bifidobacterium longum*, can stimulate trained immunity in alveolar macrophages, putatively by the NOD2 receptor and the action of histone 3 lysine 4 trimethylation (H3K4me3) to induce epigenetic changes at the level of histones. These macrophages respond more efficiently with increased IFN-γ production to respiratory pathogens like influenza as observed in mice that received these beneficial microorganisms. In these mice, NK cell activity was significantly increased, resulting in enhanced levels of IFN-γ, IL-2, IL-12, and IL-18 in both spleen and lungs resulting in decreased virus proliferation and suppression of inflammation (262). At the same time, the probiotic *Bifidobacterium longum* significantly reduced influenza-infection induced production of pro-inflammatory cytokines, including IL-6 and TNF-α.

Probiotics therefore may promote resistance against pathogens (261). Probiotics increase leukocyte, neutrophil, and NK cell counts and activity while increasing the production of IL-10 and decreasing production of pro-inflammatory TNF-α, IL-1ß, and IL-8 (263). It has been shown that probiotics inhibit Th17 differentiation and the production of IL-17F, IL-23 and TNF-α, while promoting the IL-10 secreting Treg subset (246). Thereby, probiotics inhibit inflammatory responses and regulate immune cell homeostasis. In addition, probiotics increase the frequency of IgA-positive cells in Peyer's patches in the lamina propria and thereby inhibit bacterial adherence to epithelial cells, maintain the epithelial barrier function, and facilitate the neutralization of bacterial toxins (264). Because IgA-positive B-cells were shown to be able to migrate to other mucosal tissues in the body, their potential to provide protection in the respiratory tract should be investigated further.

Likely candidates responsible for the functional impact of the microbiota and probiotics are SCFAs. Through their activity as histone deacetylase (HDAC) inhibitors, SCFAs might also induce innate immune memory. Mainly butyrate and propionate, and to a lesser extend acetate, demonstrate this inhibitory activity. SCFAs have been demonstrated to affect the immune system in the respiratory tract, for example with influenza infection (192), but also RSV infection (265). In turn, this also means that fibers and complex sugars that lead to the growth of healthy bifidobacteria and lactobacilli, as well as the production of SCFAs, can be important for antiviral immunity. Moreover, production of SCFAs also induced enhanced expression of respiratory IFN-β thereby increasing the expression of interferon-stimulated genes (ISGs) in the lung and thereby protected against RSV-induced disease involving activation of the membrane receptor GPR43 (265).

A profound imbalance in gut microbiota can drive the progression of COVID-19 symptoms towards the acute respiratory distress syndrome (ARDS), and this development might be reinforced by the use of antiviral medication (266). Respiratory infectious diseases might thus be affected by gut dysbiosis characterized by a decline in beneficial commensals and enrichment of opportunistic pathogens (242, 267). Administration of a *Lactobacillus plantarum* 06CC2 in mice with influenza-like disease, decreased expression of pro-inflammatory cytokines and increased the mRNA level of IFN-β, IFN-γ, OAS1a, and ISG15 in the lungs underscoring the antiviral activities of this probiotic (268). Bradley et al. showed that the intestinal microbiota influences IFN-α/β receptor expression on the surface of respiratory epithelial cells, which in the case of respiratory virus infection are able to respond more efficiently to type I IFNs stimulation with enhanced ISG levels and suppressed early virus replication (269). Some studies suggested there is transfer of bacterial strains like *Lactobacillus plantarum* from the gut to the lungs upon development of COVID-19 (270). Next to these demonstrated effects, probiotics are also anticipated to dampen the cytokine storm observed in COVID-19 patients, especially in severe stages of the disease, which was associated with increased levels of cytokines and chemokines like IP-10, MCP-1, MIP-1α, TNF-α, IL-1β, IFN-γ, IL-4 and IL-10 (268).

Probiotic supplementation could inhibit the COVID-19 cytokine storm by simultaneously boosting innate immunity and avoiding the exaggeration of adaptive immunity by the suppression of the inflammatory cytokine response. This way, probiotics can prevent the severity and the occurrence of ARDS. Taken together, modulation of gut microbiota by probiotics is suggested as a possible strategy to improve the clinical manifestations of respiratory tract infections and crucially also of COVID-19 (271).

Probiotics and prebiotics, as described above, individually have roles in improving immune health and in combination (synbiotics) might even synergize. The use of synbiotics, often a combination of *Bifidobacterium breve, Lactobacillus casei*, Streptococcus and galacto-oligosaccharides (GOS) or fructo-oligosaccharides (FOS) and inulin, is primarily based on the assumption that the included prebiotics enhance the survival of the probiotics present in the supplement in the gut as well as stimulating indigenous anaerobic bacteria. Gut microbial components and metabolites (postbiotics) including SCFAs, for instance, are involved in gut-lung immune communication (272).

As indicated earlier, the elderly population is particularly affected by a decline in immune function including the antibody response to influenza vaccination although in this population no beneficial effects in reversal of immunosenescence by synbiotics were found (273). In contrast, a synbiotic (Lactocare©) consisting of a mixture of *Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium breve, Lactobacillus bulgaricus, Bifidobacterium longum, Streptococcus thermophilus* (each at 10^9CFU) plus fructooligosacharide FOS was shown to reduce episodes of viral infection in asthmatic children (274).

A recent systematic review and meta-analysis identified 16 studies including 10,443 participants examining the effects of synbiotics on RTI incidence, duration, and/or severity. Results demonstrated that synbiotic interventions reduced the incidence rate of RTIs and the proportion of participants who experienced a RTI by 16% (275). Three separate large randomized controlled trials compared similar synbiotic supplements (containing 3 to 5 strains of *Lactobacillus plantarum, Lactobacillus rhamnosus*, and *Bifidobacterium lactis*, lactoferrin and prebiotics such as either FOS (short-chain fructooligosaccharides) or GOS (galactooligosaccharides)) to placebo, and all three of these trials showed reductions in the incidence, duration, and severity of RTI with the synbiotic (276). It was also proposed that this synbiotic could reduce SARS-Cov2 infection in high-risk medical staff working in COVID-19 hospital wards (277).

Such synbiotic interventions are suggested to be most effective in the prevention phase of a respiratory infection while they are mostly unable to influence the course of the infection once the pathogen has started to replicate in the host. They are therefore more a preventive than a therapeutic option. Nevertheless, a review describing mechanisms whereby probiotic and prebiotic interventions may link the gut microbiota to the outcome of COVID-19 infection provided arguments that synbiotics may help patients after infection with SARS-COV-2 (278) and this was based on clinical evidence indicating that modulation of the gut microbiota can positively influence COVID-19 progression. The consumption of synbiotic products may thus reduce incidence, duration and severity of respiratory tract infections (279). It would be very interesting to see if the consumption of pre- pro- or synbiotics – or other components discussed in this review - can partially reverse the loss of smell, that is associated with a loss of appetite, and hinders recovery after SARS-CoV-2 infection.

ANATOMICAL AND PHYSIOLOGICAL ASPECTS OF INTERACTION OF NUTRITION WITH THE IMMUNE SYSTEM IN THE RESPIRATORY TRACT

Micronutrients, milk-derived components, plant derived NDPs and pre-, pro- and synbiotics have all been demonstrated to support anti-viral immunity in *in vitro* and *in vivo* animal models as well as in some clinical studies.

Likewise, oral vaccines can lead to immune responses in the airways, suggesting that the oral route of administration is interconnected with the upper airways. Oral immunization with cholera toxin results in specific IgA being produced in the upper airways, the lymphoid tissues in the head/neck area, the digestive system, as well as in breast tissue [(280) and **Figure 2**]. These data indicate that oral intake of cholera toxin can induce immune responses that also target the upper respiratory tract. Several studies have since then confirmed that oral administration of antigens result in IgA production in the upper respiratory tract (281–283).

However, there appears to be a knowledge gap on how nutritional components in the gastrointestinal tract can affect (viral) infections in the respiratory tract. Here, we introduce a

hypothetical model of how many of these food components can have an effect on respiratory tract infections, keeping in mind the anatomy of the GI and respiratory tracts, interaction sites of food and food components with the immune system, and the interplay with the microbiota.

Nutritional components that are absorbed in the GI tract can exert their effects on the local immune system, but *via* systemic circulation also at distant immune sites. Mostly non-absorbed nutrients can have local effects, or can have distinct effects through instructing migratory cells of the immune system, or induction of microbial metabolites, that can become available in the circulation and can reach distant tissues. These local effects can occur before the food reaches the stomach and is digested, and can, in addition to immune effects, have anti-adhesive effects on swallowed respiratory pathogens.

The nasopharyngeal mucosa is an environment that is heavily populated with commensal organisms, some of which are associated with increased or decreased risk for respiratory infections (179, 180, 183). As discussed in the oligosaccharide section, breastmilk-derived HMOs can influence the microbiota in the nasopharynx, thus reducing the risk for respiratory infections *via* a local effect (182).

Because of their anatomical location, the immune tissues of Waldeyer's ring could have a crucial role in developing potent immune responses to respiratory pathogens (**Figure 3**). The immunological tissues in the head/neck area are mainly structured in tonsils, lymph nodes and lymph follicles. The largest area of contact between food and nasal contents and the immune system occurs in the area where nose and mouth come together, the nasopharynx. The pharynx is surrounded by a number of structured lymphoid nodules (tonsils), comprising the lingual tonsil, the nasopharyngeal tonsils (adenoids), the palatine tonsils and tubal tonsils. The tonsils contain large numbers of cells (1 x 10^9 lymphoid cells) mainly consisting of B-cells and CD4+ T-cells (284). Together these are named Waldeyer's ring, also referred to as the nasal mucosa associated lymphoid tissue (NALT).

The tonsils and adenoids have multiple antigen-retaining crypts, that greatly increase the surface area of contact between food components and the underlying cells. The reticulated epithelium lining the tonsillar crypts is probably an antigen entry portal, specialized in transporting antigens to the subepithelial tissue within on average about 10 minutes after food consumption (284–286). The adenoids, but to a much lesser extent the palatine tonsils, have epithelia that have well developed tight junctions, and that also contain M-like cells and DCs that contribute to antigen sampling (287, 288). Because of the anatomical structure and location of Waldeyer's ring, it functions as a gatekeeper for respiratory, swallowed pathogens from nasal secretions. Likewise, it is also exposed to nutritional components that locally can modulate the immune responses to respiratory pathogens sampled in Waldeyer's ring (**Figure 3**).

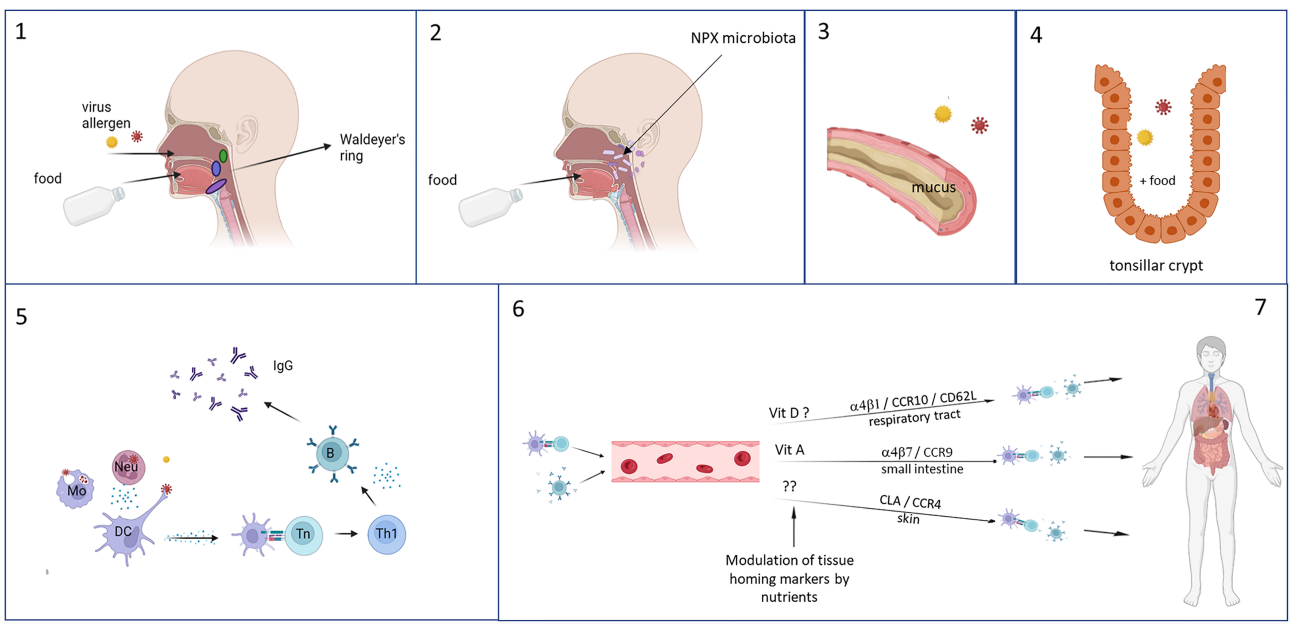

FIGURE 3 | Foods are ingested and enter the gastrointestinal tract *via* the mouth and respiratory pathogens *via* the nasal cavity, both ending up in the tonsils of Waldeyer's ring (1). Especially in infants, reflux can cause a direct interaction of the foods with the nasopharyngeal microbiota and influences their composition (2). Mixing of the food with saliva and mucus (based on charge and polarity) enhances the residence time in the nasopharynx (3), and as a result the still undigested food and respiratory pathogens can interact with each other and the immune tissues in the tonsil, where stasis occurs in the crypts which further lengthening the duration of interaction (4). Local immune responses can occur, during which can be modulated by the food components (5). Immune cell recirculate and home to different tissues – especially to the upper respiratory tract because of instruction by stromal cells in the tonsils – which is determined by the expression of homing receptors. This can also be influenced by food components, selectively homing, for example by vitamins, resulting in increased memory responses in the local tissues of the upper respiratory tract (6). This figure was created with BioRender.com.

As stromal factors determine tissue homing properties of locally activated lymphocytes, it has been suggested that antigens taken up in the tonsils of Waldeyer's ring will induce the formation of especially IgG-antibody producing memory B cells with homing capacity to lungs, upper airways and peripheral blood (289).

Selective homing of immune cells to the respiratory tract and the intestine is tightly regulated by the expression of chemokine receptors and adhesion molecules (281, 289–292). Of note, expression of homing receptors can be influenced by interactions with food components, which may also influence recirculation and homing properties of immune cells. For example, the vitamin A metabolite retinoic acid induces expression of CCR9 and thus promotes homing to the intestine (293, 294), and vitamin D3 induces CCR10 that is associated with homing to the respiratory tract (81–84). Interestingly, vitamin D3 has also been shown to block the upregulation of retinoic acid-induced gut homing markers (82, 295) and may thus promote homing to the respiratory tract.

A further aspect to consider is the food matrix. The food matrix may affect whether the nutritional components in the food can mix with saliva and the mucus overlaying the nasopharyngeal epithelium and Waldeyer's ring. This mixing will increase the duration of retention of foods in the oral and nasopharyngeal mucosa. A longer retention time will increase interaction of these food components with the underlying epithelium and cells of the immune system in Waldeyer's ring.

Two known examples that can promote residence time and mixing with mucus are charge and water in oil emulsions. Mucins have sialic acid and sulfate groups located on terminal side chains on the glycoprotein molecules, as a result of which they are anionic (negatively charged) at neutral pH (296, 297). Positively charged chitosan microparticles strongly enhance systemic as well as local immune responses to diphteria toxin when applied orally, and significantly enhance IgG production after nasal administration (298). Similar results were obtained with ovalbumin bound to cationic (i.e. positively charged) maltodextrin nanoparticles (299). In addition, water in oil emulsions and gels are other options to increase mouth and GI tract presence of compounds of interest (300). Thus, positively charged components – and ideally a matrix (particulate or viscous) that surrounds ingredients by a positive charge - can enhance the mixing behavior with mucus and saliva, and are expected to result in enhanced interaction between the food product and the mucosal immune system of Waldeyer's ring.

The above rationale suggests that foods or food components may exert their immunomodulatory function locally in Waldeyer's ring, thus explaining their effects on respiratory infections.

CONCLUDING REMARKS

Nutritional components can support immune function against respiratory pathogens *via* several mechanisms, and at several levels as described in this review. Nutritional approaches to prevent infections can thus be relevant to help prevent infections in vulnerable people like infants and the elderly, and can help to reduce the pressure on healthcare systems, especially in the winter months when respiratory infections occur frequently.

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Disparity of Gut Microbiota Composition Among Elite Athletes and Young Adults With Different Physical Activity Independent of Dietary Status: A Matching Study

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Xu Y, Zhong F, Zheng X, Lai H-Y, Wu C and Huang C (2022) Disparity of Gut Microbiota Composition Among Elite Athletes and Young Adults With Different Physical Activity Independent of Dietary Status: A Matching Study. Front. Nutr. 9:843076. doi: 10.3389/fnut.2022.843076

Objective: This study aimed to investigate the disparity of gut microbiota among elite athletes and young adults with different physical activity independent of dietary status.

Methods: In Hangzhou, China, an age and sex matching study was conducted between April and May 2021. A total of 66 Chinese young adults were recruited in this study and divided into an elite athlete group, physically active group, and physically inactive group. Fecal samples were collected to assess gut microbiota composition. Dietary status was measured using a food-frequency questionnaire. Comparisons in gut microbiota and blood biomarkers among three groups were analyzed by using the analysis of covariance.

Results: The findings depicted a tendency to form clusters for beta diversity among three groups, while no significant difference was observed in both alpha and beta diversity. In the multiple analysis model, by adjusting dietary status, a significantly higher abundance of *Clostridiaceae* ($p = 0.029$) and *Megamonas_rupellensis* ($p = 0.087$) was observed in elite athletes compared to that in general young adults. Furthermore, inflammation-related bacteria such as *Bilophila* ($p = 0.011$) and *Faecalicoccus* ($p = 0.050$) were enriched in physically inactive young adults compared to two other groups. Pearson's correlation analysis showed a positive association between *Bilophila* and circulating white body cell count ($r = 0.332$, $p = 0.006$) and its subtypes including neutrophils ($r = 0.273$, $p = 0.027$), and lymphocytes ($r = 0.327$, $p = 0.007$). *Megamonas_rupellensis* has been shown associated positively with serum lymphocytes levels ($r = 0.268$, $p = 0.03$). Although no significant differences were observed, the elite athletes tended to have lower levels of blood biomarkers of immunity within a normal range, which may reflect a better immune function.

Conclusion: This matching study indicated that physically inactive young adults are more likely to have a lower immune function and a higher abundance of pro-inflammatory gut bacteria than elite athletes and physically active young adults. Dietary status should be considered as an important factor that may affect the association of physical activity with immune function and gut microbiota.

Keywords: gut microbiota, dietary status, physical activity, elite athlete, inflammation, matching study

INTRODUCTION

Humans are superorganisms composed of human bodies and commensal microbiota (1). The human gut microbiota is approximately 100 trillion organisms, outnumbering the human cells by an estimated 10-fold (2–4). Increasing evidence suggests that there is a close relationship between gut microbiota composition, inflammation (5, 6), and immunity (7, 8). A systematic review showed that gut microbiota is thought to contribute to subacute systemic inflammatory and it may also be influenced by outcome, thereby reinforcing the disease symptoms (9). Some gut microbiota may be associated with pro-inflammatory effects, while others have anti-inflammatory properties. As potential anti-inflammatory microbiota, *Lachnospiraceae* and *Ruminococcus* are thought to prevent or ameliorate chronic hepatitis B (10). Conversely, pro-inflammatory gut microbiota, such as *Bacteroides fragilis*, were highly enriched in the gut of inflammatory arthritis (11). In addition, the *Bacteroides fragilis* toxin was also reported to be pro-inflammatory and carcinogenic (12). Thus, gut microbiota could be considered an important indicator of infectious and metabolic diseases.

It is well-known that people with high physical activity levels tend to have better immune function and lower levels of inflammation, which may help them improve health status and prevent diseases. Evidence showed that physically active adolescent girls had lower circulating IL-6 concentration compared to their sedentary counterparts (13). Moreover, a recent study reported that higher physical activity levels may be associated with lower salivary C-reactive protein in young adults (14). Studies regarding the relationship of exercise habits with inflammation-related gut microbiota are emerging. The new literature enables a better understanding of the underlying mechanisms of the association between physical activity and inflammation. Higher proportions of *Akkermansiaceae* family and *Akkermansia* genus in elite rugby athletes were detected than in overweight or obese young adults (15). Meanwhile, our previous randomized controlled trial showed that 8 weeks of combined aerobic and resistance exercise not only resulted in improved physical function but also increased abundance of anti-inflammatory bacteria and decreased pro-inflammatory bacteria in physically inactive older women (16). Even with these findings, the evidence exploring the distribution of inflammation-related gut microbiota in people with different physical activity levels is limited, and further studies are warranted.

Conversely, dietary status and nutrient intake are very important and non-negligible indicators when discussing physical activity levels and gut microbiota. Diet is considered to be a pivotal factor affecting the genes and composition of gut microbiota, of which the number, type, and balance of macronutrients (fats, proteins, and carbohydrates) all have a significant impact on gut microbiota (17). In addition, data indicated that the consumption of nuts, oily fish, fruit, vegetables, and cereals is linked to a higher abundance of anti-inflammatory bacteria (18). Meanwhile, dietary status was also shown to be associated with physical activity levels. Previous studies indicated that habitual Mediterranean dietary pattern is associated with more time spent on leisure-time physical activity among older adults (19). Momentary physical activity was concomitant with momentary consumption of both healthy and unhealthy dietary intake in college students (20). Thus, it is hypothesized that dietary status may play a contributory role in the association of physical activity with gut microbiota. However, most previous observational studies and exercise intervention trials have failed to confirm the relationship between physical activity and gut microbiota composition concerning independent of dietary status (15, 16, 21).

Therefore, this matching study aims to investigate the differences in the diversity and composition of gut microbiota among elite athletes and the general young adults with different physical activity levels, as well as explore whether the association between physical activity and gut microbiota are independent of dietary status.

MATERIALS AND METHODS

Participants

An age and sex matching study was conducted between April 2021 and May 2021 in Hangzhou, China, and aimed to examine the difference of gut microbiota, anthropometry, life-behavior habits, and physical performance between athletes and physically active or inactive young adults. All elite athletes are engaged in track and field events (including 400 m, 1,500 m, high jump, long jump, discus, javelin, and pole vault), and had at least 8 years of professional training experience as well as participated in official sports events held by the Chinese Athletics Association or International Association of Athletics Federation.

Thirty young elite athletes were included in this study according to the following inclusion criteria: (1) 18–25 years old; (2) no history of cardiovascular diseases; (3) no history of congenital diseases; (4) no history of physical dysfunction. Participants from the control group included general young adults who met the physical activity recommendation levels according to the guidelines of the American College of Sports

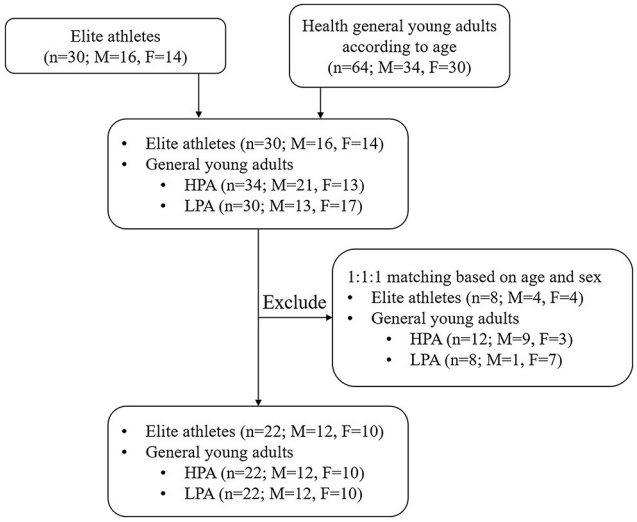

FIGURE 1 | Schematic presentation of the flow of participants through screening.

Medicine (22). A total of 64 general college students were invited to participate in this study. Among them, 34 students engaged in more than 150 min of moderate-intensity physical activity every week, and 30 students did not engage in any moderate or vigorous-intensity physical activity per day. Physical activity was assessed using the International Physical Activity Questionnaire (IPAQ), which has good reliability and validity for assessing physical activity in healthy adults (23). Thus, 94 elite athletes and college students were allowed to enter the matching process. These participants were then divided into three groups (Athlete group; High physical activity group, HPA; and Low physical activity group, LPA) according to equal proportion principle with age (±24 months) and sex. Finally, a total of 66 young adults were enrolled in the present study (**Figure 1**). The study protocol and all amendments were approved by the Ethics Committee of the Department of Psychology and Behavioral Sciences, Zhejiang University. All individuals provided written informed consent to participate in the study.

Dietary Evaluation

In the present study dietary information was collected by food-frequency questionnaire (FFQ25) which was demonstrated to have good reproducibility and validity (24). The FFQ25 primarily investigates the food intake of participants over the past week and contains 25 categories of foods. The frequency of intake of each food is divided into nine levels, from never eating to at least three times a day. The amount of food consumed is divided into five levels, from no more than 50 to at least 250 g a meal. Further, the frequency and amount categories were calculated as times per day by using a midpoint (e.g., "3–4 times per week" was used in the calculations as 3.5 times per week). Finally, all kinds of foods were divided into six groups: cereals, vegetables and fruits, meat, beans and nuts, fatty food, and alcohol.

Feces Samples Collection and DNA Extraction

The fecal samples were collected by the participants themselves, and the sample collection supplies and methods are uniformly provided and guided by an experimenter before the start of the sample collection. All samples were stored in dry ice at −80°C and transported to Huada Gene Detection Center for DNA extraction. The DNA of the intestinal microbial community was extracted using MagPure Stool DNA KF kit B (Magen, China). The Qubit® dsDNA BR Assay kit (Invitrogen, USA) was used to quantify the DNA with a Qubit Fluorometer. The regions V3–V4 of gut microbiota 16S rRNA genes were amplified using the degenerate PCR primers, 341F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Illumina adapter, pad, and linker sequences were added to both forward and reverse primers. A 50 μL reaction containing a 30-ng template, fusion PCR primer, and PCR master mix was used for PCR enrichment. The following were the PCR cycling conditions; 94°C for 3 min; subsequently, 30 cycles of 94°C for 30 s; 56°C for 45 s; 72°C for 45 s, and final extension at 72°C for 10 min. AmpureXP beads were used to purify the PCR products, which were then eluted in the Elution buffer. The Agilent 2,100 bioanalyzer (Agilent, USA) was used to qualify the libraries. Validated libraries were sequenced on the IlluminaMiSeq platform (BGI, Shenzhen, China) according to Illumina's standard procedures, and 2 × 300 bp paired-end reads were generated. The raw reads were filtered to detach adaptors, low-quality, and ambiguous bases to get the tags (25). In addition, The tags were clustered into OTUs using UPARSE software (v7.0.1090) (26). UCHIME (v4.2.40) was used to compare the chimera sequence with the Gold database (27) and classify the representative sequences of OUT by QIIME v1.8.0 (28). All tags were compared with OTU using the USEARCH global (29), and a statistical table of OTU abundance for each sample was obtained. We used MOTHUR (v1.31.2) (30) and QIIME (v1.8.0) (28) to evaluate alpha and beta diversity, respectively. Bar and Heat maps of different taxonomic levels were drawn using R package v3.4.1 and the R package "gplots," respectively. Linear discriminant analysis effect size (LefSe) was used to conducted LEfSe cluster or LDA analysis. In addition, the Wilcox-test or Kruskal-Test was used as statistical analysis for significant species.

Blood Parameters Measurements

Blood samples were collected for the measurement of biomarkers of immune function and inflammation. The laser flow cytometry was used to measure white blood cell count and the respective subtypes including basophils, eosinophils, neutrophils, lymphocytes, and monocytes. Immunoturbidimetry was used to determine C-reactive protein and immunoglobulin (Ig) G and IgM. The hematology analysis was completed by Adicon Clinical Laboratories, Hangzhou, China. In our study, 0.1 g/L was used as if the value of C-reactive protein had been measured <0.2 g/L occurs (31).

Statistical Analysis

All data analyses were performed using SPSS Statistics version 23.0 (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov

TABLE 1 | Characteristics of the participants.

Characteristics	Athlete group ($n = 22$)	HPA group ($n = 22$)	LPA group ($n = 22$)
Age, year (SD)	21.55 ± 2.42	21.27 ± 2.47	21.27 ± 2.51
Male (%)	12 (54.5)	12 (54.5)	12 (54.5)
BMI, kg/m^2 (SD)	20.67 ± 5.26	22.28 ± 2.71	21.89 ± 2.81
PA, METs· hour/week	138.50 ± 108.26	59.13 ± 29.96	20.25 ± 13.17
Muscle mass, kg (SD)	27.45 ± 6.51	23.31 ± 6.44	20.52 ± 6.02
Fat mass, kg (SD)	9.70 ± 4.41	12.99 ± 4.76	15.06 ± 6.72
Fat percentage, % (SD)	14.30 ± 5.26	20.47 ± 6.67	24.08 ± 8.46
Grip strength, kg (SD)			
Left	37.34 ± 9.81	33.41 ± 10.42	29.22 ± 9.33
Right	39.15 ± 10.58	34.84 ± 11.70	29.71 ± 7.77
Standing long jump, m (SD)	2.46 ± 0.29	1.89 ± 0.32	1.77 ± 0.34
Sit-and-reach, cm (SD)	18.26 ± 8.68	14.54 ± 9.07	8.33 ± 7.82
One-leg standing with eyes closed, sec. (SD)			
Left	30.42 ± 30.07	24.67 ± 23.21	24.39 ± 26.41
Right	35.13 ± 31.24	24.72 ± 23.27	15.57 ± 10.36
Harvard step test (SD)			
Completed, *n* (%)	21 (95.45)	15 (68.18)	7 (31.82)
Harvard step index	96.56 ± 18.43	76.75 ± 18.81	56.39 ± 24.75
Daily diet, g/d (SD)			
Cereals	164.07 ± 142.82	122.70 ± 111.51	44.09 ± 29.50
Vegetables and fruits	320.38 ± 225.33	201.82 ± 128.26	120.08 ± 107.19
Meat	642.65 ± 438.48	319.17 ± 207.61	390.34 ± 339.50
Beans and nuts	31.06 ± 36.27	18.41 ± 22.43	34.55 ± 46.32
Fatty food	62.35 ± 74.98	39.47 ± 49.02	37.95 ± 45.93
Alcohol	41.36 ± 99.61	10.98 ± 29.67	24.31 ± 101.23

PA, physical activity; HPA, high physical activity; LPA, low physical activity; BMI, body mass index; SD, Standard deviation.

test was used to assess the normal distribution of variables before statistical analysis. One-way analysis of variance with normally distributed data or the Kruskal-Wallis test with non-normally distributed data was used to compare the composition of gut microbiota and blood biomarkers of immune function among elite athletes and general college students. In addition, analysis of covariance (ANCOVA) was used to evaluate whether the difference of gut microbiota and blood immune indicators among participants is independent of dietary status which included weekly intake of cereals, vegetables and fruits, meat, beans and nuts, fatty food, and alcohol. The ANCOVA is usually applied to test whether the independent variable still influences the dependent variable after the influence of the covariate has been removed (32). Pearson's correlation was used to examine the relationship between blood immune or inflammatory biomarkers and gut microbiota composition in all participants. The continuous variables were expressed as mean ± SD or SEM as appropriate, and percentages for categorical variables. A p-value < 0.05 was considered as an indication of a significant difference.

RESULTS

Characteristics of Participants

A total of 66 young adults were enrolled in the present study. Basic information of all participants is shown in **Table 1**. There was no significant difference in the body mass index among the three groups. However, the Athlete group had higher muscle mass ($p = 0.002$) and lower fat mass ($p = 0.002$) than other groups. In terms of physical activity, there was a significant difference in total physical activity per week among the three groups ($p < 0.001$). As shown in **Supplementary Table S1**, only the HPA group had job-related physical activity, and the HPA group also participated for a longer total time in transportation and housework physical activity per week. The total time of participating in moderate and high-intensity physical activities in the Athlete group was longer than that in other groups per week (estimated to be higher than in the HPA group by 4-fold and in the LPA group by 150-fold) concerning leisure-time physical activity according to IPAQ. However, the LPA group participated for a longer total time in walking and sitting per week. With regard to physical function, the performance of both hands grip strength ($p = 0.008$ for the left hand, $p = 0.002$ for right hand), standing long jump ($p < 0.001$), sit-and-reach ($p < 0.001$), one-leg standing (right) with eyes closed ($p = 0.007$), and Harvard step test ($p < 0.001$) in the Athlete group were significantly better than the LPA group. Meanwhile, the Athlete group performed significantly better than the HPA group in the standing long jump ($p < 0.001$) and Harvard step test ($p = 0.003$). In addition, there were significant differences in the sit-and-reach ($p = 0.019$) and Harvard step test ($p = 0.002$) in the HPA group compared with the LPA group. In terms of diet, athletes tended to have higher

TABLE 2 | Difference in alpha diversity of gut microbiota composition among athletes and young adults.

	Athlete group ($n = 22$)	HPA group ($n = 22$)	LPA group ($n = 22$)	
Alpha diversity				
	mean ± SEM	**mean ± SEM**	**mean ± SEM**	***p-value***
sobs†	175.14 ± 12.50	165.27 ± 8.30	169.23 ± 10.29	0.934
chao†	202.94 ± 13.55	197.07 ± 9.56	200.53 ± 13.24	0.925
Ace†	207.91 ± 13.63	196.60 ± 9.16	194.87 ± 12.59	0.712
Shannon†	2.52 ± 0.16	2.60 ± 0.15	2.64 ± 0.14	0.924
Simpson†	0.20 ± 0.03	0.19 ± 0.03	0.19 ± 0.03	0.936
Coverage‡	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.744

HPA, high physical activity; LPA, low physical activity. SEM, Standard error of the mean.
†Kruskal–Wallis test; ‡One-way analysis of variance.

intakes of almost all food items than the other two groups, except beans and nuts. Specifically, the intake of cereals in the Athlete ($p < 0.001$) and HPA groups ($p = 0.017$) was significantly higher than that in the LPA group. Furthermore, the intake of vegetables and fruits, as well as meat in the Athlete group was significantly higher than in the HPA group ($p = 0.018$, $p < 0.001$, respectively) and the LPA group ($p < 0.001$, $p < 0.001$, respectively).

Gut Microbial Diversity

There were a total of 9,512,790 (9.5 million) 16S rRNA reads from all sixty-three isolated fecal DNA. After quality filtering, 136,836 effective sequences were collected from each fecal sample and the effective rate is 95.80%. After removal of singletons, a total of 11,212 (Athlete = 3,853, HPA = 3,636, LPA = 3,723) operational taxonomic units (OTUs) were identified using 97% sequence similarity. The average number of observed OTUs was 170 per sample. No significant differences in the OTUs were observed among the three groups ($p = 0.934$).

Alpha diversity is the analysis of species diversity in a single microbiota sample, including observed species index, richness (Chao and Ace index), diversity (Shannon and Simpson index), and good coverage index (30). **Table 2** shows that there was no statistically significant difference in alpha diversity among the Athlete, HPA, and LPA groups.

Regarding beta diversity, no significant differences were observed in the Principal Coordinates Analysis (PCoA) based on unweighted-Unifrac distance metrics ($p = 0.845$) or weighted-Unifrac distance metrics ($p = 0.372$) (**Supplementary Figure S1**). However, the beta weighted-Unifrac heatmap showed a trend of the cluster among the three groups (**Supplementary Figure S2**). The taxonomy of the three groups of gut microbiomes is different. Linear discriminant analysis effect size (LEfSe) showed that the Athlete group enriched nine taxa, the HPA group enriched one taxon, and the LPA group enriched two taxa ($p < 0.05$). Specifically, the most abundant microbiota of the Athlete and LPA group was the *Firmicutes*, while the *Proteobacteria* in the HPA group (**Supplementary Figure S3**). These bacterial taxa indicated significant enrichment due to the linear discriminant analysis (LDA) score >2 or <-2 (33).

Gut Microbiota Composition at Phylum and Class Levels

The gut microbiota composition at the phylum level among three groups was represented in **Figure 2A**. In total, 16 phyla were observed in the intestines of different groups. Of these, 12 phyla were observed in Athlete and HPA groups, while 14 phyla were observed in the LPA group. The abundant phyla were the *Bacteroidetes* (52.53 in the Athlete group, 55.35 in the HPA group, and 62.81% in the LPA group) and the *Firmicutes* (43.99 in the Athlete group, 39.67 in the HPA group, and 32.14% in the LPA group) among three groups. However, there were no significant differences in the abundance of the *Bacteroidetes* and the *Firmicutes* among the three groups ($p = 0.278$, $p = 0.164$, respectively, data not shown). Similarly, the relative abundance of *Firmicutes*: *Bacteroidetes* ratio was evaluated, and no difference was observed among the three groups ($p = 0.417$, data not shown).

Figure 2B shows the gut microbiota composition at the class level among the three groups. *Bacteroidia* (52.63 in the Athlete group, 55.34 in the HPA group, and 62.71% in the LPA group), *Clostridia* (25.68 in the Athlete group, 28.46 in the HPA group, and 24.03% in the LPA group), and *Negativicutes* (17.84 in the Athlete group, 10.39 in the HPA group, and 7.62% in the LPA group) were the dominant genus among the three groups.

Gut Microbiota Composition at Order and Family Levels

At the order level, *Bacteroidales* (52.53 in the Athlete group, 55.34 in the HPA group, and 62.71% in the LPA group), *Clostridiales* (25.76 in the Athlete group, 28.46 in the HPA group, and 24.03% in the LPA group), *Selenomonadales* (17.84 in the Athlete group, 10.39 in the HPA group, and 7.62% in the LPA group) were the dominant genus among the three groups (**Figure 2C**). For family level, *Prevotellaceae* (29.60 in the Athlete group, 25.32 in the HPA group, and 28.61% in the LPA group), *Bacteroidaceae* (20.88 in the Athlete group, 26.81 in the HPA group, and 31.92% in the LPA group), *Ruminococcaceae* (12.28 in the Athlete group, 14.67 in the HPA group, and 11.56% in the LPA group) were the dominant genus among the three groups (**Figure 2D**).

Gut Microbiota Composition at Genus and Species Levels

Figure 3A indicated the gut microbiota composition at the genus level among the three groups. *Prevotella* (20.88 in Athlete group, 26.81 in the HPA group, and 31.92% in the LPA group), *Bacteroides* (24.96 in Athlete group, 25.01 in the HPA group, and 27.41% in the LPA group), *Faecalibacterium* (6.86 in Athlete group, 10.57 in the HPA group and 6.88% in LPA group) and *Megamonas* (11.67 in the Athlete group, 5.15 in the HPA group and 2.24% in the LPA group) were the dominant genus among three groups.

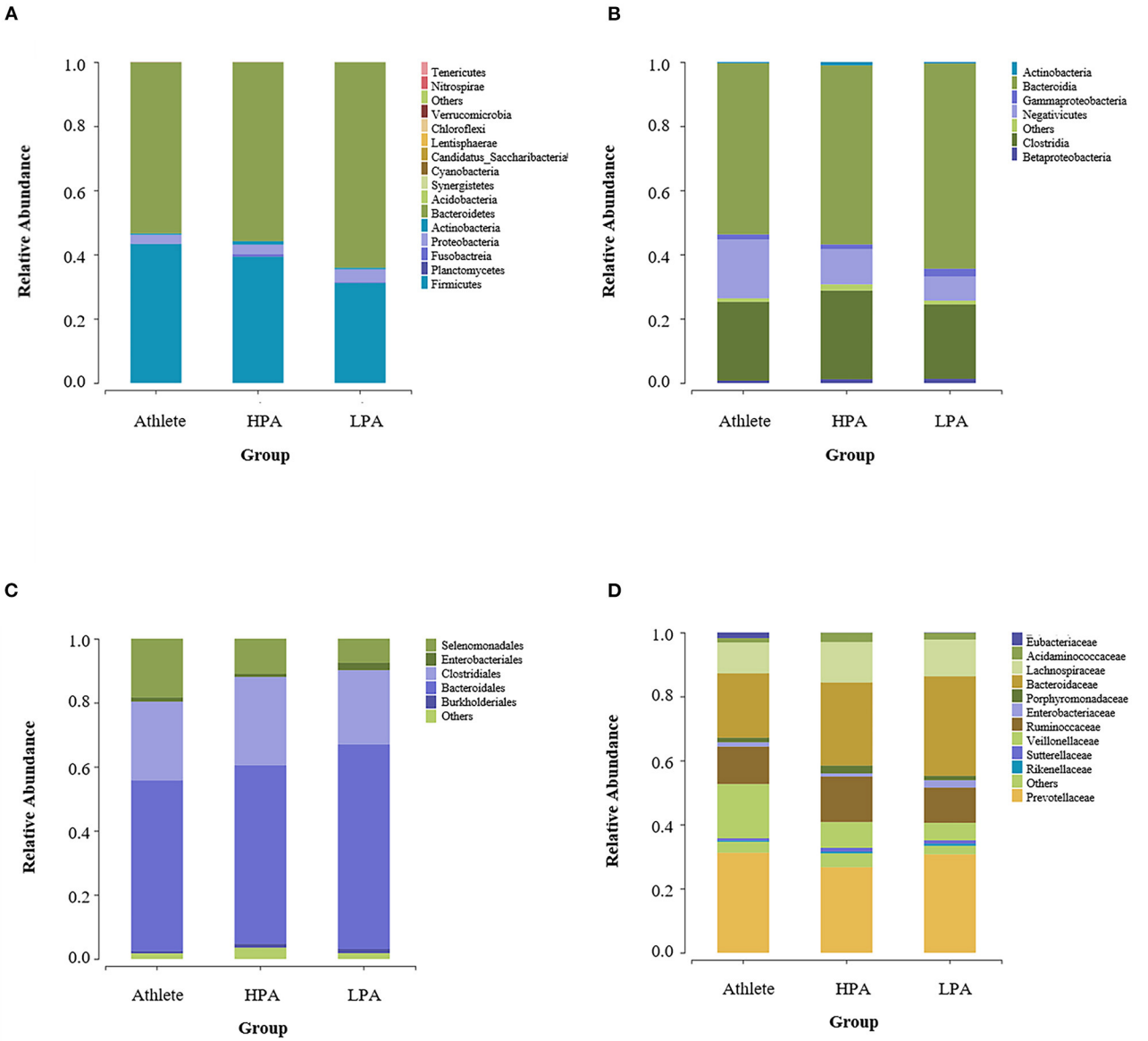

FIGURE 2 | Differences of gut microbiota relative abundance at the phylum, class, order, and family levels among three groups. Relative abundance represents the proportion of each species in each group. **(A)** phylum level; **(B)** class level; **(C)** order level; and **(D)** family level. Athlete, Athlete group; HPA, High physical activity group; LPA, Low physical activity group.

At the species level, *Prevotella_copri* (21.80 in the Athlete group, 24.64 in the HPA group, and 26.11% in the LPA group), *Faecalibacterium_prausnitzii* (6.86 in the Athlete group, 10.57 in the HPA group, and 6.88% in the LPA group), *Bacteroides_vulgatus* (7.74 in Athlete group, 6.78 in HPA group, and 6.73% in LPA group), *Bacteroides_plebeius* (3.32% in the Athlete group, 6.87 in the HPA group, and 8.61% in the LPA group), and *Megamonas_funiformis* (11.07 in the Athlete group, 5.09 in the HPA group, and 2.11% in the LPA group) were the dominant genus among three groups (**Figure 3B**).

Differences in Gut Microbiota Composition Among Athletes and Young Adults With Different Physical Activity Levels

A total of 25 taxa of gut microbiota were observed significant differences among the groups in the non-adjusted model. In the Athlete group, the abundance of *Lentisphaerae (Victivallaceae* belongs to *Lentisphaerae*; $p = 0.045$), *Clostridiaceae* ($p = 0.029$), *Megamonas* ($p = 0.020$), *Romboutsia* ($p = 0.038$), *Campylobacter_jejuni* ($p = 0.045$), and *Paraprevotella_xylaniphila* ($p = 0.045$), were

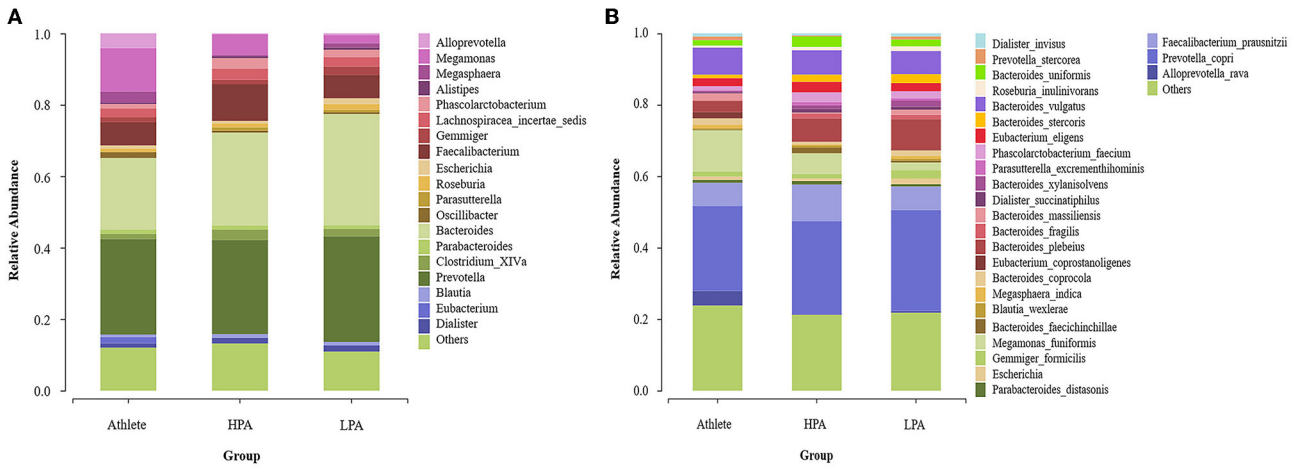

FIGURE 3 | Differences of gut microbiota relative abundance at the genus and species level among three groups. Relative abundance represents the proportion of each species in each group; **(A)** genus level; **(B)** species level; Athlete, Athlete group; HPA,High physical activity group; LPA, Low physical activity group.

higher. In the LPA group, the abundance of *Erysipelotrichia* ($p = 0.040$), *Bilophila* ($p = 0.034$), and *Faecalicoccus* ($p = 0.008$) were higher. In addition, the abundance of *Parasutterella* ($p = 0.026$), *Arcobacter_halophilus* ($p = 0.045$), *Parasutterella_excrementihominis* ($p = 0.023$) and *Clostridium_spiroforme* ($p = 0.017$) were higher in the HPA group.

Moreover, we re-analyzed the bacteria with differences between groups after dietary adjustment, and the results show that there were no group differences in most bacteria after adjusted diet intake. Only the abundance of *Clostridiaceae* ($p = 0.029$), *Bilophila* ($p = 0.011$), *Faecalicoccus* ($p = 0.050$), and *Bilophila_wadsworthia* ($p = 0.011$) were still different between groups. In addition, although no differences were observed between the three groups, two bacteria (*Campylobacter_jejuni* and *Megamonas_rupellensis*) showed differences among participants. *Campylobacter_jejuni* was higher in the Athlete group than in the LPA group ($p = 0.050$), and *Megamonas_rupellensis* sp. was higher in the Athlete group than in the HPA group ($p = 0.029$) (**Table 3**).

Association of Serum Immune Function Biomarkers With Gut Microbiota

Concerning immune function biomarkers, a significantly higher absolute neutrophil count ($p = 0.004$) and white blood cell count ($p = 0.014$) were observed in the LPA group than in other groups in the non-adjusted model. In addition, the HPA group had a higher absolute basophil count ($p = 0.022$). When adjusting for dietary status, almost all these significant differences disappeared and only a significantly higher absolute count of basophils was observed in the HPA group than in the athlete group ($p = 0.037$) (**Table 4**).

Pearson's correlation was conducted to further explore the relationship between gut microbiota, inflammation, and immunity (**Figure 4**). It was shown that *Bilophila* genus (*Bilophila_wadsworthia*) had positive linear correlation with the absolute neutrophil ($r = 0.273$, p=0.027), lymphocyte count ($r = 0.327$, $p = 0.007$), and white blood cell count ($r = 0.332$, $p = 0.006$). At the species level, *Megamonas_rupellensis* was also positively correlated with the absolute lymphocyte count ($r = 0.268$, $p = 0.030$).

DISCUSSION

This matching study investigated the characteristics of gut microbiota at all levels (including phylum, class, order, family, genus, and species) among elite athletes and general young adults with different physical activity levels. Beta diversity tended to cluster between groups with different physical activity levels, while no significant difference was observed in both alpha and beta diversity. Meanwhile, a higher abundance of several bacteria was observed in elite athletes or physically inactive young adults. Moreover, it is inferred that dietary status may be a key factor to be considered when analyzing the association between physical activity and gut microbiota.

In this study, the elite athletes had a significantly higher level of total physical activity and moderate-to-vigorous intensity physical activity, as well as lower sitting time compared to general young adults. It is worth noting that these gut microbiological differences between people with different levels of physical activity have also been observed in previous studies. Specifically, the *Bacteroides* and *Prevotella* were more abundant in top Polish endurance athletes compared to sedentary participants (34). The elite athletes and youth non-elite athletes have different taxonomical functional and phenotypic compositions of the gut microbial community. *Clostridiales* and *Faecalibacterium* were particularly enriched in the elite athletes (35). Senior orienteers were found to have a more homogeneous microbiota and more abundant *Faecalibacterium prausnitzii* than the community-dwelling older adults (36). Indeed, our results indicate partial

TABLE 3 | Phylotypes with significant difference among athletes and young adults before and after adjusting dietary status.

Taxa	specific	Non-adjusted model†				Dietary status adjusted model‡			
		Athlete group ($n = 22$) Mean ± SEM	HPA group ($n = 22$) Mean ± SEM	LPA group ($n = 22$) Mean ± SEM	*p-value*	Athlete group ($n = 22$) Mean ± SEM	HPA group ($n = 22$) Mean ± SEM	LPA group (n =22) Mean ± SEM	*p-value*
Phylum	Lentisphaerae	0.004 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	0.045	0.005 ± 0.002	0.000 ± 0.002	−0.001 ± 0.002	0.242
Class	Erysipelotrichia	0.143 ± 0.036	0.110 ± 0.028	0.221 ± 0.051$^{\#}$	0.040	0.129 ± 0.045	0.115 ± 0.042	0.231 ± 0.044	0.148
	Lentisphaeria	0.004 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	0.045	0.005 ± 0.002	0.000 ± 0.002	−0.001 ± 0.002	0.242
Order	Erysipelotrichales	0.143 ± 0.036	0.110 ± 0.028	0.221 ± 0.051$^{\#}$	0.040	0.129 ± 0.045	0.115 ± 0.042	0.231 ± 0.044	0.148
	Victivallales	0.004 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	0.045	0.005 ± 0.002	0.000 ± 0.002	−0.001 ± 0.002	0.242
Family	Clostridiaceae	0.144 ± 0.070	0.023 ± 0.008*	0.026 ± 0.014	0.029	0.180 ± 0.049	0.014 ± 0.046*	−0.002 ± 0.048*	0.029
	Erysipelotrichaceae	0.143 ± 0.036	0.110 ± 0.028	0.221 ± 0.051$^{\#}$	0.040	0.129 ± 0.045	0.115 ± 0.042	0.231 ± 0.044	0.148
	Victivallaceae	0.004 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	0.045	0.005 ± 0.002	0.000 ± 0.002	−0.001 ± 0.002	0.242
Genus	Bilophila	0.100 ± 0.032	0.101 ± 0.029	0.249 ± 0.053	0.034	0.113 ± 0.044	0.076 ± 0.041	0.261 ± 0.043$^{*\#\#}$	0.011
	Faecalicoccus	0.037 ± 0.024	0.005 ± 0.002	0.092 ± 0.038$^{\#\#}$	0.008	0.022 ± 0.028	0.010 ± 0.026	0.103 ± 0.028*	0.050
	Megamonas	11.670 ± 3.898	5.154 ± 4.276*	2.239 ± 0.776	0.020	11.254 ± 3.871	6.970 ± 3.622	0.839 ± 3.794	0.206
	Parasutterella	0.257 ± 0.144	0.969 ± 0.276*	0.703 ± 0.344	0.026	0.360 ± 0.316	1.029 ± 0.295	0.539 ± 0.309	0.290
	Romboutsia	0.134 ± 0.034	0.062 ± 0.017*	0.111 ± 0.056	0.038	0.127 ± 0.046	0.071 ± 0.043	0.109 ± 0.045	0.661
	Victivallis	0.004 ± 0.004	0.000 ± 0.000	0.000 ± 0.000	0.045	0.005 ± 0.002	0.000 ± 0.002	−0.001 ± 0.002	0.242
Species	Arcobacter_halophilus	0.000 ± 0.000	0.001 ± 0.001	0.000 ± 0.000	0.045	0.000 ± 0.000	0.001 ± 0.000	0.000 ± 0.000	0.350
	Bilophila_wadsworthia	0.100 ± 0.032	0.101 ± 0.029	0.249 ± 0.053	0.034	0.113 ± 0.044	0.076 ± 0.041	0.261 ± 0.043$^{*\#\#}$	0.011
	Campylobacter_jejuni	0.046 ± 0.045	0.000 ± 0.000	0.000 ± 0.000	0.045	0.072 ± 0.030	−0.007 ± 0.028	−0.020 ± 0.030*	0.103
	Clostridium_spiroforme	0.000 ± 0.000	0.002 ± 0.001*	0.000 ± 0.000	0.017	0.000 ± 0.001	0.002 ± 0.001	0.000 ± 0.001	0.139
	Megamonas_funiformis	11.065 ± 3.721	5.092 ± 4.240*	2.109 ± 0.733	0.019	10.661 ± 3.773	6.879 ± 3.530	0.726 ± 3.698	0.216
	Megamonas_rupellensis	0.125 ± 0.046	0.004 ± 0.003*	0.041 ± 0.032	0.036	0.125 ± 0.038	0.005 ± 0.035*	0.039 ± 0.037	0.087
	Paraprevotella_xylaniphila	0.100 ± 0.062	0.000 ± 0.000	0.000 ± 0.000	0.045	0.038 ± 0.035	−0.015 ± 0.033	0.077 ± 0.034	0.167
	Parasutterella_excrementihominis	0.253 ± 0.144	0.969 ± 0.276*	0.699 ± 0.344	0.023	0.357 ± 0.316	1.030 ± 0.295	0.533 ± 0.309	0.285
	Romboutsia_sedimentorum	0.134 ± 0.034	0.062 ± 0.017*	0.111 ± 0.056	0.038	0.127 ± 0.046	0.071 ± 0.043	0.109 ± 0.045	0.661
	Rothia_aeria	0.000 ± 0.000	0.004 ± 0.000	0.000 ± 0.000	0.045	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.166
	Victivallis_vadensis	0.004 ± 0.003	0.000 ± 0.000	0.000 ± 0.000	0.045	0.005 ± 0.002	0.000 ± 0.002	−0.001 ± 0.002	0.242

HPA, high physical activity; LPA, low physical activity; SEM, Standard error of the mean.
$^{*}p < 0.05$ when HPA or LPA group compared with Athlete group.
$^{\#}p < 0.05$ when LPA group compared with HPA group; $^{\#\#}p < 0.01$ when LPA group compared with HPA group.
†Kruskal–Wallis test; ‡Analysis of covariance.

TABLE 4 | Blood biomarkers with significant difference among athletes and young adults before and after adjusting dietary status.

Blood parameters	Non-adjusted model				Dietary status adjusted model$^{\Psi}$			
	Athlete group ($n = 22$) Mean ± SEM	HPA group ($n = 22$) Mean ± SEM	LPA group ($n = 22$) Mean ± SEM	*p-value*	Athlete group ($n = 22$) Mean ± SEM	HPA group ($n = 22$) Mean ± SEM	LPA group (n =22) Mean ± SEM	*p-value*
White body cell, (10^9/L)†	4.96 ± 0.20	5.53 ± 0.23	6.45 ± 0.41*	0.014	5.18 ± 0.34	5.57 ± 0.32	6.19 ± 0.33	0.148
Basophils, (10^9/L)†	0.027 ± 0.002	0.039 ± 0.003*	0.034 ± 0.003	0.022	0.028 ± 0.003	0.038 ± 0.003*	0.034 ± 0.003	0.111
Eosinophils, (10^9/L)†	0.136 ± 0.020	0.136 ± 0.015	0.142 ± 0.019	0.886	0.130 ± 0.022	0.141 ± 0.020	0.144 ± 0.021	0.902
Neutrophils, (10^9/L)‡	2.30 ± 0.15	2.93 ± 0.18*	3.32 ± 0.27*	0.004	2.46 ± 0.23	2.94 ± 0.22	3.15 ± 0.23	0.147
Lymphocytes, (10^9/L)†	2.19 ± 0.10	2.10 ± 0.10	2.62 ± 0.17	0.062	2.25 ± 0.15	2.13 ± 0.14	2.54 ± 0.15	0.151
Monocytes, (10^9/L)‡	0.303 ± 0.016	0.326 ± 0.022	0.343 ± 0.023	0.392	0.311 ± 0.024	0.330 ± 0.022	0.331 ± 0.023	0.818
Immunoglobulin G, (g/L)‡	11.29 ± 0.26	12.07 ± 0.37	11.97 ± 0.47	0.285	11.18 ± 0.44	12.14 ± 0.41	12.00 ± 0.43	0.279
Immunoglobulin M, (g/L)‡	1.46 ± 0.09	1.34 ± 0.09	1.52 ± 0.12	0.412	1.46 ± 0.12	1.34 ± 0.11	1.53 ± 0.12	0.490
C-reactive protein, (g/L)†	0.81 ± 0.22	1.14 ± 0.53	0.74 ± 0.10	0.122	0.97 ± 0.39	1.27 ± 0.37	0.45 ± 0.39	0.324

HPA, high physical activity; LPA, low physical activity; SEM, Standard error of the mean.
$^{*}p < 0.05$ when HPA or LPA group compared with Athlete group.
†Kruskal–Wallis test in non-adjusted model; ‡One-way analysis of variance in non-adjusted model; $^{\Psi}$Analysis of covariance in dietary status adjusted model.

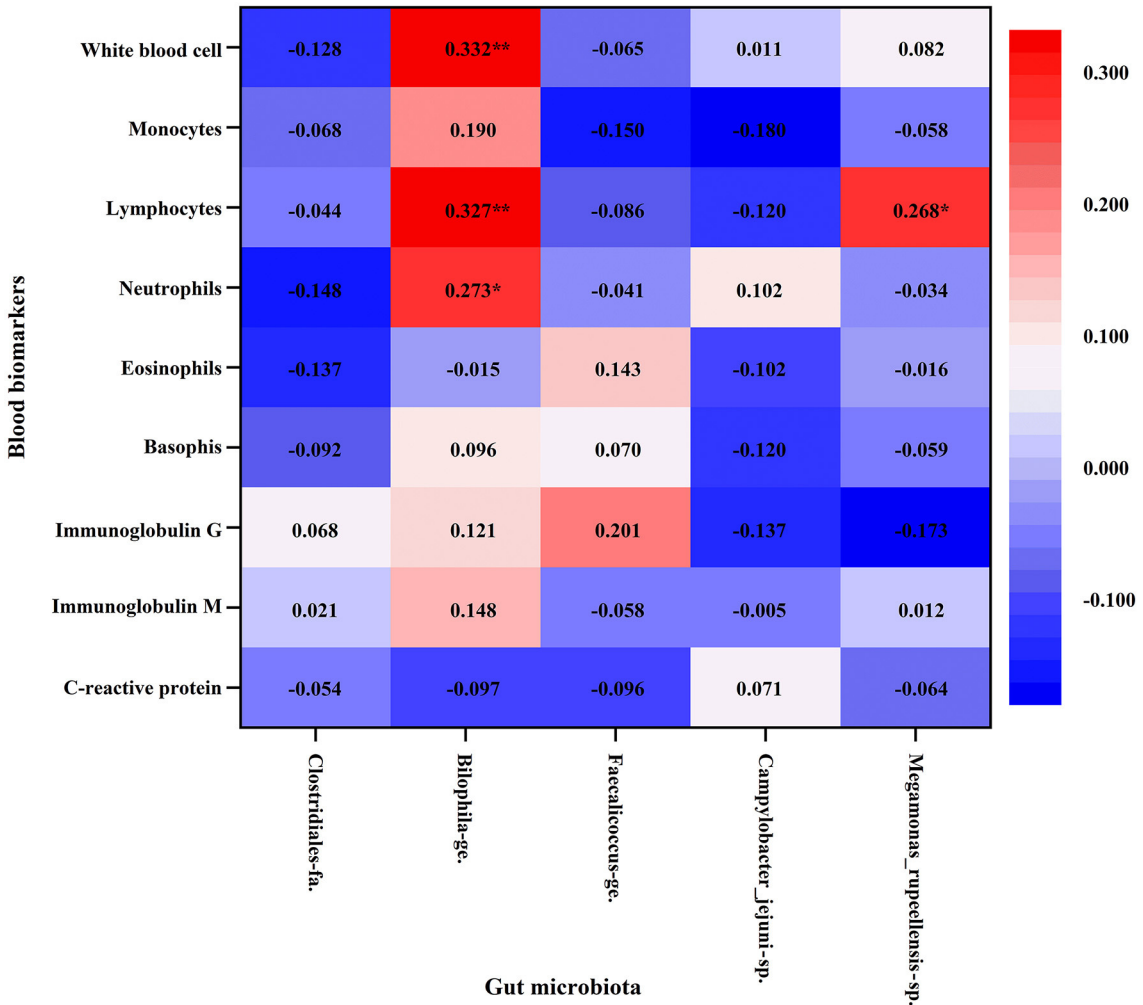

FIGURE 4 | Association between blood biomarkers of immune function and gut microbiota with Pearson's correlation analysis. Athlete, Athlete group; HPA, High physical activity group; LPA, Low physical activity group. *$p < 0.05$; **$p < 0.01$.

disparity of gut microbiota composition among elite athletes and young adults with different physical activity, particularly after adjusting dietary status.

Diet is an important factor affecting gut microbiota (17). Some previous studies confirmed that the different dietary ingredients have a substantial impact on the gut microbiota (37). Specifically, high-fiber feeding can reshape gut microbiota and promote the release of short-chain fatty acids (SCFAs), which play a role in maintaining the normal functions of the innate and adaptive immune system (38, 39). The intervention of low-calorie weight loss on a high-fat diet induced the growth of bile-resistant bacteria and reduction of bacteria associated with inflammation in humans (40). Moreover, in athletes, high-protein diets may be negatively associated with the diversity of gut microbiota, and a decreased relative abundance of short-chain fatty acid-producing commensal bacteria was observed in a high protein low carbohydrates diet for athletes in resistance sports (41). It is worth noting that athletes often consume a diet that differs from the general population, including in dietary diversity and total energy consumption. The eating habits of athletes are even different between different events (e.g., increased protein intake in resistance-trained athletes or carbohydrate intake in endurance athletes) (42). Previous studies have shown that there is a rapid change in the composition of the gut microbial and increased abundance of *Alistipes*, *Bilophila*, and *Bacteroides* after consuming a high-fat/protein diet for 5 days (43). Thus, there is a need to consider the influence of an athletic diet when comparing the differences in gut microbiota among athletes of different sports, as well as between athletes and the general population. In our study, 19 taxa of gut microbiota showed no statistical differences, the remaining 6 taxa (*Clostridiaceae*, *Bilophila*, *Faecalicoccus*, *Bilophila_wadsworthia*, *Campylobacter_jejuni*, *Megamonas_rupellensis*) observed significant differences among the groups after adjusting the covariates of dietary intake. These changes show that dietary intake is also a particularly important factor in regulating

microbiota composition of the gut between elite athletes and general young adults.

Previous studies indicated that some abundant gut microbiota in physically inactive young adults of this study may be closely associated with inflammation and immunity. For example, a high abundance of *Bilophila_wadsworthia* (belong to *Bilophila* genus) caused a systemic inflammatory response in mice that included elevated IL-6 (44), which was enriched in patients with Behcet's disease (45) and can promote a Th1-mediated immune response in dietary-fat-induced colitis (46). In addition, a recent study demonstrated that *Faecalicoccus* was considered to be an important factor in the classification of subjects in Crohn's disease and enriched in patients with Crohn's disease (47). Our study showed that a higher abundance of *Bilophila* and *Faecalicoccus* was observed in young adults with insufficient physical activity. It is worth noting that *Bilophila* genus was positively associated with the counts of circulating white blood cells and its subtypes such as neutrophils and lymphocytes. The bacterium *Bilophila_wadsworthia* can erode the mucus layer on colon surfaces and allow more bacterial flora to approach lining cells, then resulting in inflammation (48). These findings suggest that physical inactivity may be correlated to pro-inflammatory gut bacteria.

It is well-known that physical inactivity is associated with high-grade systemic inflammation (49) and low immune function (50). However, the mechanism by which physical inactivity induces a reduction of immune function has not been fully elucidated. One possible underlying mechanism is that physical inactivity or low physical activity may lead to impaired ability to store fat and inflammation of subcutaneous adipose tissue (51, 52). In addition, the subjects with low physical activity have a higher susceptibility to infection compared to regular moderate exercise subjects (53). The reason may be that the growth hormone, cortisol, adrenaline, prolactin, and other factors showed a decreased release, leading to attenuated immunomodulatory effects in the subjects with physical inactivity (54).

It is well known that, the white blood cell, comprising neutrophils, eosinophils, basophils, lymphocytes, and monocytes, play an important role in the implementation of immune function and anti-infection in humans (55). The leukocyte count can increase in response to infections. Evidence indicated that patients with severe COVID-19 infection tend to have a higher leukocyte count in the Chinese population (56). Moreover, the elevated leukocyte count, particularly neutrophil and monocyte count were reliable inflammatory markers within the normal range, even in physically healthy individuals (57–59). Thus, it may reduce the body's inflammation and the risk of inflammation-induced diseases if the number of leukocytes can be kept at an appropriate level. Studies have reported regular exercise, particularly in more moderate-to-vigorous physical activity, can have an anti-inflammatory effect and reduce the odds of elevated white blood cell count (51, 60, 61). Although previous studies focused on elite athletes who practice water sports (62), cyclists who participate in intense endurance exercise (63) have higher levels of inflammation and low immune function. The reason for these findings could be that exercise-induced immunosuppression makes elite athletes more susceptible to infection symptoms after short-term acute exercise. Our results showed that athletes participating in regular exercise training have better immune function reflected by lower leukocyte count. Conversely, the *Clostridiaceae*, a bacterium associated with inflammation (64) was more abundant in athletes. As another bacterium relatively abundant in athletes, *Megamonas_rupellensis* also showed a positive correlation with circulating lymphocytes. These results may be interpreted as injury-induced local inflammation when the athletes are susceptible to sports injury. Evidence showed that muscle injury in basketball players can lead to increased levels of inflammation (65). Due to the lack of data on acute or chronic sports injury among athletes, these speculations could not be confirmed in this study and need to be further clarified in subsequent work.

We have to admit that there are some limitations to this study. First, our elite athletes only include track and field athletes, and the lack of participants from other sports may affect the results of the gut microbiota in different groups, hence similar findings may not necessarily be observed. Second, there was no data on the nutrient supplementation of participants, particularly in elite athletes, hence we cannot rule out the effect of these factors on main observation results (66). Third, we did not analyze the metabolites of gut microbes, which might normally be directly involved in the human metabolism process (67), hence the in-depth interpretation of results was limited.

CONCLUSION

These findings indicated that several gut bacteria are inversely associated with immune function and tended to be abundant in physically inactive people, while elite athletes are more likely to have a better immune function and specific gut bacteria composition than general young adults. It is important to note that dietary intakes are potential confounders that may affect the observed association of physical activity with gut microbiota and immune function. Further studies are warranted to determine the correlation between physical activity and gut microbiota. Moreover, considering diet, the effects and mechanisms of long-term habits with moderate and vigorous-intensity physical activity on immune indices and gut microbiota need to be further clarified.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available without the consent and permission of the study participants. Requests to access the datasets should be directed to Fei Zhong, zf358235743@zju.edu.cn.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Department of Psychology and Behavioral Sciences, Zhejiang University. The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YX, XZ, CW, and CH: designing the experiment. YX, FZ, XZ, and H-YL: acquisition of subjects and data. YX, FZ, XZ, H-YL, CW, and CH: statistical analyses and interpretation of data and revision of the manuscript. YX, FZ, XZ, and CH: drafting the manuscript. CH: study supervision. All authors have revised and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.843076/full#supplementary-material

Supplementary Figure S1 | Principal Coordinate Analysis (PCoA) of gut microbiota. **(A)** unweighted-Unifrac distance metrics; **(B)** weighted-Unifrac distance metrics. Each point represents a sample, athlete group in blue, high physical activity group in orange, and low physical activity group in green. The two coordinates are plotted and further indicated the percentage of variability on the axis.

Supplementary Figure S2 | Beta weighted-Unifrac heatmap. The Gradient color in the heatmap represents the degree of difference of each sample among athletes, high physical activity group, and low physical activity group. The distance between samples indicated increases gradually with the color from blue to red.

Supplementary Figure S3 | Differential taxon features were analyzed by linear discriminant analysis (LDA) effect size (LEfSe) analysis among three groups. **(A)** showing differentially abundant bacterial taxa. **(B)** indicating LDA scores. Bacterial taxa indicated significantly high when LDA score >2 or LDA score <-2. Athlete, Athlete group; HPA, High physical activity group; LPA, Low physical activity group.

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Development and Validation of a Nomogram Based on Geriatric Nutritional Risk Index to Predict Surgical Site Infection Among Gynecologic Oncology Patients

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Background: The geriatric nutritional risk index (GNRI) is a commonly used method to assess nutritional risk for predicting potential surgical site infections (SSI) in cancer patients. This study aims to create and verify a simple nomogram and a dynamic web-based calculator for predicting the risk of SSI among gynecologic oncology patients.

Methods: A retrospective evaluation was conducted on patients who were admitted into a tertiary hospital in China with confirmed diagnosis of gynecologic cancer between 01 August 2017 and 30 November 2021. A two-piecewise linear regression model with a smoothing function was used to investigate the non-linear association between GNRI and SSI to determine the ideal cut-off point. Three models were developed on the basis of different variables to predict SSI in gynecologic oncology patients. Through a nomogram the concordance index (C-index), the Akaike information criterion (AIC), and the integrated discrimination index (IDI) were used to determine the final model. Finally, the performance of the nomogram was validated using the 1,000-bootstrap resamples method and analyzed using C-index, GiViTI calibration belts, and decision curve. Also, a user-friendly dynamic web-based calculator was developed.

Results: A total of 1,221 patients were included in the analysis. A non-linear association could be observed between GNRI and SSI risk with a GNRI cut-off value of 101.7. After adding GNRI to Model 2 (which comprised Morse Fall Scale score, preoperative length of stay, operation time, and estimated blood loss), the AIC value decreased, the C-index value increased and IDI increased significantly. The nomogram C-index in the development cohort and internal validation cohort demonstrates a moderate-high degree of discrimination. The GiViTI calibrated belt showed a good agreement between

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the observed and predicted probabilities of SSI. The decision curve validates the clinical feasibility of the nomogram with a threshold value between 0 and 49%.

Conclusion: The GNRI cut-off value of 101.7 allowed for appropriate stratification of patients into distinct SSI risk groups. This study found that including GNRI in the above nomogram (Model 2) would enhance its potential to predict SSI in gynecologic oncology patients.

Keywords: geriatric nutritional risk index, gynecologic oncology, surgical site infection, infection prevention, nomogram, prediction model

INTRODUCTION

With an incidence rate of approximately 5–35% following a gynecologic oncology surgery, surgical site infections (SSIs) are considered one of the most prevalent postoperative complications reported among patients globally (1–4). Also considered as one of the primary etiological factors that leads to postoperative nosocomial infections, SSI will bring about an increased morbidity and mortality rate as well as prolonged hospitalization, and higher healthcare expenditures (5–7). Therefore, it is important to identify patients highly susceptible to SSI.

SSI is the outcome of a complex combination of factors relating to the patient, environment, and to the surgery itself (6). Recent studies have also shown and elucidated that nutritional status is also considered a critical factor that is closely associated with the development of SSI in gynecologic oncology patients (8, 9). This suggests that the assessment of malnutrition could provide clinically valuable information regarding the prediction of SSI in gynecologic oncology patients. The Geriatric Nutritional Risk Index (GNRI) is one of a few established scoring systems for measuring the severity of malnutrition in clinical settings. The GNRI calculation takes into account the serum albumin level, current weight, and optimum body weight based on gender and height, all of which are accessible to most patients prior to therapy (10). GNRI is a simple-to-calculate technique that is sensitive to detecting malnutrition (11–13) and has only recently gained popularity in assessing a patient's nutritional status and in predicting the risk of SSI in cancer patients (14, 15). Some studies have used the GNRI in conjunction with other traditional indicators to predict the development of SSI in gynecologic oncology patients. In combination with the usage of a nomogram as a means of visualization of a mathematical model, may prove beneficial as it incorporates many critical variables rather than analyzing individual risk factors (16). This method provides each patient with personalized, evidence-based, and highly accurate risk estimations that are presented intuitively.

The primary objectives of this study are to (1) investigate potential associations between the GNRI and the risk of SSI in gynecologic oncology patients; (2) investigate the predictive value of including the GNRI in the study models; (3) to develop and validate a nutrition-based nomogram that incorporates the GNRI and clinical risk factors to predict SSI in gynecologic oncology patients.

MATERIALS AND METHODS

Study Design and Participants

To establish the development cohort herein, a total of 1,561 consecutive gynecologic oncology patients who underwent surgery at Wenzhou People's Hospital between 01 August 2017 and 30 November 2021, were retrospectively evaluated (**Figure 1**). The following are the inclusion criteria: medically confirmed diagnoses of gynecological (cervix, corpus, ovarian/tubal/peritoneal, vulva, or vaginal) cancer; patients who have received surgery and postoperative care at our institution. Patients under the age of 18 years, patients with a length of stay of less than 48 h, patients who were postoperatively hospitalized for less than 24 h, and pregnant women were excluded from this analysis. For patients admitted more than once during the study period, only the first admission was analyzed. The entirety of this study was approved by the Ethics Review Committee of Wenzhou People's Hospital (approval no. KY-2022-010). Waiver of informed consent was granted due to the retrospective nature of the study.

Candidate Predictors

Expert judgment, extensive literature studies, and the availability of clinical data were used to select all possible candidate predictors for potential operative site infections in gynecologic oncology patients (5–9). The following candidate predictors were included in the study: general information [age, body mass index (BMI), season of admission, surgical history in recent 3 months, comorbidities, and the age-adjusted Charlson Comorbidity Index (aCCI)], preoperative variables [FIGO stage, ASA class, site of cancer, Barthel Index, Morse Fall Scale (MFS) score, preoperative steroid use, laboratory values, preoperative hair removal, preoperative length of stay (LOS), antibiotic prophylaxis within 0.5–1 h before operation, and the modified surgical complexity score (MSCS)], and intraoperative variables (surgical approach, operative time, estimated blood loss, blood transfusion, emergent surgery, and NNIS index). Furthermore, the GNRI was also included as a candidate predictor.

Preoperative peripheral blood samples were drawn from patients no more than 7 days prior to surgery. The comorbidity burden was assessed using aCCI (17). Furthermore, the MSCS was used to classify the extent of surgery into two groups (mild and moderate/severe) with respect to the number and complexity of each surgical procedure (5). According to the

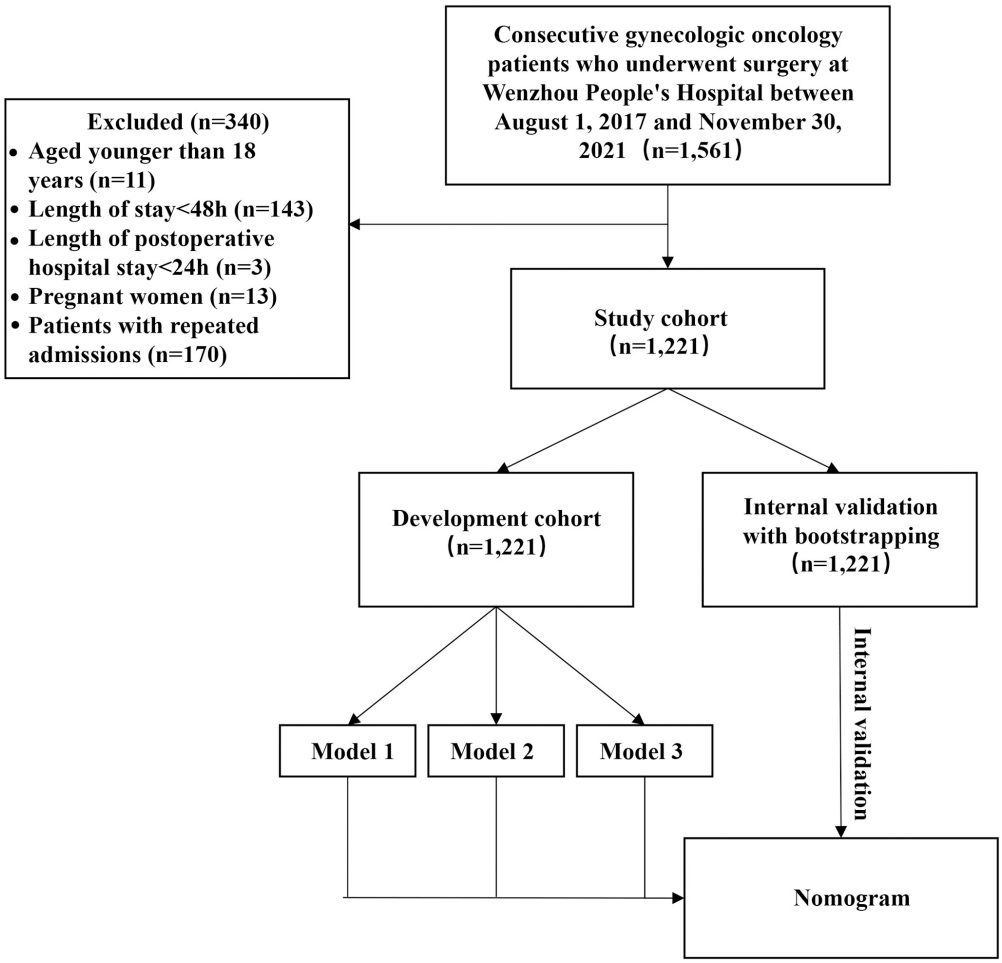

FIGURE 1 | Flow chart of the study population.

US National Nosocomial Infection Surveillance System (NNIS), the risk index for nosocomial infections is a coherent additive scale that takes into account patient variability in underlying illness severity, surgical wound contamination level, and surgical process complexity (as measured by surgical procedure duration) (18, 19). This risk index is validated to adequately stratify the risk of SSI in some specific surgeries (20). The GNRI was calculated using the following formulas: GNRI = (1.519 × serum albumin, g/L) + 41.7 × (current body weight [kg]/ideal body weight [kg]) (10). Patients' current body weight was evaluated by competent nurses within 7 days preoperatively following a standardized protocol. The optimal weight for women was determined using the following Lorenz formula: ideal body weight = height (cm) – 100 – ([height (cm) – 150]/2.5) (21). When a patient's body weight surpasses the optimal weight, the actual body weight/ideal weight ratio was adjusted to 1. Additional information detailing other candidate predictors is listed in **Supplementary Table 1**.

Outcome

The SSIs were characterized according to the definitions set by the Centers for Disease Control (22). The SSIs were classified into incisional and organ/space infections. Incisional infections were further subclassified into superficial and deep incisional infections, whereas organ/space infections were further subcategorized into pelvic cellulitis, pelvic abscess, as well as vaginal cuff infections. The detailed criteria for the abovementioned SSIs can be found in **Supplementary Table 2**. The SSIs data were acquired and analyzed via the Xinglin real-time nosocomial infection monitoring system. In addition, this system is capable of automatically generating infection warning notices in real-time based on the database of different hospitals [Hospital Information System (HIS), Laboratory Information System (LIS), and Anesthesia Information Management System (AIMS)]. Cases of nosocomial infection documented within the Xinglin system, such as SSIs, must be confirmed after verification by a clinician and a senior infection control practitioner, respectively. Inconsistent opinions were resolved through extensive discussions.

Statistical Analysis

This study follows the guidelines outlined in the Transparent Reporting of a Multivariable Prediction Model for Individual

Prognosis or Diagnosis (TRIPOD) statement (23). To compare categorical variables [summarized as number (%)], the Chi-square test or Fisher's exact tests were used where applicable. Two-sample Student *t*-test or Mann-Whitney *U* test was employed to compare continuous variables expressed as mean ± standard deviation (SD) or median [interquartile range (IQR)]. All statistical analyses were analyzed using the R software (version 3.6.3)[1] and a two-tailed *P*-value of ≤ 0.05 was considered to be statistically significant.

Missing Data

Prior to data analysis, the distribution of data and missing values for all predictors were examined. The percentage of missing data varied between 0 and 16.5% for each candidate predictor variable. Furthermore, to maximize statistical power and decrease selection bias caused by missing data, we performed multiple imputation with the Multivariate Imputations by Chained Equation (MICE) package in R (**Supplementary Table 3**) (24).

Sample Size Calculation

Based on clinical factors such as MFS score, preoperative LOS, operative time, estimated blood loss, and GNRI, we established a model capable of predicting SSIs in gynecological surgery. The incidence of operative site infection in gynecological surgery is estimated to be 5.3% in this model. The multivariable logistic model included five predictor parameters and had a C statistic of 0.77. We performed a power analysis using the formula developed by Riley et al. (25) and obtained a required minimum sample size for the development of a new model consisting of 851 patients, 46 events (assuming an outcome prevalence = 0.053), and an EPP of 9.02.

The Cut-Off Point for Continuous Variables

All participants were divided into three groups using BMI for Chinese men and women as the criteria (26): underweight (BMI < 18.5 kg/m^2), normal weight (BMI 18.5–24.0 kg/m^2), and overweight/obese (BMI ≥ 24 kg/m^2). To categorize the patients, the cut-off point for laboratory values was determined as the upper or lower limit of the normal value. The cut-off point for glucose, albumin, ALT, total bilirubin, platelet count, hematocrit, TLC, WBC was 110 mg/dL, 3.0 g/dL, 40 U/L, 1.1 mg/dL, 350×10^9/L, 36%, 0.8×10^9 /L, and 10×10^9 /L, respectively. The two-piecewise linear regression model with a smoothing function was performed using the GAM package in R to investigate the non-linear relationship between GNRI, preoperative LOS, operative time, estimated blood loss, and SSI. The cut-off point was determined through trial and error, which included selecting turning points within a pre-defined interval and then selecting the ideal cut-off point that produced the highest model likelihood. Furthermore, we ran a log-likelihood ratio test to compare the one-line linear regression model and the two-piecewise linear model.

Variables Selection and Model Development

On the basis of different inclusions of variables, we have developed three models to predict the potential operative site infections in a gynecologic oncology surgery:(a) candidate predictor for Model 1 was limited to only the NNIS risk index; (b) candidate predictors for Model 2 included all factors indicated in **Table 1** except GNRI; (c) candidate predictors for Model 3 included all variables listed in **Table 1**. To select the most optimal predictive features from the candidate predictors, the least absolute shrinkage and selection operator (LASSO) method was performed using the R package glmnet (9). This method is well-suited for reducing large datasets and reducing the likelihood of collinearity between the variables obtained from the same subject. The 10-fold cross-validation method was also used to determine the ideal value of the penalty parameter λ. Furthermore, the variables with non-zero coefficients in the fitted LASSO model were considered significant predictors. Candidate predictors selected through the LASSO method were used to construct a multivariate logistic regression model, and the Akaike Information Criterion (AIC) was referenced to find the optimal prediction model (12). The above analysis was performed using function glm in the "stats" package and function stepAIC in the "MASS" package. Finally, the Harrell concordance index (C-index), AIC, and the integrated discrimination index (IDI) were used to determine the final model (13, 14).

Based on the regression coefficients of the chosen independent variables, a simple nomogram was established by R package "regplot" to predict the SSIs in gynecologic oncology patients.

Accuracy and Reliability Evaluation of Prediction Model

In order to obtain an unbiased assessment of model performance, 1,000-bootstrap resamples were used for internal validation (27). Discrimination and calibration were used to describe the model's prediction performance. The nomogram's discrimination ability was evaluated using the concordance index (C-index; equal to the area under the receiver operating curve). A value higher than 0.75 indicates relatively good discrimination in the C-index value, which ranges from 0.5 to 1.0. An evaluation of the nomogram's calibration was carried out by plotting the GiViTI calibration belts (28). The 0.95 confidence band of the calibration curve and calibration test was used to detect the discrepancy between predicted and observed probabilities. The 95% CI did not cross the bisector, indicating a statistically significant deviation from the predicted probabilities. The calibration test (*P*-value > 0.05) suggests that there was no evidence of poor fit of the developed model. In addition, the existing predictors were also evaluated for their accuracy and suitability. A variety of statistical techniques, including the Cook's distance, the studentized residuals, the variance inflation factor (VIF), and the hat value were used to identify the outliers, collinearity, influential observations, as well as data with high leverage.

Clinical Utility

The current study utilizes the decision curve analysis (DCA) to evaluate the clinical efficacy of the established nomogram (29). DCA is a novel method that could be applied to a clinical setting

[1]https://www.R-project.org

TABLE 1 | Baseline characteristics of development cohort after imputation.

Characteristic	Non-SSI (*n* = 1,156)	SSI (*n* = 65)	*P*
General information			
Age (years), median (IQR)	50 (18)	56 (18)	0.010
BMI (kg/m^2), *n* (%)			0.786
<18.5	44 (3.8)	2 (3.1)	
18.5–24.0	662 (57.3)	40 (61.5)	
≥24	450 (38.9)	23 (35.4)	
Season of admission, *n* (%)			0.652
Spring	218 (18.9)	11 (16.9)	
Summer	259 (22.4)	12 (18.5)	
Fall	383 (33.1)	21 (32.3)	
Winter	296 (25.6)	21 (32.3)	
Surgical history in recent 3 months, *n* (%)	45 (3.9)	4 (6.2)	0.366
Comorbidities			
Hypertension, *n* (%)	290 (25.1)	26 (40.0)	0.008
Diabetes, *n* (%)	135 (11.7)	14 (21.5)	0.018
Coronary artery disease, *n* (%)	20 (1.7)	3 (4.6)	0.096
COPD/emphysema, *n* (%)	13 (1.1)	2 (3.1)	0.164
Moderate or severe renal disease, *n* (%)	32 (2.8)	3 (4.6)	0.385
Liver disease, *n* (%)	275 (23.8)	23 (35.4)	0.034
Bacterial vaginosis, *n* (%)	43 (3.7)	1 (1.5)	0.359
aCCI (points), median (IQR)	1 (4)	3 (5)	0.002
Preoperative variables			
FIGO stage ≥ III, *n* (%)	92 (8.0)	6 (9.2)	0.713
ASA class ≥ III, *n* (%)	58 (5.0)	4 (6.2)	0.685
Site of cancer, *n* (%)			0.109
Cervix	792 (68.5)	43 (66.2)	
Ovary/Fallopia	144 (12.5)	4 (6.2)	
Tube/Peritoneum uterus	220 (19.0)	18 (27.7)	
Barthel index, *n* (%)			0.102
Independent	1,076 (93.1)	57 (87.7)	
Partially/Totally dependent	80 (6.9)	8 (12.3)	
MFS score, *n* (%)			0.002
No risk	891 (77.1)	39 (60.0)	
Low/High risk	265 (22.9)	26 (40.0)	
Preoperative steroid use, *n* (%)	13 (1.1)	1 (1.5)	0.760
Laboratory values			
Glucose > 110 mg/dL, *n* (%)	891 (17.5)	14 (21.5)	0.403
Albumin ≤ 3.0 g/dL, *n* (%)	86 (7.4)	6 (9.2)	0.594
ALT > 40 U/L, *n* (%)	88 (7.6)	5 (7.7)	0.981
Total bilirubin ≥ 1.1 mg/dL, *n* (%)	79 (6.8)	4 (6.2)	0.832
Platelet count > 350 × 10^9/L, *n* (%)	47 (4.1)	6 (9.2)	0.047
Hematocrit < 36%, *n* (%)	377 (32.6)	24 (36.9)	0.472
TLC < 0.8 × 10^9 /L, *n* (%)	38 (3.3)	2 (3.1)	0.926
WBC > 10 × 10^9 /L, *n* (%)	48 (4.2)	5 (7.7)	0.173
Preoperative hair removal, *n* (%)	1,119 (96.8)	64 (98.5)	0.453
Preoperative LOS (d), median (IQR)	2.0 (2.0)	4.0 (5.0)	<0.001
Antibiotic prophylaxis within 0.5–1 h before operation, *n* (%)	815 (70.5)	53 (81.5)	0.056
MSCS>2, *n* (%)	40 (3.5)	3 (4.6)	0.623
Intraoperative variables			
Surgical approach, *n* (%)			0.417
Laparotomy	611 (52.9)	31 (47.7)	
Laparoscopy	545 (47.1)	34 (52.3)	
Operative time (min), median (IQR)	88.0 (130.5)	180.0 (152.0)	<0.001
Estimated blood loss (mL), median (IQR)	50.0 (90.0)	100.0 (250.0)	<0.001
Blood transfusion, *n* (%)	55 (4.8)	5 (7.7)	0.287
Emergent surgery, *n* (%)	185 (16.0)	8 (12.3)	0.427
NNIS risk index ≥ 1, *n* (%)	253 (21.9)	33 (50.8)	<0.001
Nutrition risk screening tools			
GNRI (points), median (IQR)	102.6 (8.5)	99.9 (6.7)	<0.001

IQR, interquartile range; SSI, surgical site infection; BMI, body mass index; COPD, chronic obstructive pulmonary disease; aCCI, age-adjusted Charlson comorbidity index; FIGO, International Federation of Gynecology and Obstetrics; ASA, American Society of Anesthesiology; MFS, Morse Fall Scale; ALT, alanine aminotransferase; TLC, total lymphocyte count; WBC, white cell count; LOS, length of stay; MSCS, modified surgical complexity score; NNIS, National Nosocomial Infection Surveillance; GNRI, geriatric nutritional risk index.

to assess the potential advantages of a risk prediction model, and it is derived using the formula provided below:

$$\text{Net benefit} = \text{true positive rate} - \text{false positive rate} \times \frac{p_t}{1 - p_t}$$

where, p_t represents the threshold probability at which the expected benefit of intervention-all-patients is equal to the expected benefit of intervention-none.

RESULTS

Characteristics of the Study Cohort

Among the 1,561 cohort participants, a total of 340 patients of which were individuals who were under 18 years of age (11 patients, 0.7%), patients with the length of stay less than 48 h (143 patients, 9.2%), patients who were postoperatively hospitalized for less than 24 h (3 patients, 0.2%), pregnant patients (13 patients, 0.8%), and as to not introduce bias due to hospital readmissions, another 170 patients (10.9%) were excluded from the study. Finally, the final analysis consisted of 1,221 participants (**Figure 1**). Among them, 65 patients (5.3%) had an SSI within 30 days of gynecologic oncology surgery. Baseline characteristics of the study cohort after imputation in regard to SSI vs. no SSI are listed in **Table 1** and **Supplementary Table 3**.

Association Between Geriatric Nutritional Risk Index and Surgical Site Infections

Before and after adjusting for the factors that impact the association between GNRI and SSI, the results of the logistic regression analysis both revealed that GNRI remained strongly linked with SSI when examined as continuous variables (**Table 2**). Furthermore, a smooth curve fitting graph was used before and

TABLE 2 | Cut-off point of GNRI before and after adjustment of the effect modifier.

		GNRI	
		Crude	**Adjusted†**
One-line linear regression model	OR (95% CI)	0.95 (0.92, 0.98)	0.92 (0.87, 0.97)
Two-piecewise linear model	Cut-off point	101.7	101.7
	OR1 (95% CI)	1.01 (0.95, 1.08)	1.00 (0.91, 1.11)
	OR2 (95% CI)	0.83 (0.74, 0.93)	0.83 (0.73, 0.94)
	OR2/OR1 (95% CI)	0.82 (0.71, 0.96)	0.83 (0.69, 1.00)
	Logarithmic likelihood ratio test	0.006	0.036

OR, odds ratio; CI, confidence interval; GNRI, geriatric nutritional risk index.
†Adjusted for age, BMI, season of admission, surgical history in recent 3 months, hypertension, diabetes, coronary artery disease, COPD/emphysema, moderate or severe renal disease, liver disease, bacterial vaginosis, aCCI, FIGO stage, ASA class, site of cancer, Barthel Index, MFS score, preoperative steroid use, glucose, albumin, ALT, total bilirubin, platelet count, hematocrit, TLC, WBC, preoperative hair removal, preoperative LOS, antibiotic prophylaxis within 0.5–1 h before operation, surgical approach, operative time, estimated blood loss, blood transfusion, emergent surgery, and NNIS risk index.

after all variables were adjusted, and the resultant curve exhibited a two-stage change and a cut-off point (**Figures 2A,B**). Although the SSI risk is lower when GNRI is higher than the cut-off point, there is no discernible difference when GNRI is lower than the cut-off point. The saturation effects were analyzed based on the curve, and the data indicated that the cut-off point was 101.7 (**Table 2**).

Moreover, we used a smoothing function to investigate the non-linear relationship between additional continuous variables (preoperative LOS, operative time, and estimated blood loss), and SSI in a two-piecewise linear regression model in which the results demonstrated a linear relationship between preoperative LOS and the risk of SSI. In contrast, a non-linear relationship could be seen between the duration of surgery and estimated blood loss with the risk of SSI (**Supplementary Figures 1A–C**). Based on the saturation effect of the curve analysis, the operating duration and estimated blood loss thresholds were 145 and 40, respectively (**Supplementary Table 4**).

Feature Selection

Using the LASSO method, five predictive features with non-zero coefficients (including MFS score, preoperative LOS, operation time, NNIS risk index, and estimated blood loss) were screened from the 36 candidate predictors (**Figures 3A,B**), and six predictive features with non-zero coefficients (including MFS score, preoperative LOS, operation time, NNIS risk index, estimated blood loss, and GNRI) were screened from the 37 candidate predictors (**Figures 3C,D**). Besides, the NNIS risk index was excluded in the final constructed multivariate regression model (Model 2 and Model 3) according to the lowest principle of AIC (**Table 3**).

Table 3 compares the predictive effectiveness of adding preoperative and intraoperative indexes, as well as the GNRI, to Model 1 (which only comprised the NNIS risk index) in predicting SSI. Model 1 exhibits the lowest C-index and the highest AIC values of 0.644 and 487.44, respectively. In contrast, there were four variables (MFS score, preoperative LOS, operation time, and estimated blood loss) that led to a greater risk of SSI in gynecologic oncology patients in Model 2 based on the LASSO and multivariate logistic regression analysis (**Figure 3** and **Table 3**). The elevated C-index value of 0.745–0.770 as well as a decrease of AIC from 468.23 to 458.99 in the development cohort may be attributable to the addition of GNRI to Model 2 containing the aforementioned four variables. To further evaluate whether the addition of GNRI data into the predictors would improve the risk classification with regard to SSI development, IDI was used. After the addition of another factor into the prediction model, the IDI value significantly improved [Model 3 vs. Model 2: 5.42% (1.80–9.03%)].

Development of a Nutrition-Based Nomogram

A unique nutrition-based nomogram was constructed to predict the likelihood of SSI in gynecologic oncology patients using the five independent variables aforementioned (**Figure 4**). In addition, for this particular model, a visual and operational dynamic web-based calculator was created by using the R package shiny Through the use of this calculator, users may easily acquire the SSI prediction probability simply by inputting or selecting a variable in the graphical user interface[2]. For instance, the estimated SSI risk was assessed to be approximately 12% (**Figure 4** and **Supplementary Figure 2**) in the dynamic nomogram for SSI in gynecologic oncology patients with MFS score = Low /High Risk ($\geq$ 25 points), preoperative LOS = 14 d, operative time = 160 min, estimated blood loss = 30 ml, and GNRI = 100.9.

Prediction Model Validation

According to **Figures 5A,B**, the nomogram C-index in the development cohort was at 0.770 (95% CI: 0.718–0.821) and 0.768 (95% CI: 0.716–0.820) in the internal validation cohort, respectively. Based on these findings, the nomogram demonstrates a moderate to high degree of discrimination.

As seen in **Figures 6A,B**, the 95% CI region of the calibrated GiViTI belt did not cross the 45-degree diagonal bisector line in either the development or internal validation cohorts ($P = 0.573$, $P = 0.316$, respectively), indicating that the observed and predicted probabilities of SSI in the prediction model were in good agreement. Because the VIF in all predictors was less than 5, no multicollinearity was seen. Moreover, there were also no observable changes and high leverage cases since both Cook's distances and hat values were no more than 0.1 and 0.2, respectively (**Supplementary Figures 3A,B**). Taken together, these findings revealed that the nutrition-based nomogram was a good model for predicting SSI in gynecologic oncology patients.

Clinical Usefulness of the Nomogram

According to DCA, using the nomogram to predict SSI risk is more beneficial than using the "intervention-all-patients"

[2]https://ssi-prediction.shinyapps.io/DynNomapp/

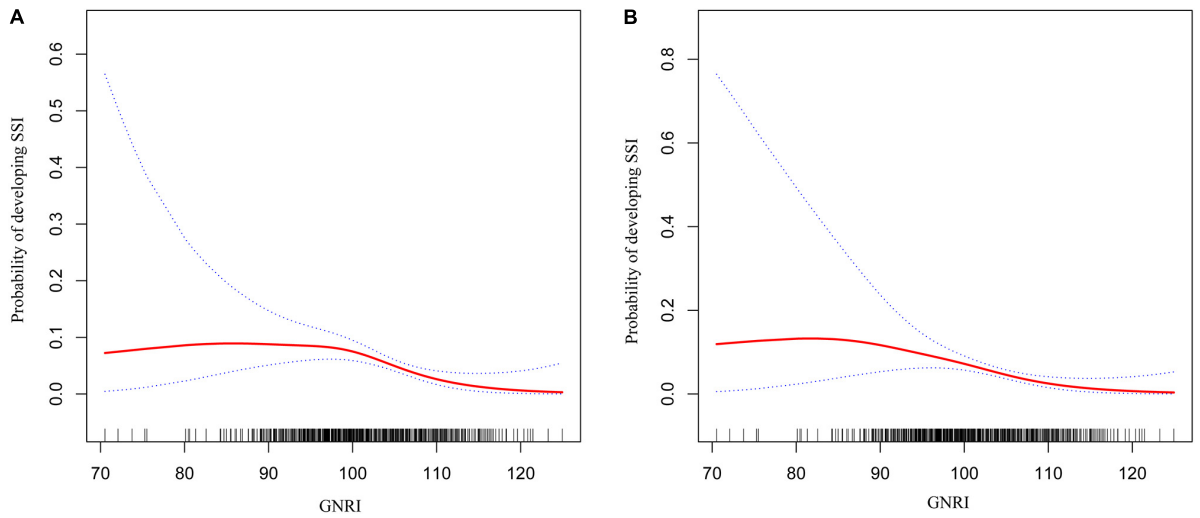

FIGURE 2 | Two-piece piecewise regression and smooth curve-ftting for association between GNRI and the risk of SSI in gynecologic oncology patients. **(A)** The two-piece wise models unadjusted for any variables. **(B)** The two-piece wise models adjusted for age, BMI, season of admission, surgical history in recent 3 months, hypertension, diabetes, coronary artery disease, COPD/emphysema, moderate or severe renal disease, liver disease, bacterial vaginosis, aCCI, FIGO stage, ASA class, site of cancer, Barthel Index, MFS score, preoperative steroid use, glucose, albumin, ALT, total bilirubin, platelet count, hematocrit, TLC, WBC, preoperative hair removal, preoperative LOS, antibiotic prophylaxis within 0.5–1 h before operation, surgical approach, operative time, estimated blood loss, blood transfusion, emergent surgery, NNIS risk index, and GNRI. Note: the red line represents the best-fit line, and the blue lines are the 95% confidence intervals.

or "intervention-none" methods within a range of 0–0.49 (**Figures 7A,B**).

DISCUSSION

The rise of SSIs has now become a serious safety problem for gynecologic oncology patients since it not only jeopardizes their health with the increase in mortality rate but will also necessitate much greater treatment costs and longer hospitalization. The ability to accurately identify individuals with a high risk of infection and alter therapy measures appropriately offers an improved preventative approach that may improve health outcomes. As a result, it is imperative that those at greater risk should be promptly identified.

Using the five independent predictors listed above (with reference to Results: Development of a nutrition-based nomogram), we developed a simple yet efficient nomogram to predict surgical site infection in gynecologic oncology patients to aid in medical decision-making. In both the training and validation cohorts, the nomogram demonstrated excellent calibration and discrimination performance. Furthermore, as compared to Model 1 (only NNIS risk index included) and Model 2 (without GNRI), the nomogram based on GNRI demonstrated higher prediction reliability, accuracy, and net benefit. This nomogram can assist physicians in making more precise clinical judgments. By only incorporating these five factors in our nomogram and accounting for varied proportions of those variables, we were able to simply and effortlessly provide an accurate forecast of a patient's likelihood of acquiring SSI in a systematic and thorough manner. In addition, the serum albumin level, height, as well as the patient's body weight that make up the GNRI in this study, were routinely assessed before surgery and did not necessitate a special examination, highlighting its feasibility in clinical settings. The GNRI was initially designed to assess malnutrition, as well as its associated morbidity and mortality in elderly patients (10). Numerous studies have shown that GNRI is a suitable predictor for the development of SSI in individuals with various forms of cancer (10, 30, 31). However, the optimal cut-off point value of GNRI for predicting SSIs in cancer patients is still unknown. In a previous study, Sasaki et al. (30) utilized the ROC curve to derive a GNRI cut-off point value of 98 to predict potential prognosis and postoperative complications of elderly patients with colorectal cancer. A similar approach was used to obtain a GNRI cut-off point value of 94 by Funamizu et al. (31) in their study to predict the post-pancreatoduodenectomy development of SSI. In this study, we investigated the non-linear association between GNRI and SSI in gynecologic oncology patients using a two-piecewise linear regression model fitted with a smoothing function and ultimately found the ideal cut-off point value for GNRI was 101.7. There are several possible reasons for the uncertainty surrounding the GNRI cut-off point: Firstly, different malignant tumors exhibit unique biological characteristics; Secondly, the limited number of patients may lead to the bias of the best cut-off value; and finally because there are numerous statistical methods for calculating the best cut-off point value, different cut-off points may be chosen in different studies. For instance, when defining the cut-off value, the ROC curve cannot correct the potential confounding factors within the model, but the two-piecewise linear regression can fully adjust the confounding factors and explain the non-linear connection between the variables (30, 32, 33).

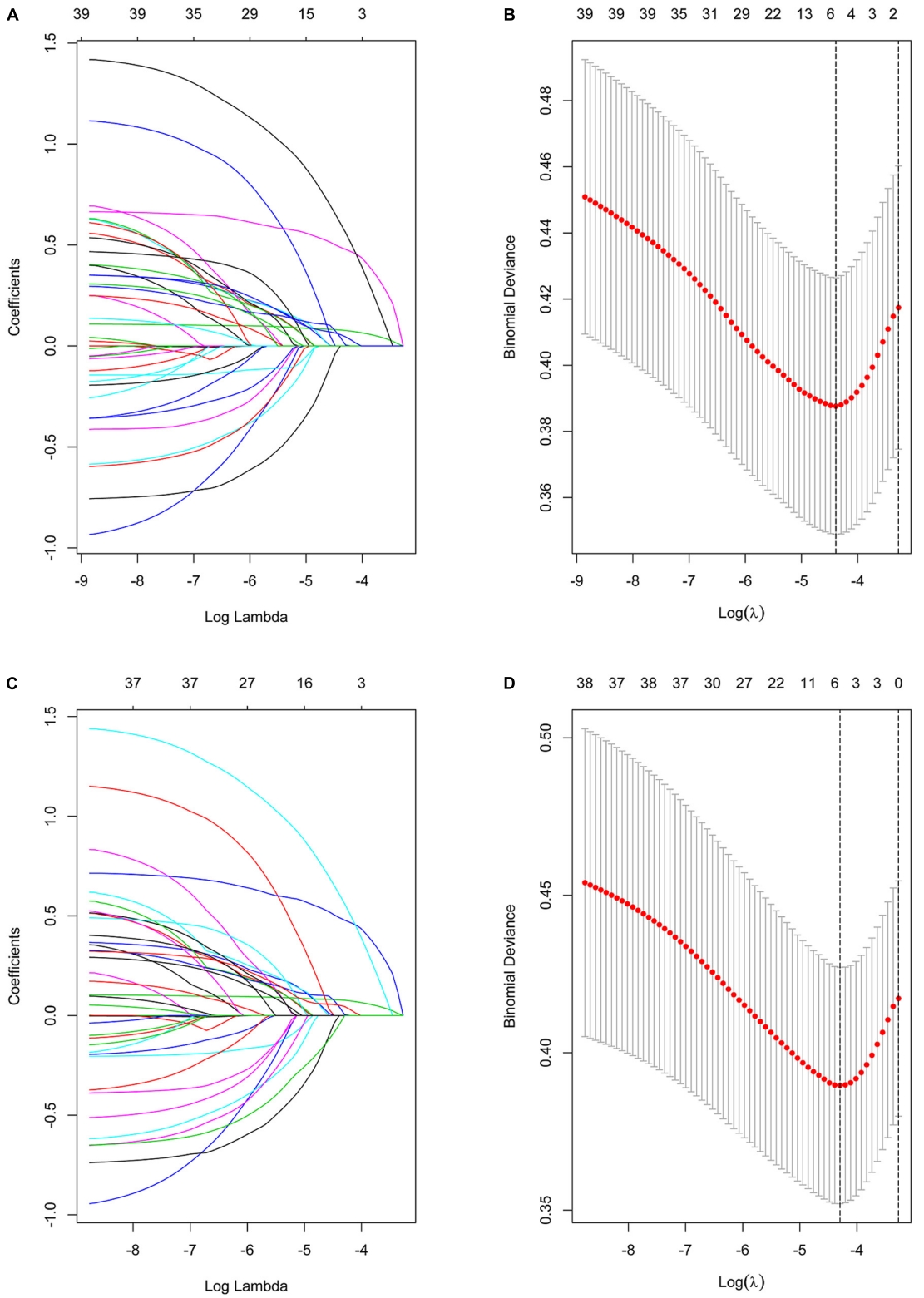

FIGURE 3 | Variable selection using the LASSO binary logistic regression model. **(A)** Profiles of the LASSO coefficients for the 36 candidate variables. **(B)** Optimal penalization coefficient (λ) selection in the LASSO model used 10-fold cross-validation via minimum criteria. **(C)** Profiles of the LASSO coefficients for the 37 (Plus GNRI) candidate variables. **(D)** Optimal penalization coefficient (λ) selection in the LASSO model used 10-fold cross-validation via minimum criteria. Note: the left vertical line represents the minimum error, and the right vertical line represents the one standard error of the minimum criteria (1-SE criterion).

TABLE 3 | Prediction effect of the three models.

Intercept and variable	Model 1†		Model 2‡		Model 3§	
	β	Adjusted OR (95% CI)	β	Adjusted OR (95% CI)	β	Adjusted OR(95% CI)
Intercept	−3.34		−4.49	–	−4.49	–
MFS score (Low /High Risk)	–	–	0.50	1.65(0.96–2.78)	0.41	1.51(0.87–2.57)
Preoperative LOS (increase per day)	–	–	0.27	1.32(0.66–2.76)	0.10	1.10(1.02–1.18)
Operative time ≥ 145 min	–	–	0.69	1.99(0.91–4.20)	0.70	2.01(1.14–3.65)
Estimated blood loss ≥ 40 ml	–	–	1.39	4.01(1.79–10.22)	1.31	3.69(1.65–9.42)
NNIS risk index ≥ 1	1.30	3.68(2.22–6.12)	–	–	–	–
GNRI ≥ 101.7	–	–	–	–	−0.52	0.60(0.35–1.00)
		C_1-index		C_2-index		C_3-index
Primary cohort		0.644		0.745		0.770
Internal validation with 1000 bootstrapping		0.644		0.743		0.768
AIC		487.44		468.23		458.99
IDI (95%CI)		7.28% (3.46%–11.09%)*		5.42% (1.80%–9.03%)**		

LOS, length of stay; NNIS, National Nosocomial Infection Surveillance; GNRI, geriatric nutritional risk index; OR, odds ratio; CI, confidence interval; C-index, concordance index; IDI, net reclassification improvement; AIC, akaike information criterion.
†*Candidate predictor for Model 1 was limited to only the NNIS risk index.*
‡*Candidate predictors for Model 2 included all factors indicated in* ***Table 1*** *except GNRI.*
§*Candidate predictors for Model 3 included all variables listed in* ***Table 1****.*
**for Model 3 vs Model 1.*
***for Model 3 vs Model 2.*

The Morse Fall Scale score was also included in our nomogram. This score is often regarded as a significant indicator of frailty (34–36). Additionally, this study demonstrated that surgical time might independently predict the development of SSI in patients with gynecologic cancer, with 145 min serving as the cut-off value for categorization. It has been proven that a prolonged surgical duration increases the incidence of SSI (37). Also, many prior studies have found a link between estimated blood loss and the risk of SSI (38, 39). According to our study, the development of SSI in gynecologic oncology patients may also be determined using predicted blood loss as an independent variable, with the classification cut-off value set as 40 ml. In addition, the preoperative length of stay was also regarded as an independent predictor of the development of SSI in gynecological oncology patients and was included in the model in accordance with a linear relationship. It should also be noted that, although the NNIS risk index was retained in the model after LASSO dimension reduction using, this factor was excluded in the final constructed multivariate regression model according to the lowest principle of AIC. This further establishes that the NNIS risk index is unsuitable for stratifying the risk of surgical site infection across all types of surgery (19, 40). Mahdi et al. (5) found that the risk of developing SSI for endometrial cancer was 0.8 times higher than that for other types of gynecologic oncology patients in the laparotomy group by multivariate analysis (OR, 1.8; 95% CI,1.2–2.6; P = 0.02). However, by multivariate analysis, the above association was not observed in the laparoscopic surgery group (5). Our study showed that the site of cancer was not an independent predictor for SSI in gynecologic oncology patients, which may be attributed to the inclusion of a subset of laparoscopic surgery patients in our cohort.

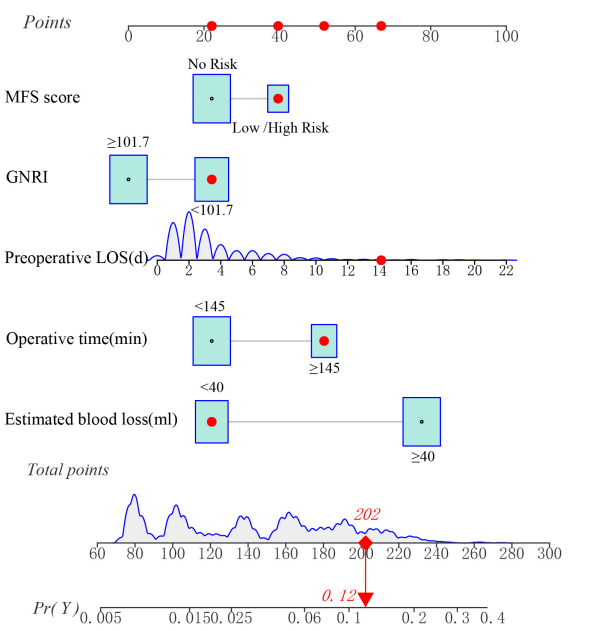

FIGURE 4 | A dynamic nomogram for predicting the risk of SSI in gynecologic oncology patients. Note: the SSI risk nomogram was developed with the MFS score, preoperative LOS, operative time, estimated blood loss, and GNRI as predictors.

Additionally, a dynamic web-based calculator was developed to facilitate the use of the nomogram (see text footnote 2). For this particular model, users may easily acquire the SSI prediction probability simply by inputting or selecting a variable in the graphical user interface. Consequently. healthcare providers may conduct a preliminary assessment of the risk of development

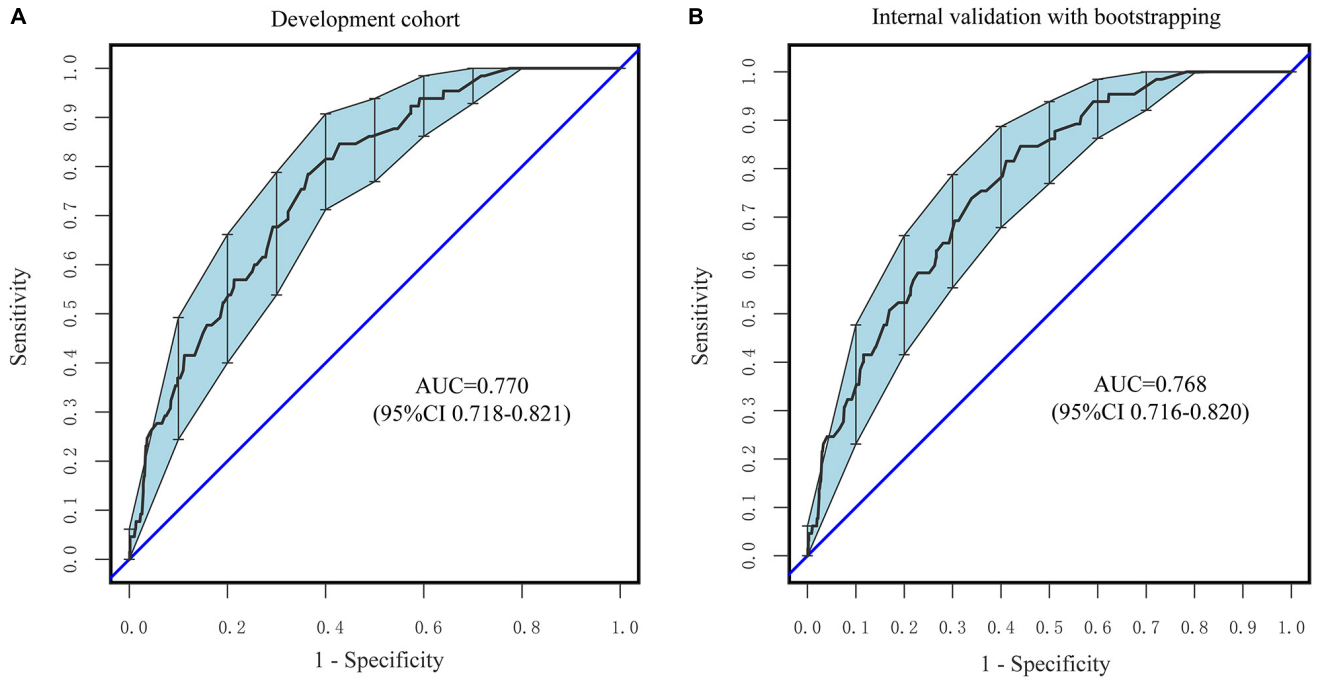

FIGURE 5 | ROC curves of the nomogram. **(A)** The development Cohort. **(B)** The internal validation cohort. The *X*-axis represents the false-positive rate of the risk prediction. The *Y*-axis meant the true-positive rate of the risk prediction.

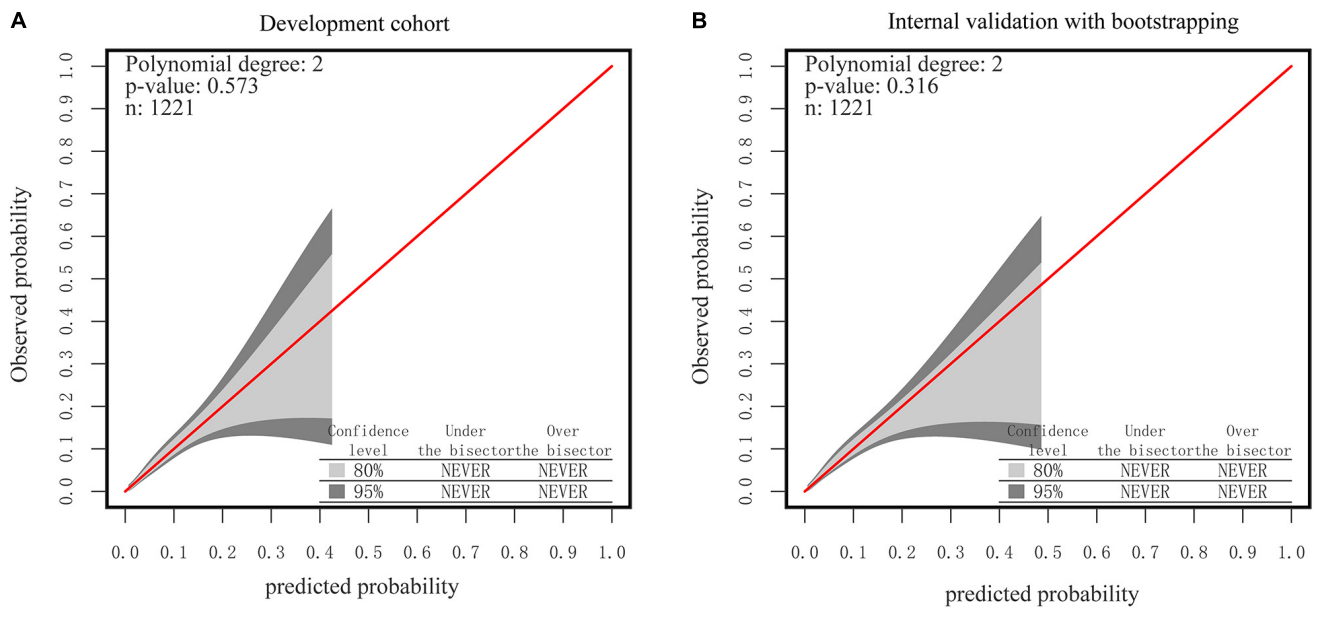

FIGURE 6 | The GiViTI calibration belt for the nomogram. **(A)** The development Cohort. **(B)** The internal validation cohort. Note: the 80%CI and 95%CI calibration belt are plotted, in light and dark gray, respectively. The red diagonal line is the reference line indicating perfect calibration. Note: the red line represents the best-fit line, and the blue lines are 95% confidence intervals.

of SSI in gynecologic oncology individuals and closely monitor those who are more susceptible. A subset of people who would benefit the most from more regular examinations is the high-risk population. In addition, interventions (e.g., nutritional interventions) can be taken more aggressively in high-risk individuals.

There are, however, several limitations that must be taken into account. To begin, this is a retrospective single-center

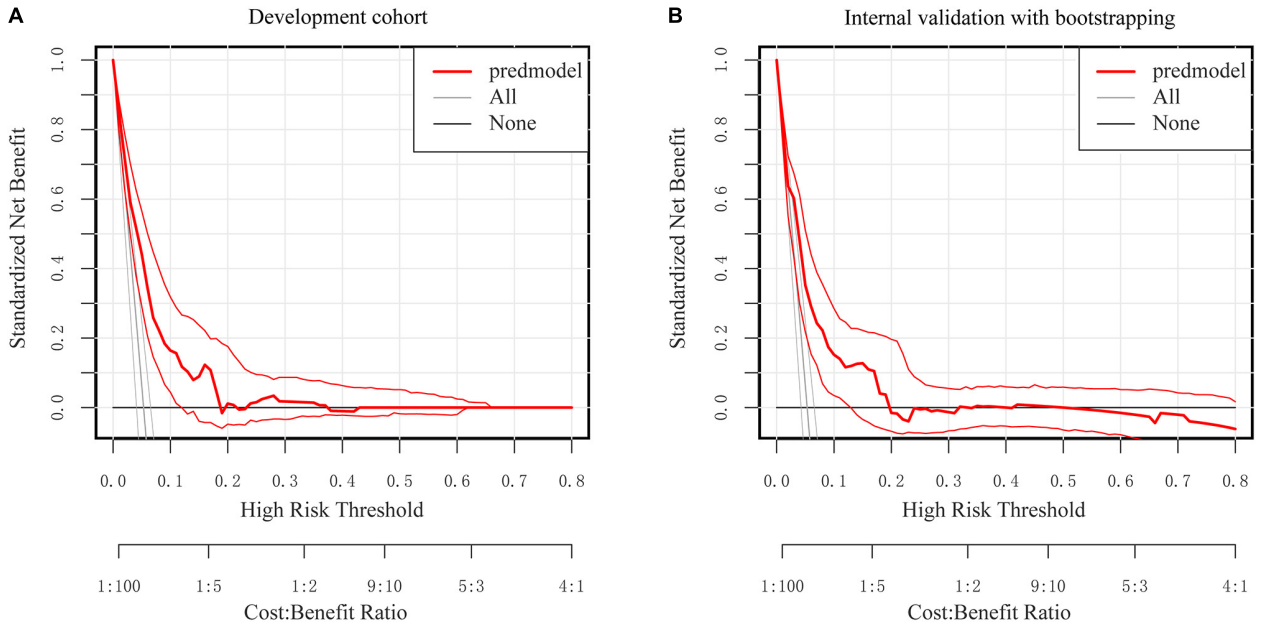

FIGURE 7 | Decision curve analysis for the nomogram. **(A)** The development Cohort. **(B)** The internal validation cohort. The *Y*-axis represents the standardized net benefit. The thick red solid line is the nomogram to predict SSI risk. The thin red solid line represents the 95% credible interval. The black solid line represents the assumption that all patients had no SSI. The gray solid line represents the assumption that all patients had SSI.

research, which inevitably has limitations due to its methodology and sample size. Therefore, further research is needed to externally validate the suggested nomogram despite the fact that we employed 1,000-bootstrap resamples for internal validation. Secondly, knowing that the cause of SSI is multifactorial, our research was primarily concerned with patient-related and surgical variables, excluding other elements like the operating room setting. Thirdly, there were too many missing data (≥50%) or too few positives in this study to incorporate previously established risk factors for SSI, such as current smokers and intraoperative hypothermia. Fourthly, while each case of SSI was thoroughly evaluated and co-confirmed by a physician and a senior infection control practitioner to avoid misclassification bias in the current investigation, misdiagnosis and missed diagnoses could still have occurred. Finally, even though various assessment tools, such as Barthel Index, Morse Fall Scale, weight, and height, were evaluated by competent nurses in our hospital, we could not rule out the possibility of measurement bias as a result of the screening being performed by different nurses.

CONCLUSION

The current research investigates the use of preoperative GNRI as an independent predictor of SSI in gynecologic oncology patients. We found that a cut-off value of 101.7 for the GNRI allowed for appropriate stratification of patients into distinct SSI risk groups. Moreover, we established and validated a nomogram based on the GNRI to estimate the risk of SSI in gynecologic oncology patients, and proved that incorporating the GNRI into the model may further increase its prediction power.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Review Committee of Wenzhou People's Hospital. Written informed consent for participation was not required for this study in accordance with the National Legislation and the Institutional Requirements.

AUTHOR CONTRIBUTIONS

ZC and MZ analyzed the data and drafted the manuscript. ZX, QY, WX, SG, LC, LQ, JJ, and HWu collected and analyzed the data. XL and HWa conceived and designed the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.864761/full#supplementary-material

Supplementary Figure 1 | Two-piece piecewise regression and smooth curve-ftting to analyze the association between preoperative LOS, operative time, estimated blood loss and the risk of SSI in gynecologic oncology patients. **(A)** The association between preoperative LOS and the risk of SSI. **(B)** The association between operative time and the risk of SSI. **(C)** The association between estimated blood loss and the risk of SSI. All variables (preoperative LOS, operative time, estimated blood loss) in **Table 1** were adjusted except self.

Supplementary Figure 2 | Screenshot of the online program used for the prediction of SSI risk.

Supplementary Figure 3 | Diagnostic plots of accuracy and suitability of existing predictors. **(A)** The Cook's distance, the studentized residuals, and the hat value of the model. **(B)** The variance inflation factor of the model.

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