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Evidence-based on health benefits: Probiotics, micronutrients, and edible plants

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Published in
Frontiers in Nutrition

ISSN 1664-8714
ISBN 978-2-8325-3720-6
DOI 10.3389/978-2-8325-3720-6

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Evidence-based on health benefits: Probiotics, micronutrients, and edible plants

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Citation

Saokaew, S., Dilokthornsakul, P., Goh, B. H., Lee, L.-H., eds. (2023). *Evidence-based on health benefits: Probiotics, micronutrients, and edible plants*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3720-6

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frontiers | Frontiers in Nutrition

TYPE Editorial
PUBLISHED 02 October 2023
DOI 10.3389/fnut.2023.1272456

OPEN ACCESS

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RECEIVED 04 August 2023
ACCEPTED 21 September 2023
PUBLISHED 02 October 2023

CITATION
Lee L-H, Goh B-H, Dilokthornsakul P and Saokaew S (2023) Editorial: Evidence-based on health benefits: probiotics, micronutrients, and edible plants. *Front. Nutr.* 10:1272456. doi: 10.3389/fnut.2023.1272456

Editorial: Evidence-based on health benefits: probiotics, micronutrients, and edible plants

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KEYWORDS

probiotics, micronutrients, edible plants, health benefits, evidence-based, SDG 3 good health and wellbeing

Editorial on the Research Topic
Evidence-based on health benefits: probiotics, micronutrients, and edible plants

Nutrition is critical in preventing, treating, and prognosis acute and chronic disorders. The field of nutritional epidemiology, which has emerged from public health sciences, has been the cornerstone of nutrition research for several decades, significantly influencing dietitian practices and dietary counseling worldwide (1). Evidence-based nutrition involves a conscientious approach, working with patient's preferences and values to help them address physical, mental, and social health challenges by integrating the best available nutrition evidence with clinical expertise. In recent years, there has been growing interest in understanding the therapeutic role of probiotics, micronutrients and bioactive compounds derived from edible plants in various health conditions (2–4).

This Research Topic aims to consolidate the literature on the efficacy of probiotics, micronutrients, and edible plants in managing acute and chronic diseases. A total of 18 studies have been published on this Research Topic. Primarily, these studies contribute to the growing body of research exploring the potential benefits of nutrition-based interventions and their impact on human health. Notably, numerous research investigated the associations between dietary components and specific health outcomes, shedding light on potential interventions and preventive measures. The overarching area of interest for these 18 studies is the exploration of how dietary components influence various health conditions. Researchers have focused on examining micronutrients like zinc, copper, selenium, and manganese, as well as vitamins and antioxidants. Additionally, studies have looked at the effects of probiotics, prebiotics, and synbiotics on liver enzymes, neurological diseases, and metabolic disorders. The use of natural products derived from edible plants to ameliorate health conditions has also been a common theme. These studies utilized different methodologies, including meta-analyses, systematic reviews, experimental studies, and quasi-experimental designs, to investigate the relationships between nutrition and specific health conditions.

Nutrition plays a central role in the context of the discussed studies on the healing potential of edible plants and their bioactive natural compounds (5, 6). In this Research Topic, four research papers emphasized the importance of consuming a well-balanced diet rich in nutrients, including vitamins, minerals, antioxidants, and other bioactive compounds found in edible plants. For instance, Salari-Moghaddam et al. identified that the dietary total antioxidant capacity (TAC) could be considered a proper measure for assessing diet quality, highlighting that antioxidants are among the important nutrients in foods included in a high-quality diet. Rawangkan et al. investigated the antimicrobial activity of coffee beans and coffee by-products against drug-resistant *Vibrio cholerae*. The study shows that various phytochemicals, such as caffeic acid and chlorogenic acid, are effective in treating multidrug-resistant *V. cholerae* infections. Barakat et al. demonstrated lentils (*Lens culinaris* M.) as a superfood rich in bioactive phytochemicals that could potentially confer antioxidative, hepatoprotective, and nephroprotective effects. Meanwhile, Saadat et al. consolidated an extensive list of preclinical and clinical evidence on the effects of natural products derived from various plant sources against noxious agents-induced lung injuries, highlighting their therapeutic potential in the clinical management of lung disorders.

There are six publications that investigated the impact of vitamins, namely vitamins C and D, on various health outcomes. These studies focused on their supplementation, dietary intake, and deficiency in relation to various health risks, including colorectal cancer, brain tumor, ischemic stroke, bacterial vaginosis during pregnancy and spinal cord injury recovery. Han et al. conducted a meta-analysis, suggesting that dietary high intake of β-carotene may have a protective effect against colorectal cancer. Zhang W. et al. reviewed the evidence on vitamins and brain tumors, finding that higher intake or serum concentration of vitamins C, β-carotene, and folate may significantly reduce brain tumor risk, providing new perspectives on prevention. Tang et al. reviewed the evidence on vitamin C and ischemic stroke risk, highlighting its protective effects through various mechanisms, including regulating vascular tone and reducing oxidative stress. Ma et al. conducted a meta-analysis, finding that vitamin D deficiency is positively associated with the risk of bacterial vaginosis during pregnancy. Wang et al. reviewed the literature on vitamin D and spinal cord injury (SCI), revealing a high prevalence of vitamin D insufficiency in SCI patients, which may impair functional restoration. Vitamin D supplemental treatment could potentially aid post-injury rehabilitation and has neuroprotective effects.

Lastly, Jittat et al. conducted a quasi-experimental study, finding that oral multi-vitamin multi-mineral (MVMM) supplement formulations, (1) Hydro-Cell-Key (HCK) granule and (2) VTL-7 capsule, increased serum levels of vitamin D and β-carotene. The study suggested that these formulations could be a good reference for future studies on micronutrient supplementation, primarily benefitting those individuals with vitamin A or D deficiency. Collectively, these research papers contribute valuable insights into the potential roles of vitamins in promoting health and preventing diseases. These findings underscore the importance of ensuring adequate vitamin intake for overall wellbeing and highlight potential avenues for therapeutic interventions. However, further research and well-designed clinical trials are necessary to establish definitive causal relationships and optimize vitamin-based interventions for specific health conditions.

The significance of micronutrients cannot be overstated in the realm of nutrition and their impact on overall human health. These essential elements, found in trace amounts within our diets, play a vital role in the proper functioning of our bodies and contribute to overall wellbeing. There were four studies that shed light on the associations between micronutrients and various health outcomes, ranging from thyroid cancer and metabolic syndrome to depression and urologic cancers.

Zhang X. et al. explored the association between iodine nutrition and papillary thyroid cancer (PTC) based on evidence from epidemiological and experimental studies investigating the prevalence, distribution and aggressiveness of PTC in relation to iodine intake. The findings illustrated the U-shaped relationship between iodine and papillary thyroid cancer, indicating the importance of maintaining an optimal and balanced intake of iodine to mitigate potential health risks. Meta-analyses were performed on observational studies to examine the associations between dietary micronutrient intake and metabolic syndromes and depression. Ding et al. unveiled the inverse associations between dietary zinc intake and metabolic syndrome. Similarly, Ding and Zhang demonstrated the negative relationship between dietary copper, selenium, and manganese with depression. These studies provide valuable insights into the potential benefits of these micronutrients in promoting metabolic health and emotional wellbeing.

In contrast, Lu et al. revealed differential roles of micronutrients (copper, iron, and zinc) in influencing the risk for urologic cancers using a two-sample Mendelian randomization study. The study genetically predicted that the increase in serum copper and iron levels was causally associated with an increased risk of renal cell carcinoma (RCC). Meanwhile, an increase in serum zinc level was related to decreased risks of RCC but increased risk of prostate cancer. Evidently, these findings indicate that further exploration and well-designed prospective cohort studies are essential in unraveling the intricate roles of micronutrients in health and disease. By gaining a deeper understanding of the roles and interactions of these trace elements, we can refine our nutritional approaches to support health and wellbeing. Empowering individuals with evidence-based information will enable them to make informed dietary choices, ensuring adequate intake of essential micronutrients. Together, micronutrients hold immense potential to be developed into targeted and personalized nutritional interventions, becoming a cornerstone of public health preventive and therapeutic strategies.

The gut microbiome is a key player in mediating the effects of dietary interventions on various health outcomes (7). The gut microbiome refers to the diverse community of microorganisms, including bacteria, viruses, fungi, and other microbes, that reside in the gastrointestinal tract (8). It plays a critical role in various aspects of health, including digestion, metabolism, immune function, and even mental health (9). Probiotics have been shown to modulate the gut-brain axis, the bidirectional communication system between the gut and the brain (10). In the study by Mahboobi et al., the

researchers evaluated the effects of probiotic and magnesium co-supplementation on mood, cognition, intestinal barrier function, and inflammation in individuals with obesity and depressed mood. The randomized, double-blind placebo-control trial concluded that probiotic and magnesium co-supplementation resulted in reduced serum C reactive protein in obese and depressed patients. Similarly, Lee et al. reviewed the use of probiotic therapy in metabolic and neurological diseases, emphasizing the role of the gut microbiome in influencing health outcomes.

Besides that, Kanchanasurakit et al. systematically evaluated the effects of synbiotics, probiotics, and prebiotics on liver enzymes and other clinical parameters in patients with non-alcoholic fatty liver disease (NAFLD). The gut microbiome has been linked to the pathogenesis of NAFLD, and these interventions have the potential to alter the gut microbial community, leading to improvements in liver function. Lastly, Kaewdech et al. explored the effect of fiber supplementation on the prevention of diarrhea in hospitalized patients receiving enteral nutrition. Fiber serves as a prebiotic that nourishes beneficial gut bacteria and confers positive effects on colonocytes. The meta-analysis highlighted that specific fiber types, such as mixed soluble/insoluble fiber and hydrolysed guar gum, are associated with a more evident reduction of diarrhea among hospitalized patients receiving enteral nutrition. Therefore, these studies reveal that gut microbiota modulation-based nutritional interventions are promising avenues for disease prevention and management.

While these research papers contribute valuable insights, there are some potential gaps that future studies could address. First, many of these studies are based on observational data, which can show associations but not causation. Future research should focus more on well-designed randomized controlled trials to establish the therapeutic efficacy of specific dietary components or interventions for various acute and chronic diseases. Moreover, the studies predominantly focus on specific dietary components, but the synergistic effects of a balanced diet on overall health remain to be explored further. Another potential gap is the lack of representation of diverse populations in some of the studies. Nutrition and health outcomes can be influenced by genetic, cultural, and lifestyle factors, which could vary across different populations. Conducting research on diverse populations would provide a more comprehensive understanding of the relationships between nutrition and health (11, 12). Furthermore, the studies primarily focused on the impact of dietary components on disease risk or progression. Exploring the mechanisms by which these components exert their effects at the molecular and cellular levels would enhance our understanding of the underlying biology and facilitate targeted interventions.

In summary, the collection of publications reviewed in this Research Topic sheds light on the intricate relationship between dietary factors and various health conditions. By translating these findings into practice and conducting further research in emerging directions, we can harness the power of nutrition to address global health challenges effectively. While providing valuable insights, these studies also highlight potential gaps in knowledge that warrant further investigation. Future research in this area should aim to establish causal relationships, consider diverse populations, explore mechanisms of action, and emphasize the importance of a balanced diet for overall health and disease prevention. Such endeavors would pave the way for more effective and personalized nutritional interventions to improve public health outcomes.

Author contributions

L-HL: Conceptualization, Validation, Writing—original draft. B-HG: Conceptualization, Validation, Writing—review and editing. PD: Conceptualization, Validation, Writing—review and editing. SS: Conceptualization, Validation, Writing—review and editing.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers in Nutrition

SYSTEMATIC REVIEW
published: 03 February 2022
doi: 10.3389/fnut.2022.825913

Association Between Dietary Zinc Intake and Metabolic Syndrome. A Meta-Analysis of Observational Studies

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Background: Epidemiological studies have investigated the association between dietary zinc intake and metabolic syndrome (MetS). However, their results are conflicting. This meta-analysis was therefore employed to investigate the associations further.

Methods: A comprehensive literature search was employed by using the electronic database of PubMed, Web of Science, and Embase up to November 2021. The pooled relative risk (RR) of MetS for the highest vs. lowest dietary zinc intake category, and the weighted mean difference (WMD) of dietary zinc intake for MetS vs. control subjects as well as their corresponding 95% confidence interval (CI) were calculated.

Results: A total of 13 observational studies (18,073 participants) were identified in this meta-analysis. The overall multi-variable adjusted RR demonstrated that the dietary zinc intake was inversely associated with MetS (RR = 0.75, 95%CI: 0.61 to 0.93; $P = 0.009$). The subgroup analysis confirmed such findings in cross-sectional (RR = 0.70, 95%CI: 0.55 to 0.87; $P = 0.002$), NCEP-ATP III (RR = 0.64, 95%CI: 0.48 to 0.84; $P = 0.002$), adult (RR = 0.77, 95%CI: 0.62 to 0.96; $P = 0.02$), dietary recall method (RR = 0.70, 95%CI: 0.55 to 0.87; $P = 0.002$), and >500 sample-sized study (RR = 0.79, 95%CI: 0.64 to 0.99; $P = 0.002$), respectively. On the other hand, the overall combined WMD showed that the dietary zinc intake in MetS was also lower than that in control subjects (WMD = −0.21, 95%CI: −0.42 to 0.00; $P = 0.05$).

Conclusions: Our results suggest that the dietary zinc intake is negatively associated with MetS. However, due to the limitation of available evidence. More well-designed prospective cohort studies are still needed.

Keywords: dietary zinc intake, metabolic syndrome, meta-analysis, observational studies, clinical nutrition

OPEN ACCESS

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *30 November 2021*
Accepted: *07 January 2022*
Published: *03 February 2022*

Citation:
Ding J, Liu Q, Liu Z, Guo H, Liang J and Zhang Y (2022) Association Between Dietary Zinc Intake and Metabolic Syndrome. A Meta-Analysis of Observational Studies. Front. Nutr. 9:825913. doi: 10.3389/fnut.2022.825913

INTRODUCTION

Metabolic syndrome (MetS) is defined as a cluster of elevated fasting blood glucose, triglycerides, blood pressure, waist circumference, and decreased high-density lipoprotein cholesterol (at least three of the above metabolic abnormalities) (1). Metabolic syndrome is closely associated with diabetes mellitus, stroke and coronary heart disorders (2–4). The global prevalence of MetS is

between 11.6 and 62.5%, which is still progressively growing (5). The etiology of MetS is not well-understood yet. However, the dietary factors are deemed to be significantly involved in MetS (6–10).

As the second most common trace metal in the body, zinc is associated with DNA replication and transcriptions, protein synthesis, and cellular division and differentiation (11). Zinc is an important antioxidant, which stabilizes membrane, prevents cellular apoptosis, and is also important for endothelial integrity (12, 13). It is widely accepted that zinc improves chronic inflammation, oxidative stress, and insulin resistance (14, 15), which is closely associated with the pathogenesis of MetS. Moreover, epidemiological data have indicated a negative relationship between dietary zinc intake and MetS-related context (e.g., diabetes) (16). Therefore, the dietary zinc intake is speculated to be inversely associated with MetS.

As far as we know, a number of observational studies have explored the association between dietary zinc intake and MetS (17–29). However, their results are still conflicting. Thus, this meta-analysis of observational studies is employed to investigate the issue further. It is hypothesized that the dietary zinc intake is inversely associated with MetS.

MATERIALS AND METHODS

Search Strategy

Our meta-analysis was employed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (30). Combine the keywords that related to MetS ("metabolic syndrome") and zinc ("zinc," "zn"), the electronic database of PubMed, Web of Science, and Embase were searched up to November 2021. No language restriction was set in the search strategy. The titles and abstracts of all articles were screened firstly, and the full articles were then read to identify the eligible studies.

Study Selection

The titles, abstracts and full texts of all retrieved studies were comprehensively reviewed by two researchers independently. Disagreements were resolved by discussions. The included studies were required to meet the following criteria: (1) the study design is observational study; (2) the association between dietary zinc intake and MetS; (3) the relative risk (RR), odds ratio (OR), or weighted mean difference (WMD) with 95% confidence interval (CI) were reported. The exclusion criteria were listed as follows: (1) duplicated or irrelevant articles; (2) randomized controlled trials; (3) reviews, letters, or case reports; (4) non-human studies.

Data Extraction

The effect estimates from each included studies were extracted by two researchers independently, and disagreements were resolved by discussion. The information about the first author, year of publication, location, age, gender, sample size, study design, adjustments, dietary zinc assessment, exposure, effect estimates, and diagnostic criteria of MetS, was collected. The corresponding effect estimates of MetS for the highest vs. lowest dietary zinc intake category that adjusted for the maximum number of confounding variables were extracted for analysis. Moreover, the dietary zinc intake in MetS vs. control was also extracted to calculate the WMD (mean ± SD).

Quality Assessment

We employed a quality assessment according to the Newcastle-Ottawa (NOS) criteria for non-randomized studies, which is based on three broad perspectives: the selection process of study cohorts, the comparability among different cohorts, and the

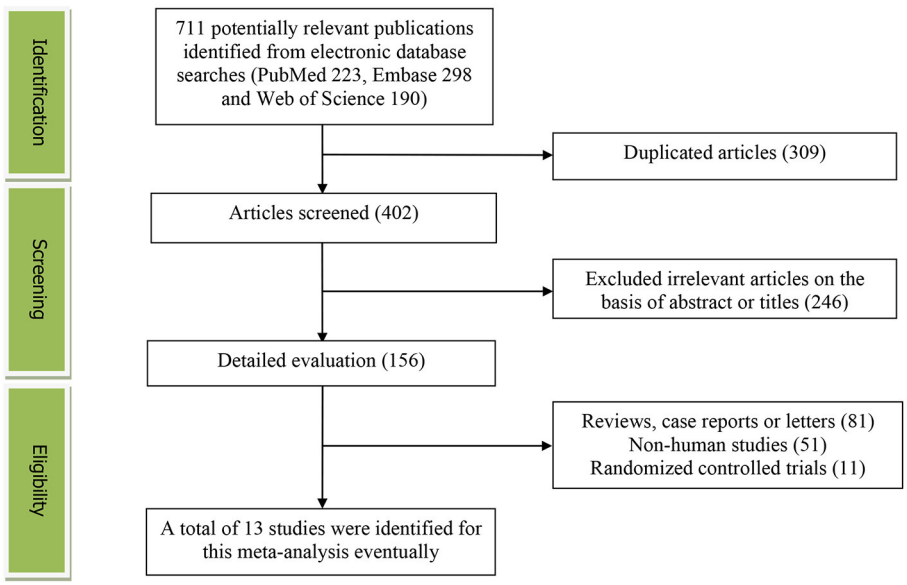

FIGURE 1 | The detailed flow diagram of the study identification and selection in this meta-analysis.

TABLE 1 | Characteristics of the individual studies included in this meta-analysis.

References	Location	Age years	Gender	Sample size	Study design	Adjustments	Dietary zinc assessment	Exposure	Effect estimates	Diagnostic criteria of MetS	NOS
Kim (17)	Korea	Middle-aged	Both	688	Cross-sectional	NA	FFQ	Control MetS Control MetS	Dietary zinc intake Male 5.50 (5.38, 5.62) 5.60 (5.46, 5.74) Female 5.80 (5.66, 5.94) 5.50 (5.36, 5.64)	NCEP-ATP III	6
Bruscato (18)	Brazil	69.3 ± 6.3	Female	284	Cross-sectional	Age, smoking, years of education, physical activity, and dietary fiber	Dietary recall	Dietary zinc intake Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4 Control MetS	1.00 0.73 (0.36, 1.47) 0.54 (0.25, 1.13) 0,98 (0.47, 2.00) Dietary zinc intake 11.40 (10.65, 12.15) 11.00 (9.83, 12.17)	IDF	7
Kouki (19)	Finland	57–78	Both	1334	Cross-sectional	Age, alcohol consumption, smoking, education, and VO_2max	Dietary recall	Dietary zinc intake Male Per mg/day Female Per mg/day Male Control MetS Female Control MetS	0.97 (0.90, 1.06) 0.99 (0.94, 1.05) 5.50 (5.38, 5.62) 5.60 (5.46, 5.74) 5.80 (5.66, 5.94) 5.50 (5.36, 5.64)	NCEP-ATP III	6
Otto (20)	US	45–84	Both	3828	Cohort	Energy intake, age, sex, race-ethnicity, education, study center, alcohol intake, physical activity, BMI, fiber intake, cigarette smoking, dietary supplement use the ratio of polyunsaturated fat intake: saturated fat intake and mutual adjustment for Mg, heme iron, non-heme iron, and antioxidant intake.	FFQ	Dietary zinc intake Quintiles 1 Quintiles 2 Quintiles 3 Quintiles 4 Quintiles 5	1.00 (0.78, 1.28) 1.20 (0.93, 1.55) 1.13 (0.85, 1.49) 1.33 (0.97, 1.82)	AHA	8

(Continued)

TABLE 1 | Continued

References	Location	Age years	Gender	Sample size	Study design	Adjustments	Dietary zinc assessment	Exposure	Effect estimates	Diagnostic criteria of MetS	NOS
Al-Daghri (21)	UK	19–60	Both	185	Cross-sectional	Age, BMI, and physical activity	Dietary recall	Dietary zinc intake Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4 Control MetS	 1.00 0.11 (0.04, 0.31) 0.17 (0.06, 0.50) 0.20 (0.07, 0.57) Dietary zinc intake 7.1 (6.5, 7.7) 6.1 (5.3, 6.6)	IDF	7
Bian (22)	China	30–70	Both	258	Cross-sectional	NA	Dietary recall	 Control MetS	Dietary zinc intake 11.9 (11.5, 12.3) 12.5 (12.0, 13.0)	NCEP-ATP III	7
Li (23)	China	18–65	Both	550	Cross-sectional	Age, sex, and energy intake	Dietary recall	Dietary zinc intake Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4 Control MetS	 1.00 0.33 (0.20–0.56) 0.34 (0.20–0.57) 0.18 (0.10–0.32) Dietary zinc intake 8.01 (7.64, 8.38) 7.22 (6.85, 7.59)	NCEP-ATP III	7
Motamed (24)	Iran	35–65	Both	3800	Cross-sectional	Sex, age, physical activity level, smoking, past medical history, energy intake, and BMI;	Dietary recall	Dietary zinc intake Quintiles 1 Quintiles 2 Quintiles 3 Quintiles 4 Quintiles 5 Male Control MetS Female Control MetS	 1.00 1.06 (0.80, 1.30) 1.37 (1.09, 1.70) 1.19 (0.90, 1.40) 1.20 (0.97, 1.50) Dietary zinc intake 7.07 (6.87, 7.27) 7.02 (6.82, 7.22) Dietary zinc intake 6.98 (6.95, 7.01) 7.15 (7.03, 7.27)	IDF	8
Suarez (25)	Colombia	11–16	Both	1311	Cross-sectional	Age, BMI, socioeconomic status, and intakes of fat, carbohydrates, protein, and ascorbic acid	Dietary recall	Dietary zinc intake Male Tertiles 1 Tertiles 2 Tertiles 3 Female Tertiles 1 Tertiles 2 Tertiles 3	 1.00 NA 0.20 (0.05, 0.80) 1.00 NA 1.29 (0.56, 2.97)	Ferranti's criteria	7
Lim (26)	Korea	52.5	Both	143	Cross-sectional	NA	Dietary recall	 Control MetS	Dietary zinc intake 8.50 (7.89, 9.11) 8.10 (7.41, 8.79)	NCEP-ATP III	6

(Continued)

TABLE 1 | Continued

References	Location	Age years	Gender	Sample size	Study design	Adjustments	Dietary zinc assessment	Exposure	Effect estimates	Diagnostic criteria of MetS	NOS
Zhu (27)	China	>18	Both	5323	Cross-sectional	Age, sex, region, years of education, physical activity level, intended physical exercises, smoking status, alcohol use, daily energy intake, iron, and magnesium	Dietary recall	Dietary zinc intake Quartiles 1 Quartiles 2 Quartiles 3 Quartiles 4	 1.00 0.76 (0.63, 0.92) 0.55 (0.44, 0.69) 0.46 (0.35, 0.61)	NCEP-ATP III	7
Batista (28)	Brazil	<18	Both	327	Cross-sectional	Sex, age, maternal education, family income, physical activity, and alcohol intake	Dietary recall	Dietary zinc intake Tertiles 1 Tertiles 2 Tertiles 3	 1.00 0.54 (0.21, 1.37) 0.46 (0.13, 1.63)	Cook's criteria	7
Zaeemzadeh (29)	Iran	18–40	Both	42	Case-control	NA	FFQ	Control MetS	Dietary zinc intake 10.46 (8.68, 12.24) 6.76 (3.05, 10.47)	NCEP-ATP III	5

identification of either the exposure or outcome of study cohorts. Disagreements were resolved by discussion.

Statistical Analyses

The RR for MetS and WMD for dietary zinc intake were the outcome measures in the present study. The I^2 statistic was employed to measure the heterogeneity by the percentage of total variation across studies ($I^2 > 50\%$ was considered as heterogeneity). If significant heterogeneity was observed among the studies, the random-effects model was used; otherwise, the fixed effects model was accepted. Begg's test was employed to assess the publication bias (31). A p-value < 0.05 was considered as statistically significant. Moreover, a subgroup analysis was performed for study design, diagnostic criteria of MetS, population, exposure assessment, and sample size, respectively.

RESULTS

Study Identification and Selection

Figure 1 presented the flow diagram of study identification and selection. Initially, a total of 711 articles (PubMed: 223, Embase: 298, and Web of Science: 190) were retrieved from the database during the literature search. After eliminating 309 duplicated articles, 402 articles were screened according to the titles and abstracts. Thereafter, 246 irrelevant studies were removed. Then, 81 reviews, case reports or letters, 51 non-human studies, 11 randomized controlled trials studies were excluded. Eventually, a total of 13 studies were identified for this meta-analysis.

Study Characteristics

The main characteristics of the identified studies were presented in **Table 1**. These studies were published between 2008 and 2021. Seven of them were employed in Asian countries [China (22, 23, 27), Korea (17, 26), and Iran (24, 29)], and the other six ones were conducted in Brazil (18, 28), US (20), UK (21), Finland (19), and Columbia (25), respectively. Most studies considered both male and female participants, whereas Bruscato's study only recruited females (18). The sample size ranged from 42 to 5,323 for a total of 18,073. The dietary zinc intake was assessed by food-frequency questionnaire (FFQ) in three studies (17, 20, 29), and dietary recall method in 10 studies (18, 19, 21–28). The criteria for MetS were National Cholesterol Education Program-Adult Treatment Panel III (NCEP ATP III) (17, 19, 22, 23, 26, 27, 29), International Diabetes Federation (IDF) (18, 21, 24), and American Heart Association (AHA) (20) in 7, 3, and 1 studies, respectively. Moreover, the Ferranti's (32) and Cook's (33) criteria were employed for adolescent population (25, 28).

Relative Risk of MetS for the Highest vs. Lowest Dietary Zinc Intake Category

The overall multi-variable adjusted RR showed that the dietary zinc intake was inversely associated with MetS (RR = 0.75, 95%CI: 0.61 to 0.93; $P = 0.009$) (**Figure 2**). A substantial level of heterogeneity was observed among the various studies ($P < 0.001$, $I^2 = 89.4\%$). No evidence of publication bias was observed among the included studies according to Begg's rank-correlation test ($P = 0.276$). The results of subgroup analysis were presented

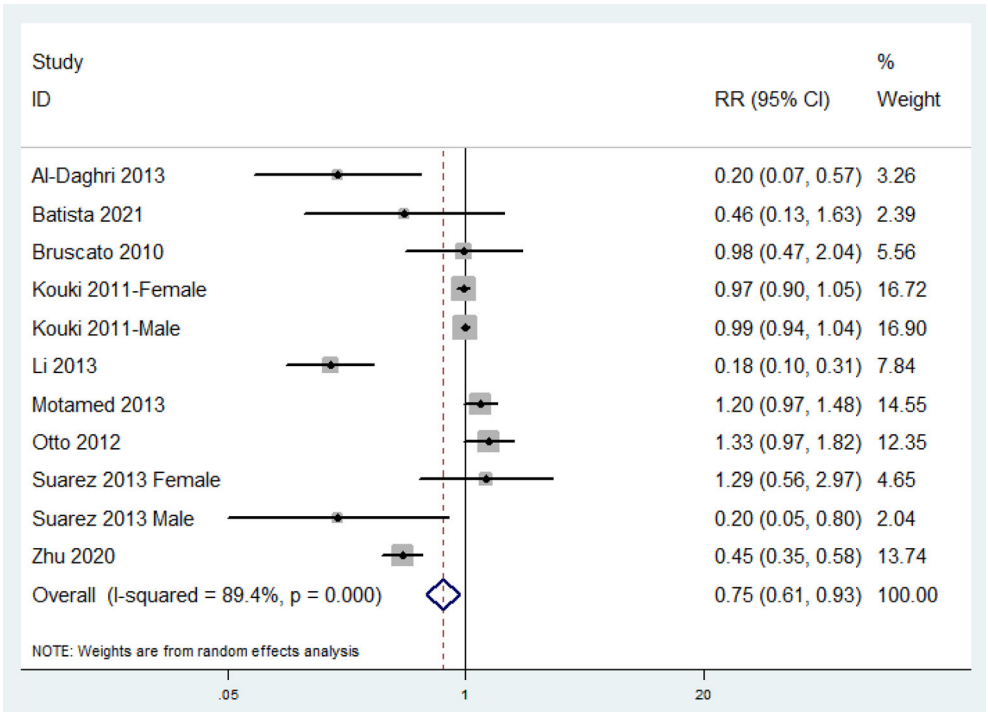

FIGURE 2 | Forest plot of meta-analysis: Overall multi-variable adjusted RR of MetS for the highest vs. lowest dietary zinc intake category.

in **Table 2**. Such findings were confirmed in cross-sectional (RR = 0.70, 95%CI: 0.55 to 0.87; $P = 0.002$), NCEP-ATP III (RR = 0.64, 95%CI: 0.48 to 0.84; $P = 0.002$), adult (RR = 0.77, 95%CI: 0.62 to 0.96; $P = 0.02$), dietary recall method (RR = 0.70, 95%CI: 0.55 to 0.87; $P = 0.002$), and >500 sample sized study (RR = 0.79, 95%CI: 0.64 to 0.99; $P = 0.002$), but not cohort (RR = 1.33, 95%CI: 0.97 to 1.82), other criteria of MetS (RR = 0.83, 95%CI: 0.55 to 1.26; $P = 0.38$), adolescent (RR = 0.55, 95%CI: 0.18 to 1.66; $P = 0.29$), FFQ (RR = 1.33, 95%CI: 0.97 to 1.82), and <500 sample sized (RR = 0.47, 95%CI: 0.17 to 1.29; $P = 0.14$) study.

Weighted Mean Difference of the Dietary Zinc Intake for MetS vs. Control Subjects

The combined WMD demonstrated that the dietary zinc intake in MetS was lower than that in control subjects (WMD = −0.21, 95%CI: −0.42 to 0.00; $P = 0.05$) (**Figure 3**). A substantial level of heterogeneity was observed among the various studies ($P = 0.001$, $I^2 = 65.1\%$). No evidence of publication bias was observed according to Begg's rank-correlation test ($P = 0.304$).

DISCUSSION

In this study, a total of 13 observational studies are identified for meta-analysis. The results show that the dietary zinc intake is inversely associated with MetS. Moreover, the dietary zinc intake in MetS is lower than that in control either.

It is well known that both oxidative stress and inflammation plays significant role in the pathophysiology of MetS (34), and the antioxidant and anti-inflammatory property of zinc may mainly account for the negative relationship between dietary zinc intake and MetS. Consistently, several randomized controlled trials have revealed that zinc supplementation improves insulin resistance, oxidative stress, and inflammation in MetS subjects (35, 36). Moreover, zinc supplementation also leads to a higher level of TNF-α bound monocytes, which may benefit the immune response system (37). On the other hand, some fundamental experimental evidence indicates that long term zinc supplementation directly improves MetS in animal model (38), and decreases several metabolic disorder makers, lipid accumulation, and toxicity (39–41). Above all, the existing clinical and experimental data are strongly consistent with our results.

Interestingly, the inverse relationship between dietary zinc intake and MetS is only obtained in cross-sectional studies. Nevertheless, the number of cohort studies is rather small (only one), which may inevitably reduce the reliability. Moreover, the inconsistent result with regard to diagnostic criteria of MetS, exposure assessment and sample size is also acquired. It is speculated that NCEP ATP III criteria, dietary recall method, and lager sample size (>500) are more precise and suitable for this analysis. On the other hand, our findings only exist in adult, but not adolescent population. Indeed, the adolescent is a less concerned population for MetS (MetS is a chronic disorder, and only two studies are identified for adolescent). Our results preliminarily suggest a potential effect of age on the relationship between dietary zinc intake and MetS. Taken together, more

TABLE 2 | Subgroup analysis of MetS for the highest vs. lowest dietary zinc intake category.

Stratification	Number of studies	Pooled RR	95% CI	*P*-value	Heterogeneity
All studies	9	0.75	0.61, 0.93	$P = 0.009$	$P < 0.001$; $I^2 = 89\%$
Study design					
Cross-sectional	8	0.70	0.55, 0.87	$P = 0.002$	$P < 0.001$; $I^2 = 90\%$
Cohort	1	1.33	0.97, 1.82	/	/
Diagnostic criteria of MetS					
NCEP-ATP III	3	0.64	0.48, 0.84	$P = 0.002$	$P < 0.001$; $I^2 = 96\%$
Other	6	0.83	0.55, 1.26	P = 0.38	$P = 0.003$; $I^2 = 70\%$
Population					
Adult	7	0.77	0.62, 0.96	$P = 0.02$	$P < 0.001$; $I^2 = 92\%$
Adolescent	2	0.55	0.18, 1.66	$P = 0.29$	$P = 0.06$; $I^2 = 64\%$
Exposure assessment					
FFQ	1	1.33	0.97, 1.82	/	/
Dietary recall method	8	0.70	0.55, 0.87	$P = 0.002$	$P < 0.001$; $I^2 = 90\%$
Sample size					
<500	3	0.47	0.17, 1.29	$P = 0.14$	$P = 0.05$; $I^2 = 67\%$
>500	6	0.79	0.64, 0.99	$P = 0.04$	$P < 0.001$; $I^2 = 92\%$

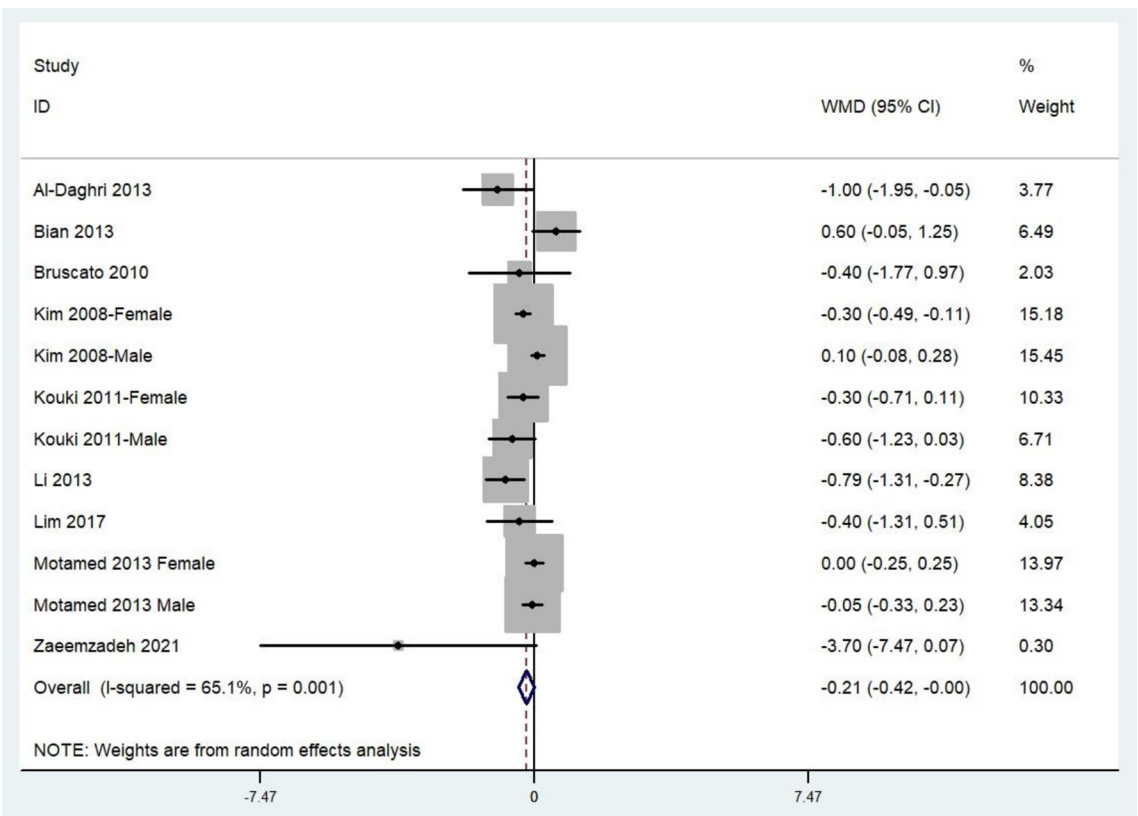

FIGURE 3 | Forest plot of meta-analysis: Weighted mean difference of dietary zinc intake for MetS vs. control subjects.

well-designed prospective cohort study with the specification of population age (adult/adolescent) is still needed.

Several similar meta-analysis studies should also be noted. Capdor et al. find that zinc supplementation reduces glucose concentrations and HbA1c, which may contribute to the management of hyperglycemia in individuals with MetS (42). Moreover, Khazdouz et al. further indicates that zinc supplementation has beneficial effects on glycemic indices and

lipid profile, which contributes to a reduction in risk of atherosclerosis (43). In addition, Karamali et al. demonstrates that 30 mg/day zinc supplementation for 6 weeks has beneficial effects on metabolic profiles in gestational diabetes subjects (44). These evidences strongly suggest a potential beneficial effect of zinc supplementation on MetS, which is a significant supplement for our results.

The relationship between serum zinc level and MetS has been deeply discussed in our previous work (45). It demonstrates that the serum zinc level in MetS is slightly higher than that in control, and an increased serum zinc level might be associated with a higher risk of MetS. However, these results seem to be limited by available evidence. More importantly, the development of MetS is associated with the chronic inflammation and oxidative stress (46–48), which lead to a lower serum zinc level. In turn, zinc can also reduce inflammatory cytokine production and oxidative stress (14, 45). As a consequence, the level of serum zinc might be dynamic in MetS condition. Alternatively, the dietary zinc intake is also served as a valid and reliable indicator for zinc status (49–53). Interestingly, a negative relationship between dietary zinc intake and MetS was obtained in our present study, which may encourage to build a collaboration between physicians and nutritionists to reinforce the dietary education in MetS subjects. Nevertheless, the toxicity of excess zinc intake should not be ignored neither. Excess zinc intake leads to the aggravation of renal function and an increase in systemic blood pressure predominantly through the oxidative stress (54). Moreover, excess dietary zinc intake may have negative impacts on epithelial signaling pathways, barrier function, and luminal ecology in the intestine, which may have long-term consequences on intestinal health (55). Therefore, a careful clinical validation is still needed before its application.

Our study has several strengths. First, this is the first meta-analysis of observational studies on the association between dietary zinc intake and MetS. Second, the included studies are analyzed based on the adjusted results and large samples. Third, our results may be beneficial for the nutritional management in MetS. The limitations of the present study should also be acknowledged. First, the reliability of our results might be influenced by the substantial level of heterogeneity. Second, due to the limitation in the relevant literature, only one prospective cohort study is identified (precludes causal relationships). Third, the classification of exposure varies greatly among individuals. Fourth, the selection of adjusted factors and definition of MetS are not uniform. Finally, only two studies have considered the adolescent population. These limitations may weaken the significance of this study.

CONCLUSIONS

Our results suggest that the dietary zinc intake is negatively associated with MetS. However, due to the limited evidence, more well-designed prospective cohort study with the specification of population age is still needed to elaborate the issues examined in this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

YZ was the guarantor of the overall content, conceived the idea, and assessed each study. JD and YZ drafted this study. ZL and QL performed the statistical analysis. HG and JL selected and retrieved relevant papers. All authors revised and approved the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by National Natural Science Foundation of China (82102581), National Postdoctoral Science Foundation of China (2021M693562), Provincial Outstanding Postdoctoral Innovative Talents Program of Hunan (2021RC2020), Provincial Natural Science Foundation of Hunan (2019JJ40517), Young Investigator Grant of Xiangya Hospital, Central South University (2020Q14), and FuQing Postdoc Program of Xiangya Hospital, Central South University (176).

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frontiers in Nutrition

SYSTEMATIC REVIEW
published: 15 March 2022
doi: 10.3389/fnut.2022.854774

Associations of Dietary Copper, Selenium, and Manganese Intake With Depression: A Meta-Analysis of Observational Studies

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *14 January 2022*
Accepted: *17 February 2022*
Published: *15 March 2022*

Citation:
Ding J and Zhang Y (2022) Associations of Dietary Copper, Selenium, and Manganese Intake With Depression: A Meta-Analysis of Observational Studies. Front. Nutr. 9:854774. doi: 10.3389/fnut.2022.854774

Objective: To comprehensively summarize the evidence on the associations of dietary copper, selenium, and manganese intake with depression based on a meta-analysis of observational studies.

Methods: The electronic database of PubMed, Web of Science, and Embase were searched up to January 7, 2022, for observational studies on the associations of dietary copper, selenium and manganese intake with depression (no restriction was set for the initiate time). The pooled relative risk (RR) of depression for the highest vs. lowest dietary copper, selenium, and manganese intake category were calculated.

Results: A total of 11 observational studies (61,430 participants) were identified as meeting the inclusion criteria. Specifically, five studies were related to the dietary copper intake. The overall multi-variable adjusted RR demonstrated that dietary copper intake was inversely associated with depression (RR = 0.63, 95% CI: 0.52–0.76; $P < 0.001$; $I^2 = 2.4\%$). With regard to the dietary selenium intake, six studies were identified for meta-analysis. The overall multi-variable adjusted RR showed that dietary selenium intake was also negatively associated with depression (RR = 0.63, 95% CI: 0.54–0.74; $P < 0.001$; $I^2 = 37.8\%$). In addition, four studies were specified for the dietary manganese intake, and the overall multi-variable adjusted RR indicated a negative relationship between dietary manganese intake and depression (RR = 0.71, 95% CI: 0.58–0.86; $P < 0.001$; $I^2 = 0.0\%$).

Conclusions: Our results suggest a negative relationship between dietary copper, selenium and manganese intake and depression, respectively. However, due to the limited prospective evidence, our results are restricted to cross-sectional design that precludes causal relationships. More well-designed prospective cohort studies are still needed.

Keywords: dietary copper intake, dietary selenium intake, dietary manganese intake, depression, meta-analysis, observational studies

INTRODUCTION

Depression is one of the most common global mental disorders (affecting females twice as much as males) (1), which usually presents as exhaustion, sadness, and lack of interest in daily activities (2). As a global burden of disease affecting ∼300 million people (3), depression is estimated to be the leading cause of disability worldwide by 2030 (4). Nevertheless, the current treatment for depression may be limited to the following issues: costly pharmacotherapy, adverse side effects and unsatisfactory curative effect (5). Emerging evidence suggests that dietary factors are associated with depression (6, 7). Thus, the identification of modifiable dietary factors for depression appears to be an important step in its clinical prevention and management.

Micronutrients are important factors for cellular biochemical functions. Among them, copper, selenium, and manganese are considered to be significant ones. As a component of extracellular superoxide dismutase (8), copper is essential for iron uptake and signaling in energy metabolism, reactive oxygen species detoxification and eukaryotic organisms (9). In addition, copper plays a significant role in signaling involving mitophagy, bioenergetics, and dynamics and mitochondrial function, which determine cellular fate by metabolic reprogramming (9). In addition, selenium is severed as an essential micronutrient that maintain the different cellular functions, such as immune-endocrine function and signaling transduction pathways (10). Moreover, selenium incorporates into selenoproteins and selenium-dependent enzymes (e.g., glutathione peroxidases), which is closely related to intracellular redox regulation and modulation (11). On the other hand, as another essential nutrient for the body, manganese is an important component of manganese superoxide dismutase (MnSOD, SOD-2), which is the primary antioxidant enzyme that protects cells from oxidative stress (catalyze the dismutation of superoxide to hydrogen peroxide and oxygen in the mitochondria) (12). Since the oxidative stress is considered to play a significant role in the pathophysiology of depression (13, 14), the dietary copper, selenium, and manganese intake is considered to be beneficial to depression.

As far as we know, a number of observational studies have been employed to investigate the associations of dietary copper, selenium, and manganese intake with depression (15–25). However, their results are still conflicting. Thus, this meta-analysis of observational studies is employed to investigate the issues further. It is hypothesized that the dietary copper, selenium, and manganese intake is inversely associated with depression, respectively.

MATERIALS AND METHODS

Search Strategy

Our meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (26). The electronic database of PubMed, Web of Science and Embase were searched up to January 7, 2022 (no restriction was set for the initiate time) by using a combination of keywords that related to depression ("depression," "depressive"), copper ("copper"), selenium ("selenium"), and manganese ("manganese"). No language restriction was set in the search strategy. We screened the titles and abstracts of all articles, and then read the full articles to identify the eligible studies.

Study Selection

Two researchers reviewed the titles, abstracts, and full texts of all retrieved studies independently. Disagreements were resolved by discussions. The inclusion criteria were listed as follows: (1) observational studies; (2) the associations of dietary copper, selenium and manganese intake with depression; and (3) relative risk (RR) or odds ratio (OR) with 95% confidence interval (CI) was reported. The exclusion criteria were listed as follows: (1) duplicated or irrelevant articles; (2) reviews, letters, or case reports; (3) randomized controlled trials; and (4) non-human studies.

Data Extraction

The data was extracted by two researchers independently, and disagreements were resolved by discussions. The information about the first author and year of publication, location, age, sex, sample size, study design, adjustments, exposure assessment, category of exposure, effect estimates, and diagnostic criteria of depression, was collected. The corresponding effect estimates of depression with 95% CIs for the highest vs. lowest dietary copper, selenium and manganese intake category were extracted (adjusted for the maximum number of confounding variables).

Quality Assessment

The Newcastle-Ottawa (NOS) criteria for non-randomized studies was employed to assess the quality of each included study. NOS is based on three broad perspectives: (1) the selection process of study cohorts; (2) the comparability among different cohorts; (3) the identification of exposure or outcome of study cohorts. Disagreements with respect to the methodological quality were resolved by discussion.

Statistical Analyses

The RR for depression were the outcome measures in this meta-analysis. The I^2 statistic, which measures the percentage of total variation across studies due to heterogeneity, was examined ($I^2 > 50\%$ was considered heterogeneity). If significant heterogeneity was observed among the studies, the random-effects model was used; otherwise, the fixed effects model was accepted. Begg's test was employed to assess the publication bias (27). Moreover, subgroup analysis for sex, geographical region, sample size, diagnostic criteria of depression, exposure assessment, population, and study design were employed.

RESULTS

Study Identification and Selection

The detailed flow diagram of the study identification and selection were presented in **Figure 1**. Initially, a total of 755 potentially relevant articles (179 for PubMed, 198 for Embase,

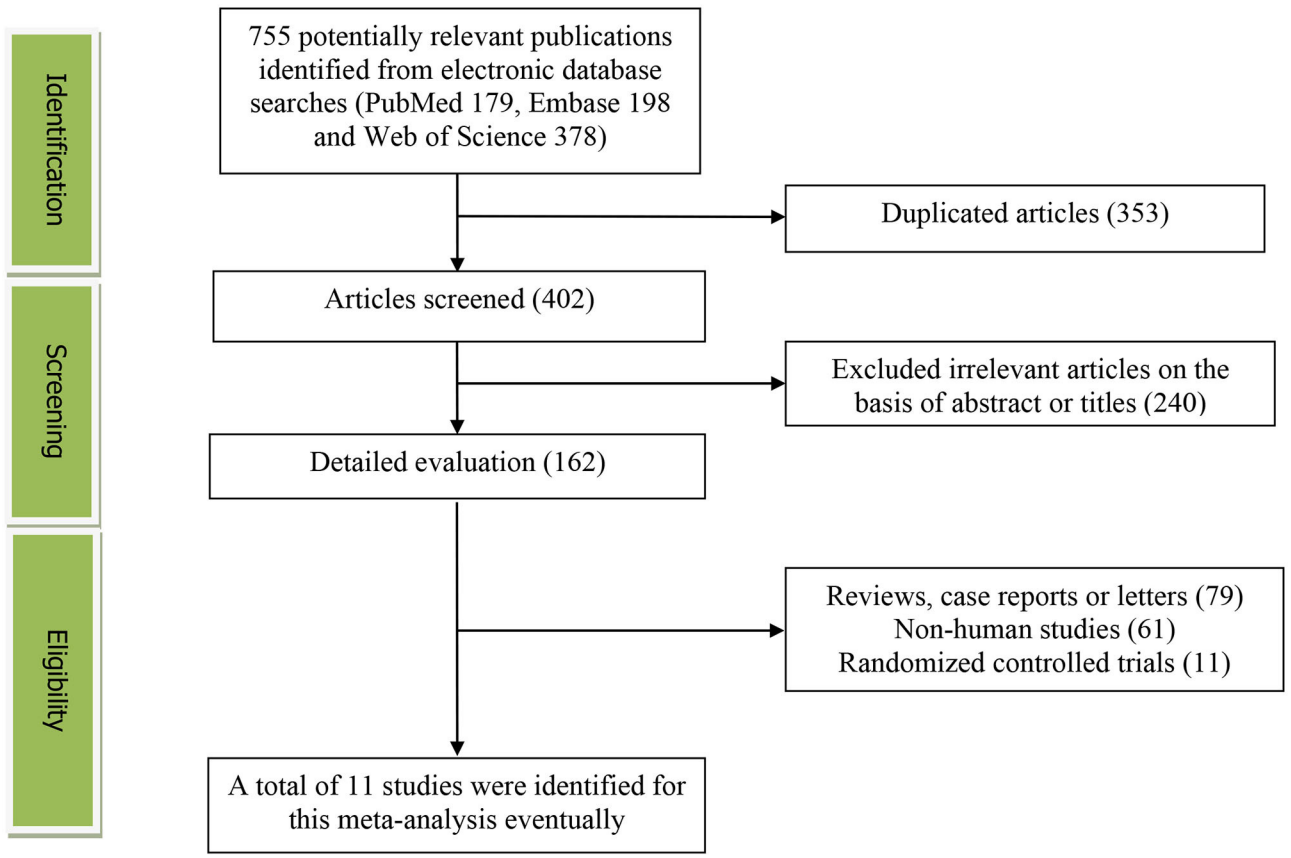

FIGURE 1 | The detailed flow diagram of the study identification and selection in this meta-analysis.

and 378 for Web of Science) were retrieved during the literature search. After eliminating 353 duplicated articles, 402 articles were screened according to the titles and abstracts, and then, 240 irrelevant studies were excluded. Thereafter, 79 reviews, case reports or letters, 61 non-human studies, 11 randomized controlled trials studies were removed. Eventually, 11 studies were selected for this meta-analysis (15–25).

Study Characteristics

The main characteristics of the included studies were presented in **Table 1**. These studies were published between 2012 and 2022. Among them, five studies were performed in Asian countries [Japan (17, 21, 22), China (23) and Korea (16)], and the other six ones were from US (19, 20), Brazil (24, 25), Spain (18), and Australia (15). Four articles included only females (15–17, 23), and the other seven studies recruited both males and females (18–22, 24, 25). With regard to the study design, nine studies were cross-sectional/case-control (16, 17, 19–25) and two ones were prospective cohort (15, 18) studies. The sample size ranged from 316 to 14,834 for a total number of 61,430. The dietary micronutrients were assessed by food-frequency questionnaire (FFQ) in eight studies (15–18, 21–23, 25), and recall method in three studies (19, 20, 24). The diagnostic criteria of depression were Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) (15, 18), Patient Health Questionnaire-9 (PHQ-9) (19, 20), Center for Epidemiological Studies Depression Scale (CES-D) (17, 23), Beck Depression Inventory (BDI) (16), Kessler's six-item psychological distress scale (K6) (21), Geriatric Depression Scale (GDS) (22), Mini-International Neuropsychiatric Interview (MINI) (24), and Clinical Interview Schedule Revised (CIS-R) (25), respectively.

RR of Depression for the Highest vs. Lowest Dietary Copper Intake Category

The overall multi-variable adjusted RR showed that the dietary copper intake was inversely associated with depression (RR = 0.63, 95% CI: 0.52–0.76; $P < 0.001$; **Figure 2**). No substantial level of heterogeneity was obtained among various studies ($P = 0.401$, $I^2 = 2.4\%$). No evidence of publication bias existed according to the Begg's rank-correlation test ($P = 0.707$). **Table 2** presented the results of subgroup analysis. The above findings were confirmed in female (RR = 0.60, 95% CI: 0.40–0.80; $P < 0.001$), but not in male (RR = 0.64, 95% CI: 0.36–1.11).

RR of Depression for the Highest vs. Lowest Dietary Selenium Intake Category

The overall multi-variable adjusted RR showed that the dietary selenium intake was inversely associated with depression (RR =

TABLE 1 | Characteristics of the individual studies included in this meta-analysis.

References	Location	Age (years)	Sex	Sample size	Study design	Adjustments	Exposure assessment	Category of exposure	Effect estimates	Diagnostic criteria of depression	NOS
Pasco et al. (15)	Australia	20–89	Female	316	Cohort	Age and socioeconomic status	FFQ	Selenium Low intake High intake	 1.00 0.34 (0.12, 0.96)	DSM-IV	7
Kim et al. (16)	Korea	12–18	Female	849	Case-control	Menstrual regularity and energy	FFQ	Copper Tertile 1 Tertile 2 Tertile 3	 1.00 0.78 (0.48, 1.38) 0.41 (0.17, 0.96)	BDI	7
Miyake et al. (17)	Japan	31	Female	1,745	Cross-sectional	Age, gestation, region of residence, number of children, family structure, history of depression, family history of depression, smoking, secondhand smoke exposure at home and at work, employment, household income, education, BMI, intake of saturated fatty acids, eicosapentaenoic acid plus docosahexaenoic acid, calcium, vitamin D and isoflavones	FFQ	Copper Quartile 1 Quartile 2 Quartile 3 Quartile 4 Manganese Quartile 1 Quartile 2 Quartile 3 Quartile 4	 1.00 0.74 (0.57, 0.96) 0.80 (0.60, 1.06) 0.73 (0.51, 1.05) 1.00 0.93 (0.72, 1.19) 0.94 (0.73, 1.21) 0.74 (0.56, 0.97)	CES-D	6
Sánchez-Villegas et al. (18)	Spain	38	Both	13,983	Cohort	Sex, age, physical activity, BMI, energy intake, special diets, smoking, alcohol intake and prevalence of CVD, HTA, or T2DM	FFQ	Selenium Inadequacy Adequacy	 1.00 0.78 (0.57, 1.07)	DSM-IV	8
Li et al. (19)	US	>18	Both	14,834	Cross-sectional	Age, gender, BMI, race, educational level, smoking status, family income, work activity, recreational activity, hypertension, diabetes, and total daily energy intake	Recall method	Copper Quartile 1 Quartile 2 Quartile 3 Quartile 4 Selenium Quartile 1 Quartile 2 Quartile 3 Quartile 4	 1.00 0.81 (0.65, 1.03) 0.78 (0.62, 0.98) 0.68 (0.49, 0.94) 1.00 0.69 (0.53, 0.91) 0.52 (0.39, 0.69) 0.46 (0.32, 0.67)	PHQ-9	8

(Continued)

TABLE 1 | Continued

References	Location	Age (years)	Sex	Sample size	Study design	Adjustments	Exposure assessment	Category of exposure	Effect estimates	Diagnostic criteria of depression	NOS
Ghimire et al. (20)	US	>18	Both	7,725	Cross-sectional	Age, sex, race ethnicity, marital status, educational status, family poverty income ratio, BMI, smoking, alcohol use, physical activity, and use of dietary supplements, diabetes, kidney disease, cancer, and heart disease and total energy intake	Recall method	Selenium Quintile 1 Quintile 2 Quintile 3 Quintile 4 Quintile 5	 1.00 0.64 (0.48, 0.85) 0.69 (0.49, 0.96) 0.57 (0.36, 0.90) 0.60 (0.39, 0.94)	PHQ-9	8
Nakamura et al. (21)	Japan	18–79	Both	2,089	Cross-sectional	Age, sex, smoking, alcohol drinking, BMI, shift work, and intake of Vitamin C, B6, B12, folic acid, PUFA, medications for hypertension, hyperlipidemia, and diabetes	FFQ	Copper Quartile 1 Quartile 2 Quartile 3 Quartile 4 Manganese Quartile 1 Quartile 2 Quartile 3 Quartile 4	 1.00 0.60 (0.31, 1.16) 0.52, (0.28, 0.97) 0.43 (0.22, 0.84) 1.00 0.56 (0.27, 1.16) 0.51 (0.27, 0.96) 0.51, (0.24, 1.08)	K6	7
Nguyen et al. (22)	Japan	>65	Both	1,423	Cross-sectional	Age, BMI, living status, having a job status, married status, smoking status, alcohol consumption, total energy, hypertension, diabetes, and hyperlipidemia	FFQ	Male Copper Quartile 1 Quartile 2 Quartile 3 Quartile 4 Manganese Quartile 1 Quartile 2 Quartile 3 Quartile 4 Female Copper Quartile 1 Quartile 2 Quartile 3 Quartile 4 Manganese Quartile 1 Quartile 2 Quartile 3 Quartile 4	 1.00 0.78 (0.42, 1.42) 0.78 (0.43, 1.41) 0.78 (0.42, 1.42) 1.00 1.21 (0.67, 2.18) 1.51 (0.84, 2.71) 0.83 (0.45, 1.53) 1.00 0.81 (0.49, 1.34) 0.61 (0.36, 1.02) 0.43 (0.25, 0.77) 1.00 1.08 (0.65, 1.82) 0.80 (0.47, 1.37) 0.75 (0.43, 1.30)	GDS	7

(Continued)

TABLE 1 | Continued

References	Location	Age (years)	Sex	Sample size	Study design	Adjustments	Exposure assessment	Category of exposure	Effect estimates	Diagnostic criteria of depression	NOS
Li et al. (23)	China	42–52	Female	2,993	Cross-sectional	Energy intake, saturated fatty acids intake, unsaturated fatty acids intake, *n*−3 PUFA intake, vitamin B6 intake, vitamin B12 intake, vitamin C intake, vitamin D intake, calcium intake, copper intake, zinc intake, age, race/ethnicity, education, income, financial strain, physical activity, BMI, VMS, chronic stress, use of antidepressant, estradiol, testosterone, and SHBG	FFQ	Manganese Early perimenopausal Quartile 1 Quartile 2 Quartile 3 Quartile 4 Premenopausal Quartile 1 Quartile 2 Quartile 3 Quartile 4	 1.00 0.87 (0.58, 1.31) 0.79 (0.49, 1.27) 0.80 (0.46, 1.39) 1.00 0.97 (0.66, 1.43) 0.71 (0.45, 1.11) 0.51 (0.29–0.91)	CES-D	7
Almeida et al. (24)	Brazil	18–59	Both	736	Cross-sectional	Gender, marital status, socioeconomic class, alcohol consumption, and pesticide poisoning	Recall method	Selenium Low intake High intake	 1.00 0.46 (0.24, 0.90)	MINI	7
Ferriani et al. (25)	Brazil	35–74	Both	14,737	Cross-sectional	Age, race, total cholesterol, HDL cholesterol, systolic blood pressure, antihypertensive drug, diabetes, and smoking, cardiovascular disease, physical activity, and calorie	FFQ	Selenium Quintile 1 Quintile 2 Quintile 3 Quintile 4 Quintile 5	 1.00 0.88 (0.69, 1.12) 0.80 (0.62, 1.03) 0.76 (0.59, 0.98) 0.72 (0.56, 0.94)	CIS-R	8

BMI, Body mass index; FFQ, Food frequency questionnaire; CVD, Cardiovascular disease; HDL, High density lipoprotein; HTA, Hypertension; T2DM, Type 2 diabetes mellitus; PUFA, Polyunsaturated fatty acid; VMS, Vasomotor symptoms; SHBG, Sex hormone binding globulin; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders-IV; PHQ-9, Patient Health Questionnaire-9; CES-D, Center for Epidemiological Studies Depression Scale; BDI, Beck Depression Inventory; K6, Kessler's six-item psychological distress scale; GDS, Geriatric Depression Scale; MINI, Mini-International Neuropsychiatric Interview; CIS-R, Clinical Interview Schedule Revised.

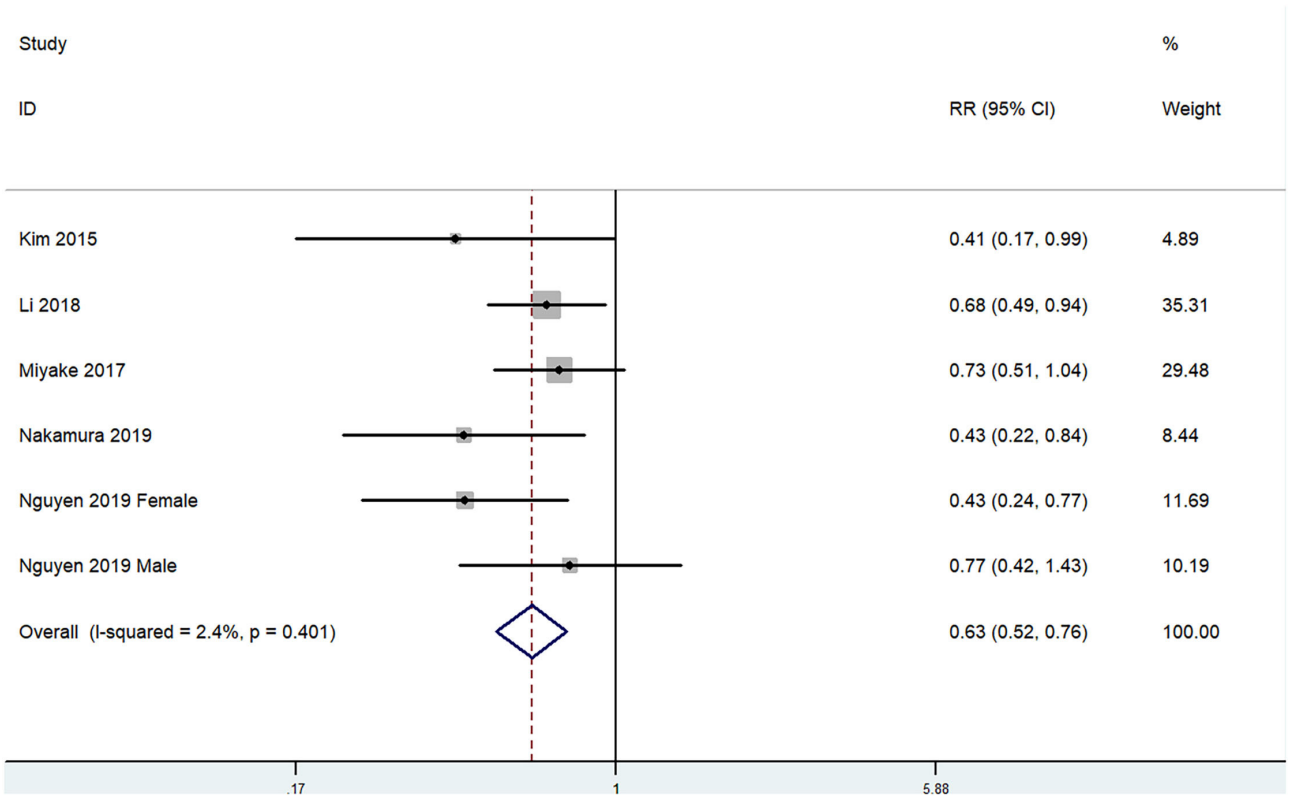

FIGURE 2 | Forest plot of meta-analysis: overall multi-variable adjusted RR of depression for the highest vs. lowest category of dietary copper intake.

TABLE 2 | Subgroup analysis of depression for the highest vs. lowest dietary copper intake category.

Stratification	Number of studies	Pooled RR	95% CI	*P*-value	Heterogeneity
All studies	5	0.63	0.52, 0.76	$P < 0.001$	$P = 0.40$; $I^2 = 2\%$
Sex					
Male	1	0.77	0.42, 1.43	/	/
Female	3	0.60	0.40, 0.80	$P < 0.001$	$P = 0.21$; $I^2 = 36\%$
Geographical region					
Asia	4	0.60	0.47, 0.76	$P < 0.001$	$P = 0.31$; $I^2 = 16\%$
Non-Asia	1	0.68	0.49, 0.94	/	/
Sample size					
<2,000	3	0.63	0.49, 0.82	$P < 0.001$	$P = 0.30$; $I^2 = 18\%$
>2,000	2	0.62	0.46, 0.84	$P = 0.002$	$P = 0.23$; $I^2 = 31\%$
Exposure assessment					
FFQ	4	0.60	0.47, 0.76	P < 0.001	$P = 0.31$; $I^2 = 16\%$
Recall method	1	0.68	0.49, 0.94	/	/
Population					
Adolescent	1	0.41	0.17, 0.99	/	/
Middle aged and elderly	4	0.64	0.52, 0.78	$P < 0.001$	$P = 0.38$; $I^2 = 4\%$

RR, Relative risk; CI, Confidence interval; FFQ, Food frequency questionnaire.

0.63, 95% CI: 0.54–0.74; $P < 0.001$) (**Figure 3**). No substantial level of heterogeneity was obtained among various studies ($P = 0.154$, $I^2 = 37.8\%$). No evidence of publication bias existed according to the Begg's rank-correlation test ($P = 0.260$). **Table 3** presented the results of subgroup analysis. The above findings were confirmed in female (RR = 0.63, 95% CI: 0.47–0.85; $P = 0.003$), PHQ-9 (RR = 0.51, 95% CI: 0.39–0.68; $P < 0.001$), cross-sectional (RR = 0.60, 95% CI: 0.51–0.72; $P < 0.001$) studies, but

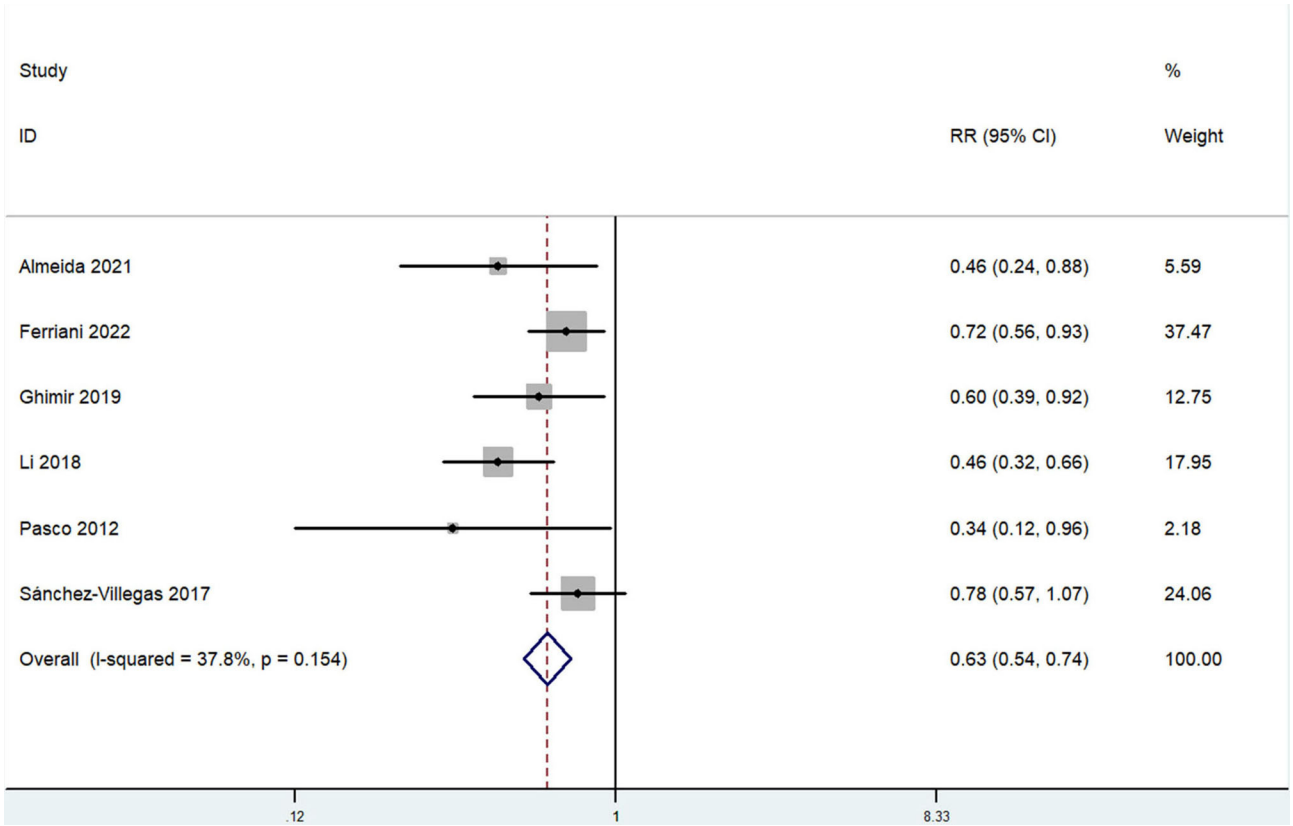

FIGURE 3 | Forest plot of meta-analysis: overall multi-variable adjusted RR of depression for the highest vs. lowest category of dietary selenium intake.

TABLE 3 | Subgroup analysis of depression for the highest vs. lowest dietary selenium intake category.

Stratification	Number of studies	Pooled RR	95% CI	*P*-value	Heterogeneity
All studies	6	0.63	0.54, 0.74	$P < 0.001$	$P = 0.15$; $I^2 = 38\%$
Sex					
Male	1	0.64	0.36, 1.11	/	/
Female	2	0.63	0.47, 0.85	$P = 0.003$	$P = 0.22$; $I^2 = 33\%$
Diagnostic criteria of depression					
DSM-IV	2	0.60	0.28, 1.28	$P = 0.19$	$P = 0.13$; $I^2 = 55\%$
PHQ-9	2	0.51	0.39, 0.68	$P < 0.001$	$P = 0.36$; $I^2 = 0\%$
Geographical region					
US	2	0.51	0.39, 0.68	$P < 0.001$	$P = 0.36$; $I^2 = 0\%$
Brazil	2	0.68	0.54, 0.86	$P = 0.001$	$P = 0.21$; $I^2 = 37\%$
Sample size					
<2,000	2	0.42	0.24, 0.73	$P = 0.002$	$P = 0.63$; $I^2 = 0\%$
>2,000	4	0.66	0.56, 0.77	$P < 0.001$	$P = 0.14$; $I^2 = 46\%$
Exposure assessment					
FFQ	3	0.72	0.60, 0.88	$P = 0.001$	$P = 0.33$; $I^2 = 11\%$
Recall method	3	0.51	0.39, 0.65	$P < 0.001$	$P = 0.62$; $I^2 = 0\%$
Study design					
Cross-sectional	4	0.60	0.51, 0.72	$P < 0.001$	$P = 0.19$; $I^2 = 36\%$
Cohort	2	0.60	0.28, 1.28	$P = 0.19$	$P = 0.13$; $I^2 = 55\%$

RR, Relative risk; CI, Confidence interval; FFQ, Food frequency questionnaire; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders-IV; PHQ-9, Patient Health Questionnaire-9.

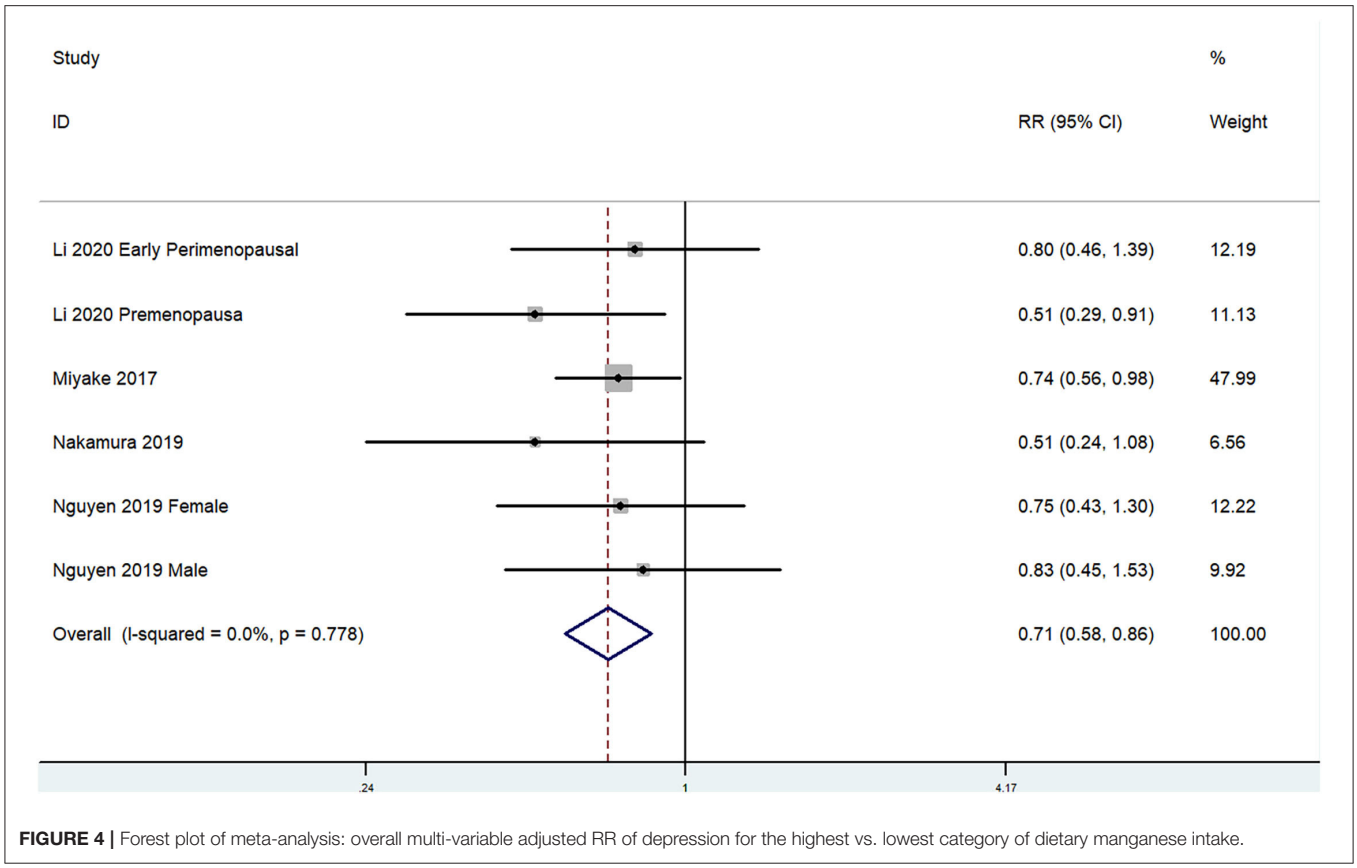

FIGURE 4 | Forest plot of meta-analysis: overall multi-variable adjusted RR of depression for the highest vs. lowest category of dietary manganese intake.

not in male (RR = 0.77, 95% CI: 0.42–1.43; $P = 0.001$), DSM-IV (RR = 0.60, 95% CI: 0.28–1.28; $P = 0.19$) and prospective cohort studies (RR = 0.60, 95% CI: 0.28–1.28; $P = 0.19$).

RR of Depression for the Highest vs. Lowest Dietary Manganese Intake Category

The overall multi-variable adjusted RR showed that the dietary manganese intake was inversely associated with depression (RR = 0.71, 95% CI: 0.58–0.86; $P < 0.001$; **Figure 4**). No substantial level of heterogeneity was obtained among various studies ($P = 0.778$, $I^2 = 0.0\%$). No evidence of publication bias existed according to the Begg's rank-correlation test ($P = 1.000$). **Table 4** presented the results of subgroup analysis. The above findings were confirmed in female (RR = 0.71, 95% CI: 0.58–0.88; $P = 0.002$) and CES-D (RR = 0.71, 95% CI: 0.56–0.89; $P = 0.003$) studies, but not in male (RR = 0.83, 95% CI: 0.45–1.53) and other criteria studies (RR = 0.71, 95% CI: 0.49–1.02; $P = 0.06$).

DISCUSSION

A total of 11 observational studies were identified in the present meta-analysis. The pooled results demonstrated a negative relationship between dietary copper, selenium, and manganese intake and depression, respectively.

The pathophysiology of depression is involved in oxidative stress, whereas copper, selenium, and manganese are served as important antioxidants that act against oxidative stress. Copper is a cofactor of the copper/zinc superoxide dismutase, a protein located in both the cytosol and mitochondrial inner membrane space to relieve the electron transport chain-generated reactive oxygen species (9). On the other hand, copper may drive the activity of the two neurotrophic factors Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (28), which further influence the activity-dependent neural plasticity and neural network (29). Indeed, experimental evidence demonstrates that low-dose copper exacerbates depression-like behavior in ApoE4 transgenic mice (30). Differently from other metals, selenium incorporates into selenoproteins (glutathione per-oxidases and thioredoxin reductases) and protects from lipoperoxidation and oxidative cell damage (the glutathione antioxidant system is implicated in the pathophysiology of mood disorders) (31). Consistently, the selenocompound 1-methyl-3-(phenylselany1)-1H-indole attenuates depression-like behavior, oxidative stress, and neuroinflammation in streptozotocin-treated mice (32). Moreover, manganese is an important component of MnSOD, an antioxidant mitochondrial metalloenzyme that protects cells from oxidative stress (12, 33). Reduced MnSOD activity due to manganese deficiency might contribute to the development of depression. These above may significantly account for the major findings of our study.

TABLE 4 | Subgroup analysis of depression for the highest vs. lowest dietary manganese intake category.

Stratification	Number of studies	Pooled RR	95% CI	*P*-value	Heterogeneity
All studies	4	0.71	0.58, 0.86	$P < 0.001$	$P = 0.78$; $I^2 = 0\%$
Sex					
Male	1	0.83	0.45, 1.53	/	/
Female	3	0.71	0.58, 0.88	$P = 0.002$	$P = 0.68$; $I^2 = 0\%$
Diagnostic criteria of depression					
CES-D	2	0.71	0.56, 0.89	$P = 0.003$	$P = 0.48$; $I^2 = 0\%$
Other	2	0.71	0.49, 1.02	$P = 0.06$	$P = 0.60$; $I^2 = 0\%$
Geographical region					
Japan	3	0.73	0.58, 0.91	$P = 0.005$	$P = 0.79$; $I^2 = 0\%$
China	1	0.65	0.43, 0.96	/	/
Sample size					
<2,000	2	0.75	0.60, 0.95	$P = 0.02$	$P = 0.95$; $I^2 = 0\%$
>2,000	2	0.61	0.43, 0.87	$P = 0.007$	$P = 0.48$; $I^2 = 0\%$

RR, Relative risk; CI, Confidence interval; FFQ, Food frequency questionnaire; CES-D, Center for Epidemiological Studies Depression Scale.

Interestingly, our findings are only confirmed in females, but not males. It suggests that some genetic sexual differences with the diet-related pathology of depression should be considered. For example, the genetic contributions of the serotonin transporter in depression may be different (34), and the process of some serotonin systems may be more apparent in females than that in males either (35). Importantly, the inverse relationship between dietary selenium intake and depression is lost in prospective cohort study, which might be attributed to the potential reversed causality (e.g., depressive subjects may consume less dietary copper, selenium and manganese due to the reduced appetite). Moreover, the diagnostic criteria of depression vary greatly among individuals, which may also influence the reliability of subgroup analysis. Overall, very small number of studies are qualified for subgroup analysis, and the corresponding results should be considered very carefully. More well-designed prospective cohort studies with sexual specification are still needed.

It should also be noted that a very recent meta-analysis study has investigated the role of selenium in depression (36). The authors fail to demonstrate any significant differences in serum selenium levels between depressive and healthy subjects. On the contrary, they find the selenium supplementation significantly reduces depressive symptoms. The inconsistent results may be explained as follow: (1) The selenium in serum may not reflect the issues of dietary selenium intake (37). In fact, demographic variables, health status, and some other factors may also influence serum selenium levels (38), and only one study has adjusted these confounding variables (39). (2) Given that the long-term exposure to low serum selenium level may impair brain function (40), the duration of low selenium intake is ignored in most studies. Importantly, their overall OR result shows no significant relationship between dietary selenium intake and depression. However, their search was performed on June 30, 2020 (updated on April 12, 2021) and two recent published studies were not specified for analysis (24, 25). Moreover, the category of exposure was unclear in one included study either (excluded in the present meta-analysis) (41). Most importantly, the effect estimates for the highest vs. lowest and lowest vs. highest (inadequate vs. adequate) exposure category was also pooled directly. Above all, our study is an important advance and supplement to their study.

Another relevant meta-analysis study has also comprehensively evaluated the relationship between body burden of copper and depression (42). They demonstrate that the blood copper level in depressive subjects is higher than that in controls, which implies that blood copper may be served as a biomarker for depression. On this basis, our study further demonstrates that dietary copper intake is inversely associated with depression either. Interestingly, Johnson et al. further found an inverse relationship between selenium level in household groundwater and depression, and GPX1 gene is related to depression risk and significantly influences the protective impact of selenium (43), which indicates a gene-environment interaction.

Although our findings may encourage to build an awareness with the collaboration between physicians and nutritionists, our results might be influenced by environmental and medical treatment factors, the interaction of multiple dietary factors, and the reversed causality (depressed individuals may have irregular/inadequate nutrition patterns that lead to nutritional inadequacy of these micronutrients intake). Moreover, the toxicity of these micronutrients should also be recognized. For instance, excess copper intake is reported to induce oxidative stress, damage to the mitochondrial, and leads to apoptosis, DNA damage and inflammatory responses (44, 45). In addition, selenium exposure is associated with increased risk for type 2 diabetes (46). Elevated selenium exposure has also been suspected to be a risk factor for the development of several neurodegenerative and neuropsychiatric diseases (47, 48). Moreover, long-term exposure to manganese may have adverse effects on mood state, neurobehavior, and peripheral

neurotransmitters (49). Therefore, a careful validation by high-quality randomized controlled trial/prospective cohort study is still needed.

Our study has several strengthens. First, this is the first meta-analysis of observational studies on the associations of dietary copper, selenium, and manganese intake with depression. In addition, the included studies are analyzed based on the adjusted results and large samples. Moreover, the limited heterogeneity level may reflect a decent reliability of our results. Finally, our findings may provide significant information to better consider the dietary effects on depression. The limitations of our study should also be acknowledged. First, only two prospective cohort studies were identified due to the limited relevant literature, which precludes causal relationships (depressive subjects may consume less dietary copper, selenium, and manganese due to the reduced appetite). Second, the classification of exposure and diagnostic criteria of depression varies greatly among individuals. Third, the adjusted factors were not uniform. Fourth, the environmental and medical treatment factors are considered in few studies, their impact cannot be clearly clarified and our topic might be over-simplified (the interaction of multiple dietary factors). Last but not the lease, the circulating level of these micronutrients is not considered due to the limited evidence, and the issue of microelement deficiency cannot be addressed. These limitations may weaken the significance of our study.

CONCLUSIONS

Our results suggest a negative relationship between dietary copper, selenium, and manganese intake and depression, respectively. However, due to the limited prospective evidence, our results are restricted to cross-sectional design that precludes causal relationships. More well-designed prospective cohort studies with sexual specification are still needed.

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

YZ and JD conceived the idea, drafted this manuscript, selected and retrieved relevant papers, and assessed each study. JD performed the statistical analysis. YZ was the guarantor of the overall content. All authors revised and approved the final manuscript.

FUNDING

This study was supported by National Natural Science Foundation of China (82102581), National Postdoctoral Science Foundation of China (2021M693562), Provincial Outstanding Postdoctoral Innovative Talents Program of Hunan (2021RC2020), Young Investigator Grant of Xiangya Hospital, Central South University (2020Q14), and FuQing Postdoc Program of Xiangya Hospital, Central South University (176).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.854774/full#supplementary-material

Supplementary Figure 1 | Funnel plot with pseudo 95% confidence limits for the analysis of dietary copper intake and depression.

Supplementary Figure 2 | Funnel plot with pseudo 95% confidence limits for the analysis of dietary selenium intake and depression.

Supplementary Figure 3 | Funnel plot with pseudo 95% confidence limits for the analysis of dietary manganese intake and depression.

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ORIGINAL RESEARCH
published: 04 April 2022
doi: 10.3389/fnut.2022.838752

Association Between Dietary Total Antioxidant Capacity and Diet Quality in Adults

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Background: Diet quality is a major contributor to human health. In addition, antioxidants have a great contribution to several chronic conditions. The purpose of this study was to evaluate if dietary total antioxidant capacity (TAC) can be considered as a measure of diet quality in a Middle Eastern country.

Methods: In this cross-sectional study on 6,724 Iranian adults, we used a validated food frequency questionnaire (FFQ) to assess dietary intakes. Data derived from the FFQ was used to calculate dietary TAC and well-known diet quality scores including alternate healthy eating index (AHEI) and dietary diversity score (DDS). Dietary TAC was calculated based on the ferric reducing-antioxidant power (FRAP) values reported in earlier publications. AHEI and DDS have also been constructed based on previous publications. Cross-classification was used to examine the agreement between these measures.

Results: Mean age and BMI of study participants were 36.89 ± 8.08 y and 24.97 ± 3.87 kg/m^2, respectively. We found that individuals in the highest tertile of dietary TAC had higher scores of AHEI (57.53 ± 0.20 vs. 52.03 ± 0.20, $P < 0.001$) and DDS (5.56 ± 0.03 vs. 4.15 ± 0.03, $P < 0.001$) compared with those in the lowest tertile. Participants' distribution on the basis of the cross-classification analysis indicated that the classifications were in exact agreement for 42.6%, within an adjacent tertile for 33.05%, and in gross misclassification for 20% of individuals. When this was examined between dietary TAC and DDS, we found that exact agreement in the classifications was for 59.2% of participants. Notably, a very low proportion of gross misclassification was seen in this regard such that only 6% of participants were classified in the opposing tertiles, indicating additional support for a good agreement.

Conclusion: We found that dietary TAC might be considered as a proper measure for the assessment of diet quality because it was well correlated with well-known measures of diet quality including DDS and AHEI scores.

Keywords: total antioxidant capacity, diet quality, dietary diversity score, alternate healthy eating index, TAC

OPEN ACCESS

Edited by:
Learn-Han Lee,
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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *18 December 2021*
Accepted: *21 February 2022*
Published: *04 April 2022*

Citation:
Salari-Moghaddam A, Nouri-Majd S, Keshteli AH, Emami F, Esmaillzadeh A and Adibi P (2022) Association Between Dietary Total Antioxidant Capacity and Diet Quality in Adults. Front. Nutr. 9:838752. doi: 10.3389/fnut.2022.838752

INTRODUCTION

Diet quality is a major determinant of the increased incidence of chronic diseases (1). It has been well-established that individuals with greater diet quality had a lower risk of cancer (2), diabetes (3) and cardiovascular diseases (4). Although several studies examined diet quality in relation to chronic conditions, the characteristics of a high-quality diet are not well established. Diets with a high quality include high amounts of fruit and vegetables, nuts, fish, legumes, and whole grains (5, 6). The beneficial effects of such diets in disease prevention cannot be attributed to a single nutrient and their effects are likely due to the interactions of all nutrients (7). Antioxidants are among important nutrients in foods included in the high quality diets. Most previous studies examining diet-disease relations have focused on a single antioxidant; however, dietary total antioxidant capacity (TAC) has been developed to assess cumulative, synergic and protective activities of all the antioxidants present in the diet (8). Similar to diets with a high quality, high dietary TAC was also inversely associated with stroke (9, 10), various types of cancer (11–14), cardiovascular diseases (15), diabetes (16), metabolic syndrome (17), and inflammation (18). Therefore, it seems that the effect of high quality diets on disease prevention might be mediated through its high dietary TAC.

Previous studies found a positive association between dietary TAC and dietary quality scores (19, 20). The application of suggested scoring methods for definition of high quality diets is important in the Middle Eastern countries, where the people's dietary intakes have its own characteristics. Given the different nature of diets in different geographical regions along with lack of information in the understudied region of the Middle East, the present study was done to investigate the association between dietary TAC and diet quality in a large sample of Iranian adults.

METHODS AND MATERIALS

Study Design and Population

This cross-sectional study was performed based on data from the Study on the Epidemiology of Psychological, Alimentary Health and Nutrition (SEPAHAN) project, which was a cross-sectional study looking at the prevalence of functional gastrointestinal disorders (FGIDs) and their relationship with lifestyle factors and psychological disorders. Details about SEPAHAN project have been published earlier (21). This study was performed among Iranian general adults working in 50 different healthcare centers affiliated to Isfahan University of Medical Sciences (IUMS) across Isfahan province. To collect information about anthropometric measures, demographic and lifestyle factors, including dietary intakes and physical activity, self-administered questionnaires were distributed among 10,087 subjects, and 8,691 participants returned the completed questionnaires (response rate: 86.16%). In the current analysis, we excluded subjects who reported their total daily energy intake outside the range of 800–4,200 kcal/d. We also excluded those who had missing data on any relevant variable. These exclusions resulted in a dataset of 6,724. All participants provided written inform consent forms. Although the protocol of SEPAHAN study was approved by the Regional Bioethics Committee of Isfahan University of Medical Sciences, the current study was separately approved by the Research Council of School of Nutritional Sciences and Dietetics of Tehran University of Medical Sciences, Tehran, Iran (Ethics code: IR.TUMS.VCR.REC.1398.131).

Dietary Intakes Assessment

A self-administered, Willett-format, Dish-based, 106- item Semi-quantitative Food Frequency Questionnaire (DS-FFQ), was used to assess dietary intakes. The questionnaire was designed and validated for use in Iranian adults. Details on design, foods included, and the validity of this questionnaire has been reported elsewhere (22). Briefly, we provided a comprehensive list of foods and dishes commonly consumed by Iranian adults. Then, those foods that were nutrient-rich, often consumed, or contributed to between-person variation were selected. Eventually, this process led to remaining of the 106 food items in 5 various categories in the questionnaire: (1) mixed dishes (cooked or canned, 29 items); (2) grains (different types of bread, biscuits, cakes and potato, 10 items); (3) dairy products (dairy, butter and cream, 9 items); (4) fruits and vegetables (22 items); and (5) miscellaneous food items and beverages (including fast foods, nuts, sweets, desserts and beverages, 36 items). In order to provide precise and accurate estimates, the portion size of foods and mixed dishes as a unit with the same perception were given to all people. Nine multiple choice frequency response categories ranging from "never or $<$1/month" to "$\geq$12/day" were provided for reporting dietary intakes of participants. The number of response categories for the food list varied from 6 to 9 choices. For foods consumed infrequently, we omitted the high-frequency categories, while the number of multiple choice categories increased for common foods with a high intake. Finally, daily intake of all food items was computed and then converted to grams per day using household measures (23). Daily nutrient intakes of each participant were estimated based on the US Department of Agriculture's (USDA) national nutrient databank (24). The validity of DS-FFQ was examined in a subgroup of 200 participants randomly selected for the SEPAHAN project. All participants in the validation study completed the DS-FFQ at study baseline and 6 months later. During this validation study, participants provided three detailed dietary records that were used as the gold standard. As shown in earlier studies (22), it seems that this questionnaire provides reasonably valid measures of long-term dietary intakes. Also, some recent studies have shown that FFQ was a valid questionnaire for assessing dietary quality scores (25).

Dietary TAC Assessment

Dietary TAC was obtained from previous studies, based on the ferric reducing-antioxidant power (FRAP) values of 100 food items from the 106 selected food items. The food parameters that were not included in the TAC construction included salt, pepper, sugar, sugar loaf, gaz and nabat (traditional Iranian sweets), because the TAC value for these dietary factors was zero. The FRAP assay is a tool measuring the ability of dietary antioxidants to reduce ferric to ferrous ions. The FRAP values express as mmol per 100 grams of foods (mmol/100 g) (26). For similar food items

in Iranian culture (e.g., several types of breads), we calculated the overall mean value. Finally, the frequencies of consumption of each food item were multiplied by their related FRAP values and then summed up to obtain dietary TAC for each participant.

Dietary Diversity Score

A method described by Kant et al. (27, 28) was used for scoring dietary diversity. This method was based on five groups including grains, vegetables, fruits, meats, and dairy, all food groups in the USDA food guide pyramid. The grains group was composed of seven components: refined bread, macaroni, whole grain bread, corn flakes, rice, biscuit, and refined flour. As we had no data about intake of corn flakes, we decided to consider six components. Fruit was defined by summing up fruit and fruit juice, berries, and citrus fruits. In terms of vegetables, we summed up mixed vegetables, potato, tomato, other starchy vegetables, legumes, yellow vegetables, and green vegetables. The group of meat was composed of red meat, poultry, fish, and eggs and the group of dairy was composed of milk, yogurt, and cheese.

Participants were considered as a "consumer," and scored as 1, for each component of food groups if they had intakes higher than median levels; otherwise they were given the score of 0. Then the scores for components in each food group were summed up to have total score of that food group. Then, we divided total scores obtained in each group to the number of components in that group. This value was then multiplied by 2. Total DDS for each participant was then computed by summing up the figures for food groups. For example, in the grains group, if a person had dietary intakes of whole grain bread, macaroni, and rice higher than the median values, her or his score was calculated as $(3/6) \times 2 = 1$. Therefore, the diversity score for the grains group would be 1 for that person. After computing the diversity score for the other four groups in that person, total DDS was computed. Therefore, minimum and maximum scores of total dietary diversity for each participant were between 0 and 10.

Alternate Healthy Eating Index

To calculate AHEI-2010, a method designed by Kennedy et al. was used (29–31). AHEI-2010 consisted of eleven components: fruit, vegetables, whole grains, nuts and legumes, long-chain n-3 fats (DHA and EPA), PUFA, alcohol consumption, sugar-sweetened drinks and fruit juice, red and processed meats, trans-fat and sodium. In the current study, alcohol consumption was not included into the score, because of the lack of information in the original dataset. To construct the index, first we obtained energy-adjusted intakes of the above-mentioned components by using the residual method (32). Next, participants were classified based on decile categories of energy-adjusted intakes of these components. As scoring by deciles would be least prone to misclassification, we used decile categories of components instead of other classifications. Individuals in the highest deciles of fruits, vegetables, whole grains, nuts and legumes, long-chain n-3 fats and PUFA were given a score of 10, and those in the lowest decile of these items were given a score of 1. Individuals in the other deciles of these components were assigned the corresponding scores. Regarding sugar-sweetened drinks and fruit juice, red and processed meat, trans-fatty acids, and sodium intake, the lowest decile was given a score of 10 and the highest decile was given a score of 1. Those in deciles 9, 8, 7, 6, 5, 4, 3, and 2 of these components were given scores of 2, 3, 4, 5, 6, 7, 8, and 9, respectively. The whole AHEI-2010 was computed through summing up the scores of its components ranging from 10 to 100.

Assessment of Other Variables

Required information on other variables including age, sex, marital status, smoking status, and education was obtained from demographic and medical history questionnaires. Physical activity was assessed using the General Practice Physical Activity Questionnaire (GPPAQ) (18). Based on participants' responses, they were classified into 4 categories; (1) inactive, (2) moderately inactive, (3) moderately active, (4) active. However, in the current study, due to low number of subjects in some of the above-mentioned categories, individuals in the "inactive" and "moderately inactive" groups were combined and were defined as those with "sedentary physical activity". Similarly, individuals in the "moderately active" and "active" categories were combined and then defined as "physically active". Anthropometric measures including weight and height were assessed using a self-administered questionnaire. Body Mass Index (BMI) was calculated by dividing weight (kg) to height (m^2). The correlation coefficient for computed BMI from self-reported values, and the one from measured values was 0.70 ($P < 0.001$).

Statistical Analysis

We classified participants based on tertiles cut-off points of dietary TAC. General characteristics of study participants across tertiles of dietary TAC were presented as means ± SDs for continuous variables and percentages for categorical variables. To examine the differences across tertiles, we used ANOVA for continuous variables and chi-square test for categorical variables. The multivariable-adjusted means for AHEI and DDS across tertiles of dietary TAC were computed and compared using ANCOVA. In these analyses, energy intake was controlled for in the first model. Further adjustments were made for age (continuous) and sex (male/female) in the second model. BMI (continuous) was controlled for in the third model. Cross-classification of participants across tertiles of TAC, DDS and AHEI was examined. In this analysis, exact agreement was defined when individuals were classified in the same tertiles based on TAC and DDS or AHEI. When individuals were classified in the opposing tertiles, this was considered as gross misclassification. All statistical analyses were done using the Statistical Package for Social Sciences (version 20; SPSS Inc.). $P < 0.05$ was considered as statistically significant.

RESULTS

Median and range of TAC, DDS, and AHEI was 2010.68 (range: 418.09–5,247.56), 4.92 (range: 0–10), and 55 (range: 26–90), respectively. General characteristics of study participants across tertiles of dietary TAC are shown in **Table 1**. Participants in the top tertile of dietary TAC were more likely to be older, physically

TABLE 1 | General characteristics of study participants across tertiles of dietary TAC.

	Tertiles of dietary TAC			
	T_1 (418.1–1,720.4)	T_2 (1,720.5–2,332.4)	T_3 (2,333.2–5,247.5)	*P*-value[a]
Age, y	36.48 ± 8.05	36.61 ± 7.87	37.58 ± 8.29	<0.001
BMI, kg/m^2	24.92 ± 3.93	24.88 ± 3.80	25.10 ± 3.86	0.12
Energy intake, kcal/d	1,616.5 ± 473	2,375.5 ± 572	3,122.9 ± 631	<0.001
Female, %	62.5 (1,401)	59.7 (1,339)	54.8 (1,229)	<0.001
Married, %	83.3 (1,820)	82.8 (1,816)	82.5 (1,811)	0.46
Physically active (≥1 h/week), %	32.5 (663)	32.4 (675)	36.0 (752)	0.02
Overweight or obese, %	45.6 (1,018)	45.9 (1,026)	48.6 (1,083)	0.08
Current smokers, %	3.0 (58)	3.3 (64)	5.3 (105)	<0.001
Education (university graduate), %	56.3 (1,234)	63.5 (1,393)	60.8 (1,330)	<0.001

Data are mean ± standard deviation (SD) or percent (number).
[a]Obtained from ANOVA or chi-square test, where appropriate.
TAC, total antioxidant capacity.

TABLE 2 | Mean scores of AHEI and DDS across tertiles of dietary TAC.

	Tertiles of dietary TAC			
	T_1	T_2	T_3	*P*-value[a]
Subjects, *n*	**2,241**	**2,242**	**2,241**	
AHEI				
Crude	52.06 ± 0.16	54.87 ± 0.16	57.52 ± 0.16	<0.001
Model I	51.99 ± 0.20	54.87 ± 0.16	57.58 ± 0.20	<0.001
Model II	52.00 ± 0.20	54.87 ± 0.16	57.57 ± 0.20	<0.001
Model III	52.03 ± 0.20	54.87 ± 0.16	57.53 ± 0.20	<0.001
DDS				
Crude	3.33 ± 0.03	5.03 ± 0.03	6.37 ± 0.03	<0.001
Model I	4.15 ± 0.03	5.03 ± 0.02	5.56 ± 0.03	<0.001
Model II	4.15 ± 0.03	5.03 ± 0.02	5.55 ± 0.03	<0.001
Model III	4.15 ± 0.03	5.03 ± 0.02	5.56 ± 0.03	<0.001

Data are mean ± standard error (SE).
[a]Obtained from ANCOVA.
Model I: adjusted for energy intake.
Model II: additionally, adjusted for age and sex.
Model III: additionally, adjusted for BMI.
AHEI, alternate healthy eating index; DDS, dietary diversity score; TAC, total antioxidant capacity.

TABLE 3 | Participants' distribution across tertiles of dietary TAC, AHEI, and DDS.

	Tertiles of dietary TAC			
	T_1	T_2	T_3	*P*-value[a]
AHEI				<0.001
T_1	47.7 (1,070)	33.6 (753)	21.4 (479)	
T_2	33.7 (755)	34.3 (769)	32.8 (735)	
T_3	18.6 (416)	32.1 (719)	45.8 (1,027)	
DDS				<0.001
T_1	68.1 (1,525)	24.7 (553)	7.7 (173)	
T_2	27.6 (619)	44.3 (993)	27.0 (605)	
T_3	4.3 (97)	31.0 (696)	65.3 (1,463)	

Data are percent (number).
[a]Obtained from chi-square test.
AHEI, alternate healthy eating index; DDS, dietary diversity score; TAC, total antioxidant capacity.

active, current smokers, and university graduated and less likely to be female. No significant difference was found in terms of other variables.

Crude and multivariable-adjusted means for AHEI and DDS across tertiles of dietary TAC are shown in **Table 2**. After controlling for energy intake, age, sex, and BMI, we found that individuals in the highest tertile of dietary TAC had higher scores of AHEI compared with those in the lowest tertile (57.53 ± 0.20 vs. 52.03 ± 0.20, $P < 0.001$). In the fully adjusted model, individuals in the top tertile of dietary TAC had higher scores of DDS (5.56 ± 0.03 vs. 4.15 ± 0.03, $P < 0.001$) compared with those in the bottom tertile.

Participants' distribution on the basis of cross-classification analysis between tertiles of dietary TAC and AHEI indicated that the classifications were in exact agreement for 42.6%, within an adjacent tertile for 33.05%, and in gross misclassification for 20% of individuals. When this was examined between dietary TAC and DDS, we found that exact agreement in the classifications was for 59.2% of participants. Notably, a very low proportion of gross misclassification was seen in this regard such that only 6% of participants were classified in the opposing tertiles, indicating additional support for a good agreement (**Table 3**).

DISCUSSION

In this cross-sectional study, we investigated if dietary TAC can be considered as a measure for healthy eating. We found that participants in the highest tertiles of dietary TAC had higher scores of AHEI and DDS as well. Given the proper agreements

between dietary TAC and AHEI and DDS, we concluded that dietary TAC can be a good and appropriate measure for healthy eating.

Dietary TAC was used to assess dietary antioxidants. It was associated with reduced risk of various chronic diseases (9, 10, 14–17). On the other hand, indicators of healthy eating were also previously linked with a lower risk of mortality and several chronic conditions. Therefore, we assumed that dietary TAC might be a good indicator for diet quality as well. Poor diet quality has been associated with increased risk of chronic diseases (33). Identification of nutrients involved in increasing the quality of the diet might help individuals to make accurate food choices. Investigating the association between dietary TAC and diet quality is a novel concept and few studies have been done in this regard so far. We found that high quality diets contained greater dietary TAC. In line with our study, Ha et al. reported that high dietary TAC was correlated with greater adherence to the diet quality index scores (DQIS) including Healthy Eating Index (HEI), Alternative Healthy Eating Index (AHEI), alternate Mediterranean Diet (aMED), and Dietary Approaches to Stop Hypertension (DASH) (20). Similar findings were also reported by Puchau et al. in which dietary TAC was positively associated with dietary quality scores including HEI, AHEI, Diet Quality Index-International (DQII), Diet Quality Index-Revised (DQIR), Mediterranean Diet Score (MDS), Alternate Mediterranean Diet Score (AMDS), Modified Mediterranean Diet Score (MMDS), Quantitative Index for Dietary Diversity (QIDD), and Recommended Food Score (RFS) (19). Overall, it seems that dietary TAC can be considered as a measure of diet quality. In other words, recommending general population to increase their dietary antioxidants intake might result in increased quality of their diet, as measured by indicators of diet quality.

An imbalance between antioxidants and pro-oxidants in the body leads to oxidative stress, which is the basis for several diseases. Oxidative stress occurs when there is an overproduction of ROS or an enzymatic or non-enzymatic deficiency of antioxidants in the body. Fruits and vegetables as rich sources of antioxidants in the diet can act as anti-aging agents and are beneficial for health (34). Greater dietary TAC, as compared with a low dietary TAC, has been associated with lower levels of inflammation, as measured by high-sensitivity C-reactive protein (35). In addition, dietary TAC was positively associated with food group intake including fruits, vegetables, whole grains, legumes, nuts, seeds, and seafood, and inversely related to red and processed meat consumption (20). Therefore, it seems that dietary TAC is a good measure to classify food groups in terms of their contribution to human health and may also be considered to measure the quality of the diet. Application of TAC assessment might be that, instead of the whole diet assessment, we can assess limited numbers of dietary factors to assess peoples' adherence to a healthy diet.

The use of cross-classification of participants in terms of dietary TAC and AHEI and large sample size of the study might be considered as some strengths of this study. In addition, it must be kept in mind that these findings came from a region where data on dietary information and dietary measurements by valid methods are scarce. Given the unique characteristics of diet in Middle Eastern countries, having information about appropriate measures to define quality diets in this area is of importance. However, this study has some limitations that should be considered when interpreting our results. Due to the application of FFQ for dietary assessment, measurement errors and misclassification of participants is unavoidable. However, we used a validated FFQ for assessment of dietary intakes to minimize the bias in dietary assessments. In addition, we did not examine the validity of this questionnaire for assessment of dietary TAC. However, earlier publications based on this questionnaire have revealed that data on dietary TAC from this questionnaire can be used to predict the chronic conditions (36). Moreover, some components of DDS and AHEI including corn flakes and alcohol consumption were not considered in this study, because of the lack of data in the original data set. The usage of FRAP to assess the TAC of the food items is another limitation. This essay has an inherent error where the reagents react with atmospheric Oxygen, thereby, rendering the values interfered.

In conclusion, we found that dietary TAC might be considered as a proper measure for assessment of diet quality, because it was well correlated with well-known measures of diet quality including DDS and AHEI scores.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, via email request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Bioethics Committee of Isfahan University of Medical Sciences. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS-M, SN-M, FE, and AK: conceptualization, formal analysis, writing—original draft, and writing—review and editing. AE: supervision, conceptualization, methodology, investigation, funding acquisition, formal analysis, writing—original draft, and writing—review and editing. PA: conceptualization, investigation, and methodology. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors are thankful to participants of SEPAHAN project and authorities of Isfahan University of Medical Sciences for their excellent cooperation.

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ORIGINAL RESEARCH
published: 25 April 2022
doi: 10.3389/fnut.2022.865684

Potential Antimicrobial Properties of Coffee Beans and Coffee By-Products Against Drug-Resistant *Vibrio cholerae*

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *30 January 2022*
Accepted: *21 March 2022*
Published: *25 April 2022*

Citation:
Rawangkan A, Siriphap A, Yosboonruang A, Kiddee A, Pook-In G, Saokaew S, Sutheinkul O and Duangjai A (2022) Potential Antimicrobial Properties of Coffee Beans and Coffee By-Products Against Drug-Resistant Vibrio cholerae. Front. Nutr. 9:865684. doi: 10.3389/fnut.2022.865684

Vibrio cholerae is the causative organism of the cholera epidemic, and it remains a serious global health problem, particularly the multidrug-resistant strain, despite the development of several generic drugs and vaccines over time. Natural products have long been exploited for the treatment of various diseases, and this study aimed to evaluate the *in vitro* antibacterial activity of coffee beans and coffee by-products against *V. cholerae* antimicrobial resistant strains. A total of 9 aqueous extracts were investigated, including light coffee (LC), medium coffee (MC), dark coffee (DC), dried green coffee (DGC), dried red coffee (DRC), fresh red coffee (FRC), Arabica leaf (AL), Robusta leaf (RL), and coffee pulp (CP). The influential coffee phytochemicals, i.e., chlorogenic acid (CGA), caffeic acid (CA), and caffeine, were determined using HPLC. The antibacterial properties were tested by agar well-diffusion techniques, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were further determined against 20 *V. cholerae* isolates. The results revealed that all tested strains were sensitive to coffee extracts, with MIC and MBC values in the range of 3.125–25.0 mg/mL and 12.5–50.0 mg/mL, respectively. With a MIC of 6.25 mg/mL, DGC, DRC, and CP appeared to be the most effective compounds against 65, 60, and 55% of clinical strains, respectively. The checkerboard assay revealed that the combination of coffee extract and tetracycline was greater than either treatment alone, with the fractional inhibitory concentration index (FICI) ranging from 0.005 to 0.258. It is important to note that CP had the lowest FICI (0.005) when combined with tetracycline at 60 ng/mL, which is the most effective dose against *V. cholerae* six-drug resistance strains (azithromycin, colistin, nalidixic acid, sulfamethoxazole, tetracycline, and trimethoprim), with a MIC of 47.5 μg/mL (MIC alone = 12.5 mg/mL). Time killing kinetics analysis suggested that CA might be the most effective treatment for drug-resistant *V. cholerae* as it reduced bacterial growth by

3 $\log_{10}$ CFU/mL at a concentration of 8 mg/mL within 1 h, via disrupting membrane permeability, as confirmed by scanning electron microscopy (SEM). This is the first report showing that coffee beans and coffee by-product extracts are an alternative for multidrug-resistant *V. cholerae* treatment.

Keywords: antimicrobial activity, coffee by-products, coffee extract, drug-resistant, *Vibrio cholerae*

INTRODUCTION

Cholera is an acute diarrheal infection caused by the consumption of contaminated food or water containing the gram-negative bacteria *Vibrio cholerae*, especially the serogroups O1 and O139, which are capable of causing cholera outbreaks that can kill within hours if left untreated. Seven cholera pandemics have already been reported throughout the world (1). According to the most recent global burden estimate, there are approximately 1.3–4.0 million cholera cases per year, with 21,000–143,000 deaths worldwide (2). Despite the availability of a vaccine, 923,037 cases were reported from 31 countries in 2019, with 1,911 deaths (a mortality rate of 0.2%) (3). According to World Health Organization (WHO) reports, a global cholera control strategy called "Ending Cholera: A Global Roadmap to 2030" was created with the goal of reducing the mortality rate by 90% (4).

Oral rehydration therapy, supplemented with antibiotics such as tetracycline, fluoroquinolones, and azithromycin, is the primary treatment for *V. cholerae* (5). Due to its extraordinary genomic plasticity, treatment failures have become more common in recent years, with the recurrence of antimicrobial resistant *V. cholerae* (6–11). The rise of drug-resistant *V. cholerae* is a major public health concern because the illnesses that occur are often more severe and difficult to treat. Infections with drug-resistant *V. cholerae* lead to greater mortality rates, longer hospital stays, more secondary infections, and higher medical expenses (12). In Thailand, 61.5% (48 of 78 isolates) of *V. cholerae* isolates between 1991 and 2013 were reported to be antimicrobial resistant strains, with 56.3% of them being multidrug-resistant (MDR) and conferring resistance to three or more antimicrobial classes (13). It is important to note that the development of antibiotic resistance outpaces the development of new drugs, resulting in a global problem with long-term negative consequences. Therefore, the development of new anti-*Vibrio* compounds, particularly those derived from plants, has become critical.

Natural compounds against *V. cholerae* have been shown to inhibit bacterial growth or the secreted cholera toxin, including catechins from green tea (*Camellia sinensis*) (14), procyanidins from *Guazuma* (*Guazuma ulmifolia*) (15), gallate analogs from Daio (*Rhei rhizoma*) (16), apelphenon from apple (*Malus* spp.) (17), procyanidins from hop (*Humulus lupulus*) (18), oil (diallyl sulfides) from elephant garlic (*Allium ampeloprasum*) (19), and capsaicin from red chili (*Capsicum annum*) (20, 21). Piperidine, chlorogenic acid (CGA), and eugenyl acetate derived from *Piper betel* have also been shown to be equally effective against MDR strains of *V. cholerae* (22–24). Carvacrol, a major essential oil fraction of Oregano (*Origanum vulgare*), inhibited the virulence of *V. cholerae* by inhibiting mucin penetration, adhesion, and the expression of virulence-associated genes (*tcpA*, *ctxB*, *hlyA*, and *toxT*), resulting in reduced fluid accumulation (25). On the other hand, cranberry (*Vaccinium macrocarpon*) extract inhibited *V. cholerae* biofilm formation, possibly by modulating the cyclic dimeric guanosine monophosphate (c-di-GMP) level (26). Furthermore, methanolic extracts of basil (*Ocimum basilicum* L.), nopal cactus (*Opuntia ficus-indica* var. Villanueva L.), sweet acacia (*Acacia farnesiana* L.), and white sagebrush (*Artemisia ludoviciana* Nutt.) were found to be the most active against *V. cholerae* via cell membrane disruption (27). However, there has been no mention of coffee extract.

Coffee (*Coffea* L.) is one of the world's most valuable primary products (28). *C. arabica* L. cv. Caturra (Arabica) is the most popular and preferred coffee cultivar worldwide. Coffee processing generates a large amount of solid by-products during coffee cultivation and preparation, such as spent coffee grounds, the by-products of coffee fruit and bean processing (coffee husks, peel, pulp), and so on (29). Recently, we revealed that coffee beans or coffee by-product extract, which are high in phenolic compounds and antioxidant activity, seem to have a wide-range of health benefits, including anti-hyperglycaemic and anti-hyperlipidaemic activities (30), anti-adipogenic and lipolytic properties (31), anti-diabetic, cholesterol-lowering, and anti-hepatic steatosis activity (32–34), anti-hepatic steatosis activity (35, 36), as well as antibacterial activity against both gram-positive and gram-negative bacteria (37). Therefore, the use of coffee extracts with medicinal properties could be an alternative treatment for various diseases.

Coffee beans contain a variety of compounds with powerful bioactive activities, i.e., caffeine, CGA, diterpenes, and trigonelline (38). Several studies have shown that coffee extracts have strong antibacterial activity (39–42). Flavonoids, CGA, caffeic acid (CA), trigonelline, caffeine, and protocatechuic acid play a key role as potential natural antimicrobial agents against enteric bacteria (40, 43, 44), but there is no relevant data on *V. cholerae*. Nevertheless, the efficacy varies, depending on the species, degree of roasting, brewing procedure, and decaffeination (45). Coffee varieties from various origins differ significantly in terms of their constituents, and multiple agricultural geography conditions of the coffee plant, such as the soil type, altitude, and harvest season, as well as the pre- and post-harvest management practices, influence coffee bean bioactivity (46, 47).

For the first time, we shed light on the potential antimicrobial properties of coffee beans and coffee by-products against MDR *V. cholerae* in health improvement treatments. With this goal,

this work seeks (1) to examine the antimicrobial activity of coffee beans, classified by temperature and roasting time, as well as coffee by-product extracts, such as coffee fruits and leaf extracts, and coffee pulp (CP) extract, (2) to investigate the synergistic effects of the crude extract compounds of coffee with the antibiotic tetracycline, and (3) to assess the pharmacological mode of action of coffee bioactive molecules with respect to potential disruption in the membrane of microorganisms and their effect on bacterial morphology, which may be helpful to bring about new opportunities in complementary and alternative medicine.

MATERIALS AND METHODS

Preparation and Phytochemical Characterization of Coffee Beans and Coffee By-Products

Plant Materials and Extract Preparation

The Chao-Thai-Pukao Factory (Chiang Mai, Thailand) provided coffee beans and coffee by-products. As indicated in the previous report, NU003806 was the coffee tree's voucher number (30).

Roasted Coffee Extracts: Light Coffee, Medium Coffee, and Dark Coffee

The roasted coffee extracts were prepared from green coffee beans (*Coffea arabica* L.), with the degree of roasting performed in accordance with previous studies (30, 48). Light coffee (LC), medium coffee (MC), and dark coffee (DC) are classified by the roasting temperature and roasting time (176.7–232.2°C and 10–20 min). The roasted coffee was extracted with water (1:5; w/v) using an ultrasonic bath at 35 kHz at 40°C for 5 min. The filtered samples were dried using a freeze dryer (CoolSafe 110-4 Pro, LaboGeneTM, Allerød, Denmark), and the LC, MC, and DC extracts were then stored at −20°C for further study.

Coffee Fruit Extracts: Dried Green Coffee, Dried Red Coffee, and Fresh Red Coffee

Coffee fruit extracts were prepared according to previous studies (31, 32). Briefly, fresh and dried coffee fruits were extracted with boiling distilled water for 30 min (1:10; w/v). The aqueous solution was dried by a freeze dryer (CoolSafe 110-4 Pro, LaboGeneTM, Allerød, Denmark), and the powder of dried green coffee (DGC), dried red coffee (DRC), and fresh red coffee (FRC) were stored at −20°C until use.

Coffee Leaf Extracts: Arabica Leaf and Robusta Leaf

Coffea arabica L. cv. Caturra (Arabica) and *C. canephora* var. robusta (Robusta) leaves were extracted with boiling water (1:5; w/v) for 10 min. This step was repeated three times, and then the filtered solutions were freeze dried. The powder was stored at −20°C until further examination.

Coffee Pulp Extract

The coffee pulp (CP) was extracted according to a previous study (37). Briefly, dried pulps were extracted with boiling water (1:5; w/v) for 10 min. This step was repeated twice before the solutions were freeze dried. The pulp powder was stored at −20°C for later use.

Determination of Coffee Phytochemical Content by Chromatographic Analysis

Six coffee extracts were subjected to high-performance liquid chromatography (HPLC) to determine the levels of CGA, CA, and caffeine, according to previous studies (31, 48). In brief, the HPLC separation of the LC, MC, DC, CP, AL, and RL extracts was performed on a C18 column (4.6 × 150 mm, 5 μm) using mobile phase A (15% methanol) and mobile phase B (85% methanol:distilled water [30:70], 2% acetic acid; pH 3.4). The flow rate was set at 0.5 mL/min for 30 min, with detection at 280 and 320 nm for CA, caffeine, and CGA. The peaks were identified by the reference standards. DGC, DRC, and FRC extracts were run with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) using HPLC and coupled to LC-ESI-Q-TOF-MS according to previously reported (31).

Bacterial Strains and Growth Conditions

The clinical strains of 20 representative isolates of *V. cholerae* serogroups O1 and O139 were obtained from previous studies (13), in which they were isolated from feces and rectal swabs of patients in Thailand between 1994 and 2004. The 7th pandemic *V. cholerae* N16961 strain from Bangladesh in 1975 was used as a standard reference strain. The antibiotic resistance pattern of all strains has previously been characterized and can be found in **Supplementary Table 1**.

To perform the preliminary antimicrobial screening of the effect of each crude extract on *V. cholerae* growth inhibition, the N16961 strain was grown overnight in Mueller Hinton Broth (MHB) containing 1% NaCl at 37°C. The 0.5 McFarland turbidity standard cultures (1–1.5 × 10^8 colony-forming units; CFU) were spread onto Mueller Hinton Agar (MHA) plates using sterile cotton swabs, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (49). The extracted compounds were diluted in MHB. Then, 50 μL of filtered extracts were allowed to diffuse into a 6-mm cork borer well in MHA containing 1% NaCl medium at 500 mg/mL. The plates were kept at room temperature for 30 min to allow diffusion of the test solution into the surrounding media. The plates were then incubated at 37°C for 18 h. Each plate was examined for the inhibition zone. Tetracycline, the first line treatment for cholera disease, was used as a positive control at a concentration of 30 μg/mL, and media solution was used as a negative control (50).

Determination of the Minimum Inhibitory Concentration and the Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) values were determined using a 96-well microtiter plate and the CLSI protocol (51, 52). Freshly prepared stock solutions of the extracts or their phytochemical compounds were serially diluted twice using MHB with 1% NaCl. All wells were inoculated with *V. cholerae* at a final volume of 100 μL of bacterial inoculum (5 × 10^5 CFU/mL). After incubation for 24 h at 37°C, 1 mg/mL resazurin was added to all wells (10 μL per well), and the plates

were further incubated for 4 h to observe the color change. On completion of the incubation, columns with no color change (blue resazurin color remained unchanged) were scored as being above the MIC value (53).

The MBC was determined using the MHA plates with 1% NaCl by dropping 10 μL of test solution directly into the content of the wells that had concentrations higher than the MIC value, and then incubating at 37°C for 24 h. The MBC value was determined when there was no colony growth from the contents of the 10 μL directly-plated wells. In addition, the contents of the wells showing indications of growth inhibition were serially diluted to quantify the end-point killing of the bacteria, as detailed in the results section.

Antimicrobial Synergy Testing

The checkerboard assay was used to determine the potential synergistic activity of the extracts and tetracycline on *V. cholerae* N16961 and P48 *V. cholerae* El Tor Ogawa strains, which are a reference and tetracycline resistance strain, respectively (54). The extract compounds were serially diluted to 1/128 MIC, while the drug was serially diluted to 1/516 MIC. Compounds and antibiotics were prepared in 96-well microtiter plates using 2-fold serial dilutions based on the MIC of each substance. A final bacterial suspension at 5×10^5 CFU/mL was added to each well. After incubation for 24 h at 37°C, the wells were visually inspected, and the synergistic MIC (compound in combination with antibiotic) was determined as the first well with no visible turbidity. The observed MIC values were used to calculate the fractional inhibitory concentration index (FICI), which allows evaluation of the combined effects of an antibiotic and a compound according to the following formula: FICI = FIC (a) + FIC (b), where FIC (a) = MIC of extract in the combination/MIC of extract alone, and FIC (b) = MIC of tetracycline in the combination/MIC of tetracycline alone. These values were interpreted as follows: for FICI $\leq$0.5: a synergistic effect; for FICI $>$0.5 and $\leq$4: an additive effect; and for FICI $>$4: an antagonistic effect (55, 56).

Time-Kill Kinetics Assay

The killing kinetics of the potent coffee phytochemical compounds, including CGA, CA, and caffeine, at 1x, 2x, 4x, and 8x MIC values were determined using the method described previously (57–59), with slight modifications. Different concentrations of compounds were added to reach the final volume of 100 μL with 1×10^5 CFU/mL of *V. cholerae* N16961 reference stain grown in MHB containing 1% NaCl and kept at 37°C. Bacterial growth was monitored over a time-course of 24 h (0, 1, 2, 4, 8, 16, 24 h). A sample without the compound served as a growth control. To evaluate the survival of the pandemic strains during the observation period, aliquots of serial dilutions of the bacterial suspensions were determined by a spread plate technique on MHA with 1% NaCl, and the plates were incubated at 37°C for 24 h to evaluate the viable bacterial colony counts. Data was analyzed as killing curves by plotting the $\log_{10}$ CFU/mL vs. time (h), and the change in bacterial concentration was determined. The viable bacterial cell count for the time-kill end point determination, i.e., bactericidal activity, was defined as a reduction of $\geq$3 $\log_{10}$ CFU/mL relative to the initial inoculum, whereas bacteriostatic activity corresponded to a $<$3 $\log_{10}$ CFU/mL decrease relative to the initial inoculum (60).

Outer Membrane Permeabilization Analysis

Determination of Nucleotide and Protein Leakage

The leakage of cytoplasmic elements from the cell was used to evaluate the integrity of the cell membrane using the method described by Lou et al. (61), with some modifications. In brief, the *V. cholerae* N16961 cells were cultured overnight at 37°C, and the cells were washed and resuspended at a concentration of 1×10^7 CFU/mL in phosphate buffer saline (PBS), pH 7.2. Then, 1 mL of these suspensions was incubated with CGA, CA, and caffeine at concentrations of 1, 2, 4, and 8x MIC at 37°C for 1 h. After centrifugation, the supernatant samples were immediately filtered through a 0.2 μm organic membrane, and the optical density measured at 260 nm using a NANO-400A Micro Spectrophotometer, to determine the amounts of DNA released from the cytoplasm. The cell integrity was further examined by determining the release of proteins into the supernatant. The Bradford dye-binding reagent of the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Inc., USA) was used to determine the amount of protein by measuring the optical density of the resulting solution at 750 nm within 5 min. The protein quantity of each sample was determined from the equation of the best-fit linear regression obtained from the Bovine Serum Albumin (BSA) standard curve. Triton X-100 (0.1%; v/v) was used as a positive control, while PBS inoculated with the same inoculum was used as a negative control.

Determination of Outer Membrane Disruption

The effect of the potent coffee phytochemical compounds on the bacterial outer membrane permeability was determined using an N-Phenyl-1-naphthylamine (NPN) uptake assay (62, 63). Briefly, *V. cholerae* N16961 cells were treated with 0, 1, 2, 4, and 8 MIC at a final volume of 1 mL and incubated for 1 h at 37°C. The cell suspensions were then washed and resuspended in 1 mL of 0.5% NaCl. NPN solution (TCI, Japan) in ethanol (100 mM) was added to 200 μL of cells to give a final concentration of 0.75 mM. The background fluorescence was recorded for subtraction, using the Cytation 5 Cell Imaging Multi-Mode Reader with an excitation wavelength of 350 nm and an emission wavelength of 420 nm at room temperature. As the outer membrane permeability increased due to the addition of the coffee phytochemical compound, NPN incorporated into the membrane resulted in an increase in fluorescence. Triton X-100 (0.1%; v/v) was used as a positive control for the 100% maximum dye leakage release. Values were converted to % NPN uptake using the following equation: % NPN uptake = (Fobs-F0)/(F100-F0)x100, where Fobs is the observed fluorescence at a given compound concentration, F0 is the initial fluorescence of NPN with the cells in the absence of compound, and F100 is the fluorescence of NPN with the cells upon addition of TritonX-100 (64).

Determination of Cell Membrane Potential

To measure the changes in membrane polarity caused by the coffee, bioactive compounds were adapted through the incorporation of Rhodamine 123 (Rh123) (Sigma-Aldrich, USA) (65–67). *V. cholerae* N16961 cells were treated with 0, 1, 2, 4, and 8 MIC at a final volume of 1 mL and incubated for 1 h at 37°C. The cell suspension was mixed with a freshly-prepared Rh123 solution (final Rh123 concentration, 5 μg/mL), kept at 37°C for 10 min, and centrifuged at 1,500 rpm for 10 min. The cell pellets were then diluted in 0.5% NaCl, and the fluorescence signal measured at the excitation and emission wavelengths of 480 and 530 nm, respectively. The fluorescence intensity were calculated using the equation: Relative fluorescence intensity = F1/F0 × 100%, where F0 is the fluorescence intensity of untreated cells, and F1 is the fluorescence intensity of CA-treated cells.

Analysis of Scanning Electron Microscopy

Vibrio cholerae N16961 was treated with CA at a concentration of 8x MIC for 2 h at 37°C. The appropriate treatment was harvested by centrifugation at 5,000 rpm for 5 min, washed with PBS, dropped onto a filter membrane of 0.2 μm, and air dried. The samples were fixed using 2.5% (v/v) glutaraldehyde in PBS at 4°C overnight. Thereafter, the bacteria were washed with 0.1M PO_4 buffer and re-fixed with 1% OsO_4 for 1 h. After dehydration with a graded ethanol series (50, 70, 90, and 100%) for 10 min each, the bacterial samples were transferred to absolute ethanol for 20 min. After drying by critical-point drying (CPD), the bacterial sample was mounted and coated with gold, before examination by scanning electron microscopy (SEM) (JSM 5910 LV, Oxford Instrument) (62).

Statistical Analysis

Values are presented as the mean ± standard deviation (SD) of three independent experiments. The significance of differences between the average values of different experimental treatments and controls was assessed by ANOVA, considering that statistical significance was set at a $p < 0.05$. When ANOVA revealed significant differences among treatments, *post-hoc* tests were carried out with Dunnett's Multiple Comparison Test from GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Characterization of Coffee Beans and Coffee By-Products

As indicated in **Table 1**, coffee beans and coffee by-products including the roasted beans, fruits, leaves, and pulp extracts, represented bioactive substances with CGA, CA, and caffeine. Calibration curves were linear over a large concentration range of 3.125–400 μg/mL for caffeine and CGA, and 3.125–200 μg/mL for CA, and exhibited good linear regressions ($r^2 = 0.9997$ for caffeine, $r^2 = 0.9989$ for CGA, $r^2 = 0.9986$ for CA), data not shown. CGA was found to be more abundant in CP (13.45 mg/g extract), DGC (12.56 mg/g extract), and RL (12.04 mg/g extract) than in the other extracts. Additionally, roasted coffee had higher levels of CA than coffee leaves, pulp, and fruits. CA (2.66 mg/g extract) was detected in higher concentrations in LC than in the other extracts. Interestingly, all extracts, especially MC, had a high caffeine content (13.08–26.80 mg/g extract).

TABLE 1 | The phytochemical profile of the extracts of coffee beans and coffee by-products.

Samples	CGA (mg/g extract)	CA (mg/g extract)	Caffeine (mg/g extract)
Roasted coffee extracts			
LC	11.21	2.66	23.39
MC	5.53	1.20	26.80
DC	2.69	1.01	22.77
Coffee fruit extracts*			
DGC	12.56*	0.25*	ND
DRC	7.21*	0.21*	ND
FRC	6.97*	0.08*	ND
Coffee leaf extracts			
AL	1.99	0.80	17.72
RL	12.04	1.85	13.08
CP extract	13.45	1.10	16.88

LC, light coffee; MC, medium coffee; DC, dark coffee; DGC, dried green coffee; DRC, dried red coffee; FRC, fresh red coffee; AL, Arabica leaf; RL, Robusta leaf; CP, coffee pulp; CGA, chlorogenic acid; CA, caffeic acid; ND, not determined.
**Our previous report (31).*

As a result, the extract of coffee beans and coffee by-products containing coffee phytochemicals were used to determine the biological activity of the antibacterial analysis in future studies.

The Extracts of Coffee Beans and Coffee By-Products Inhibit Drug Resistant *V. cholerae* Strains

The 7th pandemic N16961 reference strain was used to screen the growth inhibition effect of each extract on *V. cholerae*. **Table 2** shows the diameter of the inhibition zones in treatments with 500 mg/mL of the extract. The inhibition zones range from 10.67 ± 3.79 to 16.67 ± 1.15 mm in the agar well-diffusion assay. Treatment with AL extract inhibited bacterial growth the most effectively, followed by CP and MC, respectively. The inhibition zones of AL, CP, and MC were 16.67 ± 1.15, 13.33 ± 1.53, and 13.00 ± 1.00 mm, respectively, which is approximately half that of tetracycline (26.00 ± 1.73 mm). Therefore, the extracts may have the potential to act as natural antibiotics.

We then investigated the MIC and MBC of the extracts on the representatives of 20 *V. cholerae* clinical strains that maintain an antibiotic resistant pattern, such as streptomycin (STM), colistin (COL), nalidixic acid (NAL), sulfamethoxazole (SMX), tetracycline (TET), trimethoprim (TMP), ciprofloxacin (CIP), and azithromycin (AZI). The results, which were expressed as MIC and MBC values in **Table 3**, show that all of the extracts were active against all of the strains. The most effective against the representative clinical strains had a MIC of 6.25 mg/mL, referring to DGC (65%), DRC (60%), and CP (55%), respectively.

As part of roasted coffee extracts, the trend of MIC and MBC values were determined to be 12.5 (mean 83.33 ± 10.41%) and 50.0 (mean 86.67 ± 15.28%) mg/mL, respectively. The

findings of this research show that 95% of the tested strains were sensitive to DC, followed by LC (80%), and MC (75%), respectively. Furthermore, treating P36 *V. cholerae* El Tor Ogawa, which carried five drug resistances, i.e., COL, NAL, SMX, TET, and TMP, with DC gave a MIC value of 6.25 mg/mL (**Supplementary Table 2**). Similarly, the results also revealed that two of three coffee fruit extracts (DGC and DRC) efficiently suppressed the growth of pathogens, with the MIC at 6.25 mg/mL (65% for DGC and 60% for DRC), while 55% of treated strains expressed a MIC of 25.0 mg/mL after treatment with FRC. Treatment of P46 *V. cholerae* El Tor Ogawa with FRC gave the lowest MIC value at 3.125 mg/mL, despite carrying five drug resistance genes, namely AZI, NAL, SMX, TET, and TMP. Furthermore, Arabica and Robusta coffee leaf extracts with MICs of 12.5 mg/mL suppressed *V. cholerae* growth conditions by 75% (AL) and 70% (RL), respectively, whereas the MIC of CP extract was 6.25 (55%), 12.5 (40%), and 25 (5%) mg/mL. These results suggest that the effects of coffee beans and coffee by-product extracts on antimicrobial activities are varied depending on the extract and the *V. cholerae* clinical strain.

TABLE 2 | Diameters of inhibition zones obtained with coffee beans and coffee by-products at 500 mg/mL on the 7th pandemic *V. cholerae* O1 El Tor N16961 strain.

Samples	Diameters of inhibition zones (mm)
Roasted coffee extracts	
LC	12.33 ± 0.58**
MC	13.00 ± 1.00**
DC	11.67 ± 1.15*
Coffee fruits extracts	
DGC	11.33 ± 4.04*
DRC	11.00 ± 2.65
FRC	10.67 ± 3.79
Coffee leaf extracts	
AL	16.67 ± 1.15***
RL	12.00 ± 1.00*
CP extract	13.33 ± 1.53**
Controls	
Tetracycline	26.00 ± 1.73***
MHB	6.00 ± 0

*LC, light coffee; MC, medium coffee; DC, dark coffee; DGC, dried green coffee; DRC, dried red coffee; FRC, fresh red coffee; AL, Arabica leaf; RL, Robusta leaf; CP, coffee pulp; MHB, Mueller Hinton Broth. Values are means of triplicate determination (n = 3) ± standard deviations. Significant differences are as follows: *p < 0.05, **p < 0.01, and ***p < 0.001 compared with negative control (MHB).*

Regarding MDR *V. cholerae* O1, which is a major public health concern, we investigated the synergistic effect of each extract and tetracycline in the N16961 and P48 *V. cholerae* El Tor Ogawa strains, a reference and six-drug resistance strain (AZI, COL, NAL, SMX, TET, and TMP), respectively. In double-dose response (checkerboard) experiments, the extract combinations were used to determine the nature of their interaction with tetracycline. **Table 4** shows the evaluation of the synergistic effect of the extracts and antibiotics. In both standard and multidrug resistance strains, the combination effect on bacterial growth appeared to be greater than treatment alone, with FICI ranging from 0.005 to 0.258. It is important to note that CP had the lowest FIC index (0.005) when combined with 47.5 μg/mL and tetracycline 60 ng/mL. These results indicate that combining the extracts with tetracycline might be a more effective treatment for *V. cholerae* infection.

Analysis of Bacterial Killing Kinetics

Considering the main bacteriostatic activity of coffee bioactive compounds, the standard CGA, CA, and caffeine, were applied to the N16961 reference strain. **Table 5** shows the MIC and MBC data. The MIC for CGA was 0.5 mg/mL, while CA and caffeine had MICs of 1 mg/mL. In contrast, the MBC of all compounds was >4 mg/mL.

We then investigated the time kill kinetics of each coffee phytochemical on the viability of *V. cholerae*, in order to define the bactericidal level using a 1- to 8-fold MIC treatment. **Figure 1**

TABLE 3 | The susceptibility of a total of 20 *V. cholerae* clinical strains to the extracts of coffee beans and coffee by-products.

Coffee beans or coffee by-products	No. of strains (%)					
	MIC [mg/mL]			MBC [mg/mL]		
	6.25	12.5	25	12.5	25	50
LC	2 (10.0)	16 (80.0)	2 (10.0)	0	2 (10.0)	18 (90.0)
MC	4 (20.0)	15 (75.0)	1 (5.0)	0	6 (30.0)	14 (70.0)
DC	1 (5.0)	19 (95.0)	0	0	0	20 (100.0)
DGC	13 (65.0)	4 (20.0)	3 (15.0)	0	14 (70.0)	6 (30.0)
DRC	12 (60.0)	7 (35.0)	1 (5.0)	4 (20.0)	9 (45.0)	6 (30.0)
FRC	1 (5.0)	8 (40.0)	11 (55.0)	5 (25.0)	5 (25.0)	10 (50.0)
AL	3 (15.0)	15 (75.0)	2 (10.0)	0	17 (85.0)	3 (15.0)
RL	6 (30.0)	14 (70.0)	0	0	0	20 (100.0)
CP	11 (55.0)	8 (40.0)	1 (5.0)	0	18 (90.0)	2 (10.0)

LC, light coffee; MC, medium coffee; DC, dark coffee; DGC, dried green coffee; DRC, dried red coffee; FRC, fresh red coffee; AL, Arabica leaf; RL, Robusta leaf; CP, coffee pulp. The bold values indicates MIC and MBC.

TABLE 4 | Synergistic effect of coffee beans and coffee by-products in combination with tetracycline against standard and multidrug-resistant *V. cholerae*.

Samples	MIC (mg/mL) of extracts [a]		FIC a	MIC (mg/mL) of tetracycline [b]		FIC b	FICI	Outcome
	Alone	Combination		Alone	Combination			
N16961 *V. cholerae* El Tor O1								
LC	12.50	0.095	0.0076	0.00039	0.000012	0.031	0.038	Synergistic
MC	6.25	0.046	0.0073	0.00039	0.000048	0.123	0.130	Synergistic
DC	12.50	0.095	0.008	0.00039	0.000006	0.015	0.023	Synergistic
DGC	6.25	0.048	0.0076	0.00039	0.0000975	0.250	0.258	Synergistic
DRC	12.50	0.095	0.0076	0.00039	0.00000038	0.001	0.009	Synergistic
FRC	12.50	0.095	0.0076	0.00039	0.000048	0.123	0.131	Synergistic
AL	25.00	0.190	0.0076	0.00039	0.00000038	0.001	0.009	Synergistic
RL	12.50	0.095	0.0076	0.00039	0.00000038	0.001	0.009	Synergistic
CP	12.50	3.125	0.25	0.00039	0.00000038	0.001	0.251	Synergistic
P48 *V. cholerae* El Tor O1 Ogawa								
LC	12.50	0.090	0.0072	0.0625	0.0078	0.125	0.132	Synergistic
MC	6.25	0.090	0.0144	0.0625	0.0039	0.062	0.077	Synergistic
DC	12.50	0.090	0.0072	0.0625	0.000006	0.008	0.015	Synergistic
DGC	6.25	0.0475	0.0076	0.0625	0.0019	0.030	0.038	Synergistic
DRC	12.50	0.0475	0.0038	0.0625	0.0039	0.062	0.066	Synergistic
FRC	12.50	0.090	0.0072	0.0625	0.00012	0.002	0.009	Synergistic
AL	25.00	0.090	0.0036	0.0625	0.00000038	0.016	0.019	Synergistic
RL	12.50	0.090	0.0072	0.0625	0.00000038	0.004	0.011	Synergistic
CP	12.50	0.0475	0.0038	0.0625	0.00006	0.001	0.005	Synergistic

LC, light coffee; MC, medium coffee; DC, dark coffee; DGC, dried green coffee; DRC, dried red coffee; FRC, fresh red coffee; AL, Arabica leaf; RL, Robusta leaf; CP, coffee pulp; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.

TABLE 5 | The MIC and MBC values of coffee phytochemicals of the *V. cholerae* O1 El Tor N16961.

Phytochemical	MIC (mg/mL)	MBC (mg/mL)
CGA	0.5	>4
CA	1	>4
Caffeine	1	>4

CGA, chlorogenic acid; CA, caffeic acid.

demonstrates the time-killing curve analysis. The kill kinetic profiles of the bacterial cultures had no effect when treated with CGA at a concentration of 8x MIC (4 mg/mL) (**Figure 1A**) or 16x MIC (8 mg/mL), data not shown. Whereas, CA demonstrated rapid bactericidal activity, with an approximate reduction of CFU by 3 log units in viable cell count relative to the initial inoculum at all tested concentrations within 1 h (**Figure 1B**), 8x MIC (8 mg/mL) of caffeine demonstrated a dose-dependent killing property after 16 h (**Figure 1C**). As a result, CA may be the most potent bioactive compound in coffee against *V. cholerae*.

CA Disrupts *V. cholerae* Membrane Permeability

To investigate the mechanism of CA on the damaged bacterial cell membrane, an effective drug permeability barrier of the gram-negative cell wall, we measured nucleotide and protein leakage, NPN uptake, and Rh123 incorporation, as shown in **Figure 2**. Bacterial cells were treated for 1 h with CA at concentrations of 1, 2, 4, and 8 mg/mL, referred to as 1x to 8x MIC.

The leakage of genetic materials, i.e., DNA, and the amount of proteins passing over the cell membrane was used to investigate the effect of CA on the integrity of the membrane via assessment of the absorbance in the CA-treated supernatant. The results are summarized as the DNA content and protein concentration, in **Figures 2A,B**, respectively, and indicate that the release of cell constituents increased significantly in a CA concentration-dependent manner. Indeed, 8 mg/mL of CA increased DNA and protein leakage more than 0.1% Triton X-100, by about 2.3- and 14.2-fold, respectively.

The NPN uptake assay was used to assess *V. cholerae* outer membrane permeabilization. NPN cannot normally insert into intact bacterial membranes (68); however, when CA disrupts the outer membrane, NPN penetrates the lipid layers, increasing the intensity of its fluorescence emission. CA easily permeabilized the outer membrane in a dose-dependent manner, as indicated by a rise in the intensity of NPN fluorescence (**Figure 2C**).

We also investigated the transmembrane potential activity by staining Rh123. Considering that Rh123 uptake was proportional to the membrane potential, the results showed that CA treatment increased membrane potential at all tested concentrations (**Figure 2D**). The highest fluorescence intensity of Rh123 was at 8 mg/mL CA concentration.

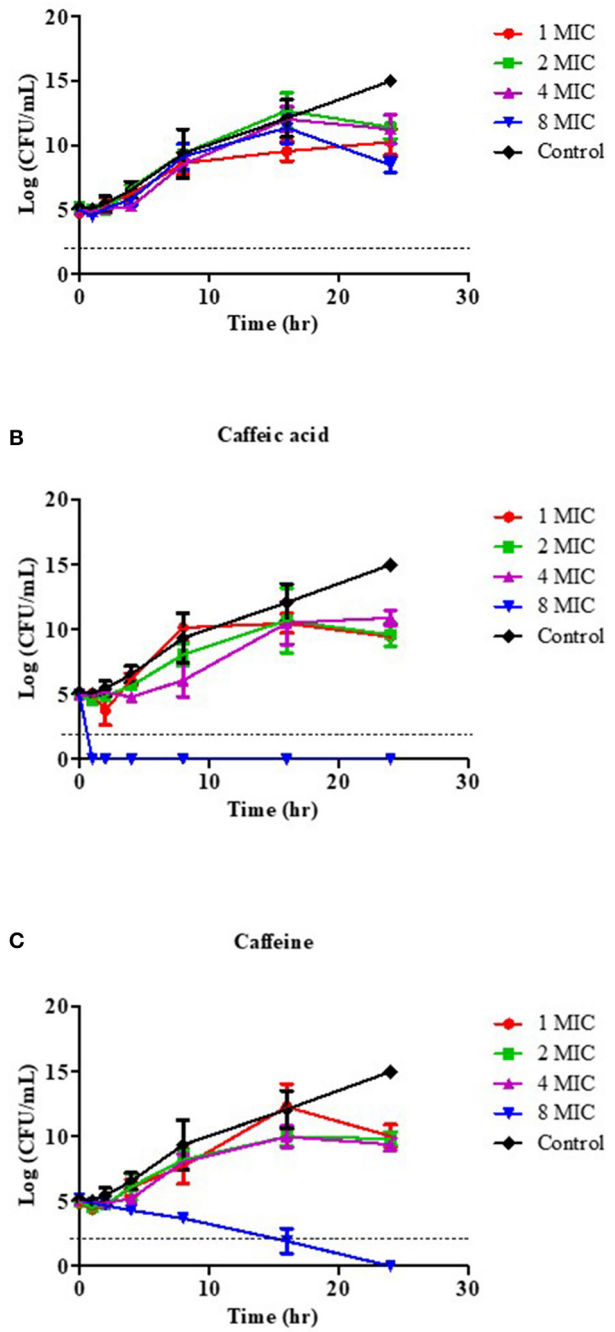

FIGURE 1 | Effect of coffee phytochemical on the viability of *V. cholerae* O1 El Tor N16961. Time-kill kinetics of CGA **(A)**, CA **(B)**, and caffeine **(C)** at concentrations of 1–8 MIC against *V. cholerae* were investigated over a 24 h incubation period at 37°C. The MIC for CGA was 0.5 mg/mL, while the MICs for CA and caffeine were 1 mg/mL. MHB was used as the control instead of compound. Samples were taken at 1, 2, 4, 8, 16, and 24 h to determine viable bacterial numbers. The bactericidal level is indicated by the dashed lines.

These findings suggest that CA may increase membrane potential activity, resulting in increased membrane permeability, which causes intracellular ingredient leakage and cell death.

CA Altered the Morphological Characterization of *V. cholerae*

Finally, SEM was used to compare morphological changes in the appearance of cells with and without 8 mg/mL of CA exposure. **Figure 3** shows the SEM images of bacterial cells at x10,000 and 20,000 magnifications. The untreated control bacteria had a smooth, compact surface with an intact cell membrane and no surface ruptures (**Figures 3A,B**). In contrast, after 2 h of exposure to CA, the cell was found to be severely disrupted (**Figure 3C**), with membrane corrugations due to withering wrinkling and damage, as indicated by the red circled portions in **Figure 3D**. Thus, CA treatment of bacterial cells typically interferes with the integrity of the cell membranes, resulting in morphological changes that allow for intracellular material leakage, cell membrane shrinkage, and ultimately cell death.

DISCUSSION

The efficacy of antibiotics is currently decreasing due to an increase in bacterial antimicrobial resistance. According to a WHO report, antimicrobial resistance is one of the top 10 global public health threats facing humanity due to the misuse and overuse of drugs, including anti-cholera drugs (69). As a consequence, alternative therapeutic approaches are in high demand. Here, we have demonstrated that coffee beans and coffee by-products extract have anti-cholera properties, and CA showed the most effective treatment for *V. cholerae* by involving membrane permeability disruption.

Coffee and its bioactive compounds have been shown to have a variety of pharmacologically beneficial effects on humans. The phytochemical profiles of the extracts tend to vary considerably in terms of CGA and CA content, but not in terms of caffeine. It should be noted that CGA, which has potential anti-MDR *V. cholerae* activity (22–24), was found in the most abundant phenolic compounds in the CP extract, which is consistent with previous findings (70–72). The productivity, chemical composition, and biological activity of coffee extract are all known to be influenced by the brewing process (73). In the current study, three types of roasted coffee beans (light, medium, and dark) were extracted at different temperatures and time durations using ultrasonic-assisted extraction (UAE), an efficient method for retrieving natural antioxidants (30, 48).

Hence, we have demonstrated that roasting circumstances have a considerable impact on the features of the physicochemistry of CGA, but do not significantly affect caffeine thermally, which is consistent with the findings of most of the aforementioned studies (48, 74, 75), while this is the first report of a 2.5-fold decrease in CA in medium and dark coffee. Several studies have previously reported that CGA levels are lost during the roasting process of coffee beans.

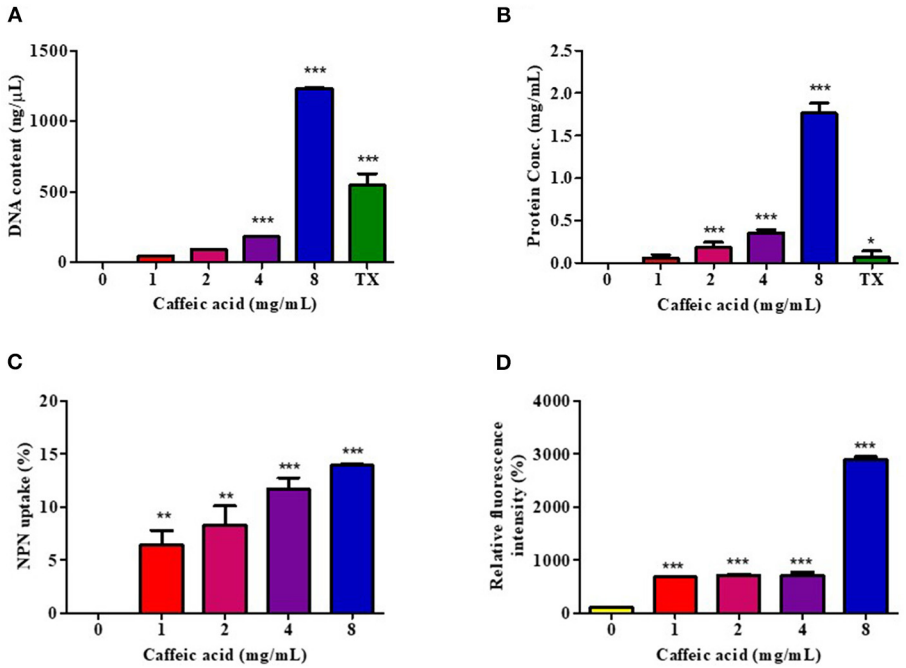

FIGURE 2 | Effect of CA on membrane permeability. *V. cholerae* O1 El Tor N16961 was treated with CA at a concentration of 1–8 mg/mL for 1 h at 37°C. The intracellular leakage of nucleotides **(A)** and proteins **(B)** were measured, and 0.1% Triton X-100 (TX) was used as a positive control. The outer membrane disruption and membrane potential dissipation were investigated by the percentages of NPN uptake **(C)** and Rh123 relative fluorescence intensity **(D)**, respectively. Significant differences compared to untreated controls are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

Using high temperatures during the roasting process has been shown to convert CGA into CGA lactone due to the breaking of carbon-carbon bonds in the CGA structure, resulting in thermal degradation and isomerization (76–78). On the other hand, some studies claim that the level of caffeine increases as the degree of roasting increases, reaching a peak in light and medium-roasted coffee before beginning to decline in dark-roasted coffee. It is anticipated that increasing the temperature can reduce the water content of the coffee beans, thus helping to release volatile compounds (e.g., caffeine) from coffee; indeed, the caffeine levels were reduced significantly compared to the light and medium roast coffee after increasing the temperature to higher limits (dark roast) (79–81).

Our findings are consistent with previous studies, since we demonstrated that green beans (unripe) contain approximately twice as much CGA as red fruits (ripe). The variation in CA content, on the other hand, could be due to a difference in extract solvent: 95% ethanol yielded 38.73 and 26.70 mg/g of green and red fruits, respectively (82). Interestingly, with old coffee leaf, RL provided 6-fold more CGA than AL and 2.3-fold more CA. According to previous studies, Robusta has a higher total phenolic content than Arabica, and old leaf has a higher total phenolic content than young leaf (83–85). Nonetheless, the age of the coffee leaves and the method of processing have an impact on their phytochemical profiles and bioactivity (86).

Although several studies have reported anti-cholera activity with natural product extracts, to the best of our knowledge, this is the first report on the antibacterial activity of coffee beans and coffee-by products extract against *V. cholerae*, particularly with regard to the MDR strain. In this study, we have shown that DGC and DRC fruits, as well as their CP, are the most effective against *V. cholerae*. Furthermore, CP, the first by-product of coffee processing, has been shown to be very effective in the treatment of MDR strains in combination with tetracycline. The effects on antimicrobial activity varied depending on the sample and the clinical strain of *V. cholerae*. However, there is a scarcity of data on the antibacterial activity of coffee beans and coffee by-products extract against *V. cholerae*. Green coffee beans, in particular, had greater antimicrobial activity than roasted coffee. According to many studies, the differences in antibacterial activity between extracts are primarily due to the phenotype and genotype diversity of coffee plants, brewing conditions, roasting temperature, quality of field processing, laboratory extraction processes, and solvents utilized (45, 46, 87). The usefulness of determining the major active components against this bacterium led to a time-killing kinetics study, which revealed that CA had bactericidal activity against *V. cholerae* within 1 h of exposure. This is unexpected, because a previous study found that CGA from *Piper betel* plants had antimicrobial activity against MDR *V. cholerae* at a concentration of the MIC value of 5.5 ± 0.5 mg/mL (22).

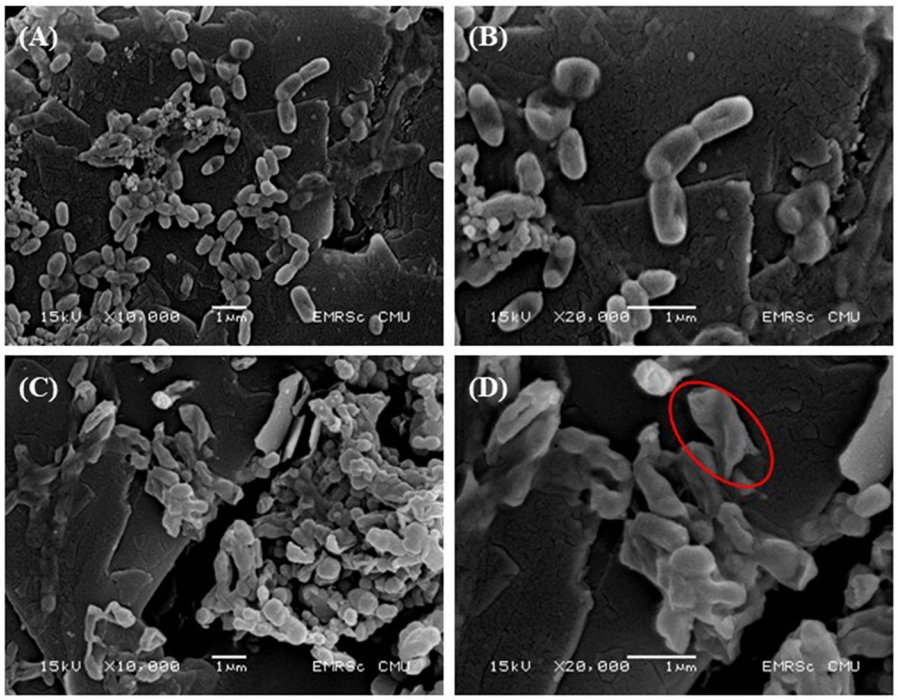

FIGURE 3 | Effect of CA on bacterial cell morphology. *V. cholerae* O1 El Tor N16961 was treated with CA at a concentration of 8 mg/mL for 2 h at 37°C. SEM images at x10,000 and x20,000 magnifications were demonstrated. **(A,B)** Negative controls and **(C,D)** effective treatments. The cell membrane disruption is represented by the red circles.

CA or 3,4-dihydroxy cinnamic acid, is a phenolic compound found in many plant products, including fruits, wine, coffee, olive oil, and legumes (88). It has been widely used as an alternative strategy to combat microbial pathogenesis and chronic infection caused by microbes such as bacteria, fungi, and viruses, via changing the membrane permeability, inhibition of enzyme activity, damage to the DNA and protein structure, and so on (44). However, the mechanism of antibacterial action of CA in *V. cholerae* has not yet been reported. Many virulent factors are involved in *V. cholerae* infection, including cholera toxin (hemolysins), toxin coregulated plus (TCP), adhesin factor (ACF), hemagglutination-protease (hap, mucinase), neuraminidase, siderophores and outer membrane proteins, and lipopolysaccharides (89). Therefore, the modes of action and target sites of CA might vary considerably. During bacterial infection, the outer membrane prevents the entry of noxious compounds into the cell, helping them recognize the host and facilitate colonization. This prompted us to speculate that CA may influence bacterial membrane permeabilization. As expected, CA disrupted the integrity of *V. cholerae* cell membranes by causing the intracellular material leakage of both proteins and nucleotides, resulting in cell membrane shrinkage and morphological changes that allow for cell death. Similar to a previous study, CA had an effect on the membrane by changing cell permeability, leaking intracellular components, causing membrane damage, and decreasing efflux activity, which has been found in both gram-negative and gram-positive bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (44, 90–92). It is worth noting that the bacterial cells were exposed to a higher concentration of CA (up to 8 mg/mL) than the extract. Because the extract contains CA in concentrations ranging from 0.08 to 2.66 mg/g of extract, the combination effect may be more potent than CA alone. Furthermore, an agar well-diffusion assay used to screen the effect of crude extract revealed that the AL treatment had the largest diameters of inhibition zones, despite the fact that it contained less CA (0.8 mg/g extract). It's possible that this is due to the synergistic effect of CA and other bioactive compounds in the extract. However, this critical point we need to confirm to future study. CA is an excellent synergy compound (93). In drug resistant *Listeria monocytogenes*, 1.5 mg/mL of CA in combination treatment with 50 mg/L of fosfomycin enhanced the antimicrobial activity from 5% of fosfomycin alone to 82% of the fosfomycin and CA combination, which might be by acting as the *FosX* gene inhibitor (94). Besides, CA treatment at 0.5 mg/mL in combination with UV-A LEDs effectively inhibited the survival of foodborne bacteria such as *Escherichia coli* O157: H7, *Salmonella enterica* serovar Typhimurium, and *L. monocytogenes* by inducing cell membrane damage (95).

The cell membrane is an active structure that regulates internal conditions for metabolism and energy transfer. It serves as a primary barrier between the cytoplasm and the extracellular medium. Once this barrier is breached, the bacterial cells cease to function (27). SEM, a powerful tool for investigating the

effects of CA on bacterial cells, revealed its inhibitory effects, as confirmed by the severe morphological changes in the tested *V. cholerae*. Similar morphological alterations have also been observed in *V. cholerae* cells treated with the polyphenolic fraction of Kombucha or zinc oxide nanoparticles (96–98). One bacteriostatic mechanism of phenolic compounds is to cause irreversible changes in the cell membrane by altering hydrophobicity and causing local rupture or pore formation in the cell membrane, resulting in an increase in the permeability of the cell membrane, giving rise to the leakage of cellular contents, disrupting the proton-motive force and electron influx, and ultimately destroying cell integrity (99).

Our research has some limitations, because the number of clinical *V. cholerae* strains was small. Furthermore, since CA has synergistic effects with various pharmaceutical entities (93), antimicrobial activity in combination with CGA or caffeine could be tested. This work, however, was intended as a pilot screening, to assess the antibacterial potential of extract against *V. cholerae* clinical drug-resistant strains. For future studies, we need to investigate other modes of action, efficacy, and safety of coffee extracts in animal models and finally in clinical trials.

CONCLUSION

The findings of the present study highlight the promising role of the extracts of coffee beans and coffee by-products, especially in combination treatment with tetracycline, as novel anti-cholera compounds, which can be promoted as an alternative therapeutic agent to treat drug-resistant *V. cholerae* infections.

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.

AUTHOR CONTRIBUTIONS

The experiments were conceived and designed by AR, AS, AY, AK, GP-I, OS, SS, and AD. AR, AS, and AD contributed to the experimental design and data analysis. The first draft of the manuscript was written by AR. AR, AS, AY, AK, GP-I, OS, SS, and AD edited the manuscript draft. The published version of the manuscript has been read and approved by all authors.

FUNDING

This research was funded by the Unit of Excellence in Research and Product Development of Coffee (Grant No. FF64-UoE64002), and Unit of Excellence on Clinical Outcomes Research and IntegratioN (UNICORN) (Grant No. FF65-UoE005), University of Phayao, Thailand.

ACKNOWLEDGMENTS

We are grateful to Dr. Supaporn Lamlertthon of Naresuan University for their stimulating discussions. We also thank Ms. Chatchaya Sumana from the University of Phayao for her technical assistance. We thank Emma Taylor, Ph.D., from the PRS Group (www.proof-reading-service.com/en/) for editing a draft of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.865684/full#supplementary-material

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frontiers | Frontiers in Nutrition

MINI REVIEW
published: 03 May 2022
doi: 10.3389/fnut.2022.887019

The Use of Probiotic Therapy in Metabolic and Neurological Diseases

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The human gut is home to trillions of microbes that interact with host cells to influence and contribute to body functions. The number of scientific studies focusing on the gut microbiome has exponentially increased in recent years. Studies investigating factors that may potentially affect the gut microbiome and may be used for therapeutic purposes in diseases where dysbioses in the gut microbiome have been shown are of particular interest. This review compiles current evidence available in the scientific literature on the use of probiotics to treat metabolic diseases and autism spectrum disorders (ASDs) to analyze the efficacy of probiotics in these diseases. To do this, we must first define the healthy gut microbiome before looking at the interplay between the gut microbiome and diseases, and how probiotics affect this interaction. In metabolic diseases, such as obesity and diabetes, probiotic supplementation positively impacts pathological parameters. Conversely, the gut–brain axis significantly impacts neurodevelopmental disorders such as ASDs. However, manipulating the gut microbiome and disease symptoms using probiotics has less pronounced effects on neurodevelopmental diseases. This may be due to a more complex interplay between genetics and the environment in these diseases. In conclusion, the use of microbe-based probiotic therapy may potentially have beneficial effects in ameliorating the pathology of various diseases. Validation of available data for the development of personalized treatment regimens for affected patients is still required.

Keywords: therapy, microbes, obesity, probiotics, diabetes, neurodegenerative diseases

OPEN ACCESS

Edited by:
Surasak Saokaew, University of Phayao, Thailand

Reviewed by:
Anchalee Rawangkan, University of Phayao, Thailand
Eric Banan-Mwine Daliri, Kangwon National University, South Korea

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *01 March 2022*
Accepted: *29 March 2022*
Published: *03 May 2022*

Citation:
Lee SHF, Ahmad SR, Lim YC and Zulkipli IN (2022) The Use of Probiotic Therapy in Metabolic and Neurological Diseases. Front. Nutr. 9:887019. doi: 10.3389/fnut.2022.887019

INTRODUCTION

The gut is a natural habitat for trillions of diverse microbes (anaerobic bacteria, yeasts, viruses, and bacteriophages) where the phyla Firmicutes, Bacteroidetes, and Actinobacteria are the most common (1). The gut microbiome is a complex ecosystem where microbes and their metabolites interact with host cells to influence body functions. General health is associated with a "healthy" microbiome, defined by the diversity and types of species of bacteria within the gut.

Fecal microbiome analysis has shown that the gut microbiota composition is influenced by various factors such as age, genetics, types of food consumed, economic development, and immediate environment (2–7). Dysbiosis of the microbiome is associated with a reduction in the diversity of microbes within the gut. The altered diversity of gut microbes is correlated with various diseases such as metabolic diseases, autism spectrum disorders (ASDs), and other brain disorders (8–16). Changes in the microbiome have been also linked with infection risk and susceptibility (17), including COVID-19 (18).

Recently, products containing supposedly "healthy" bacteria are touted as being beneficial to health by restoring balance to the microbiome within the gut. These products are generally termed "probiotics," but have also generated other related products, all of which are proposed to act to enhance healthy bacteria within the gut. The term "probiotics" was coined in the 1970s while food containing beneficial bacteria have been consumed even earlier. Recently added interest in the commercialization of probiotic foods meant that there has been a need to define what can be claimed as a probiotic.

The generally accepted definition of probiotic was generated together by the Food and Agriculture Organization of the United Nations (FAO) and WHO—"live microorganisms which when administered in adequate amounts confer a health benefit on the host" (19). Other related products include "prebiotics," "synbiotics," "postbiotics," and "metabiotics" (20–23). The term "live and active cultures" is sometimes used for fermented or functional foods with live microorganisms within them but those microorganisms may not prove to be probiotic yet (19).

With the plethora of probiotics and associated products now available commercially, it is no wonder that there have been many misconceptions regarding probiotics, their usage, and their health benefits, which we will address in the subsequent sections of this review.

Constituents of Probiotics

Specific health benefits have been ascribed to particular probiotic strains, and therefore, not all probiotic supplements are equal, even if they list the same species of probiotic bacteria. Therefore, it is essential to ensure that the correct strain is used to treat the underlying clinical issue. Additionally, supplements that contain multiple strains of bacteria may also lack the scientific evidence for the claimed benefits.

The most common bacterial species used in current probiotic products are lactic acid bacteria such as *Bifidobacterium* and *Lactobacillus* strains (24). However, recent studies have identified other species of bacteria that may also confer benefits when used as probiotics, such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* (25), and the use of these bacteria in probiotic products is rising as well (26). The effectiveness of probiotic supplementation can be measured through the bacterial load in the feces, or other measures within the body (27, 28), and is essential to establish the efficacy of any treatment.

Evidence of Therapeutic Effects of Probiotics

The benefits of probiotic supplementation result from either inhibition of pathogen growth in the large intestine or augmented immune response and intestinal barrier function in both small and large intestines (29). As most probiotics are beneficial bacteria found naturally within the gut, ingested probiotics within the gut interact with immune cells to sustain an immunologic balance within the gastrointestinal tract (30, 31). Therefore, the interplay between the gut microbiome, probiotics, and human health is *via* the modulation of immune responses at the epithelial cells constituting the mucosal interface between host and microorganisms.

The gut microbiome also produces a wide range of metabolites due to the anaerobic fermentation of undigested materials and endogenous compounds found within the microbes and host. The metabolites produced by the microbiome serve as agents that modulate the host cells' responses, thus its immune system and disease probability. Rooks and Garrett have reviewed how these metabolites modulate the immune responses and disease risk (32). We have summarized probiotic strains, their resulting metabolites, and their effects on health in **Table 1**.

Regular consumption of probiotic supplements and foods has ascribed numerous scientifically backed benefits, including effects on the gut such as amelioration of diarrhea and other digestive symptoms (33–38), reduction of inflammation (33, 39), as well as benefits to various conditions ranging from emotional imbalance to autoimmune diseases (40–45). Some groups have even shown the benefits of consuming probiotics for patients with cancer (28, 46, 47). However, it must be noted that while many clinical benefits have been rigorously tested, in many cases, probiotics cannot be considered an alternative to medicine, particularly in severe diseases.

HEALTHY GUT MICROBIOME PROFILES AND CHANGES IN DISEASE

Knowledge of a healthy gut microbiome is necessary before addressing the diseases triggered by the dysregulation of the gut microbiome. Hou et al. (51) established three enterotypes comprising specific species and functional composition: Bacteroides, Blautia, and Prevotella enterotypes. These different gut microbiome diversity signatures have different risks for different diseases (48–50). Additionally, the efficacy of probiotic supplementation is also affected by enterotype (51). Therefore, these enterotypes may form a basal gut microbiome that is independent of geographical location as well as nutrition.

Gut community profiles have also shown that healthy pre-adolescents have more significant numbers of species and greater diversity than adults, with increased *Firmicutes* and *Actinobacteria* (52). Both *Bacteroidetes* and *Firmicutes* bacteria are SCFAs producers, specifically acetic acid and propionic acid by *Bacteroidetes* and butyric acid by *Firmicutes* (53). Functionally, the diversity of microbial genes detected in the gut microbiome in children was responsible for the ensuing growth and development, such as vitamin synthesis. In contrast, the enriched microbial genes detected in the gut microbiome of adults are associated with inflammation and fat deposition (52). Findings from a further study to understand the gut microbiome of pre-adolescents in different geographical areas and conditions showed that the distal guts of children living in the Bangladeshi slum have significantly higher bacterial gut microbiome diversity with enrichment in *Prevotella*, *Butyrivibrio*, and *Oscillospira* together with a depletion in *Bacteroides* (54). However, this microbial diversity was more prone to changes, unlike the microbiota found in children living in the suburban community.

TABLE 1 | List of probiotic strains and the metabolites produced and their effects.

Probiotic (bacterial) strain(s)	Metabolites produced	Effects	References
Bacteroides thetaiotaomicron	Acetate	Increase mucus production	(65)
Bacteroides thetaiotaomicron and *Faecalibacterium prausnitzii*	Acetate and Butyrate	Ensure maintenance of appropriate secretory cells proportion	(65)
Bifidobacterium longum	Acetate	Fortifies intestinal epithelial cells integrity and prevent toxins entry into circulation	(66)
Bifidobacterium dentium	Acetate γ-aminobutyric acid (GABA)	Stimulates MUC2 synthesis, Promotes autophagy and calcium mobilization to release mucus	(67)
Bifidobacterium Lactis sp. *420*	Acetate Lactate	Modulate Cox expression profile, resulting in anti-inflammatory and anticarcinogenic properties	(68)
Lactobacillus rhamnosus GG and *Saccharomyces cerevisiae boulardii*	Butyrate Propionate Ethanol	Protects against pathogenic Escherichia coli	(69)
Lactobacillus casei	Butyrate Acetate	Increase secretion of Glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) secretion	(70)
Lactobacillus. johnsonii L531	Butyrate Acetate Lactate	Reduces pathogen load	(71)
Lactobacillus gasseri	Butyrate	Exerts anti-obesity effects	(72)
Saccharomyces boulardii	Acetate	Antibiotic potency	(73)

A reference profile comprising the abundance and list of microbes in a healthy human was constructed, with 157 organisms classified as healthy gut microbes in the Fecal Biome Population Report (55). Additionally, Kong et al. (56) studied the gut microbiome of healthy centenarians as a benchmark for a healthy microbiome model. They found that short-chain fatty acids (SCFAs)-producing bacteria were more abundant in the long-living Chinese cohort. SCFAs such as butyrate, propionate, and acetate, produced by the gut microbiome, are beneficial for health. SCFAs act by stimulating the expansion of regulatory T cells, inhibiting inflammation *via* reducing histone deacetylase-9 gene expression (57). Thus, SCFAs maintain the gut barrier's integrity, stimulate immunity in the intestines, and prevent pathogen infection (32, 58). Hence, metabolites produced by the gut microbiome can also modulate a person's health status (59).

Interestingly, the follow-up study revealed that the long-living healthy people in the study (both Chinese and Italian cohorts) had more diverse microbiota structures than younger age groups (60). This result contrasts with previous studies whose results have suggested that gut microbiome diversity in a person tends to decrease as the person ages (61, 62). This suggests that the changes in your gut microbiome are not set in stone and can be modulated with environmental factors and diet.

The potential of the dysbiosis of the gut microbiome in the establishment of metabolic diseases should be obvious. However, the gut microbiome is also able to communicate with the nervous system *via* the gut-brain axis (GBA) and thus affects neurological diseases as well. The GBA involves bidirectional interaction between the central and the enteric nervous systems, connecting the cognitive and emotional centers of the brain with peripheral intestinal functions. Bacteria in the gastrointestinal (GI) tract influence the signaling of neural pathways and the central nervous system (CNS) (63–67). Evidence of microbiota-GBA communications emerged from the association of dysbiosis with central nervous disorders (63, 68, 69). From this, we note that healthy gut microbiota is essential for brain development and function.

Consequently, a healthy gut microbiome is essential for both metabolic and neurological health. In the following sub-sections, we will be addressing the use of probiotics in metabolic diseases (obesity and type II diabetes) and neurodegenerative diseases.

Gut Dysbiosis and Probiotics and Obesity

Obesity is defined by excessive fat accumulation in the body, which may increase the risk of non-communicable diseases such as diabetes, cardiovascular diseases, some cancers, and hypertension (70). The gut microbiome and the composition of dietary intake are profoundly linked (71). For example, the intake of animal-based foods provided up to 5 consecutive days of increased bile-tolerant microbes (*Alistipes*, *Bilophila*, and *Bacteroides*) and reduced the amount of fiber-fermenting bacteria (72).

Gut microbiota profiles in overweight and obese individuals show higher amounts of *Bacteroides, Bifidobacteria, Staphylococcus aureus,* and *Lactobacilli Clostridia* (73, 74). Among overweight individuals, the baseline ratio of gut microflora, *Firmicutes* to *Bacteroidetes* was disturbed (75). *Firmicutes* bacteria potentially are able to affect the modulation of gene expression and hormones involved in metabolism (76). Therefore, the change in the ratio of different bacteria species may affect human metabolism, leading to obesity.

Probiotics may act as anti-obesity agents by various modes of action, including modulation of specific gut microbiota strains, gastrointestinal and immune system modulation, lowering insulin resistance, and greater satiety. The use of probiotics containing *Lactobacillus* and *Bifidobacterium* species in obesity treatment is promising (77). Some of the positive changes which resulted from the intake of probiotics include lower

waist circumference, lower body fat deposition, lower body weight, lower weight gain, and improved lipid profile. However, Vajro et al. showed that *L. salivalis* supplementation in obese adolescents led to no improvement in obesity parameters (78). Another study with the consumption of one capsule of *L. rhamnosus* G showed a lower weight gain at 1 year of life and up to 4 years old in children but observed no weight changes after that period (79). This difference in weight gain patterns may be due to the colonization of the gut microflora, which begins during the first few years of life (80, 81). Unless various scientific groups consistently match the age of controls and subjects, together with consistent bacterial strains utilized in probiotics, the conclusion derived from the comparison of these studies remains murky.

A. muciniphila is negatively correlated to obesity development, as well as other diseases such as type-2 diabetes and hypertension (82). A human clinical trial looking at the impact of *A. muciniphila* supplementation for over 3 months showed that the treatment led to improved insulin sensitivity, insulinemic, and reduction of total cholesterol (83). The evidence of *A. muciniphila* as a probiotic that confers a protective effect against metabolic disorders has been accumulating over the past few years (84) and may merit further study.

Hence, probiotics positively impact the reduction of relevant obesity parameters, although the effect varies across the different age groups and genders. More standardized studies are needed to investigate how the different mixtures of bacterial species in probiotics affect different age groups and genders.

Gut Dysbiosis, Probiotics, and Diabetes

Type-2 diabetes is a metabolic disorder in which individuals display abnormally high blood glucose, resulting from inadequate insulin secretion and resistance (85). Type 2 diabetes results from the interaction between environmental factors and genetic factors (86). One of the primary risk factors of type-2 diabetes is being overweight or obese (87).

A change in the composition of the gut microbiota may result in increased susceptibility to prediabetic conditions such as insulin resistance (87–89). Reports have revealed that the intestinal microbiome of individuals with type-2 diabetes has reduced butyrate-producing bacteria (87, 90), a lower frequency of Firmicutes, and a higher frequency of Bacteroidetes and Proteobacteria (88). The metabolites produced by gut microbes also affect insulin sensitivity and glucose homeostasis, with metabolites like SCFA improving insulin secretion (91). Therefore, butyrate-producing bacteria affect insulin secretion and therefore, the blood sugar level of a person. Further exploration of the bacterial strain or administration of butyrate may be beneficial to a diabetic.

Probiotic intake, such as *Lactobacillus rhamnosus* GG, leads to improvement in intestinal integrity, reduced lipopolysaccharide level, reduced endoplasmic reticulum stress, and improved insulin sensitivity (91–93). Animal and clinical trials have shown that both single probiotic strains or mixtures of probiotics have the potential to improve type-2 diabetes parameters (87, 94). More research is required to dissect the most suitable species impacting gut metabolism, as well as exposure time, and dose.

Gut Dysbiosis, Probiotics, and Autism Spectrum Disorder

Autism spectrum disorder is a group of neurodevelopmental disorders defined by deficits in communication and social interaction, and stereotyped behaviors (65). GI abnormalities are common among individuals with ASD (95, 96), with a strong correlation of GI symptoms with ASD severity (97).

The gut microbiota of children with ASD is less diverse, with decreased levels of health-promoting gut bacteria, and an increased abundance of species that produce neurotoxins (65). Metabolites from the gut microbiota may play vital roles in the pathogenesis of ASD (95, 96). Altered fecal SCFAs have been linked to constipation in ASD (97), where lower levels of acetic acid and butyrate and an elevated level of valeric acid have been reported in subjects with ASD (96). It has also been shown that SCFAs can induce autistic-like symptoms upon injection into rats (98).

Maternal immune activation (MIA) mouse models that display features of ASD have altered microbiota and GI barrier defects. Oral treatment of MIA offspring with the human commensal bacteria *Bacteroides fragilis* improves gut permeability, alters the microbial composition, and corrects behavioral defects in MIA animals. Therefore, it has been proposed that targeting the gut microbiota may be a potential therapy for specific symptoms in ASDs (95).

Probiotics potentially impact gut microbiota communities to alter the levels of harmful metabolites in ASD children, reducing GI inflammation and intestinal permeability (1, 99). However, the results of probiotic supplementation in individuals with ASD remain inconclusive and controversial. Current probiotics are mainly aerobic, short-lived, milk-derived cultures, which are not usually a significant part of the primarily anaerobic human gut microbiome (1). A review based on four studies concluded that current evidence does not support the use of probiotics to modify behavior in patients with ASD (100). Probiotics did not exert a significant effect to restore most of the beneficial bacteria upon assessment of stool samples from 58 individuals with ASD and 39 age-matched typically developing children (97). On the other hand, it has also been reported that probiotics treatment seems to improve ASD-associated behavioral symptoms (101).

Autism spectrum disorder individuals are highly selective eaters (102, 103); therefore dietary factors remain a strong confounding factor for these individuals. The complex interplay between host genetics, environment, and the microbiome although challenging to dissect are important factors to consider. Larger longitudinal trials as well as optimizing dosage, formulation (single vs. multispecies probiotics), timing (101), route of administration as well as toxicity concerns remain to be addressed to validate the efficacy of probiotics for ASD, taking into consideration age and population-specific differences in gut microbiota/metabolites produced (6, 7).

Gut Dysbiosis and Probiotics in Neurodegenerative Diseases

It is well-established that age is a primary risk factor for neurodegenerative diseases due to increased insults including

decreased neurotransmitter levels, chronic inflammation, oxidative stress, and apoptosis (104). There is also a high prevalence of GI comorbidities among patients with Parkinson's and Alzheimer's diseases (105, 106). Dysbiosis in the intestinal microbiota in the elderly may result in a leaky gut, and subsequently, promote systemic and neuroinflammation (107).

Gut microbiota secretes neurometabolites, which include neurotransmitters that regulate the signaling cascades of the CNS. A comprehensive review of neurotransmitters directly secreted by various probiotics has been published (105). Altered levels of neurotransmitters result in behavioral changes in neurodegenerative diseases. Restoring the balance of neurotransmitters by targeting gut microbiota is therefore central to the management of neurodegenerative disease.

Parkinson's disease (PD) is characterized by loss of dopaminergic neurons and intraneuronal alpha-synuclein accumulation, in the basal ganglia and at peripheral sites, including the gut (108). GI dysfunction has been reported to be a potential contributor to the pathogenesis of PD with evidence that alpha-synuclein inclusions appear early in the enteric nervous system and travel to the brain *via* the vagal nerves (109, 110). A review on altered gut microbiota compositions in patients with PD is available (111). Probiotics administration in independent studies improves GI symptoms and the metabolic profile of patients with PD (108, 111, 112).

Alzheimer's disease (AD) is one of the most common irreversible, neurodegenerative disorders in the elderly, which leads to cognitive decline and dementia. Inflammatory response at the site of beta-amyloid (one of the hallmark features of AD) accumulation in the brain has been linked to the gut microbiota (66). Current studies on the efficacy of probiotics in AD, although limited, seems promising. In a transgenic mice model of Alzheimer's Disease (AD), modulation of the gut microbiota through exercise and probiotic treatment alleviated the progress of AD (113). Rats injected with probiotics (*L. acidophilus, L. fermentum, B. lactis, B. longum*) for 8 weeks elicit an improvement in memory deficit and AD-associated pathology (114). However, it remains to be determined whether these findings are replicable in humans. Another randomized, double-blind, and controlled clinical trial among 60 patients with AD revealed that a 12-week probiotic (*L. acidophilus, L. casei, B. bifidum*, and *L. fermentum*) consumption improved cognitive function and certain metabolic markers (115). There is also an ongoing clinical trial (randomized, placebo-controlled) to investigate the effect of probiotics on 58 participants with dementia (116). Therefore, the efficacy of probiotics to restore gut dysbiosis in patients with AD awaits further validation.

A key limitation of current probiotic studies for PD and AD is the small sample sizes ($n < 100$). Consistent study designs in larger human trials with validated safety and efficacy are required before translation into clinical settings.

FUTURE DIRECTIONS

Manipulation of the gut microbiota and microbial metabolites to address challenging questions in metabolic and brain disorders is difficult due to the complex relationship between host genetics and environmental factors to influence the gut microbiota. A healthy diet and exercise positively modify the gut microbiota (117–119), therefore it remains inevitable to tackle these key modifiable factors to ensure a healthy community of microbes.

Utilizing data from the NIH Human Microbiome Project (HMP) for resources and insights on the human microbiome provides an opportunity to further understand the complex relationship between human health and diseases, which will serve as a pedestal for novel approaches toward the development of therapeutics to tackle relevant diseases. Large scale, harmonized multi-center studies, and freely accessible data are imperative to validate the role of probiotics as potential therapeutics before translating research into clinical practice.

The long-term effects of probiotics and their corresponding metabolites/substances on health are needed to fully understand the mechanisms of each probiotic strain on health (120). Delineation of the precise role and effect of each probiotic strain may just be the beginning of introducing precise probiotic strain for an exact clinical disease. This delineation may be followed by combined efforts of various strains of probiotics. In short, the journey into the gut microbiome is just the tip of the iceberg at the moment.

AUTHOR CONTRIBUTIONS

IZ provided the concept of the manuscript and finalized the manuscript. All authors wrote, provided revisions to the manuscript, read and approved the final manuscript, reviewed the manuscript, and consented for it to be sent for publication.

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frontiers | Frontiers in Nutrition

REVIEW
published: 18 May 2022
doi: 10.3389/fnut.2022.867914

Experimental and Clinical Studies on the Effects of Natural Products on Noxious Agents-Induced Lung Disorders, a Review

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OPEN ACCESS

Edited by:
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Reviewed by:
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Sontaya Sookying, University of Phayao, Thailand

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *01 February 2022*
Accepted: *16 March 2022*
Published: *18 May 2022*

Citation:
Saadat S, Beigoli S, Khazdair MR, Amin F and Boskabady MH (2022) Experimental and Clinical Studies on the Effects of Natural Products on Noxious Agents-Induced Lung Disorders, a Review. Front. Nutr. 9:867914. doi: 10.3389/fnut.2022.867914

The harmful effects of various noxious agents (NA) are well-known and there are reports regarding the induction of various lung disorders due to exposure to these agents both in animal and human studies. In addition, various studies have shown the effects of natural products (NP) on NA-induced lung disorders. The effects of various NP, including medicinal plants and their derivatives, on lung injury induced by NA, were reviewed in this study. The improving effects of various NP including medicinal plants, such as *Aloe vera, Anemarrhena asphodeloides, Avena sativa, Crocus sativus, Curcuma longa, Dioscorea batatas, Glycyrrhiza glabra, Gentiana veitchiorum, Gentiopicroside, Houttuynia cordata, Hibiscus sabdariffa, Hochu-ekki-to, Hippophae rhamnoides, Juglans regia, Melanocarpa fruit juice, Mikania glomerata, Mikania laevigata, Moringa oleifera, Myrtus communis L., Lamiaceae, Myrtle, Mosla scabra leaves, Nectandra leucantha, Nigella sativa, Origanum vulgare L, Pulicaria petiolaris, Paulownia tomentosa, Pomegranate seed oil, Raphanus sativus L. var niger, Rosa canina, Schizonepeta tenuifolia, Thymus vulgaris, Taraxacum mongolicum, Tribulus Terrestris, Telfairia occidentalis, Taraxacum officinale, TADIOS, Xuebijing, Viola yedoensis, Zataria multiflora, Zingiber officinale, Yin-Chiao-San,* and their derivatives, on lung injury induced by NA were shown by their effects on lung inflammatory cells and mediators, oxidative stress markers, immune responses, and pathological changes in the experimental studies. Some clinical studies also showed the therapeutic effects of NP on respiratory symptoms, pulmonary function tests (PFT), and inflammatory markers. Therefore, the results of this study showed the possible therapeutic effects of various NP on NA-induced lung disorders by the amelioration of various features of lung injury. However, further clinical studies are needed to support the therapeutic effects of NP on NA-induced lung disorders for clinical practice purposes.

Keywords: natural product, medicinal plants, bleomycin, cadmium, dust, lipopolysaccharide, sulfur mustard, lung injury

INTRODUCTION

The respiratory function of the lung is critical and important for survival because oxygen is a vital molecule for the production of energy that is essential for the life of organisms (1). Studies have shown the effects of exposure to air pollutants on respiratory disorders such as COPD, asthma, and lung cancer (2). Specific alterations of passive and active non-respiratory functions generate functional or anatomical disorders that compromise breathing later. The basic scientific and clinical research of various diseases generated by alterations of these functions can produce knowledge on the pathophysiology, biochemistry, genetics and immunology (3).

Respiratory disorders such as COPD and asthma are related to immune and inflammatory reactions and the status of oxidants which were remarkably enhanced in respiratory disorders (4). Allergic disorders have increased in recent decades due to increased allergens and air pollutants in the environment and workplace (5). In allergic disorders such as skin and respiratory allergies, the reaction of the immune system to exposure and re-exposure to allergens releases allergy-related mediators (6).

Bleomycin (BLM) is a type of antibiotic used for cancer chemotherapy. This drug reduces or stops the growth of cancer cells in the body. It inhibits DNA metabolism and is used as an antineoplastic (anticancer) agent, especially for solid tumors. At high concentrations of the drug, protein production and cellular RNA are also inhibited. It has the least toxic effect on blood-forming tissues and the immune system. Unfortunately, due to the complication of pulmonary fibrosis (PF), the use of this drug is clinically limited (7).

Cadmium (Cd) is a naturally occurring toxic element. Several studies have shown that exposure to Cd from cigarette smoking and occupational resources causes lung disorders. There are reports regarding the involvement of pro-inflammatory chemokines and cytokines, such as interleukins, growth factors, and nuclear factor kappa B (NF-κB), a transcription factor that regulates the expression of genes of cytokines which play an important role in pulmonary fibrosis due to Cd exposure (8, 9).

Studies have shown that dust particles may penetrate deep into the lungs, throat, and airways and cause respiratory disorders. The entry of dust into the lung parenchymal macrophage cells leads to the chemical secretion of chemotaxis and inflammatory mediators, leukotrienes and thromboxane, causing the invasion of inflammatory cells from the vessels to the lung damage area. This process, in turn, stimulates the synthesis of the fibroblasts and causes fibrotic pulmonary parenchyma (10). It was also reported that lipopolysaccharide (LPS) causes lung damage (11) through several inflammatory mechanisms (12).

Paraquat (PQ) causes human or animal toxicity and the lungs are the primitive target organ due to being the main exposed organ to this toxin (13). The effects of PQ on the lung result in lung edema, hypoxia, and lung fibrosis (14, 15). Also, the effects of PQ on interleukin 6 (IL 6) and tumor necrosis factor-α (TNF α) in the macrophages have been reported (16, 17). It was also shown that the mechanisms of pulmonary injuries caused by the PQ are mainly related to the inflammatory and oxidative stress processes (18). Chemical agents such as sulfur mustard (SM) might cause acute and chronic injuries in the lung tissue (19) due to enhanced inflammatory oxidant stress mechanisms (20, 21).

Medicinal herbs are applied for the medical treatment of various disorders (22). People use different products from plant resources traditionally for the treatment of respiratory disorders including asthma and bronchitis (22). Several natural ingredients such as polyphenols, flavonoids, and alkaloids derived from medicinal plants showed potent anticancer activity (23).

Natural products (NP) can be considered as the alternative therapeutic potential for respiratory diseases caused by toxic agents since different inflammatory mediators are involved in these disorders and several NP showed anti-inflammatory effects. Most of the studies are pointing out the effects of NP on the inhibition of NF-κB and MAPK pathways, besides the antioxidant effects associated with these products. However, clinical trials using these compounds are scarce in the literature and the safety and efficacy should be confirmed for further studies.

Since no study has been done on the effect of NP on the noxious agents-induced lung disorders so far, this review article is to present available basic and clinical evidence about the efficacy of the mentioned NP and the herbal constituents in the prevention or treatment of lung disorders induced by the noxious agents-induced similar inflammatory and pathological changes in the lung as induced by BLM, Cd, dust, and LPS in experimental and SM in clinical models. Therefore, the effects of NP and their constituents on noxious agents-induced lung changes were also suggested in the present review to support their effect on lung changes induced by noxious agents in clinical studies.

METHOD

In this review, the keywords including "chemical agent" and "medicinal plants" or "natural products" and "lung injury" or "respiratory system" were searched on different databases such as Web of Science, PubMed, and Scopus from 1989 to the end of September 2021.

In total, 224 articles were retrieved including 115 duplicates articles. Therefore, 109 articles (14 reviews, 4 book chapters, and 91 original articles) related to the described topic were included in this review article (**Figure 1**).

RESULTS

Bleomycin-Induced Lung Disorders

Experimental Studies

The prophylactic effect of common walnut *(Juglans regia)* (150 mg/kg) on rats exposed to BLM was shown by increased glutathione reductase (GR) and catalase (CAT) levels and decreased lung inflammation and apoptosis through regulation of NF-κB activity. The treatment with *Juglans regia* also causes modulated lung injury through markers of cellular injury including lactate dehydrogenase (LDH), alkaline phosphatase, and reduced glutathione (GSH) (24).

Gentiana veitchiorum treatment decreased collagen VI and improved the lung injury induced by BLM. The treatment with *Gentiana veitchiorum* also decreased the malondialdehyde

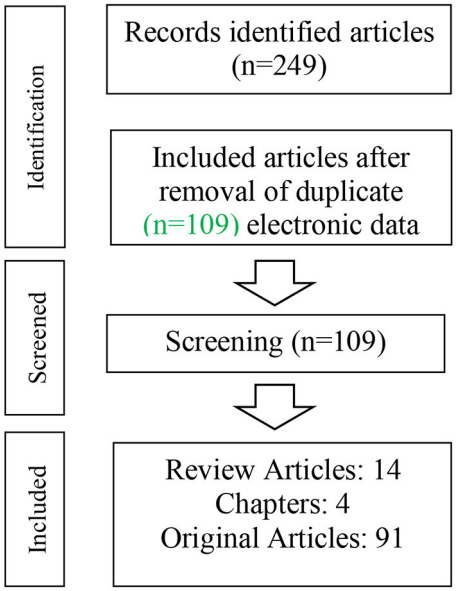

FIGURE 1 | Flowchart of the process of selecting studies for review.

(MDA) level and increased the superoxide dismutase (SOD) and GSH activities, which correlated with oxidation resistance and scavenging of free radicals. Finally, *Gentiana veitchiorum* deceased the inflammatory lung damages through the alleviation of tumor necrosis factor-alpha (TNF-α) expression (25).

In the BLM-induced lung inflammation and pulmonary fibrosis (PF) mouse model, treatment with *Juglanin* (80 mg/kg), that was mainly divided from the green walnut husks of *Juglans mandshuric*, improved the survival rate in the treated mice. Also, the PF induced by the BLM was markedly attenuated by *Juglanin* with the decreasing of the expression of the transforming growth factor-β1 (TGF-β1), metallo-proteinase-9 (MMP-9), α-smooth muscle actin (α-SMA), and collagen I (26).

The treatment with a Japanese herbal medicine, Hochu-ekki-to (TJ-41), in a mouse model of BLM-induced PF, for 2 months before and 1 month after receiving BLM, prevented PF through the modification of the Th_1/Th_2 imbalance toward the Th_2 balance (27). *Feitai* is a Chinese herb used for the treatment of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS). In the BLM-induced pulmonary fibrosis in rats, *feitai* blocked lung p38 MAPK, NF-κB65, HIF-1α, p-IκB-α, and TGF-β1 expression, and enhanced the Nrf2 and IkB expression (28).

The administration of *Rosmarinus officinalis* L. extract (75 mg/kg) protected against the BLM-induced acute lung injury in the animal model *via* declines in lung edema, septal thickening, alveolar subsidens, hemorrhage, and oxidative stress (29). The findings of the other study showed that apigenin (4, 5, 7-trihydroxyflavone) with doses 10, 15, and 20 mg/kg is a potent anti-inflammatory and antifibrotic agent against the BLM-induced PF (30).

The effects of indirubin, a compound derived from mollusks of the family Muricidae, on the BLM-induced PF were examined by pathological staining, western blot, RT-PCR, and immunofluorescent staining. The treatment with indirubin protected the mice against the BLM-induced PF by alleviated fibroblast differentiation indicating its possible therapeutic effect on PF (31). In a model of PF induced by a single endotracheal injection of BLM, the extract of *Nigella sativa* (500 mg/kg) was effective for early and late prevention of PF and inflammation (32).

The administration of *Raphanus sativus L. var niger* (RSN), from the black radish plant, ameliorated the BLM-induced acute lung injury. The post-treatment of rats with intravenously administered RSN (75, 150, 300 mg/kg) protected the lung against the BLM-induced oxidative stress and reduced the number of neutrophils and lymphocytes as well as the IL-6, TNF-α, IL-1β, and TGF-β1 levels (33). *Houttuynia cordata* (HC) has shown antioxidant activity, free radical scavenging capacity as well as anti-inflammatory and anticancer activities. These results suggested that *Houttuynia cordata* has a protective effect against BLM-induced PF (34).

The *Myrtus communis* L. extract (50 mg/kg) effectively inhibited the inflammation and fibrosis of lung parenchyma in a rat model of BLM-induced pulmonary injuries. This impact might be due to the decrement of tissue inflammation and inhibition of oxidative stress (35). The treatment with resveratrol (10 mg/kg), a phenolic compound, prevented the BLM-induced PF in the rats by the suppression of oxidative stress and endothelin-1 (ET-1) expression. The results demonstrated that resveratrol with its potent free radical scavenging and antioxidant properties seems to be a highly promising agent in protecting lung tissue against oxidative damage and in preventing PF due to BLM treatment (36).

Ganoderic acid A has been shown to mitigate the increment in NF-κB p65, TNF-α, and IL-1β and IL-6 mRNA expression, and improved the expression of the anti-inflammatory cytokine IL-10 following the BLM injection. The treatment with ganoderic acid A (25 and 50 mg/kg, for 3 weeks) significantly improved the MPO activity and lung histopathology in the mice. Also, the protective effect of ganoderic acid A may be related to a decrease in TNF-α, IL-1β, IL-6, and MDA and an increment of SOD (37). The gallic acid (75, 150, and 300 mg/kg, for 3 weeks) in an animal model of BLM-induced PF reduced the inflammation process to some extent and could exert its effects through TGF β1/Smad2-signaling pathway and balancing NOX4/factor erythroid-2-related factor 2 (Nrf2) (38). Yin-Chiao-San (YCS), a Kampo medicine, is widely applied for pulmonary diseases. The treatment of rats with YCS (1,000 mg/kg/day, i.v.) protected the lung against the BLM-induced and reduced the lung index, MDA, HP, and TNF-α as well as significantly enhanced the CAT activity (39).

Several reports have evaluated the effects of NP on the BLB-induced lung disorders in experimental models and it is suggested that the herbs and their active ingredients are a promising source of compounds that can play pivotal roles in the alternative adjuvant chemotherapy in reducing the pulmonary fibrosis of BLM. However, clinical trials in this field are not found and should be performed in the future. The therapeutic effects of

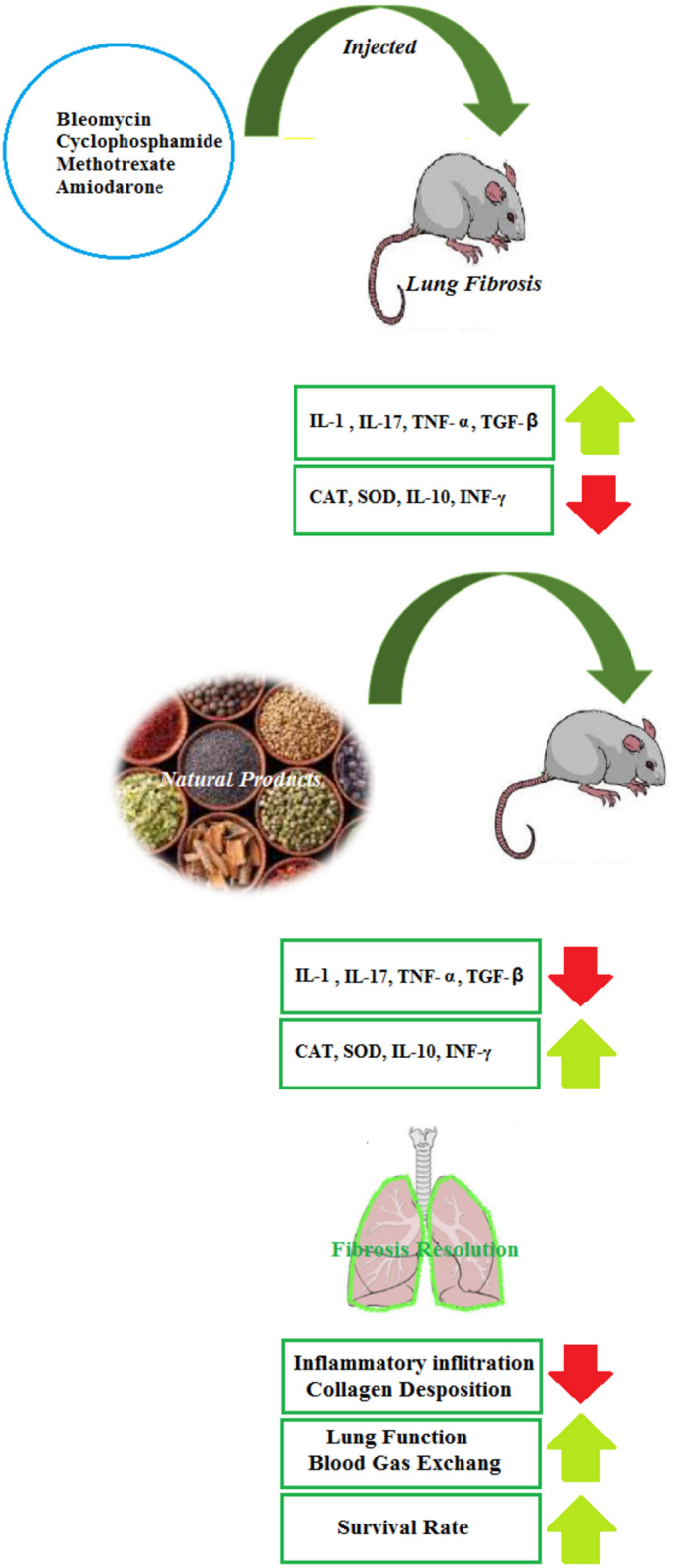

FIGURE 2 | Inhibitory effects of NP on pulmonary fibrosis induced by various noxious agents.

TABLE 1 | The possible therapeutic effects of NP in the BLM-induced lung injury.

Study type	Study design	NP	Dose	Effects	References
In vivo	BLM-exposed rats	*Juglans regia*	150 mg/kg, for 14 days	↑ GR and CAT activities ↓ LDH, ALP, GSH Apoptosis via regulate the NF-κB signaling pathway	(24)
	BLM-exposed rats	*Gentiana veitchiorum*	-	↓ Inflammatory lung injury by decreasing TNF-α expression and MDA ↑ SOD, GSH	(25)
	BLM-exposed mice	Juglanin	80 mg/kg, i.p. for 4 weeks	↓ Expression of TGF-β1, MMP-9, α-SMA, collagen I	(26)
	BLM-exposed mice	TJ-41	1g/kg, orally for 13 weeks	Prevented experimental lung fibrosis through the correction of the Th_1/Th_2 imbalance	(27)
	BLM-exposed rats	*Feitai*	-	↓ Oxidative stress and lung inflammation	(28)
	BLM-exposed rats	*Rosmarinus*	75 mg/kg, i.p. 15 days	↓ Lung edema, septal thickening, alveolar subsidence, hemorrhage and oxidative stress	(29)
	BLM-exposed rats	Apigenin	10, 15 and 20 mg/kg, orally for 14 days	↑ CAT, SOD activities, IL-10 and INF-γ	(30)
	BLM-exposed rats	Indirubin	12.5 mg/kg, or 25 mg/kg, i.p. for 14 days	Alleviated fibroblast differentiation	(31)
	BLM-exposed rats	*Nigella sativa*	500 mg/kg, i.p. for 14 days	Prevented pulmonary fibrosis and inflammation	(32)
	BLM-exposed rats	RSN	150 mg/kg, orally for 7 days	↓ TGF-β1 level	(33)
	BLM-exposed mice	HC	50 and 100 mg/kg, i.g. for 5 weeks	↓ Oxidative damage	(34)
	BLM-exposed mice	Resveratrol	10 mg/kg, orally for 14 days	↓ Oxidative damage Prevented pulmonary fibrosis	(36)
	BLM-exposed rats	*Myrtus communis*	50 mg/kg, i.g for 14 days	↓ Tissue inflammation Inhibition of oxidative stress	(35)
	BLM-exposed rats	Gallic acid	50, 100 and 200 mg/kg, orally for 14 days	↓ Serum levels of IL-4, IL-17A, IFN γ	(40)
	BLM-exposed rats	GAA	25 and 50 mg/kg, i.g. for 21 days	↑ NF-κB, TNF-α, IL-1β and IL-6	(37)
	BLM-exposed rats	Gallic acid	75, 150 300 mg/kg, i.g. for 21 days	↑ CAT, SOD activities, IL-10 and INF-γ	(38)
	BLM-exposed mice	YCS	1,000 mg/kg for 5 days i.p.	Antioxidant and anti-inflammatory activities and also inhibited collagen formation	(39)

NP, natural products; Ext, extract; BLM, bleomycin; TGF-β1, transforming growth factor-β1; MMP-9, metallo-proteinase-9; α-SMA, α-smooth muscle actin; TNF- α, tumor necrosis factor alpha; IFNγ, Interferon gamma; IL-10, Interleukin-10; IL-17, Interleukin-17; SOD, superoxide dismutase; CAT, catalase; RSN, Raphanus sativus L. var niger; HC, Houttuynia cordata; TJ-41, hochu-ekki-to; GR, glutathione reductase; GSH, glutathione; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; GAA, Ganoderic acid A; i.g., intragastrically; YCS, Yin-Chiao-San.

The up arrow (↑) indicates an increase in the variable, and a down arrow (↓) indicates a decrease.

NP in the BLM-induced lung injury are summarized in **Table 1** and **Figure 2**.

Cadmium-Induced Lung Disorders

Experimental Studies

The administration of *Nigella sativa* oil (1 ml/kg, i.p.) ameliorated the Cd-induced lung damage with the reduction of histopathological changes in the lung architecture (41). The treatment effects of *Tribulus Terrestris* against the Cd-induced toxicity in the mice showed that the alcoholic extract of *Tribulus Terrestris* fruit (200 mg/kg, for 10 days) eliminated the free radicals and increased the antioxidant enzymes expression as well as the down-regulation of pro-inflammatory markers in cellular injuries (42). The anti-inflammatory effects of the phenolic compounds from grape seeds were associated with their regulatory effect on the expression of the pro-inflammatory genes, such as cyclooxygenase and lipoxygenase and also by acting on the NF-κB signaling and MAPK. The findings showed that the phenolic compounds of the grape seeds ameliorate the toxic impacts of Cd in the lung tissue *via* its free radical scavenging property, antioxidant activity, and antiapoptotic potential (43). In the rabbits challenged with Cd (6 mg/kg, i.p.) and treated with pomegranate seed oil (0.8 ml/kg), a significant decrease in the blood volume and hemoglobin was seen (44). Some herbs and NP exhibited significant protection on Cd-induced respiratory insults in experimental animal models and pre-clinical studies but the clinical studies were not found in this regard. The findings from these studies may lead to new

TABLE 2 | The possible therapeutic effects of NP in the Cd-induced lung injury.

Study type	Study design	NP	Dose	Effects	References
In vivo	Cd-exposed mice	*Nigella sativa*	1 ml/kg, i.p. for 28 days	Ameliorated Cd-induced lung damage with minimal histopathological changes in lung architecture	(41)
	Cd-exposed mice	*Tribulus Terrestris*	200 mg/kg, i.p. for 10 days	Eliminate free radicals ↓ Antioxidant enzymes expression, down-regulated proinflammatory markers in cellular injuries	(42)
	Cd-exposed mice	Grape seeds Ext	-	Improved hazard toxic effect on the lung tissue, antioxidant activity and anti-apoptotic potential	(43)
	Cd-exposed rabbits	Pomegranate seeds oil	0.8 ml/kg, for 30 days	↓ Blood volume, hemoglobin, and improved lung function	(44)

NP, natural products; Ext, extract; Cd, Cadmium; i.p., intraperitoneal.
The up arrow (↑) indicates an increase in the variable, and a down arrow (↓) indicates a decrease.

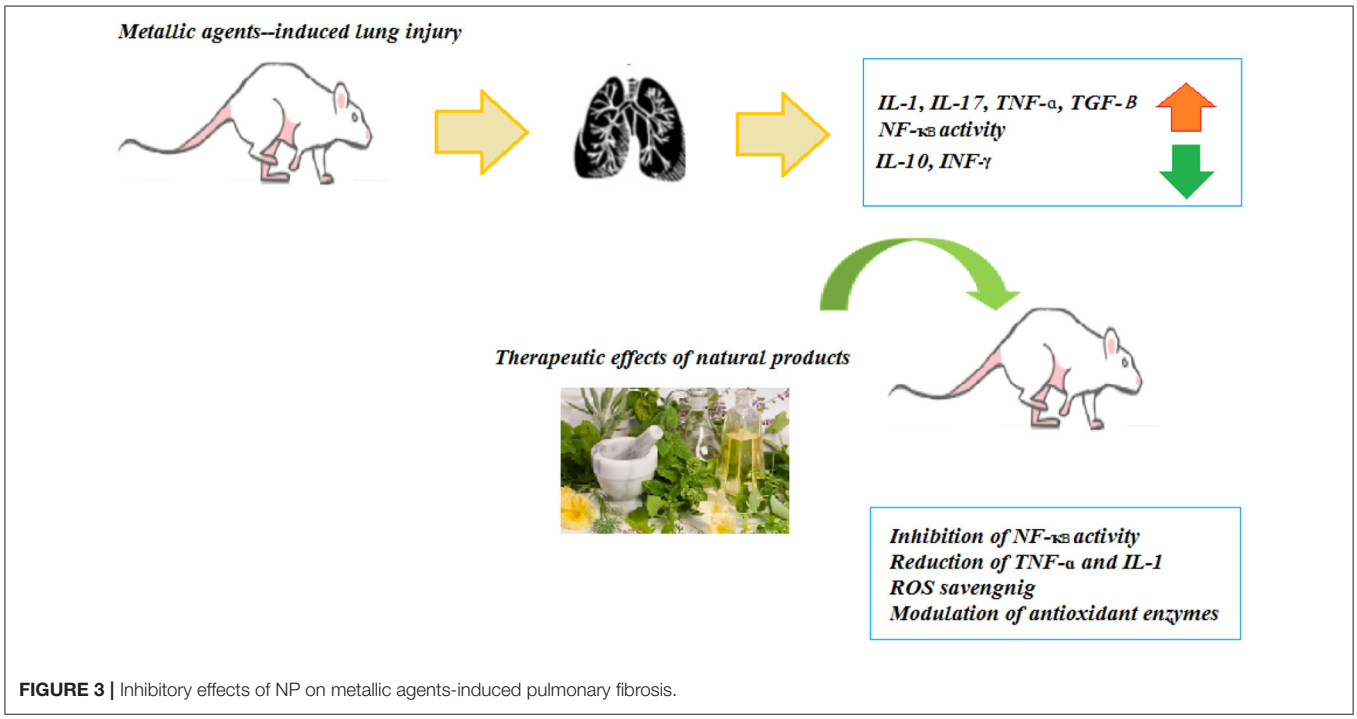

FIGURE 3 | Inhibitory effects of NP on metallic agents-induced pulmonary fibrosis.

therapeutic development for a new drug for the treatment of Cd-induced respiratory injuries. These studies may also guide other investigators to develop quality NP clinical trials in the future. The effects of NP on Cd-induced lung injury are summarized in **Table 2** and **Figure 3**.

Environmental Dust-Induced Lung Disorders

Experimental Studies

Pneumoconiosis is a lung disease caused by certain kinds of dust particles in the workplace. In rats exposed to an inoculation inside the trachea, coal dust (3 mg/0.3 ml of saline), treatment with *Mikania glomerata* and *Mikania laevigata* extracts (100 mg/kg, s.c., for 2 weeks) prevented the increase in the total cell count and LDH activity in the bronchoalveolar lavage fluid (BALF), and diminished the lung inflammatory infiltration induced by the coal dust, as assessed by the histopathologic analyses. These findings suggested that both extracts showed a protective effect on the oxidation of thiol groups (45).

In rats exposed to uranium ore dust inhalation (period of 3, 7, 30, and 60 days), the administration of licorice (*Glycyrrhiza glabra*) aqueous extract normalized the pyruvic acid contents in the lung tissue during the study periods and decreased the lactic/pyruvic acid ratio (46).

In albino rats exposed to cement dust, the antioxidant activities of roselle (*Hibiscus sabdariffa*), moringa (*Moringa oleifera*), ginger (*Zingiber officinale*), and 'ugwu' (*Telfairia occidentalis*), fed with herbal extracts (400 mg/kg) for 6 months were evaluated. The lungs of non-treated rats showed severe interstitial fibrosis and cellular debris. Moderate fibrosis was seen

TABLE 3 | The possible therapeutic effects of medicinal herbs in the dust-induced lung disorders in experimental studies.

Study type	Study design	NP	Dose	Effects	References
In vivo	Coal dust-exposed rats	MGE and MLE Exts	100 mg/kg, for each Ext, s.c., for 2 weeks	↓ Total cell count and LDH activity ↑ Protein sulfhydryl content in lung	(45)
	Uranium ore dust-exposed rats	*Glycyrrhiza glabra* aqueous Ext	100 mg/kg	Normalized pyruvic acids cand actic/pyruvic acids ratio in lung tissue	(46)
	Cement dust-exposed rats	*Hibiscus sabdariffa*, *Moringa oleifera*, *Zingiber officinale* and *Telfairia occidentalis* ethanolic Ext	400 mg/kg, orally, for 180 days ratio 1:1:1:1	Decrease in lung fibrosis	(47)
	Cement dust-exposed rats	*Hibiscus sabdariffa*, *Moringa oleifera*, *Zingiber officinale* and *Telfairia occidentalis* ethanolic Ext	400 mg/kg, orally, for 180 days (100 mg of each Ext)	↓ Serum protein, ALT, AST and ALP and lung histological changes ↓ Toxic elements accumulation in the lung	(48)

NP, natural products; Ext, extract; ALP, alkaline phosphates; ALT, alanine amino transferase; AST, aspartate amino transferase; MGE, Mikania glomerata; MLE, Mikania laevigata. The up arrow (↑) indicates an increase in the variable, and a down arrow (↓) indicates a decrease.

in the lung tissues of the rats treated with *Hibiscus sabdariffa* and *Moringa oleifera* extracts. The rats that were fed with the mixture of the extracts had mild septal fibrosis (47).

Treatment with *Hibiscus sabdariffa*, *Moringa oleifera*, *Zingiber officinale,* and *Telfairia occidentalis* in the rats exposed to cement dust showed moderate to normal biochemical parameters when compared with the non-treated rats. Higher hematological parameters were observed in the treated rats than in the non-treated rats. Overall, the mixture of extracts decreased the adverse effects of cement-dust exposure more than any individual extract alone. Individually, *Telfairia occidentalis* performed the best, followed by *Zingiber officinale*, *Moringa oleifera*, and *Hibiscus sabdariffa* (48). The elemental analysis of the cement dust shows that it contains 57% calcium, 23% silicon, 10.5% aluminum, 8.5 % chromium, and 8.0 % lead (47). The data of these studies indicated the antioxidant properties of the food plants which modulated the effects of cement dust. Hence, the plants could be used as supportive care in polluted environments to lower the health problems associated with cement-dust exposures. These experimental studies indicated that *Hibiscus sabdariffa*, *Moringa oleifera*, *Zingiber officinale, Telfairia occidentalis, Mikania glomerata, Mikania laevigata,* and *Glycyrrhiza glabra* might be candidates for the prevention of lung injury caused by swine barn, coal, uranium ore, and cement-dust exposure. These results suggested a potential role for oxidative stress pathways in mediating occupational lung diseases and antioxidants effect of the plants in reducing dust-mediated oxidative stress in lung disorders of exposed workers.

This review study indicates the therapeutic effect of NP on dust-induced lung disorders but more clinical studies are required to establish the clinical efficacy of these plants and their constituents on lung and allergic disorders. The therapeutic effects of NP including anti-inflammatory effects and reduction of airway responsiveness in the animal models of asthma and COPD were also illustrated. Different NP and their components were identified as anti-asthmatic components. We suggest the possible therapeutic effect of NP on lung disorders of dust-exposed patients *via* the decrement of inflammatory and enhanced anti-inflammatory mediators, and improved pulmonary function tests. The effects of NP on dust-induced lung disorders are summarized in **Table 3**.

Lipopolysaccharide-Induced Lung Disorders

Experimental Studies

The administration of the extract of *Paulownia tomentosa* stem bark (2.5, 5, 10, 20, and 40 μg/ml, for 24 h), represses the release of IL-6 and TNF-α in the RAW 264.7 macrophages stimulated by LPS (49). In this cell line, dehydrodieugenol B from *Nectandra leucantha* (10, 20, 30, and 60 μM) did not influence the cell viability but hindered the enhancement in IL-1β and IL-6 gene expression and NO release (50).

In vitro, Barbaloin (a major anthraquinone compound) (25, 50, or 100 μM), decreased the expression of TNF-α, IL-1β, and IL-6 as well as the activation of ROS-mediated PI3K/AKT/NF-κB pathway dose-dependently (51). The ethanolic extract of the aerial parts of *Houttuynia cordata* (30, 50, 100, and 300 μg/mL) also inhibited the iNOS-mediated NO release from the LPS-stimulated MH-S cells (a mouse alveolar macrophage cell line) concentration-dependently (52).

Incubating the RAW264.7 cells with LPS and alpinumisoflavone (1, 5, and 10 μg/mL, for 24 h), a plant-derived pyranoisoflavone remarkably inhibited the release of NO, cytokines, and ICAM-1 protein expression. Treatment with alpinumisoflavone blocked the IκBα phosphorylation and degradation and decreased the phosphorylation of IKK and NF-κB. In addition, it effectively decreased the phosphorylation of ERK, Jc-Jun-NH2 terminal kinase (JNK), and p38. In LPS activation of the NLRP3 inflammasome, caspase-1, and IL-1β proteins were inhibited by alpinumisoflavone, especially at its high dose. The alpinumisoflavone treatment also remarkably decreased the IL-17A and iNOS protein expression but it did not block the LPS-induced cyclooxygenase-2 (COX-2) induction. Furthermore, alpinumisoflavone significantly enhanced the expression of antioxidant enzymes dose-dependently. The LPS induction of intracellular ROS production was also significantly inhibited by the treatment with alpinumisoflavone (53).

The treatment of LPS-stimulated and non-stimulated splenocytes with the aqueous extract of *Curcuma longa* (0.8–500 μg/mL, for 48 h), remarkably enhanced NO, pro-inflammatory cytokines, tumor necrosis factor, interferon-gamma, and monocyte chemoattractant protein-1(MCP-1). The levels of IL-12 and PGE2 in the LPS-stimulated cells were also inhibited by the plant extract (54). In the LPS-activated epithelial cell line, the levels of NF-κB p52, NF-κB p65 transcription factors protein, IL-1ß, interleukin-8 (IL-8), and mucus secretion were significantly reduced by the hydroalcoholic extract of *Thymus vulgaris* (0.04–0.60%) (55).

Daidzein (100 μM,), a diphenolic isoflavone 15 min after LPS stimulation, obviously inhibited the expressions of myeloid differentiation factor 88 (MyD88), toll-like receptor 4 (TLR4), and the activation of NF-κB in the A549 alveolar epithelial cells stimulated by 10 μg/ml LPS (56). In the RAW264.7 cells, treatment with a mixture of *Taraxacum officinale* (a herbal formulation) (0.5, 1, and 2 mg/mL), repressed the LPS (100 ng/mL)-induced inflammatory responses (57). Also, the expression of pro-inflammatory cytokines activated the Nrf2-HO-1 axis and oxidative stress was inhibited in the treated LPS-stimulated cells (57).

Eugenol and dehydrodieugenol B from *Nectandra leucantha* (30 mg/kg) in the mice with the LPS-induced ALI, decreased lung edema, inflammatory cells, and the IL-6 and IL-1 β levels in the BALF as well as decreased inflammatory cell infiltration and those positive to iNOS, MMP-9, and TIMP-1, and decreased the collagen content and the 8-isoprostane expression in the lung tissue (50).

In LPS-challenged mice, treatment with thymol (30 and 100 mg/kg, i.p.), one of the primary active constituents derived from *Thymus vulgaris*, before or after the LPS challenge, significantly improved the pathological changes in the lung tissues. Thymol also inhibited the LPS-induced inflammatory cells influx and protein concentration in the BALF. Additionally, thymol markedly inhibited the LPS-induced elevation of MDA and MPO levels as well as reduction of the SOD activity. Thymol also effectively inhibited the NF-κB activation in the lung (58). In the LPS-induced ALI, treatment with methanolic extract of *Pulicaria petiolaris* (50 and 100 mg/kg, p.o., for 5 days) reduced pulmonary edema, ameliorated the LDH level in the BALF, improved the histopathological lesions in the lung tissue, and showed antioxidant capacity (59).

The administration of a single dose of cannabidiol (0.3, 1.0, 10, 20, 30, 40, and 80 mg/kg, i.p.), extracted from *Cannabis sativa*, before the LPS-induced ALI, decreased the migration of leukocytes into the lungs, albumin concentration in the BALF, production of pro-inflammatory cytokines and chemokines, and MPO activity in the lung tissue. In addition, in the LPS-induced ALI, ZM241385 (a selective adenosine A2A receptor antagonist), inhibited all anti-inflammatory effects of cannabidiol which indicate the contribution of adenosine A2A receptor in the anti-inflammatory effects of cannabidiol (60).

The linalool (25 mg/kg, i.p.) treatment attenuated the production of LPS—decreased the changes in TNF-α and IL-6 as well as lung histopathologic changes in the ALI mouse model (61). In the mice with the LPS-induced ALI, Barbaloin extracted from *Aloe vera* ameliorated lung pathological changes such as infiltration of inflammatory cells, alveolar hyperemia, necrosis, and lung epithelial cell detachment (51).

The treatment with the aqueous extract of *Taraxacum mongolicum* Hand.-Mazz (5 and 10 g/kg, p.o.) inhibited the LPS-induced lung injury in female BALB/c mice by reducing the inflammatory cell infiltration in the BALF, lung protein levels and PI3K/Akt/mTOR signaling. It also improved the activity of SOD and inhibited the MPO activity (62).

The *Portulaca oleracea* extract (50, 100, and 200 mg/kg, p.o., 1 h before LPS injection) suppressed the LPS-induced rat ALI by decreasing IL-6, IL-β, TNF-α, TGF-β, and, PGE2 but increasing IL-10 levels. *Portulaca oleracea* improved the levels of the white blood cells (WBC), MDA, MPO, and thiol as well as SOD and CAT activities. The lung wet/dry ratio (an index of interstitial edema) was also significantly reduced. Therefore, the *Portulaca oleracea* extract displayed antioxidant and anti-inflammatory activity dose-dependently on the LPS-induced ALI model in the rat model (63).

In the LPS-induced ALI, xanthohumol (10 or 50 mg/kg, i.p.), a prenylflavonoid extracted from the hop plants (*Humulus lupulus*) (0, 18, 35, and 70 μmol/kg, i.p., for 30 min), showed a protective effect against oxidative stress and inflammatory damage by regulation of the Nrf2 pathway through AMPK/GSK3β activation, and suppression of LPS-activated Txnip / NLRP3 inflammation and the NF-κB signaling pathway (64). In LPS-induced lung inflammation in mice, pre-treatment with luteolin (0, 18, 35, and 70 μmol/kg, i.p., for 30 min) decreased IL-6 and TNF-α levels and expression of COX-2 and iNOS. In addition, luteolin represses activation of NFκB and its upstream molecular factor, Akt (65).

The treatment of ALI mice with the ethanolic extract of *Glycyrrhiza glabra* (200 and 400 mg/kg, p.o., for 4 days) significantly reduced the exudation of protein and the total cell count into the BALF but increased the BALF SOD and CAT activities (66). The alcoholic extract of *Anemarrhena asphodeloides* decreased the inflammatory cells in the BALF and inhibited lung inflammation by its saponin-enriched fraction. The inflammatory markers in the LPS-induced ALI in mice were significantly inhibited by oral administration of timosaponin A-III (67).

The pre-treatment of mice exposed to LPS with crude extract of *Eleusine indica* (400 mg/kg) inhibited the lung neutrophil recruitment 98% dose-dependently. Vitexin (8-C-β-glucopyranosylapigenin) and schaftoside (6-C-β-glucopyranosyl-8-C-α-arabinopyranosylapigenin) isolated from aerial parts (400 μg/kg), inhibited lung neutrophil influx, by 62 and 80%, respectively (68). The pre-treatment with astragalin (25, 50, and 75 mg/kg, p.o., 1 h before LPS challenge), a flavonoid from several medicinal plants, decreased inflammatory responses and improved survival in lethal endotoxemia of a murine model of the LPS-induced ALI. The anti-inflammatory effect of astragalin was correlated with the reduction of IL-1, IL-6, and TNF-α levels produced through the inactivation of NF-κB (69).

The Jojoba oil dry (400 mg, i.t., the air flow rate of 60 L/min for the duration of 7 s) nanoemulsion powders indicated more anti-inflammatory effects on the LPS-induced ALI than

dexamethasone with a detrimental effect on the total protein content and down-regulation of TNF-α, IL-1β, IL-6, and NF-κB p65 (116).

Among carvone isomers, pre-treatment with D-carvone (25 and 50 mg/kg, i.g., 1 h before LPS challenge), significantly alleviated the LPS-induced lung injury by diminishing the lung wet/dry ratio and the number of inflammatory cells in the BALF. The serum pro-inflammatory cytokines were remarkably decreased in D-carvone-treated mice. The lung histopathological changes in the LPS-induced lung injury were improved by D-carvone. In addition, the comparable effects of D-carvone with those of dexamethasone were seen (70). Myricetin (10, 20, and 40 mg/kg, 30 min after the LPS challenge), a member of the flavonoid class of polyphenolic compounds, significantly decreased lung inflammation by reduction of the lungs' wet/dry weight ratio, protein levels in the BALF, cytokine levels, and migration of the inflammatory cells. The TLR4, MyD88, and NF-κB expressions were also decreased and the activities of MPO, SOD, GPx, and CAT were increased in the mice exposed to LPS (71).

The administration of petroleum ether fraction of *Viola yedoensis* (2, 4, and 8 mg/kg, p.o.) in the LPS-induced ALI in mice significantly reduced the wet/dry weight ratio of the lung, total inflammatory cells, the activity of MPO, and protein levels in the BALF. The lung morphology improved, the complement deposition was markedly reduced, and the expression of pro-inflammatory cytokines was suppressed in the treated group (72) and pre-treated with rhamnazin (5, 10, and 20 mg/kg, i.p., 2 days before LPS) significantly reduced the inflammatory parameters, improved lung histopathology changes, activated Nrf2 pathway, and attenuated ROS as well as H_2O_2, MDA, and hydroxyl ion in the LPS-exposed rats (73). In the LPS-induced lung inflammation and oxidative stress model, the administration of *Nigella sativa* extract (100–400 mg/kg, i.p.) decreased the total and differential WBC counts, oxidative stress, and inflammatory (TGF-β1, IFN-γ, PGE2, and IL-4) markers in the BALF and serum as well as the pathological changes of the lung tissue in the rats (74).

Therefore, the experimental studies showed that pre-treatment with various NP remarkably reduced the inflammatory markers and improved the lung histopathology in the LPS-induced ALI animal models, indicating the therapeutic effect of NP on ALI in the animal models due to their antioxidant and anti-inflammatory properties. The underlying mechanisms of the anti-inflammatory action of NP are inhibition of the Nrf2-mediated antioxidative pathway. Among the biological activities of NP derived from plants anti-inflammatory, antiviral, antitumor, antiallergic, and antioxidant activities can be pointed out. Although many reports have evaluated the effects of these compounds in the experimental models, studies evaluating clinical trials are scarce in the literature. In this section, the effects of different NP on the LPS-induced lung disorders in the experimental models and some possible mechanisms of action were shown. Some experimental data suggest that supplementation with NP may be an effective treatment for patients with the LPS-induced respiratory disorders. The effects of NP on the LPS-induced lung disorders are summarized in **Table 4**.

Paraquat-Induced Lung Disorders

Experimental Studies

The treatment with *Zataria multiflora* (200 and 800 mg/kg) and carvacrol (20 and 80 mg/kg) improved the inhaled PQ-induced systemic oxidative stress and inflammation (75). Also, treatment with carvacrol reduced the WBC (total and differential) count, oxidant biomarkers, and inflammatory cytokines, but increased the antioxidants including CAT and SOD, and anti-inflammatory cytokines in the inhaled PQ-exposed rats similar to the effects of pioglitazone and dexamethasone (76).

The administration of the bark extract of *Bathysa cuspidata* (200 and 400 mg) was shown to protect against PQ-induced acute lung injury and mortality in the rats exposed to PQ as substantiated by the significant decreases in lung edema, septal thickening, alveolar collapse, hemorrhage, cell migration, malondialdehyde, and proteins carbonyl levels (77). In another similar study, salidroside (10 mg/kg), derived from *Rhodiola rosea*, alleviated the PQ-induced lung injury in the rats *via* down-regulation of the TGF-β1 expression (78). In an experimental study, the rats were exposed to a single dose of PQ and treated with pioglitazone (5 mg/kg), pioglitazone plus *Zataria multiflora* extract (200 mg/kg), pioglitazone plus carvacrol (20 mg/kg), and dexamethasone (0.03 mg/kg). The results indicated that the treatment of lung and systemic oxidative stress and inflammation induced by the inhaled PQ in the rats with a combination of pioglitazone plus *Zataria multiflora* or carvacrol showed more effect than the effect of pioglitazone or the plant and carvacrol alone (79).

In the rats exposed to PQ and treated with *Zataria multiflora*, the levels of IL-10, IL-4, TNF-α, and IFN-γ were significantly increased and IL-6, IL-8, and IL-2 levels were decreased (80). The treatment with *Zataria multiflora* (200 and 800 mg/kg) markedly reduced the WBC (total and differential) counts, serum levels of NO_2, MDA, IL-17, and TNF-α as well as improved the PQ-induced acute lung injuries (81). *Ligustrazine* (30 mg/kg, i.g.), an active substance extracted from the Umbelliferae plant *Ligusticum chuanxiong* (30 mg/kg, i.g.), improved the lipid peroxidation damage, improved the lung injury, and induced the concentrations of NK-κB and iNOS caused by acute poisoning with PQ (82). In the lung injury induced by sub-acute exposure with PQ, treatment with curcumin (30 mg/kg, i.g.) and nano-curcumin-attenuated lung fibrosis may be associated with their antioxidant properties (83).

Diosmin (50 and 100 mg/kg), in a mouse model of PQ-lung injury, showed antioxidant, anti-inflammatory, and antifibrotic effects (84). In a similar study, the PQ-exposed mice treated with the extract of *Rosa canina* fruits (200 and 400 mg/kg) improved the oxidant-antioxidant balance in the lung tissue (85). The treatment of PQ-exposed mice with apigenin (25, 50, and 100 mg/kg) significantly reduced lung injury by inhibition of oxidative stress and inflammation (86).

The studies have shown that NP have a variety of medicinal activities including anti-inflammatory, antioxidant, and anticancer. Due to their low water solubility, NP are significantly limited in clinical application. Many potential strategies are expected to be developed to improve their pharmacokinetic values and bioavailability. The experimental

TABLE 4 | The possible therapeutic effects of medicinal herbs in the LPS-induced lung disorders in experimental studies.

Study type	Study design	NP	Dose	Effects	References
In vitro	LPS-stimulated mice RAW264.7 macrophages	*Paulownia tomentosa* methanolic Ext	2.5, 5, 10, 20 and 40 μg/ml, for 24 h	Suppressed IL-6 and TNF-α production	(49)
	LPS-stimulated mice RAW264.7 macrophages	Dehydrodieugenol B from *Nectandra leucantha*	10, 20, 30 and 60 μM	No effect on cell viability Inhibited NO release and IL-1β and IL-6 gene expression	(50)
	LPS-stimulated mice RAW264.7 macrophages	Linalool	40, 80 and 120 μg/mL	↓ TNF-α and IL-6, blocked IκBα protein phosphorylation, p38, c-Jun terminal kinase, and extracellular signal-regulated kinase	(61)
	LPS-stimulated mice RAW264.7 macrophages	Barbaloin from *Aloe vera*	25, 50, or 100 μM	Inhibited IL-1β, IL-6, and TNF-α expression, ROS-mediated PI3K/AKT/NF-κB pathway activation	(51)
	LPS-stimulated mice RAW264.7 macrophages	*Houttuynia cordata* ethanolic Ext	30, 50, 100 and 300 μg/mL	Inhibited NO production	(52)
	LPS-stimulated mice RAW264.7 macrophages	Alpinumisoflavone	1, 5 and 10 μg/mL, for 24 h	↓ NO, TNF-α, IL-6, IL-1β, and ICAM-1 protein expression, IKK and NF-κB phosphorylation, NF-κB nuclei localization, ERK, JNK and p38 phosphorylation, IL-17A and iNOS expression Blocked IκBα phosphorylation and degradation, NLRP3 inflammasome, caspase-1 activation, and IL-1β proteins, ↑ CAT, HO-1, GPx, and SOD, Inhibited intracellular ROS generation	(53)
	LPS-stimulated RAW264.7 macrophages in mice	TADIOS ethanolic Ext	0.5, 1 and 2 mg/mL	↑ Relative luciferase units Suppressed IL-6 and IL-1β, and ROS production *in vivo*	(57)
	LPS-stimulated mice splenocytes and RAW264.7 macrophages	*Curcuma longa* aqueous Ext	0.8-500 μg/mL, for 48 h	↑ NO, IL-12, IL-10, IL-6, IL-2, TNF-α, IFN-γ and MCP-1 in non-stimulated mouse splenocytes and macrophages, Inhibited production of IL-12 and PGE2 in LPS-stimulated cells	(54)
	LPS-stimulated LECL and H460 CCL	*Thymus vulgaris* Hydroalcoholic Ext	0.04-0.60%	↓ NF-κB p52 and NF-κB p65 transcription factors protein, IL-1β, IL-8 and mucus s Induced necrotic cell death in human H460 lung cancer cell line	(55)
	LPS-stimulated A549 alveolar epithelial cells	Daidzein	100 μM, 15 min after LPS stimulation	Inhibited expressions of TLR4 and MyD88 and the activation of NF-κB	(56)
	LPS-induced ALI in mouse model	TADIOS	1000 mg/kg, orally	↓ Neutrophil infiltration in BALF, inflammatory cell infiltration in lung tissue and thickening of the alveolar wall Activated Nrf2-HO-1 axis	(57)
In vivo	LPS-induced ALI in mouse model	Eugenol and Dehydrodieugenol B from *Nectandra leucantha*	30 mg/kg	↓ Lung edema, inflammatory cells, and IL-6 and IL-1 β levels in BALF, iNOS, MMP-9, and TIMP-1, collagen levels and the 8-isoprostane expression in lung tissue, Inhibited phosphorylation of JNK	(50)
	LPS-induced ALI in mouse model	Thymol from *Thymus vulgaris*	30 and 100 mg/kg, i.p.	Improved lung pathological changes ↓ Inflammatory cells influx, TNF-α and IL-6 protein, MDA and MPO l levels in BALF and NF-κB activation in lung ↑ SOD activity,	(58)
	LPS-induced ALI in mouse model	*Pulicaria petiolaris* methanolic Ext	50 and 100 mg/kg, p.o., for 5 days	↓ Lung wet/dry weight (W/D) ratio, total protein and LDH level in BALF, lung histopathological lesions, inflammatory cell infiltration, MDA and ↑ SOD and GSH	(59)

(Continued)

TABLE 4 | Continued

Study type	Study design	NP	Dose	Effects	References
	LPS-induced ALI in mouse model	Cannabidiol from *Cannabis sativa*	0.3, 1.0, 10, 20, 30, 40, and 80 mg/kg, i.p.	↓ Leukocyte and myeloperoxidase activity in lungs, albumin level in BALF, and TNF, IL-6, MCP-1 and MIP-2 production	(60)
	LPS-induced ALI in mouse model	Linalool	25 mg/kg, i.p.	↓ TNF-α and IL-6 production, total WBCs, neutrophils and macrophages in BALF Improved lung histopathologic changes	(61)
	LPS-induced ALI in mouse model	Barbaloin from *Aloe vera*	25, 50, and 100 mg/kg, i.p.	↓ Lung pathological changes, MPO activity and inflammatory neutrophil in lung tissue	(51)
	LPS-induced ALI in mouse model	*Taraxacum mongolicum* Hand.-Mazz aqueous Ext	5 and 10 g/kg, p.o.	↓ Inflammation cells in BALF, protein levels I3K/Akt/mTOR, MPO and inflammatory neutrophil accumulation in lung P ↑ SOD activity	(62)
	LPS-induced ALI in mouse model	*Portulaca oleracea* hydroethanolic Ext	50, 100 and 200 mg/kg, p.o., 1 h before LPS injection	↓ IL-β, IL-6, TNF-α, PGE2, and TGF-β, and increased IL-10 levels, lung wet/dry ratio Improved the level of WBC, MPO, MDA, thiol, SOD and CAT,	(63)
	LPS-induced ALI in mouse model	Xanthohumol from *Humulus lupulus*	10 or 50 mg/kg, i.p.	↓ Lung histopathological changes W/D ratio protein levels, neutrophil infiltration, MDA, MPO, SOD and GSH depletion, ROS, and cytokines levels, iNOS and HMGB1 expression, Txnip/NLRP3 inflammasome and NF-κB signaling pathway activation, t-BHP-stimulated cell apoptosis ↑ Anti-oxidative enzymes expression regulated by Keap1-Nrf2/ARE activation	(64)
	LPS-induced ALI in mouse model	Luteolin	0, 18, 35 and 70 μmol/kg, i.p., for 30 min	↓ Histological changes and lung tissue edema, vascular permeability, TNF-α and IL-6 levels in BALF, and expression of iNOS and COX-2 in lung, NFκB activation upstream molecular factor, Akt	(65)
	LPS-induced ALI in mouse model	*Glycyrrhiza glabra* Ethanolic Ext	200 and 400 mg/kg, p.o., for 4 days	↓ Lung wet/dry weight ratios, lung pathological changes. total cell and protein exudate in BALF, pro-inflammatory mRNA expression Improved SOD activity in BALF,	(66)
	LPS-induced ALI in mouse model	*Anemarrhena asphodeloides*, alcoholic Ext Saponin-enriched fraction, Timosaponin A-III	50 and 200 mg/kg, p.o. 10 and 50 mg/kg 25-50 mg/kg	↓ Total WBCs count, and inflammatory cell infiltration, neutrophil infiltration and macrophages in BALF, IL-1β and IL-6 production in BALF, STAT3 activation, alveolar wall thickness and infiltration of inflammatory cells	(67)
	LPS-induced ALI in mouse model	*Eleusine indica*, Schaftoside and vitexin	4, 40 and 400 mg/kg, i.p. 400 μg/kg, i.p.	Inhibited lung neutrophil influx	(68)
	LPS-induced ALI in mouse model	*Houttuynia cordata* ethanolic Ext Afzelin, hyperoside and Quercitrin	100 and 400 mg/kg, p.o. 100 mg/kg, p.o.	↓ Total cell numbers in BALF ↓ Neutrophils, macrophages and dendritic cells in BALF	(52)
	LPS-induced ALI in mouse model	Astragalin	25, 50 and 75 mg/kg, p.o., 1 h before LPS challenge	Improved animal survival rate, ↓ Lung pathological changes, lung W/D ratio, total protein levelin BALF, total WBC, neutrophils and macrophages in BALF, IκB degradation Down-regulated TNF-α, IL-1β and IL-6 production,	(69)
	LPS-induced ALI in mouse model	D-carvone	25 and 50 mg/kg, i.g., 1 h before LPS challenge	↓ Lung wet/dry ratio, total cells, macrophages, and neutrophils in BALF, TNF-α, IL-1β, and IL-6 in serum	(70)
	LPS-induced ALI in mouse model	Myricetin	10, 20 and 40 mg/kg, 30 min after LPS challenge	↓ Lung wet/dry ratio, protein concentration in BALF, MPO activity, cytokine, and inflammatory cell migration, TLR4, MyD88 and NF-κB expression, ↑ SOD, GPx and CAT levels	(71)

(Continued)

TABLE 4 | Continued

Study type	Study design	NP	Dose	Effects	References
	LPS-induced ALI in mouse model	Petroleum ether fraction of *Viola yedoensis*	2, 4, and 8 mg/kg, p.o.,	↓ Lung wet/dry ratio, total cells, RBC, protein level, and MPO activity in BALF, histopathological damage, expression of TNF-α, IL-1β, and IL-6	(72)
	LPS-induced ALI in rat model	Rhamnazin	5, 10 and 20 mg/kg, i.p., 2 days before LPS	↓ Lung wet/dry ratio, protein level in BALF, LDH and MPO activities, cytokine and oxidative stress, and histopathological damage	(73)
	LPS-induced ALI in rat model	*Nigella sativa* hydroethanolic Ext	100-400 mg/kg, i.p.	↓ Total and differential WBC, MDA, TGF-β1, IFN-γ, PGE2 and IL-4 levels in BALF, and lung pathology ↑ thiol. SOD and CAT levels in BALF and serum	(74)

NP, natural products; Ext, extract; ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; LECL, lung epithelial cell line; CCL, cancer cell line; COX-2, cyclooxygenase-2; GPx, glutathione peroxidase; GSH, glutathione; HO-1, heme oxygenase-1; i.g., intragastric; i.t., intratracheal; ICAM-1, intercellular adhesion molecule-1; JNK, Jc-Jun-NH2 terminal kinase; LDH, lactate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; MDA, malondialdehyde; MPO, myeloperoxidase; MyD88, myeloid differentiation factor 88; Nrf2, nuclear factor erythroid 2–related factor 2; p.o., per os (by way of the mouth); PGE2, prostaglandin E2; RBC, red blood cells; ROS, reactive oxygen species; SOD, superoxide dismutase; TGF-β, transforming growth factor; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; Taraxacum officinale F.H.Wigg; TADIOS, Dioscorea batatas Decaisne and Schizonepeta tenuifolia. The up arrow (↑) indicates an increase in the variable, and a down arrow (↓) indicates a decrease.

evidence supports the causal relationship between oxidative stress and various chronic diseases. Thus, numerous studies are focused on ameliorating the PQ-induced lung injury by decreasing oxidative stress. The experimental studies suggest that NP can combat oxidative stress and reduce the morbidity and mortality associated with PQ-induced lung injury. The effects of NP on PQ-induced lung injury are summarized in **Table 5** and **Figure 4**.

Sulfur Mustard-Induced Lung Disorders

Experimental Studies

The aqueous extract of *Crocus sativus* (225, 450, and 900 μg) decreased DNA damage and MDA but increased the GSH level in the SM-exposed macrophage cells (87).

The effects of antioxidants caffeic acid (CA) (250 μM) and quercetin (100 μM) on normal human epithelial keratinocytes (NHEKs) treated with SM (200 μM) showed their protective effects on the cytotoxicity induced by SM. Also, CA improved cell viability at concentrations > 250 μM dose-dependently. In addition, the treatment with CA and quercetin decreased the phosphorylation of p38 and p53 but increased the phosphorylation of JNK 1/2 induced by SM. Furthermore, CA and quercetin reduced the expression levels of cyclooxygenase 2 (COX-2), inducible NO synthase (iNOS), and the induction of oxidative stress irrespective of the p38 and lipoxygenase pathway (88).

In female mice exposed to SM, ethanolic extract of *Hippophae rhamnoides* L. leaf (HL-EOH), water and ethanolic extract of *Hippophae rhamnoides* fruit (HF-W and HF-EOH), and *H. rhamnoides* flavone from fruit (HR-flavone) significantly protected the lethal effect of SM. Treatment with HL-EOH and HR-flavone markedly protected the bodyweight loss, levels of GSH, oxidized glutathione (GSSG), and MDA. The LD_{50} of all extracts was more than 5 g/kg indicating their non-toxic property (89).

The *Nigella sativa* extract (0.08 g/day) in drinking water markedly decreased airway responsiveness to methacholine and WBC count in the guinea pigs exposed to SM (90). Similarly, the *Nigella sativa* extract at the above dose for 2 weeks also remarkably reduced airway responsiveness, neutrophil, eosinophil, lymphocyte, and monocyte percentage in the SM-exposed guinea pigs and the effects of *Nigella sativa* were similar to the onset of dexamethasone (5 mg/kg, i.p.) (91). The therapeutic effects of *Salvia miltiorrhiza* and *Anemarrhena asphodeloides* mixture (MSTF) (30, 60, 120 mg/kg) after exposure of the rats to SM (3.5 mg/kg, s.c.) significantly enhanced the survival and diminished the SM-induced morphological changes in the liver, small intestine, and testis tissues. The administration of MSTF (60 and 120 mg/kg) markedly increased the GSH level and prevented the differential expression of genes in the SM-exposed rats (92).

Clinical Studies

In a case-control study, the treatment of patients with lung disorder due to SM exposure (n=20) with boiled extract of *Nigella sativa* (0.375 ml/kg) significantly improved the PFT values, and chest wheeze 30 and 60 days after treatment compared to the placebo-treated group (n=20) and also compared to the beginning of the study (93). The treatment effects of the *Avena sativa* plant (0.1% cream twice a day for 4 weeks) on chronic pruritus in the SM-exposed patients in a double-blind clinical trial were studied. A total of seventy-five patients were divided into 3 groups including *Avena sativa* ointment, placebo, and betamethasone groups. At the end of the study period, the pruritus severity was significantly reduced in groups A and B compared to group C. The *Avena sativa* ointment treatment also improved the quality of life and quality of sleep in the patients (94).

In a randomized clinical study, the treatment of patients with lung disorder due to SM exposure with a syrup made from *Zataria multiflora* (5 and 10 mg/kg) for 2 months) reduced the WBCs (total and different) and oxidant biomarker but increased thiol, SOD, and CAT activities, and increased the PFT values (20). In a similar study, the serum levels of inflammatory mediators

TABLE 5 | The possible therapeutic effects of NP in the PQ-induced lung injury.

Study type	Study design	NP	Dose	Effects	References
In vivo	PQ-exposed rats	*Zataria multiflora*	200 and 800 mg/kg, i.g. for 16 days	Improved systemic inflammation and oxidative biomarkers	(75)
	PQ-exposed rats	Carvacrol	20 and 80 mg/kg, i.g. for 16 days	↓ Total and differential WBC, MDA, NO2, IL-17 and TNF- α ↑ CAT, SOD activities, IL-10 and INF-γ	(76)
	PQ-exposed rats	*Bathysa cuspidata*	200 and 400 mg/kg, i.g.	↓Lung edema, septal thickening, alveolar collapse, hemorrhage, cell migration, malondialdehyde and proteins carbonyl levels	(77)
	PQ-exposed rats	*Zataria multiflora*, Carvacrol	200 mg/kg 20 mg/kg, i.g. for 16 days	↓ Total and differential WBC, MDA, NO2, IL-17 and TNF- α ↑ CAT, SOD activities, IL-10 and INF-γ	(79)
	PQ-exposed rats	*Zataria multiflora*	200 and 800 mg/kg, i.g. for 16 days	Improved lung inflammation and oxidative stress	(80)
	PQ-exposed rats	*Zataria multiflora*	200 and 800 mg/kg, i.g. for 16 days	↓ Total and differential WBC, IL-17, TNF- α ↑ IL-10, INF-γ	(81)
	PQ-exposed rats	Salidroside	10 mg/kg, i.p.	Suppressed TGF-β1 expression in rat lung injury	(78)
	PQ-exposed rats	ligustrazine	30 mg/kg, i.g.	Improve the lipid peroxidation damage ↓ Lung injury, NK-κB, and iNOS	(82)
	PQ-exposed rats	Curcumin	30 mg/kg, i.g.	↓ Total and differential WBC, IL-17, TNF- α ↑ IL-10, INF-γ	(83)
	PQ-exposed mice	Diosmin	50 and 100 mg/kg, i.p. for 10 or 24 days	Protective effects against PQ-induced lung injury	(84)
	PQ-exposed mice	*Rosa canina*	200 and 400 mg/kg, orally for 14 days	↓ IL-17, TNF- α ↑ IL-10, INF-γ	(85)
	PQ-exposed mice	Apigenin	25, 50 and 100 mg/kg, orally for 7 days	↓ NF-κB, inflammation and oxidative stress	(86)

NP, natural products; Ext, extract; PQ, paraquat; WBC, white blood cell; MDA, malondialdehyde; NO_2, nitrogen dioxide; IL-17, interleukin-17; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL-10, interleukin-10; NF-κB, nuclear factor kappa B; iNOS, induced nitric oxide synthase; i.g., intragastrically; i.p., intraperitoneal.
The up arrow (↑) indicates an increase in the variable, and a down arrow (↓) indicates a decrease.

were reduced but the PFT values were increased due to a 2-month treatment with *Zataria multiflora* in the patients with lung disorders due to SM exposure (95). The treatment with *Zataria multiflora* extract in these patients also diminished cytokines, and respiratory symptoms, but increased some PFT values (96).

The 2-month treatment of the patients with lung disorder for a long time (27–30 years) exposing to SM with carvacrol (1.2 mg/kg) significantly enhanced the CAT and SOD activities, thiol level, and PEF values, but, declined the MDA level, total WBC and neutrophil count (21). The 2-month treatment with carvacrol in similar patients also remarkably reduced the respiratory symptoms and serum levels of IL-2, IL-4, IL-6, IL-8, EGF, and VEGF, but incremented the IFN-γ and IL-10 levels in the serum. In addition, the carvacrol treatment increased MEF25, 50, and 75 (maximum expiratory flow at 25, 50, and 75% of vital capacity) and MMEF (maximum mid-expiratory flow) values after 2 months of treatment (97, 98).

In the patients exposed to SM with chronic pruritic skin lesions ($n = 96$) treated by curcumin (1 g/d) or placebo for 4 weeks, the serum levels of high-sensitivity C-reactive protein (hs-CRP) and IL-8 were reduced in both groups. However, a higher effect of curcumin was observed compared to the placebo group and only treatment with curcumin reduced the calcitonin gene-related peptide (CGRP) level. In addition, in the curcumin group, IL-8 was correlated with the dermatology life quality index (DLQI) change (99). The treatment with curcumin in SM-induced chronic pruritus also improved the antioxidant status, quality of life (QoL), and pruritus (100).

The treatment of the patients with pulmonary complications induced by SM, with curcuminoids (500 mg) for 4 weeks, increased FEV1/FVC compared to placebo-treated patients. The inflammatory mediators (IL-6, IL-8, TGFβ, TNFα, hs-CRP, substance P, CGRP, and MCP-1 also improved remarkably greater than the placebo group. Therefore, in patients with SM-induced chronic pulmonary disorders, short-term curcuminoids treatment reduced lung and systemic inflammation (101).

In the other study, the SM-exposed patients were treated with standard drugs plus curcuminoids and piperine (1,500 and 15 mg/day, respectively) or placebo for 4 weeks. The serum level of GSH was increased but that of MDA decreased and the health-related quality of life (HRQoL) was significantly improved at the end of the study in both groups. However, GSH, MDA, and the HRQoL changes in the curcuminoids-piperine-treated group were markedly greater than the placebo group (102).

The above studies showed the protective effect of NP on the cell viability, inflammation, and pathological changes in

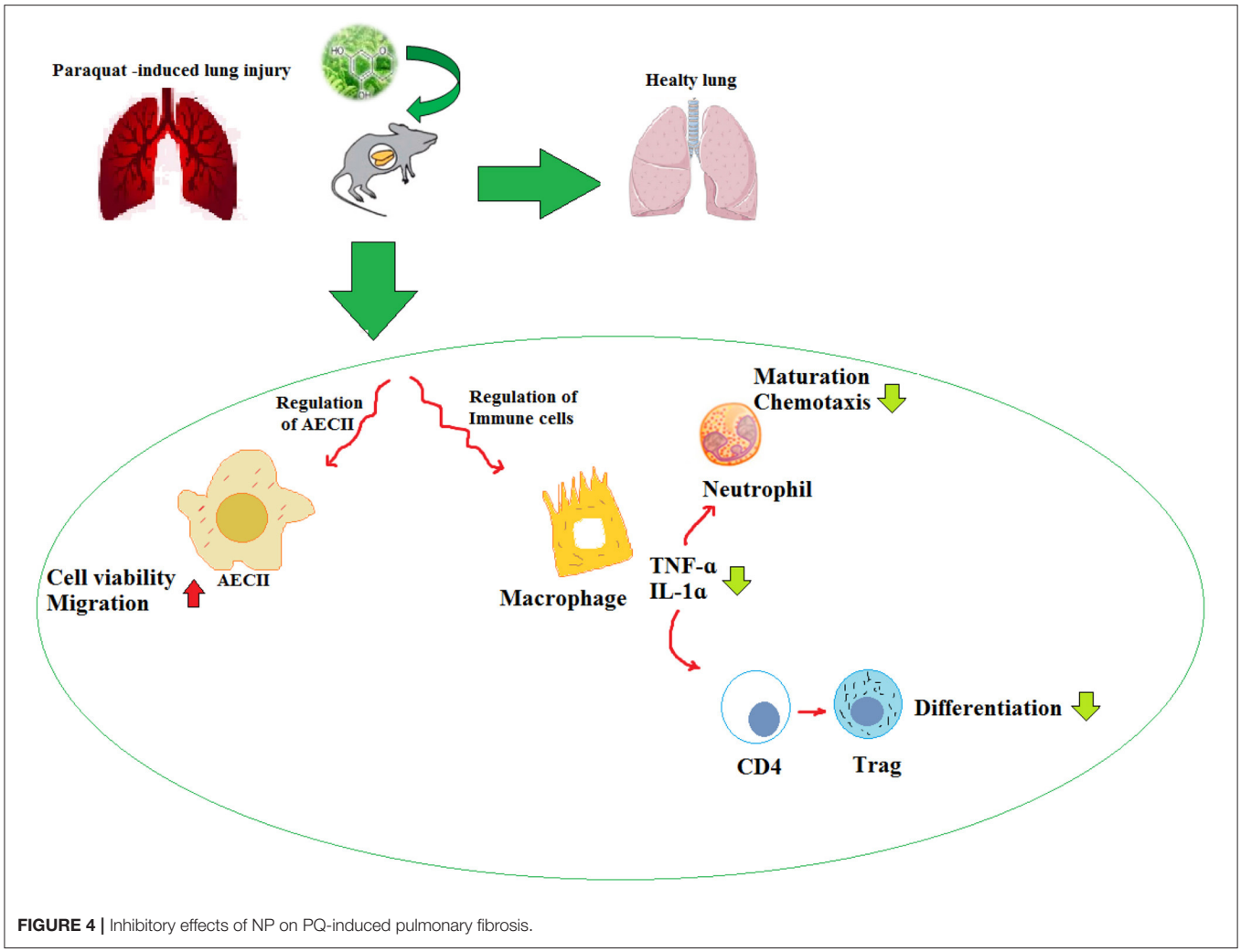

FIGURE 4 | Inhibitory effects of NP on PQ-induced pulmonary fibrosis.

the experimental studies that were exposed to SM. The clinical studies also indicated that NP improve the quality of life, PFT values, respiratory symptoms inflammatory mediators, and oxidative stress markers of SM-induced lung disorder. The therapeutic effects of NP in SM-induced lung injury are summarized in **Table 6** and **Figure 5**. The molecular mechanism of SM-induced toxicity is shown in **Figure 5**.

Other Noxious Agents-Induced Lung Disorders

Experimental Studies

The pre-treatment with *Origanum vulgare* extract (50, 100, 200, and 400 mg/kg) protected the lung tissues from cyclophosphamide (CP)-induced pulmonary damage and suggested a role for oxidative stress in the pathogenesis of the lung disease induced by CP (103). In the rats challenged with methotrexate (MTX) (20 mg/kg) and treated with alpha-lipoic acid (ALA) after MTX administration, the levels of IL-1β, MDA, GSH, TNF-α, MPO, and sodium potassium-adenosine triphosphatase (Na+/K+ATPase) were improved due to the ALA treatment (104).

In a rat model of amiodarone-induced lung insult, the serum levels of TGF-β1 and TNF-α markedly increased. The treatment with grape seed extract (150 mg/kg) ameliorated oxidative and fibrotic damages in the lung of the amiodarone-treated rats (105). The aqueous extract of caffeic acid phenethyl ester (5 and 10 μmol /day) significantly attenuated the acute lung injury induced by amiodarone (7.5 UI/kg). The activities of myeloperoxidase and SOD enzymes were significantly decreased in the group which was treated with caffeic acid phenethyl ester (106). In the amiodarone-induced lung toxicity, two phenolic acids, ferulic acid and gallic acid, showed a protective effect on the inflammatory biomarkers and oxidative stress (107).

A protective effect of *A. melanocarpa* fruit juice against amiodarone-induced pulmonary toxicity was shown by the reduction of amiodarone-induced direct toxic damage signs, oxidative stress, and fibrosis (108). Treatment with grape seed and Ginkgo biloba (100 mg/kg) ameliorated the histopathological structure, increased the contents of glycogen, and improved the ultrastructure alternations of the lung tissue in the rats exposed to a single dose of amiodarone (40 mg/kg). Grape seed was markedly more effective than Ginkgo biloba in protecting the

TABLE 6 | The possible therapeutic effects of medicinal plants and their derivatives in the SM-induced lung injuries.

Study type	Study design	NP	Doses	Effects	References
In vivo	Macrophage	*Crocus sativus*	225, 450 and 900 μg	↓ DNA damage and MDA ↑ GSH level in macrophage cells exposed to SM.	(87)
	SM-exposed NHEKs	Caffeic acid and quercetin	250 and 100 μM	↓ p38 and p53 phosphorylation, expression levels of COX2 and iNOS and oxidative stress ↑ JNK 1/2 phosphorylation	(88)
	SM-exposed Swiss female mice	*Hippophae rhamnoides* ethanolic Ext	1g/kg; 3 doses; p.o	Protected the body weight loss ↑ GSH, and GSSG levels ↓ MDA w	(89)
	SM-exposed guinea pigs	*Nigella sativa*	0.08 g/day	↓ TR to methacholine, total and differential WBC count	(90)
	SM-exposed guinea pigs	*Nigella sativa*	0.08 g/day	↑ TR and lung in?ammation similar to the effect of dexamethasone	(91)
	SM-exposed Sprague Dawley rats	*Salvia miltiorrhiza Anemarrhena asphodeloides*	30, 60, 120 mg/kg	↑ Survival levels of rats ↓ The SM-induced morphological changes in the testis, small intestine and liver tissues	(92)
Clin	SM-exposed patients	*Nigella sativa* aqueous Ext	0.375 mL/kg	Improved chest wheeze, PFT values	(93)
	SM-exposed patients	*Avena sativa*	%5 w/w	Improved disease severity, quality of life and quality of sleep	(94)
	SM-exposed patients	*Zataria multiflora*	5 and 10 mg/kg	↓ Total and different WBC, MDA ↑ Thiol, CAT, SOD, FVC and PEF	(20)
	SM-exposed patients	*Zataria multiflora*	-	Improved serum levels of various cytokines, chemokine's and PFT values	(95, 96)
	SM-exposed patients	Carvacrol	-	↑ Thiol level, CAT and SOD activity and PE ↓ Total WBC and MDA	(21)
	SM-exposed patients	Carvacrol	1.2 mg/kg/day	↓ Respiratory symptoms, EGF, VEGF, IL-8, IL-2, IL-6 and IL-4 in the serum, ↑ Serum levels of IL-10 and IFN-γ and PFT values	(97, 98)
	SM-exposed patients	Curcumin	-	↓ hs-CRP, CGRP and IL-8 serum levels and DLQI	(99)
	SM-exposed patients	Curcumin	1 g/day	Improved HQoL, pruritus, and antioxidant status	(100)
	SM-exposed patients	Curcuminoids	500 mg	Improved FEV1/FVC, IL-6, IL-8, TGFβ, TNFα, hs-CRP, SP, CGRP and MCP-1. Also	(101)
	SM-exposed patients	Curcuminoids + piperine	1500 and 15 mg/day	Improved HRQoL, GSH, MDA	(102)

NP, natural products; Clin, clinical; Ext, extract; SM, sulfur mustard; NM, nitrogen mustard; TR, tracheal responsiveness; WBC, white blood cells; GSH, glutathione; VEGF, vascular endothelial growth factor; NO, nitric oxide; MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized glutalthione; SOD, superoxide dismutase; CAT, catalase; IL, Interleukin; IFNγ, interferon γ; TNFα, tumor necrosis factor-α; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; SIL-BS, silibinin-bis-succinat; PFT, pulmonary function test; FEV1, volume in one second; MMEF, maximal mid expiratory Low; FVC, forced volume capacity; hs-CRP, high-sensitivity C-reactive protein; CGRP, calcitonin gene-related peptide; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; DLQI, dermatology life quality index; HRQoL, health-related quality of life; SP, substance P.
The up arrow (↑) indicates an increase in the variable, and a down arrow (↓) indicates a decrease.

rats against amiodarone (109). The therapeutic effects of NP in the other noxious agents-induced lung injury are summarized in **Table 7** and **Figure 3**.

DISCUSSION

The induction of various lung disorders due to exposure to NA of the general environment or in the workplace was shown, both in animal and human studies. Among the most important NA, exposure to BLM, Cd, environmental dust, LPS, PQ, SM, and amiodarone can cause lung diseases. Exposure to these NA usually leads to PF and COPD but it also can induce various other lung disorders such as emphysema. The induction of lung disorders due to exposure to NA is accomplished with lung pathological changes, wet/dry lung weight disturbance, oxidative stress in the lung, lung inflammation indicated by increased inflammatory mediators, and immune dysregulation indicated by the changes in the cytokine levels and other immune markers in the BALF or lung tissues.

The results of this review study showed the pharmacological and therapeutic effects of different NP including medicinal plants and their derivatives on lung disorders both in the experimental and clinical studies. The experimental studies indicated the effects of different medicinal plants

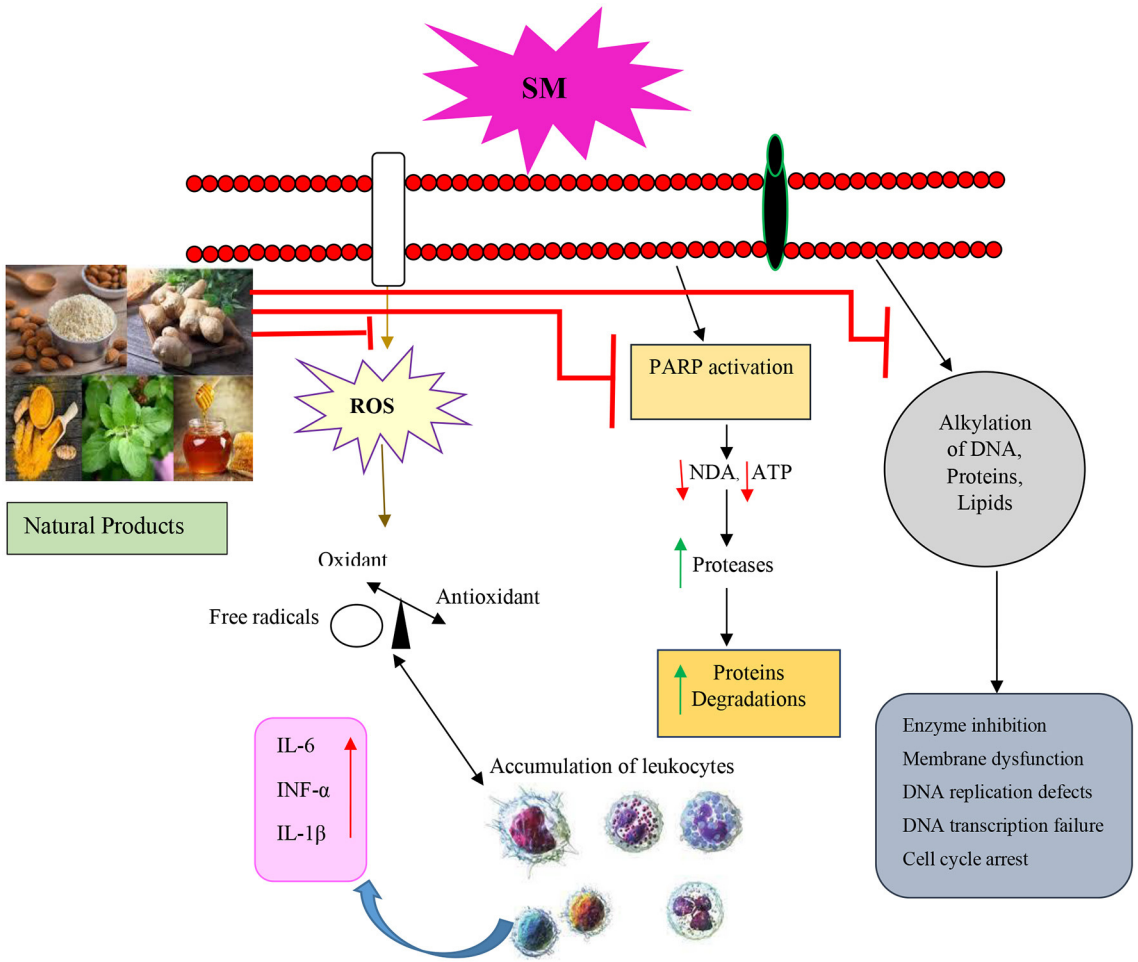

FIGURE 5 | Molecular mechanism of SM-induced toxicity of the relevant studies. SM, Sulfur mustard; ROS, Reactive oxygen species; PARP, Poly (ADP-ribose) polymerase; IL, Interleukin; TNFα, Tumor necrosis factor-α.

TABLE 7 | The effect of other noxious agents-induced lung disorders.

Study type	Study design	NP	Dose	Effects	References
In vivo	CP-exposed mice	*Origanum vulgare*	50, 100, 200 and 400 mg/kg, i.p. for 7 days	↓ IL-6, IL-8, mRNA, protein expression and NF-κB activity	(103)
	MTX-exposed rats	Alpha-lipoic acid	60 mg/kg, i.p. for 16 days	↓ IL-2, IL-4, IL-6, IL-8 ↑ IL-10, IFN-γ, IFN-γ/IL-4 ratio	(104)
	AM-exposed rats	Grape seed Ext	150 mg/kg, i.p for 14 days	↓ Serum levels of IL-4, IL-17A, IFN γ ↑ TGFβ	(105)
	AM-exposed rats	Caffeic acid phenethyl ester	5 and 10 μmol /day, i.p. for 3 weeks	↓ MDA level and the activity of myeloperoxidase ↑ SOD	(106)
	AM-exposed rats	ferulic acid, gallic acid	200 and 100 mg/kg, i.g., for 6 weeks	Improved inflammatory biomarkers and oxidative stress	(107)
	AM-exposed rats	*A. melanocarpa* fruit juice	5 and 10 mL/kg, orally for 10 days	↓ Oxidative stress, inflammation, and fibrosis	(108)
	AM-exposed rats	Grape seed and ginkgo biloba	100 mg/kg, i.g. for 8 weeks	↓ Antioxidant's and histopathological structure ↑ The contents of glycogen	(109)

NP, natural products; Ext, extract; CP, cyclophosphamide; MTX, methotrexate; AM, Amiodarone; MDA, Malondialdehyde; SOD, superoxide dismutase; IL, Interleukin; NF-κB, Nuclear factor kappa B; IFN-γ, Interferon gamma; TGFβ, Transforming growth factor beta; i.g., intragastrically; i.p., intraperitoneal.
The up arrow (↑) indicates an increase in the variable, and a down arrow (↓) indicates a decrease.

including *Aloe vera, Anemarrhena asphodeloides, Avena sativa, Crocus sativus, Curcuma longa, Dioscorea batatas, Glycyrrhiza glabra, Gentiana veitchiorum, Gentiopicroside, Houttuynia cordata, Hibiscus sabdariffa, Hochu-ekki-to, Hippophae rhamnoides, Juglans regia, Melanocarpa fruit juice, Mikania glomerata, Mikania laevigata, Moringa oleifera, Myrtus communis L., Lamiaceae, Myrtle, Mosla scabra leaves, Nectandra leucantha, Nigella sativa, Origanum vulgare L, Pulicaria petiolaris, Paulownia tomentosa, Pomegranate seed oil, Raphanus sativus L. var niger, Rosa canina, Schizonepeta tenuifolia,Thymus vulgaris, Taraxacum mongolicum, Tribulus Terrestris, Telfairia occidentalis, Taraxacum officinale, TADIOS, Xuebijing, Viola yedoensis, Zataria multiflora, Zingiber officinale, Yin-Chiao-San,* and their derivatives on the lung injury induced by NA. The treatment with NP in NA-induced lung disorders ameliorated all lung changes induced by NA such as lung pathological changes, lung oxidative stress, lung inflammation, and immune dysregulation. In clinical studies, the effects of medicinal plants and their derivatives such as *Avena sativa, Curcuma longa, Nigella sativa,* and *Zataria multiflora* on SM-induced lung disorders were shown by reducing respiratory symptoms, oxidative stress markers, inflammatory mediators, and cytokine levels as well as increasing PFT.

The results of this review study showed the possible therapeutic effects of various NP on NA-induced lung disorders by amelioration of various features of lung injury. However, further clinical studies, especially on the effect of NP on lung diseases induced by BLM, Cd, environmental dust, LPS, PQ, and other noxious agents are needed to support the therapeutic effect on NP on NA-induced lung disorders for clinical practice purposes.

AUTHOR CONTRIBUTIONS

SS, SB, and MK prepared the first draft of the manuscript and helped in the revision of the final version of the manuscript. MB designed the study, critically edited, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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GLOSSARY

Ach: acetylcholine
AHR: hyperresponsiveness
BALF: Broncho-alveolar lavage fluid
CAT: catalase
CGRP: calcitonin gene-related peptide
COPD: Chronic obstructive pulmonary disease
CW: Chemical warfare
DLQI: Dermatology Life Quality Index
DOX: Doxycycline
EPO: peroxidase
FDA: Food and Drug Administration
FEV1: Forced expiratory volume in the first second
FVC: Forced vital capacity
GSH: glutathione
HDAC: Histone deacetylase complex
hPBMCs: human peripheral blood mononuclear cells
HRQoL: health-related quality of life
hs-CRP: high-sensitivity C-reactive protein
IFNγ: Interferon-gamma
Ig: immunoglobulin
IL: Interleukin
iNOS: induced nitric oxide synthase
IPF: idiopathic pulmonary fibrosis
LPS: Lipopolysaccharide
LTC_4: leukotriene C_4
MCHC: mean corpuscular hemoglobin concentration
MCP-1: monocyte chemotactic protein-1
MCV: mean corpuscular volume
MDA: malondialdehyde
mMCP-1: mouse mast cell protease-1
MMEF: maximum mid-expiratory flow
MMPs: matrix metalloproteinase
NAC: N-Acetylcysteine
NFkB: nuclear transcription factor
NK cells: natural killer cells
NO: oxide production
OVA: Ovalbumin
$PaCO_2$: arterial blood carbon dioxide
PE: pulmonary embolism
PEF: peak expiratory flow
PF: pulmonary fibrosis
PFT: Pulmonary function tests
PQ: paraquat
QoL: quality of life
ROS: Reactive oxygen species
SaO_2: arterial blood oxygenation saturation
SIL-BS: silibinin-bis-succinat
SM: Sulfur mustard
SOD: superoxide dismutase
TAT: Thrombin anti-thrombin
TFPI: Tissue factor pathway inhibitor
TGF-β1: transforming growth factor beta1
TNFα: Tumor necrosis factor-α
tPA: Tissue plasminogen activator
TR: tracheal responsiveness
TSM: tracheal smooth muscle
UV: ultraviolet
WBC: White blood cells.

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SYSTEMATIC REVIEW
published: 20 May 2022
doi: 10.3389/fnut.2022.880014

Effects of Synbiotics, Probiotics, and Prebiotics on Liver Enzymes of Patients With Non-alcoholic Fatty Liver Disease: A Systematic Review and Network Meta-Analysis

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Background: A systematic review and network meta-analysis was primarily conducted to compare the effects of synbiotics, probiotics, and prebiotics on aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Moreover, their effects on body mass index (BMI), waist circumference (WC), lipid profile, fasting blood sugar (FBS), and homeostatic model assessment-insulin resistance (HOMA-IR) of patients with non-alcoholic fatty liver disease (NAFLD) were investigated and analyzed as secondary outcomes.

Methods: The randomized controlled trials (RCTs), limited to the English language, were searched through PubMed, the Web of Science, Embase, CLINAHL Plus, and the Cochrane Library from inception to February 2, 2022. The eligible studies were reviewed and their risk-of-bias and heterogeneity were assessed. Both direct and indirect evidence were assembled using a random-effects model. The effects of the intervention were presented as weighted mean differences (WMD) with 95% confidence interval (95% CI).

Results: Of 3,864 identified records, a total of 1,389 patients with NAFLD from 26 RCTs were included in the analyses. Among these, 241 were diagnosed with non-alcoholic steatohepatitis. The quality assessment reported a moderate risk of bias from most studies. Among adult patients with NAFLD, when compared with placebo, synbiotics provided the largest effect on reductions of AST (−12.71 IU/L; 95% CI: −16.95, −8.47), WC (−2.26 cm; 95% CI: −2.98, −1.54), total cholesterol (−22.23 mg/dl; 95% CI:

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Edited by:
Pietro Vajro, University of Salerno, Italy

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Michele Malaguarnera, Principe Felipe Research Center (CIPF), Spain

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *20 February 2022*
Accepted: *13 April 2022*
Published: *20 May 2022*

Citation:
Kanchanasurakit S, Kositamongkol C, Lanoi K, Nunta M, Saetuan T, Chaiyakunapruk N, Saokaew S and Phisalprapa P (2022) Effects of Synbiotics, Probiotics, and Prebiotics on Liver Enzymes of Patients With Non-alcoholic Fatty Liver Disease: A Systematic Review and Network Meta-Analysis. Front. Nutr. 9:880014. doi: 10.3389/fnut.2022.880014

−29.55, −14.90), low-density lipoproteins (−17.72 mg/dl; 95% CI: −25.23, −10.22), and FBS (−6.75 mg/dl; 95% CI: −10.67, −2.84). Probiotics lowered ALT (−14.46 IU/L; 95% CI: −21.33, −7.59) and triglycerides (−20.97 mg/dl; 95% CI: −40.42, −1.53) the most. None had significant impact on BMI, high-density lipoproteins, and HOMA-IR changes.

Conclusion: Synbiotics and probiotics are likely to be the most potential effective treatments for AST and ALT reduction in adult patients with NAFLD, respectively. Although liver enzymes cannot exactly define the severity of NAFLD, unlike the results from biopsy or imaging tests, they are important indicators that can monitor the status of the disease and provide benefits for clinical management.

Systematic Review Registration: [https://www.crd.york.ac.uk/prospero/display_record.php?ID], identifier [CRD42020200301].

Keywords: non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), meta-analysis, synbiotic, probiotic, prebiotic, liver enzymes

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic fatty liver disease found in approximately 25% of the population worldwide (1). The incidence of NAFLD varied from 19 to 86 per 1,000 person-year (2). Patients with a metabolic syndrome are considered a high-risk group facing NAFLD (3). NAFLD covers both non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). NAFL is defined as the presence of hepatic steatosis without hepatocellular injury, whereas NASH is a NAFL with hepatocellular injury which may involve fibrosis. NAFLD can lead to other severe diseases such as cirrhosis, liver failure, liver cancer, and non-liver-related conditions, e.g., cardiovascular diseases, chronic kidney disease, etc. (3–7). In order to prevent complications and treat the disease, the etiology and pathophysiology of it should be understood. The mechanism of NAFLD involves various pathways, including gut microbiota. Its association with liver disease has been demonstrated through animal models. The samples that were intervened with antimicrobials and controls were compared to investigated gut microbial metabolic phenotypes. Notably, more than 200 microbial-related metabolites were identified in fingerprints of urine and feces of animals exposed to antimicrobials (8). Some of microbiota-derived metabolites may trigger hepatic metabolism alteration and inflammatory reaction (9). Although the issue on a relationship between liver and intestine is not fully clarified, various studies showed that dysbiosis results in malfunction of hepatic fat deposition (10, 11).

Currently, the only treatments for NAFLD recommended in the guidelines (3, 5, 6) are lifestyle modifications including diet control, exercise, and weight reduction. These methods, especially weight reduction, are hard to achieve and maintain. All other pharmacological treatments are reserved for patients with biopsy-proven NASH and liver fibrosis. According to previous studies, numerous pathophysiologic mechanisms relating the gut microbiome and NAFLD have been indicated, including the dysbiosis-induced dysregulation of the gut endothelial barrier function that allows for the translocation of bacterial components, leading to the accumulation fat and hepatic inflammation (12, 13). Thus, using microbial therapy, including synbiotics, probiotics, and prebiotics, may help to restore the unbalanced microbiomes. Also, as proven by many randomized controlled trials (RCTs), microbial therapy is classified as one of the non-pharmacological treatments which may provide the clinical benefit of slowing down the progression of NAFLD. Nevertheless, the recommendation of using these agents in clinical practice is still inconclusive (5). The primary objective of this study was to compare the effects among synbiotics, probiotics, and prebiotics by focusing on the modification of liver enzymes, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), in patients with NAFLD. Moreover, for the secondary objectives, we explored the effects of microbial therapies on body mass index (BMI), waist circumference (WC), lipid profile, fasting blood sugar (FBS), and homeostatic model assessment-insulin resistance (HOMA-IR) in patients with NAFLD.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; FBS, fasting blood sugar; HDL, high-density lipoproteins; HOMA-IR, homeostatic model assessment-insulin resistance; LDL, low-density lipoproteins; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PRISMA, preferred reporting items for systematic reviews and meta-analyses; RCTs, randomized controlled trails; SUCRA curves, surface under the cumulative ranking curves; TC, total cholesterol; TG, triglycerides; WC, waist circumference; WMD, weighted mean difference.

METHODS

Protocol and Registration

A systematic review and network meta-analysis (NMA) were performed and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) extension statement for NMA (14). This study was registered with the trial registration

number CRD42020200301 under the international prospective register of systematic reviews (PROSPERO: www.crd.york.ac.uk/PROSPERO).

Eligibility Criteria

The RCTs that included participants with NAFLD and that which compared the effects of synbiotics, probiotics, or prebiotics against each other or with a placebo were included in the analysis. The diagnosis method of NAFLD was not restricted only to liver biopsy. Reliable imaging techniques such as ultrasound, transient elastography (Fibroscan), and proton density fat fraction on magnetic resonance imaging (MRI-PDFF) were also acceptable to include in the analyses. The primary interested effects of the interventions were the reduction of AST and ALT since they were basic biomarkers that could be used to monitor the severity of the disease. Furthermore, the studies that showed the results in other secondary outcomes that consisted of BMI, WC, lipid profile, FBS, and/or HOMA-IR were included. Our protocol had no limitations on the length of follow-up period for each trial in the inclusion criteria. We excluded studies that consisted of only abstracts presented at conferences, along with editorials, any type of reviews, and meta-analyses.

Information Sources and Search Strategy

We searched for relevant published articles from five electronic databases, namely, PubMed, the Web of Science, Embase, CLINAHL Plus, and the Cochrane Library, from the inception of the databases to February 2, 2022. The keywords included "synbiotic," "probiotic," "Lactobacillus*," "Bifidobacterium*," "*Enterococcus faecium,*" "*Streptococcus thermophiles,*" "*Bacillus clausii,*" "*Saccharomyces cerevisiae,*" "*Saccharomyces boulardii,*" "*Escherichia coli* Nissle 1917," "prebiotic," "FOS," "Fructooligosaccharide*," "Fructo-oligosaccharide*," "GOS," "Galactooligosaccharide*," "Galacto-oligosaccharide*," "XOS," "Xylooligosaccharide*," "Xylo-oligosaccharide*," "TOS," "Transgalactooligosaccharide*," "*Trans*-galactooligosaccharide*," "Inulin," "Lactitol," "Lactulose," "Lactosucrose," "Soy oligosaccharide*," "NAFLD," "NASH," "Fatty liver*," "Non-alcoholic fatty liver disease," "Non-alcoholic fatty liver disease," "Non-alcoholic fatty liver disease," "Non-alcoholic fatty liver*," and "Non-alcoholic steatohepati*." Bibliographic lists of related articles were also explored. The complete search strategy is provided in the **Supplementary Appendix 1**.

Study Selection

Four investigators independently screened the titles and the abstracts of the retrieved citations to identify potentially eligible studies. Only English articles were included. Any conflict was resolved through a subsequent team discussion and an expert consultation. Adults and children with the disease have different characteristic (15). Also, the interventions might act differently regarding age of the patients and there was a limited number of studies in children. Our network meta-analysis would only include adult patients with NAFLD. The data from studies involving with pediatric patients would be extracted, summarized, and reported descriptively.

Data Extraction and Study Appraisal

Each potentially relevant study was accessed in a full-text manner against the eligible criteria and then adopted in a data-extraction process by the same four investigators. Any inconsistent opinion along this process was settled through a discussion. We extracted the data, including the study design, the details of the interventions, such as the regimens and treatment durations, the study size, and the population characteristics and treatments' outcomes, i.e., the reported mean and/or standard deviation (SD) values of age, AST, ALT, BMI, WC, total cholesterol (TC), triglycerides (TG), low-density lipoproteins (LDL), high-density lipoproteins (HDL), FBS, and HOMA-IR, which were the representative parameters of the effects of the interventions. When mean and/or SD were not reported, continuous outcomes were estimated by using the reported statistics (e.g., median, interquartile range, etc.) (16). Furthermore, we had contacted study authors to acquire the missing outcomes of pertinent studies. However, if the authors did not respond within a month, the study was, then, excluded from the analyses.

Risk-of-Bias Assessments

The risk of bias in each individual study was assessed independently by four investigators using the instructions from the revised Cochrane risk-of-bias tool for randomized trials (RoB 2.0) (17). This tool addresses specific bias domains, including methods for generating the random sequence, allocation concealment, blinding of participants and investigators, blinding of the outcome assessment, incompleteness of the outcome data, and selective outcome reporting. Each item is adjudicated within each study, and the results are represented in the risk-of-bias summary graph and risk-of-bias summary itself. The adjudication of the risk of bias was achieved by answering pre-specified questions about the methods reported by each study in relation to the risk domain, such that the conclusion consists of a low risk of bias, an unclear risk of bias, or a high risk of bias. All disagreements among four investigators were resolved by consensus or with the consultation of the expert.

Outcomes and Definitions

The primary outcomes were the effects of synbiotics, probiotics, and prebiotics on the reduction of the AST and ALT levels in patients with NAFLD. The secondary outcomes were the effects of synbiotics, probiotics, and prebiotics on patients' BMI, WC, lipid profiles (i.e., TC, TG, LDL, and HDL), FBS, and HOMA-IR. The definitions of NASH that would be later used to classify patients for sensitivity analyses were given according to what was defined in the included studies. Those studies which did not obviously specify that they included patients with NASH in the trial would be categorized as the studies which were conducted in patients with NAFLD (5).

Synthesis and Statistical Analysis

First, we conducted pairwise meta-analyses by using the DerSimonian and Laird random effects model (18) to estimate

the outcomes. Then, we reported them in weighted mean differences and 95% confidence intervals (95% CIs). We assessed the statistical heterogeneity in each pairwise comparison by using *I*-squared statistic and Chi-squared statistic. Heterogeneity was indicated when the *p*-value was less than 0.1. We also performed a random-effects NMA to combine direct and indirect evidence of all relative options effects by using the *network* command in the Stata Statistical Software: Release 16 (StataCorp LP, College Station, TX, United States) and the methods of the NMA described by Lu and Ades (19). To rank the options hierarchy of competing for intervention in the NMA, the rankogram, the surface under the cumulative ranking (SUCRA) curves, the mean ranks, and the league tables were used (20). Network inconsistency between direct and indirect evidence was assessed using a global inconsistency test (p-value $\geq$ 0.05 indicated consistency). We also used a comparison-adjusted funnel plot to detect any small-study effects and publication bias.

In addition, to determine whether the results were affected by the variety in the studies' characteristics, we also performed sensitivity analyses, focusing on the above-mentioned outcomes of synbiotics, probiotics, and prebiotics. Multiple sensitivity analyses were performed to assess the robustness of the findings. These were based on (1) the subgroup of participants with liver biopsy-proven NASH and (2) the duration of treatment that were less than and at least 12 weeks in patients with NAFLD and patients with NASH. We use two-sided statistical testing with p-values < 0.05 to indicate the statistical significance.

RESULTS

A total of 3,864 articles were identified from PubMed, the Web of Science, Embase, CLINAHL Plus, and the Cochrane Library. Seven-hundred and forty-nine duplicated articles were removed. The full texts of 159 articles were assessed and 134 studies were excluded due to the reasons described in **Figure 1**. In addition, 1 RCT identified from reference lists was included. Ultimately, we obtained 26 eligible articles: 22 RCTs were performed focusing on the adult patients with NAFLD (21–42), and the other 4 RCTs were performed concerning the pediatric patients with NAFLD (43–46; **Table 1**). The study-selection-process flow is summarized in the PRISMA flow diagram (**Figure 1**).

Characteristics and Quality of the Included Studies

The included studies are comprised of 1,389 participants with NAFLD (1,230 adults, age $\geq$ 18 years and 159 children, age 6–18 years). Of 1,230 adults with NAFLD, 241 were confirmed as NASH by either liver biopsy or ultrasound. Liver biopsy was done in 8 of 26 RCTs (21–23, 29, 36, 39, 42, 44). Others were diagnosed the disease by ultrasound (13 studies), Fibroscan (4 studies), and MRI-PDFF (1 study). Four studies that involved pediatric patients (age $<$ 18 years) focused only on the effects of probiotics (43–46). Otherwise, the studies involving adult patients focused on probiotics, prebiotics, or synbiotics. The probiotics assessed in this systematic review

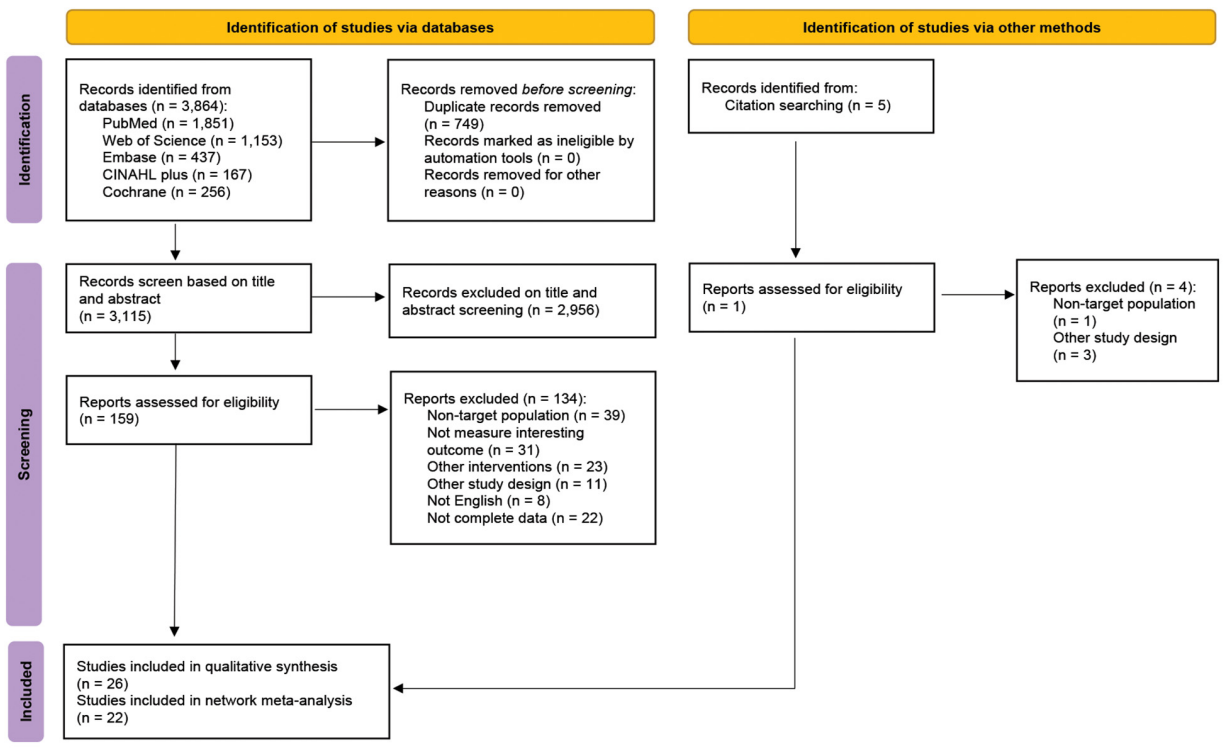

FIGURE 1 | Preferred reporting items for systematic review and meta-analyses (PRISMA) flow diagram.

TABLE 1 | Details of included trials.

ID	First author, publication year	Country	Studied population	Age (years)	Diagnosis	Study design	Interventions	Sample size	Treatment duration (weeks)	Outcomes
1	Aller et al. (21)	Spain	Adults, NAFLD	29–60	Liver biopsy	Double-blind RCT	Probiotics	14	12	AST, ALT, BMI, TC, TG, LDL, HDL, FBS, HOMA-IR
							Placebo	14		
2	Vajro et al. (43)	Italy	Children, NAFLD	11 ± 2	Ultrasound	Pilot double-blind RCT	Probiotics	10	8	ALT, BMI
							Placebo	10		
3	Malaguarnera et al. (22)	Italy	Adults, NASH	30–65	Liver biopsy	Double-blind RCT	Synbiotics	34	24	AST, ALT, BMI, TC, TG, LDL, HDL, FBS, HOMA-IR
							Placebo	32		
4	Wong et al. (23)	Hong Kong	Adults, NASH	18–70	Liver biopsy	Open-label RCT	Probiotics	10	24	AST, ALT, BMI, WC, TC, TG, LDL, HDL, FBS
							Placebo	10		
5	Alisi et al. (44)	Italy	Children, NAFLD	6–12	Liver biopsy	Double-blind RCT	Probiotics	22	16	ALT, BMI, TG, HOMA-IR
							Placebo	22		
6	Eslamparast et al. (24)	Iran	Adults, NAFLD	≥18	Fibroscan	Double-blind RCT	Synbiotics	26	28	AST, ALT, HOMA-IR
							Placebo	26		
7	Miccheli et al. (45)	Italy	Children, NAFLD	6–12	Ultrasound	Double-blind RCT	Probiotics	15	16	AST, ALT, BMI, TC, TG, LDL, HDL, FBS, HOMA-IR
							Placebo	16		
8	Sepideh et al. (25)	Iran	Adult, NAFLD	18–65	Ultrasound	Double-blind RCT	Probiotics	21	8	FBS, HOMA-IR
							Placebo	21		
9	Akbarzadeh et al. (26)	Iran	Adults, NAFLD	18–77	Fibroscan	Double-blind RCT	Prebiotics	38	10	AST, ALT, BMI, WC
							Placebo	37		
10	Asgharian et al. (27)	Iran	Adults, NAFLD	18–60	Ultrasound	Double-blind RCT	Synbiotics	38	8	AST, ALT, BMI, WC
							Placebo	36		
11	Ekhlasi et al. (28)	Iran	Adults, NAFLD	25–64	Ultrasound	Double-blind RCT	Synbiotics	15	8	AST, ALT, BMI, WC, TC, TG, LDL, HDL, FBS, HOMA-IR
							Placebo	15		
12	Ferolla et al. (29)	Brazil	Adults, NASH	25–74	Liver biopsy	Double-blind RCT	Synbiotics	27	12	AST, ALT, BMI, WC, TC, TG, LDL, HDL, FBS
							Placebo	23		
13	Asgharian et al. (30)	Iran	Adults, NAFLD	18–60	Ultrasound	Double-blind RCT	Synbiotics	38	8	BMI, WC, TC, TG, LDL, HDL, FBS
							Placebo	36		
14	Behrouz et al. (31)	Iran	Adults, NAFLD	20–60	Ultrasound	Double-blind RCT	Probiotics	30	12	BMI, FBS, HOMA-IR
							Prebiotics	29		
							Placebo	30		
15	Famouri et al. (46)	Iran	Children, NAFLD	10–18	Ultrasound	Triple-blind RCT	Probiotics	32	12	AST, ALT, WC, TC, TG, LDL, HDL
							Placebo	32		
16	Javadi et al. (32)	Iran	Adults, NAFLD	20–60	Ultrasound	Double-blind RCT	Synbiotics	17	12	AST, ALT, BMI
							Probiotics	20		
							Prebiotics	19		
							Placebo	19		
17	Javadi et al. (33)	Iran	Adults, NAFLD	20–60	Ultrasound	Double-blind RCT	Synbiotics	17	12	BMI, WC, TC, TG, LDL, HDL, FBS, HOMA-IR
							Probiotics	20		
							Prebiotics	19		
							Placebo	19		
18	Manzhalii et al. (34)	Ukraine	Adults, NASH	30–60	Ultrasound and elevated hepatic enzymes	Non-blinded RCT	Synbiotics	38	12	AST, ALT, BMI, TC, TG, LDL, FBS
							Placebo	37		
19	Mofidi et al. (35)	Iran	Adults, NAFLD	≥18	Fibroscan	Double-blind RCT	Synbiotics	21	28	AST, ALT, TC, TG, LDL, HDL, FBS, HOMA-IR
							Placebo	21		

(Continued)

TABLE 1 | (Continued)

ID	First author, publication year	Country	Studied population	Age (years)	Diagnosis	Study design	Interventions	Sample size	Treatment duration (weeks)	Outcomes
20	Monem et al. (36)	Egypt	Adults, NASH	44 ± 6	Liver biopsy	RCT	Probiotics	15	4	AST, ALT
							Placebo	15		
21	Bakhshimoghaddam et al. (37)	Iran	Adults, NAFLD	≥18	Ultrasound	Open-label RCT	Synbiotics	34	24	AST, ALT, HOMA-IR
							Placebo	34		
22	Ahn et al. (38)	South Korea	Adults, NAFLD	19–75	MRI-PDFF	Double-blind RCT	Probiotics	30	12	AST, ALT, BMI, TC, TG, HDL, FBS, HOMA-IR
							Placebo	35		
23	Duseja et al. (39)	India	Adults, NAFLD	≥18	Liver biopsy	Double-blind RCT	Probiotics	17	48	AST, ALT
							Placebo	13		
24	Abhari et al. (40)	Iran	Adults, NAFLD	18–75	Fibroscan	Double-blind RCT	Synbiotics	22	12	AST, ALT, BMI, WC, TC, TG, LDL, HDL, FBS, HOMA-IR
							Placebo	24		
25	Behrouz et al. (41)	Iran	Adults, NAFLD	20–60	Ultrasound	Double-blind RCT	Probiotics	30	12	AST, ALT, BMI, WC, TC, TG, LDL, HDL, FBS
							Prebiotics	29		
							Placebo	30		
26	Chong et al. (42)	United Kingdom	Adults, NAFLD	25–70	Liver biopsy	Double-blind RCT	Probiotics	19	10	AST, ALT, TC, TG, LDL, HDL, HOMA-IR
							Placebo	16		

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, fasting blood sugar; HDL, high-density lipoproteins; HOMA-IR, homeostatic model assessment-insulin resistance; LDL, low-density lipoproteins; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; MRI-PDFF, proton density fat fraction on magnetic resonance imaging; RCT, randomized controlled trial; TC, total cholesterol; TG, triglycerides; WC, waist circumference.

included *Lactobacillus* spp., *Bifidiobacterium*spp., *Streptococcus thermophilies*, and *Pediococcuspentosaceus*. Included prebiotics were fructooligosaccharides, inulin, and oligofructose. Synbiotics were defined as interventions when they contained both probiotics and prebiotics. Details of the general characteristics of all included microbial therapy interventions are given in the **Supplementary Appendix 2**. The duration of treatment varied between 4 and 48 weeks. The details regarding the interventions and the baseline characteristics of included patients in each study are shown in the **Supplementary Appendices 3, 4**, respectively. The networks of all option comparisons for primary and secondary outcomes were illustrated in **Figure 2** and **Supplementary Appendix 5**, respectively. A quality assessment of the risk of bias revealed some concern in most of the studies. There were 6 RCTs considered as having a low risk of bias (21, 29, 32, 33, 45, 46) and 3 RCTs had a high risk of bias (26, 34, 37), while the rest (17 studies) was categorized as moderate-risk studies (**Supplementary Appendix 6**). All data extracted for systematic review and network meta-analyses were detailed in **Supplementary Appendices 7, 8**.

Pediatric Patients With Non-alcoholic Fatty Liver Disease

Primary Outcomes

Aspartate Aminotransferase and Alanine Aminotransferase [4 Studies]

Only two studies conducted by Miccheli et al. (45) and Famouri et al. (46) investigated the effect of probiotics on AST change in pediatric patients. Both studies indicated AST level was significantly reduced after the treatment as compared to the level of the enzyme at baseline. Moreover, the effect on AST lowering were significantly greater in probiotics group than placebo group. All 4 studies focusing on pediatric patients with NAFLD (43–46) evaluated the ALT change, but 2 out of 4 reported that probiotics might not be capable to reduce ALT level compared with a placebo (44, 45).

Secondary Outcomes

Body Mass Index [4 Studies]

Half of the studies showed that probiotics did not lower the BMI of the pediatric patients with NAFLD (43, 46). The other two studies (44, 45), which conducted in the same cohort of patients, indicated that BMI of the intervention group was significantly lowered at the end of the trial.

Waist Circumference [1 Study]

Only one study by Famouri et al. (46) measured the effect of probiotics on WC change in children with obesity who were diagnosed with NAFLD. They reported that probiotics had a significant effect on WC reduction, as compared to a placebo.

Lipid Profile [Total Cholesterol: 2 Studies, Triglycerides: 3 Studies, Low-Density Lipoproteins: 2 Studies, and High-Density Lipoproteins: 2 Studies]

The study by Miccheli et al. (45) pointed out that probiotics did not have an impact on TC. In addition, even if Famouri et al. (46) reported that their intervention could significantly reduce TC, a

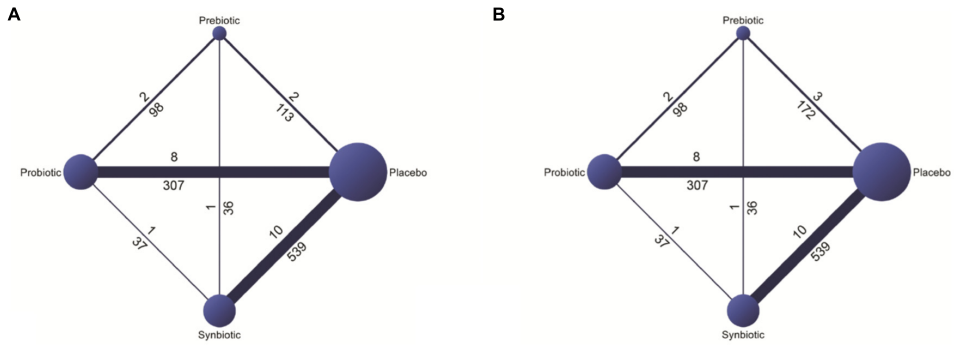

FIGURE 2 | Networks of all options comparisons for reduction in **(A)** aspartate aminotransferase (AST) and **(B)** alanine aminotransferase (ALT).

median baseline TC level of the control group was significantly lower than the probiotics group.

All tree studies by Alisi et al. (44), Miccheli et al. (45), and Famouri et al. (46) concluded that probiotics did not provide any additional benefit over a placebo in TG reduction among obese children with NAFLD.

Micheli et al. (45) did not see the effect of probiotics on LDL lowering. Nonetheless, the median LDL of the intervention group of the study by Famouri et al. (46) was significantly lower at the end of the trial than the value at the baseline. Moreover, the magnitude of LDL reduction in the intervention group was larger than the control group.

Both trials by Miccheli et al. (45) and Famouri et al. (46) did not observe any significant change in HDL level of the participants.

Fasting Blood Sugar and Homeostatic Model Assessment-Insulin Resistance [1 Study]

Only one of four included studies in children investigated the effect of probiotics on diabetes-related outcomes. Miccheli et al. (45) could not conclude any benefit of probiotics based on the outcomes of the trial.

Adults Patients With Non-alcoholic Fatty Liver Disease

Primary Outcomes

Aspartate Aminotransferase and Alanine Aminotransferase

Adults With Non-alcoholic Fatty Liver Disease [18 Studies]. Our NMA found that when compared with a placebo, all three interventions significantly decreased the levels of both AST and ALT. Synbiotics provided the best effect on AST. They reduced the AST by −12.71 IU/L (95% CI: −16.95, −8.47). The second and third best interventions were probiotics (AST: −11.62 IU/L; 95% CI: −17.15, −6.09) and prebiotics (AST: −8.42 IU/L; 95% CI: −16.27, −0.56), respectively (**Figure 3A**). When the interventions were compared against each other, there was no specific intervention that could be considered better than another (**Supplementary Appendix 9**).

Probiotics provided the most impact on ALT reduction when compared with placebo (ALT: −14.46 IU/L; 95% CI: −21.33, −7.59). Synbiotics and prebiotics significantly reduced ALT by −12.60 IU/L (95% CI: −17.22, −7.98) and −13.65 IU/L (95% CI: −22.69, −4.61), respectively (**Figure 3B**). When compared among interventions, the statistical difference did not show in any pair of interventions (**Supplementary Appendix 9**).

When interventions, including placebos, were compared with one another, as shown in SUCRA, synbiotics had the highest likelihood of being ranked first in the analysis of the effects on AST reduction, followed by probiotics, prebiotics, and placebo (**Supplementary Appendix 10**). Contrastingly, SUCRA showed that probiotics had the highest likelihood of being ranked first for ALT reduction, followed by prebiotics and synbiotics. The results indicated no possibility that placebo would provide better outcomes than other interventions (**Supplementary Appendix 10**).

Adults With Biopsy-Proven Non-alcoholic Steatohepatitis [4 Studies]. In the subgroup of patients with biopsy-proven NASH, synbiotics provided the best effects, in terms of AST reductions when compared to placebo (−22.34 IU/L; 95% CI: −38.02, −6.67). However, when synbiotics were compared against probiotics, no significance difference of AST reductions was seen. Probiotics had the most impact on ALT reduction in this subgroup. It significantly decreased more ALT than both placebo (−34.10 IU/L; 95% CI: −46.43, −21.77) and synbiotics (−17.70 IU/L; 95% CI: −34.61, −0.79). Synbiotics also significantly reduced ALT in patients with biopsy-proven NASH. When compared with a placebo, they reduced ALT by −16.40 IU/L (95% CI: −27.96, −4.83). More details were shown in **Supplementary Appendices 13, 14**.

Further results of the sensitivity analyses, which were restricted to the effects of interventions in the studies in which durations of treatments were less than 12 weeks and at least 12 weeks, separately, are presented in **Supplementary Appendices 13, 14**. Most of the sensitivity analyses showed similar results to the main analyses. Particularly, the interventions could significantly reduce hepatic enzymes when compared with placebo. However, there was no specific intervention that could considered better than the others in terms of liver enzymes reductions. Prebiotics provided the lowest magnitude of effect on AST reduction. All three microbial treatments did not provide significant effect on AST level

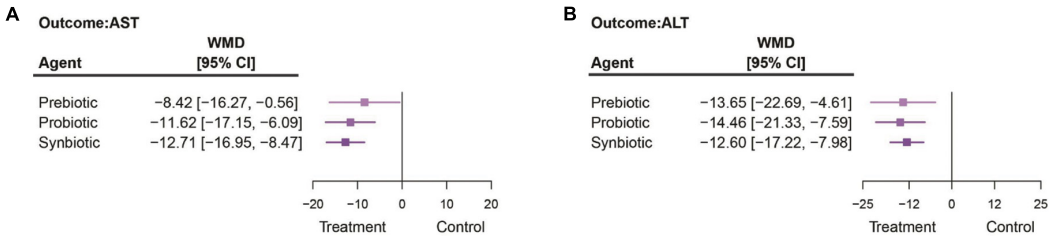

FIGURE 3 | The summarized results of using synbiotics, probiotics, and prebiotics for the reduction in **(A)** AST and **(B)** ALT.

among patients with NAFLD compared to a placebo unless the treatments were given at least 12 weeks. Both probiotics and synbiotics significantly reduced AST in patients with NASH who were treated for not less than 12 weeks, but only synbiotics could significantly decrease ALT in this subgroup.

Secondary Outcomes

Body Mass Index [13 Studies]

The pooled results showed that the interventions did not have a significant impact on BMI in adult patients with NAFLD, as shown in **Figure 4A** and **Supplementary Appendix 9**. The results from subgroup among patients with biopsy-proven NASH also showed no statistically different effect when a comparison was made between interventions and placebo. The sensitivity analyses, including the analyses among adult patients who were treated for not less than 12 weeks, revealed no statistical differences between all pairs of options. The details are shown in **Supplementary Appendix 15**.

Waist Circumference [8 Studies]

Among three microbial therapies, only synbiotics significantly reduced the WC of adults with NAFLD (synbiotics vs. placebo: −2.26 cm; 95% CI: −2.98, −1.54 and synbiotics vs. probiotics: −1.98 cm; 95% CI: −3.84, −0.11), as shown in **Figure 4B** and **Supplementary Appendix 9**. Nonetheless, this statistically significant result was not seen in any sensitivity analysis. Further details are shown in **Supplementary Appendix 16**.

Lipid Profile [Total Cholesterol: 13 Studies, Triglycerides: 13 Studies, Low-Density Lipoproteins: 11 Studies, and High-Density Lipoproteins: 12 Studies]

Synbiotics had significant effects on TC, TG, and LDL reduction when compared with a placebo (TC: −22.23 mg/dl; 95% CI: −29.55, −14.90; TG: −12.77 mg/dl; 95% CI: −20.88, −4.66; and LDL: −17.72 mg/dl; 95% CI: −25.23, −10.22), as shown in **Figures 4C–E**. When compared among the interventions, there was no specific one that could be considered significantly better than others (**Supplementary Appendix 9**). Prebiotics also significantly decreased TC by −16.42 mg/dl (95% CI: −31.57, −1.27) and LDL by −15.88 mg/dl (95% CI: −29.34, −2.42) more than placebo. Probiotics provided the largest impact on TG reduction (−20.97 mg/dl, 95% CI: −40.42, −1.53), but did not have an effect on other parameters related to patients' lipid profile. Moreover, this NMA showed that neither prebiotics, probiotics, nor synbiotics had an effect on increasing the HDL level (**Figure 4F**). The results are shown in **Supplementary Appendix 9**.

Surprisingly in the sensitivity analysis involving the biopsy-proven NASH, probiotics provided a significant reduction of the HDL level (−3.86 mg/dl; 95% CI: −7.25, −0.47), but this only involved one study. Nevertheless, the other microbial therapies did not show the significant effects on HDL, TC, TG, and LDL in patients with biopsy-proven NASH. The analyses of the studies that treated the patients for at least 12 weeks demonstrated that when compared to a placebo, synbiotics could significantly reduce the TC, TG, and LDL levels among patients with NAFLD (TC −18.04 mg/dl; 95% CI: −33.00, −3.09; TG: −16.16 mg/dl; 95% CI: −31.42, −0.90; and LDL: −14.85 mg/dl; 95% CI: −26.31, −3.38). Probiotics significantly reduced TG by −25.34 mg/dl (95% CI: −46.42, −4.27) and LDL by −11.88 mg/dl (95% CI: −21.69, −2.08) when compared to a placebo. When treated with prebiotics for at least 12 weeks, the pooled outcomes showed that in adult patients with NAFLD, prebiotics could reduce TC by −16.04 mg/dl (95% CI: −32.03, −0.05), and LDL by −16.40 mg/dl (95% CI: −27.16, −5.63) compared to a placebo. Among the studies with treatment duration of less than 12 weeks, only synbiotics could significantly lower TC, TG, and LDL in patients with NAFLD when compared to a placebo. Other details are shown in **Supplementary Appendix 17**.

Fasting Blood Sugar [14 Studies]

The pooled outcomes showed that synbiotics were the only intervention that significantly lowered the FBS by −6.75 mg/dl (95% CI: −10.67, −2.84) in patients with NAFLD when compared to a placebo (**Figure 4G** and **Supplementary Appendix 9**). Nonetheless, when compared to another microbial therapy, synbiotics did not provide any additional favorable effect on FBS. The sensitivity analyses demonstrated that in both groups of cohorts treated with at least 12 weeks and less than 12 weeks of the interventions, synbiotics would still be the only treatment option that provided a significant effect, particularly when comparing their effect with a placebo in adults with NAFLD. Also regarding the sensitivity analysis, none of the interventions had an effect on FBS, specifically for patients with NASH. The magnitudes of effects are shown in **Supplementary Appendix 18**.

Homeostatic Model Assessment-Insulin Resistance [12 Studies]

Both main and sensitivity analyses showed that there was no significant difference in the HOMA-IR change in any pair of

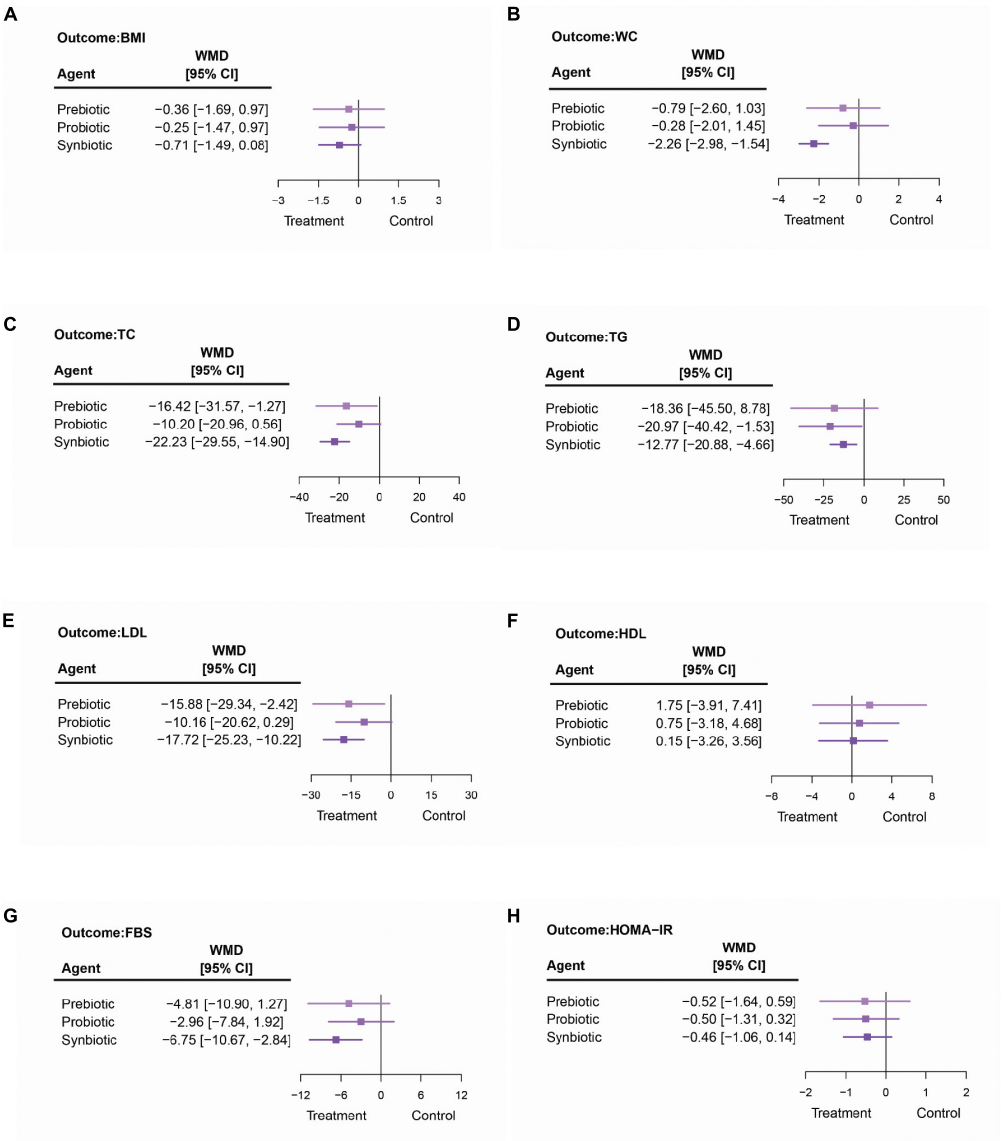

FIGURE 4 | The summarized results of using synbiotics, probiotics, and prebiotics for the modifications in **(A)** body mass index (BMI), **(B)** waist circumference (WC), **(C)** total cholesterol (TC), **(D)** triglycerides (TG), **(E)** low-density lipoproteins (LDL), **(F)** high-density lipoproteins (HDL), **(G)** fasting blood sugar (FBS), and **(H)** homeostatic model assessment-insulin resistance (HOMA-IR).

the options; neither when compared with a placebo nor among interventions (**Figure 4H** and **Supplementary Appendix 9**). Further details can be found in **Supplementary Appendix 19**.

The rank-bar chart which illustrated SUCRA cumulative probabilities of all outcomes associated with synbiotics, probiotics, prebiotics, and placebo used in patients with NAFLD are illustrated in **Figure 5**.

Network Consistency and Small-Study Effects

There was no evidence of any inconsistency in the results of our network meta-analysis. The results of the global-inconsistency assessment are shown in the **Supplementary Appendix 11**. The comparison-adjusted funnel plots revealed no evidence of small-study effects for AST, ALT, BMI, WC, TC, TG, FBS, and HOMA-IR, but there was evidence of small-study effects on LDL and HDL outcomes (**Supplementary Appendix 12**).

DISCUSSION

This systematic review summarized the data from 26 RCTs by comparing the effects of synbiotics, probiotics, and prebiotics in 1,389 patients with NAFLD. Trials conducted in adult and pediatric patients were separately analyzed and reported.

FIGURE 5 | Rank-bar chart with surface under the cumulative ranking (SUCRA) values for outcomes associated with synbiotics, probiotics, and prebiotics use in patients with non-alcoholic fatty liver disease (NAFLD).

The number of studies in pediatric patients was too small to draw any conclusion about the effect of probiotics on NAFLD. Additionally, network meta-analyses were performed to demonstrate the pooled outcomes related to NAFLD among adult patients. There was no evidence of inconsistency in our analysis. Thus, we compared the effects of synbiotics, probiotics, and prebiotics by using a consistency model. Our primary findings were that when compared to a placebo, all three interventions could significantly reduce AST and ALT. The effects of liver enzymes reduction in patients with NAFLD when microbial therapy was competed with one another was inconclusive. According to the results, there was no specific intervention that could be considered better than others. The sensitivity analyses showed similar effects. However, no study had investigated the effect of prebiotics on hepatic enzymes, particularly in patients with NASH. Interestingly, probiotics did provide a significantly superior ability to decrease ALT as compared to synbiotics among patients with biopsy-proven NASH.

The secondary outcomes showed that some interventions might improve WC, lipid profile (only TC, TG, and LDL), and FBS of patients with NAFLD. Synbiotics showed significant effects in most biomarkers including WC, TC, LDL, and FBS. Probiotics could lower only TG in adults with NAFLD. In addition, prebiotics provided the abilities of TC and LDL decrements. Neither of the interventions increased the HDL level of patients.

Regarding AST and ALT reductions, when sensitivity analyses were performed on studies which included only patients with

biopsy-proven NASH, the significant results were only seen in synbiotics and probiotics. Furthermore, when sensitivity analyses were exclusively done in trials that examined the effects of interventions which were given at least a 12 week-duration, the microbial therapies significantly performed better than a placebo in most outcomes (i.e., AST, ALT, TC, TG, LDL, and FBS).

Our results are mostly consistent with those of previous studies (47–50) which have demonstrated a significant reduction of AST and ALT by microbial therapies in patients with NAFLD, though our systematic review and NMA included more up-to-date RCTs with an overall larger sample size than previous meta-analyses. Five new RCTs were reported after the latest meta-analysis of the efficacy of microbiome-targeted therapies in NAFLD by Sharpton et al. (49) was published. A meta-analysis by Loman et al. (47) indicated that only prebiotics and probiotics, but not synbiotics, significantly decrease ALT in patients with NAFLD. The significant benefit of synbiotics in ALT modification was additionally seen in our present analysis. Moreover, Loman et al. demonstrated that all three microbial interventions could significantly decrease BMI in patients with NAFLD. However, our study showed that when incorporating indirect effects in the analysis, none of the interventions was considered to be an effective treatment for BMI reduction. Currently, the mechanism underlying NAFLD in human is not clearly known and varies with regard to the disease heterogeneity. However, one of the etiologic pathways that has been demonstrated in pre-clinical models is involved with gut microbiota (9–11).

This NMA has several strengths. First, we included both direct and indirect evidence of all comparisons relating to the interested outcomes. Second, we only included RCTs to compare the effects of synbiotics, probiotics, and prebiotics. Finally, sensitivity analyses were performed for every outcome associated with NAFLD. They were likely to yield similar results as those from the main analysis. This confirms the robustness of the study.

There were a few limitations in this study. First, the number of studies focusing on pediatric patients was too small to be pooled and to summarize the effects of microbial therapies on the interested outcomes. The sample size of adult patients was also relatively small for an NMA. Second, we did not explore the effect of each subtype of microbial therapy or the relative dose-response relationship, which may have affected the results. There were multiple types of microbial therapies and dosage recommendations. Furthermore, the dosage varied depending on a type of microbial therapy. We were not able to perform subgroup analyses due to a limited number of studies. However, according to results from the test of global inconsistency, they indicated no heterogeneity. Hence, we could infer that even if there were variations in type and dosage, the effect sizes and outcomes might be interpreted the same way as they were. On the other hand, these results should be able to apply in general. Third, most studies were considered to have at least moderate risk-of-bias. Three of which were considered high-risk-of-bias studies. Finally, our outcomes of interest were surrogate outcomes, such as liver-enzyme levels, which cannot exactly define the severity, prognosis, and treatment outcomes of NAFLD. Moreover, it is important to remark that some patients may develop the disease through different pathways. Thus, the interventions may not provide good efficacy in every patient with NAFLD. Other numerous risk factors associated with NAFLD and its complications were reported, such as age, sex, ethnicity, genetic variants, comorbidities, sociocultural, and so on (9). This might lead to some difficulty of result interpretations when the data from various studies with a variety of enrolled patients were pooled together. However, these surrogate outcomes are important basic indicators that can primarily monitor status of the disease, and which should result in higher accessibility rates of early appropriate treatment for patients. Liver fibrosis is another unfavorable outcome in patients with NAFLD. Due to limited data, in this study, we did not examine the outcomes of interested interventions on liver fibrosis. In combination with other parameters, these indicators will help both the patients and clinicians make the best choices regarding treatment. Also, currently, there is no evidence pertaining to the adverse events of taking these agents. Nevertheless, we should always carefully consider every factor, including the potential benefits, risks, and costs, before deciding to use these agents.

CONCLUSION

In conclusion, we found that synbiotics, probiotics, and prebiotics could significantly reduce hepatic enzymes of adult patients with NAFLD. However, the question of which microbial therapy provides the best effect on AST and ALT reduction is yet to be answered. The effect on other clinical parameters including WC, lipid profile, and FBS varied regarding types of microbial therapies. There was limited information about the efficacy of microbial therapy in pediatric patients with NAFLD.

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

SK, CK, KL, MN, TS, NC, SS, and PP: study concept and design. SK, KL, MN, TS, and SS: acquisition of data. SK, CK, KL, MN, TS, and SS: analysis and interpretation of data. SK, CK, KL, MN, TS, SS, and PP: drafting of the manuscript. SK, CK, NC, SS, and PP: critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from the Unit of Excellence on Clinical Outcomes Research and IntegratioN (UNICORN)

[Grant number: FF65-UoE005], School of Pharmaceutical Sciences, University of Phayao. The funding source had no role in the study design, collection, analysis, and interpretation of data.

ACKNOWLEDGMENTS

We thank the University of Phayao for its financial support of this research project. We are also grateful to Pongpol Nimitpunya for validating the data, Miss Pinyapat Ariyakunaphan for coordinating the project, and Keith Fitzgerald for his linguistic editing and proofreading.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.880014/full#supplementary-material

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frontiers | Frontiers in Nutrition

CLINICAL TRIAL
published: 27 June 2022
doi: 10.3389/fnut.2022.889910

Effects of Oral Multi-Vitamin Multi-Mineral Supplement Formulations on Laboratory Outcomes and Quality of Life: A Quasi-Experimental Study

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: 04 March 2022
Accepted: 19 May 2022
Published: 27 June 2022

Citation:
Jittat N, Pongpirul K, Tepwituksakit B, Iammaleerat P, Heath J, Lungchukiet P, Taechakraichana N and Charukitpipat A (2022) Effects of Oral Multi-Vitamin Multi-Mineral Supplement Formulations on Laboratory Outcomes and Quality of Life: A Quasi-Experimental Study. Front. Nutr. 9:889910. doi: 10.3389/fnut.2022.889910

Background: Multi-vitamin multi-mineral (MVMM) products often come in several single-substance capsules from different manufacturers. However, attempts to mix several vitamins and minerals into one MVMM product have been complicated and often involve legal concerns. This study aimed to comparatively investigate the changes in laboratory parameters and the quality of life (QOL) among individuals who received different MVMM formulations.

Methods: This three-arm non-randomized controlled trial was conducted at VitalLife Scientific Wellness Center (VSWC), Bangkok, Thailand. A total of 72 healthy adult individuals with total serum 25-(OH)D level of 20–29 ng/ml were invited to choose from the three available options, namely, (1) Hydro-Cell-Key (HCK®, Hepart AG, Switzerland) contains vitamin D3 2,000 IU, vitamin C 1,000 mg, vitamin E 166 mg, vitamin A 1 mg, coenzyme Q10 30 mg, natural carotenoids 8 mg, and citrus flavonoids 200 mg in granule formulation; (2) VTL-7 (VWSC) contains similar vitamins and minerals but in capsule formulation; and (3) placebo capsule (no supplement). The 36-Item Short-Form Health Survey (SF-36) was used to measure QOL at baseline, month 3 and 6. A generalized estimating equation (GEE) was used to compare the repeated-measure outcomes across the three groups. This study was registered at the Thai Clinical Trial Registration (TCTR20190205002) and approved by the Bumrungrad International Institutional Review Board (BI-IRB No.258-10-18PhFub).

Results: Both VTL-7 and HCK saw a significantly higher increase in vitamin D than placebo at months 3 and 6, i.e., VTL-7 from 25.15 ± 2.13 to 35.53 ± 6.11 ($p < 0.001$) and 33.38 ± 6.89 ($p < 0.001$); HCK from 24.25 ± 3.08 to 28.43 ± 5.93 ($p = 0.005$) and 27.40 ± 5.24 ($p = 0.012$); and placebo from 24.00 ± 2.73 to 23.05 ± 4.39 ($p = 0.273$) and 22.30 ± 6.23 ($p = 0.200$), respectively. Similarly, β-carotenoids of VTL-7 vs. HCK groups significantly increased from 0.88 ± 0.68 vs. 0.94 ± 0.55 at baseline to 3.03 ± 1.79 ($p < 0.001$) vs. 1.09 ± 0.61 ($p = 0.125$) and 3.26 ± 1.74 ($p < 0.001$) vs. 1.15 ± 0.66 ($p = 0.064$), respectively. These findings were corroborated through the GEE analysis.

Other micronutrients at months 3 and 6 did not increase significantly from baseline in any group. The overall QOL among the three groups in terms of physical ($p = 0.560$) and mental ($p = 0.750$) health increased but was not statistically significant.

Conclusion: The supplements of MVMM in capsule formulation increased the serum levels of some micronutrients to a higher extent than that of granule formulation. Participant adherence remains a potential confounder and should be further explored.

Clinical Trial Registration: identifier: TCTR20190205002.

Keywords: multi-vitamin supplement, quality of life, drug formulations, quasi-experiment design, multi-mineral supplement

BACKGROUND

The human diet requires both macronutrients, including carbohydrates, proteins, and fats, and micronutrients, such as vitamins and minerals. While macronutrients provide the main source of calories, micronutrients are required for developmental processes. Even though they are only required in a small amount, they have a profound impact on health and are vital to body development, disease prevention, immune function, tissue regeneration, and optimizing health (1, 2).

Micronutrient supplementation has gained popularity among individuals who want to ensure and maintain their health and wellness. Consumed by 50% of adults and one-third of children in economically advanced economies, the dietary supplement business is worth more than US$100 billion annually (3). According to the National Health and Nutrition Examination Survey (NHANES) collected between 1999 and 2014, the prevalence of any supplement use has significantly increased from 52 to 58%, whereas that of vitamin and mineral use has increased from 47 to 52% and from 47 to 51%, respectively (4). The trend of any supplement use varied by age, sex, race/ethnicity, or education but not by diabetes duration or comorbidities associated with diabetes. During 2017–2018, 57.6% of adults of at least 20 years of age reported that they had taken a dietary supplement within the past 30 days; individuals with a higher family income were more likely to consume a dietary supplement than those with a lower family income (4). Furthermore, women were significantly more likely than men to use a dietary supplement overall (63.8 vs. 50.8%) (4).

In contrast to mono-substance supplements, supplementation of multiple vitamins and minerals has significantly decreased from 36 to 32%, largely because micronutrient supplements are often manufactured in numerous single-substance capsules, which can decrease compliance. Multi-vitamin multi-mineral (MVMM) supplements, which contain numerous vitamins and minerals within one single-substance capsule, alleviate the issue of non-compliance by making it easier for consumers to supplement with multiple vitamins and minerals. However, attempts to mix several vitamins and minerals into one MVMM product have been both legally and technically complicated. Each MVMM "recipe" must be registered with the national authority of the country, such as the Food and Drug Administration (FDA) (5). Furthermore, product variation of available MVMM supplements, including varying product formulations, has led to limited evidence on the efficacy of different forms of MVMM supplements. It has been suggested that different MVMM formulations could also impact the degree of nutrient absorption in the body. There is some evidence to suggest that vitamin D buccal spray, for instance, has higher absorption than soft gel capsules (6). However, the lack of evidence looking at the formulation of MVMM supplementation and its effects on laboratory parameters makes it difficult to understand the extent to which MVMM formulation impacts its efficacy. Thus, the objective of this study is to comparatively investigate the effectiveness of different formulations of combined MVMM products, specifically Hydro-Cell-Key (HCK) granules vs. VTL-7 capsules, and their impacts on certain laboratory parameters, including serum micronutrient levels and quality of life (QOL) (focusing on the vitality domain).

A hospital with a qualified production facility might procure raw substances and produce an MVMM product that does not require FDA approval for in-house use. With this in mind, Vital-Life Scientific Wellness Center (VSWC), i.e., a medical anti-aging and wellness center in Bangkok, Thailand, developed an in-house personalized vital-life (VTL-7) MVMM capsules. As several patients preferred fewer capsules per day, along with an absorption concern, VSWC considered switching from capsule to granule formulation. To compare the efficacy of a similar MVMM product in granule formulation, the HCK®, i.e., MVMM granules of the Hepart AG Switzerland group, was included in the study. The HCK granule contains vitamin D3, vitamin C, vitamin E, vitamin A, coenzyme Q10, carotenoids, and flavonoids; hence, it was determined to be one of the best available candidates in comparison with the VTL-7 capsules. Built into a plant hydrocolloid matrix, the vitamins and minerals in granules are released in the intestines through the colloid film formed by the colloidal system after ingestion. This process mimics the absorption of micronutrients from natural fruits and vegetables, resulting in optimal nutrient distribution throughout the body, along with prolonged absorption over several hours, prevention of disturbances, and antagonism of various micronutrients. However, the formation of a dry granulation requires many more steps than capsule formulation.

The clinical efficacy (i.e., serum level of the micronutrients) of the original HCK® granules has been anticipated to be higher than conventional capsule formulation; however,

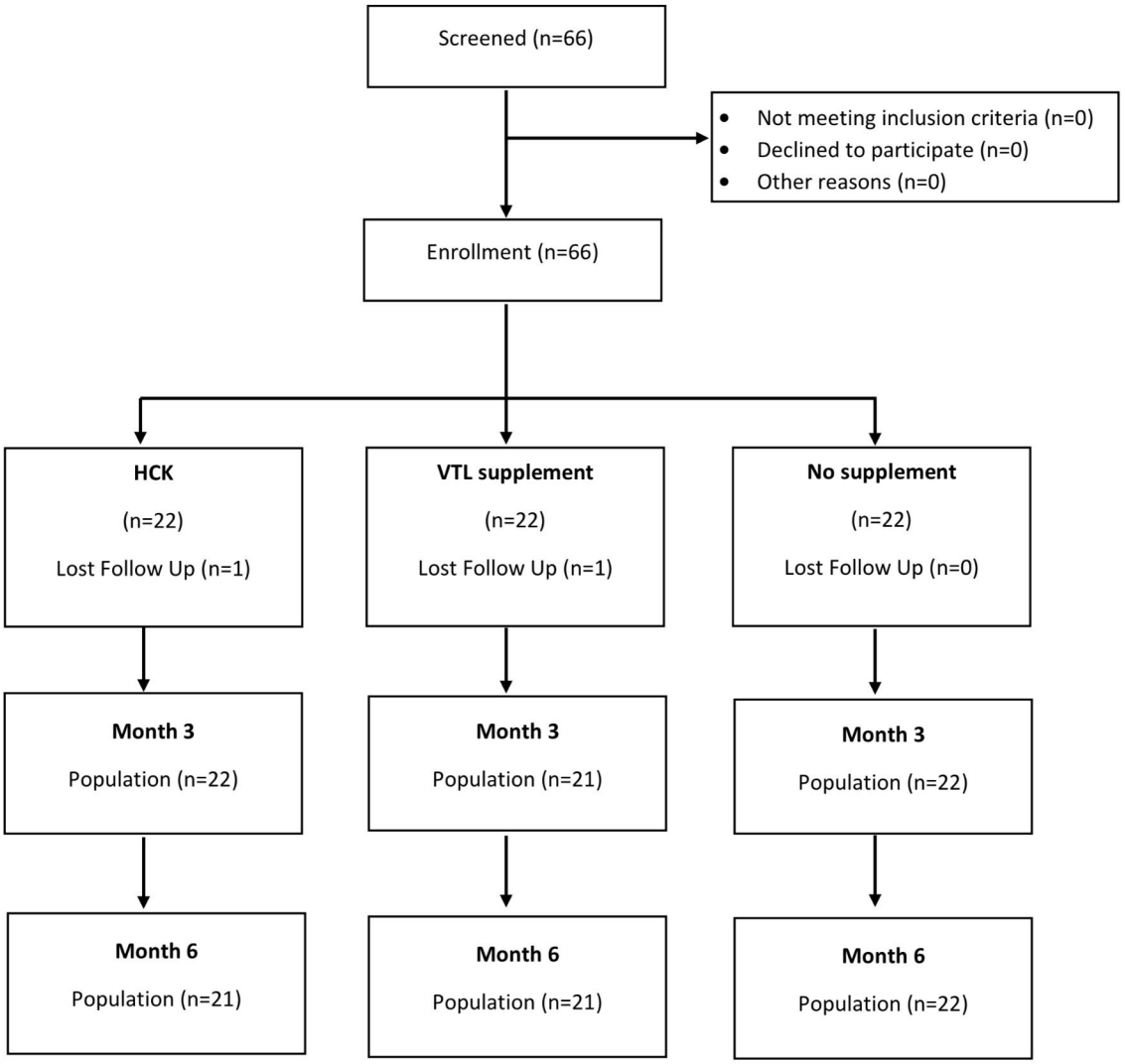

FIGURE 1 | Patient disposition.

there has been no comparative evidence of the changes in blood vitamins and minerals between the two products. As previously mentioned, product formulation could affect not only individual compliance but also laboratory changes. Thus, the methods used to examine both the effects of MVMM supplementation and the relationship between MVMM product formulation on laboratory parameters and QOL are presented in this study.

METHODS

Study Design and Setting

This three-arm non-randomized controlled trial was conducted at VSWC, a medical anti-aging and wellness center, focusing on promoting good health and preventing illness and chronic diseases. It is a subsidiary company of Bumrungrad International Hospital Public Company Limited, i.e., one of the largest international hospitals for medical tourism located in Bangkok, Thailand.

Participants

A total of 72 healthy adult individuals with insufficient levels of total serum 25-(OH)D level (20–29 ng/ml) were invited to participate in this study. Individuals with poorly controlled blood pressure, impaired kidney function (eGFR $<$ 30 ml/min/1.73 m^2), pregnancy, malabsorption, bowel surgery, and currently on medications or supplements that might affect the levels of vitamins were excluded from this study. Individuals who have underlying diseases that require vitamin D treatment (i.e., osteoporosis and hyperparathyroidism) or those who have a deficient level of total serum 25-(OH)D level ($<$20 ng/ml) were also excluded. As carotenoids could increase the risk of lung cancer (7), current and former smokers were also excluded.

TABLE 1 | Characteristics of the participants.

	Overall	HCK	VTL-7	Placebo	*p*-value
Age (years)	35.06 ± 8.47	37.64 ± 10.04	36.14 ± 7.09	31.41 ± 6.99	0.037
Women	83.30%	77.30%	77.30%	95.50%	0.175
Systolic BP (mm Hg)	109.14 ± 11.24	109.95 ± 11.39	111.64 ± 12.34	105.82 ± 9.51	0.212
Diastolic BP (mm Hg)	70.62 ± 8.72	71.27 ± 9.38	71.05 ± 9.73	69.55 ± 7.15	0.780
Pulse rate	76.45 ± 14.28	71.77 ± 10.55	76.50 ± 11.12	81.09 ± 18.73	0.095
Body temperature (°C)	36.75 ± 0.28	36.69 ± 0.18	36.90 ± 0.35	36.67 ± 0.24	0.120
Body weight (kg)	58.27 ± 11.26	58.12 ± 10.90	58.08 ± 13.16	58.60 ± 10.03	0.986
BMI	22.51 ± 3.57	21.95 ± 3.39	22.54 ± 3.75	23.05 ± 3.62	0.601

Supplements

Participants were assigned to one of the three groups (**Figure 1**), namely, HCK® (Hepart AG, Switzerland), VTL-7 (VSWC, Thailand), and placebo group (no supplement). The two study groups have similar micronutrient compositions, namely, vitamin D3 2,000 IU, vitamin C 1,000 mg, vitamin E 166 mg, vitamin A 1 mg, coenzyme Q10 30 mg, natural carotenoids 8 mg, and citrus flavonoids 200 mg; HCK was prepared in granules, while VTL-7 was the capsule formulation of the HCK product. The placebo group was used to allow for a comparison of the effects of each MVMM formulation on specified lab parameters and QOL vs. no supplementation, in addition to providing comparative evidence between the two products. Diet counseling was not included for any group as a part of this trial.

Outcomes

Primary outcomes (blood levels of the six micronutrients) and secondary outcomes (hs-CRP, homocysteine, lipid profile, ESR, CD4, CD8, and QOL) were measured at baseline, month 3, and 6. Although previous studies have found micronutrient supplementation to have a positive impact on overall mood and QOL, the 36-Items Short Form Health Survey (SF-36) was used to determine if different MVMM formulations impact the QOL in different ways (10–14). Circulatory cholesterol, lipoproteins, and homocysteine were also included as secondary outcomes since MVMM supplementation has been previously found to impact cholesterol levels and decrease homocysteine levels in certain populations (12, 18).

Sample Size

Previous trials suggest that a weekly supplementation of 50,000 IU cholecalciferol for 12 weeks resulted in an increase of 25-(OH)D (8). Given the differences in 25-(OH)D levels between the intervention and placebo control groups of 7.90 ng/ml and a pooled standard deviation of 7.85 ng/ml, an alpha SD of 0.05%, and a beta SD of 0.20, approximately 16 subjects were required per arm. Assuming a conservative dropout rate of 30%, 22 subjects were anticipated for each arm of this study.

Statistical Methods

Descriptive statistics (mean, standard deviation, and percentage) were used for demographic variables. The analysis of variance (ANOVA) and generalized estimating equation (GEE) were used to compare the changes in cross-sectional and longitudinal laboratory outcomes between the three groups, respectively. No subgroup analysis was performed. The data were analyzed based mainly on the intention to treat (ITT) principle, whereas per-protocol (PP) analysis was also performed to ensure the robustness of the analysis. In case of discrepant findings from both approaches, the ITT analysis was chosen.

Trial Registration and Ethical Approval

This study was registered at the Thai Clinical Trial Registration (Registration No. TCTR20190205002) and approved by the Bumrungrad International Institutional Review Board (BI-IRB No. 258-10-18PhFub). All participants provided written informed consent.

RESULTS

Characteristics of the Participants

The age of the participants was 35.06 ± 8.47 years, 83.3% were women, and the BMI of the participants was 22.51 ± 3.57 (**Table 1**). While almost all the characteristics of the participants were not significantly different across the three groups, the placebo control group was significantly younger than the intervention groups ($p = 0.037$). The baseline micronutrient and biomarker levels were comparable across the three groups (**Table 2** and **Figures 1**, **2**).

Primary Outcomes

Vitamin D and β-carotenoids levels increased in all three groups (**Figure 2**). Both VTL-7 and HCK observed a significantly higher increase in vitamin D than placebo, i.e., VTL-7 from 25.15 ± 2.13 to 35.53 ± 6.11 at month 3 ($p < 0.001$) and 33.38 ± 6.89 at month 6 ($p < 0.001$); HCK from 24.25 ± 3.08 to 28.43 ± 5.93 at month 3 ($p = 0.005$) and 27.40 ± 5.24 at month 6 ($p = 0.012$); and placebo from 24.00 ± 2.73 to 23.05 ± 4.39 at month 3 ($p = 0.273$) and 22.30 ± 6.23 at month 6 ($p = 0.200$). Similarly, β-carotenoids of VTL-7 vs. HCK groups significantly increased from 0.88 ± 0.68 vs. 0.94 ± 0.55 at baseline to 3.03 ± 1.79 ($p < 0.001$) vs. 1.09 ± 0.61 ($p = 0.125$) at month 3 and 3.26 ± 1.74 ($p < 0.001$) vs. 1.15 ± 0.66 ($p = 0.064$) at month 6, respectively. GEE analysis revealed a significantly higher increase in vitamin D ($p < 0.001$) and β-carotenoids ($p < 0.001$) in VTL-7 (capsule formulation) than HCK (granule formulation), both

TABLE 2 | Laboratory outcomes and quality of life at baseline, month 3 and 6.

	Overall	HCK	VTL-7	Placebo	*p*-value*
Primary outcomes					
Vitamin D (ng/mL)					
Month 0	24.47 ±2.68	24.25 ±3.08	25.15 ±2.13	24.00 ±2.73	0.325
Month 3	28.90 ±7.47	28.43 ±5.93	35.53 ±6.11	23.05 ±4.39	**<0.001**
Month 6	27.61 ±7.60	27.40 ±5.24	33.38 ±6.89	22.30 ±6.23	**<0.001**
Vitamin C (μmol/L)					
Month 0	88.99 ±32.52	86.20 ±28.35	87.71 ±35.48	93.07 ±34.40	0.768
Month 3	110.96 ±24.62	113.22 ±25.84	117.71 ±21.93	102.25 ±24.31	0.103
Month 6	120.24 ±35.82	123.62 ±37.45	126.74 ±36.36	110.82 ±33.29	0.306
Vitamin E (mg/L)					
Month 0	30.33 ±7.86	32.45 ±10.76	29.11 ±5.81	29.42 ±5.86	0.300
Month 3	34.58 ±12.39	41.60 ±16.35	31.89 ±7.85	30.13 ±7.89	**0.003**
Month 6	36.07 ±9.50	28.60 ±5.35	29.02 ±7.90	31.19 ±8.39	**0.004**
Vitamin A (μmol/L)					
Month 0	1.90 ±0.53	2.05 ±0.67	1.82 ±0.52	1.83 ±0.35	0.283
Month 3	1.86 ±0.53	2.08 ±0.64	1.82 ±0.49	1.66 ±0.33	**0.026**
Month 6	1.71 ±0.46	1.85 ±0.52	1.68 ±0.48	1.62 ±0.34	0.230
α-Carotenoid (μmol/L)					
Month 0	0.25 ±0.20	0.27 ±0.15	0.24 ±0.24	0.25 ±0.20	0.895
Month 3	0.27 ±0.24	0.39 ±0.32	0.20 ±0.17	0.21 ±0.13	**0.011**
Month 6	0.22 ±0.20	0.31 ±0.25	0.20 ±0.19	0.16 ±0.10	**0.024**
β-Carotenoid (μmol/L)					
Month 0	0.89 ±0.55	0.94 ±0.55	0.88 ±0.68	0.84 ±0.43	0.823
Month 3	1.67 ±1.44	1.09 ±0.61	3.03 ±1.79	0.95 ±0.44	**<0.001**
Month 6	1.76 ±1.52	1.15 ±0.66	3.26 ±1.74	0.93 ±0.52	**<0.001**
Co-Q10 (μmol/L)					
Month 0	1.61 ±0.75	1.71 ±0.89	1.55 ±0.85	1.57 ±0.48	0.765
Month 3	1.89 ±0.96	2.15 ±1.21	1.98 ±0.94	1.55 ±0.59	0.107
Month 6	1.73 ±0.71	1.96 ±0.72	1.67 ±0.65	1.57 ±0.74	0.181
Secondary outcomes					
hs-CRP (mg/L)					
Month 0	0.28 ±0.60	0.25 ±0.79	0.20 ±0.33	0.40 ±0.60	0.522
Month 3	0.19 ±0.30	0.15 ±0.15	0.16 ±0.28	0.24 ±0.43	0.559
Month 6	0.22 ±0.37	0.18 ±0.23	0.23 ±0.39	0.25 ±0.46	0.848
Homocysteine (μmol/L)					
Month 0	8.86 ±2.27	9.01 ±2.20	8.99 ±2.39	8.60 ±2.29	0.800
Month 3	8.67 ±2.20	8.34 ±2.13	9.15 ±2.10	8.53 ±2.38	0.466
Month 6	8.43 ±2.02	8.09 ±1.65	9.00 ±2.31	8.20 ±2.01	0.285
Total cholesterol (mg/dL)					
Month 0	202.22 ±31.16	213.09 ±34.34	190.44 ±28.90	203.13 ±26.85	0.052
Month 3	204.08 ±28.68	201.38 ±26.71	200.41 ±27.55	210.30 ±31.76	0.463
Month 6	200.54 ±33.92	193.93 ±31.14	194.59 ±26.72	212.52 ±40.22	0.123
Triglyceride (mg/dL)					
Month 0	97.23 ±98.27	125.57 ±160.99	79.26 ±40.06	86.85 ±32.82	0.248
Month 3	99.39 ±61.45	126.99 ±84.85	77.96 ±32.59	92.25 ±44.56	**0.024**
Month 6	81.93 ±34.84	87.25 ±23.12	70.27 ±32.81	87.98 ±43.64	0.174
HDL (mg/dL)					
Month 0	60.44 ±14.26	61.04 ±10.50	60.66 ±18.44	59.61 ±13.33	0.944
Month 3	59.16 ±13.31	58.01 ±10.32	60.42 ±16.68	59.10 ±12.85	0.843
Month 6	57.70 ±14.32	54.83 ±12.21	58.84 ±15.27	59.34 ±15.45	0.539

(Continued)

TABLE 2 | Continued

	Overall	HCK	VTL-7	Placebo	*p*-value*
LDL (mg/dL)					
Month 0	125.30 ±26.37	129.80 ±28.14	117.80 ±25.27	128.29 ±25.17	0.262
Month 3	126.58 ±27.70	123.13 ±27.06	124.40 ±25.68	132.12 ±30.49	0.517
Month 6	123.33 ±32.56	121.39 ±31.67	115.09 ±35.58	133.05 ±29.11	0.186
LDL (Oxidized) (U/L)					
Month 0	38.89 ±11.30	41.32 ±14.10	36.55 ±10.69	38.82 ±8.38	0.380
Month 3	44.28 ±14.10	44.18 ±13.56	45.90 ±15.56	42.82 ±13.67	0.778
Month 6	39.50 ±14.46	36.71 ±12.92	37.95 ±16.52	43.64 ±13.42	0.247
ESR (mm/hr)					
Month 0	22.64 ±14.24	20.64 ±10.89	21.45 ±14.85	25.82 ±16.50	0.438
Month 3	21.11 ±13.96	17.77 ±8.26	20.10 ±15.80	25.41 ±16.00	0.179
Month 6	20.41 ±13.64	18.67 ±10.53	18.05 ±12.85	24.32 ±16.46	0.253
CD4 (cells/mm^3)					
Month 0	772.92 ±236.59	790.95 ±270.73	812.73 ±171.79	715.09 ±254.85	0.362
Month 3	808.77 ±244.93	813.36 ±242.67	836.71 ±219.77	777.50 ±275.89	0.732
Month 6	787.25 ±232.78	751.90 ±217.81	788.48 ±219.61	819.82 ±262.84	0.639
CD8 (cells/mm^3)					
Month 0	552.20 ±201.81	560.86 ±158.28	588.05 ±215.45	507.68 ±226.04	0.412
Month 3	552.62 ±202.54	552.77 ±197.89	589.29 ±216.07	517.45 ±196.86	0.516
Month 6	546.94 ±200.19	509.71 ±169.50	565.14 ±223.15	565.09 ±208.28	0.590
CD4/CD8					
Month 0	1.69 ±1.41	1.91 ±2.32	1.54 ±0.63	1.62 ±0.50	0.659
Month 3	1.56 ±0.53	1.57 ±0.52	1.56 ±0.64	1.56 ±0.43	0.998
Month 6	1.56 ±0.52	1.55 ±0.64	1.51 ±0.39	1.54 ±0.51	0.938
Quality of life					
Physical outcomes					
Month 0	75.63 ±14.30	75.72 ±13.91	72.57 ±15.57	78.60 ±13.33	0.382
Month 3	80.69 ±14.00	83.76 ±13.74	81.54 ±12.18	76.80 ±15.50	0.246
Month 6	81.66 ±13.19	85.46 ±9.76	84.23 ±11.50	75.58 ±15.66	**0.025**
Physical functioning					
Month 0	77.12 ±20.44	79.32 ±16.35	68.64 ±23.31	83.41 ±18.99	**0.044**
Month 3	82.46 ±15.89	88.64 ±12.36	82.38 ±15.78	76.36 ±17.33	**0.035**
Month 6	86.17 ±16.49	92.14 ±9.43	87.14 ±15.21	79.55 ±20.70	**0.039**
Physical role functioning					
Month 0	88.07 ±17.12	86.08 ±19.95	86.08 ±18.49	92.05 ±11.92	0.417
Month 3	90.00 ±16.75	90.34 ±15.64	94.94 ±9.40	84.94 ±21.79	0.147
Month 6	91.21 ±13.53	90.77 ±12.12	95.54 ±8.65	87.50 ±17.47	0.148
Body pain					
Month 0	76.67 ±21.06	75.00 ±21.77	77.61 ±20.81	77.39 ±21.48	0.904
Month 3	81.04 ±20.55	80.80 ±22.89	79.76 ±19.90	82.50 ±19.53	0.910
Month 6	77.50 ±21.38	82.14 ±19.63	79.05 ±18.90	71.59 ±24.56	0.252
General health perception					
Month 0	60.67 ±18.29	62.50 ±21.17	57.95 ±16.57	61.55 ±17.34	0.692
Month 3	69.26 ±17.64	75.27 ±13.92	69.10 ±16.02	63.41 ±20.88	0.081
Month 6	71.75 ±16.76	76.76 ±15.77	75.19 ±15.46	63.68 ±16.53	**0.017**
Mental outcomes					
Month 0	71.99 ±15.17	72.41 ±16.66	69.98 ±13.85	73.58 ±15.35	0.730
Month 3	76.21 ±13.15	77.20 ±14.42	76.85 ±11.81	74.63 ±13.49	0.788
Month 6	77.80 ±11.43	79.51 ±12.36	80.42 ±10.85	73.67 ±10.35	0.108
Vitality					
Month 0	57.48 ±16.92	58.24 ±18.84	56.53 ±14.62	57.67 ±17.78	0.945

(Continued)

TABLE 2 | Continued

	Overall	HCK	VTL-7	Placebo	*p*-value*
Month 3	64.04 ± 15.78	66.48 ± 15.36	65.48 ± 15.64	60.23 ± 16.31	0.377
Month 6	68.55 ± 15.31	71.13 ± 17.51	69.05 ± 15.99	65.63 ± 12.31	0.498
Social role functioning					
Month 0	77.65 ± 19.05	76.70 ± 22.59	76.70 ± 17.80	79.55 ± 17.05	0.854
Month 3	80.77 ± 17.41	84.66 ± 17.22	78.57 ± 19.02	78.98 ± 16.08	0.441
Month 6	81.05 ± 15.59	83.33 ± 13.88	84.52 ± 18.50	75.57 ± 13.07	0.121
Role emotional					
Month 0	86.62 ± 19.66	85.61 ± 19.28	83.71 ± 20.49	90.53 ± 19.47	0.501
Month 3	88.59 ± 17.53	83.33 ± 19.42	91.67 ± 14.91	90.91 ± 17.43	0.225
Month 6	88.93 ± 15.00	88.10 ± 15.94	95.24 ± 10.06	83.71 ± 16.36	**0.037**
Mental health					
Month 0	66.21 ± 16.24	69.09 ± 17.50	62.95 ± 14.20	66.59 ± 17.00	0.459
Month 3	71.46 ± 15.43	74.32 ± 16.71	71.67 ± 12.97	68.41 ± 16.36	0.452
Month 6	72.66 ± 14.42	75.48 ± 17.39	72.86 ± 11.68	69.77 ± 13.76	0.437

**Analysis of variance.*

Bold values indicate outcome values that were statistically significant, with a p-value < 0.05.

of which were significantly higher than the control groups ($p < 0.001$). Vitamin C, vitamin E, vitamin A, α-carotenoid, and coenzyme Q10 at months 3 and 6 did not increase significantly from baseline in any of the three groups.

Secondary Outcomes

All of the secondary laboratory outcomes (hs-CRP, homocysteine, lipid profile, ESR, CD4, and CD8) and QOL did not increase significantly from baseline in any of the three groups (**Figure 3**). The overall QOL among the three groups in terms of physical health ($p = 0.560$) and mental health ($p = 0.750$) has increased but is not statistically significant.

DISCUSSION

Absorption of micronutrients could be affected by several factors such as variants of genetic polymorphisms, underlying health conditions, diet, exercise, smoking, alcohol consumption, age, and form of supplements (9). This quasi-experimental study revealed a comparably significant increase in serum levels of two out of the six micronutrients that were provided to the participants as supplements in two different oral formulations. The significant increase in only two out of the six micronutrients could be due to various reasons, including dose intake of these nutrients or individual absorption capabilities. Further and longer studies are needed to assess the reasoning behind a lack of significant increase among the other four micronutrients observed in this study.

The difference in laboratory results (vitamin D and beta carotene levels) between the study groups could be since vitamin D and beta carotenoid differed between the two products' formulations, representing two common types of Solid Oral Dosage Form (SODF). Some key differences among the differently formulated micronutrients include variations in the physicochemical state of the vitamin D (molecular forms, potency, and their physiological linkages), the complexity of the food matrix (the amount and type of fatty acids, dietary fibers, and presence/absence of vitamin D enhancer and inhibitor), and its interaction of other fat-soluble compounds with vitamin D, as well as the host-associated factors (e.g., age, disease, surgery, obesity, and genetic variation) (10). The homeostasis of vitamin C is influenced by several factors, including genetic polymorphisms and environmental and lifestyle factors, such as smoking and diet, as well as the presence of diseases (11). Excessive chronic alcohol intake is generally associated with vitamin deficiency (especially folate, thiamine, and vitamin B6) due to malnutrition, malabsorption, and ethanol toxicity. The effects of moderate alcohol use are mainly explained by a lower vitamin intake. In the case of vitamin A and beta-carotene, the effects on post-absorptive (lipoprotein) metabolism have been demonstrated (12).

Besides the effects of vitamins and minerals on blood biomarkers, previous studies showed that supplementation of multivitamin and mineral preparations has beneficial effects on mood and stress (13–15). In addition, the association between serum level of vitamin D and self-rated health in healthy male workers was observed (16). Moreover, results from a previous study revealed that elderly participants with poor physical health status assessed by the SF-36 exhibit lower alpha-tocopherol blood concentrations (17). The formulation type of the MVMM supplement used differed with each study but included both capsule and granule formulations. None of these studies, however, looked at the difference between MVMM formulation and its effect on the QOL. Nonetheless, our findings did not suggest that supplementation of vitamins and minerals may improve the QOL.

We observed that the participants who preferred no supplement were younger than the study groups. Previous studies revealed that supplement use increased with age, with 72% of adults of 65 years or older of age reporting

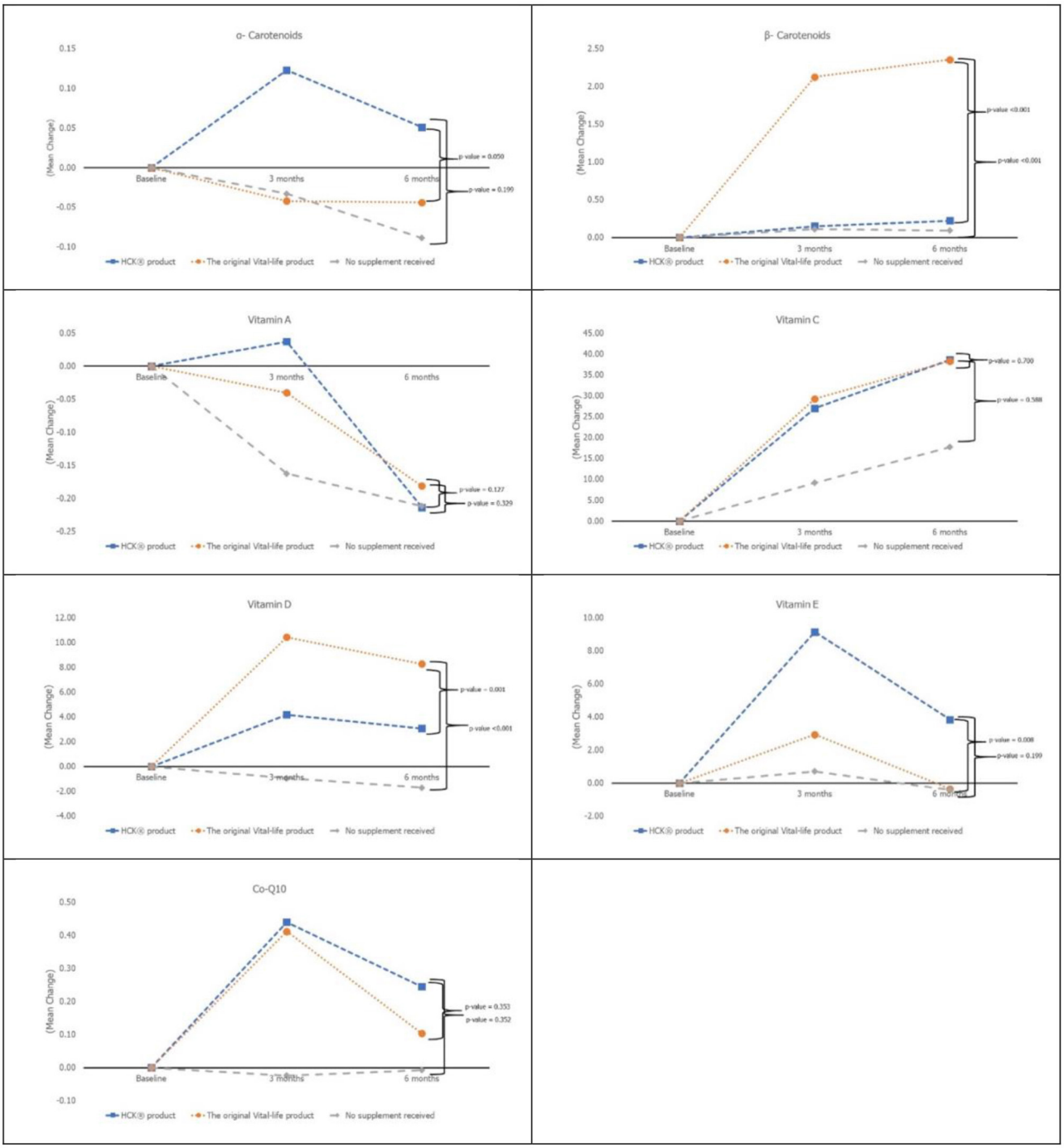

FIGURE 2 | Micronutrient levels at baseline, month 3 and 6 among all 3 groups.

an increased use compared with 40% of adults of 20–39 years of age (18). Several lifestyle and behavioral factors were associated with relatively less herb and dietary supplement use in young adults (19) for various reasons. First, they might prefer improving their diet and lifestyle rather than taking an oral supplement. Second, they might have been concerned about the potential side effects of the supplement (20).

The findings presented in this article could serve to influence the future of micronutrient supplementation, particularly for those with vitamin A or vitamin D deficiency. As the results from the GEE analysis (presented in the Results section)

revealed a significantly higher increase in vitamin D and β-carotenoids in VTL-7 than HCK, consumers looking for supplementation of these nutrients may benefit more from capsule formulation than granule formulation. Knowing the composition of different MVMM formulations can help us to better understand the differences in the mechanism of action through which the nutrients are absorbed, based on the formulation type. Furthermore, expanding our knowledge of the different MVMM formulations can influence what kind of supplements are prescribed by medical providers to help with vitamin and mineral deficiencies, as well as help consumers to determine over-the-counter counter supplements to purchase.

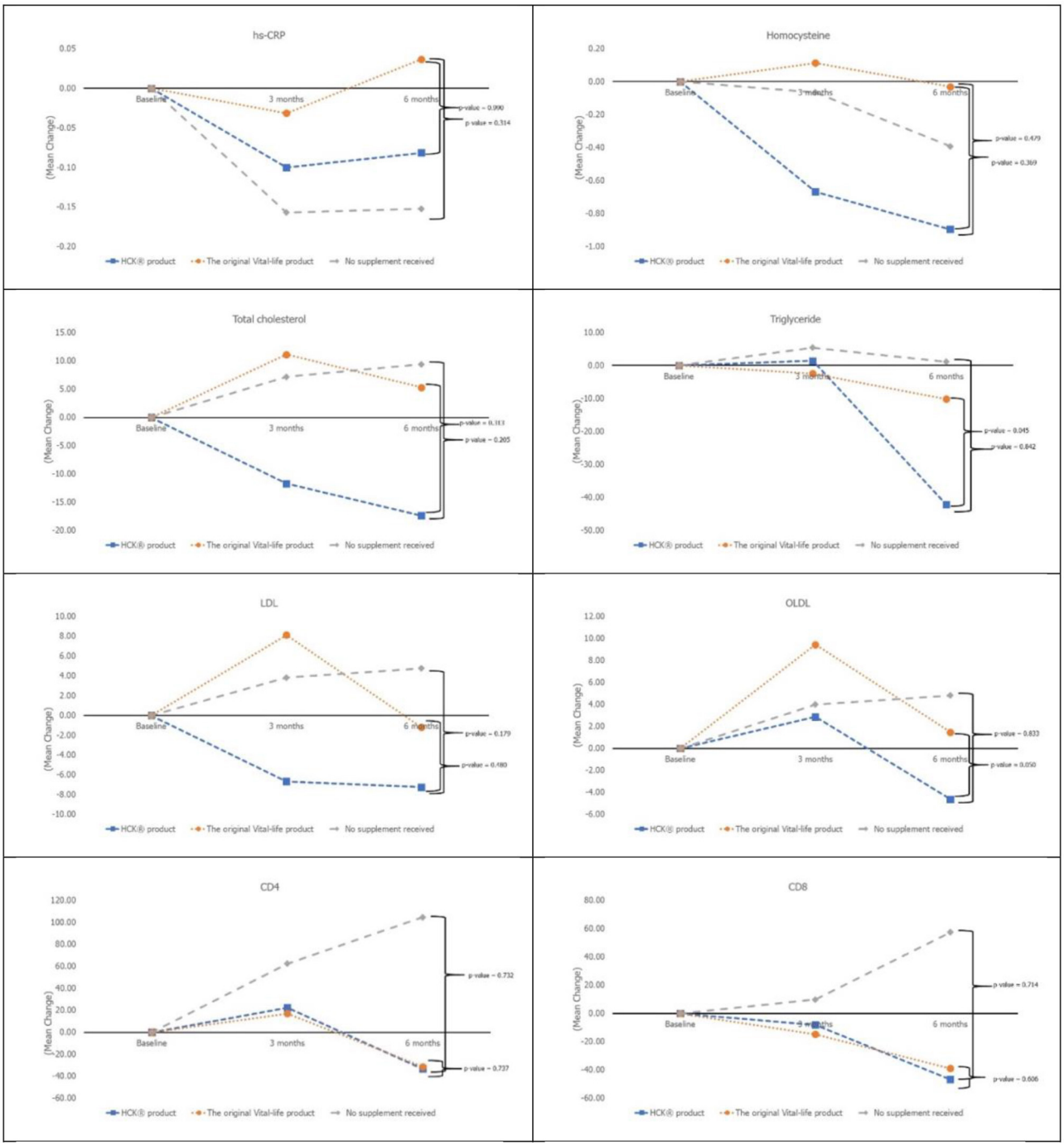

FIGURE 3 | Continued

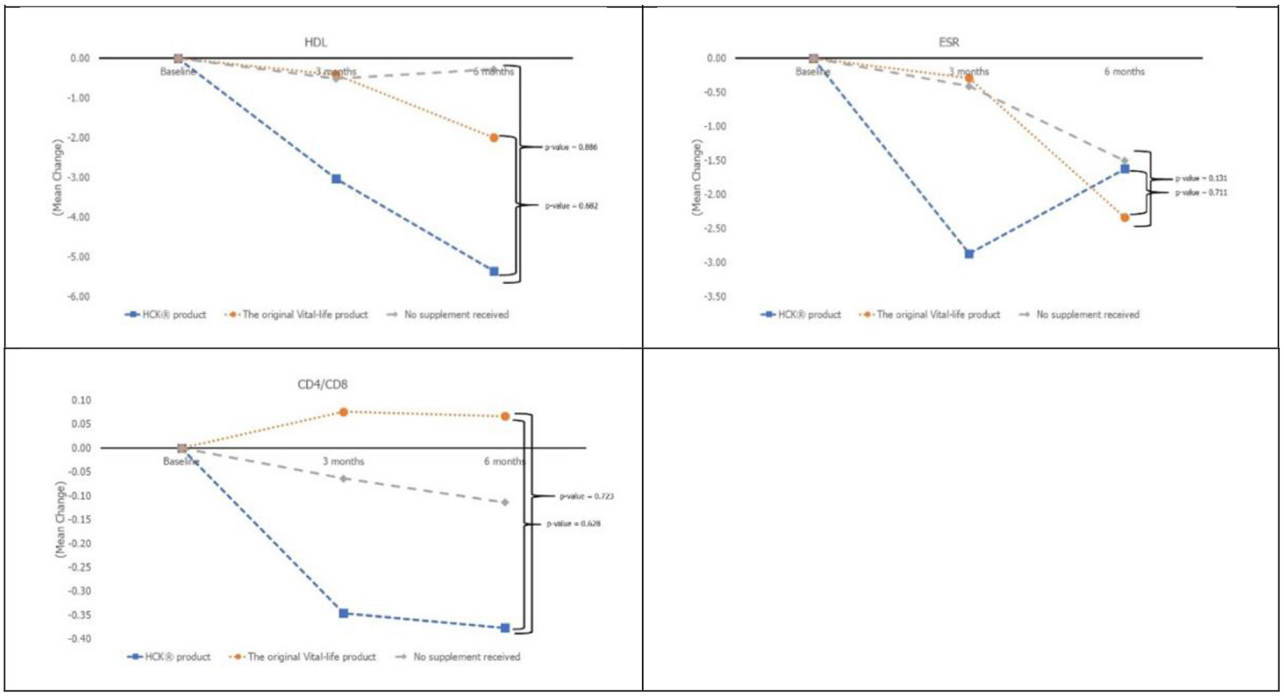

FIGURE 3 | Secondary laboratory outcomes at baseline, month 3 and 6 among all 3 groups.

Still, more research should be done in this area to further explore the differences in efficacy between the two formulations by looking at micronutrients not included in this study.

This quasi-experimental study has some limitations. First and most important is the non-randomization nature of the study. Although a random allocation of participants is usually preferred, this study was conducted in a private international hospital setting, in which the participants were healthy and did not receive financial support from a third-party payer for this type of wellness service. Second, the different product formulations, which was the primary objective of this study, could indeed introduce another bias. That is, the participants who were familiar with conventional capsules, as opposed to the new formulation, were health-conscious. Although only two formulations/products were included, the findings from this study could be suggestive of the effect of supplement formulations on the change in laboratory parameters. Finally, it was not required that subjects follow a specific diet during participation in this study and, therefore, it cannot be ascertained that the diet of the subjects did not impact the uptake of CoQ10 or vitamins into the blood. Similarly, there was no method in place to assure that subjects took their supplementation exactly as prescribed, which could also influence the laboratory outcomes. Future studies should be conducted to control these factors.

CONCLUSION

Micronutrient supplement formulation, specifically granule vs. capsule formulation, was found to impact certain laboratory outcomes but not QOL. More specifically, MVMM supplements in capsule formulation were found to increase the serum levels of some micronutrients, namely, vitamin D and β-carotenoids, to a higher extent than that of granule formulation. Nonetheless, participant adherence remains a potential confounder and should be further explored.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Bumrungrad International Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC conceived of and supervised the study. BT and PI collected and help to analyze the data. KP analyzed the data. PL and NT facilitate the data collection, data analysis, and supervised the study. KP, NJ, BT, PI, and JH drafted the manuscript. All authors read and approved the manuscript.

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Conflict of Interest: PI was employed by Asia Global Research Co., Ltd.

The remaining 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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SYSTEMATIC REVIEW
published: 30 June 2022
doi: 10.3389/fnut.2022.918777

Association of Retinol and Carotenoids Content in Diet and Serum With Risk for Colorectal Cancer: A Meta-Analysis

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: 13 April 2022
Accepted: 13 June 2022
Published: 30 June 2022

Citation:
Han X, Zhao R, Zhang G, Jiao Y, Wang Y, Wang D and Cai H (2022) Association of Retinol and Carotenoids Content in Diet and Serum With Risk for Colorectal Cancer: A Meta-Analysis. Front. Nutr. 9:918777. doi: 10.3389/fnut.2022.918777

Background: Colorectal cancer (CRC) risk is linked to serum and dietary retinol and carotenoids, according to clinical and epidemiological research. However, the findings are not consistent. As a result, we did this meta-analysis to determine the link between them.

Methods: From 2000 through 2022, the PubMed, Web of Science, and Embase databases, as well as pertinent article references, were searched and filtered based on inclusion and exclusion criteria and literature quality ratings. High and low intake were used as controls, and OR (odds ratio) or RR (relative risk) and 95% confidence interval were extracted. The extracted data were plotted and analyzed using Stata12.0 software.

Results: A total of 22 relevant studies were included, including 18 studies related to diet and 4 studies related to serum. For high and low intake or concentration controls, the pooled OR was as follows: β-carotene (OR = 0.89, 95% CI: 0.78–1.03), α-carotene (OR = 0.87, 95% CI: 0.72–1.03), lycopene (OR = 0.93, 95% CI: 0.81–1.07), lutein/zeaxanthin (OR = 0.96, 95% CI: 0.87–1.07), β-cryptoxanthin (OR = 0.70, 95% CI: 0.48–1.01), total carotenoids (OR = 0.97, 95% CI: 0.81–1.15), retinol (OR = 0.99, 95% CI: 0.89–1.10), serum carotenoids (OR = 0.73, 95% CI: 0.58–0.93), serum retinol (OR = 0.62, 95% CI: 0.26–1.49). Subgroup analysis was performed according to tumor type, study type and sex.

Conclusion: Total carotenoid intake and Lutein/Zeaxanthin intake were not associated with CRC risk. High β-carotene, α-carotene, lycopene, and β-cryptoxanthin all tended to reduce CRC risk. Serum carotenoid concentrations were significantly inversely associated with CRC risk.

Keywords: retinol, carotenoids, colorectal cancer, risk, meta-analysis

INTRODUCTION

In recent years, the incidence and mortality of malignant tumors have been increasing year by year, even exceeding other chronic diseases, becoming a veritable human health killer (1). Although the efficacy of cancer treatment has been improved due to comprehensive therapies such as surgery, chemotherapy, radiotherapy, targeted therapy, and immunotherapy, the prognosis and early diagnosis remain poor and the mortality rate remains high (2). Colorectal cancer (CRC) is the world's third most frequent cancer and the second largest cause of cancer mortality, with a significant number of new cases and deaths every year (3). CRC has caused great burden and harm to the economy and society of the country (4). Economic development and changes in lifestyle and dietary choices have increased the prevalence and mortality of CRC in China in recent years, putting a strain on the health-care system (3, 5). The etiology of CRC is heavily influenced by environmental and genetic factors. Diet, history of benign adenomatous polyps and inflammatory bowel disease, age, diabetes, obesity, lack of physical activity, and a family history of CRC are all risk factors for CRC (6). Therefore, the prevention of CRC by changing dietary habits and lifestyle is an area that we should focus on.

Fruits and vegetables are among the daily foods required for good health since they include high levels of minerals, vitamins, carbs, proteins, dietary fiber, and different substances with nutritional medicinal value that can help prevent a variety of ailments (7). Many studies have indicated that eating fruits and vegetables helps prevent cancer, with vegetable-related protection being more substantial (8, 9). Vitamin A is an unsaturated hydrocarbon group that includes retinol and its derivatives such as retinaldehyde, retinoic acid, and retinyl ester (10). Cell development and differentiation, embryogenesis, reproduction, epithelial cell integrity, and immunological function are all regulated by vitamin A (11, 12). It also has antioxidant properties (13) and helps to reduce oxidative stress damage and inflammation (11, 14). Carotenoids are a good source of vitamin A and may be turned into it by the body (15). Carotenoids are natural pigments found in a wide range of fruits and vegetables, including lycopene, β-carotene, lutein, zeaxanthin, and β-cryptoxanthin (16). Carotenoids and retinoids share many biological actions, including antioxidant capabilities, suppression of malignant tumor development, and activation of apoptosis (17). In addition, carotenoids can influence cell development, as well as gene expression and immunological responses (18, 19). Thus, retinol and carotenoids are indispensable in the human body. But retinol cannot be synthesized in the human body, and it must be obtained from the diet (20). As a result, research into the relationship between their consumption and human illnesses, including cancer, is required.

Over the last two decades, researchers have conducted substantial research on the link between nutrition and cancer. Epidemiological studies have found a link between food and cancer incidence and aggressiveness (21). A high intake of dietary carotenoids or vitamin A (retinol) has been linked to a decreased risk of CRC in several studies (22–25). However, other studies have shown no substantial link between their use and the risk of cancer onset (25–27). In addition to diet, there has been interest in the research of serum retinol and carotenoids, and some studies have shown that their levels in the blood are related to the risk of colon cancer. As a result, we completed our meta-analysis in time to incorporate the most recent relevant data, providing more credible scientific support for CRC prevention.

MATERIALS AND METHODS

Search Strategy for Literature

Two writers (Xiaoyong Han and Rangyin Zhao) separately conducted a literature search for the association between retinol, carotenoids, and related derivatives and the risk of CRC in humans using the PubMed, Web of Science and Embase databases. The following keywords were used in the search: "retinol" or "carotenoids" or "carotene" or α-carotene or "β-carotene" or "cryptoxanthin" or "lycopene" or "lutein" or "zeaxanthin" combined with "colorectal cancer" or "colon cancer" or "rectal cancer." All relevant literature was searched from 2000 to April 2022. In addition, we performed a manual search of the reference lists of reviews, meta-analyses, and other relevant publications to prevent potentially missed articles. The language of included articles was limited to English.

Inclusion and Exclusion Criteria

Studies were included according to the following criteria: (1) patients were diagnosed with colon or rectal cancer; (2) observational studies, including cohort or case-control studies; (3) The associations of interest are about the association of serum or dietary retinol or carotenoids with CRC risk, and there are comparisons of high and low content.; (4) Included studies contained relative risks (RR) or odds ratios (OR) with 95% confidence intervals for CRC. The following exclusion criteria were used: (1) reviews or conferences or abstracts or letters to the editor; (2) duplicate study populations; (3) animal studies; (4) other cancer studies; (5) lack of RR or OR data; (6) other vitamin supplement studies.

Data Extraction and Quality Evaluation

All included papers were examined and relevant data were retrieved independently by two researchers. Inclusion basic information included: name of first author, date of publication, country, type of study, vitamin type, cancer type, sample size of cases and controls, RR or OR and 95% CI for cancer, covariate correction. The disagreement between these two researchers was decided jointly by a third author. The quality of the included studies was assessed using the NOS scoring criteria (0–9 points), and those with a score > 6 were included in the meta-analysis.

Statistical Analysis

RR or OR with 95% confidence intervals were extracted from each study to assess the association of high retinol or carotenoids intake with cancer risk. The results generally combined in cohort studies are RR values, and the results generally combined in case-control studies are OR values. In order to better calculate and combine the results of studies, the difference between the two

is negligible, and all the results are expressed as OR values. In addition, heterogeneity among studies was assessed by Q-test and I^2 statistic. Q-test (P_Q) p-value < 0.1 and $I^2 > 50\%$ indicated that there was significant statistical heterogeneity between studies, and the results were analyzed using a random-effects model. Otherwise, a fixed effects model was used. We used forest plots to present the meta-analysis results and used Begg's test as well as Begg's funnel plots to assess publication bias. In addition, by eliminating each study one by one, a sensitivity analysis was performed to check the stability of the results. Analyses were performed using Stata12.0 for Windows (Stata, College Station, TX, United States) and $p < 0.05$ was considered statistically significant.

RESULTS

Screening Process for Eligible Literatures

The relevant literatures were searched in three main English databases according to the search strategy: PubMed (n = 235), Web of Science (n = 218), Embase (n = 173). After de-duplication (n = 376), the titles and abstracts of the remaining articles (n = 250) were examined and evaluated. A total of 193 articles were rejected for purpose, article type (review, case study, or conference abstract), or irrelevant findings. Fifty-seven full-text articles were downloaded, of which 35 studies were rejected after initial analysis due to lack of important data or unsatisfactory quality of NOS scores. Finally, the meta-analysis comprised 22 papers that fully fulfilled the inclusion criteria and quality evaluation. **Figure 1** depicts the search flow chart.

Characteristics of Included Research Projects

Table 1 shows the main characteristics of the 22 included studies. Regarding dietary aspects, a total of five cohort studies were included, and 399,558 individuals were followed up for 5–15 years, eventually resulting in 6,919 CRC patients. A total of 13 case-control studies involving 11,029 cases and 19,024 controls were included. With respect to serum, two cohort studies were included, with 32,428 participants and, ultimately, 272 patients with CRC. Two case-control studies involving 1,073 cases and 1,116 controls were included. Studies were published between 2000 and 2019. Eight studies were from

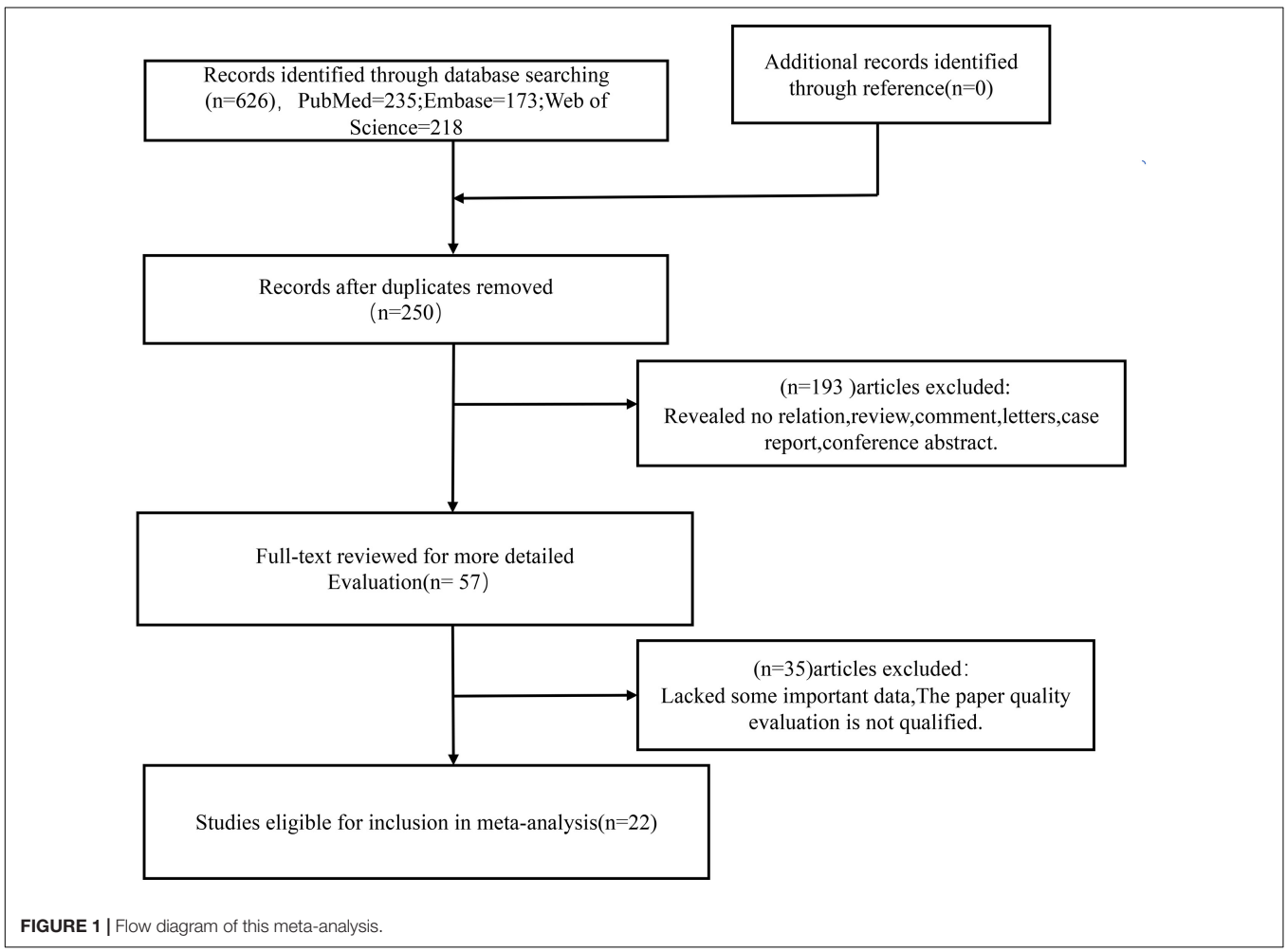

FIGURE 1 | Flow diagram of this meta-analysis.

TABLE 1 | Characteristics of included studies.

References Country	Type of cancer	Type of study	Sample size	Diet/Serum	Nutrient type	Adjustment for covariates.	NOS score
Roswall et al. (27) Denmark	Colon and rectal cancer	Cohort study	56,332/748	Diet	β-carotene	Education, alcohol consumption, consumption of red and processed meat, smoking status	7
Murtaugh et al. (23) United States	Rectal cancer	Case-control study	952/1,205	Diet	Lycopene, β-carotene, lutein	Age, body mass index, physical activity, energy intake, dietary fiber, dietary calcium, and smoking status	7
Williams et al. (53) United States	Colorectal cancer	Case-control study	945/959	Diet	β-carotene	Age, gender, education, smoking status, BMI, physical activity, family history, history of alcohol use	6
Park et al. (26) United States	Colon and rectal cancer	Cohort study	191,004/2,378	Diet	Lycopene, α-carotene, β-carotene, carotenoids, β-cryptoxanthin, Lutein	Gender, age, family history of colorectal cancer, history of intestinal polyps, number of pack-years smoked, body mass index	8
Slattery et al. (54) United States	Colon cancer	Case-control study	1,993/2,410	Diet	Lycopene, α-carotene, β-carotene, β-cryptoxanthin, Lutein, zeaxanthin	Age, gender, smoking, alcohol consumption, BMI and long term strenuous physical activity	7
Leenders et al. (22) Europe	Colon and rectal cancer	Case-control study	1,399/1,399	Diet	Lycopene, α-carotene, β-carotene, carotenoids, retinol	Smoking, alcohol consumption, BMI, physical activity, consumption level	7
Terry et al. (55) Canada	Colon and rectal cancer	Cohort study	56,837/5,681	Diet	Lycopene, α-carotene, β-carotene, carotenoids	Smoking status, relative body mass (body mass index), total fat intake, energy, alcohol, and folic acid, or menopausal status	7
Nkondjock and Ghadirian (56) Canada	Colon cancer	Case-control study	402/688	Diet	Lycopene, α-carotene, β-carotene, carotenoids, lutein/zeaxanthin, β-cryptoxanthin	Age, history of CC in first-degree relatives, marital status, gender, physical activity, fiber and folate consumption, and total energy intake	7
Wang et al. (57) Japan	Colon and rectal cancer	Case-control study	816/815	Diet	Lycopene, carotenoids	Age, residence, family history of colorectal cancer, smoking, alcohol consumption, BMI, type of work, physical activity	6
Negri et al. (58) Italy	Colorectal cancer	Case-control study	1,953/4,154	Diet	Lycopene, carotenoids, retinol	Sociodemographic characteristics, smoking, physical activity, anthropometric measurements at different ages, family history of cancer	7
Levi et al. (59) Switzerland	Colorectal cancer	Case-control study	223/491	Diet	Carotenoids, retinol	Age, sex, education, smoking, alcohol, body mass index, physical activity, and total energy and fiber intake	7
Lu et al. (60) China	Colorectal cancer	Case-control study	845/845	Diet	Lycopene, α-carotene, β-carotene, carotenoids, lutein/zeaxanthin, β-cryptoxanthin	Education, marital status, occupation, income, family history of cancer, smoking status, passive smoking, alcohol consumption, occupational activities, family and leisure activities, BMI	7
Paiva et al. (61) Portugal	Colorectal cancer	Case-control study	100/211	Diet	Carotenoids	Age, sex, marital status, work physical activity, family history of cancer, body mass index, fiber, carotene, vitamin C, and total energy	7
Rosato et al. (24) Switzerland	Colorectal cancer	Case-control study	329/1,361	Diet	β-carotene	Age, gender, family history, alcohol use, education, physical activity	6
Key et al. (62) United Kingdom	Colorectal cancer	Case-control study	565/1,951	Diet	β-carotene	Height, weight, energy intake, alcohol intake, dietary fiber, smoking, alcohol	

(Continued)

TABLE 1 | (Continued)

References Country	Type of cancer	Type of study	Sample size	Diet/Serum	Nutrient type	Adjustment for covariates.	NOS score
						consumption, physical activity, education, social class	7
Cook et al. (63) United States	Colon and rectal cancer	Cohort study	22,071/267	Diet	β-carotene	Age, education, marital status, occupation, income, family history of cancer, smoking status, passive smoking, alcohol consumption, occupational activity, BMI	7
Wakai et al. (64) Japan	Colon and rectal cancer	Case-control study	507/2,535	Diet	Carotenoids, retinol	Sex, age, family history, smoking, alcohol use, physical activity, energy intake	7
Shin et al. (25) China	Colon and rectal cancer	Cohort study	73,314/283	Diet	Carotenoids, retinol	Age, menopausal status, education, smoking, alcohol consumption, physical activity, family history of colorectal cancer, use of vitamin supplements, and total energy intake	8
Kabat et al. (65) United States	Colorectal cancer	Cohort study	5,477/88	Serum	Lycopene,α-carotene, β-carotene, Lutein + Zeaxanthin,β-Cryptoxanthin, Retinol	Age, body mass index, waist circumference, alcohol intake, physical activity, family history of colorectal cancer, ethnicity	8
Huang et al. (28) China	Colorectal cancer	Case-control study	538/564	Serum	Lycopene, α-carotene, β-carotene, lutein/zeaxanthin, β-cryptoxanthin	Living conditions, educational level, occupation, income, study, alcohol consumption, family history of colorectal cancer, physical activity	7
Luo et al. (66) China	Colon and rectal cancer	Case-control study	535/552	Serum	Retinol	Age, sex, residence, educational level, marital status, income, family and leisure activities, passive smoking, alcohol consumption, adult height, and BMI	6
Malila et al. (67) Finland	Colorectal cancer	Cohort study	26,951/184	Serum	Retinol, β-carotene	Age, body mass index (BMI), number of cigarettes smoked per day, occupational and leisure time physical activity, serum cholesterol concentration, alcohol intake	8

European countries, eight from North American countries and six from Asian countries. The major nutrient species studied were carotenoids, lycopene, α-carotene, β-carotene, lutein/zeaxanthin, β-cryptoxanthin, and retinol. The included studies were adjusted for covariates, mainly including: gender, age, smoking, alcohol consumption, family history of CRC, and physical activity. The NOS was scored from 6 to 8. The original data of the included studies are found in **Supplementary Table S1**.

Association Between Dietary Retinol and Various Carotenoids and Colorectal Cancer Risk

β-Carotene

We combined a total of 20 sets of data from 11 studies. Comparing low intakes, dietary high intake of β-carotene reduced the risk of CRC by 11% (OR = 0.89, 95% CI: 0.78–1.03, **Figure 2A**), but the association between the two was not significant (p_t = 0.113), and due to significant heterogeneity (I^2 = 63.2%, p < 0.001), we used a random-effects model for pooled analysis. According to subgroup analysis by tumor type, it can be seen that there is no significant correlation between dietary intake and the risk of colon cancer (OR = 0.96, 95% CI: 0.86–1.06, **Figure 3A**) and rectal cancer (OR = 1.06, 95% CI: 0.89–1.25, **Figure 3A**). In the subgroup analysis by study type, both the cohort study (OR = 0.95, 95% CI: 0.85–1.07, **Figure 4A**) and the case-control study (OR = 0.81, 95% CI: 0.63–1.05, **Figure 4A**) showed a trend of β-carotene to reduce the risk of CRC, but none of them were significantly associated. Finally, according to gender subgroup analysis, β-carotene intake was not significantly associated with the risk of CRC in female (OR = 0.97, 95% CI: 0.79–1.19, **Figure 5A**), but β-carotene intake was negatively associated with the risk of CRC in male (OR = 0.74, 95% CI: 0.55–0.99, **Figure 5A**).

α-Carotene

We combined a total of 10 sets of data from 5 studies. Comparing low intakes, dietary high intake of α-carotene reduced the risk of CRC by 13% (OR = 0.87, 95% CI: 0.72–1.03, **Figure 2B**), but the association between the two was not significant (p_t = 0.110), and due to significant heterogeneity (I^2 = 55.3%, p = 0.017), we used a random-effects model for pooled analysis. Subgroup analysis by tumor type showed that dietary intake was not significantly associated with the risk of colon cancer (OR = 0.96, 95% CI: 0.84–1.09, **Figure 3B**) and rectal cancer (OR = 1.01, 95% CI: 0.76–1.35, **Figure 3B**). In the subgroup analysis by study

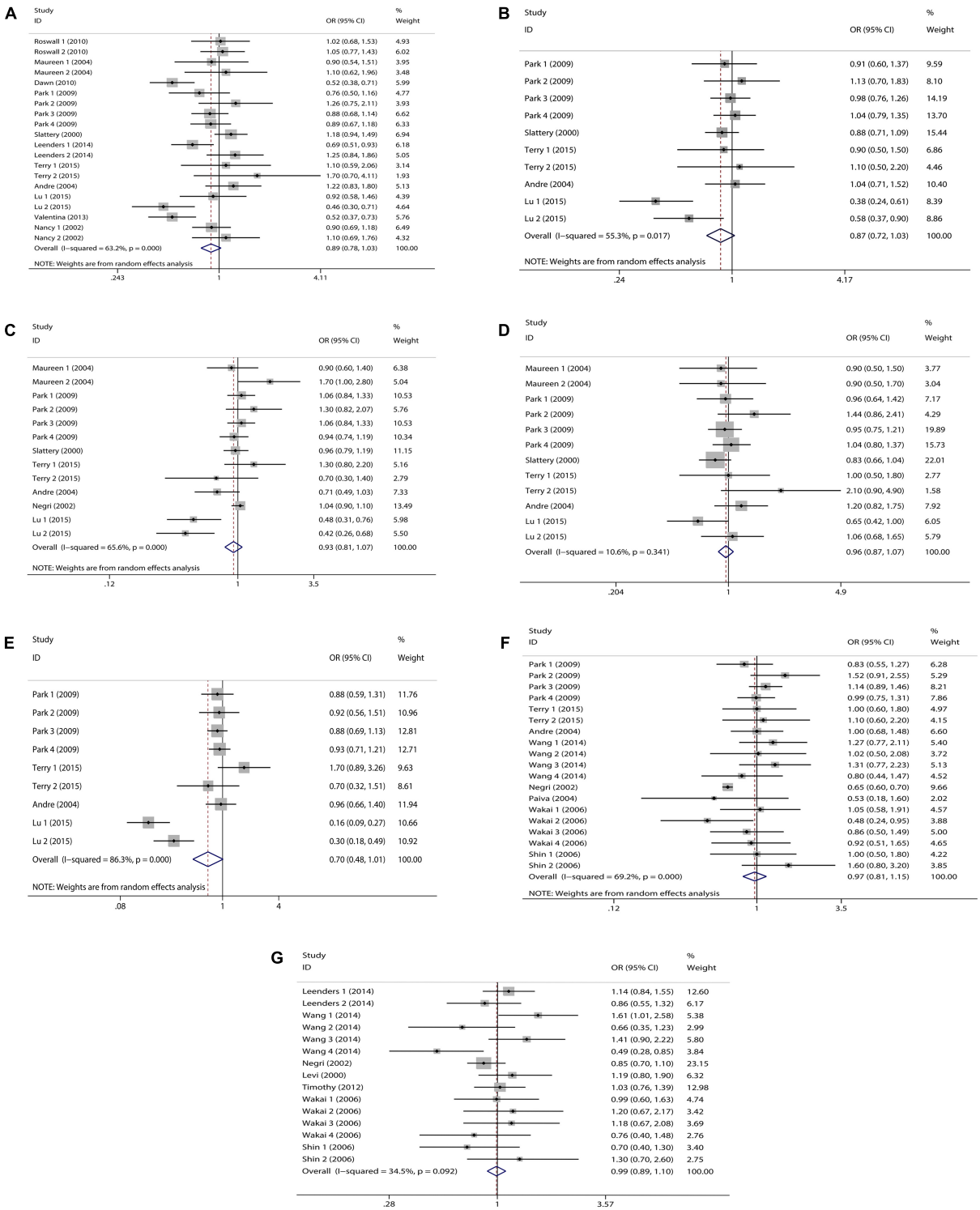

FIGURE 2 | Forest plot on dietary intake of carotenoids and retinol and colorectal cancer risk. **(A)** β-carotene; **(B)** α-carotene; **(C)** lycopene; **(D)** lutein/zeaxanthin; **(E)** β-Cryptoxanthin; **(F)** carotenoids; **(G)** retinol.

type, the cohort study (OR = 1.00, 95% CI: 0.86–1.16, **Figure 4B**) showed no significant association between their intake and CRC, and the case-control studies (OR = 0.69, 95% CI: 0.47–1.02, **Figure 4B**) showed that their high intake tended to reduce CRC risk, but there was no significant association. Finally, high intake of α-carotene tended to reduce CRC in male (OR = 0.71, 95% CI:

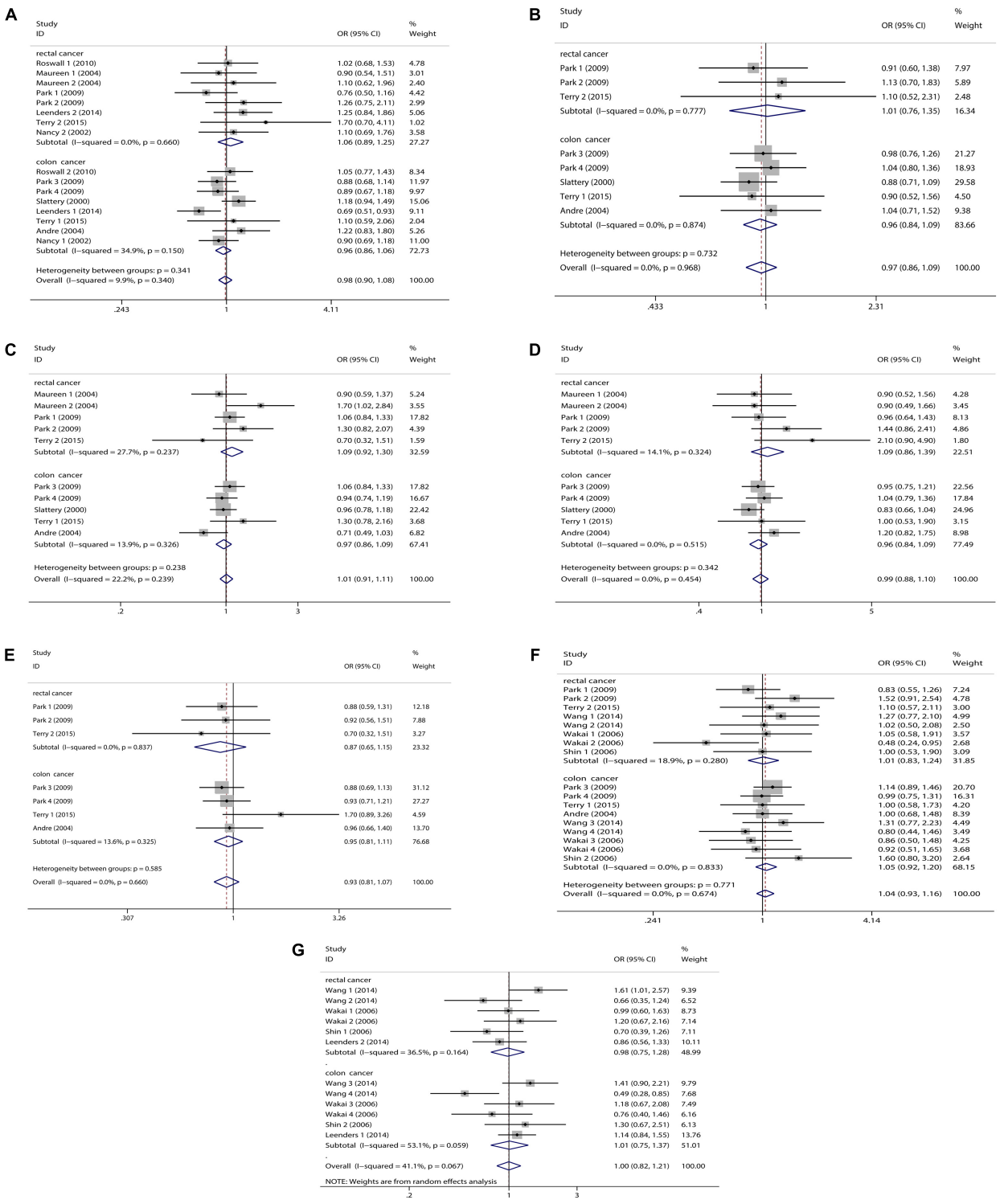

FIGURE 3 | Tumor subgroup analysis of dietary carotenoid and retinol intake and colorectal cancer risk. **(A)** β-carotene; **(B)** α-carotene; **(C)** lycopene; **(D)** lutein/zeaxanthin; **(E)** β-Cryptoxanthin; **(F)** carotenoids; **(G)** retinol.

0.42–1.22, **Figure 5B**) and female (OR = 0.89, 95% CI: 0.61–1.30, **Figure 5B**) according to gender subgroup analysis, but there was no significant association.

Lycopene

Seven studies were included to combine a total of 13 sets of data. High lycopene (OR = 0.93, 95% CI: 0.81–1.07, **Figure 2C**) intake

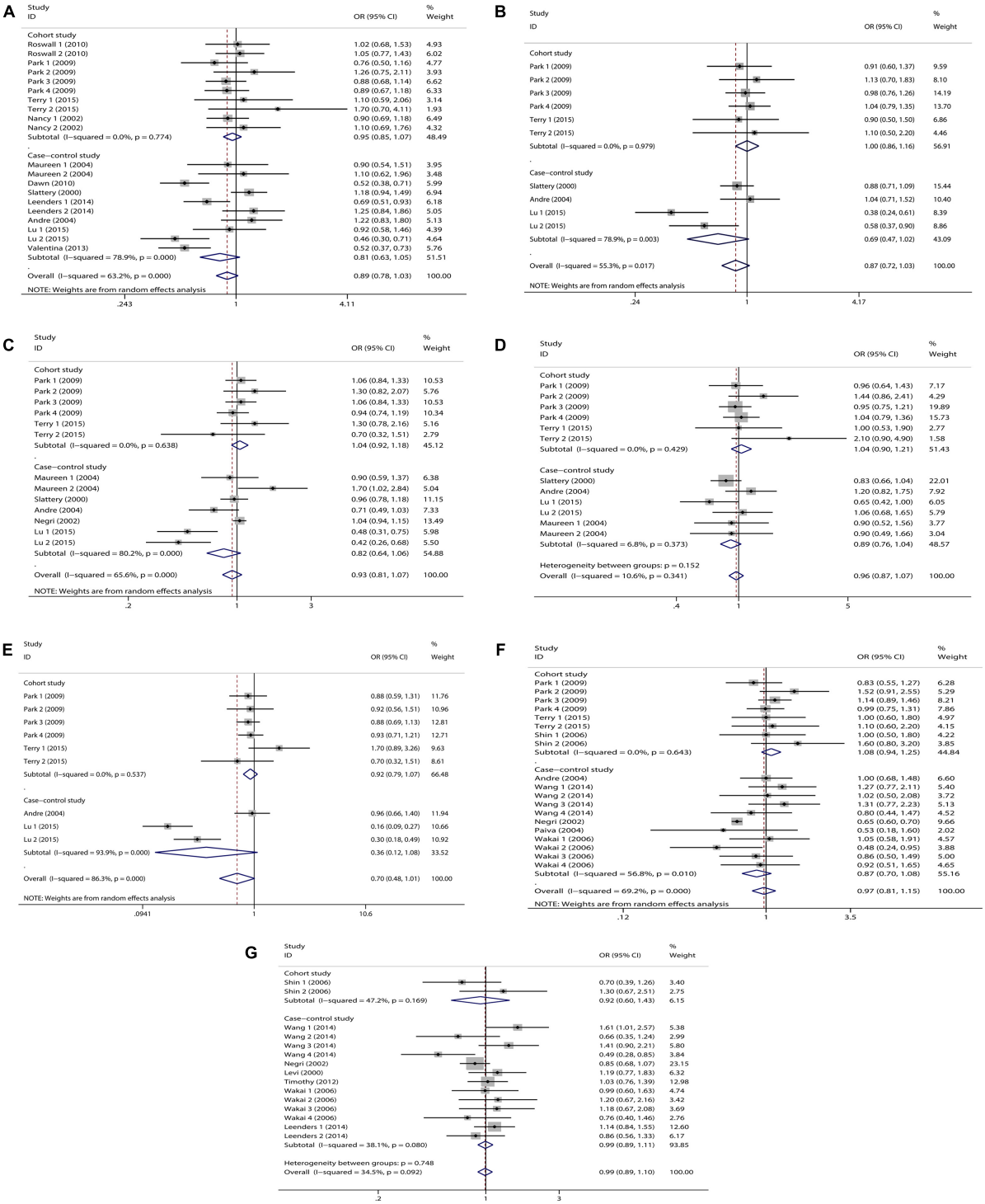

FIGURE 4 | Study type subgroup analysis of dietary carotenoid and retinol intake and colorectal cancer risk. **(A)** β-carotene; **(B)** α-carotene; **(C)** lycopene; **(D)** lutein/zeaxanthin; **(E)** β-Cryptoxanthin; **(F)** carotenoids; **(G)** retinol.

slightly, but not significantly (p_t = 0.329), reduced CRC risk. Due to significant heterogeneity (I^2 = 65.6%, p = 0.000), pooling was performed with a random-effects model. Subgroup analysis was performed according to tumor type, study type and gender. Colon cancer (OR = 0.97, 95% CI: 0.86–1.09, **Figure 3C**), rectal cancer (OR = 1.09, 95% CI: 0.92–1.30, **Figure 3C**), cohort study (OR = 1.04, 95% CI: 0.92–1.18, **Figure 4C**), case-control study (OR = 0.82, 95% CI: 0.64–1.06, **Figure 4C**), male (OR = 0.88, 95%

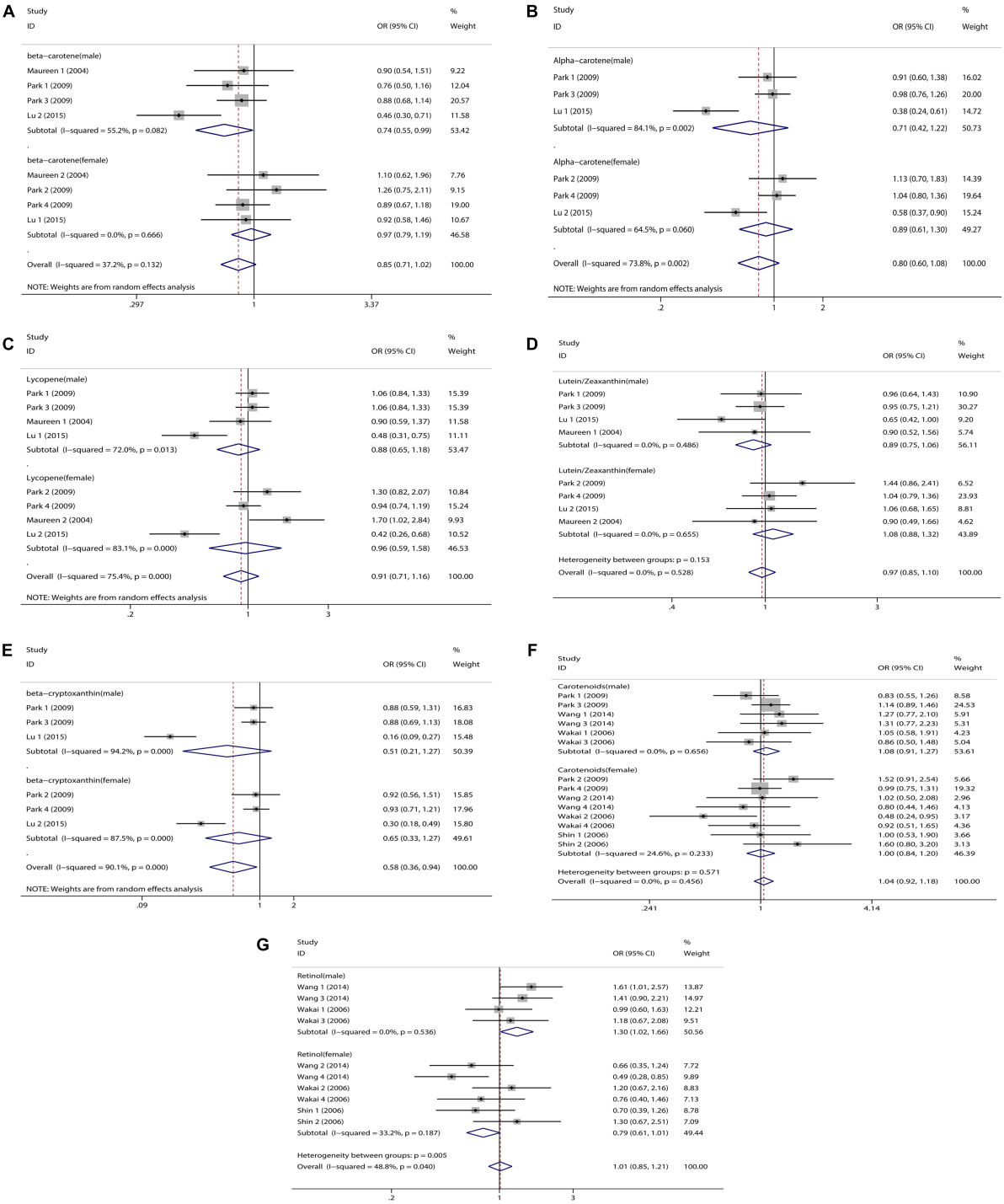

FIGURE 5 | Sex subgroup analysis of dietary carotenoid and retinol intake and colorectal cancer risk. **(A)** β-carotene; **(B)** α-carotene; **(C)** lycopene; **(D)** lutein/zeaxanthin; **(E)** β-Cryptoxanthin; **(F)** carotenoids; **(G)** retinol.

CI: 0.65–1.18, **Figure 5C**), female (OR = 0.96, 95% CI: 0.59–1.58, **Figure 5C**). In subgroup analyses, case-control studies showed a non-significant inverse association between lycopene intake and CRC risk. There was also a risk reduction effect in male, although it was not significant.

Lutein/Zeaxanthin

Six studies were included and a total of 12 sets of data were combined. There was no significant (p_t = 0.508) association between high lutein/zeaxanthin (OR = 0.96, 95% CI: 0.87–1.07, **Figure 2D**) intake and CRC risk. No significant heterogeneity was

found (I^2 = 10.6%, p = 0.341), which was summarized using a fixed-effect model. Subgroup analysis was performed according to tumor type, study type and gender. Colon cancer (OR = 0.96, 95% CI: 0.84–1.09, **Figure 3D**), rectal cancer (OR = 1.09, 95% CI: 0.86–1.39, **Figure 3D**), cohort study (OR = 1.04, 95% CI: 0.90–1.21, **Figure 4D**), case-control study (OR = 0.89, 95% CI: 0.76–1.04, **Figure 4D**), male (OR = 0.89, 95% CI: 0.75–1.06, **Figure 5D**), female (OR = 1.08, 95% CI: 0.88–1.32, **Figure 5D**). In subgroup analysis, case-control studies showed a non-significant inverse association between the intake of lutein/zeaxanthin and CRC risk. The risk reduction effect was also present in male, but was not significant.

β-Cryptoxanthin

Four studies were included and a total of 9 sets of data were combined. High β-cryptoxanthin (OR = 0.70, 95% CI: 0.48–1.01, **Figure 2E**) intake was able to reduce CRC risk by 30%, but not statistically significant (p_t = 0.058). High heterogeneity was found (I^2 = 86.3%, p = 0.000), which was combined using the random-effects model. Subgroup analysis was performed according to tumor type, study type, and gender. Colon cancer (OR = 0.95, 95% CI: 0.81–1.11, **Figure 3E**), rectal cancer (OR = 0.87, 95% CI: 0.65–1.15, **Figure 3E**), cohort study (OR = 0.92, 95% CI: 0.79–1.07, **Figure 4E**), case-control study (OR = 0.36, 95% CI: 0.12–1.08, **Figure 4E**), male (OR = 0.51, 95% CI: 0.21–1.27, **Figure 5E**), female (OR = 0.65, 95% CI: 0.33–1.27, **Figure 5E**). In subgroup analysis, high β-cryptoxanthin intake tended to decrease risk of CRC, but this was not significant.

Total Carotenoids

Eight studies were included and a total of 19 sets of data were combined. There was no significant (p_t = 0.717) association between high carotenoids (OR = 0.97, 95% CI: 0.81–1.15, **Figure 2F**) intake and CRC risk. There was significant heterogeneity (I^2 = 69.2%, p = 0.000), which was combined using the random-effects model. Subgroup analysis was performed according to tumor type, study type and gender. Colon cancer (OR = 1.05, 95% CI: 0.92–1.20, **Figure 3F**), rectal cancer (OR = 1.01, 95% CI: 0.83–1.24, **Figure 3F**), cohort study (OR = 1.08, 95% CI: 0.94–1.25, **Figure 4F**), case-control study (OR = 0.87, 95% CI: 0.70–1.08, **Figure 4F**), male (OR = 1.08, 95% CI: 0.91–1.27, **Figure 5F**), female (OR = 1.00, 95% CI: 0.84–1.20, **Figure 5F**). No association was found between high carotenoids intake and the risk of CRC in any Subgroup group.

Retinol

Seven studies were included and a total of 15 sets of data were combined. There was no significant (p_t = 0.850) association between high retinol (OR = 0.99, 95% CI: 0.89–1.10, **Figure 2G**) intake and CRC risk. There was no significant heterogeneity (I^2 = 34.5%, p = 0.092), and fixed effect model was used for combination. Subgroup analysis was performed according to tumor type, study type and gender. Colon cancer (OR = 1.01, 95% CI: 0.75–1.37, **Figure 3G**), rectal cancer (OR = 0.98, 95% CI: 0.75–1.28, **Figure 3G**), cohort study (OR = 0.92, 95% CI: 0.60–1.43, **Figure 4G**), case-control study (OR = 0.99, 95% CI: 0.89–1.11, **Figure 4G**), male (OR = 1.30, 95% CI: 1.02–1.66, **Figure 5G**), female (OR = 0.79, 95% CI: 0.61–1.01, **Figure 5G**). Retinol appeared to play a protective role in women, reducing CRC risk by 21%, although there was no significant association. For men, retinol intake was significantly positively associated with the risk of CRC.

Association of Serum Retinol and Carotenoid Levels With Colorectal Cancer Risk

With regard to serum carotenoids, three studies were included and a total of 11 sets of data were combined. Serum total carotenoids (OR = 0.73, 95% CI: 0.58–0.93, **Figure 6A**) were significantly (p_t = 0.01) negatively associated with CRC risk. The results showed significant heterogeneity (I^2 = 67.5%, p = 0.001), which was combined using the random-effects model. The subgroup analysis was performed according to the type of nutrients. Serum α-carotene (OR = 0.61, 95% CI: 0.37–0.99, **Figure 6B**) was significantly inversely associated with CRC risk. However, the serum content of β-carotene (OR = 0.83, 95% CI: 0.64–1.08, **Figure 6B**), Lycopene (OR = 0.58, 95% CI: 0.22–1.54, **Figure 6B**), and β-Cryptoxanthin (OR = 0.69, 95% CI: 0.28–1.69, **Figure 6B**), although negatively correlated with CRC risk, was not significant. There was no correlation between serum Lutein/Zeaxanthin (OR = 0.99, 95% CI: 0.63–1.56, **Figure 6B**) content and CRC risk.

With regard to serum retinol, three studies were included and a total of four sets of data were combined. High serum retinol (OR = 0.62, 95% CI: 0.26–1.49, **Figure 6C**) was inversely associated with CRC risk, but the association was not significant (p_t = 0.284). The results showed significant heterogeneity (I^2 = 90.8%, p = 0.000), and random effects model was used for combination. Subgroup analysis were also performed according to study type. Cohort studies (OR = 1.22, 95% CI: 0.80–1.86, **Figure 6D**) showed no association between serum retinol and CRC risk, but case-control studies (OR = 0.29, 95% CI: 0.16–0.54, **Figure 6D**) showed a significant inverse association between serum retinol and CRC risk. Meta-analysis results of the above various nutrients are shown in **Table 2**.

Publication Bias and Sensitivity Analysis

Due to the less in serological studies included, bias testing and sensitivity analysis were not necessary. Therefore, we performed bias test and sensitivity analysis on the combined results of dietary retinol and carotenoids. We used Begg's test as well as Begg's funnel plot to assess publication bias. Begg's test results (**Figure 7**): β-carotene (Pr > | z | = 0.417), α-carotene (Pr > | z | = 0.721), lycopene (Pr > | z | = 0.464), β-Cryptoxanthin (Pr > | z | = 0.075), Lutein/Zeaxanthin (Pr > | z | = 0.304), Carotenoids (Pr > | z | = 0.234), retinol (Pr > | z | = 0.692). The results of bias test showed that all funnel plots were symmetrical and (Pr > | z | > 0.05), indicating that no significant publication bias was found in the combined results. Sensitivity analysis (**Figure 8**) of the results was performed and the pooled OR varied in a limited range without significant change after removing each study, indicating that our results were stable.

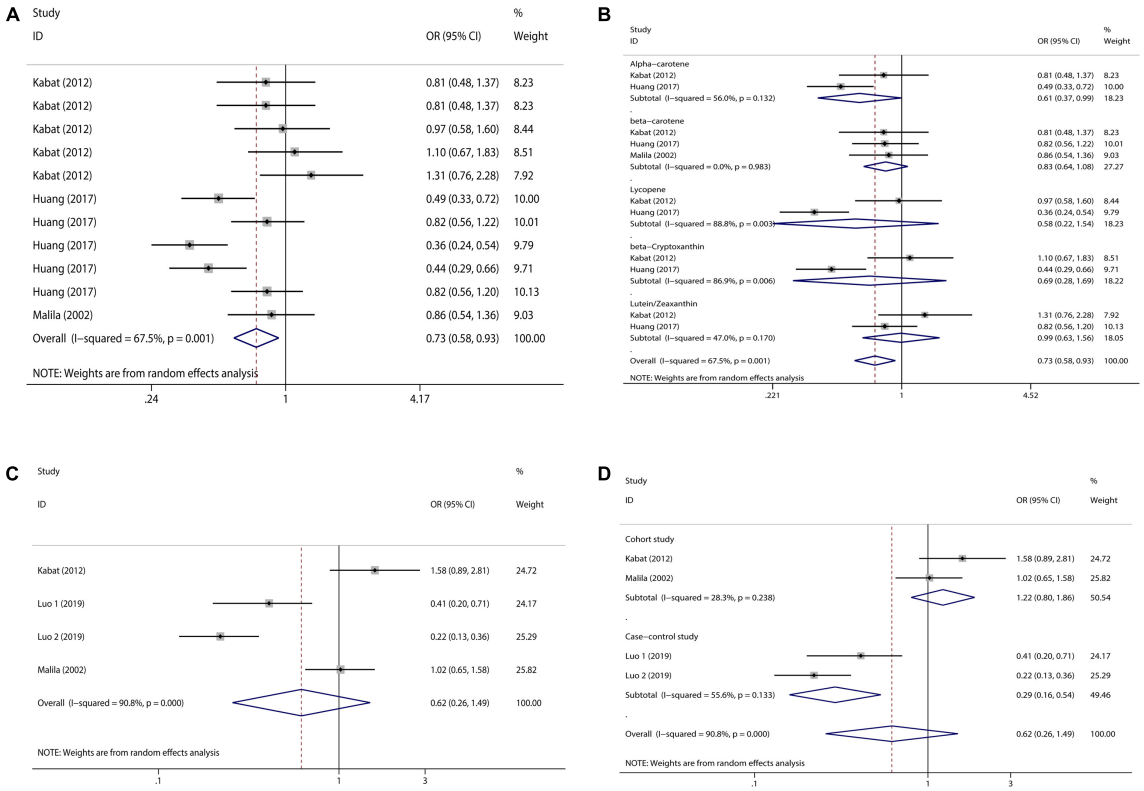

FIGURE 6 | Forest plot of serum carotenoid and retinol concentrations and colorectal cancer risk. **(A)** serum carotenoid; **(B)** subgroup analysis of serum carotenoids according to their types; **(C)** serum retinol; **(D)** subgroup analysis of serum retinol by study type.

TABLE 2 | Meta-results on intake of various nutrients and colorectal cancer risk.

						Heterogeneity		
Nutrient type	**Studies (*n*)**	**OR**	**95%CI**	***P*-value**	**Model**	**Chi^2**	**I^2**	***P*-value**
β-carotene	20	0.89	0.78–1.03	0.113	Random	51.61	63.2%	0.000
α-carotene	10	0.87	0.72–1.03	0.110	Random	20.14	55.3%	0.017
Lycopene	13	0.93	0.81–1.07	0.329	Random	34.83	65.6%	0.000
Lutein/zeaxanthin	12	0.96	0.87–1.07	0.508	Fix	12.31	10.6%	0.341
β-Cryptoxanthin	9	0.70	0.48–1.01	0.058	Random	58.36	86.3%	0.000
Carotenoids	19	0.97	0.81–1.15	0.717	Random	58.44	69.2%	0.000
Retinol	15	0.99	0.89–1.10	0.850	Fix	21.37	34.5%	0.092
Carotenoids (serum)	11	0.73	0.58–0.93	0.010	Random	30.79	67.5%	0.001
Retinol (serum)	4	0.62	0.26–1.49	0.284	Random	30.51	90.8%	0.000

From this, it can be seen that the relevant conclusions we draw are stable and reliable.

DISCUSSION

Although vitamin A (retinol) and carotenoids are widely present in a variety of vegetables and fruits, many people still lack the intake of these nutrients. Therefore, the impact of retinol and carotenoids intake on CRC risk has important public health implications. We included a total of 22 studies that pooled clinical studies on dietary and serum retinol and carotenoids and CRC risk. Subgroup analysis was performed according to tumor type, study category and sex.

The results showed that dietary β-carotene intake was not significantly negatively correlated with CRC risk, but β-carotene could significantly lower CRC risk in the male population, showing a protective and preventive effect. The consumption of α-carotene lowered the risk of CRC, however, the link was not statistically significant. A high lycopene consumption lowered CRC risk marginally but not dramatically. There was no link seen between high lutein/zeaxanthin consumption and CRC risk. High intake of β-cryptoxanthin was able to non-significantly reduce the risk of CRC. There was no link found between

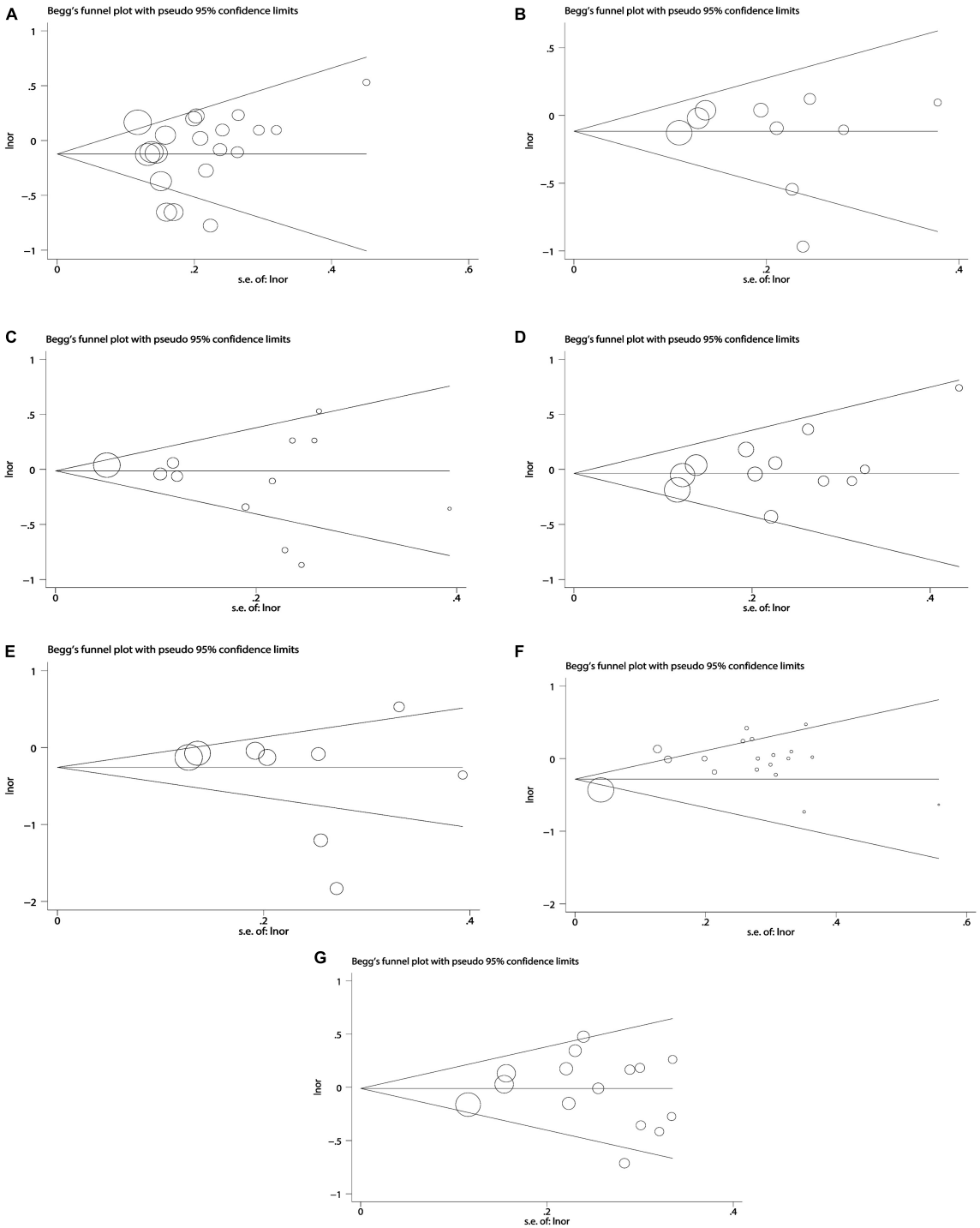

FIGURE 7 | Begg' s publication bias plots on dietary carotenoids and retinol and colorectal cancer risk. **(A)** β-carotene; **(B)** α-carotene; **(C)** lycopene; **(D)** lutein/zeaxanthin; **(E)** β-cryptoxanthin; **(F)** carotenoids; **(G)** retinol.

high total carotenoids consumption and CRC risk. High retinol consumption had no significant connection with CRC risk, and although it appeared to protect women and reduce CRC risk by 21%, high retinol intake was able to significantly increase the risk of CRC for men, and this difference was very important and could guide dietary matching. In summary, high β-carotene, α-carotene, lycopene, and β-cryptoxanthin all have a tendency to reduce CRC risk, which is more pronounced in the male population, but there is uncertainty that must be explored. Total carotenoids intake and Lutein/Zeaxanthin intake were not

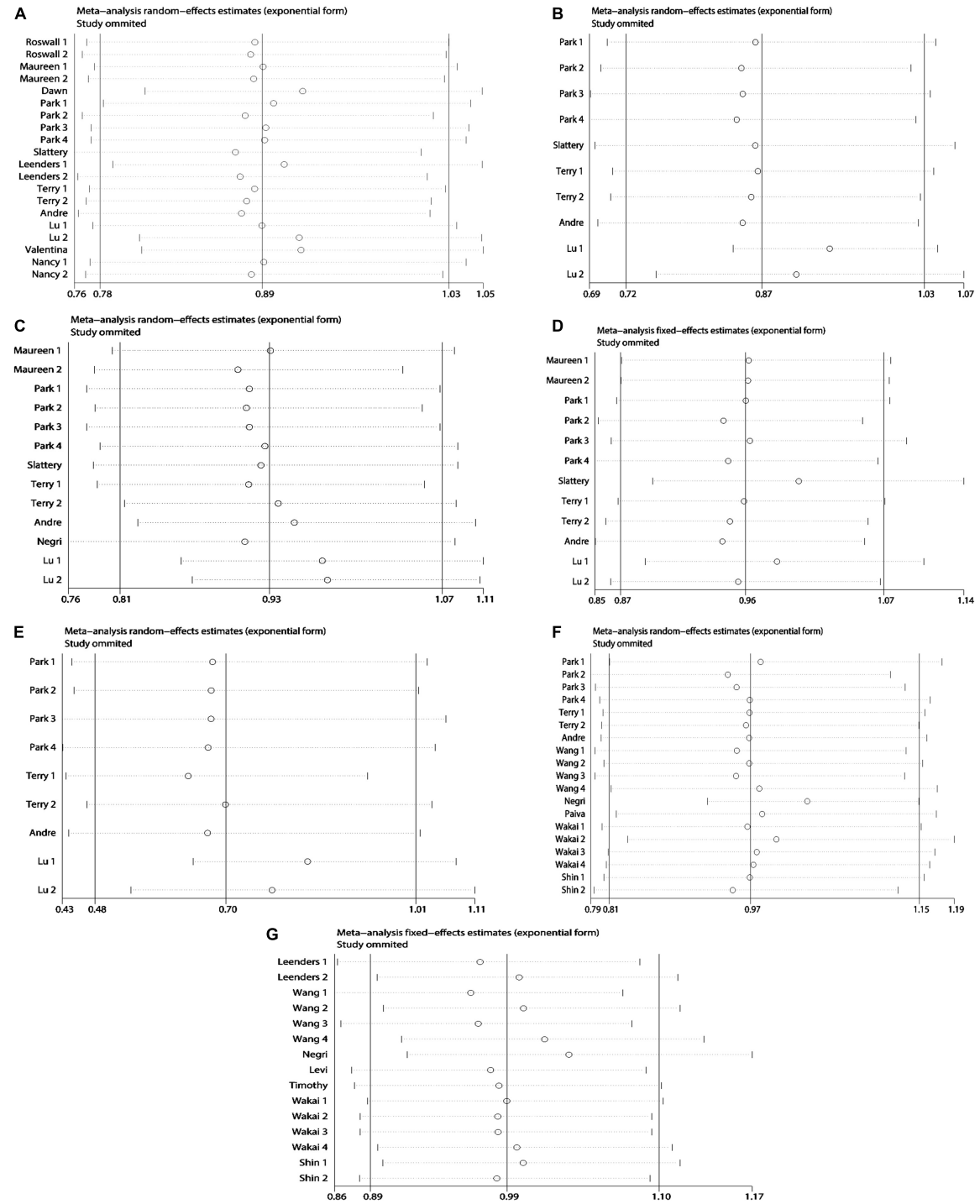

FIGURE 8 | Sensitivity analysis plots on dietary carotenoids and retinol and colorectal cancer risk. **(A)** β-carotene; **(B)** α-carotene; **(C)** lycopene; **(D)** lutein/zeaxanthin; **(E)** β-cryptoxanthin; **(F)** carotenoids; **(G)** retinol.

associated with CRC risk. Beta-carotene has a preventive effect on CRC in men and retinol seems to have a preventive effect in women and a carcinogenic effect in men, and this difference has led us to new ideas about adjusting the diet by sex.

In addition to focusing on the risk of dietary retinol and carotenoids on CRC, we also focused on serological aspects of the study. A significant inverse association was found between serum carotenoids concentrations and CRC risk. Serum β-carotene was shown to have a substantial negative relationship with CRC risk. Other carotenoids, while adversely associated, were not significant. Case-control studies have found a substantial negative link between serum retinol and CRC risk, while cohort studies

have found no significant relationship, hence the relationship between serum retinol and CRC remains unknown and must be confirmed by large prospective investigations. Most previous studies have focused on dietary carotenoids, but in recent years attention has gradually shifted to serum carotenoids, possibly due to the development of serum detection techniques and more stable and accurate quantitative assessment of serum. Serum carotenoids are widely studied, in addition to CRC (28, 29), but also associated with the risk of breast cancer (30), lung cancer (31), prostate cancer (32, 33) and hepatocellular carcinoma (34), which can be used as a key research direction in the future. If the relationship between serum carotenoids and various cancers can be clearly understood, routine admission examination can be performed in high-risk cancer population to preliminarily evaluate and screen related tumors, which has certain application prospects in clinical practice.

We will further explore the mechanisms underlying the prevention and suppression of CRC by carotenoids. β-carotene has been found in animal studies to have anti-colon cancer properties through modulating M_2 macrophages and activated fibroblasts (35). By regulating K-ras, PKB, and beta-catenin, dietary lutein can decrease colon carcinogenesis caused by p-dimethylhydrazine in rats (36). Carotenoids isolated from Chlorella ellipsoidea and Chlorella vulgaris have also been shown in cell tests to have antiproliferative and anticancer effects on human colon cancer cells (37). B-carotene has been demonstrated to decrease colon cancer cell development by reducing COX-2 production and down-regulating colon cancer cell homeostasis (38). A growing number of experimental investigations have also proven the mechanism and significance of carotenoids in anti-CRC. Our study surprised us by the finding that a high intake of β-cryptoxanthin (OR = 0.70, 95% CI: 0.48–1.01) was able to reduce CRC risk. β-cryptoxanthin is one of the six primary carotenoids. It is mostly present in citrus fruits, although it is also found in corn, peas, and other yellow animal products (16, 39). β-cryptoxanthin has been demonstrated in animal experiments to have preventative and inhibitory effects on a number of malignancies, including colon cancer (40), gastric cancer (41), lung cancer (42–44), bladder cancer (45), and liver cancer (46) through a variety of molecular mechanisms. It has been demonstrated that β-cryptoxanthin in combination with oxaliplatin dramatically increased the apoptosis of colon cancer cells *in vitro* and *in vivo*, indicating anti-tumor and therapeutic actions on CRC (40). From this point of view, although there are few studies on β-cryptoxanthin, it may have a role in preventing and inhibiting tumors in a variety of cancers, especially CRC. The conclusions about β-cryptoxanthin in this meta-analysis should be paid attention to, and strengthening the study of β-cryptoxanthin may bring fruitful results.

There have also been several earlier meta-analyses investigating the relationship between carotenoids and CRC. Männistö et al. performed a meta-analysis of cohort studies on dietary carotenoids and CRC risk in 2006, and discovered no link between any carotenoids and CRC risk (47). Conclusion may be caused by several limitations. On the one hand, we believe that the relevant studies it includes are somewhat old and not suitable for the dietary pattern of modern humans. On the other hand, the studies it included were Caucasian studies in Europe and North America with certain geographical limitations; at the time, communication technology was limited, which easily led to a loss due to follow-up bias. Wang et al. performed a meta-analysis of observational data on lycopene consumption and CRC risk in 2016 (48). The data indicate that lycopene consumption is not related with an increased risk of CRC, which is consistent with our findings. In 2016, Panic et al. performed a meta-analysis of dietary carotenoid consumption and CRC risk, which found no significant link between dietary carotenoid intake and CRC (49). The reason for the inconsistency with our findings is that on the one hand we updated and added several new studies, on the other hand our study was performed in strict accordance with the quality assessment rules and removed several unqualified studies, and his study included these low-quality articles, which may affect the results. Third, we also conducted a gender subgroup analysis that may derive the effect of gender differences, and his study did not consider gender differences. Our findings are not consistent with the above the meta-analyses, but our study is higher credible.

We found clear heterogeneity in the entire summary results for retinol and carotenoids and CRC risk. Heterogeneity is inevitable in meta-analysis, and determining the source of the heterogeneity is an important step. First, where the heterogeneity of the data was considerable, we utilized a random-effects model to combine effect sizes. Second, we conducted a subgroup analysis by tumor type, research type, and gender. Most studies' heterogeneity was greatly decreased after subgroup analysis. Third, we conducted a sensitivity analysis to exclude the one that had the biggest influence on the research outcomes, hence lowering heterogeneity. In addition, there may be many factors that can increase heterogeneity, such as differences in race, region, dietary structure, ideology, and degree of economic development. Finally, heterogeneity may ensue as a result of non-uniform methodologies and research details, as well as inconsistency between meals and vitamin A content measurement instruments or scales. Heterogeneity is exacerbated by the inconsistency of particular dosage limits for high and low intakes. As a result, the conclusions drawn should be treated with caution.

Our meta-analysis provides a number of advantages. First, for the first time, we not only evaluated the association between dietary carotenoids and retinol and CRC risk, but also performed serological aspects. Furthermore, each of the six major groups of carotenoids was thoroughly examined. Second, because this study included a large number of cases and participants, more reliable estimations of the connection between retinol and carotenoids consumption and CRC risk may be obtained. Third, there was no evidence of significant publication bias in our meta-analysis. Fourth, we conducted a detailed subgroup analysis according to tumor type, study type, and gender. Fifth, the results of our included studies were all adjusted for covariates. Sixth, we included studies from the last 20 years, avoiding that old dietary patterns influence the accuracy of study conclusions.

Our study has several limitations. First, we only included English articles, which may cause selection bias. Second, there is a large heterogeneity in the findings, although the sources

of heterogeneity have been explored. Third, study results were not subgroup analyzed by region and race. Fourth, the specific doses of retinol and carotenoids were not stated, and no dose-response meta-analysis was performed. Fifth, detection and transformation tools for retinol and carotenoids contained in ingested foods are not described. Sixth, although all results were adjusted for covariates, it is possible that there are other factors that affect the accuracy of the results.

While the efficacy of early CRC has improved, the prognosis of advanced CRC remains poor, so we must invest more effort in cancer prevention. Through the transformation of scientific research achievements, develop a set of preventive means suitable for the CRC population, strengthen people's health publicity and education from the aspects of diet, exercise, and mental psychology, and eliminate tumors in the bud. From the results of our study, it can be seen that retinol, β-cryptoxanthin, β-carotene, α-carotene, and lycopene have some value in preventing CRC. We suggest that middle-aged and older adults with a family history of CRC or other risk factors can prevent CRC by modestly increasing carotenoid intake and even by taking supplements. From our gender subgroup analysis, it can be seen that β-carotene has a preventive effect on CRC in men, while retinol seems to have a preventive effect on CRC in women, so we can develop corresponding dietary recipes according to gender, and agents with different allocation ratios can also be made when designing supplement components to make cancer prevention more accurate.

Internationally, most of the research on vitamin prevention of cancer stays at the level of observational studies, and most of them have not been studied more deeply. In the future, on the one hand, perfect inclusion and exclusion criteria can be developed to conduct multicenter large randomized controlled trials (RCTs) to clarify the preventive effect. On the other hand, rigorous animal experiments and tumor cell experiments can be designed to determine the preventive effect, as well as to clarify the preventive mechanism. If validated by multicenter RCT and cell and animal experiments, recipes for relevant populations can be developed and corresponding supplements can be manufactured for promotion and application. Vitamins are one of the essential nutrients for human beings, which contain a wide variety and have different physiological functions and have an important relationship with many diseases. While exploring retinol and carotenoids, we can also try to explore the preventive effects of vitamin B, vitamin C, vitamin D, vitamin E, and folic acid on different cancers. In addition to exploring the value of vitamins in the prevention of cancer, the value of survival and prognosis was explored. In addition, we found that most of the vitamins belong to antioxidants, and in addition to studying the value of vitamins in cancer, the relationship between other antioxidants and cancer can be explored, such as melatonin, anthocyanins, astaxanthin, and quercetin. Finally, it should be noted that excessive use of vitamins will produce corresponding toxic side effects, such as excessive intake of carotenoids will cause loss of appetite, yellow skin, poor sleep, affecting female ovulation and so on (50–52). Therefore, we recommend an appropriate increase in vitamin A intake within a safe dose range, especially an increase in dietary intake of vegetables, fruits, and animal products.

CONCLUSION

Total carotenoids intake and Lutein/Zeaxanthin intake were not associated with CRC risk. High β-carotene, α-carotene, lycopene, and β-cryptoxanthin all tended to reduce CRC risk, with a more pronounced effect in the male population. In addition, β-carotene had a significant preventive effect on CRC in men. In the female population, high dietary retinol intake can reduce CRC risk, while it has carcinogenic effects in men. On the other hand, serum carotenoids concentrations were significantly and inversely associated with CRC risk. Finally, due to the limitations, large prospective studies with adequate sample size, well-controlled confounders, and long-term follow-up are needed for further exploration.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

XH and RZ conceived the study and wrote the draft. GZ and YJ performed the literature search. DW and YW extracted the required data. XH performed the statistical analyses. HC reviewed the manuscript. All authors viewed and gave permission to publish this manuscript.

FUNDING

This study was supported by the grants from the Central to Guide Local Scientific and Technological Development (ZYYDDFFZZJ-1), Key talent project of Gansu Province of the Organization Department of Gansu provincial Party committee (2020RCXM076), Key Laboratory of gastrointestinal cancer diagnosis and treatment of National Health Commission (2019PT320005), Gansu Provincial Youth Science and Technology Fund Program (21JR7RA642), Gansu Key Laboratory of molecular diagnosis and precision treatment of surgical tumors (18JR2RA033), Guiding plan for scientific and technological development of Lanzhou (2019-ZD-102), and Natural Science Foundation of Gansu Province (21JR11RA186).

ACKNOWLEDGMENTS

We thank the researchers and study participants for their contributions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.918777/full#supplementary-material

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SYSTEMATIC REVIEW
published: 14 July 2022
doi: 10.3389/fnut.2022.935991

Vitamin C Intake and Ischemic Stroke

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Vitamin C is an essential micronutrient with important antioxidant properties. Ischemic stroke is a major public health problem worldwide. Extensive evidence demonstrates that vitamin C has protective effects against cardiovascular disease, and there is a close relationship between vitamin C intake and ischemic stroke risk. Based on the evidence, we conducted this umbrella review to clarify the relationship between vitamin C intake and ischemic stroke risk from four perspectives: cellular mechanisms, animal experiments, clinical trials, and cohort studies.

Keywords: vitamin C, ischemic stroke, review, micronutrient, CVD

OPEN ACCESS

Edited by:
Manfred Eggersdorfer, University of Groningen, Netherlands

Reviewed by:
Gloria Olaso-Gonzalez, University of Valencia, Spain
Tanase Corneliu, George Emil Palade University of Medicine, Pharmacy, Sciences and Technology of Târgu Mureș, Romania

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Specialty section:
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

Received: *04 May 2022*
Accepted: *22 June 2022*
Published: *14 July 2022*

Citation:
Tang X, Liu H, Xiao Y, Wu L and Shu P (2022) Vitamin C Intake and Ischemic Stroke. Front. Nutr. 9:935991. doi: 10.3389/fnut.2022.935991

INTRODUCTION

Stroke is the second most common cause of death and the leading cause of disability and, therefore, a major public health concern (1). Stroke is associated with high rates of morbidity, disability, mortality, and recurrence (1). Ischemic stroke is the most common type of stroke, accounting for approximately 80% of all cases of stroke (1). Although the mortality rate of stroke has decreased globally in recent years, the global disease burden of stroke has continued to increase; thus, the prevention and treatment of stroke are important components of global public health management (1). Vitamin C is an essential nutrient with antioxidant and anti-inflammatory properties, and has been shown to inhibit the inflammatory response and oxidative reactions, protect the vascular endothelium, and prevent the development of atherosclerosis (2). This review systematically discusses the relationship between vitamin C intake and ischemic stroke risk from four perspectives: cellular mechanisms, animal experiments, clinical trials, and cohort studies.

Vitamin C, also known as ascorbic acid, cannot be synthesized by the human body and must be obtained through the diet (3). It is a water-soluble vitamin that is present in a wide range of fruits and vegetables. As an essential micronutrient in the human body, an adequate intake of vitamin C helps maintain human health (3). Vitamin C is a water-soluble acidic polyhydroxy compound with six carbon atoms and a structure similar to glucose (4). It has many biological functions. It produces H^+ after being oxidized to dehydrovitamin C. The oxidized and reduced forms of vitamin C can be converted into each other to form a redox system in biological tissues (4). Many physiological activities of vitamin C are related to this property. For instance, vitamin C functions as a coenzyme or a substrate for a series of enzymes involved in various metabolic pathways: it is a coenzyme for prolyl and lysyl hydroxylases, which catalyze the hydroxylation of proline and lysine, respectively, during collagen biosynthesis, and a coenzyme in iron metabolism (4).

The circulating vitamin C concentration in healthy people is approximately 70 μM. Concentrations below 23 μM indicate vitamin C deficiency, and concentrations below 11 μM indicate severe vitamin C deficiency with a risk of scurvy (5). The amount of vitamin C intake required by the human body depends on the plasma ascorbic acid concentration (5). While the recommended dietary allowances (RDAs) provide estimates of the required vitamin C intake for humans, the optimal dietary intake is unknown and may be determined by factors such as

the dose–function relationship, the availability of vitamin C in dietary sources, the plasma and tissue steady-state concentrations after each dose of vitamin C, urinary excretion, bioavailability, toxicity, and epidemiological observations of vitamin C intake (5). The relationship between the plasma vitamin C concentration and intake dose shows an S-shaped curve (5). Plasma vitamin C concentrations of 50 μM and higher are considered to be appropriate. The RDA of vitamin C varies widely among different health organizations. The German, Austrian, and Swiss Institutes of Nutrition have stipulated an RDA of 110 mg/day for men and 95 mg/day for women, whereas the American and Canadian Institutes of Medical Research have set an RDA of 90 mg/day for men and 75 mg/day for women (6, 7).

THE POSSIBLE MECHANISM WHEREBY VITAMIN C REDUCES THE RISK OF ISCHEMIC STROKE

Vitamin C may reduce the risk of ischemic stroke through various mechanisms, such as inhibiting low-density lipoprotein (LDL) oxidation, increasing intravascular nitric oxide (NO) production, increasing vasodilation and lowering blood pressure, and reducing the adhesion of monocytes to the vascular endothelium, thereby reducing atherosclerosis (8, 9).

Vitamin C Inhibits the Inflammatory Response

Vitamin C has strong reducing properties due to it being a strong antioxidant. In the human body, it can inhibit the formation of oxygen free radicals, regulate inflammatory factors, inhibit inflammatory cell infiltration, reverse endothelial dysfunction, improve microcirculation, and alleviate the micro-inflammatory state (10). Mohammed et al. (11) found that vitamin C-sufficient mouse macrophages exhibited an obvious anti-inflammatory phenotype, whereas vitamin C-deficient mouse macrophages continued to express interleukin (IL)-1 (*IL-1*), tumor necrosis alpha (*TNF-α*), and monocyte chemoattractant protein-1 mRNAs, indicating a pro-inflammatory phenotype. Block et al. (12) found that the serum C-reactive protein concentrations were significantly reduced in active and passive smokers after oral administration of vitamin C. Mikirova et al. (13) found that the intravenous injection of vitamin C in cancer patients resulted in significant decreases in the serum concentrations of the inflammatory cytokines IL-1α, IL-2, IL-8, and TNF-α; the eosinophil chemokine eotaxin; and C-reactive protein.

Atherosclerotic plaques are important risk factors for cerebrovascular disease (14). Activation of the inflammatory response reduces the stability of a plaque, leading to its rupture (14). Importantly, secondary thrombosis and embolism are the main mechanisms of ischemic stroke (15). Early in the formation of atherosclerotic plaques, monocytes adhere to the endothelial wall, causing the vessel wall to thicken and lose its elasticity (15). Vitamin C has been found to reduce the adhesion of monocytes to the vascular endothelium (8, 9) by decreasing the expression of intercellular adhesion molecule-1, a surface glycoprotein that mediates the adhesion of monocytes to endothelial cells (16) (**Figure 1**).

Vitamin C Inhibits Oxidative Reactions

Studies have shown that oxidative stress and its related molecular events play important roles in the pathological process of ischemic stroke (17). Ischemic stroke occurs due to a sudden interruption of the cerebral arterial blood supply due to the occlusion of cerebral arteries, which in turn leads to cerebral hypoxia and the accumulation of reactive oxygen species (18). When blood flow is restored, oxidative stress in the brain may be exacerbated, leading to an imbalance between the production of oxidants and the antioxidant defense mechanisms, resulting in dysregulated cell survival mechanisms and, ultimately, nerve damage (18).

Under normal conditions, the production and elimination of free radicals in the body are balanced. When there are too many free radicals, cholesterol in lipoproteins, especially LDL, easily undergoes peroxidation, which is a risk factor for atherosclerosis and ischemic stroke (19). In addition, oxidized LDL is highly cytotoxic and can accelerate the formation of fatty streaks (20). Monocytes adhered to the endothelium are activated to differentiate into macrophages, which ingest large amounts of oxidized LDL, become enriched in cholesterol, and transform into foam cells, leading to the development of fatty streaks, thereby promoting the development of atherosclerosis (21, 22). Importantly, vitamin C inhibits LDL oxidation (**Figure 1**).

Pro-oxidative Effects of Vitamin C

Regarding the pro-oxidative effect of vitamin C, the most intuitive evidence was obtained from the experiment performed by Griffiths et al. (23). Their results showed that U937 monocytes displayed increased production of reactive oxygen species after they were co-incubated with 150 μmol/L ascorbic acid and dihydrochlorofluorescein for 40 min.

The reason for the pro-oxidative effect of vitamin C has not been determined. Some scholars believe that this effect may be the result of the interactions between vitamin C and some metal ions (such as Fe^{3+}). In 1996, Andorn et al. (24)confirmed that vitamin C can cause lipid peroxidation in the human brain, and this effect depends on the participation of iron ions, with iron at 100 $mg{\cdot}d^{-1}$ being able to cause uncontrollable lipid peroxidation. Similarly, Lachili et al. (25) found uncontrollable lipid peroxidation in pregnant women taking concurrent vitamin C (500 $mg{\cdot}d^{-1}$) and iron (100 $mg{\cdot}d^{-1}$) supplements.

In addition, some scholars believe that the pro-oxidative effect of vitamin C may be related to the ascorbic acid cycle. In this cycle, the dehydroascorbic acid transported into the cell is rapidly converted into ascorbic acid by enzymatic or non-enzymatic catalysis, and the resulting ascorbic acid causes the oxidation of other substances in the cell (23, 26). The oxidation of cellular substances was confirmed by Song et al. through a series of experiments in which the transport of dehydroascorbic acid was blocked with wortmannin (a glucose carrier-specific

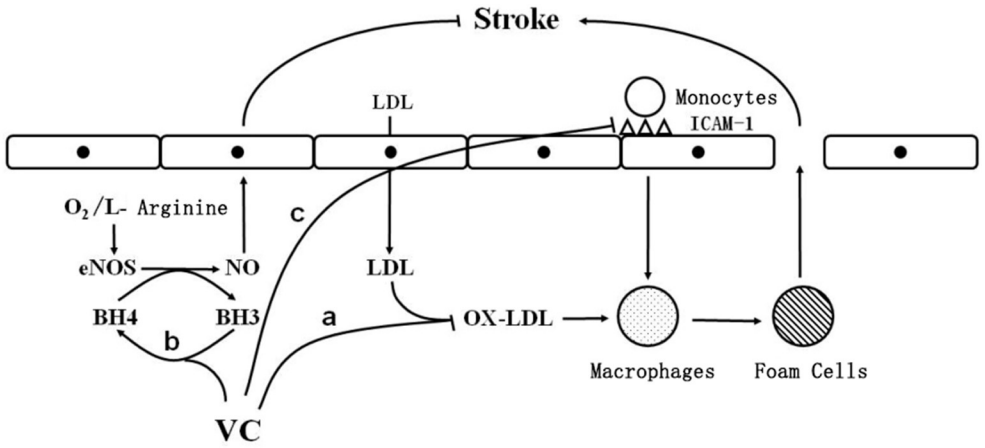

FIGURE 1 | The cellular mechanism by which vitamin C reduces the risk of ischemic stroke. O_2: oxygen; eNOS: endothelial nitric oxide synthase; BH3: trihydrobiopterin; BH4: tetrahydrobiopterin; vc: vitamin C; NO: nitric oxide; LDL: low density lipoprotein; OX-LDL: oxidized low density lipoprotein; ICAM-1: intercellular adhesion molecule-1; stroke: stroke; a: vitamin C inhibits low-density lipoprotein oxidation; b: vitamin C reduces trihydrobiopterin to tetrahydrobiopterin; c: Vitamin C reduces the expression of ICAM-1.

blocker), which reduced the vitamin C-induced production of lipid peroxidation products (26).

Vitamin C Protects the Vascular Endothelium

Vitamin C reduces the inflammatory response by protecting against endothelial dysfunction via many mechanisms, including scavenging oxygen free radicals and inducing the synthesis of NO. Levine et al. (27) found that vitamin C reverses vascular endothelial dysfunction in patients with coronary heart disease. Animal experiments (28) have shown that vitamin C stabilizes tetrahydrobiopterin without dehydrogenation to allow endothelium-derived NO synthase to remain in a normal coupled state and maintain its normal activity. Cell culture experiments (29) have shown that vitamin C increases endothelium-dependent vasodilation by increasing the glutathione concentration in endothelial cells and inducing NO synthesis in these cells in a time- and dose-dependent manner.

Endothelial dysfunction is a main cause of ischemic stroke. After endothelial cell injury, platelet adhesion and aggregation accelerate thrombosis, leading to the development of ischemic stroke (30). Vitamin C stimulates endothelial cell proliferation by increasing the synthesis and deposition of type IV collagen in the basement membrane, thereby inhibiting apoptosis, and by stabilizing the NO produced by endothelial cells to regulate vascular tone and protect the vascular endothelium (31). Vitamin C reduces trihydrobiopterin radicals to tetrahydrobiopterin (**Figure 1**), which is an important cofactor for endothelial NO synthase (31). Tetrahydrobiopterin deficiency causes endothelial NO "uncoupling" and decreases NO production, leading to endothelial cell dysfunction (31). By maintaining the tetrahydrobiopterin concentration, vitamin C enables endothelial cells to produce normal amounts of NO, thus protecting vascular endothelial cells from damage, inhibiting the development of atherosclerosis, and reducing the risk of ischemic stroke (31).

ANIMAL EXPERIMENTS ON THE EFFECT OF VITAMIN C ON ISCHEMIC STROKE

Yan et al. showed that simultaneous tetrahydrobiopterin, L-arginine, and vitamin C supplementation increased vascular perfusion after ischemia by increasing endothelial NO synthase activity and reducing oxidative stress (32). Through experiments using mice, D'Uscio et al. showed that vitamin C preserved vascular endothelial function by protecting tetrahydrobiopterin and restoring endothelial NO synthase activity (28). Using the Rice–Vannucci model, Miura et al. found that, in immature rats, intracerebroventricular injection of ascorbic acid after hypoxia–ischemia had neuroprotective effects; particularly, ascorbic acid inhibited cell necrosis and apoptosis in the brains of immature rats after hypoxia–ischemia-induced cell death (33, 34). Iwata et al. showed that during middle cerebral artery occlusion and reperfusion in rats with diabetes, ascorbic acid supplementation inhibited apoptosis and pro-inflammatory responses and alleviated brain injury and neurological deficits in the diabetic state (35). Furthermore, a study on patients with diabetes showed that ascorbic acid supplementation protected the endothelium from ischemia-induced oxidative damage (35). This is closely related to the reduction in intravascular reactive oxygen species levels mediated by ascorbic acid (36). These animal experiments show that vitamin C reduces the risk of ischemic stroke via antioxidant effects, thus protecting vascular endothelial function and inhibiting inflammation.

CLINICAL TRIALS OF VITAMIN C IN ISCHEMIC STROKE

In recent years, there have been several randomized controlled clinical trials of vitamin C for ischemic stroke. Most of the experimental results have shown that vitamin C has no significant

TABLE 1 | Cohort study of vitamin C and ischemic stroke.

References	Research type	Time	Sample size (examples)	Age (years)	Vitamin C intake (mg/ day)	Follow-uptime (years)	Relative risk (95%CI)	Adjustment factor
Gey et al. (47)	Observational (Measure vitamin C concentration in plasma)	Beginning: 1971–1973 Ending: 1985	2,974 Men	/	"Low""Normal"	12	0.24 (0.10–0.60)	Gender, smoking, blood pressure, cholesterol and beta carotene
Gale et al. (48)	Observational (7 days of dietary records, measurement of plasma vitamin C)	Beginning: 1973–1974 Deadline: Not mentioned	730 (equal number of men and women)	≥65	19.4 (T1) 53.4 (T3)	20	0.5 (0.3–0.8)	Age, sex and determined cardiovascular risk factors
Ascherio et al. (49)	Observational (Food Frequency Questionnaire)	1986–January 31, 1994	43,738 Men	40–75	95.00 (Q1) 1167.00 (Q5)	8	IS: 1.03 (0.66–1.59)	Age, smoking, hypertension, hypercholesterolemia, body mass index, physical activity
Hirvonen et al. (50)	Observational (dietary questionnaire)	Ending: 1993.4.30	26,593 Composition of male smokers	50–69	52.00 (F1) 141.00 (F4)	6.1	0.89 (0.72–1.09)	Age, BMI, blood pressure, cholesterol, height, smoking, history of diabetes or coronary heart disease, alcohol consumption and education
Yochum et al. (51)	Observational (semi-quantitative food frequency questionnaires, vitamin and mineral supplement intake)	1986–december 31, 1997	34,492 Postmenopausal women	55–69	82.40 (Q1) 678.70 (Q5)	11	1.23 (0.76–1.90)	Age, BMI, waist-to-hip ratio, smoking, diabetes, high blood pressure, physical activity, alcohol consumption, marital status and education level, intake of cholesterol, saturated fat, fish, dietary fiber, whole grains and other antioxidants
Yokoyama et al. (52)	Observational (Food Frequency Questionnaire)	1977–1997	2,121 (880 men and 1,241 women)	≥40	44.01 (F1) 52.13 (F4)	20	IS:0.71 (0.59–0.51)	Age, sex
Kurl et al. (53)	Observational (Measure vitamin C in plasma)	1984–1998.12.31	2,419 Middle-aged men	42–60	28.40 (T1) 64.96 (T4)	10.4	0.48 (0.26–0.83)	Age, BMI, systolic blood pressure, smoking, alcohol consumption, total serum cholesterol, diabetes, and exercise-induced myocardial ischemia
Voko et al. (54)	Observational (food frequency data)	1990 to 1993: before 1 January 1999	5,197 Men	≥55	T1 T3	6.4	0.66 (0.46–0.93)	Age, sex, total energy intake, smoking, hypertension, diabetes, coronary artery disease, history of TRANSIENT ischemic attack
Lee et al. (55)	Observational (Food Frequency Questionnaire)	January 1986–December 31, 2000	1,923 Postmenopausal women	55–69	85.00 (Q1) 667.00 (Q5)	15	1.89 (0.73–4.92)	Age, total energy intake, history of hypertension, BMI, waist-to-hip ratio, physical activity score, smoking, alcohol consumption, hormone replacement therapy, major type of diabetes medication use, and duration of diabetes
Myint et al. (56)	Observational (Health and Lifestyle Questionnaire (containing supplements or supplements containing vitamin C)	From 1993 to 1997 until March 2005	20,649 (Men 9,449 Women 11,200)	40–79	35.00 (Q1) 71.50 (Q5)	9.5	0.57 (0.43–0.76)	Age, sex, smoking status, BMI, systolic blood pressure, cholesterol, physical activity, myocardial infarction and diabetes mellitus

(Continued)

TABLE 1 | Continued

The first author	Research type	Time	Sample size (examples)	Age (years)	Vitamin C intake (mg/ day)	Follow-uptime (years)	Relative risk (95%CI)	Adjustment factor
Del Rio et al. (57)	Observational (Semi-quantitative food Frequency Questionnaire)	From 1993 to 1998 to 31 December 2004	41,620 (Not mentioned)	44–61	83.00 (T1) 201.00 (T3)	7.9	IS:0.53 (0.31–0.89)	Age, sex, high blood pressure, smoking, education, alcohol consumption, waist circumference, BMI and total physical activity
Kubota et al. (58)	Observational (Semi-quantitative food Frequency Questionnaire)	From 1988 to 1990 to 2006	23,119 Men/35,611 Women	40–79	M:52.00 (Q1) 145.00 (Q5) F:60.00 (Q1) 150.00 (Q5)	16.5	M:0.84 (0.62–1.13) F:0.7 (0.54–0.92)	Age, history of hypertension and diabetes, smoking, alcohol consumption, body mass index, mental stress, physical activity, education level, total dietary energy intake, cholesterol, saturated fatty acids, n-3 fatty acids and sodium
Uesugi et al. (59)	Observational (Semi-quantitative food Frequency Questionnaire)	From 1995 to 1997–as of the end of 2009	82,044	45–74	60.00 (Q1) 239.00 (Q5)	15	0.76 (0.60–0.96)	Age, sex,BMI, smoking, alcohol consumption, physical activity, medication or history of diabetes, hyperlipidemia, and hypertension
Martín-Calvo et al. (60)	Observational (Semi-quantitative food Frequency Questionnaire)	Prior to March 2014–December 2016	13,421 (Not mentioned)	≥40	148.00 (T1) 445.00 (T3)	11	0.30 (0.12–0.72)	Gender,BMI, total energy intake, total fiber intake, physical activity, TV watching, smoking, cardiovascular disease, family history of stroke, and aspirin treatment
Lee et al. (61)	Observational (Semi-quantitative food Frequency Questionnaire)	Beginning 1995-1996-as of December 31, 2017	875	25–74	F1 F4	22	0.66 (0.52–0.85)	Age, sex, BMI, smoking, hypertension, dyslipidemia, abnormal blood glucose, and baseline history of cardiovascular disease

IS: ischemic stroke; T1: the lowest third of vitamin C intake distribution; T3: the highest third of vitamin C intake distribution; F1: the lowest quartile of vitamin C intake distribution; F4: the highest quartile of vitamin C intake distribution; Q1: The lowest quintile of vitamin C intake distribution; Q5: Highest quintile of vitamin C intake; BMI: BODY mass index; CI: confidence interval; Relative risk: The highest quintile of vitamin C distribution compared with the lowest quintile.

effect on reducing the risk of ischemic stroke (37–42). In a study in which 20,536 adults with coronary heart disease, other occlusive arterial disease, or diabetes were randomly assigned to receive vitamin C supplements or placebo, plasma vitamin C concentrations increased by one third in the supplement group during the 5-year intervention period, but there was no significant difference in stroke-related mortality between the two groups (41). In studies of populations with a high risk of stroke, vitamin C supplementation has shown no significant effect on stroke risk (41). In the American Men's Physician's Health Study, an intervention consisting of 400 IU of vitamin E every other day and 500 mg of vitamin C daily was associated with protective effects against cardiovascular disease compared with placebo after 8 years of follow-up (43). However, the occurrence of cardiovascular disease (CVD) was not significantly affected by the intervention, with the overall hazard ratio for stroke in the intervention group being 0.89 [95% confidence interval (CI), 0.74–1.07] (37). Similar results were reported in the Women's Antioxidant Cardiovascular Study, in which the intervention included 500 mg of vitamin C daily, 600 IU of vitamin E on alternate days, and 50 mg of beta-carotene on alternate days in women with a high risk of CVD (38). Vitamin C was found to have no overall effect on CVD or cerebrovascular events in these women (38). Studies by Blot et al., Hercberg et al., and Brown et al. found that vitamin C supplementation did not reduce the risk of stroke (39, 40, 42). These findings are consistent with the results of meta-analyses by Myung et al. and Ye et al. (18, 44). Lena et al. also found no evidence that vitamin C supplementation reduces the risk of stroke (21).

The design, endpoint, observation time, and study population of a clinical trial have important effects on the results. Accumulating evidence indicates that well-designed clinical trials are necessary to evaluate the effects of vitamin C on the risk of stroke and CVD (37–39). The greatest clinical benefit of vitamin C can only be achieved by designing more targeted clinical trials to evaluate its effect on CVD.

COHORT STUDIES OF VITAMIN C AND ISCHEMIC STROKE

Search Strategy for Systematic Review

This systematic umbrella review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 Statement guidelines (45, 46). Studies were identified through a comprehensive search of ProQuest, MEDLINE (PubMed), EBSCOhost, Web of Science, and ScienceDirect from the inception of the respective databases to June 2022. No language restrictions were applied. The search strategy included several MeSH terms: "Vitamin C" OR "micronutrient" OR "nutrients" AND "Ischemic Stroke" AND "Stroke". The references cited in all of the eligible articles were also manually searched.

Eligibility Criteria

Cohort studies evaluating vitamin C intake and ischemic stroke risk in humans were included. The inclusion criteria were studies that (1) included adults aged ≥18 years; (2) reported dietary vitamin C intakes or measured serum vitamin C levels; and (3) assessed the occurrence of ischemic stroke as the outcome.

Study Selection and Data Collection

The selection of articles involved three stages: (1) title screening, (2) abstract screening, and (3) full-text review. Two investigators (XLT and HGL) screened the titles and abstracts independently and selected eligible articles through full-text review. Any discrepancies in selecting articles between the two researchers were resolved by a third investigator (LW).

Data extraction was performed using a data extraction table in which the following types of information were entered: (1) name of the first author, (2) journal, (3) publication year, (4) vitamin C intake, (5) outcome, (6) number of males and females, (7) number of participants in each study, (8) study design, (9) follow-up time, (10) type of comparison (highest vs. lowest intake of vitamin C), and (11) the estimated summary effect (relative risk) and corresponding 95% CIs.

Table 1 lists the cohort studies of vitamin C and ischemic stroke. Most epidemiological studies reported that vitamin C can reduce the risk of ischemic stroke. In Finland, 2,419 middle-aged men with no history of stroke were followed up for 10.4 years, and it was found that after adjusting for factors such as age, body mass index, smoking, and alcohol consumption, men with the highest plasma vitamin C concentration (64.96 μmol/L) had a reduced risk of stroke compared with men with the lowest plasma vitamin C concentration (28.40 μmol/L; hazard ratio for stroke: 0.48; 95% CI: 0.26–0.83), indicating that low plasma vitamin C concentrations are associated with an increased risk of stroke (53). A 20-year follow-up study in the United Kingdom confirmed that people with the lowest vitamin C status had the highest risk of stroke and that vitamin C concentrations in older adults were closely associated with stroke risk, regardless of whether vitamin C was measured in terms of plasma concentration or dietary intake (48). Similar findings were obtained in cohort studies in different countries (47, 52, 54–57, 59–61). However, some studies have reported inconsistent findings (49–51, 58).

In an 8-year follow-up study, Ascherio et al. assessed the risk of stroke in the top and bottom quintiles (1,167 and 95 mg/day, respectively) of vitamin C intake among 43,738 men aged 40–75 years without CVD or diabetes. The relative risk of ischemic stroke in the top quintile was 1.03 (95% CI, 0.66–1.59) compared with the bottom quintile, and vitamin C supplementation did not significantly reduce ischemic stroke incidence in this cohort, which may be due to dietary measurement errors or the study subjects being medical professionals who had healthier lifestyles and eating habits than the average man (49). Yochum et al. concluded that vitamin C is not associated with stroke risk, which may be due to the pro-oxidative effect of vitamin C. Vitamin C not only is an antioxidant but also functions as a pro-oxidant in some cases (51, 62). Kubota et al. found that vitamin C was not associated with stroke risk in men, which may be due to the lower antioxidant capacity of dietary vitamin C or other risk factors for stroke in men (58). Hirvonen et al. concluded that the risk of stroke in their study subjects was probably attributable to smoking, and the influence of vitamin

C on stroke risk may differ between smokers and non-smokers. Therefore, the results of their study cannot be generalized to non-smokers (50).

These inconsistencies between the results of different studies may be due to differences in the ethnicity of the studied populations and the adjustment of confounders. Moreover, in most cohort studies, vitamin C intake was mainly determined by dietary assessments, which are not accurate indicators of the plasma vitamin C concentration (50). Some scholars believe that the discrepant results may be attributable to the pro-oxidative effect of vitamin C, which, despite being an antioxidant, functions as a pro-oxidant in some cases (60, 62). Furthermore, the results of experimental studies differ from those of observational studies. Experimental studies may have tended to include high-risk groups and use high doses of vitamin C. Thus, from the results of these studies, it may not be possible to determine whether long-term low-dose dietary vitamin C intake affects the risk of ischemic stroke in the general population. The discrepant results may also be due to the poor lifestyle habits of participants with low vitamin C intake in cohort studies. Although most studies adjusted for multiple confounders, such as smoking, alcohol consumption, and a history of diabetes or hypertension, they did not control for key dietary confounding factors, such as the intakes of dietary fiber, whole grains, nuts, or salt, which are known to influence the risk of ischemic stroke (63).

CONCLUSIONS

The purpose of this review is to describe the research progress on the relationship between vitamin C and ischemic stroke. As an effective antioxidant, vitamin C plays an important role in reducing the risk of ischemic stroke by protecting the cardiovascular system and preventing atherosclerosis through anti-inflammatory, antioxidant and endothelial protective effects. However, it remains unknown whether the patients with stroke should be administered vitamin C to decrease their level of oxidative stress; whether long-term supplementation of vitamin C is required; what amount of supplementation is optimal; and what is the best source of supplementation. The results of many cohort studies have shown that long-term dietary intake of vitamin C can reduce the risk of ischemic stroke, but the results of the studies so far are not completely consistent; more prospective clinical trials are needed to confirm the role of optimal vitamin C status in stroke management and the effectiveness of this supplementation during stroke.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

PS and LW conceived the idea for this initiative. XT contributed to reading the literature, preparation of figures and the table, and writing the manuscript. HL and YX assisted with writing and revising the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank all authors for their contributions to the article.

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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.

frontiers | Frontiers in Nutrition

TYPE Systematic Review
PUBLISHED 29 July 2022
DOI 10.3389/fnut.2022.935706

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

RECEIVED 04 May 2022
ACCEPTED 11 July 2022
PUBLISHED 29 July 2022

CITATION
Zhang W, Jiang J, He Y, Li X, Yin S, Chen F and Li W (2022) Association between vitamins and risk of brain tumors: A systematic review and dose-response meta-analysis of observational studies.
Front. Nutr. 9:935706.
doi: 10.3389/fnut.2022.935706

Association between vitamins and risk of brain tumors: A systematic review and dose-response meta-analysis of observational studies

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Background: Brain tumor is one of the important causes of cancer mortality, and the prognosis is poor. Therefore, early prevention of brain tumors is the key to reducing mortality due to brain tumors.

Objective: This review aims to quantitatively evaluate the association between vitamins and brain tumors by meta-analysis.

Methods: We searched articles on PubMed, Cochrane Library, Web of Science, and Embase databases from inception to 19 December 2021. According to heterogeneity, the fixed-effects model or random-effects model was selected to obtain the relative risk of the merger. Based on the methods described by Greenland and Longnecker, we explored the dose-response relationship between vitamins and the risk of brain tumors. Subgroup analysis, sensitivity analysis, and publication bias were also used for the analysis.

Results: The study reviewed 23 articles, including 1,347,426 controls and 6,449 brain tumor patients. This study included vitamin intake and circulating concentration. For intake, it mainly included vitamin A, vitamin B, vitamin C, vitamin E, β-carotene, and folate. For circulating concentrations, it mainly included vitamin E and vitamin D in the serum (25-hydroxyvitamin D and α-tocopherol). For vitamin intake, compared with the lowest intakes, the highest intakes of vitamin C (RR = 0.81, 95%CI:0.66–0.99, I^2 = 54.7%, $P_{for\ heterogeneity}$ = 0.007), β-carotene (RR = 0.78, 95%CI:0.66–0.93, I^2 = 0, $P_{for\ heterogeneity}$ = 0.460), and folate (RR = 0.66, 95%CI:0.55–0.80, I^2 = 0, $P_{for\ heterogeneity}$ = 0.661) significantly reduced the risk of brain tumors. For serum vitamins, compared with the lowest concentrations, the highest concentrations of serum α-tocopherol (RR = 0.61, 95%CI:0.44–0.86, I^2 = 0, $P_{for\ heterogeneity}$ = 0.656) significantly reduced the risk of brain tumors. The results of the dose-response relationship showed that increasing the intake of 100 μg folate per day reduced the risk of brain tumors by 7% ($P_{-nonlinearity}$ = 0.534, RR = 0.93, 95%CI:0.90–0.96).

Conclusion: Our analysis suggests that the intake of vitamin C, β-carotene, and folate can reduce the risk of brain tumors, while high serum α-tocopherol concentration also has a protective effect on brain tumors. Therefore, vitamins may provide new ideas for the prevention of brain tumors.

Systematic Review Registration: PROSPERO, identifier CRD42022300683.

KEYWORDS

vitamin, brain tumor, meta-analysis, β-carotene, folate, observational study

Introduction

Brain tumors are the primary central nervous system tumors, with an annual incidence rate of 22.6/1,00,000 (1). They are a significant cause of cancer incidence rate and mortality, especially in children, accounting for 30% of cancer deaths (2). Because the prognosis of brain tumors, especially glioma, is poor, early prevention and detection are the keys to reducing brain tumor mortality (3).

Although the etiology of brain tumors had been studied for decades, the risk factors accounting for a large proportion of cases had not been found. In recent years, people had often paid attention to the relationship between diet and brain tumors. Some studies found that a diet rich in antioxidants, such as vegetables and fruits, could prevent brain tumors. Experimental studies had shown that these dietary antioxidants, could significantly inhibit the growth of cancer cells, especially brain tumor cells (4–6). Vitamins had a similar effect. Some vitamins with antioxidant properties, such as vitamin C and vitamin E, could inhibit tumor growth by eliminating free radicals and inducing apoptosis (7–9). In addition, fat-soluble vitamins, such as vitamin A and vitamin D, also played a certain preventive role by regulating cell differentiation and inhibiting cancer cell proliferation (9, 10). However, the current epidemiological results on vitamins and brain tumors were inconsistent. Chen et al. analyzed the diet of 236 patients with brain tumors through a case-control study and found that the intake of vitamin A was negatively correlated with the risk of glioma (odds ratio (OR) = 0.50, 95% confidence interval (95%CI):0.30–0.80) (11). However, Gile et al. arrived at the opposite conclusion (OR = 1.64, 95% CI:1.13–2.37) (12). A meta-analysis of seven articles showed that the highest intake of vitamin A in the diet was significantly associated with a reduced risk of glioma (relative risk (RR) = 0.80, 95% CI = 0.62-0.98, $P = 0.014$, $I^2 = 54.9\%$) (13). Tedeschi Blok et al. also found that people with a higher intake of carotene, the precursor of vitamin A, had a lower risk of brain tumors (OR = 0.72, 95% CI:0.54–0.98) (14), and vitamin C and vitamin E also had similar results in this study. However, Durrow et al. followed up with 545,770 participants for 7.2 years and found that dietary vitamin C (RR = 1.26, 95% CI: 0.96–1.66) and vitamin E (RR = 1.17, 95% CI:0.90–1.53) were not associated with the risk of brain tumors (15). Moreover, by detecting the concentrations of vitamin C and vitamin E in participants' serum, it was found that both had protective effects on brain tumors (vitamin C: OR = 0.19, 95% CI: 0.10–0.60, vitamin E: RR = 0.65, 95% CI:0.44–0.96) (16). In addition, the effect of folate on brain tumors had also attracted much attention. Studies had shown that both folate supplementation during pregnancy and children's high intake of folate could significantly reduce the risk of brain tumors (pregnant women OR = 0.60, 95% CI: 0.68–0.98, children: OR = 0.63, 95% CI: 0.41–0.97) (17).

Since the conclusions of previous studies were inconsistent, and most studies on the effects of vitamins on brain tumors included fewer cases, we quantitatively evaluated the relationship between various vitamin intake and *in vivo* exposure concentrations and brain tumor risk through the latest evidence of comprehensive observational studies. We tried to explore the dose-response relationship between vitamins and brain tumors, hoping to provide evidence for preventing brain tumors.

Methods

Search strategy

A comprehensive search was conducted for available articles published in English using databases such as the Cochrane Library, PubMed, Web of Science, and Embase up to 19 December 2021. The Cochrane Library search terms used for the title, abstract, and keywords were ("glioma" OR "brain cancer" OR "brain tumor") combined with ("diet" OR "food" OR "lifestyle" OR "nutrition" OR "nutrient" OR "vitamin" OR "carotenoid" OR "carotene" OR "ascorbic acid" OR "thiamine" OR "riboflavin" OR "tocopherol" OR "25 hydroxyvitamin D" OR "folic acid" OR "nicotinic acid" OR "antioxidant"). The same retrieval strategy was also applied to the other databases. No document type or other relevant restrictions were used in the retrieval process, and unpublished articles were excluded. Two investigators independently searched articles and reviewed all retrieved studies. A third author settled any disagreements between the two authors. In addition, we explored the references of published meta-analyses to identify other potential articles.

Inclusion and exclusion criteria

The following inclusion criteria were used: (1) the studies were using a cohort design or a case-control design; (2) the exposure of interest was vitamin intake or serum vitamin concentration; and (3) the ending outcome was brain tumors.

The exclusion criteria of the meta-analysis were as follows: (1) non-observational study (reviews, case reports, and clinical trials); (2) lack of effect size and 95%CI which were available for the highest category of vitamin vs. lowest category of vitamin; and (3) If multiple studies used data from the same population, the study with the largest sample size was included in this meta-analysis.

Data extraction

Two investigators extracted the following information from the included study independently: the first author, year of publication, country, study population, study type, age, sex, sample size, number of cases, disease, vitamin source, vitamin type, vitamin level, effect size, and 95% CI extracted from the most adjusted model. If there was disagreement between the two authors about the appropriateness of the data, it was resolved by consensus with a third author.

Quality assessment

Two investigators evaluated each study and handed it over to a third party for adjudication in case of disagreement. Since the included articles were observational studies, the Newcastle-Ottawa scale (NOS) was used to evaluate the quality of the study and the possible risk of bias (18).

Statistical analysis

Stata 14.0 software was used for data analysis. We pooled effect size estimations by combining the multivariable-adjusted effect size and 95%CI of the highest compared with the lowest vitamins. I^2 statistics assessed heterogeneity between the studies. Suppose the heterogeneity was not statistically significant ($I^2 < 50\%$ and $P > 0.10$), the fixed-effects model was used to pool them. Otherwise, the random-effects model was used. We conducted a subgroup analysis to determine whether the heterogeneity of the study came from disease (brain tumor and glioma), vitamin source (diet and supplements), study population (pregnancy exposure and self-exposure), study type (case-control study and cohort study), and study quality (>7 points and ≤7 points), to explore the potential sources of heterogeneity. We used sensitivity analysis to assess each study's relative impact on the total effect size by successively omitting one study when determining the effect size. For publication bias, Egger's test and Begg's test were used to detect it.

Subsequently, we also explored the dose-response relationship between vitamins and brain tumor risk. The method developed by Greenland and Longnecker was used to analyze the dose-response relationship in this study (19). For this method, we needed to extract at least three groups of vitamin intakes or serum vitamin concentration, number of participants, number of cases, effect size, and 95% CI in each study. The median or average vitamin corresponding to each group was used for risk estimation for each study. Suppose the median or average vitamin of each group was not provided, the midpoint of each group's upper and lower limits should be designated as the intermediate exposure level. If the highest group was open, we assumed that the interval width was the same as the second-highest category. *Q*-value was applied to assess between-study heterogeneity. Unless otherwise noted, two-tailed $P < 0.05$ was accepted as statistically significant.

Results

Study characteristics

Figure 1 shows the articles screening process of this study. A total of 3,604 articles were retrieved, including 387 from the Cochran Library, 896 from PubMed, 307 from Web of Science, and 2014 from Embase. After excluding duplicates between different databases, titles and abstracts of 2,493 articles were reviewed. A total of 2,340 articles were excluded because they were not related to the aim of the study. Then, 153 articles were reviewed in full text, and 130 articles were excluded due to non-observational studies, animal/cell experiments, reviews, lacked effect size, or duplication of the study population. A total of 23 articles were included (11, 12, 14–17, 20–36).

Table 1 summarizes the 23 articles and characteristics included in this meta-analysis. All studies included 1,347,426 controls and 6,449 patients with brain tumors. Among them, the patients in eight studies were minors, and the participants in the other studies were 18–80 years old. The included studies were mainly concentrated in North America (America and Canada) and Europe (Britain, Germany, and Sweden). A few studies were completed by Australia, China, and Iran, including 20 case-control studies and 4 cohort studies. These studies provided brain tumor-related results for 6 vitamin intakes: vitamin A, vitamin B, vitamin C, vitamin E, β-carotene, and folate. In addition, there were the results of serum 25-hydroxyvitamin D and serum α-tocopherol. Around 50% of the studies had a NOS score of eight or above.

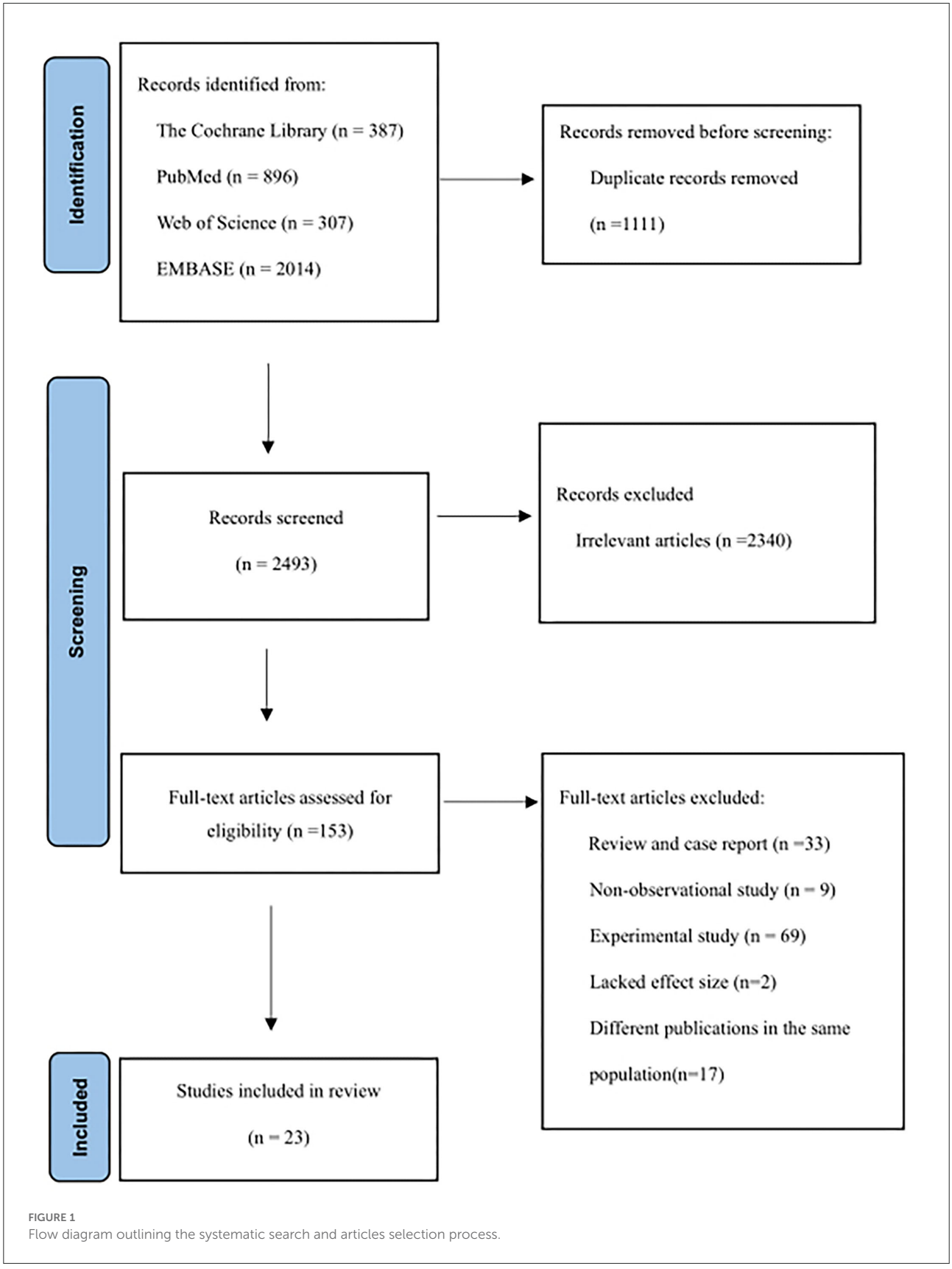

FIGURE 1
Flow diagram outlining the systematic search and articles selection process.

TABLE 1 Characteristics of studies investigating the association between vitamins and brain tumors.

Study	Year	Country	Study type	Age	Sex[a]	Population[b]	Sample size	Case	Disease	Source	Vitamin	Effect size (95%CI)	Quality score
Howe et al. (20)	1989	Canada	Case-control	<19	Both	Self-exposure	146	52	Brain tumor	Supplement	Vitamin C	0.91(0.43–1.93)	8
Boeing et al. (21)	1993	Germany	Case-control	25–75	Both	Self-exposure	470	93	Glioma	Diet	Vitamin C	0.90(0.50–1.70)	7
Bunin et al. (22)	1994	America	Case-control	0–6	Female	Pregnancy exposure	288	144	Glioma	Diet	Vitamin A	0.70(0.30–1.40)	8
											Vitamin C	0.70(0.40–1.50)	
											Vitamin E	0.70(0.30–130)	
											β-carotene	1.00(0.50–2.00)	
											Folate	1.00(0.50–2.10)	
Gile et al. (12)	1994	Australia	Case-control	20–70	Both	Self-exposure	818	409	Glioma	Diet	Vitamin A	1.64(1.13–2.37)	7
											Vitamin C	0.96(0.42–2.15)	
											Vitamin E	1.42(1.00–2.02)	
											β-carotene	0.85(0.59–1.23)	
Blowers et al. (23)	1997	America	Case-control	25–74	Female	Self-exposure	188	94	Glioma	Diet	Vitamin A	0.70(0.30–1.90)	7
											Vitamin C	1.50(0.60–4.10)	
											Vitamin E	2.20(0.80–5.70)	
Lee et al. (24)	1997	America	Case-control	>20	Both	Self-exposure	857	419	Glioma	Supplement	Vitamin C	0.76(0.56–1.01)	8
											Vitamin E	0.79(0.55–1.14)	
Preston-Martin et al. (25)	1998	Britain	Case-control	0–19	Female	Pregnancy exposure	952	373	Brain tumor	Supplement	Vitamin A	0.40(0.20–0.80)	7
											Vitamin C	0.50(0.30–0.90)	
											Vitamin E	0.50(0.30–0.80)	
											Folate	0.50(0.30–0.80)	
Hu et al. (26)	1999	China	Case-control	20–74	Both	Self-exposure	287	129	Brain tumor	Diet	Vitamin C	0.78(0.20–4.10)	6
											Vitamin E	0.16(0.10–0.50)	
											β-carotene	0.38(0.10–1.60)	
Schwartzbaum et al. (16)	2000	America	Case-control	36–69	Both	Self-exposure	69	34	Glioma	Serum	α-tocopherol	0.36(0.10–1.10)	8
Chen et al. (11)	2002	America	Case-control	≥21	Both	Self-exposure	685	236	Glioma	Diet	Vitamin A	0.50(0.30–0.80)	7
											Vitamin C	0.90(0.50–1.50)	
											Vitamin E	0.80(0.50–1.40)	
											β-carotene	0.50(0.30–0.90)	
											Folate	0.90(0.50–1.50)	
Tedeschi-Blok et al. (14)	2006	America	Case-control	≥20	Both	Self-exposure	1,648	802	Glioma	Diet	Vitamin C	0.70(0.51–0.94)	9
											Vitamin E	0.91(0.62–1.34)	
											β-carotene	0.72(0.54-0.98)	

(Continued)

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TABLE 1 Continued

Study	Year	Country	Study type	Age	Sex[a]	Population[b]	Sample size	Case	Disease	Source	Vitamin	Effect size (95%CI)	Quality score
Holick et al. (27)	2007	America	Cohort	25–75	Both	Self-exposure	2,29,637	296	Glioma	Diet	β-carotene	0.92(0.64–1.32)	8
Michaud et al. (28)	2009	America	Cohort	25–75	Both	Self-exposure	2,19,334	335	Glioma	Diet	Vitamin C	0.88(0.62–1.26)	8
											Vitamin E	0.98(0.67–1.43)	
Dubrow et al. (15)	2010	America	Cohort	50–71	Both	Self-exposure	5,45,770	585	Glioma	Diet	Vitamin C	1.26(0.96–1.66)	7
											Vitamin E	1.17(0.90–1.53)	
Stalberg et al. (29)	2010	Sweden	Case-control	0–15	Female	Pregnancy exposure	1,037	512	Brain tumor	Supplement	Folate	0.60(0.30–1.10)	9
Miline et al. (17)	2012	Australia	Case-control	0–14	Female	Pregnancy exposure	1,014	287	Brain tumor	Supplement	Vitamin A	1.17(0.72–1.90)	8
											Vitamin B	1.03(0.68–1.56)	
											Vitamin C	0.96(0.64–1.46)	
											Folate	0.60(0.38–0.98)	
Greenop et al. (30)	2014	Australia	Case-control	0-15	Female	Pregnancy exposure	1,019	293	Brain tumor	Diet	Vitamin B	1.04(0.72-1.50)	8
											Folate	0.70(0.48–1.02)	
Bhatti et al. (31)	2015	America	Case-control	0–15	Both	Self-exposure	494	247	Brain tumor	Serum	25-hydroxyvitamin D	1.30(0.80–2.20)	8
Greenop et al. (32)	2015	Australia	Case-control	3–15	Both	Self-exposure	739	216	Brain tumor	Diet	Vitamin B	1.23(0.80–1.89)	6
											Folate	0.63(0.41–0.97)	
Zigmont et al. (33)	2015	America	Case-control	20–65	Both	Self-exposure	1,704	592	Glioma	Serum	25-hydroxyvitamin D	1.04(0.73–1.47)	7
Huang et al. (34)	2017	America	Case-control	50–69	Male	Self-exposure	128	64	Glioma	Serum	α-tocopherol	0.65(0.44–0.96)	8
Heydari et al. (35)	2020	Iran	Case-control	20–75	Both	Self-exposure	384	128	Glioma	Diet	Vitamin B	0.35(0.13–0.97)	7
											Vitamin C	0.14(0.05–0.36)	
											Vitamin E	0.83(0.35–1.97)	
											β-carotene	0.99(0.45–2.18)	
Yue et al. (36)	2021	America	Cohort	40–69	Both	Self-exposure	3,46,812	444	Glioma	Serum	25-hydroxyvitamin D	0.87(0.68–1.11)	8
Yue et al. (36)	2021	America	Case-control	30–55	Both	Self-exposure	252	84	Glioma	Serum	25-hydroxyvitamin D	0.97(0.51–1.85)	8
											α-tocopherol	0.61(0.29–1.32)	

[a]Sex of exposed population.
[b]The population was divided into pregnancy exposure and self-exposure.

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TABLE 2 A meta-analysis of the association between vitamins and brain tumors.

Vitamins	Number of studies	RR (95%CI)	I^2 (%)	P for heterogeneity
Intake				
Vitamin A	6	0.79(0.48–1.29)	77.9%	<0.001
Vitamin B	4	1.03(0.82–1.29)	41.1%	0.165
Vitamin C	14	0.81(0.66–0.99)	54.7%	0.007
Vitamin E	11	0.83(0.63–1.10)	73.5%	<0.001
β-carotene	7	0.78(0.66–0.93)	0	0.460
Folate	7	0.66(0.55–0.80)	0	0.661
Serum				
Serum 25-hydroxyvitamin D	4	0.97(0.81–1.16)	0	0.533
Serum α-tocopherol	3	0.61(0.44–0.86)	0	0.656

Effect size estimations of risk for the association between vitamins and brain tumor

The effect size estimations between all vitamins and risk of brain tumors are shown in Table 2. For vitamin intake, compared with the lowest intakes, the highest intakes of vitamin C (RR = 0.81, 95%CI:0.66–0.99, I^2 = 54.7%, $P_{for\ heterogeneity}$ = 0.007), β-carotene (RR = 0.78, 95%CI:0.66–0.93, I^2 = 0, $P_{for\ heterogeneity}$ = 0.460), and folate (RR = 0.66, 95%CI:0.55–0.80, I^2 = 0, $P_{for\ heterogeneity}$ = 0.661) significantly reduced the risk of brain tumor, while the highest intakes of vitamin A (RR = 0.79, 95%CI:0.48–1.29, I^2 = 77.9%, $P_{for\ heterogeneity}$ < 0.001), vitamin B (RR = 1.03, 95%CI:0.82–1.29, I^2 = 41.1%, $P_{for\ heterogeneity}$ = 0.165), and vitamin E (RR = 0.83, 95%CI:0.63–1.10, I^2 = 73.5%, $P_{for\ heterogeneity}$ < 0.001) were not related to the incidence of brain tumor. For serum vitamin, compared with the lowest concentrations, the highest concentrations of serum α-tocopherol (RR = 0.61, 95%CI:0.44–0.86, I^2 = 0, $P_{for\ heterogeneity}$ = 0.656), while the highest concentrations of serum 25-hydroxyvitamin D (RR = 0.97, 95%CI:0.81–1.16, I^2 = 0, $P_{for\ heterogeneity}$ = 0.533) was not related to the incidence of brain tumor (Supplementary Figures 1–8).

Subgroup analysis

For disease, vitamin E was statistically significant in the brain tumor subgroup (RR = 0.30, 95%CI:0.10–0.90). For vitamin source, vitamin C was statistically significant in the supplement subgroup (RR = 0.77, 95%CI:0.60–0.98). For the study population, vitamin E was statistically significant in the pregnancy exposure subgroup (RR = 0.55, 95%CI:0.37–0.83). For the study area, vitamin A was statistically significant in the subgroups of America, Europe, and Australia (America: RR = 0.57, 95%CI:0.39–0.84; Europe: RR = 0.40, 95%CI:0.20–0.80; and Australia: RR = 1.44, 95%CI:1.04–1.99), and the heterogeneity of vitamin A decreased from 77.9 to 15.0%. For study type, vitamin C was statistically significant in the case-control study subgroup (RR = 0.75, 95%CI:0.61–0.93) (Table 3).

For vitamin C, when Heydari's study (35) was excluded, the results of all studies and brain tumor risk remained significant, but the heterogeneity decreased significantly (RR = 0.87, 95%CI:0.77–0.99, I^2 = 23.4%, $P_{for\ heterogeneity}$ = 0.207). Similarly, excluding another study (26), the heterogeneity of vitamin E was also significantly reduced (RR = 0.94, 95%CI:0.76–1.16, I^2 = 52.3%, $P_{for\ heterogeneity}$ = 0.026). It was speculated that these studies might be the main reasons for the heterogeneity of vitamin C and brain tumor risk. The sources of heterogeneity between vitamin B intake and brain tumor effect size estimations were unclear.

The heterogeneity of β-carotene, folate, serum 25-hydroxyvitamin D, and serum α-tocopherol was minimal, so no subgroup analysis was carried out.

Sensitivity analysis and publication bias

The sensitivity analysis showed that no individual study had an excessive influence on the association of vitamins and brain tumors when we removed one individual study at a time. This suggested the results of this meta-analysis were relatively stable (Table 4).

Publication bias was evaluated by Egger's regression test and Begg's rank correlation method. The *P*-value of publication bias of vitamins was more significant than 0.1, suggesting that the difference was not statistically significant, thus there was no publication bias (Table 4).

Dose-response relationship

Due to the limited number of available articles, only vitamin C, vitamin E, folate, and serum 25-hydroxyvitamin D could

10.3389/fnut.2022.935706

TABLE 3 Subgroup analysis for the association between vitamins and brain tumors.

Vitamin	Subgroup	Number	RR (95%CI)	I^2 (%)	$P_{for\ heterogeneity}$
Vitamin A	**Disease**				
	Glioma	4	0.82(0.42–1.61)	81.1	0.001
	Brain tumor	2	0.70(0.25–2.01)	83.8	0.013
	Vitamin source				
	Diet	4	0.82(0.42–1.61)	81.1	0.001
	Supplement	2	0.70(0.25–2.01)	83.8	0.013
	Study population				
	Pregnancy exposure	3	0.71(0.37–1.38)	68.3	0.043
	Self-exposure	3	0.85(0.36–2.03)	86.7	0.001
	Study area				
	America	3	0.57(0.39–0.84)	0	0.692
	Europe	1	0.40(0.20–0.80)	-	-
	Australia	2	1.44(1.04–1.99)	15.0	0.278
	Study quality				
	≤7	4	0.71(0.33–1.52)	85.8	<0.001
	>7	2	0.99(0.62–1.59)	18.3	0.269
Vitamin B	**Study population**				
	Pregnancy exposure	2	1.04(0.79–1.36)	0	0.973
	Self-exposure	2	1.01(0.68–1.50)	80.3	0.024
	Study quality				
	≤7	2	1.01(0.68–1.50)	80.3	0.024
	>7	2	1.04(0.79–1.36)	0	0.973
Vitamin C	**Disease**				
	Glioma	10	0.82(0.64–1.05)	63.5	0.003
	Brain tumor	4	0.77(0.54–1.09)	17.9	0.301
	Vitamin source				
	Diet	10	0.82(0.62–1.09)	62.1	0.005
	Supplement	4	0.77(0.60–0.98)	18.3	0.299
	Study population				
	Pregnancy exposure	3	0.72(0.48–1.08)	43.0	0.173
	Self-exposure	11	0.84(0.66–1.07)	58.8	0.007
	Study area				
	America	8	0.89(0.73–1.08)	38.1	0.126
	Europe	2	0.66(0.37–1.17)	49.1	0.161
	Australia	2	0.96(0.66–1.39)	0	1.000
	Asia	2	0.30(0.06–1.59)	71.3	0.062
	Study type				
	Case-control	12	0.75(0.61–0.93)	39.5	0.077
	Cohort	2	1.07(0.76–1.52)	59.4	0.116
	Study quality				
	≤7	8	0.77(0.50-1.19)	71.8	0.001
	>7	6	0.79(0.68-0.93)	0	0.829
Vitamin E	**Disease**				
	Glioma	9	1.02(0.85–1.21)	28.0	0.195
	Brain tumor	2	0.30(0.10–0.90)	82.2	0.018
	Vitamin source				

(Continued)

TABLE 3 Continued

Vitamin	Subgroup	Number	RR (95%CI)	I^2 (%)	$P_{for\ heterogeneity}$
	Diet	9	0.89(0.65–1.23)	73.0	<0.001
	Supplement	2	0.65(0.42–1.01)	53.6	0.142
	Study population				
	Pregnancy exposure	2	0.55(0.37–0.83)	0	0.455
	Self-exposure	9	0.90(0.67–1.21)	73.6	<0.001
	Study area				
	America	7	0.97(0.81–1.15)	16.8	0.302
	Europe	1	0.50(0.31–0.82)	-	-
	Australia	1	1.42(1.00–2.02)	-	-
	Asia	2	0.36(0.07–1.81)	86.6	0.006
	Study type				
	Case-control	9	0.77(0.53–1.10)	76.1	<0.001
	Cohort	2	1.10(0.89–1.37)	0	0.453
	Study quality				
	≤7	7	0.80(0.50–1.29)	83.4	<0.001
	>7	4	0.87(0.71–1.07)	0	0.792
β-carotene	**Study quality**				
	≤7	4	0.73(0.55–0.97)	23.2	0.272
	>7	3	0.81(0.65–1.01)	0	0.489
Folate	**Disease**				
	Glioma	2	0.94(0.60–1.45)	0	0.819
	Brain tumor	5	0.62(0.50–0.76)	0	0.884
	Vitamin source				
	Diet	5	0.71(0.57–0.88)	0	0.667
	Supplement	2	0.53(0.36–0.79)	0	0.661
	Study population				
	Pregnancy exposure	5	0.64(0.51–0.80)	0	0.597
	Self-exposure	2	0.72(0.51–1.01)	0.3	0.317
	Study quality				
	≤7	3	0.64(0.48–0.85)	18.6	0.293
	>7	4	0.68(0.53–0.88)	0	0.673

be analyzed for dose-response relationship from nine articles. The dose-response relationship between vitamins and the risk of brain tumor is shown in Figure 2. There was a significant linear dose-response relationship between folate and brain tumor, and increasing 100 μg folate per day reduced brain tumor risk by 7% ($P_{-nonlinearity} = 0.534$, 95%CI:0.90–0.96). Although vitamin C, vitamin E, and serum 25-hydroxyvitamin D had similar linear trends, the results were insignificant due to insufficient studies.

Discussion

Based on 23 articles on vitamins and brain tumors published from 1989 to 2021, a total of 1,347,426 controls and 6,449 patients with brain tumors were included. Our meta-analysis results showed that for vitamin intake, higher intakes of vitamin C, β-carotene, and folate had a significant protective effect on brain tumors. For vitamin concentration *in vivo*, high serum α-tocopherol concentration could significantly reduce the risk of brain tumors. There was no significant correlation between vitamin A, vitamin B, vitamin E, and serum 25-hydroxyvitamin D and the incidence of brain tumors. There was a significant linear dose-response relationship between folate and brain tumor, and increasing per 100 μg/day folate intake reduced brain tumor risk by 7%. Although there were similar linear trends between vitamin C, vitamin E, serum 25-hydroxyvitamin D, and brain tumor risk, the results were insignificant. This might be due to the limited number of articles that analyzed the dose-response relationship between these vitamins and brain tumors.

We explored the sources of heterogeneity through disease conditions, vitamin sources, study population, study area, study

TABLE 4 Sensitivity analysis and publication bias.

	Influential analysis	Egger's test	Begg's test
Vitamin A	0.38–1.48	0.169	0.707
Vitamin B	0.74–1.37	0.101	0.734
Vitamin C	0.63–1.03	0.296	0.743
Vitamin E	0.58–1.17	0.170	0.276
β-carotene	0.61–1.00	0.658	0.764
Folate	0.52–0.85	0.462	0.764
Serum 25-hydroxyvitamin D	0.76–1.42	0.300	0.734
Serum α-tocopherol	0.28–1.00	0.302	0.296

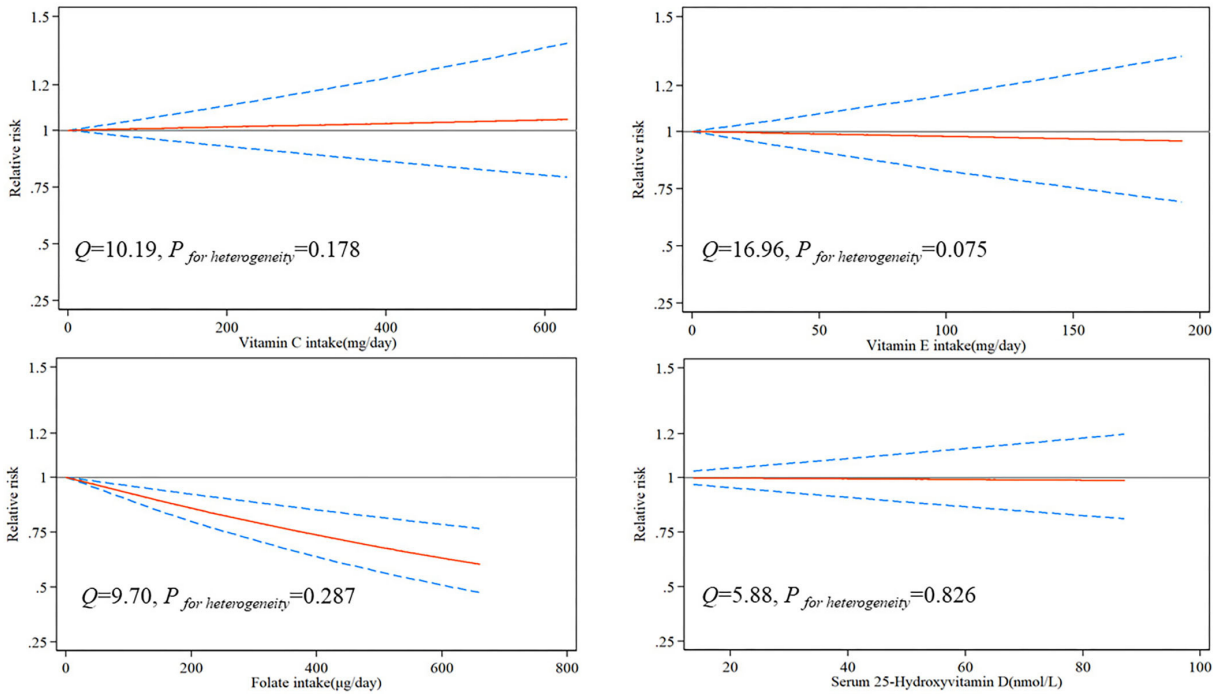

FIGURE 2
Risk between vitamins and brain tumors estimates from dose-response meta-analysis.

type, and study quality. The results of vitamin A were not significant, but through subgroup analysis, it was found that its heterogeneity mainly comes from the study area. The results of European and American studies showed that vitamin A had a protective effect on brain tumors. In contrast, the results of Australian studies suggested that excessive intake of vitamin A could significantly increase the risk of brain tumors. It was speculated that there was a significant difference in the intake of vitamin A due to different eating habits in the northern and southern hemispheres. Heydari's study contributed the most heterogeneity to the meta-analysis of vitamin C and brain tumors (35). It is well-known that the primary dietary sources of vitamin C are vegetables and fruits. Heydari's research showed that about 60% of Iranian adults had low fruit and vegetable intake, and the average intake of vitamin C in the control population was 143 mg/day (35), while half of the American people in Michaud et al.'s (28) study had more than 232 mg/day. Therefore, there were significant differences in vitamin C intake between the Iranian and other populations, resulting in considerable heterogeneity in this study. In addition, in the study of Hu et al. (26) only 57 kinds of food were investigated. In comparison, most of the food types investigated were more than 80 kinds in other studies, which might not obtain accurate vitamin intake, resulting in the heterogeneity of vitamin E.

Compared with other tissues, the brain has active metabolism and can produce many reactive oxygen species.

Still, the brain has low antioxidant defense ability, leading to DNA loss and tumor development (37). A case-control study of dietary antioxidants and glioma conducted by Tedeschi Blok et al. found that a higher intake of vitamin C was associated with a reduced risk of glioma (RR = 0.70, 95% CI:0.51–0.94) (14). Preston-martin et al. found that prenatal vitamin C supplementation could significantly reduce the risk of brain tumors in children (RR = 0.50, 95% CI:0.30–0.90). There was a dose-response relationship between intake and brain tumor risk (25). On the one hand, vitamin C could inhibit and reduce N-acetyltransferase activity and the formation of 2-aminofluorene-DNA adduct in rat C6 glioma cells in a dose-dependent manner (38). On the other hand, the rat experiment found that two markers related to brain tumor proliferation, platelet-derived growth factor receptor (PDGFRb), were found in rats fed with antioxidants such as vitamin C. Furthermore, Ki-67 transcripts were significantly reduced, suggesting that vitamin C could limit the invasiveness of brain tumors (39). In addition, some studies had found that vitamin C could inhibit the growth of glioblastoma through the caspase-3 death pathway and then assist the treatment of glioblastoma with methotrexate (40). Although our results did not find the protective effect of vitamin E intake on brain tumors, which was consistent with the results of two cohort studies in the United States (15, 28), the survival rate of patients with high vitamin E intake was higher in patients with grade III malignant glioma (41). Moreover, vitamin E derivatives reduced the incidence of pituitary tumors in X-ray-irradiated mice (42). We could not rule out the individual metabolic differences of vitamin E, resulting in inconsistent results. The results of prospective glioma serum metabolomics showed that serum α-tocopherol (the most bioactive form of vitamin E) concentrations were significantly negatively correlated with glioma risk (34), which was consistent with the results of the meta-analysis of serum α-tocopherol. We found that vitamin A intake had no significant effect on brain tumors. At present, there was no cohort study to explore the association between vitamin A and brain tumors, and the conclusions of case-control studies were inconsistent. Still, the previous meta-analysis showed that vitamin A could reduce the risk of glioma (RR = 0.80, 95% CI = 0.62–0.98, I^2 = 54.9%) (13). There were few studies on the mechanism of vitamin A and brain tumors. Some studies believed that brain tumors were closely related to retinoic acid, the metabolite of vitamin A and the level of retinoic acid-binding protein 2 in brain tumors were low related to the survival rate of patients (43). Although the relationship between vitamin A and brain tumors was not clear, β-carotene, as a precursor of vitamin A, showed a protective effect on brain tumors. Tedeschi Blok et al. found that the average intake of β-carotene in the control population was 252.8 mg/day (RR = 0.72, 95% CI:0.54–0.98) higher than that in patients with brain tumors, and the serum β-carotene concentration in patients with brain tumors was also significantly lower than that in healthy people (44). Cell experiments confirmed that β-carotene could effectively inhibit DNA synthesis in growing C-6 glioma cells (45). In addition, in the study of vegetable intake and brain tumors, it was also found that compared with other vegetables, orange vegetables rich in β-carotene had a stronger protective effect on brain tumors (11, 46). This study was the first time that folate could reduce the risk of brain tumors in the meta-analysis, which was consistent with the results of many epidemiological studies (17, 32). In recent years, the effect of folate on brain tumors had attracted much attention, especially in children. It had been found that the deficiency of folate metabolism might play an important role in the pathogenesis of some specific subtypes of brain tumors in children, especially embryonic central nervous system tumors (47). The mechanism might be related to the folate receptor. On the one hand, the folate receptor was found to be overexpressed in ependymoma, medulloblastoma, and other common malignant tumors of children's central nervous system (48, 49). Moreover, folate supplementation can enhance DNA remethylation through SP1/SP3 mediated transcriptional upregulation of DNMT3a and DNMT3b protein-coding genes to limit the invasiveness of glioma (50). In addition, targeted folate metabolism had selective cytotoxicity to glioma stem cells and can effectively cooperate with differentiation therapy to eliminate tumor-initiating cells in xenogeneic glioma grafts (51). However, only a few studies had reported the association between vitamin B and brain tumors, and the results were not significant. We also did not find any relevant research on dietary vitamin D and brain tumors. Since sunlight could promote vitamin D synthesis *in vivo*, it seemed more scientific to evaluate its effect on brain tumors through vitamin D concentration *in vivo*. Although experimental studies had shown that Vitamin D could promote cell cycle arrest and induce cell death to suppress tumor growth in glioblastoma (52, 53). However, no significant effect of vitamin D on brain tumors was found in epidemiological studies (31, 33).

So far, this was the largest meta-analysis of vitamins and brain tumors. Therefore, this study had several advantages. First, this study was the first meta-analysis involving the effects of multivitamins on brain tumors, including seven vitamins. The protective effects of β-carotene and folate on brain tumors were found in a meta-analysis for the first time. The dose-response relationship between folate and the risk of brain tumors was explored, which provided new evidence for preventing brain tumors. Second, this study also explored the relationship between vitamin concentration in serum and brain tumors to confirm the actual effect of vitamin intake. Third, we thoroughly discussed the sources of heterogeneity of the research results and improved the accuracy of the significant results. However, the study also had limitations. This study failed to further explore the relationship between vitamin and brain tumor subtypes. The incidence rate of brain tumors is very low, and the annual incidence rate was only 22.6/10 million (1). Although our current study included most observational studies of vitamins

and brain tumors, the sample size was still limited compared with other tumor studies. In addition, glioma is the most common brain tumor. Therefore, most of the current related studies focused on gliomas or brain tumors, especially in meta-analyses and systematic reviews (54, 55). In the search process, we did not find any studies that met the inclusion criteria, and the subjects had meningioma, germ cell tumor, or other brain tumor diseases. As a considerable part of the exposed population was pregnant women, and the outcomes of relevant studies were child brain tumors, this might cause some heterogeneity in the analysis process. However, we discussed the results of pregnancy exposure and self-exposure in the subgroup analysis and obtained similar results in some vitamins (such as folate). Most studies could only provide the source of intake of a particular vitamin (diet or supplement), so it was impossible to comprehensively evaluate the relationship between the overall intake of vitamins and brain tumors. Next, for the study of vitamin concentrations *in vivo*, only vitamin D and vitamin E provide sufficient articles, and there were too few studies on the concentrations of other vitamins to explore their correlation fully. We hope to improve the relevant analysis by adding more articles in future research.

Conclusion

In summary, the current meta-analysis shows that higher intakes of vitamin C, β-carotene, and folate can reduce the risk of brain tumors. At the same time, high serum α-tocopherol concentration also has a protective effect on brain tumors. Therefore, vitamins may provide new ideas for the prevention of brain tumors. In the future, we should pay attention to the compounds with antioxidant effects in the diet to further discover their effects on brain tumors.

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

WL and WZ contributed to the conception or design of the work, WZ, JJ, and YH contributed to searching the databases. WZ, JJ, and XL contributed to the acquisition, analysis, or interpretation of data for the work. WZ, XL, and SY proofread and modified the language. WL and FC reviewed and edited the manuscript. All authors have read and approved the final manuscript.

Funding

This study was supported by the National Natural Science Foundation of Beijing (No. J200003) and the National Science and Technology Major Project of China (No. 2016ZX09101017).

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.

Publisher's Note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.935706/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 28 September 2022
DOI 10.3389/fnut.2022.1018357

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Nutrition, a section of the journal Frontiers in Nutrition

RECEIVED 13 August 2022
ACCEPTED 01 September 2022
PUBLISHED 28 September 2022

CITATION
Mahboobi S, Ghasvarian M, Ghaem H, Alipour H, Alipour S and Eftekhari MH (2022) Effects of probiotic and magnesium co-supplementation on mood, cognition, intestinal barrier function and inflammation in individuals with obesity and depressed mood: A randomized, double-blind placebo-controlled clinical trial. *Front. Nutr.* 9:1018357. doi: 10.3389/fnut.2022.1018357

Effects of probiotic and magnesium co-supplementation on mood, cognition, intestinal barrier function and inflammation in individuals with obesity and depressed mood: A randomized, double-blind placebo-controlled clinical trial

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Background: The co-occurrence of obesity and mood impairments named as "metabolic mood syndrome" (MMS) is often neglected in the obesity management. This study aimed to evaluate effects of Probio-Tec®BG-VCap-6.5 and magnesium co-supplementation on mood, cognition, intestinal barrier function and serum C reactive protein (CRP) levels in participants with obesity and depressed mood.

Design: Seventy-four eligible participants were randomly allocated to either Probio-Tec®BG-VCap-6.5 [containing *Lactobacillus rhamnosus* (LGG®) and *Bifidobacterium animalis* subsp. *Lactis* (BB-12®)] + Magnesium chloride or placebo for 9 weeks. Sociodemographic data were collected in the beginning. Anthropometric, dietary and physical activity (PA) assessments were carried out. Beck Depression Inventory-II (BDI-II) and Montreal Cognitive Assessment (MoCA) scores were assessed through validated questionnaires. Fasting plasma zonulin, lipopolysaccharide (LPS) and (CRP) were measured by ELIZA kits.

Results: Of seventy-four participants (mean age 37.51 ± 8.10), 52 completed the study. Changes in serum LPS and zonulin were not different significantly between groups (−3.04 ± 44.75 ng/dl, 0.11 ± 5.13, ng/dl, $p > 0.05$ for LPS and 1.40 ± 48.78 ng/dl, −0.17 ± 6.60, $p > 0.05$ for zonulin, respectively).

CRP levels reduced significantly in intervention group compared to placebo [−474.75 (−1,300.00, −125.00) mg/l vs. 175.20 (−957.75, 1,683.25) mg/l, $p = 0.016$]. Changes in BDI-II and MoCA scores were not significantly different between intervention (−7.13 ± 5.67, 1.20 ± 2.16, respectively) and placebo (−5.42 ± 6.71, 1.94 ± 1.86, respectively) groups ($p > 0.05$).

Conclusion: Nine weeks of probiotic and magnesium co-supplementation resulted in decreased CRP levels as an indicator of inflammatory state with no significant effects on mood, cognition and intestinal integrity in individuals with obesity and depressed mood.

KEYWORDS

obesity, mood, intestinal integrity, inflammation, cognition, probiotics

Introduction

Obesity, characterized by excessive body fat accumulation (1), is one of the most important features of metabolic syndrome (METs) associated with multiple comorbidities contributing to a lower life expectancy (2, 3). Obesity was responsible for 120 million disability-adjusted life years (DALYs), equal to 4.9% of all DALYs in 2015 (4).

According to World Health Organization (WHO), the worldwide prevalence of overweight and obesity was 39 and 13%, respectively (5). Obesity prevalence was estimated to be 22.7% in the Iranian population (6).

Research has revealed that obesity is not just a simple imbalance between calorie intake and expenditure, but a more complex neurobiological condition manifesting anxiety, depression, binge eating, and mild cognitive impairment (7, 8). A bidirectional relationship has been shown between obesity and neuropsychiatric status (9), which constitutes an illness subtype named "metabolic-mood-syndrome" (MMS) with distinct pathophysiological mechanisms, different clinical manifestation and treatment response compared to each condition, separately (10–12).

Mood disorders manifest several pathological features most of which overlap with obesity, making them powerful candidates for the etiology of MMS. Gut dysbiosis, impaired intestinal permeability, cytokine imbalances and chronic low grade systemic inflammation can be regarded as some important key players in the etiology of MMS (13–15).

The human gastrointestinal (GI) tract is resided by a large microbial community named as gut microbiota (16). Metagenomic analysis of this microbial population has revealed that intestinal microbiota can act as a metabolic organ with a variety of physiological functions including immune modulation and metabolic function (17). Several studies has reported an association between obesity and changes in both composition and function of gut microbiota including an increase in opportunistic pathogens, reduced short chain fatty acid (SCFA) producer genera and increased capacity to harvest energy from diet (18). These alterations namely gut dysbiosis (19) can impair gut physiology and disrupt intestinal barrier integrity (20–22). Gut dysbiosis is directly associated with obesity (19) and can negatively impact gut physiology and disrupt intestinal barrier integrity (20–22). Impaired intestinal permeability leads to elevated circulating bacterial derived Lipopolysaccharides (LPS) which activates Toll Like Receptor-4 (TLR-4) located on the surface of macrophages (23–25) which in turn triggers systemic and neuro-inflammation (22). Zonulin reversibly regulates intestinal tight junction proteins (occludin and zonula occludens-1) (26, 27) and is strongly correlated with the lactulose: mannitol ratio (28) which makes it a useful marker of intestinal permeability (26).

Modern world, including developing countries, has experienced a shift to more consumption of high calorie, high fat westernized diets (29, 30) which not only impair gut microbial diversity (19), but also lead to inadequate intake of micronutrients, such as vitamin B-6, magnesium, calcium and zinc, in the long run (31, 32). Magnesium is an essential micronutrient with a variety of functions in metabolism, neurotransmission and immunomodulation (33, 34). Magnesium deficiency can contribute to systemic and neuro-inflammation and involves in the pathogenesis of metabolic and psychiatric disorders (34–36). New studies are indicative of a direct association between gut microbiota and the variations in dietary magnesium intake. Some animal studies have shown that magnesium administration can enhance SCFA concentrations and gut microbiota diversity (37, 38). Magnesium deficient diet on the other hand, resulted in decreased gut *Bifidobacterium*, lower mRNA levels of tight junction proteins, as well as increased levels of Tumor Necrosis Factor-α (TNF-α) and IL-6 (39). Inadequate magnesium intake is also associated with elevated CRP levels, a common indicator of inflammatory state (40). A very recent review focused on studies in the last 3 years, has reported the possibility of adding magnesium orotate and probiotic as an adjunct treatment in

individuals suffering from both GI and psychiatric disorders focusing on their ability to modulate gut-brain axis (37).

Probiotics are beneficial micro-organisms that can improve their host's health through restoration of gut microbial communities, improving intestinal barrier integrity and immunomodulation (41, 42) and suppression of body weight gain (43). Some probiotics can positively impact mental health and alleviate depressive symptoms, which are specifically called psychobiotics (44, 45). Based on evidence, several pathways can be hypothesized through which probiotics exert beneficial effects on gut-brain axis making them capable of alleviating MMS; SCFAs as a major metabolite of probiotics, participate in anti-inflammatory processes leading to increased production of IL-8 and improved gut barrier tightness (44). These SCFAs can also exert anti-obesity functions by increasing insulin sensitivity and fatty acid oxidation and decreasing fat accumulation, through the activation of AMP kinase (Adenosine Monophosphate activated Kinase) in muscles (46). Probiotic supplements suppress the expression of pro-inflammatory cytokines such as IL-6 and IL-17, and promote the expression of tight junction proteins (Zo-1, claudin-1, and occludin) (47).

Species from *Bifidobacteria* and *Lactobacilli* genera have gained the most interest in probiotic and psychobiotic- related studies (48, 49).

Studies regarding effects of probiotics on weight management, inflammation, intestinal permeability, depression and cognition, have reported inconsistent findings (42, 44, 45, 50–53). Although many clinical trials exist in the field of probiotics and obesity, to our knowledge no clinical study has targeted psychology and gut brain axis for probiotic interventions in MMS management. The anti-depressant role of magnesium has been well established in previous research (54). However, clinical studies investigating its role in improving gut barrier function is scarce. Since both magnesium and probiotics have the ability to improve gut-brain axis, we assumed their combination might exert more beneficial effects than each intervention, separately.

Gathering all these evidence and assumptions together, we designed a clinical trial evaluating effects of probiotic and magnesium co-supplementation on some parameters related to gut-brain axis in individuals with MMS.

Subjects, material, and methods

Sample size determination

A sample size of 60 (30 per group) was calculated based on a previous study by Steenburgen et al. (55), considering BDI-II as main variable, type I error 0.05 and type II error of 0.20 (power 80%). With a predicted attrition rate of 20%, the sample size was increased to 72 (36 per group).

Participants, randomization, and procedures

Seventy-four men and women with obesity and depressed mood participated in this 9-week, double-blind, placebo controlled randomized clinical trial. Through local advertisements and social media, recruitment was conducted consecutively from October 2020 to February 2021, in Nutrition clinic, Imam Reza Hospital, affiliated with Shiraz University of Medical Sciences, Shiraz, Iran. In an initial screening phase, volunteers were evaluated for eligibility based on the following criteria: age 18–50 years, body mass index (BMI) $\geq$ 30 kg/m^2, waist circumference (WC) $\geq$ 88 cm for women and 102 cm for men, and BDI-II scores between 14 and 28 (with the approval of a clinical psychiatrist). Further criteria were as follows: being non-smoker, non-alcohol/opioid addict, not suffering from any chronic condition (renal/liver/gastrointestinal/lung diseases, diabetes, severe neuropsychological or mental disorders and infections), not having a history of stroke, not taking anti-depressants, anti-inflammatory drugs and corticosteroids and not being in pregnancy, lactation and menopause states. Furthermore, participants must have not taken antibiotics, probiotic, magnesium and omega 3 supplements, at least 1 month prior to the study commencement. Because of COVID-19 pandemic, confirmed cases of COVID-19 and those with any usual symptoms of COVID-19 were not included in the study. Exclusion criteria were: any changes in usual diet, medication and physical activity, starting antibiotic therapy, the occurrence of any side effects which would stop by discontinuation of intervention and non-compliance to research instructions. The study protocol was drafted and conducted according to Declaration of Helsinki (56) and CONSORT statements (57), registered in Iranian Registry of Clinical Trials (IRCT ID: IRCT20191127045525N1) and approved by the ethics committee of Shiraz University of Medical Sciences (approval code:IR.SUMS.REC.1398.1375). After providing a written informed consent, subjects started a 2-week run- in period. In this phase, for ethical reasons, all participants were first given a general consultation for lifestyle improvement and were asked to keep their diet, physical activity and usual medications constant during run-in and intervention periods. No weight loss or specific diets were provided. Participants were also asked to avoid taking probiotic products, magnesium and omega3, as well as any anti-inflammatory or pain relieving medications during the study.

After an overnight fast and providing blood samples, participants were assessed for demographic characteristics, diet, physical activity, anthropometric parameters and cognition, and were then randomly allocated into either intervention ($n = 39$) or placebo ($n = 35$) groups through block randomization with blocks of four. Allocation order was concealed from research executors by sealed opaque envelopes containing A or B, by

a third party who was not actively involved in recruitment process. Subjects in the intervention group received two separate Probio-Tec®BG-VCap-6.5 and magnesium chloride capsules (one capsule each) while those in placebo group received two placebo capsules for 9 weeks, on a daily basis. Subjects were instructed to store the capsules in refrigerator and take one of each after main meal. Products and compliance checklists were distributed in the start and middle (forth week) of the study. Participants were asked to record any adverse events and were in every-day contact with a trained executor through phone calls and text messages to keep compliance and discuss any probable questions. After 9 weeks of intervention a final visit was arranged to obtain post- intervention fasting blood samples, psychological and cognitive assessments, evaluating compliance and gathering dietary and physical activity data.

Study products and blinding

Probiotics (Probio-Tec®BG-VCap-6.5) were a research fund received from Chr. Hansen company (Copenhagen, Demark) and contained *Lactobacillus rhamnosus* (LGG®) and *Bifidobacterium animalis* subsp. *Lactis* (BB-12®) in a ratio of 1:1 with a potency of 1.8×10^{10} CFU (Colony Forming Unit) per cap. Magnesium chloride powder was purchased (Pharmbio Inc., Korea), processed and capsulated in laboratory of pharmacy department, Shiraz University of Medical Sciences, Shiraz, Iran. Each capsule contained 500 mg magnesium chloride which provided 125 mg elemental magnesium (∼31 and 41% RDA for women and men, respectively). While the original protocol was to provide 250 mg elemental magnesium, which needed participants to take magnesium capsules twice per day, for better compliance we decided to provide 125 mg elemental magnesium once per day. Placebos contained maltodextrin and were similar in shape, color, weight and packaging to either probiotic or magnesium chloride capsules. Therefore, neither participants nor research executors were capable of distinguishing active products vs. placebos until the analyses were completed.

Demographic, dietary, and physical activity assessment

Data regarding general demographic, medical history and socioeconomic factors were gathered using a questionnaire designed by research team. For dietary assessments, participants filled three 24-h food records (two weekdays and one weekend day) in the start and in the last week of study period. Daily calorie and nutrient intakes were then calculated by Nutritionist IV software (First Databank, San Bruno, CA, USA) using Iranian food composition database.

Three 24-h physical activity (PA) dairies were completed by participants before and at the end of study duration (58). PA was then calculated as metabolic equivalents in hour per day (METs-hrs/day). To compute METs for each activity we calculated daily hours a person had spent on that specific activity. MET-hrs of all daily activities were then summed to calculate daily physical activity.

Anthropometric assessment

Body weight was measured with 100 g precision using a Seca scale (Seca, Germany) while subjects were in light clothing and barefoot. Height was measured with 0.1 cm precision using wall mountable height rod on a flat surface with barefoot. The narrowest part between lowest rib and iliac crest was marked for measuring WC with an un-stretchable tape with 0.1 cm precision. BMI was calculated by dividing weight (kg) to height squared (m^2).

Psychologic and cognitive assessments

For mood assessment we used Beck Depression Inventory II (BDI-II), a 21-item self-administered questionnaire. For each item participants were instructed to choose the best option that described their mood during the last 2 weeks. Options of each item are rated from 0 to 3 based on symptom severity and the final score is a sum of all scores ranging from 0 to 63. Scores between 14 and 28 are indicative of mild to moderate depression. BDI-II is the most commonly used instrument for screening of depression in general population. It has a high internal consistency, reliability and structural validity and has shown the capacity to discriminate between depressed and non-depressed subjects and can be applicable for research and clinical practice worldwide (59–61). The reliability and validity of Persian BDI-II was confirmed in previous studies (60). Cognition was evaluated by Montreal Cognitive Assessment (MoCA) tool. The scoring is based on Visuospatial and executive functioning (5 points), animal naming (3 points), attention (6 points), language (3 points), abstraction (2 points), delayed recall (5 points), orientation (6 points) plus 1 extra point for those who have <12 years of formal education. Persian MoCA is validated by Z. Nasreddin and is available on www.mocatest.org.

Biochemical analyses

After an overnight fast (10 h), 5 cc blood samples were collected between 07:30 to 9:30 a.m. Samples were then centrifuged at 3,000×, serum was separated and stored at −70°C till analysis. Serum Zonulin and LPS were analyzed by Enzyme-linked Immunosorbent Assay (ELIZA) kits (both:

Shanghai Crystal Day Biotech Co., China) following the instruction manual. Serum CRP was analyzed by ELIZA kit (LDN, Nordhorn, Germany) according to the manufacturer's instruction. Serum magnesium was measured by a commercially available kit (ZistChem Diagnostics, Tehran, Iran) using colorimetric method with autoanalyzer.

Statistical analysis

Data was analyzed using SPSS software (ver.17, for windows, SPSS Inc., Chicago, USA). Normal distribution of quantitative variables was assessed using Shapiro-Wilk test as well as normality curves. Mean ± SD and median (Q1, Q3) were used to present normally and non-normally distributed variables, respectively. Categorical variables were presented as numbers and percentages. To calculate missing data for dropouts, imputation technique was carried out using mean differences obtained from existing data. In case of normal distribution, Within-group and between group comparisons were conducted using paired sample *t*-test and independent sample *t*-test, respectively. For skewed variables we applied their equivalent non-parametric tests including Wilcoxon signed ranked test and Mann-Whitney U test. Categorical variables were compared between groups by applying chi-2 test. For all tests, p-value ≤ 0.05 was considered significant.

Results

Of 207 volunteers, 74 eligible subjects (58 women and 16 men) were randomized to either intervention or placebo groups. Fifty- two participants completed the study and were included in the final analysis. However, due to dropouts, data for missing values were computed based on imputation method explained in the previous section. Figure 1 demonstrates the study CONSORT flowchart. Mean ± SD age, BMI and BDI-II scores of study participants were 37.51 ± 8.10, 34.42 ± 3.60, 21.95 ± 7.77, respectively. Participants' baseline characteristics are presented in Table 1 compared by study group. As shown in the table, no significant differences exist between study groups in terms of age, sex BMI, WC, BDI-II, serum magnesium and sociodemographic factors at the baseline which is indicative of appropriate randomization process. Table 2 demonstrates data on calorie and nutrient intakes as well as PA of participants in the beginning and after 9 weeks. No significant within-group and between group changes were observed regarding calorie, macronutrient and micronutrient intake as well as PA, during the study. Effects of probiotic and magnesium co-supplementation on study outcomes are shown in Table 3. Serum levels of LPS and Zonulin did not significantly change in intervention (−3.04 ± 44.75 ng/dl, $p > 0.05$; 0.11 ± 5.13 ng/dl, $p > 0.05$, respectively) or placebo (1.40 ± 48.78 ng/dl, $p > 0.05$; −0.17 ± 6.60, $p > 0.05$, respectively) groups during the study. Between-group differences were also non-significant ($p > 0.05$). Our intervention resulted in reduction in serum CRP levels [−0.047 (−0.13, −0.012) mg/l] which was significantly different from its change in placebo group [0.017 (−0.095, 0.160) mg/l] ($p = 0.016$). BDI-II and MoCA scores significantly improved in both intervention (−7.13 ± 5.67, $p < 0.001$; 1.20 ± 2.16, $p = 0.001$, respectively) and placebo (−5.42 ± 6.71, $p < 0.001$; 1.94 ± 1.86, $p < 0.001$, respectively). However, between-group differences for these two outcomes were non-significant. Serum magnesium was also measured as a secondary outcome which did not significantly change post-intervention in either groups (−0.03 ± 0.16, $p > 0.05$ for intervention and 0.03 ± 0.13 for placebo group). Furthermore, these changes were not significantly different between groups.

In order to adjust potential confounders, multiple linear regression model was conducted for evaluation of between group comparison. In this model, changes in outcome variable (post-intervention minus baseline) were entered as dependent variable while participants' BMI, education, job, income, BDI-II as well as energy and macronutrient intakes were regarded as covariates. Although after adjustment, no differences were observed in the study results.

Discussion

Effects of probiotic and magnesium co-supplementation on intestinal barrier function and systemic inflammation

Our study revealed that 9 weeks' supplementation with probiotic and magnesium in individuals with obesity and depression might improve CRP levels with no significant effects on serum zonulin and LPS concentrations as markers of intestinal integrity. Our findings are consistent with a previous study conducted by Lee et al. evaluating effects of herbal medicine with or without probiotics on gut microbiota, gut permeability and endotoxin levels in subjects with overweight/obesity. Similar to our results, no significant changes were observed in LPS, intestinal barrier function and other metabolic markers (62). In a 12-week trial of post-menopausal women with obesity, it was shown that probiotic supplementation might beneficially affect LPS levels in a dose-response manner (63). In another 4-month clinical trial carried out on individuals undergoing gastric bypass surgery, multispecies probiotic could improve levels of LPS binding protein, TNF-α and weight loss (64). Amirani et al. conducted a metaanalysis on the effects of probiotics on inflammatory markers in participants with psychiatric disorders. A significant reduction in CRP and Interleukine-10 (IL-10) levels was seen following probiotic consumption (65). Exact mechanisms through which probiotics exert beneficial

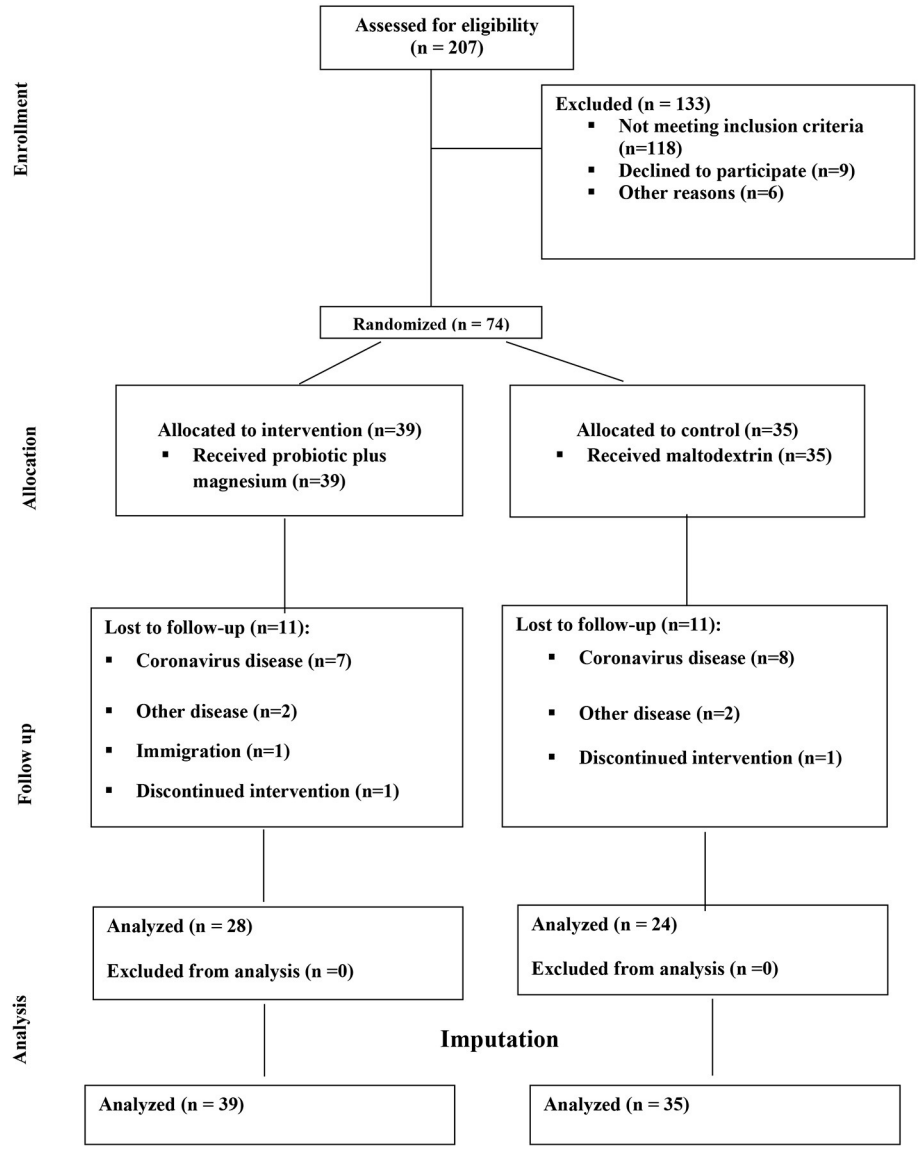

FIGURE 1
Study CONSORT flowchart.

effects on inflammation and gut barrier function are not completely elucidated. Probiotic strains have the potential to enhance epithelial barrier integrity through modulating gene expression of adhesion proteins (47, 66) and production of health promoting molecules and anti-microbial peptides which prevent pathogen growth (67, 68). Probiotics also modulate host immune system by microbe-associated molecular patterns (MAMPs) which interact with pattern recognition receptors (PRRs) present on the surface of intestinal epithelial and immune cells and maintain immune homeostasis (69, 70) which might not only improve intestinal barrier integrity, but also play a role in regulation of inflammatory state (70). Probiotics produce surface-layer proteins (SLPs) that reduce LPS induced inflammation through decreased translocation of NF-Kβ into nucleus which eventually attenuates TNF-α, IL-1β and oxidative stress (70).

Magnesium deficiency is associated with an inflammatory state characterized by elevated levels of acute phase proteins (71). The inverse relationship between magnesium intake and inflammatory state have been reported in several studies as reviewed by Belin and He (72). However, studies regarding direct effects of magnesium intake on intestinal integrity and gut

TABLE 1 Baseline characteristics of study participants.

Variable	Study groups		*p*-value
	Group A[a]	Group B[b]	
Sex (*n*, %)			0.411[§]
Male	10, 25.6	6, 17.1	
Female	29, 74.4	29, 82.9	
Age (year)	38.94 ± 7.19	35.90 ± 8.64	0.108[¶]
Education (*n*, %)			0.541[§]
≤6 years of official education	3, 7.7	6, 17.1	
6–10 years of official education	18, 46.2	17, 48.6	
B.Sc. degree	14, 35.9	10, 28.6	
M.Sc. degree and above	4, 10.3	2, 5.	
Weight (Kg)	96.92 ± 17.27	91.20 ± 13.73	0.121[¶]
BMI (kg/m^2)	34.59 ± 3.97	34.24 ± 3.16	0.685[¶]
WC (cm)	115.42 ± 10.13	113.39 ± 8.38	0.337[¶]
BDI-II	21 (15, 29)	20.50 (15.00, 25.25)	0.685[R]
MoCA	24.92 ± 2.99	24.08 ± 3.49	0.271[¶]
Serum magnesium (mg/dl)	2.00 ± 0.16	1.97 ± 0.22	0.879[¶]
Serum LPS (ng/ml)	217.00 (179.50, 256.50)	217.00 (181.00, 264.00)	0.860[R]
Serum zonulin (ng/ml)	13.73 (8.92, 18.88)	13.02 (10.00, 18.25)	0.808[R]
Serum CRP (ng/ml)	5705.94 ± 3583.72	6201.82 ± 4705.63	0.899

[a]Group A: intervention group, received one probiotic capsule (Probio-Tec® BG-VCap-6.5, containing 1.8×10^{10} CFU *Lactobacillus rhamnosus* and *Bifidobacterium animalis* subsp. *Lactis*) plus one magnesium chloride capsule (containing 125 mg elemental magnesium), on a daily basis for 9 weeks.
[b]Group B: received two placebo capsules containing maltodextrin on a daily basis for 9 weeks.
[§] *P*-values obtained from Chi-2 test.
[¶] *p*-values obtained from independent samples t- test.
[R] *p*-values obtained from Mann-Whitney U test.
[*] $P \leq 0.05$ was considered as statistically significant.
B.Sc., bachelor of science; M.Sc., master of science; BMI, body mass index; WC, waist circumference; BDI-II, Beck depression inventory-II test; MoCA, Montreal Cognitive Assessment tool; LPS, lipopolysaccharide; CRP, C-reactive protein; CFU, Colony Forming Unit.

microbiota are rare. Mice fed with magnesium deficient diet had a lower gut *Bifidobacteria* content, lower mRNA levels encoding factors involved with intestinal barrier integrity (zonula-occludens-1, occluding, proglucagon), increased expression of TNF-α, IL-6 and activating transcription factor-4, a reflection of inflammatory and cellular stress (39).

Based on previous studies, a longer study duration might be needed to observe potential improvements in gut barrier function while inflammatory markers such as CRP levels take less time to be influenced by probiotics or dietary supplements. Furthermore, since our intervention contained magnesium, inflammatory status might be improved by pathways related to weight reduction which occurred in our intervention group; After analyzing anthropometric findings, we realized that participants in intervention group had considerable reductions in weight (-4.99 ± 1.32 kg, $p = 0.012$), BMI (-1.95 ± 0.51 kg/m^2, $p = 0.012$) and WC (-1.58 ± 1.51 cm, $p < 0.001$). Therefore, reduced adiposity might be a potential explanation for at least part of inflammation improvement in our study.

Effects of probiotic and magnesium co-supplementation on mood and cognition

BDI-II and MoCA as indicators of mood and cognitive performance improved in both groups with no significant between-group differences. This finding can be justified by a couple of logics; As stated by evidence, BDI-II is a standardized self-report measure to identify depressive disorders and categorize the severity of depressive symptoms (73). We assume that subjective nature of this tool and placebo effect might be the reason for significant improvements in control group. Actually a recent 2022 publication has clearly stated that "depression is a highly placebo responsive condition" (74).

Regarding cognitive assessment, MoCA is an excellent and simple tool which evaluates multiple cognitive domains with great sensitivity and specificity for detecting mild cognitive impairment (MCI) (75). However, since participants performed MoCA before and after 9 weeks, and regarding the fact that

TABLE 2 Dietary intake and physical activity levels at baseline and after 9 weeks' intervention.

Variable	Group A[a]				Group B[b]				*P*-value[e]
	Baseline	Post-intervention	Δ[c]	*P*-value[d]	Baseline	Post-intervention	Δ[c]	*P*-value[d]	
Energy (kcal/d)	2029.24 ± 452.80	2120.24 ± 468.05	91.00 ± 429.63	0.460	1734.66 ± 390.95	1805.78 747.49	71.12 ± 797.34	0.744	0.937
Carbohydrate (g/d)	289.51 ± 98.72	313.33 ± 76.97	57.76 ± 160.95	0.223	247.71 ± 58.78	258.71 ± 89.25	54.40 ± 136.44	0.701	0.954
Protein (g/d)	72.78 ± 15.39	75.83 ± 22.35	3.05 ± 23.08	0.642	70.24 ± 25.22	73.43 ± 29.64	3.18 ± 43.16	0.787	0.992
Fat (g/d)	64.71 (49.71, 84.51)	65.89 (49.87, 83.25)	0.97 ± 25.91	0.917	55.60 (43.68, 71.18)	65.93 (39.69, 88.68)	12.46 59.35	0.397	0.526
SFA (g/d)	17.18 ± 6.68	16.04 ± 4.20	−1.13 ± 8.00	0.620	13.10 ± 3.60	16.29 ± 8.15	3.19 ± 7.80	0.150	0.168
MUFA (g/d)	17.95 ± 6.24	18.59 ± 5.78	0.64 ± 7.88	0.773	16.07 ± 3.77	18.26 ± 7.25	2.18 ± 6.78	0.249	0.589
PUFA (g/d)	23.48 ± 12.35	22.22 ± 10.33	−1.26 ± 15.72	0.777	19.99 ± 5.14	22.05 ± 13.97	2.06 ± 14.28	0.597	0.569
Magnesium (mg/d)	184.37 (157.59, 227.56)	167.85 (142.18, 226.11)	−3.54 (−62.69, 25.08)	0.507	163.19 (138.64, 222.65)	176.44 (135.19, 238.14)	14.18 (−61.95, 72.24)	0.778	0.192
Fiber (g/d)	12.84 ± 4.13	12.56 ± 3.26	0.45 ± 4.24	0.78	11.87 ± 5.46	13.70 ± 4.16	4.38 ± 11.77	0.787	0.267
Sugar (g/d)	42.55 (30.79, 60.31)	46.91 (35.77, 62.56)	−5.75 ± 21.63	0.507	37.64 (23.45, 58.94)	45.66 (38.46, 69.62)	9.51 ± 38.82	0.470	0.223
PA (METs-hr/d)	34.52 ± 4.55	31.68 ± 4.66	−2.84 ± 5.96	0.190	32.91 ± 4.57	31.12 ± 6.67	−1.79 ± 8.04	0.522	0.757

Data are presented as mean ± SD and median (Q1, Q3) for normally and non-normally distributed variables, respectively.

[a]Group A: intervention group, received one probiotic capsule (Probio-Tec® BG-VCap-6.5 Pla V2, containing 1.8×10^{10} CFU *Lactobacillus rhamnosus* and *Bifidobacterium animalis* subsp. *Lactis*) plus one magnesium chloride capsule (containing 125 mg elemental magnesium), on a daily basis for 9 weeks.

[b]Group B: received two placebo capsules containing maltodextrin on a daily basis for 9 weeks.

[C]Δcalculated as: (post-intervention – baseline) in each study group.

[d]*P*-value for within-group comparisons, obtained from paired samples *t*-Test and Wilcoxon signed ranked test for normally and non-normally distributed variables, respectively.

[e]*P*-value for between-group comparisons, obtained from independent samples *t*-Test and Mann-Whitney U test for normally and non-normally distributed variables, respectively.

[*]$P \leq 0.05$ was considered as statistically significant.

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; PA, physical activity; METs, metabolic equivalents; CFU, Colony Forming Unit.

10.3389/fnut.2022.1018357

10.3389/fnut.2022.1018357

TABLE 3 Outcome variables at baseline and after 9 weeks' intervention.

Variable	Group A[a]				Group B[b]				*p*-value
	Baseline	Post-intervention	Δ[c]	*P*-value	Baseline	Post-intervention	Δ[c]	*p*-value	
Serum Magnesium (mg/dl)	2.00 ± 0.16	1.97 ± 0.16	−0.03 ± 0.16	0.179[$]	1.97 ± 0.22	2.00 ± 0.23	0.03 ± 0.13	0.155[$]	0.053[¶]
Serum Zonulin (ng/ml)	13.73 (8.92, 18.88)	15.06 (9.63, 18.53)	0.11 ± 5.13	0.858[Ω]	13.02 (10.00, 18.25)	13.30 (10.48, 16.80)	−0.17 ± 6.60	0.455[Ω]	0.873[¶]
Serum LPS (ng/ml)	217 (179.50, 256.50)	210.50 (171.75, 262.25)	−3.04 ± 44.75	0.485[Ω]	217.00 (181.00, 264.00)	229 (178.75, 264.75)	1.40 ± 48.78	0.661[Ω]	0.754[¶]
CRP (mg/l)	5705.95 ± 3583.72	5231.19 ± 3576.39	−474.75 (−1300,−125)	0.090[$]	6201.82±4705.63	6701.18±4235.99	175.20 (−957.75, 1683.25)	0.240[$]	0.016[R]
BDI-II (score)	21.00 (15.00, 29.00)	15.00 (7.00, 22.00)	−7.13 ± 5.67	<0.001[Ω]	20.50 (15.00, 25.00)	13 (8.00, 24.50)	−5.42 ± 6.71	<0.001 [Ω]	0.246[¶]
MoCA (score)	24.99 ± 2.99	26.12 ± 2.66	1.20 ± 2.16	0.001[$]	24.20 ± 3.47	26.15 ± 2.88	1.94 ± 1.86	<0.001[$]	0.124[¶]

Data are presented as mean ± SD and median (Q1, Q3) for normally and non-normally distributed variables, respectively.

[a]Group A: intervention group, received one probiotic capsule (Probio-Tec® BG-VCap-6.5 Pla V2, containing 1.8×10^{10} CFU *Lactobacillus rhamnosus* and *Bifidobacterium animalis* subsp. *Lactis*) plus one magnesium chloride capsule (containing 125 mg elemental magnesium), on a daily basis for 9 weeks.

[b]Group B: received two placebo capsules containing maltodextrin on a daily basis for 9 weeks.

[c] Δ calculated as: [post-intervention − baseline] in each study group.

[$] *p*-values obtained from paired samples *t*-test.

[Ω] *p*-values obtained from Wilcoxon signed ranked test.

[¶] *p*-values obtained from independent samples t- test.

[R] *p*-value obtained from Mann-Whitney U test.

* $P \leq 0.05$ was considered as statistically significant.

BMI, body mass index; WC, waist circumference; Mg, Magnesium; CRP, C-reactive protein; LPS, lipopolysaccharide; MoCA, Montreal cognitive assessment test; BDI-II, Beck depression inventory-II test; CFU, Colony Forming Unit.

several sections of MoCA test are memory based, elevated MoCA scores in control group might be due to participants' task learning and memorization.

Several clinical trials have been conducted in this area with various results. In a 12-week randomized clinical trial, Akbari et al. demonstrated that a mixture of probiotics can significantly improve cognition evaluated by Mini- mental state examination (MMSE) in patients with Alzhimer's disease (AD) (76). Furthermore, a metaanalysis of four randomized trials, revealed beneficial effects of probiotic supplementation in Hamilton Depression Rating Scale (HAMD) (65). Probiotic supplementation along with magnesium was carried out in one small pilot study. Eight weeks' consumption of probiotics and magnesium orotate significantly improved depression scores and quality of life in 12 participants with drug resistant depression (77).

Probiotics can exert promoting effects on mood and cognition through several mechanisms. Since persistent low grade inflammation is associated with existence and severity of depressive symptoms, probiotics might relieve such symptoms *via* anti-inflammatory activities explained earlier in this section (78). Furthermore, gut microbiota and probiotics are known to synthetize neurotransmitters responsible for maintaining proper brain function including gamma amino butyric acid (GABA), serotonin (5-HT), glutamate (Glu), dopamine and norepinephrine (NE). Gut microbiota also regulates the bioavailability of precursors for these neurotransmitters (79). Besides these mechanisms, research has indicated that gut microbiota elicits signals to the brain *via* vagus nerve and vice versa (80).

Magnesium has long been used to treat depression and relieve a variety of emotional problems even in homeopathic medicine (81). Studies in this area has been going on so far. Tarleton et al. showed that 6 weeks consumption of magnesium chloride can improve depressive symptoms (82). In another study, consumption of 500 mg magnesium oxide for 8 weeks led to improvements in depressive symptoms and serum magnesium levels in participants with depression and hypomagnesemia (83). In the present study, serum magnesium levels did not change significantly following magnesium chloride consumption. It might be due to our finding that mean baseline serum magnesium levels of participants was 1.99 ± 0.19 mg/dl, which already drops within normal range (84).

The role of magnesium in intestinal barrier function remains to be elucidated. In a study by Pachikian et al., 4 days of magnesium deficiency resulted in decreased ileal expression of Occludins, Zo-1 and Zo-2 in mice. Magnesium deficient mice also exhibited discontinuous Zo-1 and Occludin staining in the ileum compared with control group (39). In addition to the crucial role of magnesium in ATP metabolism which is essential for normal neurological function and neurotransmission (85), it has been regarded as one of the modulators of N-methyl-D-aspartate (NMDA), a receptor complex involved in

pathophysiology of depression and is considered as a target for anti-depressant therapy (86).

Our study has some limitations. Although we started our study with an adequate sample size, our dropout rate was a bit higher than our expectation which was partly due to COVID-19 pandemic. Nevertheless, despite dropouts, an acceptable number of participants remained in each group and for missing data, imputation method was carried out. We had to decrease daily dose of magnesium chloride from two to one 500 mg capsule per day for better compliance, which might have attenuated probable effects of magnesium on study outcomes. Markers such as IL-6 and TNF-α could be measured besides CRP as parameters of inflammatory status but due to financial limitations we did not include such parameters as outcomes. Although BDI-II and MoCA tools have high reliability and validity due to their subjective nature they might have been affected by different conditions.

In spite of these limitations our study has several strengths. To the best of our knowledge, this was the first study which has evaluated effects of a combination of probiotic and magnesium on markers of intestinal integrity, mood, cognition and serum CRP levels in individuals with obesity and depressed mood. We did our best to control as many potential confounders as possible to increase the validity of our findings. Probiotics used in this study were Probio-Tec® BG-VCap-6.5 with accurate product information and analysis manufactured by Chr. Hansen according to food laws and legislations. For magnesium, we used its chloride salt which has a higher bioavailability and tolerability than other magnesium salts (87).

Overall, 9 weeks of probiotic and magnesium supplementation resulted in decreased CRP levels in individuals with obesity and depressed mood. However, this intervention was ineffective in improving intestinal barrier function, mood and cognition. It is suggested that future research in this area consider longer durations, higher doses of magnesium and apply objective tools for neurocognitive assessments.

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 study protocol was drafted and conducted according to Declaration of Helsinki and CONSORT statements, and approved by the Ethics Committee of Shiraz University of Medical Sciences (approval code: IR.SUMS.REC.1398.1375). All participants provided written informed consent prior to the study commencement.

Author contributions

SM and ME contributed in research conceptualization. SM, ME, and HG developed study design, methodology, validation, and took part in the manuscript finalization and improvement. SM and MG participated in investigation process, experiments, data collection, data entry, drafted the original manuscript, and contributed to data visualization. HG and SM contributed in data analysis and curation. SA contributed in providing study resources. All study phases were conducted with the supervision of ME. All authors contributed to the article and approved the submitted version.

Funding

This manuscript was extracted from a PhD dissertation funded by Vice President of Research and Technology (Research Code: 20052-84-01-98), Shiraz University of Medical Sciences, Shiraz, Iran.

Acknowledgments

Authors would like to thank staff members of Imam Reza Clinic, Nutrition Research Laboratory, Boqrat Laboratory and all participants for their valuable cooperation. We would also like to thank Dr. Arvin Hedayati (Research Centre for Psychiatry and Behavioral Sciences, Shiraz University of Medical Sciences, Shiraz, Iran) for her valuable consultations in mood and psychiatric assessments. Furthermore, we kindly thank Chr. Hansen company for funding our research by providing probiotic capsules and their placebo.

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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frontiers | Frontiers in Nutrition

TYPE Review
PUBLISHED 20 October 2022
DOI 10.3389/fnut.2022.1022650

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

RECEIVED 18 August 2022
ACCEPTED 30 September 2022
PUBLISHED 20 October 2022

CITATION
Zhang X, Zhang F, Li Q, Feng C and Teng W (2022) Iodine nutrition and papillary thyroid cancer. *Front. Nutr.* 9:1022650. doi: 10.3389/fnut.2022.1022650

Iodine nutrition and papillary thyroid cancer

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Thyroid cancer (TC) is the most frequent endocrine malignancy. The incidence of TC, especially papillary thyroid carcinoma (PTC), has continued to rise all over the world during the past few years, for reasons that are not entirely clear. Though the phenomenon of overdiagnosis is occurring, it is not the sole driver of the substantial increase in incidence. Lifestyle, environmental factors, or complications are considered to be potential risk factors. Among these factors, iodine is a micronutrient that is vital to thyroid function. The effect of iodine intake on PTC has been controversial for many years and the epidemiological or experimental studies provided diametrically opposite conclusions. Combining all these studies, we found that iodine nutrition may affect the overall prevalence, distribution of the histological types, and clinicopathological aggressiveness of TC, especially PTC. However, the available evidence is poor due to the impact of various internal and external related factors. Therefore, this article sums up available results from both epidemiological and experimental studies, future studies are also warranted to expound on the relationship between overall PTC prevalence and iodine intake.

KEYWORDS

iodine, papillary thyroid cancer, iodine nutrition, thyroid cancer, epidemiological studies

Introduction

Though the global thyroid cancer (TC) incidence has grown remarkably over the past few years (1–3), the mortality rate remains static (4, 5). There are four main kinds of TC: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC), and anaplastic thyroid cancer (ATC) (6). ATC, one of the fatal and rare forms of TC (1–2%) that generally presents as

a rapidly growing neck tumor (7), needs early, accurate identification and timely treatment (8). However, most TC especially PTC differentiates well and has a low risk of becoming malignant. Thus, it is necessary to adjust the treatment plan according to the specific situation to avoid overtreatments and identify controllable risk factors to conduct preventive programs. Risk factors including radiation exposure, dietary nutrition, BMI (9), metabolic syndrome (10), environmental pollutants, family history of thyroid nodules, and overdiagnosis have been reported (11). Despite overdiagnosis, environmental/lifestyle factors do contribute to some increase in TC prevalence (12–16).

Dietary iodine intake has also been speculated as a risk factor that may influence the occurrence and development of PTC (17), but inconsistencies in research results have led to great controversy throughout these years. Iodine is a crucial micronutrient and a vital composition for the biosynthesis of thyroid hormone which plays a part in various biochemical and metabolic pathways throughout the human body (18, 19). The thyroid can maintain normal function, and keep thyroid hormone and thyroid stimulating hormone (TSH) in an appropriate ratio through automatic regulation even though daily iodine intake fluctuates widely. A U curve has been come up by many studies (20–22), both chronic iodine deficiency and excess can lead to thyroid dysfunction by interfering with homeostasis (23, 24), which means that the dose-based effects of iodine nutrition on the prevalence of TC need to be considered.

At the population level, the main sources of iodine intake include salt (25), water, milk, and seaweed (26, 27). The thyroid gland actively uptakes about 120 μg of iodine per day, which is distributed to a reservoir in the thyroid that contains about 5,000–10,000 μg of iodine. Monoiodothyronine and diiodothyronine are deiodinated in the periphery and T4 is converted to T3, resulting in the return of 60 μg of iodine per day to the external thyroid reservoir. Approximately 110 μg of iodine (about 97% of daily intake) is excreted in the urine, preserving the normal daily equilibrium (28). Thus urinary iodine concentration (UIC) has been considered a sensitive indicator of recent iodine intake (29–31). The status of iodine nutrition can be divided into four stages based on UIC according to WHO iodine recommendations: UIC $<$ 100 μg/L (insufficient), 100–199 μg/L (adequate), 200–299 μg/L (above requirements), and $\geq$ 300 μg/L (excessive) (32, 33). To adjust for the influences from dilution of the urine, the proportion of urinary I/Cr is also used to evaluate the iodine status (30). Creatinine-adjusted UIC: $<$ 85 μg/g Cr (deficiency), 85–219 μg/g Cr (adequate), and $\geq$ 220 μg/g Cr (excessive). So as a satisfying bioindicator of the iodine level (34, 35), UIC and urinary I/Cr ratio have been examined in various epidemiological studies aimed to clarify the association between iodine intake and PTC risk.

Therefore, we overview the standpoints from relevant epidemiological studies and experiments to clarify the correlations between iodine nutrition and PTC.

Epidemiological studies

Effect of iodine intake on thyroid cancer

Mandatory universal salt iodization (USI) has been put into practice since the 1990s (33), which meets the iodine requirements and gained notable success in preventing iodine deficiency in the general population (36). The ensuing question is whether iodine affects the onset of TC. Numerous epidemiological researches have evaluated the relationship between iodine intake and TC, and have presented a variety of views. Although some studies supported that there is no clear association between iodine nutrition and TC (37–39), a large number of studies these years have provided evidence for the relationship. Most studies were affected by many factors such as ethnic differences, diet customs (23), lifestyle, complications, and other environmental factors which can influence the development of TC. For example, the occurrence rate of TC was increased in two areas of high iodine intake: Iceland (40) and Hawaii (41). However, the natural radiation here is higher than in many other areas, so the radiation here can also drive the development of TC, especially in childhood (42, 43). Therefore, these studies cannot offer persuasive evidence to prove that high iodine intake can be a hazardous factor for TC.

Credible evidence was also presented in some epidemiological studies. A 1992 study evaluated the prevalence of TC in patients with goiter in iodine excess and iodine deficiency areas (44), which proved that individuals with excessive iodine intake had a considerably higher risk of TC than individuals in iodine deficiency regions. What's more, our epidemiological study in 2006 also investigated thyroid diseases over 5 years in three representative regions with insufficient, normal, and excessive iodine intake (45). No cases of TC were diagnosed in insufficient and normal iodine supplementation areas at baseline; however, 10 subjects were found to have PTC in Huanghua, one region with excessive intake of iodine. Between 1999 and 2004, 13 cases of PTC were found in Huanghua but none were identified in the other two areas. A retrospective analysis of the association between daily iodized salt intake and TC conducted by another research group in Hunan province also showed that consuming more than 5 g of iodized salt daily increased the risk of TC (46). Consistent with the above conclusions, a study examining TC trends in populations from three different geographic areas in Thailand between 1990 and 2009 showed an increase in PTC

prevalence and a decrease in FTC prevalence as population iodine deficiency levels declined (47).

At the same time, several studies presented other viewpoints. Some studies supported the protective effect of iodine intake on TC risk (48). For example, an ecological study of epidemiology showed that low consumption of iodized salt with mild iodine deficiency may be responsible for the high prevalence of TC in Daishan Country (49). The results of a meta-analysis also suggested that dietary iodine has a protective effect on TC (50). The drawback, however, is the lack of data on iodine intake. French Polynesia, a mild iodine deficiency area, has one of the highest TC occurrence rates in the world, so iodine was suspected to play a part in this phenomenon. In 2012, a case-control study was conducted among the inhabitants of French Polynesia (51), which showed that in this region, higher consumption of seafood and an iodine-rich diet were associated with a reduced risk of TC. However, a limitation of this study that can not be ignored is that their iodine intake was calculated by the amount and composition of the participants' daily food intake, using a composition table established in metropolitan France, which may be unsuitable for French Polynesia. What's more, cooking or other factors also have an uncontrollable influence on the final iodine intake, it would be more accurate by measuring 24-h UIC.

What's more, previous studies have indicated the prevalence of thyroid diseases may raise with both insufficient and overmuch iodine intake (52, 53). A study in 2016 indicated that compared to patients with benign thyroid nodules, TC patients tended to be distributed in UIC < 300 μg/L and UIC ≥ 2500 μg/L, this suggested that UIC may be involved in predicting TC risk in patients with thyroid nodules (54). The study supported that both low and overmuch iodine intakes can be related to TC in the iodine-replete region, so there may exist a U-shaped relationship between iodine intake and TC. Further research in the future also ought to reveal the mechanism of how iodine works and help to guide iodine intake.

A retrospective study based on patients who underwent thyroidectomy at Peking Union Medical College Hospital (PUMCH) from 1986 to 2018 implied that PTC has become the predominant type in TC surgery after USI, while the proportion of other histological subtypes has remained stable during this period (55). Therefore, we focused on the effect of iodine levels on PTC.

Effect of iodine intake on papillary thyroid carcinoma

Current studies reflected that iodine intake has a significant impact on PTC though some studies did not agree with this conclusion (56). One study that followed TC prevalence before and after iodine prevention in Argentina shows that PTC patients increased significantly after iodine supplementation, PTC/FTC ratio also increased significantly (57). Therefore, it is speculated that high iodine intake may be associated with a high prevalence of PTC (58, 59).

The prevalence of thyroid diseases in Shenyang has also raised obviously with the iodine intake increased since USI was implemented in China in 1996 (60). The diagnosis of TC and the proportion of PTC raised notably, and the proportion of FTC and UTC reduced while the ratio of MTC was not changed after USI. This study did find a correlation between iodine intake and TC, especially PTC, but there are also advances in detection technology and overdiagnosis, which need to be further verified. UIC differences between patients with PTC and nodular goiter were not statistically significant in another study (61), while in female PTC patients, extremely excessive iodine intake was independently related to the increased tumor size. This study supported that high iodine intake may be associated with the increase of tumor volume rather than its oncogenesis. Contrary to the above conclusions, a study conducted in a multiethnic group, investigated dietary iodine exposure among TC women in the San Francisco Bay area and women in the general population and concluded that an increase in dietary iodine is most likely associated with a reduced risk of PTC in those "low-risk" women (women with no risk factors) (62).

Iodine intake and combined factors

Nowadays, some studies have indicated that the combined effect of iodine and other factors plays a certain role in the occurrence and development of PTC. For example, Bisphenol A (BPA) is a kind of organic material that is widely applied to manufacturing processes (63). It has been reported that as an effective endocrine disruptor, free BPA can inhibit the expression of thyroid hormone-regulated genes by binding to thyroid hormone receptors (64). One study investigated whether BPA levels and excessive iodine intake were linked to PTC (65). The results indicated that the PTC groups' UIC and Urinary BPA concentrations (UBC) were higher than those in the control group, which suggested that high levels of UBC and iodine intake may be the predictive factors for PTC. What's more, BPA and iodine may interact with each other through some common pathways in the process of the occurrence and development of PTC.

A 2020 study tested UIC and thyroid function in patients with PTC, patients with benign thyroid tumors, and healthy individuals (66). The median UIC of the PTC and benign thyroid tumor group was markedly higher than that of healthy control groups. The regression analysis in this study also indicated that thyroglobulin antibody (TgAb) was an independent risk factor for PTC (67). What's more,

the association between TgAb and UIC was noteworthy, indicating that excessive iodine in patients with thyroid tumors may affect TgAb, which may contribute to the development of thyroid damage and subsequent malignancy (such as PTC) (68). Another case-control study in 2021 evaluated the cooperative effect of iodine intake and thyroid function on the risk of developing PTC and papillary thyroid microcarcinoma (PTMC) (69), indicating that excessive iodine intake using creatinine-adjusted UIC and high free T4 levels may have a synergistic effect on PTC and PTMC. Therefore, it is of interest to consider thyroid function in addition to iodine intake to predict the risk of PTC and PTMC. This also suggests that the combined effect of UIC and hormones on PTC risk needs to be verified in future larger studies.

Iodine intake and lymphatic metastasis in papillary thyroid carcinoma

A study in 2014 assessed the median urine iodine (MUI) of participants in Qingdao (70) and found that patients with benign thyroid nodules (MUI = 331.33 μg/l) and patients with PTC (MUI = 466.23 μg/l) had higher iodine intake than people in the control (MUI = 174.30 μg/l), which was in the iodine-replete region. In terms of MUI level, PTC patients with lymph node metastasis were higher than PTC patients without lymph node metastasis. The clinical data of 359 PTC patients who underwent surgical treatment in PUMCH from May 2015 to November 2020 were retrospectively analyzed (71). Consistent with the conclusions of previous studies, they demonstrated that low iodine was a protective factor for central lymph node metastasis in PTC, which indicated that iodine may not only be a promoter of tumorigenesis, but also a predictive factor for the aggressiveness of PTC. Another study also raised the point that high iodine intake does not seem to be a trigger, but may be a weak promoter for PTC progression in women patients, which needs further validation (72). The above data are consistent with most epidemiological studies that show an association between high iodine intake and PTC and its aggressiveness.

Iodine intake and BRAF mutation in papillary thyroid carcinoma

The familiar PTC mutation types include BRAF mutation, RET rearrangement, and RAS mutation. Among these alterations, BRAF mutations occur most frequently in PTC (73–75). Some studies proved that the BRAF V600E mutation plays a part in the biological behaviors of PTMC ($\leq$ 1 cm) and small PTC (1–1.5 cm) (76). However, the correlation between these alterations and iodine intake remains controversial (77). Kowalska's institution diagnosed an increased prevalence of BRAFV600E alterations in PTC, then they speculated that changes in iodine intake might contribute to the increased prevalence of TC (78). To clarify the above perspective, Guan and her team (79) assessed and compared the prevalence of the T1799A BRAF mutation in 1,032 PTC patients from five areas with different dietary iodine content in China. This study indicated that the frequency of BRAF mutation and the tumorigenesis of PTC are cogently associated with high iodine intake. The BRAF mutation was also confirmed to be a prognostic marker of PTC. Another study in Korea also investigated the correlation between iodine intake and BRAF mutation in PTC patients (80). BRAF mutation was the lowest in the 300–499 μg/L UIC group, which was different from that in the rarely low iodine intake (UIC $<$ 300 μg/L) and excessive iodine intake (UIC $\geq$ 500 μg/L) groups, confirming that UIC can be used as the predictor of BRAF mutation in PTC. Their results verified the U-shaped curves again.

Some studies hold contrary views. For instance, one 2016 study conducted molecular analyses of two differentiated TCs, PTC, and FTC in two countries with different iodine intake: the iodine-rich country (Japan) and the iodine-poor country (Vietnam) (77). Their study indicated that there was no difference in genetic mutations between patients from iodine-rich and iodine-poor countries, the conclusion may support that iodine status does not influence the genetic changes of PTC and FTC. Another study also investigated the iodine intake of PTC patients with or without BRAFV600E mutation and that of healthy participants in 2018 (81). Though their results indicated that iodine status differs significantly between PTC patients and healthy participants, the correlation between iodine status and BRAF alteration was not statistically significant.

Many epidemiological studies and meta-analyses showed inconsonant conclusions because of dietary information bias, measurement error, and differences in ethnic groups and regions. It is also uncertain whether there is publication bias (82) or other factors influencing thyroid carcinogenesis (83). So definitive epidemiological studies are still warranted in the future.

In vitro studies

Most of the current studies focused on epidemiological investigation, the molecular biological effect of iodine promoting PTC is unclear until now. Here we review the mechanism of iodine-induced biological behavior of PTC cells. Studies have supported the protective function of excessive iodine on thyroid follicular cells through specific pathways. For instance, RET, a proto-oncogene involved in the carcinogenesis of PTC, can be activated by the fusion of the tyrosine kinase domain with the 5′ region of another gene. This process can produce chimeric products, collectively known as RET/PTC (84–86), leading to the activation of the MAPK pathway, which plays a part in driving PTC. So a study once evaluated the effect

of high iodine concentrations on RET/PTC3-activated thyroid cells and indicated an antioncogenic role for excess iodine during thyroid oncogenic activation (87). Consistent with this viewpoint, another study in 2014 cultured thyroid follicular cells with doxycycline for 2 days, with or without 10 μM sodium iodide (88), then they found that high iodine inhibited miR-19, the newly discovered regulator of Smad4, which was activated by BRAFV600E, and restored the response to TGF-β signaling *via* the Notch pathway. This study indicated that iodine has a protective influence on thyroid cells, alleviating microRNA deregulation mediated by the BRAF oncogene, which contributes to the understanding of the physiological role of iodine on PTC. In addition, a recent study found that BRAF kinase can induce autophagy in PTC cells to participate in anti-apoptosis, and promote cell proliferation and migration under high iodine concentration (89), which support the view that high concentration of iodine can inhibit cell proliferation and promote cell apoptosis and migration.

Other studies have shown that excess iodine has adverse effects on thyroid cells. A study found that with a high iodine treatment, the miR-422a/MAPK1 pathway was complicated in the procedures of cell migration and proliferation, thus regulating tumorigenesis (90). With a high iodine concentration (100 μM), the MAPK1 signaling pathway was activated significantly in thyroid follicular epithelial cells, which means that in normal thyroid cells, high iodine may lead to the imbalance of the miR-422a/MAPK1 pathway. Considering that they only conducted functional experiments in two iodine concentrations, more studies are in the future.

Several studies have found that iodine has a double influence on thyroid cells' behaviors, depending on the iodine concentration. A study assessed the influences of different iodine concentrations on the proliferation and migration of two well-differentiated thyroid cell lines *in vitro* (91). The results supported that when iodine concentration was at a certain level, it could play a role in promoting the proliferation of thyroid cells. Iodine under 1.0×10^{-3} mM promotes the growth of thyroid cells while iodine higher than this concentration has the opposite effect. Besides, the mRNA level of VEGF-A was up-regulated in thyroid cells cultured in low iodine concentration (1.0×10^{-5}, 1.0×10^{-4}, and 1.0×10^{-3} mM) and down-regulated in thyroid cells cultured in high iodine concentration (1.0×10^{-2} and 1.0×10^{-1} mM), which indicated that the Akt, Erk, and the cytokine VEGF-A are the important mechanisms. However, iodine concentration in the human thyroid is usually from 1.0×10^{-6} to 1.0×10^{-5} mM, so in the human body, the high level of iodine intake may promote the proliferation and migration of PTC cells. Another study

TABLE 1 Characteristics of the studies included in this review.

	First author	Publication year	Study period (year/month)	Location	Sample size (*n*)	Research type
Prevalence	Belfior et al. (44)	1992	1980–1990	Italy	5637	Retrospective study
	Teng et al. (45)	2006	1999–2004	China	3018	Prospective study
	Wang et al. (46)	2021	2017/01-2019/12	China	51637	Retrospective study
	Mitro et al. (47)	2016	2001–2009	Thailand	2749	Prospective study
	Zhang et al. (49)	2019	2014–2018	China	2495	Prospective study
	Clero et al. (51)	2012	1979–2004	France	600	Retrospective study
	Kim et al. (54)	2016	2010/11-2013/05	Korea	1170	Retrospective study
	Zeng et al. (55)	2020	1986–2018	China	34213	Retrospective study
	Dong et al. (60)	2013	1992/01-2009/12	China	1239	Prospective study
	Zhao et al. (61)	2017	2013/11-2015/03	China	2041	Retrospective study
	Horn-Ross et al. (62)	2001	1992–1998	America	1166	Retrospective study
	Zhou et al. (65)	2017	2013/02-2013/09	China	261	Retrospective study
	Hou et al. (66)	2020	2017/01-2019/03	China	506	Retrospective study
	Kim et al. (69)	2021	2010/04-2014/12	Korea	946	Retrospective study
Lymphatic metastasis	Wang et al. (70)	2014	2010/06-2011/06	China	460	Retrospective study
	Zeng et al. (71)	2021	2015/05-2020/11	China	359	Retrospective study
	Zhao et al. (72)	2019	2013/11-2018/02	China	4040	Retrospective study
BRAF mutation	Vuong et al. (77)	2016	2006–2014	Japan	194	Retrospective study
	Kowalska et al. (78)	2016	2000–2013	Poland	723	Retrospective study
	Guan et al. (79)	2009	–	China	1032	Cross-sectional study
	Kim et al. (80)	2017	2010/11-2015/03	Korea	215	Retrospective study
	Lee et al. (81)	2017	2015/03-2015/12	Korea	300	Retrospective study

in 2019 also illustrated this dual effect, they investigated how iodine affected the physiological features of TC cells *in vitro*, including proliferation and apoptosis (92). Compared with the control group, extra-high doses of iodine (1.0×10^{-3} mol/l) inhibited cell proliferation and promoted cell apoptosis, while extra-low doses of iodine (1.0×10^{-4}–1.0×10^{-8} mol/l) showed opposing effects. Their results also indicated that the level of SPANXA1 was increased in cells treated with a certain concentration of iodine. The SPANXA1 (93) can also be one of the key genes, which enhanced the process of tumor growth in cells treated with an extra-low dose of iodine. Cell proliferation can be promoted by high expression of SPANXA1 while cell apoptosis can be inhibited by SPANXA1. In addition, PI3K/AKT was supposed to be a key signaling pathway through which SPANXA1 mediates its effects. Thus, SPANXA1 can be a biomarker in PTC and help in guiding dietary plans for patients with TC, which remind us that patients' iodine intake should be restricted.

Though these studies suggested some possible mechanisms for how iodine affected thyroid carcinogenesis, many other confounding factors cannot be ruled out. The effects of iodine on PTC patients are also complex and influenced by many chemical agents *in vivo*, so it is hard to clarify the interaction and feedback mechanisms of so many hormones by conducting cell experiments. Therefore, more *in vivo* studies are needed to clarify the function and mechanism of iodine on PTC.

In vivo studies

Animal studies examining the effect of different levels of iodine intake on the development of PTC were still rare. But earlier studies have shown that the development of iodine deficiency can cause PTC. The long-term effects on the thyroid with low iodine intake were assessed in 98 Sherman albino female rats (94), iodine deficiency was shown to be attributed to the production of tumors in thyroid glands. One study also found that iodine deficiency can cause goiter, hyperplasia, or malignant change as iodine deficiency time goes on (95), which also speculated that iodine deficiency can lead to reduced thyroid hormone synthesis, while the increased TSH drove chronic overstimulation of the thyroid. Proliferating thyroid cells, meanwhile, can also be more susceptible to radiation, chemical carcinogens, and oxidative stress, so more genetic mutations will show up in these cells. In addition, thyroid hyperplasia caused by insufficient iodine can lead to the change of chromosomes in the thyroid and increase the number of aneuploid cells in rats (96). Therefore, it is speculated that chronic stimulation in iodine deficiency may be one of the vital mechanisms of PTC. However, another study proved the U-shaped relationship by investigating the influence of iodine intake on p14ARF and p16INK4a expression of PTC in rats (97). This study suggested that both low and high iodine intake can decrease the expression of p14ARF and p16INK4a and drive tumor development.

The association between human iodine intake and PTC still cannot be explained because iodine deficiency or excess is much more severe in most animal models than in the human diet.

Discussion

Over these years, the occurrence rate of TC, especially PTC is increasing significantly in the world (1). Although overdiagnosis has been reported to increase the prevalence of PTC (98), there has also been a true increase. It is therefore meaningful to illustrate the role of these suspected risk factors, especially iodine intake. TC has been reported in iodine deficiency areas in earlier years. The prevalence of PTC also increased after iodine intake increased due to salt iodization (99) and iodine supplementation. In contrast, iodine was a protective factor for PTC in some studies, which lead to controversy about the correlation between iodine intake and PTC. Previous epidemiologic studies' results could be influenced by different test standards, study methods, dietary habits, measurement errors, information bias or so many other factors. For example, it is difficult to measure 24-h UIC, the gold standard for iodine intake (31). Therefore, some studies may use random spot urine UIC as an alternative indicator. While the studies *in vitro* or *in vivo* cannot reflect the true human iodine status, the evidence is far from sufficient. Hence it is still unclear the true iodine interval that directly induces PTC development, or indirectly contributes to PTC risk through interaction with other factors.

Many previous studies have addressed this controversial issue, showing that different iodine nutritional status has different effects on the development of PTC. We discuss their views in this article and summarize the basic information in **Table 1** to help readers think objectively. Taking the present studies into consideration, we speculate that the relationship between iodine nutrition and PTC may be intricate and the effect of iodine should be considered dose-based. Besides, the combined action of more variables are ought to be considered in the research of iodine and PTC.

The relationship between iodine and PTC is complex, we are still unclear about the specific role of iodine, let alone the mechanism of its activities due to the disagreement of current research results. The present studies have yielded mixed results which indicated that iodine intake may influence the development or progression of PTC, change the proportion of several subtypes of TC in the crowd, or affect the invasiveness of PTC especially lymphatic metastasis and BRAF mutation. These data provide further evidence supporting that it makes sense to achieve the appropriate level of iodine intake to satisfy the body's normal nutritional needs while avoiding either deficient or excessive iodine supplementation.

Author contributions

WT supervised the work. XZ drafted the manuscript. FZ provided the major technical support. QL and CF assisted in the literature review. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Research Fund for Public Welfare, National Health and Family Planning Commission of China (Grant No. 201402005) and the Clinical Research Fund of Chinese Medical Association (Grant No. 15010010589).

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.

Publisher's note

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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 11 November 2022
DOI 10.3389/fnut.2022.1029793

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Nutrition, a section of the journal Frontiers in Nutrition

RECEIVED 27 August 2022
ACCEPTED 27 October 2022
PUBLISHED 11 November 2022

CITATION
Barakat H, Alshimali SI, Almutairi AS, Alkhurayji RI, Almutiri SM, Aljutaily T, Algheshairy RM, Alhomaid RM, Aljalis RA, Alkhidhr MF and Abdellatif AAH (2022) Antioxidative potential and ameliorative effects of green lentil (*Lens culinaris* M.) sprouts against CCl_4-induced oxidative stress in rats. *Front. Nutr.* 9:1029793. doi: 10.3389/fnut.2022.1029793

Antioxidative potential and ameliorative effects of green lentil (*Lens culinaris* M.) sprouts against CCl_4-induced oxidative stress in rats

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The present study is aimed to investigate the antioxidative potential and ameliorative effects of *Lens culinaris* Medikus sprouts hydroalcoholic extract (LSHE) on CCl_4-induced oxidative stress in rats. The research has been carried out in two successive stages. Firstly, the highest phenolic content and antioxidant activity of *L. culinaris* sprouts were assessed at 20 ± 1°C and 90–93% RH during sprouting. Total phenolic content (TPC), total carotenoids (TC), total flavonoids (TF), total flavonols (TFL), DPPH-RSA, and vitamin C contents of *L. culinaris* seeds and 6-days sprouts were determined. Subsequently, phenolics by HPLC analysis of *L. culinaris* seeds, 3rd and 6th-day sprouts were identified and quantified. Results indicated that 6th-day sprouts contained considerable phenolics with superior antioxidant capacity, thus selected to be examined for biological activity in a rat's module consisting of five groups. G1, normal rats orally received distilled water. G2 received 1.0 mL kg^{-1} of CCl_4 and olive oil (1:1) intraperitoneally (i.p.) twice a week. G3 received CCl_4 (i.p.) and 50 mg GAE kg^{-1} of LSHE daily/orally. G4 received CCl_4 (i.p.) 100 mg kg^{-1} of LSHE orally/daily. G5 (reference group) treated by intramuscular injection (i.m.) of vit. E+Selenium (Vit. E+Se, 50 mg kg^{-1} twice a week). The weight gain, relative weight of organs, hypoglycemic and hypolipidemic efficiencies, liver's and kidneys' functions, and antioxidant biomarkers were examined. LSHE enhanced the weight gain recovery % and significantly reduced fasting blood glucose. The hypolipidemic effect of LSHE was dramatically reduced triglycerides (TG), total cholesterol (CHO), high- and low-density lipoproteins (HDL-c and LDL-c), and very-low-density lipoproteins (VLDL-c). Administration of 50 and 100 LSHE mg kg^{-1} ameliorated liver and kidney function in dose-dependent manure. Intriguingly, LSHE considerably reduced

malondialdehyde (MDA) while significantly raising reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) in a dose-dependent manner. In conclusion, biochemical examinations confirmed the therapeutic efficacy of LSHE as a functional product. It encouraged us to recommend *L. culinaris* sprout production for attenuating hepatotoxicity and nephrotoxicity, as well as being beneficial and profitable for controlling oxidative stress complications.

KEYWORDS

***Lens culinaris*, sprouts, antioxidative potential, hepatoprotection, nephroprotection**

Introduction

An imbalance among the production of reactive oxygen species (ROS) and the body's detoxification or repair mechanisms is known as oxidative stress. When the cell's redox state is disturbed, toxic peroxides and free radicals are produced, harming lipids and DNA (1). DNA strands can be broken by oxidative stress, which causes underlying damage. Indirect base damage is caused by ROS, which generates the harmful free radicals. Because some reactive oxidative species serve as cellular messengers in redox signaling, oxidative stress can interfere with cellular communication. Oxidative stress is viewed as a potential factor in the development of cancer and Alzheimer's disease in humans (2), atherosclerosis (3), and depression (4). Researchers are looking for safe and effective plant-based bioactive inhibitory compounds to combat oxidative stress.

Pulses demonstrated a significant relationship between total polyphenol and tocopherol concentration and antioxidant activity (5, 6). The amount of polyphenol, carotenoid, and tocopherol bioactive in pulses varies depending on type and cultivar (5). Lentils are among the most nutrient-dense and health-beneficial foods, according to Faris et al. (6). It includes many necessary macronutrients, such as functional proteins and carbs, essential micronutrients, and bioactive phytochemicals. Recent classifications classify lentils as a preventive and therapeutic functional food (6, 7). As one of the top five superfoods, lentils (*L. culinaris*) have recently attracted more and more attention. In addition to being high in protein, they also contain significant amounts of dietary fiber, folate, iron, and potassium (8). The abilities of green lentil extracts to act as an antioxidant and as a radical scavenger were initially documented by Amarowicz et al. (9). Using HPLC-PAD and HPLC-ESI-MS methods, phenolics were also discovered in the crude extracts. Green lentils mainly included quercetin diglycoside, *trans-p*-coumaric acid, procyanidin dimers, catechin and epicatechin glucosides, quercetin, and epicatechin glucosides. Xu et al. (10) examined 11 samples of Lentils to determine their antioxidant capacities and four main phenolic groups, phenolic acids, anthocyanins, flavones, and flavan-3-ols—in each sample. Different cultivars of lentils showed considerable variations in their unique phenolic compounds and chemical and cellular antioxidant activities. The highest levels of total polyphenol and antioxidant activity were found in the large and small green lentils, while the split red lentil was among the cultivars of pulses with the least amount of antioxidant activity and the lowest overall polyphenol content (5). To make functional foods or nutraceuticals to enhance consumer health, food processors may use lentils with high phenolic content and antioxidant capabilities (7, 10, 11).

A remarkable variety of secondary metabolites, minerals, and bioactive substances found in lentils have shown promise in treating and preventing numerous chronic human diseases. *In vivo* and *in vivo* studies confirmed the positive correlation between their bioactive compounds, antioxidant capacity, and related health effects (5, 6, 11, 12). However, lentils' anticancer, hypoglycemic, hypocholesterolemic, and blood pressure-reducing properties and their potential to alleviate disease were examined (6, 7, 13). It has been claimed that lentil extracts have a variety of pharmacological effects both *in vivo* and *in vitro*, including antidiabetic, hypotensive, hypolipidemic, and cardioprotective effects (7). A study has also shown that the phenolic extract from lentils possesses direct ROS scavenging abilities (14, 15). According to Jung et al. (15), the phenolic extract of *L. culinaris* can serve as a possible source of nutraceuticals with hepatoprotective properties since it partially protects liver cells from oxidative stress by triggering the cellular

Abbreviations: ALT, Alanine aminotransferase; AOA, Antioxidant activity; AST, Aspartate aminotransferase; CAT, Catalase; CHO, Total cholesterol; DPPH, 1,1-diphenyl-2-picryl hydrazine; dw, Dry weight; GA, Gallic acid; GAE, Gallic acid equivalent; GSH, Reduced-glutathione; HDL-c, High-density lipoproteins; LDL-c, Low-density lipoproteins; LSHE, *Lens culinaris* Medikus sprouts hydroalcoholic extract; MDA, Malonaldehyde; QE, Quercetin equivalent; RSA, Radical scavenging activity; SE, Standard error; SOD, Superoxide dismutase; TBA, Thiobarbituric acid; TC, Total carotenoids; TE, Trolox equivalent; TF, Total flavonoids; TFL, Total flavonols; TG, Triglycerides; TPC, Total phenolic compounds; VLDL-c, Very Low-density lipoproteins.

antioxidant system. According to Goudarzi et al. (12), sodium arsenite (SA) can significantly increase oxidative stress while also depleting antioxidant reserves and blocking the actions of antioxidant enzymes. The oxidative hepatotoxicity caused by SA may be considerably reduced with red lentil extract (RLE). Its medicinal potential might be a cheap, secure, herbal antioxidative medication to treat SA toxicity.

Researchers struggle to improve the nutritional quality of lentils and ensure their abundant supply of bioactive phytochemicals that have health-promoting activity. Sprouts are a phytonutrient-rich vegetable food that is a good source of proteins, minerals, vitamins, flavonoids, polyphenols, glucosinolates, and isothiocyanates (16). Sprouting processes an applicable procedure to enhance phenolic content, antioxidant capacity, glycemic index, and potential bioaccessibility (17). Swieca et al. (18) confirmed that sprouting improved the nutraceutical value of lentil sprouts regarding their antioxidant potential. Interestingly, according to Reed et al. (19), sprouts are regarded as "functional foods," defined as foods with additional health-promoting or disease-prevention benefits to their primary nutritional value. Studies on sprouts' nutritional value, phytochemical makeup during production or storage, and investigations into their microbiological, bioactive, and technological aspects have been suggested (20). The nutritional advantages and sensory acceptance of food products created with other sprouts were recently reviewed in this context (21).

Until now, nobody has looked into the bio-changes in phytochemicals during lentil sprouting. Therefore, the TPC, AOA, TC, TF, TFL, and vitamin C, as well as HPLC analysis of phenolic compounds during sprouting, were studied first. Consequently, a hydroalcoholic extract of 6-days green lentil sprouts (LSHE) was prepared.

Secondly, the antioxidative potential, hepatoprotective, and nephroprotective efficacy of green lentil sprouts hydroalcoholic extract (LSHE) on CCl_4-induced oxidative stress were investigated.

Materials and methods

Raw lentil seeds

Green lentil seeds (*L. culinaris* Medikus) were purchased from the Al-Tamimi market (https://www.tamimimarkets.com) in Al Qassim region, K.S.A. Plant expert (Dr. Mokded Rabhi) from the College of Agriculture and Veterinary Medicine at Qassim University in K.S.A., carried out the plant's verification. The broken, sick, and dusty seeds were eliminated. Green lentil sprouts were made from cleaned seeds. Until they were used for analysis, raw or milled seeds (American model laboratory mill, model ES2097) were kept in freezer-plastic bags at a temperature of 18 ± 1°C.

Sprouting of *L. culinaris* and hydroalcoholic extract preparation

The seeds were sanitized into a solution of sodium hypochlorite (1%) for 3 min before sprouting in batches of 500 g. The seeds were evenly distributed on 7 × 35 cm plastic trays after being rinsed three times in sterilized distilled water (sd.H_2O). The seed germinator was then filled with the seeds. The germination procedure was done in an atomizer-equipped temperature-controlled seed germinator (Easygreen, Canada) with a relative humidity of 90–93% with faith light. The temperature of the germinator was kept at 20 ± 1°C. For the first 3 days, 10.0 mL of sd.H_2O/tray was sprayed onto lentil samples thrice daily. Beginning at the germination process and continuing for up to 6 days, appropriate samples were taken daily. Lentil sprouts were frozen overnight at −18 ± 1°C, then freeze-dried (CHRIST, Alpha 1-2 LD plus, Germany) for 96 h at −48°C under the pressure of 0.032 mbar. Freeze-dried sprouts were crumbled in a small mill (Thomas Wiley, USA) to obtain a homogenous powder, then kept in the dark containers at 4 ± 1°C for HPLC and phytochemical analysis. Lentil sprouts were individually germinated in identical settings for 6 days. Progressively, Lentil sprouts were dried using a 24-hdrying program according to Barakat et al. (22) and Al-Qabba et al. (23). The dried sprouts were milled, sieved, and kept under cold storage until extraction. Lentil sprouts hydroalcoholic extract (LSHE) was carried out by extracting about 500 g of lentil sprouts three times with 2,500 mL of 50% ethanol. The filtered extract was concentrated in a rotary evaporator at 40°C, then frozen overnight and freeze-dried (CHRIST, Alpha 1–2 LD plus, Germany) (24). Freeze-dried samples were powdered by porcelain morsel to make homogeneous powder which was kept under cooling conditions until used.

Determination of TPC, TC, TF, and TFL in *L. culinaris* seeds and sprouts

According to Yawadio Nsimba et al. (25), the TPC of *L. culinaris* seeds and sprouts was determined using the Folin-Ciocalteu reagent. In brief, a suitable sample was extracted with 70% methanol. Aliquots of clear supernatant were mixed with (1:10) diluted Folin-Ciocalteu reagent for 5 min before being stopped with Na_2CO_3 (7.5 %). The optical density (OD) was measured after 60 min and compared to the standard curve of Gallic acid (GA) solution ($R^2 = 0.99$), and the TPC content was expressed as milligrams of Gallic acid equivalents (GAE) per 100 g (mg of GAE 100 g^{-1} DW). For TC determination, 1 g of the freeze-dried sample was repeatedly extracted with a mixture of acetone and petroleum ether (1:1, v/v), according to Yuan et al. (26). The upper phase was collected, washed with water several times, and combined with crude extracts. The petroleum

ether will be added to the solution to prepare a known volume. The TC content was determined spectrophotometrically at 451 nm and expressed as mg 100 g^{-1} dw. The upper phase was collected, washed with water several times, and combined with crude extracts. The petroleum ether will be added to the solution to prepare a known volume. The TC content was determined spectrophotometrically at 451 nm and expressed as mg 100 g^{-1} dw. The TF content of *L. culinaris* seeds and sprouts using a methanolic extract was determined. Aliquots of clear extract were mixed with 2% $AlCl_3$, then measured after 60 min at 420 nm. The TFL content of *L. culinaris* seeds and sprouts was determined by combining aliquots of methanolic extracts with sodium acetate (5 %). After 5 min, $AlCl_3$ (2%) was added, and the OD was measured after 150 min at 440 nm, according to Mohdaly et al. (27). The content of TF and TFL were expressed as mg quercetin equivalent (QE) per g^{-1} (mg QE 100 g^{-1}).

Vitamin C and antioxidant capacity determination

The vitamin C content using the 2,6-dichloro phenol indophenol titrimetric method was determined according to Silva et al. (28); data were expressed as mg 100 g^{-1} fw. The radical scavenging activity (DPPH-RSA) of *L. culinaris* seeds and sprouts was examined spectrophotometrically according to Barakat and Rohn (29), and antiradical activity value was presented as μmol TE 100 g^{-1}.

Quantification of phenolic compounds in *L. culinaris* and its sprouts by HPLC-DAD

According to Kim et al. (30), using an HPLC system HP1100 (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler, quaternary pump, and diode array detector DAD, as well as an Altima C18, 5 × 150 mm, 4.6 mm ID column and an Altima C18, 5 mm guard column (Alltech), the phenolic compounds in *L. culinaris* and its sprouts were determined. At a flow rate of 1 mL min-1, 10 μl of the extracted samples were injected, and separation was carried out at 25°C. The gradient of acetic acid concentrations in the solvent system was A (acetic acid 2.5%), B (acetic acid 8%), and C (Acetonitrile 100%). For identification and quantification, each peak's retention times and mass spectra were compared to external standards and stored; then, phenolic compounds were expressed in mg Kg^{-1}.

Animals and experimental design

This study used Wistar rats (40 adult males) weighing 175–200 g. All the experiments received approval from the Institutional Animal Ethics Committee (IAEC) of QU, KSA (No. 21-18-09 on Thursday, May 19, 2022), Qassim University, SA. Under typical laboratory conditions, animals were housed in polypropylene cages with air conditioning and kept at 24°C. Rats were placed in new cages under controlled circumstances of 24°C, 40–45% relative humidity, and a 12-h light/dark cycle after being exposed to the environment for 10 days. Randomly, five groups of eight rats each were assigned to the groups. The rats' body weight (BW) was noted along with their identification labels. Rats were given a commercial standard pellet diet and unlimited access to water (31). The following procedures were used to treat the rats for six consecutive weeks. Group 1 (normal rats, NR) received 2 mL of distilled water orally/daily and an intraperitoneal injection (i.p.) of olive oil (1.0 mL kg^{-1}) twice a week. For oxidative stress and hepatotoxicity induction in experimental animals. Rats were administrated (i.p.) with a fresh solution of CCl_4 and olive oil (1:1) at a dose of 1.0 mL kg^{-1} twice a week and 2 ml of distilled water orally/daily (32). After 1 week, rats were randomly divided into four groups, eight each, and one of those groups was labeled as Group 2 and located and positive control. Group 3 received CCl_4 (i.p.) twice a week in addition to 50 mg GAE kg^{-1} of LSHE given orally daily. Group 4 received CCl_4 (i.p.) twice a week in addition to 100 mg kg^{-1} of LSHE administered orally daily. According to Asuku et al. (33) and Gaber et al. (34), Group 5 (reference group) received an intramuscular injection (i.m.) of Vit. E+Se (Selepherol, Vetoquinol Co., France) at 50 mg kg^{-1} twice a week and 2 mL distilled water orally daily. At the end of the 6th week, animals fasted for 12 h with free access to water. According to Leila et al. (35), rats were anesthetized. Blood was collected from the heart puncture and then treated to separate blood serum by centrifugation at 4,000 x g for 30 min for use in various biochemical measures. Appropriate kits and a blood chemistry analyzer (HumaLyzer 4000, Germany) were used to determine the biochemical parameters. Rats' liver, kidneys, and spleen were removed during the dissection of sacrificed animals. The following equation was used to determine the relative weight (RW) of the organs:

$$RW = \frac{\text{Weight of the organ}}{\text{Weight of the rat}} x100 \quad (1)$$

Determination of liver and kidney functions, lipid profile, and fasting blood glucose level

Alanine aminotransferase (ALT, UL^{-1}), aspartate aminotransferase (AST, UL^{-1}), alkaline phosphatase (ALP, UL^{-1}), and total bilirubin (T. Bili, mg dL^{-1}) in blood serum were measured using specific and approved kits following the manufacturing instructions. According to the manufacturer's instructions, kidney functions such as albumin (g dL^{-1}), total protein (T. Protein, g dL^{-1}), urea (mg dL^{-1}), and

creatinine (mg dL^{-1}) concentrations were measured. Albumin concentrations were subtracted from T. Protein concentrations to calculate globulin (g dL^{-1}). Dividing the urea concentration by 0.47, blood urea nitrogen (BUN, mg dL^{-1}) was calculated. All biochemical test kits were bought from Human Co. in Wiesbaden, Germany. According to Nwagha et al. (36), the atherogenic index (AI) was calculated. An enzymatic colorimetric test kit was used to determine fasting blood glucose (mg dL^{-1}). High-density lipoproteins (HDL-c, mg dL^{-1}) and total cholesterol (CHO, mg dL^{-1}), and triglycerides (TG, mg dL^{-1}), according to manufacturer instructions, were examined. According to Friedewald et al. (37), low-density lipoproteins (LDL, mg dL^{-1}) and very-low-density lipoproteins (VLDL, mg dL^{-1}) were mathematically calculated.

Oxidative stress biomarkers

According to the described method by Beutler et al. (38), reduced-glutathione (GSH, μg dL^{-1}) was determined. According to Ohkawa et al. (39), lipid peroxidation was evaluated by detecting thiobarbituric acid reactive substance (TBARS), and the measured malondialdehyde (MDA) concentration was expressed as nmol mL^{-1}. Superoxide dismutase (SOD, U L^{-1}) activity was determined following the protocol of Giannopolitis and Ries (40). Catalase (CAT, U L-1) activity was assessed using the Aebi technique (41). All oxidative-stress biomarkers were determined using a blood chemistry analyzer (HumaLyzer 4000, Germany).

Data analysis

The SPSS (Ver. 22.0 for Windows) was used to conduct the statistical analysis. According to Steel et al. (42), one-way ANOVA was used to assess the statistical significance, *p*-values of 0.05 were used for the *post-hoc* test, and means of the experimental results and standard error were presented.

Results

The phytochemicals and antioxidant activity of *L. culinaris* sprouts

The quantitative analysis of phytochemicals such as TPC, TC, TF, and TFL, as well as related antioxidant activity using DPPH radical scavenging and vitamin C content in *L. culinaris* sprouts, was performed. The TPC content of green lentil seeds (GLS) was 379.76 mg GAE 100 g^{-1}, as illustrated in Table 1. The TC content of GLS was 14.15 g 100 g^{-1}. Both TF and TFL contents in GLS were 16.32 and 11.17 mg QE 100 g^{-1}, respectively. Furthermore, the development of antioxidant activity was tracked using DPPH-RSA. The results showed 479.42 mol of TE 100 g^{-1} in GLS. The vitamin C content of GLS was 42.91 mg 100 g^{-1}. On the 6th day, significant increases in TPC, TC, TF, and TFL, as well as Vit. C were observed.

Quantification of phenolic compounds in *L. culinaris* seeds and sprouts

The quantitative analysis of phenolics in extracts of *L. culinaris* seeds and sprouts was carried out; the data are illustrated in Table 2. Thirteen separated phenolic acids and five flavonoids were identified in GLS and its sprouts in detectable amounts. The most abundant phenolics were *p*-Hydroxy benzoic acid (71.34 mg Kg^{-1}), followed by *p*-coumaric acid (38.46 mg Kg^{-1}) and Vanillic acid (21.40 mg Kg^{-1}). The GLS is abundant in TF content, as demonstrated in Table 2. TF such as Naringenin (112.62 mg Kg^{-1}) and Quercetin (35.29 mg Kg^{-1}) were identified and found in higher amounts, followed by Myricetin (28.58 mg Kg^{-1}), Resveratrol (19.00 mg Kg^{-1}) and Kaempferol (15.27 mg Kg^{-1}). On the 3rd day of sprouting, Benzoic acid, Rosmarinic acid, and Syringic acid as phenolic acids were presented in 42.73, 28.48, and 28.48 mg Kg^{-1}, respectively. Similarly, new flavonoids have been shown in reasonable amounts with an increase in detected flavonoids in GLS except for Naringenin. Rutin and Catechin were quantified at 50.02 and 4.19 mg Kg^{-1} in the 3rd day's sprouts. Seven flavonoids, eleven phenolic acids, and their derivatives were identified and measured on the 6-day. The most abundant phenolic acids were Rosmarinic acid, *p*-Hydroxy benzoic acid, and *p*-coumaric acid. In contrast, the most abundant flavonoids were Kaempferol, followed by Myricetin, Naringenin, Resveratrol, Quercetin, Rutin, and Catechin. On the 6th day, phenolic acids and flavonoids were remarkably increased, whereas the flavonoids recorded increases higher than phenolic acids and their derivatives.

The weight gain, the RW of organs, and hypoglycemic efficiency

The weight gain, RW of organs, and hypoglycemic efficiency of GLR extracts in CCl_4-induced oxidative stress and hepatotoxicity in rats were scrutinized; data are presented in Table 3. Injection of CCl4 directly affected the rats' weight during the 1st week; however, virtually little weight gain was observed in G2 rats in the 6th week. The most efficient treatment in recovering rats' weight was administering 100 mg GAE Kg^{-1} LSHE compared with G1 or G5 at the end of the 6th week. LSHE significantly improved weight gain associatively in a dose-dependent manner. For organs' relative weight, the injected group exhibited significant increases in organs' weight.

TABLE 1 Total phenolic, carotenoids, flavonoids, flavonols contents, and potential antioxidant capacities of *L. culinaris* during sprouting at 20 ± 1°C and 90–93% RH (mean ± SE), $n = 6$.

Item	Sprouting period (day)						
	0	1	2	3	4	5	6
TPC (mg meq GAE 100 g^{-1})	379.76 ± 14.15^{f}	461.78 ± 20.82^{e}	557.55 ± 11.89^{d}	620.45 ± 14.66^{c}	678.89 ± 17.85^{b}	770.87 ± 6.36^{a}	788.78 ± 5.26^{a}
TC (μg 100 g^{-1})	13.00 ± 0.83^{d}	10.89 ± 0.28^{d}	16.47 ± 0.88^{c}	19.71 ± 0.20^{b}	21.15 ± 0.63^{b}	24.48 ± 1.16^{a}	25.17 ± 1.80^{a}
TF (mg QE 100 g^{-1})	16.32 ± 4.38^{d}	20.43 ± 6.45^{cd}	39.47 ± 7.42^{bc}	52.91 ± 4.31^{ab}	56.88 ± 9.50^{ab}	64.89 ± 10.93^{a}	52.40 ± 5.42^{ab}
TFL (mg QE 100 g^{-1})	11.17 ± 3.64^{b}	14.41 ± 4.50^{b}	17.40 ± 1.62^{b}	36.28 ± 6.75^{a}	38.30 ± 3.65^{a}	44.42 ± 5.85^{a}	45.59 ± 2.29^{a}
DPPH (μmol of TE 100 g^{-1})	479.42 ± 25.39^{c}	515.19 ± 27.58^{bc}	547.04 ± 37.76^{bc}	565.01 ± 22.14^{bc}	594.24 ± 35.74^{bc}	679.57 ± 31.31^{a}	539.73 ± 45.21^{ab}
Vitamin C (mg 100 g^{-1})	42.91 ± 1.10^{e}	40.76 ± 1.04^{e}	60.07 ± 1.54^{d}	72.94 ± 1.87^{c}	92.25 ± 2.36^{b}	93.75 ± 2.40^{b}	122.54 ± 3.14^{a}

TPC, Total phenolic content; TC, Total carotenoids; TF, Total flavonoids; TFL, Total flavonols; DPPH, Antioxidant activity using DPPH assay; RH, Relative humidity; ^{a,b,c,d,e,f}, Means with the same superscripted letters in the same row are not statistically different at $p < 0.05$.

TABLE 2 Quantitative analysis of phenolic compounds in *L. culinaris* during sprouting at 20±1 °C and 90–93% RH (mean ± SE), $n = 3$.

Item	No.	Compound	Phenolics (mg Kg^{-1}) *		
			Sprouting period (day)		
			0	3	6
Phenolic acids	1	Pyrogallol		–	
	2	Quinol	–	–	–
	3	3–Hydroxytyrosol catechol	–	–	–
	4	*p*–Hydroxy benzoic acid	71.34 ± 3.24	82.05 ± 1.10	73.93 ± 2.57
	5	Caffeic acid	1.25 ± 0.24	2.70 ± 0.87	3.34 ± 1.24
	6	Chlorogenic acid	2.16 ± 0.29	2.56 ± 0.19	4.19 ± 0.54
	7	Cinnamic acid	0.69 ± 0.12	0.41 ± 0.21	0.28 ± 0.08
	8	Ellagic acid	–	–	–
	9	Vanillic acid	21.40 ± 0.21	0.91 ± 0.14	59.20 ± 4.25
	10	Ferulic acid	6.45 ± 1.02	2.96 ± 0.85	10.32 ± 1.98
	11	Gallic acid	–	–	–
	12	*O* – coumaric acid	7.61 ± 0.97	6.33 ± 0.71	5.14 ± 0.26
	13	*p*–coumaric acid	38.46 ± 2.18	76.61 ± 3.48	59.75 ± 6.18
	14	Benzoic acid	–	42.73 ± 2.98	61.48 ± 2.78
	15	Rosmarinic acid	–	28.48 ± 1.97	114.88 ± 5.19
	16	Syringic acid	–	3.83 ± 0.58	5.19 ± 0.79
Flavonoids	1	Catechin	–	4.19 ± 0.25	14.90 ± 2.97
	2	Kaempferol	15.27 ± 1.57	166.86 ± 6.27	4439.54 ± 10.24
	3	Myricetin	28.58 ± 1.25	134.72 ± 4.97	224.16 ± 5.27
	4	Quercetin	35.29 ± 2.21	41.89 ± 3.19	54.12 ± 5.27
	5	Rutin	–	50.02 ± 4.87	39.01 ± 5.02
	6	Resveratrol	19.00 ± 2.75	54.62 ± 2.97	80.64 ± 3.97
	7	Naringenin	112.62 ± 4.21	89.78 ± 6.12	142.57 ± 8.02

*: Phenolic acids were identified at 280 nm, and flavonoids were identified at 365 nm, Not detected.

Treating rats with LSHE or Vit. E+Se showed a positive attenuation. After the 6th week, LSHE at 50 or 100 mg GAE Kg^{-1} exhibited a potent efficacy in reducing fasting blood glucose but not better Vit. E+Se at 50 mg Kg^{-1}, as shown in Table 3.

The hypolipidemic efficiency

The hypolipidemic efficiency of LSHE at 50 and 100 mg GAE Kg^{-1} and Vit. E+Se at 50 mg kg^{-1} on CCl_4-induced oxidative stress and hepatotoxicity in rats were determined;

10.3389/fnut.2022.1029793

TABLE 3 Effect of *L. culinaris* sprouts hydroalcoholic extract on weight gain%, organs' weight, and FBG in CCl_4-induced oxidative stress and hepatotoxicity in rats (mean ± SE), $n = 8$.

Groups	Weight gain %		Organs' relative weight (%)			FBG
	3rd weak	6th weak	Liver	Kidneys	Spleen	
G1	31.41 ± 2.35^{a}	44.23 ± 3.27^{a}	3.24 ± 0.02^{b}	0.79 ± 0.04^{a}	0.39 ± 0.02^{a}	78.43 ± 4.63^{b}
G2	-0.33 ± 0.24^{d}	3.63 ± 1.25^{d}	3.71 ± 0.10^{a}	0.84 ± 0.02^{a}	0.42 ± 0.01^{a}	133.02 ± 7.34^{a}
G3	16.08 ± 3.24^{c}	22.64 ± 2.48^{c}	3.24 ± 0.02^{b}	0.67 ± 0.02^{b}	0.33 ± 0.01^{b}	106.82 ± 6.00^{b}
G4	23.67 ± 2.87^{b}	36.78 ± 4.28^{b}	2.91 ± 0.15^{b}	0.66 ± 0.03^{b}	0.33 ± 0.01^{b}	94.25 ± 5.38^{b}
G5	26.59 ± 1.49^{b}	36.82 ± 3.98^{b}	3.15 ± 0.10^{b}	0.78 ± 0.01^{a}	0.39 ± 0.01^{a}	84.49 ± 5.49^{b}

G1–G5, Experimental groups see materials and methods; section 2.6, FBG, Fasting blood glucose, ^{a,b,c,d}: Means with the same superscripted letters in the same column are not statistically different at $p < 0.05$.

TABLE 4 Effect of hydroalcoholic extract of *L. culinaris* sprouts on lipid profile and Atherogenic index in CCl_4-induced oxidative stress and hepatotoxicity in rats (mean ± SE), $n = 8$.

Groups	Lipid profile parameters					
	TG	CHO	HDL-c	LDL-c	VLDL-c	AI
G1	85.5 ± 1.80^{bc}	102.30 ± 10.34^{b}	39.29 ± 02.80^{bc}	45.92 ± 11.67^{b}	17.10 ± 0.36^{bc}	0.34 ± 0.02^{b}
G2	137.19 ± 4.4^{a}	170.87 ± 7.71^{a}	33.21 ± 2.58^{b}	110.22 ± 8.51^{a}	27.44 ± 0.88^{a}	0.62 ± 0.04^{a}
G3	92.52 ± 1.94^{b}	114.29 ± 4.61^{b}	37.50 ± 0.82^{b}	58.29 ± 4.37^{b}	18.51 ± 0.39^{b}	0.39 ± 0.03^{b}
G4	$89.94 \pm 3.28b^{c}$	103.29 ± 5.74^{b}	47.14 ± 1.05^{a}	38.16 ± 5.42^{b}	17.99 ± 0.66^{bc}	0.28 ± 0.01^{c}
G5	80.19 ± 2.26^{c}	99.78 ± 4.47^{b}	44.76 ± 2.03^{a}	38.97 ± 5.45^{b}	16.04 ± 0.45^{c}	0.25 ± 0.05^{c}

G1–G5, Experimental groups see materials and methods; TG, Triglycerides; CHO, total cholesterols; HDL-c, High-density lipoprotein-cholesterols; LDL-c, Low-density lipoprotein-cholesterols; VLDL-c, Very low-density lipoprotein-cholesterols; AI, Atherogenic index, ^{a,b,c}: Means with the same superscripted letters in the same column are not statistically different at $p < 0.05$.

data are shown in Table 4. The CCl_4-induced oxidative stress and hepatotoxicity in rats were observed to significantly raise the levels of TG, CHO, LDL-c, and VLDL-c. However, CCl_4 injection resulted in significantly lower HDL-c levels than in control rats (G1). The lipid profile was improved in dose-dependent manure by administering LSHE at 50 or 100 mg GAE Kg^{-1}. The most effective therapy for enhancing the blood profile was LSHE with 100 mg GAE Kg^{-1}, which showed no significance when compared to G1 or G5. However, comparing G5 (41.55%), administrating LSHE at 50 or 100 mg GAE Kg^{-1} reduced the TG level by 33.56 and 34.44%, respectively. Interestingly, the rate of CHO reduction was 33.11, 39.55, and 41.61% for treating rats with 50, 100 mg GAE Kg^{-1} of LSHE and 50 mg Kg^{-1} Vit. E+Se, respectively. HDL-c increase was recorded as 12.92, 41.95, and 34.78%, whereas LDL-c decrease was noted as 47.12, 65.37, and 64.64% after LSHE at 50, 100 mg GAE Kg^{-1} or 50 mg Kg^{-1} Vit. E+Se treatments, respectively. VLDL-c level was improved with treatments associatively in a type and dose-dependent manner. LSHE with 100 mg LSHE Kg^{-1} was significantly better than 50 mg LSHE Kg^{-1}. Fascinatingly, when CCl_4 was injected, the AI significantly raised compared to normal rats (G1). Indeed the most efficient treatments for attenuating the atherogenicity complication were those giving 100 mg GAE Kg^{-1} of LSHE, which presented better attenuation than 50 mg GAE Kg^{-1} of LSHE and even normal rats. The superior effect was recorded for using 50 mg Kg^{-1} Vit. E+Se did not differ significantly from using 100 mg GAE Kg^{-1} of LSHE.

The liver's functions

CCl_4 injection considerably raised serum AST, ALT, and ALP enzyme levels in G2 rats as oxidative stress and hepatotoxicity complications compared to normal rats (GI). The T. Bili level was significantly increased in CCl_4-treated rats (Figure 1). Administration of LSHE at 50 or 100 mg GAE Kg^{-1} and Vit. E+Se at 50 mg Kg^{-1} improved the liver's function. A high level of LSHE was better than a low level of LSHE or Vit. E+Se to improve liver functions. Interestingly, giving LSHE reduced the modifications in liver functions caused by CCl_4 injection to be close to typical values in GI (Figure 1). The ALT level attenuated by 28.68, 38.22, and 35.94% when 50 and 100 mg GAE LSHE Kg^{-1} and 50 Kg^{-1} Vit. E+Se were given, respectively. Similarly, ATS and ALP improved by 20.01, 30.44, and 32.71% and 19.83, 28.48, and 28.80%, respectively. However, in comparison to NR in G1, LSHE, and Vit. E+Se significantly enhanced some liver functions such as T. Bili and

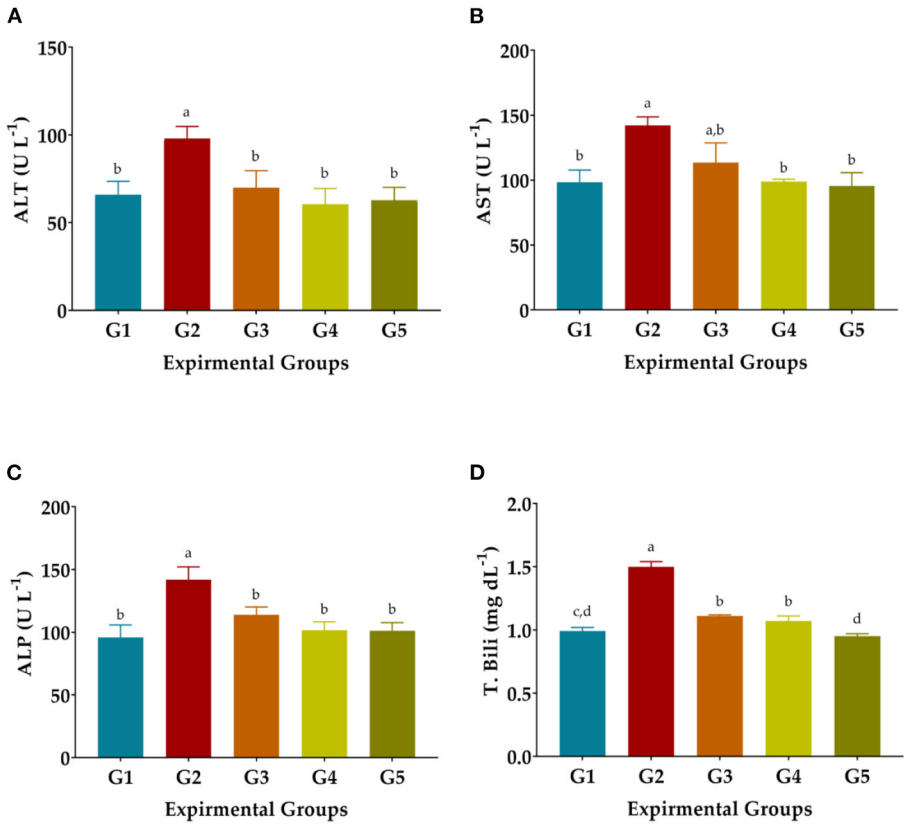

FIGURE 1
Effect of hydroalcoholic extract of *L. culinaris* sprouts on liver's functions in CCl_4-induced oxidative stress and hepatotoxicity in rats (mean ± SE), $n = 8$. **(A)** ALT: Alanine aminotransferase. **(B)** AST, Aspartate Aminotransferase. **(C)** ALP, Alkaline phosphatase. **(D)** T. Bili, Total bilirubin, ^{a,b,c,d}: Bars not sharing similar letters are statistically different at $p < 0.05$.

the liver enzymes as shown in (ALT, ALP, and AST). T. Bili level was attenuated by 26.00, 28.67, and 36.67% when rats were administrated when 50 and 100 mg GAE LSHE Kg^{-1} and 50 Kg^{-1} Vit. E+Se, respectively.

The kidneys' functions

The nephroprotective efficiency of LSHE at 50 or 100 mg GAE Kg^{-1} and Vit. E+Se at 50 mg Kg^{-1} on CCl_4-induced oxidative stress and hepatotoxicity in rats were studied; results are demonstrated in Table 5. CCl_4 injection considerably increased serum urea, BUN, and creatinine levels in G2 rats compared to NR in GI. Conversely, albumin, T. Protein, and globulin levels were drastically decreased in CCl_4-treated rats (Table 5). LSHE at 50 or 100 mg GAE Kg^{-1} and Vit. E+Se at 50 mg Kg^{-1} treatments significantly attenuated urea, creatinine, and BUN alterations caused by CCl_4 problems. Albumin, T. Protein, and Globulin levels were also raised to nearly normal levels in the GI (Table 5). The most effective enhancement was evidently recorded with LSHE at 100 mg GAE Kg^{-1} even sometimes better than using Vit. E+Se at 50 mg Kg^{-1} compared to normal rats (G1).

Antioxidant biomarkers

Injection of CCl4 dramatically decreased GSH, SOD, and CAT levels and elevated MDA levels in the blood serum of G2 compared to NR in G1, as shown in Figure 2. The activity of the antioxidant enzymes GSH, CAT, and SOD was significantly improved in the treated rats treated with 50 or 100 mg GAE Kg^{-1} and Vit. E+Se at 50 mg Kg^{-1} and the levels of MDA were significantly decreased, as shown in Figure 2. On the other hand, treatment of 50 mg LSHE Kg^{-1} exuded minimal diminution in GSH, CAT, and SOD and inhibited the autoxidation process, resulting in low MDA levels. Compared to the CCl_4-group (G2), the most effective treatment for GSH, DMA, CAT, and SOD was LSHE with 100 mg Kg^{-1}, which showed improved rates of 76.36, 29.47, 37.82, and 31.72 %, respectively. Comparing rats treated with CCl_4 and normal rats (G1), it is interesting to note that

TABLE 5 Effect of hydroalcoholic extract of *L. culinaris* sprouts on kidneys' functions in CCl_4-induced oxidative stress and hepatotoxicity in rats (mean ± SE), $n = 8$.

Group	Kidneys' functions					
	T. Protein (g dL^{-1})	Albumin (g dL^{-1})	Globulin (g dL^{-1})	Creatinine (mg dL^{-1})	Urea (mg dL^{-1})	BUN (mg dL^{-1})
G1	9.80± 1.00^{a}	4.59± 0.23^{a}	5.21± 0.86^{a}	0.89± 0.09^{b}	86.96± 8.03^{bc}	40.87± 3.77^{bc}
G2	7.44± 0.21^{a}	3.08± 0.27^{b}	4.36± 0.37^{a}	1.47± 0.22^{a}	156.52± 5.01^{a}	73.57± 2.36^{a}
G3	8.72± 0.43^{a}	3.90± 0.37^{ab}	4.82± 0.71^{a}	0.70± 0.04^{b}	105.31± 8.52^{b}	49.50± 4.01^{b}
G4	9.51± 0.61^{a}	4.49± 0.62^{ab}	5.02± 0.69^{a}	0.76± 0.02^{b}	90.10± 10.77^{b}	42.35± 5.06^{b}
G5	9.13± 0.87^{a}	4.69± 0.34^{a}	4.44± 0.88^{a}	0.81± 0.03^{b}	80.96± 3.19^{c}	38.05 ± 1.50^{b}

G1–G5, Experimental groups see materials and methods, BUN; Blood urea nitrogen, ^{a,b,c}, Means with the same superscripted letters in the same column are not statistically different at $p < 0.05$.

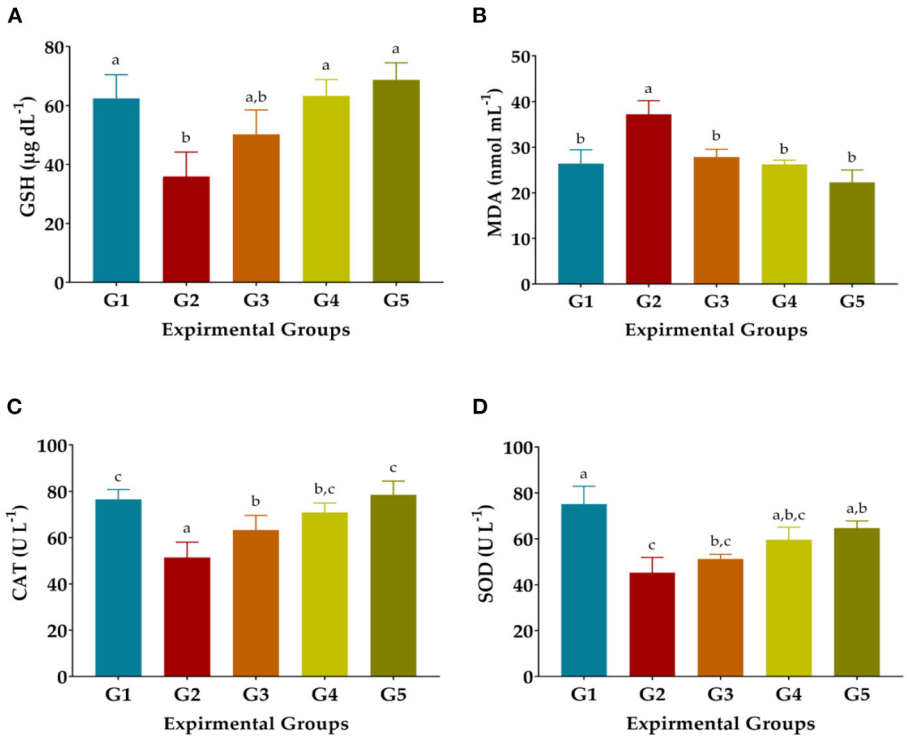

FIGURE 2
Effects of oral administration of hydroalcoholic extract of *L. culinaris* sprouts on antioxidant biomarkers in CCl_4-induced oxidative stress and hepatotoxicity in rats (mean ± SE), $n = 8$. **(A)** GSH: Reduced glutathione, **(B)** Malondialdehyde, **(C)** CAT: Catalase, and **(D)** SOD: Superoxide dismutase. G1–G5: Experimental groups see materials and methods, ^{a,b,c,d}: Bars not sharing similar letters are statistically different at $p < 0.05$.

rats treated with 50 mg Kg^{-1} of vit. E+Se dramatically improved their enzymatic defense system (G2).

Discussion

Functional foods regulate diabetes through blood pressure regulation, activation of antioxidant enzymes, interaction with gut microbiota, suppressing of pro-inflammatory cytokine overproduction, and presenting antioxidative potential (23, 43, 44). A capable strategy, particularly when phenolics are incorporated, has been described (32) with superior antioxidant activity (45). The valuable phytochemical content and antioxidant activities of LSHE corresponded to Amarowicz et al. and Xu and Chang (9, 10). Indeed, biologically active components, such as phenolic acids and flavonoids, demonstrate antioxidant capacity by stopping lipid oxidation chain reactions *in vitro* and *in vivo* (9, 10). The phenolics' ability to scavenge and inhibit free radicals is caused by the phenolic hydroxyl

groups located in polyphenols (46, 47). An efficient antioxidant component has been presented in various phenolic acids inhibiting hydrogen peroxide formation, superoxide anion, and hydroxyl radicals (48, 49). A direct correlation exists between abundant polyphenols concentration and their antioxidant function (9, 10, 23, 50).

Consumption of sprout extracts could help reduce cellular oxidation, as confirmed in the current study (23, 51). Interestingly during sprouting, phenolics and antioxidants increased (18). Zhang et al. (52) evaluated the total phenolic composition and contents, antioxidant activities (DPPH, FRAP, ORAC), and inhibitory properties of phenolic extracts from 20 Canadian lentil cultivars (*L. culinaris*) against α-glucosidase and pancreatic lipase (52). All extracts showed antioxidant and radical-scavenging properties as revealed by the total antioxidant activity (TAA) method, a β-carotene-linoleate model system, a reducing power assay, and the DPPH scavenging activity assay (9).

The increased number of phenolics in *L. culinaris* sprouts increased than its seeds with the progression of the sprouting period; results were agreed with Swieca and Gawlik-Dziki (18). Remarkably, present research noticed a considerable amount of identified phenolics higher than confirmed previously (9). However, Amarowicz et al. (9) indicated increased Rosmarinic acid, *p*-coumaric acid, and *p*-Hydroxy benzoic acid, which was consistently observed in the current study. It is worth mentioning that the phenolics profile in the cotyledon and seed coat of lentils vary and are affected by numerous factors (53, 54). *L. culinaris* seeds and sprouts show superior flavonoids content, similarly presented in 4 lentil varieties (55). Zhang et al. (52) supported our study regarding identified phenolics in *L. culinaris* seeds. In the present study, twenty-one phenolic compounds were identified, with the majority being flavonoids, including catechin/epicatechin glucosides, kaempeferol glycosides, and procyanidins. Amarowicz et al. (9). Twenty compounds (procyanidins, hydroxycinnamates, flavonols, gallates, dihydroflavonols, and dihydrochalcones) were detected and quantified in the crude extracts by HPLC-PAD and HPLC–ESI-MS procedures. The dominant phenolics in GL were epicatechin glucosides, catechin, procyanidin dimers, quercetin diglycoside, and trans-*p*-coumaric acid.

In addition to being beneficial against various metabolic illnesses, biologically active substances such as phenolic components have been defined as useful antioxidant substances, including hydroxyl radicals, hydrogen peroxide, and superoxide anion (56, 57). Interestingly, quantifying phenolics in *L. culinaris* sprouts indicated considerable numbers of phenolic acids and flavonoids, which increased significantly with increased sprouting time as confirmed (17, 18, 23) to process biological and nutritional benefits (20, 21).

Indeed, oxidative stress is thought to lead to many metabolic impairments, such as hypoglycemia (58–60). Our recent *in vivo* study indicated that LSHE stated substantial reductions in FBG in rats in a dose-dependent manner, as similarly indicated (13, 23, 51). These results strengthen our analysis, which confirms that LSHE possesses hypoglycemic effects because of rich polyphenols as effective antioxidants capable of modulating glucose levels (9, 15). Practically, the administration of LSHE was extremely beneficial in body weight recovery in a dose-dependent manner (57, 61).

Our results demonstrated the efficacy of LSHE as a rich source of antioxidants that alleviated liver malfunctions and elevated serum lipids among CCl_4-intoxicated rat groups. It might be due to increasing and supporting rats' serum with bioactive dietary antioxidants (9, 15, 62). Administration of LSHE at 50 or 100 mg GAE Kg^{-1} improved the lipid profile in dose-dependent manure. The highly efficient therapy for improving the blood profile was LSHE with 100 mg GAE Kg^{-1} which presented no significance compared to normal rats (G1) or (G5). Obviously, administrating 50 mg Kg^{-1} Vit. E+Se showed the highest improvement rate (41.55%) in lipid profile because of high antioxidant content, as similarly observed (15, 62). Excitingly, the VLDL-c level was adjusted associatively in a dose-dependent manner with LSHE treatments. Our results align with Morise et al. (63), who explained that flaxseed oil rich in α-linolenic acid caused an elevated cholesterol secretion, causing depletion of the intrahepatic pool of cholesterol resulting in cholesterol synthesis increases. Additionally, α-linolenic acid reduced hepatic lipid accumulation by stimulating β-oxidation and suppressing fatty acid synthesis (12, 15, 64). Concerning TG, high antioxidants can be assigned to a decrease in the hepatic synthesis of fatty acids, decreasing the triacylglycerol concentration in the liver and reducing autoxidation, which attenuates VLDL-c accumulation (15, 65). Interestingly, 100 mg GAE Kg^{-1} of LSHE was more effective than 50 mg GAE Kg^{-1} of LSHE or even normal rats in attenuating the atherogenicity issue. Better results were obtained with 50 mg Kg^{-1} vit. E+Se, which did not significantly differ from 100 mg GAE Kg^{-1} of LSHE (15).

CCl_4 insertion in a rat enlarged its liver by accumulating fats inside liver cells (66). Elevated serum enzymes activities levels (AST, ALT) signify cellular leak and loss of efficient integrity of cell membranes in the liver because of CCl_4 intoxication. Administration of LSHE or Vit. E+Se significantly improved the levels of liver enzymes (ALT, AST) which consistently agreed (51, 62). Similarly, Saxena et al. (67) and Jung et al. (15) have confirmed the effects of the plant-based extract on elevated serum AST and ALT enzymes against oxidative stress induced by CCl_4. T.Bili indicates that liver damage and CCl_4 had a considerably higher level than in treated groups (Vit. E+Si and LSHE) or the NR group. Incidentally, LSHE was also efficient as Vit. E+Se presented. A current study has also indicated that in valuable amounts, LSHE contains rosmarinic acid, *p*-coumaric acid, vanillic acid, *p*-Hydroxy benzoic acid, and Benzoic acid, as well as high content of flavonoids such as kaempferol, myricetin, quercetin, resveratrol, Naringenin, and rutin. Antioxidative

and anti-inflammatory efficiency in rats with hepatic damage has been proven with these compounds (68). Due to more polyphenols (20, 21), LSHE effectively attenuated oxidative stress complications. Therefore, LSHE may offer superior liver protection by blocking the development of liver fibrosis and suppressing TGF-β1 (68).

A correlation between nephrotoxicity and oxidative stress has been exhibited in many investigational models (69), and our study's results in kidney functions proved the same pattern as the organ function markers (61). Presented data clearly showed the recovery in all Kidney functions with orally administered LSHE for up to 6 weeks in dose-dependent manure. The elevated levels of Albumin, T. Protein, and Globulin and decreases in Urea, Creatinine, and BUN were highly meaningful in G4 and G5 compared to all other treated groups. An enhancement of kidney function parameters to the normal level in CCl_4-injected rats fed *Anastatica hierochuntica* ethanolic and aqueous extracts (61) Samsum Ant *Pachycondyla sennaarensis* Venom (69) was demonstrated. Concerning the positive impact of LSHE on Kidneys' function attenuation, it was previously described that caffeic acid, carnosic acid, rosemarinic acid, and essential oil are accountable for the body's protection against free radical attack through occurred oxidative stress (6, 9, 15).

As established by the catabolite MDA indicator, tissue damage and lipid peroxidation are mediated by generated ROS (70). ROS enhances the risk of tissue injury and causes lipid peroxidation, as ascertained by the catabolite malondialdehyde marker (70). Earlier experiments indicated that CCl_4 i.p. injection drastically decreased SOD, CAT, GPx, and GSH activities but considerably enhanced the MDA level (23, 71). Owning rich polyphenols content and AOA in LSHE, administrating 100 mg LSHE kg^{-1} was more efficient in attenuating autoxidation. The enzymatic antioxidant defense system such as SOD, CAT, and glutathione enzymes are essential scavengers of active radicals (68). LSHE attenuated GSH, CAT, SOD, and MDA levels close to the NR and equal to administrating Vit. E+Se. Previous studies have reported that consuming *L. culinaris* (6, 9, 15) increased serum antioxidant enzymes (51, 72). Concerning the present study's observations, administering LSHE orally increased the antioxidant enzymes SOD and CAT levels and decreased lipid peroxidation in CCl_4-injected rats (57). The efficiency was significantly improved when LSHE was given at 100 mg Kg^{-1}. As phenolics and antioxidants increase during sprouting (18), the consumption of sprouts extracts could help reduce cellular oxidation (23, 51). Also, LSHE attenuated the MDA and restored the total antioxidant defense in the CCl_4-treated rats. This protective efficiency may be due to the potent antioxidative capacity of LSHE in the presence of high polyphenols, which effectively diminishes the complications related to oxidative stress (20, 21, 49, 73, 74).

Conclusions

This study investigated and confirmed the antioxidative potential of *L. culinaris* Medikus. The current study looked into a rat module's antioxidative, hepatoprotective and nephroprotective properties of L. culinaris sprouts extract. It is possible to conclude that the *L. culinaris* sprouts extract is high in phenolic compounds, particularly flavonoids with high antioxidant capacity. Phenolic analysis revealed that *L. culinaris* sprouts contained significant amounts of TF, which support its functional and therapeutic properties. Compared to vit. E+Se administration of LSHE at 50 and 100 mg Kg^{-1} protects rats against CCl_4 oxidative stress. The protective efficacy could be attributed to the high concentration of phenolics (e.g., rosmarinic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, benzoic acid, kaempferol, naringenin, myricetin, resveratrol, and quercetin) which may modulate glucose levels and reduce hepatotoxicity complications. In addition, biochemical examinations have confirmed this superior activity. As a result, the findings could aid in explaining the therapeutic efficacy of LSHE as a functional product. It encouraged us to recommend *L. culinaris* sprout production for combining oxidative stress, as well as being beneficial and profitable for controlling oxidative stress complications.

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 animal study was reviewed and approved by the Committee of Research Ethics, Deanship of Scientific Research, Qassim University (No. 21-18-09 on Thursday, May 19, 2022), SA, governed by the Control and Supervision of Experiments on Animals (CPCSEA) Committee of the National Committee of BioEthics (NCBE), which implements regulations related to the ethics of research on living creatures.

Author contributions

SIA and HB: research design. ASA and RIA: experiment performance. SIA and HB: experiment operation assistance. SIA and RMA: main supervision and research leadership. RAA, RMG, and MA: draft manuscript writing. AAHA: validation and formal analysis. TA and HB: manuscript writing. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education, Saudi Arabia for funding this research work through the project number (QU-IF-2-1-2-26891). The authors also thank to Qassim University for technical support.

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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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 22 November 2022
DOI 10.3389/fnut.2022.1016592

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

RECEIVED 11 August 2022
ACCEPTED 07 November 2022
PUBLISHED 22 November 2022

CITATION
Ma L, Zhang Z, Li L, Zhang L, Lin Z and Qin H (2022) Vitamin D deficiency increases the risk of bacterial vaginosis during pregnancy: Evidence from a meta-analysis based on observational studies.
Front. Nutr. 9:1016592.
doi: 10.3389/fnut.2022.1016592

Vitamin D deficiency increases the risk of bacterial vaginosis during pregnancy: Evidence from a meta-analysis based on observational studies

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Background: Bacterial vaginosis (BV) is the most common microbiological syndrome in women of childbearing age, causing numerous adverse health issues in pregnant women. Several observational studies have discussed the association between vitamin D deficiency and the risk of BV during pregnancy, but the results were inconclusive. Therefore, this meta-analysis aimed to explore the association between vitamin D deficiency and BV risk in pregnant women.

Materials and methods: We searched four databases, including PubMed, Embase, Cochrane Library, and Web of Science, from their inception to July 2022. Pooled odds ratios (OR) with corresponding 95% confidence intervals (CI) were estimated using random effects models. Additionally, we conducted subgroup analyses to identify the potential sources of between-study heterogeneity. Sensitivity analysis was performed using the method of exclusion, one study at a time. Publication bias was examined using Egger's test and funnel plot.

Results: A total of 14 studies from 13 articles including 4,793 participants were eligible for this meta-analysis. The outcome showed that vitamin D deficiency may increase the risk of BV during pregnancy by 54% (OR, 1.54; 95% CI, 1.25–1.91; $P < 0.001$). In subgroup analyses, positive associations were also found in studies that were: conducted in black women (OR, 1.56; 95% CI, 0.98–2.48; $P = 0.060$), focused on the first trimester of pregnancy (OR, 2.22; 95% CI, 1.35–3.64; $P = 0.002$), of high quality (OR, 3.05; 95% CI, 1.26–7.41; $P = 0.014$), and adjusted for confounders (OR, 1.28; 95% CI, 1.06–1.55; $P = 0.012$). Sensitivity analysis reported that BV risk during pregnancy resulting from vitamin D deficiency increased by 157% (OR, 2.57; 95% CI, 1.50–4.42; $P = 0.001$) when removing the first two high-weight studies. Publication bias was observed using Egger's test ($t = 3.43$, $P = 0.005$) and a visual funnel plot.

Conclusion: This meta-analysis showed that vitamin D deficiency is positively associated with the risk of BV during pregnancy. Further high-quality prospective cohort studies are needed to determine whether vitamin D intake reduces the prevalence of BV in pregnant women.

KEYWORDS

bacterial vaginosis, vitamin D deficiency, pregnant women, infections in pregnancy, observational study, meta-analysis

Introduction

Bacterial vaginosis (BV), the most common vaginal infection among women of childbearing age, is characterized by the disruption of vaginal flora consisting of dominant physiologic *Lactobacillus* species to pathologic anaerobic and facultative bacterial species, such as *Gardnerella vaginalis, Prevotella bivia,* and *Atopobium vaginae* (1). The estimated prevalence of BV in the general population is between 23 and 26% worldwide and reaches up to 33 and 31% in black and Hispanic women, respectively (2). Pregnant women may be more susceptible to BV than the general population, particularly during early pregnancy (3). Aside from causing urogenital infections and pelvic inflammatory diseases, having BV during pregnancy may lead to numerous adverse obstetric outcomes, such as preterm birth, late miscarriage, intrauterine fetal death, chorioamnionitis, and low birth weight (4, 5). Additionally, the treatment of symptomatic BV leads to an economic burden that amounts to approximately $4.8 billion worldwide (2). However, the pathogenesis of BV remains poorly understood (5). Given its multiple adverse health outcomes, high recurrence rate, and enormous medical costs, it is pertinent to identify the associated risk factors for this condition, particularly among pregnant women, since this is the first step to preventing infection. Many factors play a role in the development of this infection, such as a higher number of sexual partners, young age at first intercourse, regular vaginal douching, and cigarette smoking (5, 6).

In addition, low vitamin D levels during pregnancy may increase the occurrence of BV (7). Vitamin D not only plays a crucial role in bone development, but also in immune-modulation, which includes triggering anti-inflammatory responses, such as cathelicidin expression and reducing pro-inflammatory cytokine production (e.g., IL-1β) (8). It was estimated that the prevalence of Vitamin D deficiency was about 30% in children and adults worldwide (9, 10). Considering that vitamin D deficiency is highly prevalent among pregnant women worldwide (10), the role of vitamin D in BV risk needs to be examined. To date, there are only a handful of epidemiological studies that have explored the relationship between vitamin D deficiency and the risk of BV in pregnancy (11–23).

However, despite the growing body of research on the relationship between vitamin D and BV risk in pregnancy, the existing literature has yielded inconsistent results. Some studies have reported a positive association between vitamin D deficiency and the occurrence of BV during pregnancy (11–18). Conversely, other studies failed to confirm this association (19–23). Although the association between vitamin D levels and the risk of BV during pregnancy has been mentioned in some systematic review articles (24, 25), these studies have only included a few studies that fulfilled the minimum requirement for meta-analysis. To address this controversial issue further, we gathered relevant data for a meta-analysis that quantitatively assesses the relationship between vitamin D deficiency and BV risk during pregnancy.

Materials and methods

Search strategy

We carried out an overall literature search from inception up to July 2022 using four databases: PubMed, Embase, Cochrane Library, and Web of Science. A search strategy was developed involving a combination of keywords and MeSH (Medical Subject Headings) or Emtree terms with boolean operators "OR" and "AND" in all databases to enhance the probability of obtaining related studies. The complete electronic search strategy is presented in **Supplementary Table 1**.

Inclusion criteria

For studies included in this meta-analysis, the following criteria were met: (1) original papers published in English; (2) the exposure of interest was vitamin D measurement during pregnancy; (3) the outcome of interest was BV; (4) odds ratio (OR), relative risk (RR), or hazard ratio with 95% confidence interval (CI) (or data/figure to estimate them); (5) observational studies (cohort, case-control, or cross-sectional design); and (6) the most recent and complete

study was selected if data from the same population had been published more than once. Meanwhile, if effect sizes were available for meta-analysis in the conference paper, these should also be extracted. In addition, the reference lists of the retrieved articles were carefully examined to avoid missing any relevant literature. All retrieved studies were carefully and independently reviewed by three investigators to determine whether an individual study met the inclusion criteria. If the three investigators (LM, ZZ, and LL) were disputable regarding the eligibility of an article, they were resolved by having a consensus or consultation with a fourth investigator (HQ).

Data extraction

During the process of literature screening, the title and abstract were reviewed first, before the full texts were further read to determine whether they should be included in the analysis. The extracted data that were obtained included the following details: the first author's last name, year of publication, country where the study was conducted, research type, sample size, mean age or age range of participants, gestational age when vitamin D was measured, vitamin D and BV determination methods, threshold of vitamin D deficiency, and adjusted confounding factors, the ORs (we used OR to represent the effect size for simplicity) with corresponding CIs of BV for vitamin D deficiency. When multiple ORs (95% CIs) were reported, we only extracted the effect estimates after adjusting for most confounders. In addition, as pregnant women in early stage may be more susceptible to BV and tend to suffer more from BV-induced adverse pregnancy outcomes than women in middle and late pregnancy (3–5), we preferentially used the OR (95% CI) of BV risk for vitamin D deficiency during early pregnancy in individual studies to calculate the pooled effect estimate if several ORs (95% CIs) were provided at different gestational ages (e.g., early, middle, and late pregnancy).

Quality assessment

The Newcastle-Ottawa Scale (NOS), a scoring system developed to assess the risk of bias, was used to evaluate the quality of the studies. The NOS covers three domains: selection, comparability, and exposure/outcome. A study can be awarded a maximum of one star for each numbered item within the selection and exposure categories. A maximum of two stars can be assigned for comparability. Each star represents one point; thus, the maximum possible score is nine. Generally, a total score of seven or more indicates high quality and a score of less than seven represents low quality.

Statistical analyses

To determine the strength of the association between vitamin D deficiency and the risk of BV during pregnancy, the DerSimonian and Laird random effects model was used to calculate the pooled OR (95% CI) in view of inevitable between-study variance (26). Between-study heterogeneity was assessed using the I^2 statistic (I^2 values of 0—25%, 25–50%, 50–75%, and 75–100% indicate no, low, medium, and high heterogeneity, respectively) (27). To explore the possible sources of heterogeneity, subgroup analyses were performed to examine the role of potential confounding factors, such as study type, geographic location where studies were conducted, race, gestational age, vitamin D assay methods, adjustment for confounders, study quality and climate characteristic of area of included paper. Sensitivity analyses were conducted, with one study excluded at a time, to assess the stability of the pooled OR (95% CI). Additionally, the Egger regression asymmetry test and visual inspection of funnel plots were used to evaluate publication bias (28).

We used Stata 14.2 software (Stata Corporation, College Station, TX, USA) to perform data analyses. All 2-tailed *P*-values < 0.05 were considered statistically significant.

Results

Initially, the database search allowed the investigators collect 617 articles (PubMed 36, Embase 174, Cochrane Library 346, and Web of Science 61). A total of 541 articles were examined through their titles and abstracts after excluding 76 duplicates. Subsequently, 513 articles were removed because they explicitly did not meet the inclusion criteria. From the remaining 28 articles, which were carefully reviewed to assess if they fit the criteria, 15 were rejected for the following reasons: 3 articles focused on non-pregnant women (29–31); 3 articles lacked ORs and corresponding 95% CIs, which could not be obtained from the available data (7, 32, 33). Among these three studies (7, 32, 33), two studies supported that vitamin D deficiency in the first (33) and second (7) trimesters of pregnancy increased BV occurrence, respectively, while one study (32) considered that neither vitamin D deficiency in early pregnancy nor supplementation reduced BV risk during pregnancy. Four studies did not quantitatively evaluate the association between vitamin D deficiency and BV risk (34–37). Among these four studies (34–37), one study was a letter to the editor (34), one was a review (35), one focused on the association between vitamin D status and the vaginal microbiome (36), and one study concentrated on complications of gestation (37). Four articles were not published in English (38–41). One study used a similar population, with the most recent studies included (42). Fourteen studies from 13 articles published from 2009 to 2021 were eligible for this meta-analysis. Detailed

information regarding the literature retrieval process is shown in Figure 1.

Baseline characteristics

Among the included studies, eight studies were carried out in North America 8 (11–13, 16, 17, 21, 23), 2 in Asia (15, 20), 3 in Europe (14, 18, 22) and 1 in Africa (19). Regarding the study type, one study had a case-control design (10), four had a cohort design (14, 18, 19, 21), and nine had a cross-sectional design (11, 13, 15–17, 20, 22, 23). Regarding the adjustment for confounding factors, 10 were adjusted (11–13, 15, 16, 19–21, 23) and 4 unadjusted (14, 17, 18, 22). With respect to BV determination methods, 10 studies were based on the Nugent score of Gram staining (11–13, 15–17, 19, 21, 22), one used 16S rRNA sequencing technology (23), and three did not report measurement methods (14, 18, 20). For the timing of blood sample collection to measure vitamin D levels, 10 studies focused on the first trimester (11, 13, 15–19, 21, 22), 2 focused on the second trimester (20, 22), and 3 focused on the third trimester (12, 14, 21). According to the scores based on the Newcastle-Ottawa scale, five were considered to be of high quality (11, 13, 15, 17, 19), and nine were classified as low quality (12, 14, 16, 18, 20–23). As for climate characteristic of area of included paper, 12 studies were belonged to temperate zone (11–18, 21–23), two were categorized as tropical zone (19, 20). The baseline characteristics of the included studies are presented in Table 1.

Quantitative synthesis

This meta-analysis used data from 14 studies in 13 articles covering 4,793 participants to assess the association between vitamin D deficiency and the risk of BV during pregnancy. Of the 14 studies, six reported no relationship between vitamin D deficiency and BV prevalence during pregnancy, while eight showed a positive association between the two. Our results showed a positive association between vitamin D deficiency and the risk of BV during pregnancy (OR, 1.54; 95% CI, 1.25–1.91, $P < 0.001$; $I^2 = 84.9\%$, $P_{heterogeneity} < 0.01$; Figure 2).

Subgroup analyses

In view of the high heterogeneity among the included studies, subgroup analyses were performed to examine possible confounders. As shown in Table 2, the pooled OR (95% CI) for subgroups stratified by study type was 1.78 (0.87–3.64), 5.11

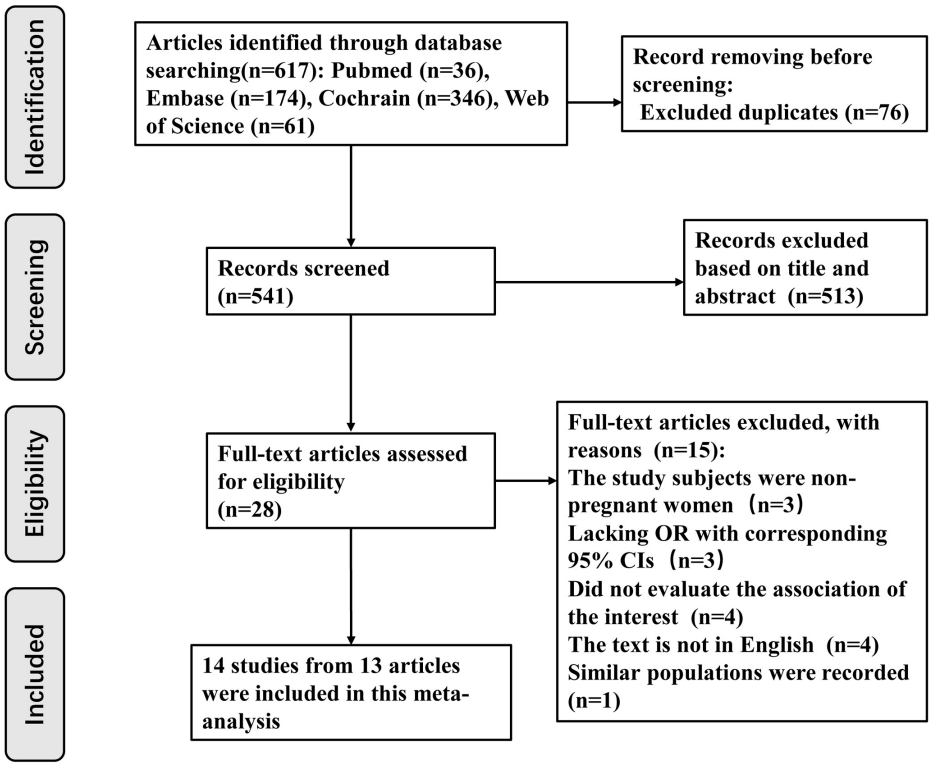

FIGURE 1
Flow chart of the selection of studies included in this meta-analysis.

TABLE 1 Baseline characteristics of the included studies.

Ref.	Country	Population	Study design	Age (years)	Gestational age (weeks)	No. of Participants (case)	Measurement method of vitamin D	Determination method of BV	Threshold of vitamin D deficiency	Evaluation of vitamin D	OR (95% CI) for vitamin D deficiency	Adjustment for covariates	Quality assessment
Bodnar et al. (11)	United States	Black and White	Cross-sectional	20–29	<16	469 (192)	RIA	Nugent score of Gram staining	<20 nmol/L	25-hydroxy-vitamin D	Total population: 1.65 (1.01, 2.69); black women: 1.47 (1.02, 2.13); white women: 1.09 (0.62, 1.92)	Sexually transmitted diseases	7
Dunlop et al. (12)	United States	Non-Hispanic black, Non-Hispanic white	Case-control	24.14 ± 6.04	31–37	160 (14)	ELISA	Nugent score of Gram staining	<12 ng/ml	25-hydroxy-vitamin D	Total population: 5.11 (1.19, 21.97)	Race, age, smoking status, BMI, gestational age at delivery, payor source	6
Hensel et al. (13)	United States	Non-Hispanic white, Non-Hispanic black, Mexican American	Cross-sectional	14–49	<13	440 (NR)	Microbiological method	Nugent score of Gram staining	<30 ng/ml	25-hydroxy-vitamin D	Total population: 2.87 (1.13, 7.28)	Age, race, education, poverty index, marital status, age at first sex, number lifetime partners, ever have female sex partner, unprotected sex, pregnancy status, oral contraception use, douching frequency last six months, cotinine level, BMI	7
Skowrońska et al. (14)	Poland	Polish	Cohort	30.5 ± 4.9	28–40	102 (NR)	ECLIA	NR	<20 ng/ml	Vitamin D supplement	White women: 10.77 (2.09, 55.40)	NR	4
Rahmanpour et al. (15)	Iran	Persian	Cross-sectional	NR	<20	204 (55)	Microbiological method	Nugent score of Gram staining	<20 nmol/L	25-hydroxy-vitamin D	White women: 16.30 (6.00, 45.50)	BMI, maternal age	7
Turner et al. (19)	Zimbabwe	Zimbabwean	Cohort	22–28	<13	141 (38)	RIA	Nugent score of Gram staining	<30 ng/ml	25-hydroxy-vitamin D	Black women: 0.88 (0.51, 1.54)	Age, education, parity, HSV-2 status, circumcision status of primary male partner, sex in the last three months, vaginal hygiene habits, sexual frequency, condom use, number of male sex partners	7
Tabatabaei et al. (16)	Canada	Montrealer	Cross-sectional	NR	8–14	433 (NR)	LC-MS	Nugent score of Gram staining	<50 nmol/L	25-hydroxy-vitamin D	Ethnic minority (black women): 5.60 (1.58, 19.84); non-ethnic minority (white women): 1.31 (0.73, 2.35)	Season of conception, age, pre-pregnancy BMI, parity, marital status, smoking, education, present history of sexually transmitted disease	5

(Continued)

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TABLE 1 (Continued)

Ref.	Country	Population	Study design	Age (years)	Gestational age (weeks)	No. of Participants (case)	Measurement method of vitamin D	Determination method of BV	Threshold of vitamin D deficiency	Evaluation of vitamin D	OR (95% CI) for vitamin D deficiency	Adjustment for covariates	Quality assessment
Powell et al. (17)	United States	African American	Cross-sectional	NR	8–12	245 (63)	Microbiological method	Nugent score of Gram staining	<40 ng/ml	Vitamin D supplement	Black women: 5.26 (3.20, 12.82)	NR	7
Lee et al. (20)	Malaysia	Malay, Chinese, Indian, other ethnicity	Cross-sectional	30.0 ± 4.36	>37	575 (13)	HPLC	NR	<20 ng/ml	25-hydroxy-vitamin D	Total population: 1.01 (0.95, 1.08)	Maternal age, BMI	4
Dunlop et al. (21)	United States	African American	Cohort	24.3 ± 4.3	8–14	137 (57)	CLIA	Nugent score of Gram staining	<20 ng/ml	Total and free 25 (OH)D	Black women: 1.04 (0.99, 1.10) First trimester: 1.04 (0.99, 1.10); last trimester: 1.06 (1.01, 1.12)	Maternal age, parity, insurance status, first prenatal BMI, gestational age of visit, receipt of antibiotics in the month prior to the visit	5
Christoph et al. (22)	Switzerland	European, Northern Africa, Middle East, South West Asia, Sub-Saharan Africa, Indian	Cross-sectional	22–38	8–16	1153 (36)	CLIA	Amsel criteria and Nugent scoring	<25 nmol/L	Vitamin D supplements	Total population: 0.69 (0.27, 1.52)	NR	4
Maliar (18)	Ukraine	Ukraninian	Cohort	25.1 ± 2.6	10–12	100 (19)	ECLIA	Nugent score of Gram staining	<30ng/ml	25-hydroxy-vitamin D	White women: 4.93 (1.50, 16.16)	NR	5
Rosen et al. (23)	United States	Black and White	Cross-sectional	26.6 ± 6.9	24–29	634 (76)	FFQ	16S rRNA sequencing technology	NR	Dietary vitamin D	Total population: 0.83 (0.51, 1.37); black women: 0.83 (0.38, 1.85); white women: 0.88 (0.47, 1.67)	Race, age, parity, BMI, maternal stress	5

Ref., reference; CLIA, chemiluminescent immunoassay; ECLIA, electrochemical luminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; FFQ, food frequency questionnaire; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; RIA, radioimmunoassay; NR, not reported; OR, odds ratio; CI, confidence interval; BV, bacterial vaginosis; BMI, body mass index; HSV-2, herpes simplex virus type 2.

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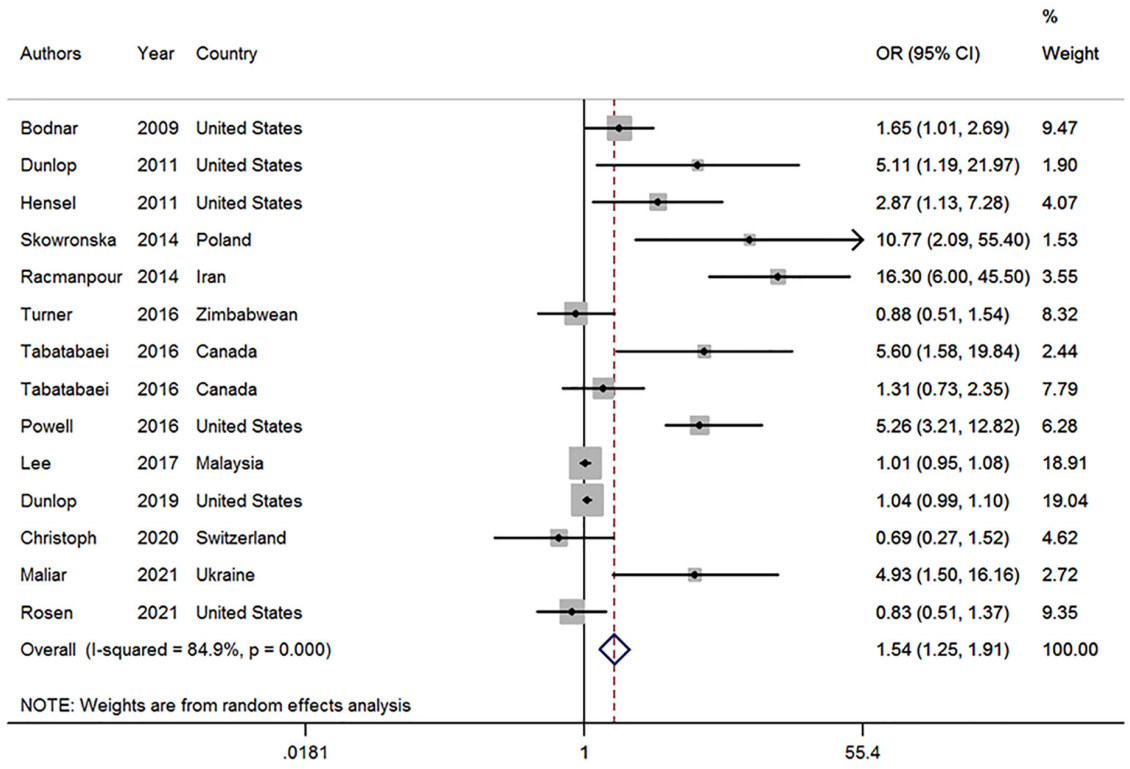

FIGURE 2
Forest plot of included studies on the association between vitamin D deficiency and bacterial vaginosis risk. OR, odds ratio; CI, confidence interval. The size of the grey box is positively proportional to the weight assigned to each study, which is inversely proportional to the SE of the OR. The horizontal lines represent the 95% CI.

(1.19–21.96), and 2.04 (1.23–3.39) in cohort, case-control, and cross-sectional studies, respectively. The combined OR (95% CI) for subgroups by gestation age was 2.22 (1.35–3.64) in the first trimester, 1.01 (0.95–1.08) in the second trimester, and 3.31 (0.69–15.94) in the third trimester. The pooled OR (95% CI) was 1.56 (0.98–2.48) for studies performed in the black population, 1.92 (1.22–3.03) for studies executed in North America, 2.51 (1.55, 4.06) for studies conducted in temperate zone, and 2.20 (1.34–3.61) for studies using Nugent Score of Gram staining. Additionally, the pooled ORs (95% CIs) in high-quality studies and studies adjusted for confounders were 3.05 (1.26–7.41) and 1.28 (1.06–1.55), respectively.

Sensitivity analysis

To assess the robustness of our meta-analysis, a sensitivity analysis was conducted by excluding one study at a time (**Supplementary Figure 1**). The findings of the sensitivity analysis suggest that the first two high-weight studies considerably lowered the pooled effect size (18, 19). After removing the two studies, the pooled OR (95% CI) of BV prevalence for vitamin D deficiency was from 1.54 (1.25–1.91, $P < 0.001$) to 2.57 (1.50–4.42, $P = 0.001$) (**Supplementary Figure 2**) and was relatively stable (**Supplementary Figure 3**).

Publication bias

Publication bias was observed using Egger's test ($t = 3.43$, $P = 0.005$) and visual inspection of the funnel plot for the effect of vitamin D deficiency on BV risk (**Supplementary Figure 4**).

Discussion

The current meta-analysis quantitatively evaluated the association between vitamin D deficiency and BV risk in pregnant women. In contrast, previous published reviews mainly narratively described the findings of original studies, partly due to limiting sample size (each review covering three studies) (24, 25).This meta-analysis, including 14 studies from 13 articles covering 4,793 participants, showed that vitamin D deficiency could increase the risk of BV by 54% during pregnancy. More specifically, for vitamin D deficiency in the first trimester and for black women, the BV risks were elevated

TABLE 2 Pooled ORs of subgroup analyses for the association between Vitamin D deficiency and the risk of bacterial vaginosis (BV).

Subgroups	No. of studies (ref.)	Pooled ORs (95% CIs)	*P*-values for pooled ORs	*P*-values for subgroup differences	Study heterogeneity	
					I^2 (%)	*P*-value
All studies	14 (11–23)	1.54 (1.25–1.91)	<0.001	–	84.9	<0.001
Study design						
Cohort	4 (14, 18, 19, 21)	1.78 (0.87–3.64)	0.117	0.957	79.6	0.002
Case-control	1 (12)	5.11 (1.19–21.96)	0.028		–	–
Cross-sectional	9 (11, 13, 15–17, 20, 21, 23)	2.04 (1.23–3.39)	0.006		88.1	<0.001
Geographic location						
North America	8 (11–13, 16, 17, 21, 23)	1.92 (1.22–3.03)	0.005	0.794	82.9	<0.001
Asia	2 (15, 20)	3.87 (0.25–58.86)	0.329		96.5	<0.001
Europe	3 (14, 18, 22)	3.01 (0.55–16.49)	0.203		83.5	0.002
Africa	1 (19)	0.88 (0.51–1.53)	0.650		–	–
Race/Ethnicity						
Total population	6 (11–13, 20, 22, 23)	1.25 (0.86–1.82)	0.234	0.265	65.7	0.012
Black women	6 (11, 16, 17, 19, 21, 23)	1.56 (0.98–2.48)	0.060		84.1	<0.001
White women	6 (11, 14–16, 18, 23)	2.77 (1.16–6.59)	0.021		85.5	<0.001
Trimester of blood collection						
First	10 (11, 13, 15–19, 21, 22)	2.22 (1.35–3.64)	0.002	0.974	87.4	<0.001
Second	2 (20, 23)	1.01 (0.95–1.08)	0.778		0.0	0.435
Last	3 (12, 14, 21)	3.31 (0.69–15.94)	0.135		83.5	0.002
Vitamin D assay methods						
Instrumental method	10 (11, 12, 14, 16, 18–22)	1.17 (0.99–1.38)	0.060	0.759	71.6	<0.001
Microbiological method	3 (13, 15, 17)	6.09 (2.50–14.85)	<0.001		68.1	0.044
Food frequency questionnaire	1 (23)	0.83 (0.51–1.36)	0.462		–	–
Determination of BV						
Nugent score of Gram staining	10 (11–13, 15–17, 19, 21, 22)	2.20 (1.34–3.61)	0.002	0.557	87.0	<0.001
16srDNA sequencing technology	1 (23)	0.83 (0.51–1.36)	0.462		–	–
NR	3 (14, 18, 20)	3.24 (0.70–15.13)	0.134		86.4	0.001
Adjusted for confound factors						
Adjusted	10 (11–13, 15, 16, 19–21, 23)	1.28 (1.06–1.55)	0.012	0.386	82.0	<0.001
Unadjusted	4 (14, 17, 18, 22)	3.44 (1.03–11.44)	0.044		82.1	0.001
Study quality						
High quality	5 (11, 13, 15, 17, 19)	3.05 (1.26–7.41)	0.014	0.382	88.0	<0.001
Low quality	9 (12, 14, 16, 18, 20–23)	1.12 (0.95–1.32)	0.196		72.1	<0.001
Climate characteristic						
Temperate zone	12 (11–18, 21–23)	2.51 (1.55, 4.06)	<0.001	0.166	86.9	<0.001
Tropical zone	2 (19, 20)	1.01 (0.95, 1.08)	0.744		0.0	0.620

Ref., reference; OR, odds ratio; CI, confidence interval; BV, bacterial vaginosis; NR, not reported.

up to 122 and 56%, respectively. Furthermore, a similar trend was found in the high-quality (OR, 3.05; 95% CI, 1.26–7.41; $P = 0.014$), adjustment for confounders (OR, 1.28; 95% CI, 1.06–1.55; $P = 0.012$), and cohort study (OR, 1.78; 95% CI, 0.87–3.64; $P = 0.117$) subgroups. In addition, our findings are partly supported by a randomized clinical trial by Taheri et al., who reported that the treatment of vitamin D deficiency might eliminate asymptomatic BV in non-pregnant women (43). Thus,

according to the recommendations from Institute of Medicine, pregnant women had better ingest on average 600 IUs of vitamin D daily and maintain the serum vitamin D at least 30 ng/mL (10).

The exact biological mechanism by which vitamin D deficiency increases susceptibility to BV is not yet well established. To date, several possible biological pathways have been proposed to elucidate the role of vitamin D in the prevalence of BV. First, vitamin D is implicated in the regulation of the proliferation and differentiation of various cells (44), particularly in stratified squamous epithelium, such as the vaginal epithelium (45). One of the mechanisms underlying this may be that vitamin D triggers the VDR (vitamin D receptor)/p-RhoA (ras homolog gene family)/p-Ezrin (cell junction proteins) pathway, which may increase cell-to-cell junctions of the vaginal epithelium and decrease the PH value of the vaginal microbial environment (46, 47). Additionally, vitamin D deficiency may induce vaginal atrophy, decrease barrier function, and increase BV risk.

Second, vitamin D is linked to diverse immunomodulatory actions, including the enhancement of the innate immune system and regulation of the adaptive immune responses, through binding to VDRs expressed by a number of different immune cell subsets (44). On the one hand, with the activation of toll-like receptors, vitamin D and VDR binding enhances the antimicrobial activities of key innate immunocytes, such as neutrophils, monocytes, and macrophages. These effects are principally mediated by up-regulating the synthesis of antimicrobial peptides, such as cathelicidins and beta-defensins, which could prevent and control invasive bacterial infections and increase genital tract immune capacity (44, 48–50). In contrast, VDR ligation by vitamin D enhances anti-inflammatory cytokine production (51, 52), such as interleukin-4 (IL-4) and interleukin-10 (IL-10), and inhibits the expression of pro-inflammatory cytokines (44), such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12), tumor necrosis factor-alpha (TNF-α), and the development of pro-inflammatory T helper 1 (Th1) and T helper 17 (Th17) cells (53). Vitamin D also inhibits the production of interleukin-2 (IL-2), which is essential for lymphocyte clonal expansion and interferon-gamma (IFN-γ) (54). Thus, given the decrease of antimicrobial peptide synthesis and anti-inflammatory cytokine production, and the increase in pro-inflammatory cytokine expression, vitamin D deficiency may promote the occurrence of BV.

Additionally, vitamin D may play a role in influencing the vaginal microbial environment. By elevating calcium concentration, vitamin D may stimulate insulin secretion and increase glycogen synthesis, which induces glycogen deposition in the vagina (36, 55). A higher concentration of free glycogen in the lower genital tract promotes *Lactobacillus* species colonization, decreases vaginal pH, and inhibits the growth of other bacteria (56). A pilot study including black adolescent women also showed that higher vaginal glycogen levels were positively related to the dominance of *Lactobacillus* (57). Therefore, vitamin D deficiency may alter glucose homeostasis in the vagina and enhance BV prevalence.

In the current study, subgroup analyses stratified by race revealed that vitamin D deficiency might increase BV risk in black women. The potential reason may be that most black women usually suffer from a higher burden of vitamin D deficiency than white women (58). Additionally, vitamin D intake from diet and supplementation for black women is relatively low (59). In contrast, darker skin pigmentation may inhibit conversion from 7-dehydrocholesterol (provitamin D_3) to pre-calciferol (pre-vitamin D_3 form) following sun exposure (60). In addition, lifestyle factors such as regular vaginal douching and cigarette smoking, which are known risk factors for BV (5, 6), are likely to differ among races. These findings suggest that race is not an independent factor for BV occurrence. Nonetheless, some studies have reported that race/ethnicity exerts an effect on the diversity and predominance of the vaginal microbiome (61, 62). In addition, it is puzzling that there was statistical association between vitamin D deficiency and BV risk in subgroup analysis on white women. Thus, further studies are needed to clarify the association between race and BV.

Strengths and limitations

Our study has several strengths. To our knowledge, this is the first meta-analysis in recent years to examine the relationship between vitamin D deficiency and BV risk during pregnancy. Second, based on potential confounders, such as race and gestational age, and adjustment for confounders, various subgroup analyses were performed.

Our study has some limitations. First, the cross-sectional or case-control design used in some original studies limits the establishment of causality due to inevitable recall and selection biases. Second, the studies included in this meta-analysis were biased toward North America and Europe, which might reduce the generalizability of our outcomes. Third, the threshold for vitamin D deficiency defined in the included studies was inconsistent, which may have underestimated the pooled ORs (95% CIs). Fourth, I^2 values of between-study heterogeneity remained high even though numerous subgroup analyses were conducted, suggesting that unaccounted potential confounders may exist. Thus, we structured a random effects model to minimize the influence of between-study heterogeneity. Fifth, some extracted ORs from the original studies used to calculate combined effect estimates were estimated based on a frequency table or figure, which may lead to unavoidable bias. Sixth, the methods used

to measure vitamin D levels and diagnose BV were not entirely consistent, which may have influenced the stability of the results. Seventh, publication bias was observed using Egger's test and funnel plot. Finally, we were unable to investigate a dose-response relationship between vitamin D levels and BV risk owing to the lack of sufficient data.

Conclusion

Our meta-analysis, involving 14 studies, showed that vitamin D deficiency contributes to the risk of BV during pregnancy. Most subgroup analyses also supported this finding, especially in studies that were focused on the first trimester of pregnancy, considered high quality, and adjusted for confounders. Considering the high prevalence and adverse health outcomes of vitamin D deficiency and BV, these findings have potential clinical implications. Additional studies, especially large prospective cohort studies in various races, are required to further assess the association between vitamin D deficiency and BV risk.

Data availability statement

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

Author contributions

LM and HQ contributed to conception and design of the study. LM, ZZ, and LL extracted data and wrote the first draft of the manuscript. LM, HQ, and LZ performed the statistical analysis. HQ and ZL reviewed and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (No. 32000495), National Natural Science Foundation of Shandong Province (No. ZR2020MH202), A Project of Shandong Province Higher Educational Science and Technology Program (No. J18KA290), Doctoral Research Start-up Foundation of Weifang Medical University, and A Project of Quality Course of Shandong Province Graduate Education–Health Management (No. SDYKC20152). The sponsors played no role in study design, collection, analysis or interpretation of the data, writing of the report, or decision to submit the manuscript 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1016592/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Systematic Review
PUBLISHED 25 November 2022
DOI 10.3389/fnut.2022.1008464

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Nutritional Epidemiology, a section of the journal Frontiers in Nutrition

RECEIVED 31 July 2022
ACCEPTED 27 October 2022
PUBLISHED 25 November 2022

CITATION
Kaewdech A, Sripongpun P, Wetwittayakhlang P and Churuangsuk C (2022) The effect of fiber supplementation on the prevention of diarrhea in hospitalized patients receiving enteral nutrition: A meta-analysis of randomized controlled trials with the GRADE assessment. *Front. Nutr.* 9:1008464. doi: 10.3389/fnut.2022.1008464

The effect of fiber supplementation on the prevention of diarrhea in hospitalized patients receiving enteral nutrition: A meta-analysis of randomized controlled trials with the GRADE assessment

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Introduction: Enteral nutrition (EN) in hospitalized patients has several advantages. However, post-feeding diarrhea occurs frequently and has been linked to negative outcomes. The EN formula itself may have an impact on how diarrhea develops, and fiber supplements may theoretically help patients experience less diarrhea. This study aimed to thoroughly evaluate whether adding fiber to EN decreases the likelihood of developing diarrhea and whether different types of fibers pose different effects on diarrhea (PROSPERO CRD 42021279971).

Methods: We conducted a meta-analysis on fiber supplementation in hospitalized adult patients receiving EN. We thoroughly searched PubMed, Medline, Embase, Scopus, Web of Science, CENTRAL, and ClinicalTrials.gov databases from inception to 1 September 2022. Only randomized controlled trials (RCTs) were included. Pooled results on the incidence of diarrhea were calculated using a random-effects model. The Grading of Recommendations, Assessment, Development, and Evaluations (GRADE) approach was applied. Only fiber types from soy polysaccharides ($n = 4$), psyllium ($n = 3$), mixed soluble/insoluble fiber (mixed fiber, $n = 3$), pectin ($n = 2$), and partially hydrolyzed guar gum (PHGG, $n = 2$) were examined in the sensitivity analysis.

Results: Among the 4,469 titles found, a total of 16 RCTs were included. Overall, compared to fiber-free formulas, fiber supplementation reduced the occurrence of diarrhea in patients receiving EN by 36% (pooled risk ratio [RR] of 0.64 [95% confidence interval (CI): 0.49–0.82, $p = 0.005$; $I^2 = 45\%$]), with GRADE showing the evidence of moderate certainty. Only mixed fiber and PHGG significantly decreased the incidence of diarrhea according to the sensitivity analyses for fiber types (RR 0.54, 95%CI: 0.39–0.75, $I^2 = 0\%$ and RR 0.47, 95%CI: 0.27–0.83, $I^2 = 0\%$, respectively). The results for the remaining fiber types were unclear.

Conclusion: According to a meta-analysis, fiber supplements help lessen post-feeding diarrhea in hospitalized patients receiving EN. However, not all fiber types produced successful outcomes. Diarrhea was significantly reduced by PHGG and mixed soluble/insoluble fiber.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=279971, identifier: PROSPERO CRD 42021279971.

KEYWORDS

nosocomial diarrhea, dietary fiber (DF), soluble fiber, psyllium, guar gum (GG), tube feeding, enteral nutrition

Introduction

Enteral nutrition (EN), a form of nutritional support delivered *via* the gastrointestinal tract, is preferred for hospitalized patients whose caloric and nutritional requirements cannot be adequately met by oral intake. EN has been proven to offer several benefits in such patients over parenteral nutrition, e.g., the maintenance of gut mucosal integrity, the reduction of bacterial translocation from the gut lumen to the blood stream, and the prevention of infection. Nonetheless, some gastrointestinal problems may occur in patients receiving EN. Diarrhea is one of the common conditions encountered, as observed in 29–39% of enterally fed patients (1–3), and can lead to unfavorable sequelae, such as volume and electrolyte disturbances, perianal dermatitis, and a longer duration of hospital stay (3–5).

Dietary fibers are parts of carbohydrates derived from plant cell wall components, which are neither digested nor absorbed in the small intestine. They have a degree of polymerization of $\geq$10 monomeric units, as defined by the World Health Organization (WHO), or three or more monomeric units, as specified by the European Food Safety Authority and by the US Food and Drug Administration (6). There are a variety of dietary fibers with different physiochemical characteristics. Dietary fibers consist of water-soluble and water-insoluble fibers. Soluble fibers, such as soy polysaccharides, psyllium, partially hydrolyzed guar gum (PHGG), pectin, banana flakes, Shen jia, and polydextrose, have been demonstrated to improve the regularity of bowel movement due to the luminal water-holding property of fibers to form bulky, soft, and easy-to-pass stools (7). In addition to improving regularity, insoluble fibers (e.g., wheat bran) can stimulate water and mucous secretion by irritating the large bowel mucosa (7).

In terms of tube-feeding diarrhea, several mechanisms proposed that dietary fiber supplementation in EN may yield a benefit in reducing the occurrence of diarrhea, e.g., increased viscosity of the stool content leading to bulk formation, prolongation of intestinal transit time, fermentability to produce short-chain fatty acids (SCFA), and exertion of several positive effects on colonocytes and colonic microbiota (6, 8–11). In the present meta-analysis, we aimed to systematically review the evidence from randomized controlled studies evaluating dietary fiber supplementation in the prevention of diarrhea in hospitalized patients requiring tube feeding.

Methods

This systematic review and meta-analysis was conducted following a protocol registered in PROSPERO (CRD42021279971) and reported in accordance with the Preferred Reporting Items of Systematic Reviews and Meta-Analyses (PRISMA) guideline (12).

Search and information sources

We systematically searched the Web of Science Core Collection, PubMed, Medline (OVID), Embase (OVID), and Scopus databases from inception to 1 September 2022. Cochrane Central Register of Controlled Trials (CENTRAL) and ClinicalTrials.gov were also searched for the trial registry. We also searched the reference lists of included full texts for additional articles. The search was limited to adult patients. No language limit was applied.

Search terms as free texts and MeSH terms related to "tube feeding" or "enteral nutrition," "fiber," and "diarrhea" or "bowel movement" were used. The following fibers reported in the literature were also used as search terms: inulin, psyllium, fructooligosaccharides (FOS), oligofructose, oligosaccharides, wheat brans, soy polysaccharides, lignin, and resistant starch. The full search strategy is available in Supplementary Table S1.

Eligibility criteria

Studies were included if they were randomized controlled trials (RCTs), comparing fiber supplementation

or fiber-enriched EN formula (any fiber type) with fiber-free EN formula and reporting the incidence/event outcome of diarrhea. Study participants were adults (aged ≥18 years old) and hospitalized in the intensive care unit (ICU) or non-ICU, receiving EN support with or without parenteral nutrition. Studies were excluded if there was no control arm or if patients received EN of <1,000 kcal/day.

Study selection and data extraction

All searched records were exported to EndNote (EndNote X8, Thomson Reuters, NY, USA) and deduplicated. Two reviewers (PS and AK) independently screened the titles and abstracts of eligible papers. When there were disagreements between the two reviewers, a consensus was reached out and the third reviewer (CC or PW) was consulted. Data extraction was performed independently by PS and AK. CC was consulted when there were any problems related to data extraction. Data extraction was performed for authors, years, title, population characteristics and setting, fiber types and dosage, the duration of EN, energy intake, the definition of diarrhea and/or methods for measuring diarrhea, and the incidence or event rate of diarrhea.

Risk of bias (quality) assessment

Two reviewers (PS and AK) independently assessed the risk of bias among the included papers using the Cochrane Risk of Bias 2.0 tool (RoB2) for RCTs (13). The RoB2 comprises five domains: bias arising from the randomization process, bias due to deviations from intended interventions, bias due to lack of outcome data, bias in outcome measurement, and bias in the selection of the reported result.

Data synthesis

The incidence or event rate of diarrhea was pooled using the Mantel–Haenszel methods (for the binary outcome) and presented as risk ratio (RR) and 95% confidence interval (CI). A random-effects model was applied for pooled estimates due to the increased chance of high heterogeneity among included studies. The I^2 statistic was used to assess heterogeneity. A heterogeneity of >50% will be judged as high, with a p-value of < 0.10 for significance. Sources of heterogeneity were explored by subgroup analysis/sensitivity analysis. Pre-priori subgroup analyses were planned for the non-ICU and ICU settings, low vs. high RoB studies, and fiber types. All statistical analyses were performed using R software, version 4.1.0 (R Foundation, Austria) with the Metafor package. A two-sided p-value of < 0.05 was considered statistically significant.

Publication bias was analyzed using the funnel plot and Egger's test for funnel plot asymmetry. The Grading of Recommendations, Assessment, Development, and Evaluations (GRADE) system was applied for pooled results, which comprises types of study, quality of methodology, consistency of outcomes, directness, effect size, and publication bias (14, 15).

Results

A total of 4,469 records were retrieved from the literature search, and three records were from additional sources. After removing duplicates, two reviewers independently screened 3,569 records for titles and abstracts, resulting in 27 full texts that were assessed for eligibility criteria. There were 17 RCTs evaluating the role of fiber supplementation on the outcomes of diarrhea in hospitalized tube-fed patients. Of these 17, only one RCT explored the role of fiber (banana flakes) vs. routine medical treatment in patients who already developed diarrhea (16), and the remaining 16 RCTs were conducted in a general tube-feeding setting to evaluate the occurrence of diarrhea. The last 16 RCTs were included in the present meta-analysis (Figure 1). Excluded full texts are shown in Supplementary Table S2, with reasons.

Study characteristics and bias of included RCTs

Most RCTs were conducted in ICU patients ($n = 11$) (17–27), followed by postoperative patients ($n = 3$) (28–30) and hospitalized patients ($n = 2$) (31, 32). The majority of RCTs investigated fiber supplements in EN using soy polysaccharides ($n = 4$), followed by mixed soluble/insoluble fiber ($n = 3$), psyllium ($n = 3$), PHGG ($n = 2$), pectin ($n = 2$), Shen jia ($n = 1$), and polydextrose ($n = 1$). The median duration of fiber supplementation was 10 days, ranging from 5 to 21 days. Diarrhea was defined based on diarrhea score, number of bowel movements per day, and Bristol or King stool chart (Table 1).

More than half of the included RCTs ($n = 9$) are at high risk of bias (Table 2). Of these, eight RCTs did not report information about whether outcome assessors were aware of the intervention received by study participants. For such reasons, the assessment of the outcome could have been influenced by knowledge of the intervention received. The funnel plot of 16 RCTs shows no publication bias, with Egger's p-value being 0.216 (Supplementary Figure S1).

Effect of fiber supplementation on the incidence of diarrhea

A meta-analysis of all 16 RCTs showed that fiber supplementation prevented the occurrence of diarrhea in

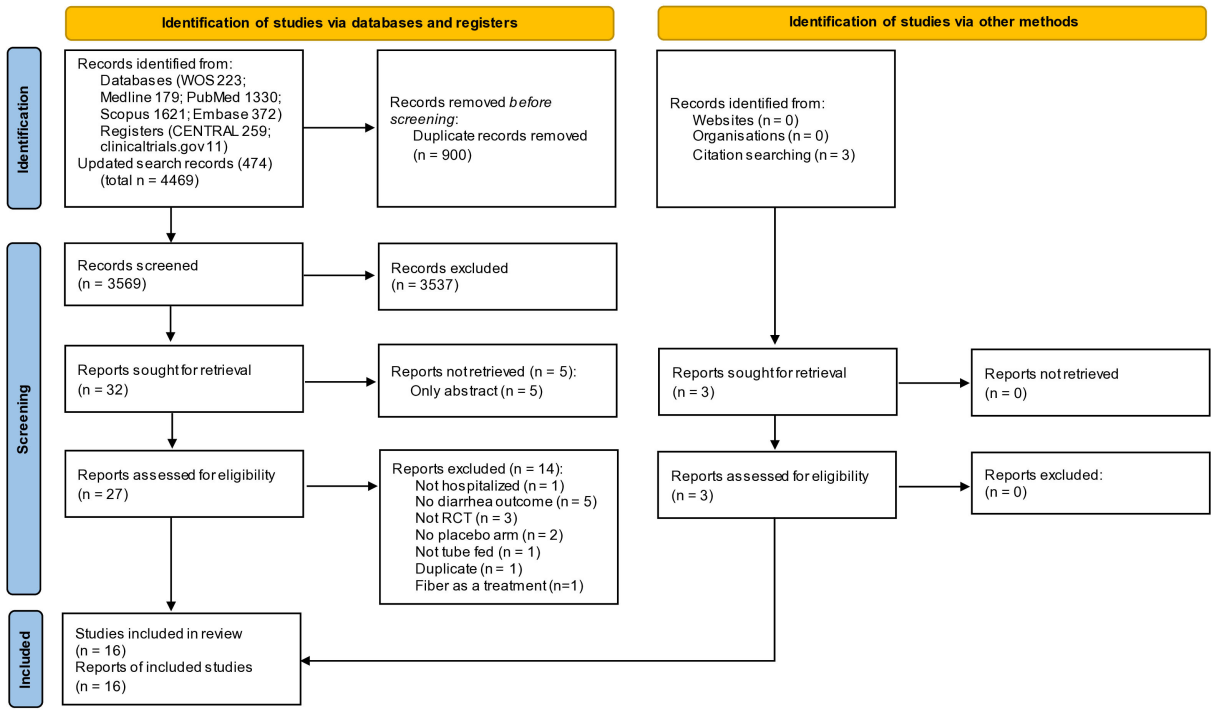

FIGURE 1
Preferred reporting items of systematic reviews and meta-analyses (PRISMA) flow diagram.

hospitalized patients receiving EN by 36% compared to the fiber-free formula (pooled RR of 0.64 [95%CI: 0.49–0.82, $p = 0.005$]; $I^2 = 45.1\%$; Figure 2), with the GRADE assessment of moderate certainty (Table 3).

Among the 11 RCTs conducted in the ICU setting, there was a 36% significant reduction in the incidence of diarrhea after fiber supplementation (pooled RR 0.64, 95%CI 0.47–0.87, $I^2 = 41.7\%$; Figure 2). In the non-ICU setting, a large effect size was observed in reducing the incidence of diarrhea (pooled RR 0.61, 95%CI 0.37–1.02), with high heterogeneity ($I^2 = 57\%$, $p = 0.05$; Figure 2). Similarly, the subgroup analysis between RCTs with a low risk of bias showed a large magnitude of effect size for the prevention of diarrhea (pooled RR 0.59, 95%CI 0.34–1.02), consistent with the pooled result of RCTs with some concerns or a high risk of bias (pooled RR 0.65, 95%CI 0.48–0.88; Figure 3).

According to sensitivity analyses, we analyzed fiber types with at least two RCTs to explore whether fiber types posed different outcomes (Figure 4). These included soy polysaccharides ($n = 4$), psyllium ($n = 3$), mixed soluble/insoluble fiber ($n = 3$), pectin ($n = 2$), and PHGG ($n = 2$). There were reductions in post-feeding diarrhea in patients receiving EN containing mixed soluble/insoluble fiber and PHGG (pooled RR 0.54, 95%CI: 0.39–0.75, $I^2 = 0\%$ and pooled RR 0.47, 95%CI: 0.27–0.83, $I^2 = 0\%$, respectively), while the remaining fiber types posed no benefits (Figure 4).

Discussion

The current systematic review and meta-analysis examines the efficacy of fiber supplementation on the outcomes of diarrhea in hospitalized patients receiving tube feeding. We included only data from randomized control studies. Overall, fiber supplementation was significantly associated with a reduced risk of developing diarrhea in such patients (pooled RR of 0.64 [95% CI: 0.49–0.82, $p = 0.005$]), but with moderate heterogeneity ($I^2 = 45.1\%$).

We further performed sensitivity analyses to identify plausible explanations for the heterogeneity of the results. As determined *a priori*, sensitivity analyses regarding patient settings (ICU vs. non-ICU), low vs. high RoB studies, and fiber types were conducted. Regarding the patient settings, the benefit of fiber supplementation was observed in both critically ill patients and patients admitted to general medical or surgical wards, with similarly pooled RRs of 0.64 and 0.61, respectively. Although only patients in the ICU group reached a statistically significant level, patients in the non-ICU setting had a 95% CI slightly above 1 (95%CI: 0.37–1.02). Moreover, moderate heterogeneity persisted in both ICU and non-ICU patients.

Similar results were observed when we conducted sensitivity analyses of studies with low and high RoB; the effect sizes of fiber

TABLE 1 Characteristics of included randomized controlled trials (RCTs).

Author	Intervention			Control			Setting	Outcome time	Diarrhea definition	Route of EN	Fiber dosage
	Fiber types	No. diarrhea	No. total	Control	No. diarrhea	No. total					
ICU setting											
Frankenfield and Beyer (18)	soy polysaccharide	3	9	Ensure	4	9	ICU head injury	6 days	1/3 criteria	NG	14 gm/L
Dobb and Towler (19)	soy polysaccharide	16	45	Ensure	13	46	ICU	18 days	diarrhea score >12	NG/PEG	21 gm/L
Tuncay et al. (26)	soy polysaccharide	2	23	Osmolite	13	23	Neurological ICU	21 days	not defined	NG/PEG	14.4 gm/L
Chittawatanarat et al. (23)	mixed soluble/insoluble	4	17	Nutren Optimum	8	17	ICU	14 days	diarrhea score >12	no defined	15.1 gm/L
Yagmurdur and Leblebici (24)	mixed soluble/insoluble	22	60	Nutrison	38	60	MICU	5 days	diarrhea score >12	NG	15 gm/L
Hart and Dobb (17)	psyllium	19	35	Osmolite	19	33	ICU	18 days	diarrhea score >12	NG	7 gm/d
Belknap et al. (20)	psyllium hydrophilic mucilloid	8	37	Ensure / Osmolite	7	23	Medical and surgical ICU	7 days	≥3 bowel movement a day	NG/PEG	14 gm/d
Schultz et al. (21)	pectin	4	11	Osmolite	1	11	ICU	8 days	diarrhea score >12	feeding tube	1.07 g/d
Xi et al. (25)	pectin	7	62	Peptisorb	16	63	ICU	6 days	not defined	NJ	24 gm/day
Spapen et al. (22)	PHGG	6	13	no label	11	12	Medical ICU	21 days	diarrhea score >12	NG	22 gm/L
Chen et al. (27)	polydextrose	2	24	no label	9	22	ICU	7 days	≥3 bowel movement a day	NJ	20 gm/d
Non-ICU											
de Kruif and Vos (28)	soy polysaccharide	8	30	Osmolite	14	30	post-operative patients	5 days	diarrhea score >6 x 2 days	NG/NJ	20 gm/L
Jakobsen et al. (31)	mixed soluble/insoluble	5	26	no label	12	25	hospitalized patients	14 days	Daily defecation score >15	NG/PEG	15 gm/L
Lertpipopmetha et al. (32)	psyllium	18	42	Blendera	13	41	hospitalized medical patients	10 days	King's stool chart ≥15	NG	15.2 gm/L
Homann et al. (29)	PHGG	2	15	Nutrodrip	6	15	upper gastrointestinal surgery	10 days	≥3 bowel movements a day	jejunostomy	20 gm/L
Zhao et al. (30)	Shen jia	12	40	no label	24	40	gastric cancer post distal gastrectomy	7 days	King's stool chart ≥15	NJ	30 gm/day

ICU, intensive care unit; PHGG, partially hydrolyzed guar gum; EN, enteral nutrition; NG, naso-gastric; PEG, percutaneous endoscopic gastrostomy; NJ, naso-jejunostomy.

10.3389/fnut.2022.1008464

TABLE 2 Risk of bias of included RCTs.

References	Experimental	Randomization process	Deviations from intended interventions	Missing outcome data	Measurement of the outcome	Selection of the reported result	Overall bas
Hart and Dobb (17)	Psyllium	Some concerns	Low	Low	High	Low	High
Frankenfield and Beyer (18)	Soy polysaccharide	Some concerns	Low	Low	High	High	High
Dobb and Towler (19)	Soy polysaccharide	Low	Low	Low	High	Low	High
de Kruif and Vos (28)	Soy polysaccharide	Low	Low	Low	High	Low	High
Homann et al. (29)	PHGG	Some concerns	Low	Low	Low	Low	Some concerns
Belknap et al. (20)	Psyllium	Low	Some concerns	Low	High	Low	High
Schultz et al. (21)	Pectin	Some concerns	Low	Low	Low	Low	Some concerns
Spapen et al. (22)	PHGG	Some concerns	Low	High	Low	Low	High
Chittawatanarat et al. (23)	Mixed soluble/insoluble	Low	Low	Low	Low	Low	Low
Yagmurdur and Leblebici (24)	Mixed soluble/insoluble	Low	Low	Low	Low	Low	Low
Jakobsen et al. (31)	Mixed soluble/insoluble	Low	Low	Low	Low	Low	Low
Xi et al. (25)	Pectin	Some concerns	Low	High	High	Low	High
Zhao et al. (30)	Shen jia	Low	Low	Low	High	Low	High
Tuncay et al. (26)	Soy polysaccharide	Some concerns	Low	High	High	Low	High
Lertpipopmetha et al. (32)	Psyllium	Low	Low	Low	Low	Low	Low
Chen et al. (27)	Polydextrose	Low	Low	Low	Low	Low	Low

PHGG, partially hydrolyzed guar gum.

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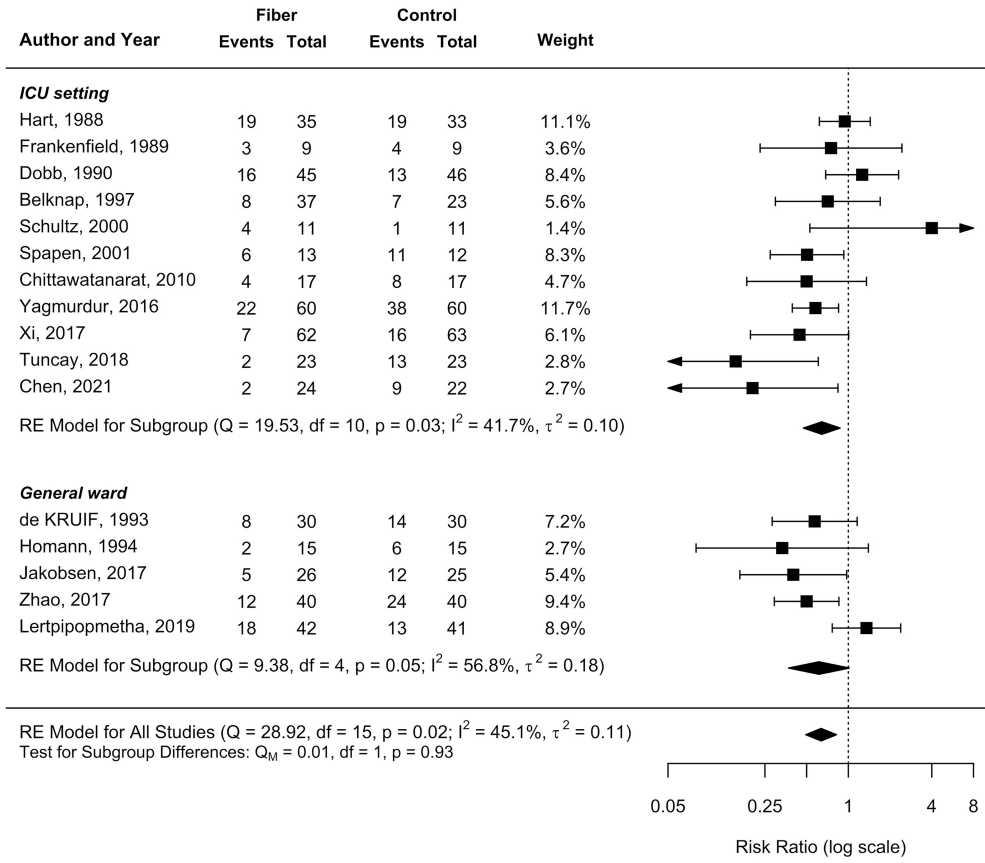

FIGURE 2
Effect of enteral nutrition (EN) containing fiber supplements on the incidence of diarrhea.

TABLE 3 Summary of findings with the grading of recommendations, assessment, development, and evaluations (GRADE) assessment.

Summary of findings

Fiber in EN compared to non-fiber formula for prevention of diarrhea in patients with tube-feeding

Patient or population: prevention of diarrhea in tube-fed patients

Setting: in hospital

Intervention: fiber in EN

Comparison: non-fiber formula

Outcome	Relative effect	Anticipated absolute effects* (95% CI)			Certainty	What happens
No of participants (studies)	(95% CI)	Without fiber	With fiber	Difference		
Diarrhea No of participants: 959 (16 RCTs)[a]	**RR 0.64** (0.49 to 0.82)	44.3%	**28.3%** (21.7 to 36.3)	**15.9% fewer** (22.6 fewer to 8 fewer)	⨁⨁⨁◯ Moderate[a,b]	Fiber supplementation in enteral nutrition likely reduces diarrhea in tube-fed hospitalized patients

*The risk in the intervention group (95% CI) is based on the assumed risk in the comparison group and the relative effect of the intervention (95% CI).
EN, enteral nutrition; CI, confidence interval; RR, risk ratio.
GRADE working group grades of evidence.
High certainty: we are very confident that the true effect lies close to that of the estimate of the effect. Moderate certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different. Low certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect. Very low certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.
[a]Subgroup analysis between "low" and "some concern or high" risk of bias shows a similar direction and magnitude of pooled results. We did not downgrade for this domain.
[b]Downgrade one level due to inconsistency. The pooled result shows moderate heterogeneity, with fiber type as a possible source of heterogeneity.

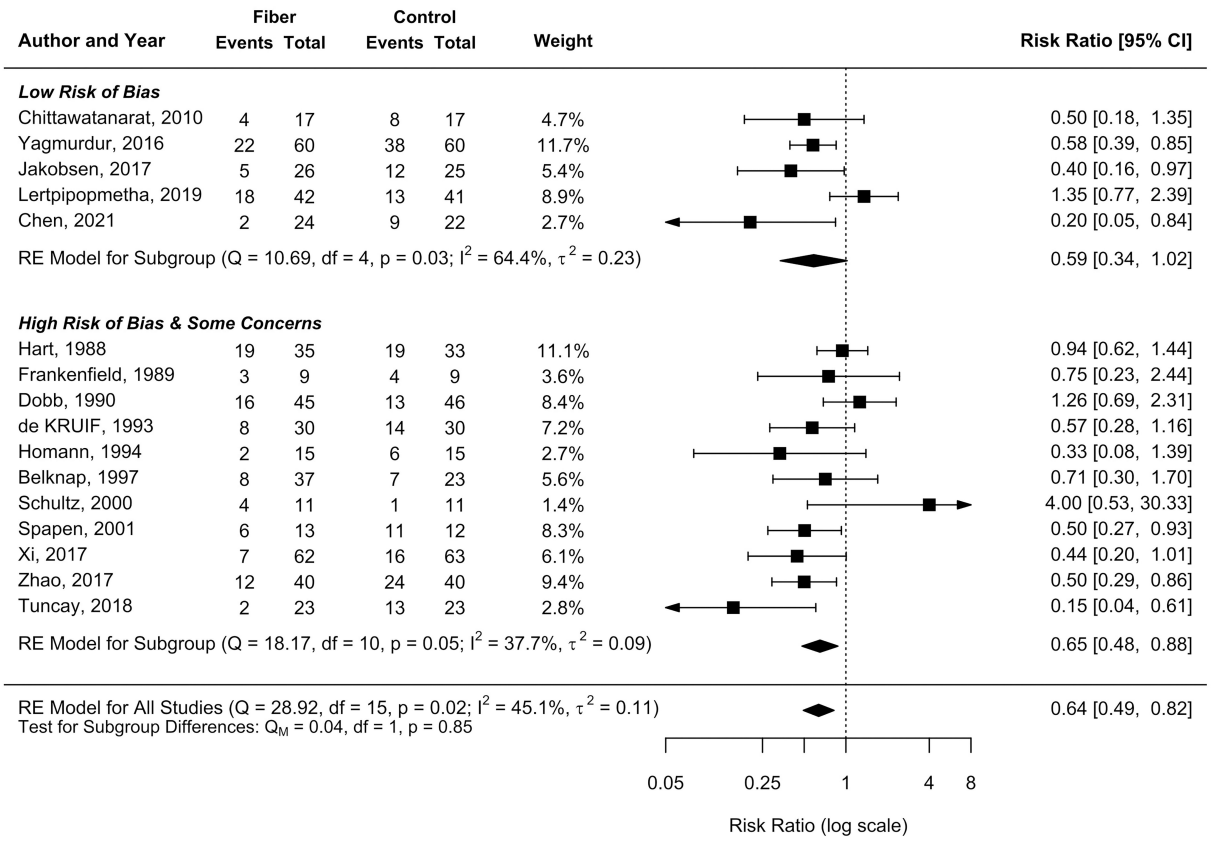

FIGURE 3
Subgroup by risk of bias on the effect of EN containing fiber supplements on the incidence of diarrhea.

supplementation in reducing the risk of occurrence of diarrhea were allied and reached a statistically significant in the high RoB study group with moderate heterogeneity, while the upper level of 95%CI in the low RoB study group was only 1.02, with high heterogeneity.

Interesting findings were observed in sensitivity analyses by fiber type. Soy polysaccharides, the most frequently studied fiber in the literature, resulted in a non-significant reduction in the outcome of diarrhea, with a high degree of heterogeneity. Psyllium and mixed soluble/insoluble fiber were evaluated in the following order, and each had three RCT data. Intriguingly, the pooled RR of both types of fiber showed no heterogeneity (I^2 = 0%) when sensitivity analyses were executed, and psyllium consistently showed no benefits in reducing the occurrence of diarrhea in patients receiving tube feeding, whereas mixed soluble/insoluble fiber significantly reduced the risk of developing diarrhea in such patients by 46% (RR 0.54 [95% CI: 0.39, 0.75]). The PHGG fiber type also showed a significant reduction in the incidence of diarrhea by 53% without heterogeneity. Compared with a recent meta-analysis by Cara et al. (33), mixed soluble/insoluble fiber did not reduce the incidence of diarrhea (RR 0.61 [95% CI: 0.37, 1.00]). However, such findings might be due to the high rate of diarrhea in a study by Schultz et al. (21).

To the best of our knowledge, this is the first study to show a novel finding of the significance of fiber types on the outcomes of diarrhea in hospitalized patients receiving EN. Our study results contradict a previous meta-analysis published in 2015, in which the benefit of fiber supplementation was observed only in non-critically ill patients and not in the ICU setting (34). Nevertheless, the current study results on the benefit of fiber supplementation in critically ill patients were consistent with a recent meta-analysis of dietary fiber in critical care patients published in 2021 (35). From our point of view, differences in the results between our meta-analysis and the prior meta-analysis by Kamarul Zaman et al. (34) might be due to differences in the study inclusion criteria, as we only included randomized control studies, and seven RCTs conducted after 2015 were added to our recent meta-analysis. Moreover, as shown in the aforementioned sensitivity analysis, the root cause of a variety of outcomes among RCTs might lay in the different types of fiber rather than in the critical care setting of patients.

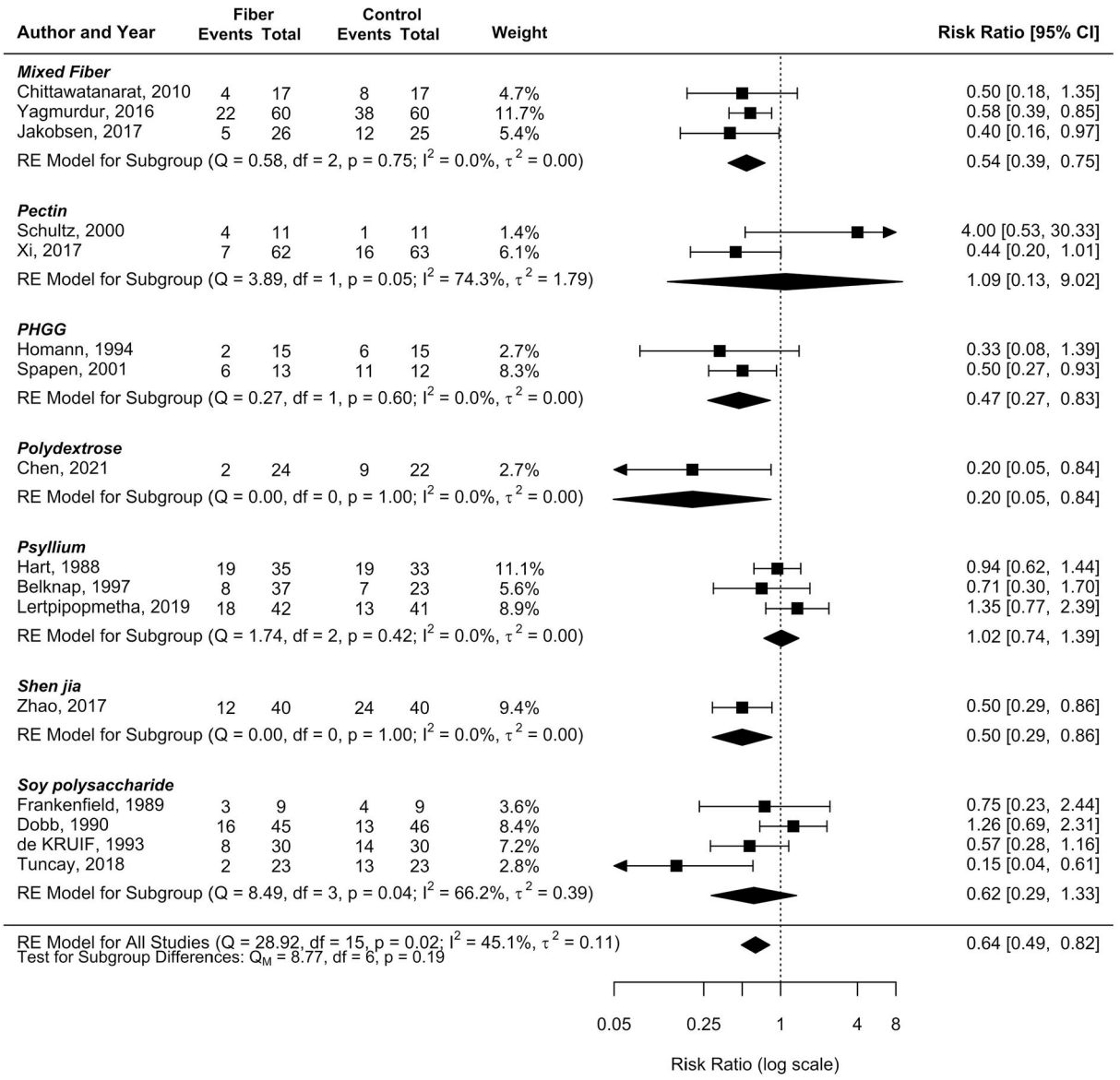

FIGURE 4
Effect of fiber types in EN on the incidence of diarrhea.

Theoretically, soluble fibers have the beneficial properties of reducing diarrhea with their water-holding capacity and increasing gut transit time, and they can be fermented by colonic bacteria to produce SCFAs and stimulate the uptake of water and electrolytes in the colon (8, 9, 11). However, when it comes to the results of clinical studies, not all soluble fibers yielded the same benefit on the outcomes of diarrhea. This might be due to the diversity of physiochemical characteristics of each fiber type. The presence of either a soluble or insoluble fiber in the ileum can stimulate the ileal brake, resulting in decreased gastric emptying and increased small intestinal transit time, making the whole gut to be delayed (6). Despite being a soluble fiber, psyllium is considered to have moderate viscosity and low fermentability (6). Guar gum, on the other hand, owes the characteristics of medium to high viscosity and high fermentability (6). A higher degree of viscosity may result in increased stool volume and longer colonic transit time, and increased fermentability, as well as increased integrity of colonic tight junctions, may provide a better microbiota environment in the colon, together leading to a better outcome for some types of fiber over others. This benefit may further minimize patients' morbidity, length of hospital stay, investigation cost, and

healthcare burden (3–5). Additionally, fiber supplementation is safe in hospitalized patients with stable hemodynamics (33). As such, our findings encourage healthcare professionals to recognize the beneficial effects of fiber supplementation in hospitalized patients receiving EN.

The strengths of our systematic review and meta-analysis are that we only included randomized controlled studies with a high-quality study design, from inception to the most recent timeframe, with over 700 patients from both critical and non-critical care settings, both surgical and medical patients. The source of heterogeneity can also be identified and minimized to the level of no heterogeneity in psyllium, PHGG, and mixed soluble/insoluble fiber subgroups using sensitivity analyses on fiber types. This novel finding and possible underlying mechanisms can be important in aiding the management of diarrhea in patients receiving EN in the future and for further studies.

Our meta-analysis also has limitations. There is a variation in the definition of diarrhea; some studies used scoring systems, while others counted the frequency of bowel movements or did not mention the definition of diarrhea in the study. This may influence the rates of occurrence of diarrhea in the included studies. Additionally, the fiber dosage varied; in some studies, the daily dosage of fiber was fixed in all patients in the fiber arm, whereas the fiber dosage administered to patients was dependent on the amount of calorie intake in a day in many studies, making an evaluation of the fiber dosage and the outcomes of diarrhea unattainable. Furthermore, there was a small number of participants in each fiber type; therefore, the power of performance assessment to determine the efficacy of different fiber types may be limited. Lastly, the variety of causes of critically ill patients could potentially affect the severity of post-feeding diarrhea, so further studies with a homogenous population should be conducted.

In conclusion, our recent systematic review and meta-analysis demonstrated a beneficial effect of fiber supplementation in minimizing diarrhea in hospitalized patients receiving tube feeding. However, not all fiber types yielded the same benefit; mixed soluble/insoluble fiber and PHGG are associated with a significant reduction in the risk of developing diarrhea, whereas studies on psyllium consistently showed no benefit over the fiber-free formula. For other types of fiber, no conclusion can be drawn at this time.

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.

Author contributions

Protocol development: PS, PW, CC, and AK. Systematic literature search: PW and CC. Study selection and data extraction and risk of bias assessment: PS and AK. Data analysis and manuscript writing: PS and CC. Critical revision of the manuscript: AK, PS, and CC. All authors approved the final version of the manuscript.

Acknowledgments

The content of this manuscript has been presented in part at the Digestive Disease Week 2022, San Diego, CA, USA (36).

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1008464/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Systematic Review
PUBLISHED 14 February 2023
DOI 10.3389/fnut.2023.920998

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Clinical Nutrition,
a section of the journal
Frontiers in Nutrition

RECEIVED 15 April 2022
ACCEPTED 18 January 2023
PUBLISHED 14 February 2023

CITATION
Wang L, Gan J, Wu J, Zhou Y and Lei D (2023)
Impact of vitamin D on the prognosis after
spinal cord injury: A systematic review.
Front. Nutr. 10:920998.
doi: 10.3389/fnut.2023.920998

Impact of vitamin D on the prognosis after spinal cord injury: A systematic review

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Vitamin D (VitD) insufficiency is a worldwide health problem and affects billions of people. Spinal cord injury (SCI) patients seem more susceptible to developing suboptimal levels of VitD. However, the literature regarding its impact on the prognosis of SCI is limited. Thus, in this review, we systematically investigated the published studies *via* a combination of keywords associated with SCI and VitD in four medical databases (Medline, Embase, Scopus, and Web of Science). All included studies were analyzed, and selected clinical data on the prevalence of VitD insufficiency (serum 25-hydroxyvitamin D < 30 ng/ml) and deficiency (serum 25-hydroxyvitamin D < 20 ng/ml) were collected for further meta-analysis *via* random effects. Through literature review, a total of 35 studies were eligible and included. The meta-analysis of VitD status (13 studies, 1,962 patients) indicated high prevalence of insufficiency (81.6% [75.7, 87.5]) and deficiency (52.5% [38.1, 66.9]) after SCI. Besides, low levels of VitD were reported to be associated with a higher risk of skeletal diseases, venous thromboembolism, psychoneurological syndromes, and chest illness after injury. Existing literature suggested that supplemental therapy might act as an adjuvant treatment to facilitate post-injury rehabilitation. Non-human experimental studies highlighted the neuroprotective effect of VitD, which was associated with enhancing axonal and neuronal survival, suppressing neuroinflammation, and modulating autophagy. Therefore, the current evidence suggests that the prevalence of VitD insufficiency is high in the SCI population, and low-level VitD may impair functional restoration after SCI. VitD supplemental treatment may have potential benefits to accelerate rehabilitation in mechanistically related processes after SCI. However, due to the limitation of the available evidence, more well-designed randomized controlled trials and mechanism experimental research are still needed to validate its therapeutic effect, elucidate its neuroprotective mechanism, and develop novel treatments.

KEYWORDS

spinal cord injury, vitamin D, deficiency, neuroprotection, progonosis, insufficiency

1. Introduction

Vitamin D (VitD) insufficiency and deficiency are described as a pandemic associated with various chronic diseases in all age populations despite the commercialization of VitD supplements and ongoing prophylaxis projects in the general population (1, 2). The national health and nutrition examination survey in the United States indicated that 23–24% and 64% population had a serum total of 25(OH)D less than 20 and 30 ng/mL, respectively (3, 4). In the European Union, Cashman et al. (5) summarized multiple national surveys and indicated that 40.4% of European individuals had serum 25(OH)D concentrations less than 20 ng/ml on average. Spinal

cord injury (SCI) is a potentially devastating event featured by severe sensorimotor deficits and autonomic dysfunction with a high burden on both family and society. SCI patients seem to be more susceptible to VitD insufficiency and deficiency due to an unbalanced diet, co-existing diseases, obesity, and lack of sunlight exposure secondary to physical inactivity (6, 7). Besides, several researchers indicated that post-injury autonomic dysfunction usually caused malfunctioning of endocrinological feedback systems, such as VitD-parathyroid hormone axis, and impaired VitD absorption through skin gastrointestinal tract, which directly led to low levels of VitD in SCI individuals (8–10). However, the mechanism underlying post-SCI VitD level alternation is still poorly understood, and literature summarizing its status and impact on the post-injury prognosis is limited.

Despite its classical role in calcium and phosphorus metabolism, VitD is a neurosteroid and exerts a neuroprotective effect in various neurological diseases. In an animal model of Parkinson's disease, VitD treatment attenuated the injury of dopaminergic neurons *via* suppressing the release of proinflammatory cytokines and upregulating anti-inflammatory signaling (11). Similar protective findings were also observed in the facial nerve injury model, in which the administration of $VitD_3$ could facilitate functional restoration by increasing myelination after 12 weeks of treatment (12).

Recent evidence highlighted the unique value of VitD in post-SCI rehabilitation. VitD deficiency in individuals with SCI has been implicated as a primary etiologic, and environmental factor responsible for multiple musculoskeletal issues (e.g., osteoporosis, fracture, chronic pain, etc.) (6, 7). At the same time, low-level VitD is associated with an increased risk of several neuropsychic diseases (e.g., chronic pain, depression, anxiety, PTSD, etc.) (13, 14). New findings also suggest that VitD insufficiency and deficiency were associated with malfunctioning of autonomic nerve system (e.g., a change of endocrinological feedback systems, impaired cardiac autonomic functions, etc.), which might delay the SCI rehabilitation (8, 15). In addition, the pressure injury, an often-occurring complication after SCI, was also reported to be associated with VitD status (16). Notably, Aminmansour et al. (17) applied a combination therapy of progesterone and VitD in a randomized, double-blinded, placebo-controlled study of acute traumatic SCI. They observed that this treatment plan was associated with better functional outcomes. Nevertheless, the published clinical and experimental studies regarding the effect of VitD in SCI are few, and its therapeutic effect remains to be determined.

In this review, we hypothesized that VitD might play an essential role in post-SCI rehabilitation. To validate this hypothesis, we performed a meta-analysis of published studies regarding the prevalence of VitD insufficiency and deficiency in SCI patients, and systematically summarized the clinical and experimental evidence of VitD's effect in post-SCI rehabilitation.

Abbreviations: BDNF, Brain-derived neurotrophic factor; CNS, Central nerves system; IL, Interleukin; NIH, National Institutes of Health; NMDA, N-methyl-D-aspartic acid; PTH, Parathyroid hormone; RXR, Retinoid X receptor; SCI, Spinal cord injury; TNF-α, Tumor necrosis factor-alpha; VDBP, Vitamin D binding protein; VDR, Vitamin D receptor; VDRE, Vitamin D response element; VEGF, Vascular endothelial growth factor; VitD, Vitamin D; UVB, Ultraviolet B.

2. Methods

2.1. Search strategy, study selection, and eligibility criteria

This study was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA, PRISMA 2020 checklist in supplement materials), and approved by the Ethics Committee of the Tongji Medical College, Huazhong University of Science and Technology (18). Details of this systematic review and meta-analysis were registered on PROSPERO (registration number 2021: CRD42021262207), and can be accessed at[1]. It was a secondary analysis of the completed studies, and the written consent was waived, respectively. A combination of keywords on VitD and SCI and Boolean Operators were applied in four medical databases (Medline, Embase, Scopus, and Web of Science) to retrieve English literature in September 2021. Per the formulated literature search (search strategy in supplement materials), we sought studies (published between 1974 and August 31, 2021) on the VitD status and its impact on the prognosis after spinal cord injury. Both clinical and non-human experimental studies were included in this review to investigate the impact of VitD on the prognosis of SCI and its underlying neuroprotective mechanism.

The retrieved studies were verified and quantified before merging *via* Endnote X9 software (Clarivate Analytics), and the duplicates were removed accordingly. The titles, abstracts, and full texts of identified publications were further evaluated to search for eligible studies. The search was supplemented by the reference list of review articles and selected publications. Reviews, book chapters, case reports, small case series (case number less than 10), conference abstracts, editorial notes, and letters were excluded. Other exclusion criteria included repeated research on the same cohort of patients and unavailable full text. Two independent investigators comprehensively reviewed all selected publications to ensure their eligibility for inclusion in the review. Any disagreement regarding the eligibility of the literature was resolved by thoroughly discussing the publication with a third independent investigator.

2.2. Inclusion and exclusion criteria, data extraction, and quality evaluation of meta-analysis

Among the eligible articles, the studies that reported VitD status (VitD deficiency and insufficiency) in the SCI population were selected for meta-analysis. The spinal cord injury (SCI) is defined as damage to the spinal cord resulting from trauma (e.g., result of accident, fall, etc.) or from nontraumatic disease (e.g., degeneration of the spinal column, tumor, etc.). Both traumatic and non-traumatic SCI were included in this review.

The exclusion criteria include (1) studies in a specific population (i.e., athletes), (2) research without qualified data, (3) and the majority of the patients already received VitD treatment. We applied the assessment tool for case series studies from the National Institutes of Health (NIH) to rate the quality of eligible studies in the metanalysis (19). There are nine criteria in the score. For each item, we scored one

1 www.crd.york.ac.uk/prospero/display_record.php?RecordID=262207

point for "yes" and zero point for "no," "not available," "not reported," and "cannot determine." The scores are gauged from all nine criteria (0–9) and generated to represent the overall quality of the study. Data were extracted by two reviewers and included: first author; publication year; country; case number; mean or media age; gender ratio; study design; VitD measure parameter; mean or media injury duration; injury type (acute and chronic); injury extent (incomplete and complete motor function impairment); injury level (paraplegia and tetraplegia); serum VitD status (insufficiency and deficiency).

Based on the international standard for neurological classification spinal cord injury (ISNCSCI), the ASIA Impairment Scale (AIS) is applied to evaluate the injury extent. Unless otherwise stated, complete motor function impairment is used when no motor preserved below the neurological level (AIS A and B) with incomplete motor function impairment referring to AIS C and D (20). Unless otherwise stated, tetraplegia is defined as the impairment or loss of sensorimotor function in the cervical levels of the spinal cord, and paraplegia refers to the impairment or loss of sensorimotor function in the thoracic, lumbar or sacral (but not cervical) segments of the spinal cord (20).

Per clinical practice guidelines of Endocrine Society, we used the serum 25-hydroxyvitamin D [25(OH)D] level to evaluate VitD status in SCI patients (21). We defined VitD insufficiency as serum concentration of 25(OH)D less than 30 ng/ml and VitD deficiency as serum concentration of 25(OH)D less than 20 ng/ml in this study (21, 22).

2.3. Statistical analysis

A single-arm meta-analysis was performed *via* Stata (version 15.1). The combined prevalence and 95% confidence interval of VitD deficiency and insufficiency were calculated by the random-effects model. Egger's and Begger's tests were performed to assess publication bias, and $p < 0.05$ was considered statistically significant. Subgroup analyses (i.e., case number, age, gender, in/out-patient, acute/chronic injury, injury level, injury duration, injury extent, etc.) were carried out to discuss and clarify the source of heterogeneity.

3. Results

3.1. Literature selection

We initially identified 1,341 publications after the systematic search in the four databases, including 135 from Medline, 491 from Embase, 429 from Scopus, and 286 from Web of Science. Among these, 634 papers were removed as duplicates and 707 unique studies were assessed for eligibility according to article type, title, abstract, and full texts (Figure 1). Two papers were supplemented from the references list of existing publications. Ultimately, 35 studies were included for the further systematic review and meta-analysis and presented in the supplemental material. There were 21 studies showing post-SCI VitD status (Table 1, (22–34) and Table 2, (35–42)), seven studies regarding the adverse effects associated with a low level of VitD (Table 3, (22, 23, 27, 28, 33, 43, 44)), eight studies that evaluated the potential therapeutic effect of VitD supplement (Table 4 (17, 35, 45–50)), and five non-human experimental researches exploring the underlying mechanism of VitD after SCI (Table 5, (51–55)).

3.2. Meta-analysis of vitamin D status in the patients with SCI

3.2.1. Included literature for the meta-analysis

In the 21 studies regarding VitD status after SCI, three studies investigated specific populations (e.g., children, athletes, etc.), one study involved patients receiving extra VitD supplements, and four studies were unqualified for meta-analysis, which were excluded accordingly. Thus, the remaining 13 investigations were included for further analysis, and their details were summarized in Table 1.

3.2.2. The prevalence of insufficient and deficient VitD status after SCI

Among the selected articles, 12 studies reported the incidence of VitD insufficiency, and 11 studies recorded the prevalence of VitD deficiency, which were analyzed, respectively. The quality score of the selected studies varied from 3 to 7 points (Supplementary Table S1).

The prevalence of insufficient VitD status after SCI was reported from 61.2% to 96.0% in the 12 eligible studies with 1,920 participants. The pooled prevalence determined by the random-effects model was 81.6% (95% CI: 75.7–87.5) with significant heterogeneity ($I^2 = 92.5\%$, $p = 0.00$, Figure 2A). The p values of Egger's and Begg's tests were 0.091 and 0.537 respectively, suggesting no significant publication bias. Subgroup analysis was performed as described (Supplementary Tables S2–S8) to further investigate potential heterogeneity sources. There was more substantial heterogeneity among studies with less than 200 patients, and years of injury less than 10 years. Additionally, insufficient VitD status were more prevalent in the studies with participants less than 200 (patients ≤ 200, $p = 0.01$), more female patients (male/female ≤ 3, $p < 0.01$) and media or mean years of injury less than 10 years ($p < 0.01$).

Regarding the prevalence of VitD deficiency after SCI, 11 articles with 1,580 participants were involved in the meta-analysis. The reported incidence ranged from 24.9 to 83.3%. The overall incidence calculated *via* the random-effects model was 52.5% (95% CI: 38.1–66.9). The I^2 was 97.6%, which suggested significant heterogeneity among selected studies ($p < 0.01$, Figure 2B). The Egger's and Begg's tests exhibited that p values were 0.051 and 0.213 respectively, suggesting no significant publication bias. A similar subgroup analysis was performed to evaluate potential heterogeneity (Supplementary Tables S2–S8). More substantial heterogeneity was also observed in the research with less than 200 patients, and years of injury less than 10 years. Besides, VitD deficiency was more severe in the studies with smaller sample size (patients ≤ 200, $p < 0.01$), younger patients (mean or media Age ≤ 46 years, $p < 0.01$), more female patients (male/female ≤ 3, $p < 0.01$), and media or mean years of injury less than 10 years ($p < 0.01$).

3.3. Potential adverse impact of VitD insufficiency and deficiency on the post-SCI complications

Through this systematic literature search, we found seven eligible studies regarding on the post-SCI complications which might be associated with low levels of VitD (Table 3). The potential adverse impact included poor physical function (23), depressive symptoms (43), venous thromboembolism (28), heterotopic ossification and hyperparathyroidism (44), chest and respiratory symptoms (22, 27, 33).

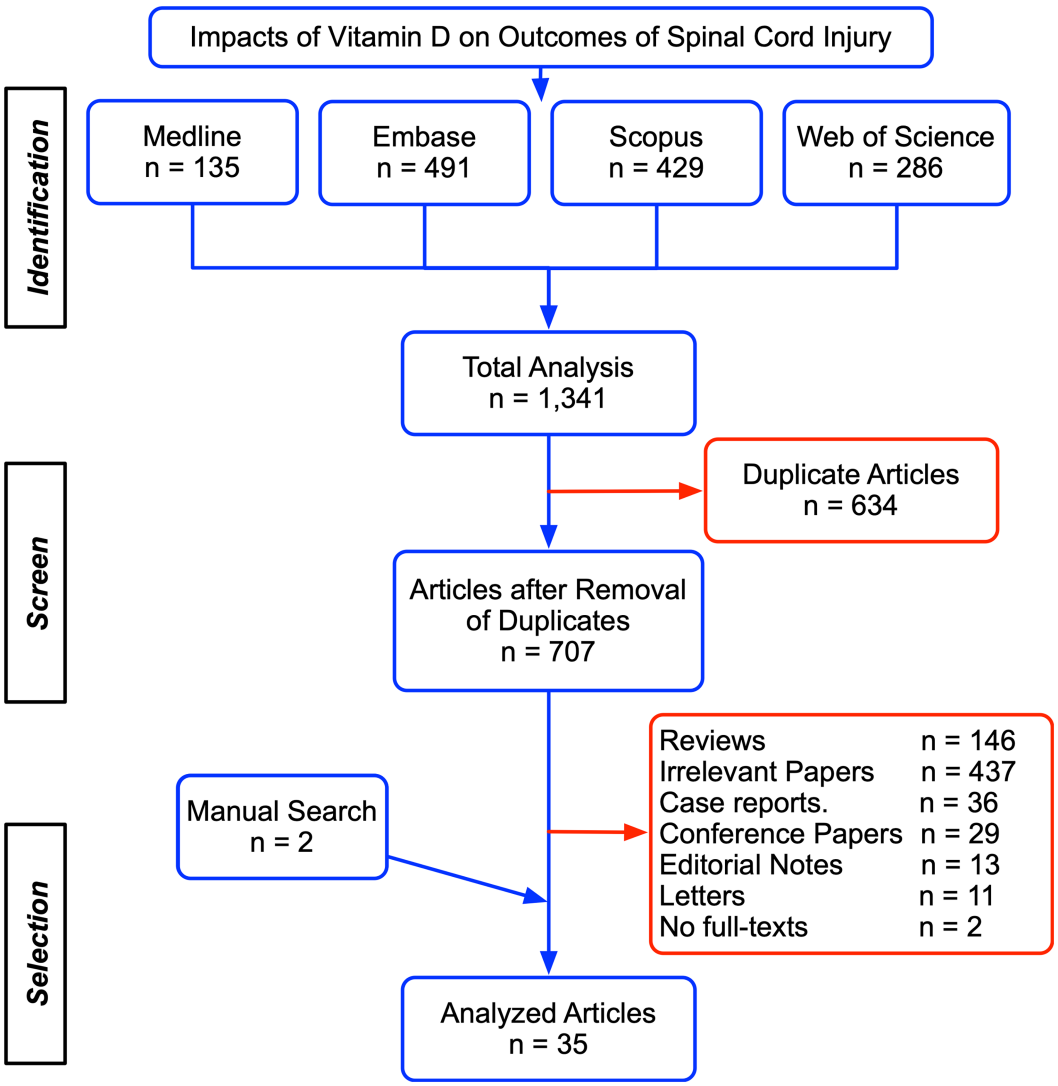

FIGURE 1
Study selection and characteristics.

3.4. Therapeutic effects of vitamin D supplements on the outcome of SCI

Earlier research suggested insufficient levels of VitD were prevalent among persons with SCI. The recommended dietary allowance for VitD is 600 IU/d for ages 1–70 years and 800 IU/d for those >70 years per the dietary reference intake report for calcium and VitD from the Institute of Medicine (56). Supplemental treatments with a dose similar to or higher than the VitD recommended dietary intakes were adopted in SCI patients to maintain an appropriate VitD level. Among the selected studies, there was eight clinical reports exhibiting the therapeutic effects of VitD supplements on the outcome of acute and chronic SCI. The existing literature suggested the VitD supplements had multiple beneficial effects on post-SCI rehabilitation, including correcting dysregulation of VitD-PTH axis (35, 45, 49, 50), reducing bone resorption (46, 48), facilitating motor and sensory functional restoration (17, 35, 50), improving carbohydrate metabolism (47), etc. (Table 4).

3.5. Neuroprotective effect of vitamin D in non-human experimental SCI

In the non-human experimental SCI, we retrieved five studies supporting the neuroprotective effect of VitD supplementation (51–55). The proposed mechanisms are multidimensional, involving promoting axonal survival (51, 52), reducing neuronal loss by attenuating oxidative stress (53, 55), suppressing neuroinflammation (54) and modulation of autophagy ((55), Table 5).

4. Discussion

In this systematic review, we included a total of 35 studies regarding its impact on the prognosis of SCI. The limited available studies suggest that the prevalence of VitD insufficiency and deficiency is relatively high in the SCI population, which may be associated with delayed neurofunctional restoration and several systematic complications. Besides, the current evidence from clinical and experimental studies

TABLE 1 The summarization of included studies in meta-analysis regarding the prevalence of vitamin D insufficiency and deficiency in SCI patients.

Author, year	Country	Case	Age	Gender (M/F)	Design	Measure.	Participants	Year of injury	Spinal cord injury		Injury extent (Motor)		Injury level		VitD levels	
									Acute	Chronic	Comp.	Incomp.	Tetra.	Para.	Ins.	Def.
Barbonetti, 2016 (23)	Italy	100	51.7	72/28	Cross-section	25(OH)D	Outpatients	6.4	0	100	60	40	39	61	96	78
Bassuino, 2018 (24)	Brazil	39	35.5	39/0	Cross-section	25(OH)D	Outpatients	6.1	0	39	23[a]	16[a]	9	30	34	24
Bauman, 1995 (25)	USA	100	51.0	N.A.	Cross-section	25(OH)D; 1, $25(OH)_2D$	N.A.	20	0	100	N.A.	N.A.	51	49	78	48
Coskun, 2016 (26)	Turkey	42	33.5	28/14	Case–Control	25(OH)D	N.A.	1.4	N.A.	N.A.	10	32	14	28	N.A.	35
Clark, 2020 (27)	USA	253	53.1	208/45	Cohort study	25(OH)D	Outpatients	13.8	0	253	N.A.	N.A.	59	194	195	64
Ehsanian 2019 (28)	USA	282	45.0	202/80	Cohort study	25(OH)D	Inpatients	N.A.	282	0	123	159	144	138	227	N.A.
Garshick, 2019 (22)	USA	312	53.9	260/52	Cross-section	25(OH)D	Outpatients	17.4	0	312	N.A.	N.A.	76	236	237	84
Khammeree 2016 (29)	Thailand	85	N.A.	64/21	Cross-section	25(OH)D	N.A.	N.A.	21	64	34	51	30	55	52	27
Nemunaitis, 2010 (30)	USA	100	48.4	70/30	Case series	25(OH)D	Inpatients	N.A.	100	0	47[a]	53[a]	57	43	93	21[b]
Oleson, 2010 (31)	USA	96	36.8	67/29	Cohort study	25(OH)D	Inpatients & outpatients	N.A.	42[d]	54	96	0	41	55	78[c]	55
Özgirgin, 2016 (32)	Turkey	125	35.2	76/49	Case–control	25(OH)D	Inpatients & outpatients	1.4	45[d]	80	86	39	31	94	119	103
Walia, 2018 (33)	USA	343	54.2	282/61	Cross-section	25(OH)D	Outpatients	17.4	0	343	N.A.	N.A.	82	261	257	93
Waliullah, 2021 (34)	India	85	30.8	60/25	Cross-section	25(OH)D	Inpatients	N.A.	85	0	28[a]	57[a]	0	85	65	50

[a]It represents the number of patients with complete and incomplete injury.

[b]This number refers to the patients with severe VitD deficiency (serum 25(OH)D ≤ 10 ng/mL).

[c]This number refers to the patients with VitD insufficiency (serum 25(OH)D ≤ 32 ng/mL).

[d]Acute SCI duration < 6 months.

25(OH)D, 25-hydroxyvitamin D; 1, $25(OH)_2D$, 1,25-hydroxyvitamin D; Comp., complete injury; incomp., incomplete; N.A., not available; Ins., insufficiency; Def., deficiency; Tetra, tetraplegia; Para, paraplegia.

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TABLE 2 The summary of the literature regarding VitD status after spinal cord injury (not involved in the meta-analysis).

Author, year	Case No.	Design	Population	Main findings
Flueck, 2016 (35)	19	PCS	Elite Athletes with SCI	All participants in the study showed an insufficient or deficient VitD status at the baseline measurement.
Hummel, 2012 (36)	65	CSS	Chronic and traumatic SCI	Disruption of the VitD-PTH axis was prevalent in SCI patients, which might lead to bone loss.
Javidan, 2014 (37)	160	CSS	Chronic and traumatic SCI	In Iranian patients with SCI, there was a high prevalence of VitD deficiency.
Mechanick, 1997 (38)	49	CCS	SCI	The suppressed levels of $1,25(OH)_2$-VitD were more frequently observed in the SCI population (66%).
Pritchett, 2016 (39)	39	CSS	Elite Athletes with SCI	A substantial proportion (~60%) of elite athletes with SCI have insufficient and deficient levels of 25(OH)-VitD in the autumn and winter.
Vaziri, 1994 (40)	40	CCS	Chronic SCI	The serum concentration of calcitriol was significantly lower in the SCI patients. However, there was no significant difference in plasma concentration of 25(OH)-VitD between the SCI and the control group.
Zebracki, 2013 (41)	82	CSS	Pediatric SCI	In comparison with the general pediatric population, pediatric SCI patients exhibited a higher prevalence of VitD insufficiency.
Zhou, 1993 (42)	92	CSS	Chronic SCI	Lower concentration of 25(OH)-VitD was observed in SCI patients, especially in the patients with pressure ulcers.

VitD, vitamin D; SCI, spinal cord injury; CCS, case–control study; CSS, cross-sectional study; PCS, prospective cohort study; PTH, parathyroid hormone.

TABLE 3 Literature summarization of the adverse effects associated with low levels of vitamin D after spinal cord injury.

Author, year	Case No.	Design	Population	Main findings
Barbonetti, 2016 (23)	100	CSS	Chronic SCI	In chronic SCI, low levels of 25(OH)D predicted poor physical function.
Barbonetti, 2017 (43)	100	CSS	Chronic SCI	Among the patients with chronic SCI, serum 25(OH)D levels were inversely associated with depressive symptoms.
Clark, 2020 (27)	253	PCS	Chronic SCI in veterans	In chronic SCI, patients with VitD deficiency had a higher risk of future chest diseases.
Ehsanian, 2019 (28)	282	RCS	Acute inpatient SCI	In acute SCI, without adequate VitD supplement, individuals with low levels of VitD had a higher risk of venous thromboembolism.
Garshick, 2019 (22)	312	CSS	Chronic SCI veterans	No cross-section relationship between VitD deficiency and reduced pulmonary function was observed in this cohort with chronic SCI.
Oleson, 2013 (44)	96	CSS	Acute and chronic SCI	The authors reported a significant correlation between hyperparathyroidism and heterotopic ossification as well as hyperparathyroidism and vitamin D deficiency.
Walia, 2018 (33)	343	CSS	Chronic SCI	The levels of VitD were not associated with respiratory symptoms in chronic SCI.

SCI, spinal cord injury; CSS, cross-sectional study; PCS, prospective cohort study; RCS, retrospective cohort study.

shows that VitD supplement treatment may have potential benefits to accelerate rehabilitation in mechanistically related processes after SCI.

4.1. The prevalence of insufficient and deficient VitD status after SCI

Previous literature regarding the VitD status after SCI was limited, and existing data remains controversial. Several reports from Veterans Affairs hospitals in the US observed that the prevalence of VitD deficiency was around 25% in chronic SCI populations (22, 27). In contrast, Coskun Benlidayi et al. (26) and Özgirgin et al. (32) reported the rate could reach 80%–90% in SCI participants. We thus performed the meta-analysis in this review to estimate the prevalence of insufficient and deficient VitD status in the general SCI population worldwide. Overall, we retrieved 13 eligible papers with 1,962 participants. Among them, 81.6% of SCI patients had a serum total 25(OH)D less than 30 ng/ml and 52.5% of participants with VitD deficiency. Compared with the general population, pediatric SCI patients exhibited a higher prevalence of VitD insufficiency (41). Additionally, Pritchett et al. (39) noticed that a substantial proportion (~60%) of elite athletes with SCI also had insufficient and deficient levels of 25(OH)D in the autumn and winter. Taken together, there was a high prevalence of VitD insufficiency and deficiency in the SCI population.

4.2. Potential adverse impact of VitD insufficiency and deficiency on the post-SCI complications

VitD plays a fundamental role in calcium and phosphate metabolism. Its insufficiency or deficiency is associated with a higher risk of bone diseases (57). Histological investigations recently indicated that VDRs have a wide distribution in non-skeletal tissues, including vessels, skin, muscles, endocrine glands, kidneys, neural tissue, etc.,

TABLE 4 The summary of clinical studies administrating different vitamin D supplemental regimens in SCI patients.

Author, year	Age (case No)	Gender (M/F)	Design	Injury (years)	Treatment	Therapeutic effect
Aminmansour, 2016 (17)	T: 42 ± 14 (n = 32); C: 45 ± 14 (n = 32)	T: 19/14; C: 16/16	RCT	Acute SCI within 8 h	Intramuscular injection of progesterone 0.5 mg/kg and oral 25(OH)D_3 200 IU/kg twice a day for 5 days on admission	The treatment group had significantly higher motor and sensory function after 6 months of therapy. Early administration (<4 h) showed additional benefits in motor and sensory function recovery.
Bauman, 2005 (45)	Study1: 53 ± 15 (n = 10); Study2: 43 ± 13 (n = 40)	N.A.	PCS	Study 1: 26 ± 13 years; Study 2: 12 ± 10 years	Study 1: Twice a week 2,000 IU 25(OH)D_3 for 2 weeks; Study 2: daily 800 IU 25(OH)D_3 supplementation for 12 months	Serum 25(OH)D_3 ↑; Serum parathyroid hormone ↓
Bauman, 2005 (46)	T: 43 ± 11 (n = 19); C: 42 ± 14 (n = 21)	T: 19/0; C: 20/1	RCT	T: 14 ± 10 years C: 9 ± 9 years	4 μg 1α(OH)D_2 with calcium (1.3 g/d) and 25(OH)D_3 (800 IU/d) supplementation	Bone mineral density with reduced bone resorption was observed in the lower limb of the treatment group.
Beal, 2018 (47)	47 ± 10 (n = 20)	20/0	CCS	17 ± 12 years	Oral intake of 25(OH)D_3 (213 ± 166 IU, [66–573])	A significant decrease in total cholesterol and improvement in glucose homeostasis were observed in the patients with a high dietary intake of vitamin D.
Chen, 2001 (48)	34 (16–78, n = 21)	17/4	RCS	Acute and subacute SCI with bone hyper-resorption: 26 days (6–122)	0.5 μg oral calcitriol once daily throughout the treatment with intravenous administration of 30 mg pamidronate on days 4 through 6 (total of 3 doses)	Serum 1, 25(OH)D_3 ↑; Serum parathyroid hormone ↑; Bone resorption↓
Flueck, 2016 (35)	37 ± 12 (n = 19)	19/0	PCS	Chronic SCI Athletes	6,000 IU daily cholecalciferol supplement over 12 weeks	Serum 25(OH)D_3 ↑. The treatment improved upper body performance and muscle strength.
Mailhot, 2018 (49)	44.2 ± 16.1 (n = 29)	21/8	PCS	Acute and subacute SCI: 29 days (15–90)	1,000 IU daily vitamin D_3 with weekly additional administration of 10,000 IU vitamin D_3 in the patients with Vit D insufficiency	The treatment increased serum 25(OH)D_3 but was unsuccessful in improving the impaired VitD status during inpatient rehabilitation of individuals with a recent SCI.
Pritchett, 2019 (50)	33 ± 15 (n = 35)	30/5	PCS	Chronic SCI Athletes:	Patients with sufficient 25(OH)D: 15,000 IU/week of vitamin D_3 for 12–16 weeks. Patients with insufficiency status: 35,000 IU/week of vitamin D_3 for the first 4 weeks and 15,000 IU/week for the rest of the study. Patients with deficient status: 50,000 IU/week of vitamin D_3 for the first 8 weeks and 15,000 IU/week for the rest of the study.	The treatment increased serum 25(OH)D_3 and improved handgrip strength post supplementation.

T, treatment; C, control; RCT, Randomized Controlled Trial; PCS, prospective cohort study; CCS, case–control study; RCS, retrospective case series; 25(OH)D, 25-hydroxyvitamin D; 1, 25(OH)2D, 1,25-hydroxyvitamin D.

which highlights its unique role in extra-skeletal disease (58). In the SCI population, the previous analysis showed a high prevalence of VitD insufficiency, and deficiency was associated with poor physical functions (23, 59). More importantly, this systematic review showed that VitD abnormality might be associated with several complications, which hampered functional restoration (Table 3).

TABLE 5 The literature investigating the neuroprotective mechanism of Vitamin D in non-human experimental spinal cord injury.

Author, year	Species and Cells	Injury	Treatment	Duration	Effect	Mechanism
Bianco, 2011 (51)	Female SD Rats	Compression SCI at T10	Oral delivery of 50 or 200 IU/kg vitamin D_3	Daily dose for 12 weeks after injury	Ventilatory response to fatigue↑; Normalization of Hoffman reflex	Axon survival within lesion epicenter and distal region
Gueye, 2015 (52)	Male SD Rats	Hemisection SCI at C2	Oral delivery of 500 IU/kg vitamin D_3	Weekly dose form day 1 or 7 after injury (total for 12 weeks)	Locomotor Function↑; Ventilatory response to fatigue↑; Phrenic nerve response↑	Axon survival in the proximal stump
Gurer, 2017 (53)	Rabbits	I/R injury of spinal cord	Intraperitoneal injections of 0.5 μg/kg calcitriol	Administration for 7 days before SCI	Improvement of histopathological change; Demyelination↓ Neurological function↑;	Caspase-3, Apoptosis↓; Serum and tissue MPO, Inflammation↓; MDA, lipid peroxidation↓; CAT↑ and XO↓, ROS↓
Khajoueinejad, 2019 (54)	Female SD Rats	Contusion SCI at T9-10	Intraperitoneal injections of 1 μg/kg calcitriol	Administration for 7 days after SCI	Improvement of histopathological change; Immunomodulatory effects Neurological function↑;	Motoneurons survival↑; IFN-γ and IL-17A ↓; Leukocytes infiltration↓
Zhou, 2016 (55)	Female SD Rats	Crushing SCI at T9	Intraperitoneal injections of 2 μg/kg calcitriol	Administration for 7 days after SCI	Improvement of histopathological change; Neurological function↑	Motoneurons survival↑; MDA↓, GSH and SOD↑, Oxidative stress↓; Caspase-3, Apoptosis↓; LC3-II and Beclin1↑, Autophagy↑;

SD rats, Sprague–Dawley rats; SCI, spinal cord injury; I/R, ischemia/reperfusion; MPO, myeloperoxidase; MDA, malondialdehyde; CAT, catalase; XO, xanthine oxidase; ROS, reactive oxygen species; GSH, glutathione.

The low serum VitD is associated with a higher risk of skeletal diseases after SCI (60). In the subjects with long-standing complete SCI, Frotzler et al. (61) observed considerable declines in bone density and a higher risk of historical fractures. Their findings demonstrated that bone loss after SCI could persist for an extended period. The mechanism underlying post-SCI skeletal abnormality is complicated, and recent evidence suggests that the disruption of the VitD-parathyroid hormone (PTH) axis contributes to this pathological process (36, 60). First of all, *via* interaction with VDR, lack of VitD may affect these osteoblasts and osteoclasts and lead to the disruption of bone microstructure and mass. Besides, low-level VitD results in dysregulation of calcium and phosphate homeostasis and abnormal fluctuations of PTH, which has an adverse effect on bones. In the chronic stage of SCI (more than 1 year), Bauman et al. (25) demonstrated a depressed level of VitD and reduction of the serum calcium concentration, which might lead to mild secondary hyperparathyroidism and accelerate the development of osteoporosis. Furthermore, in a study involving 96 SCI individuals, Oleson et al. (44) found that there was a correlation between hyperparathyroidism and heterotopic ossification as well as hyperparathyroidism and VitD deficiency, in which they inferred that low VitD and elevated PTH might increase the risk of heterotopic ossification.

VitD insufficiency and deficiency were also associated with a high risk of venous thromboembolism. Experimental data exhibited that VitD and VDR modulate the expression and activity of multiple coagulation-related proteins (e.g., plasminogen activator inhibitor-1, thrombospondin-1, etc.), which serve as adjunctive antithrombotic agents (62, 63). In a retrospective cohort study involving 282 acute SCI patients, a higher incidence of venous thromboembolism was noted in the subjects with VitD levels <30 ng/ml and an absence of VitD supplementation, which was consistent with the findings in other neurological injuries (28, 64).

Another emerging field of interest regarding VitD-related complications is secondary neurological disorders. VDR is known to express in both neuronal and glial cells in CNS, and VitD is involved in the regulation of neural differentiation and development, modulation of neuroinflammation, maintaining neuroplasticity, and expression of neurotrophins in various physiological and pathological contexts, which provide a rationale for the link between VitD and neurological comorbidities after SCI (65). Barbonetti et al. (43) recently looked into depression after SCI, and they observed that serum 25(OH)D levels were inversely associated with the psychiatric symptoms in the chronic stage. Whereas data is still limited, and future research is needed to clarify the impact of VitD on neurological comorbidity after SCI.

Previous studies in non-SCI populations reported a positive correlation between low VitD levels and increased risk of respiratory diseases (66, 67). Based on this, several research groups looked into the potential association between chest illness and VitD status after SCI. In 2018, Garshick et al. (22) and Walia et al. (33) accessed the cross-sectional associations between respiratory symptoms/pulmonary function and serum VitD levels in chronic SCI patients, and their analysis failed to establish the association. However, the researchers indeed observed that chronic obstructive pulmonary disease and low VitD levels coexisted in some SCI individuals and VitD supplementation might be beneficial in maintaining respiratory health (22, 33). Therefore, to further investigate the association between VitD levels and chest illness, Clark et al. (27) performed a prospective observational study, and they revealed that the reduction of VitD levels might be associated with an increased risk of future chest illness in chronic SCI, particularly in persons with deficient levels. Currently, high-quality evidence is still

lacking in the field, and more clinical studies with rigorous design are needed to validate the association between VitD levels and chest illness.

Taken together, we think that SCI patients are at higher risk of developing VitD insufficiency and deficiency, and its underlying mechanism may be associated with the lifestyle change, post-SCI complications, and the corresponding vicious cycle (Figure 3). First of all, the primary and secondary injury in the spinal cord leads to severe motor and sensory dysfunction. Due to the impaired mobility and pain, adequate exposure to sunlight is difficult to achieve in those individuals as most have to stay indoors with decreased physical activity, which has a negative impact on the $VitD_3$ synthesis in the skin (68). Secondly, there is converging evidence indicating that dramatic changes in social and family environments after SCI may trigger significant psychiatric stress, which may lead to comorbid psychiatric disorders (such as depression, anxiety, post-traumatic stress disorder, etc.) and makes them more vulnerable to VitD insufficiency (69, 70). It is noteworthy that the researchers also indicated that VitD was a negative acute phase reactant, and post-SCI systematic inflammation might decrease the levels of plasma VDBP, which exacerbated VitD insufficiency (71). Thirdly, SCI patients usually develop multiple complications and comorbidities, such as skeletal diseases, chest illness, neurological sequela, venous thromboembolism, intestinal dysfunction, etc., which may directly result in the lack of VitD or change the lifestyle and affect the level of VitD secondarily (72, 73). For example, persons with SCI are likely to develop pressure ulcers and dysregulation of intestinal microflora with significant diet change, which may affect VitD level synergically *via* impairing its food supply and absorption (42, 74). Notably, the low levels of VitD can also aggravate functional deficits, psychological stress and co-existing diseases, which start up a vicious cycle and hamper functional restoration after SCI.

4.3. The therapeutic effects of vitamin D supplements on the outcome of SCI

In the acute and chronic stages of SCI, VitD supplemental treatment can substantially increase VitD concentration and modulate abnormal PTH fluctuation. In 2005, Bauman et al. (45) postulated two supplemental regimens for chronic SCI: the short-term regimen, 2,000 IU $25(OH)D_3$ twice a week for 2 weeks, and the long-term regimen, 800 IU $25(OH)D_3$ daily for 12 months, both of which led to a significant increase of plasma 25(OH)D levels and suppression of plasma PTH. However, they also noticed that the two plans failed to normalize serum VitD levels, which indicated higher doses and longer administration periods were required for the supplementation (45). Notably, later in 2013, they successfully developed an oral regimen for VitD replacement in the chronic SCI population. The patients were administered $VitD_3$ at a dose of 2,000 IU daily for 3 months with 1.3 g oral calcium supplementation per day. Normal levels of VitD with a significant decrease in PTH in six of seven participants were restored at the end of the experiment (75). Meanwhile, in recent SCI with complete or incomplete sensorimotor impairments, Mailhot et al. (49) evaluated a VitD repletion protocol, in which participants were given 1,000 IU $25(OH)D_3$ daily for approximately 6 weeks with extra weekly administration of 10,000 IU $25(OH)D_3$ in the patients with VitD insufficiency. They found that the treatment increased serum $25(OH)D_3$ but was unsuccessful in improving the impaired VitD status.

Administration of VitD in SCI facilitates motor and sensory functional restoration. Recently, clinicians performed a randomized trial to assess the effects of progesterone and VitD on functional restoration after acute traumatic SCI. Their findings indicated that the synergic administration improved motor and sensory function after SCI (17). VitD treatment in wheelchair athletes with chronic SCI not only helps them maintain an adequate level of serum VitD but also improves their muscle strength. In a double-blinded study involving 20 indoor wheelchair athletes with VitD insufficiency, researchers administrated 6,000 IU cholecalciferol supplements daily over 12 weeks. The supplemental therapy restored VitD status to an optimal level and seemed to improve upper body performance and muscle strength (35). Similarly, Pritchett et al. (50) adopted a refined and hierarchical protocol pending on the baseline VitD status that exhibited VitD supplementation could increase the serum level of VitD, and improve handgrip strength in the elite athletes with chronic SCI.

There was also evidence supporting the beneficial effect of VitD supplementation on skeletal diseases. The addition of $1\alpha(OH)D_2$ on the basis of routine calcium and VitD supplementation for 1 year was reported to increase the bone mineral density of lower limbs in chronic SCI at 6 months after treatment when compared with the placebo administration (46). In acute and subacute SCI, Chen et al. (48) combined calcitriol and pamidronate therapy, and the treatment significantly inhibited bone hyper-resorption *via* normalization of the VitD-PTH axis. Additionally, a case–control study investigating dietary VitD intakes in chronic SCI suggested that a higher dietary intake of VitD could influence cholesterol and glucose homeostasis, which improved carbohydrate metabolism (47).

4.4. Neuroprotective effect of vitamin D in non-human experimental SCI

In the non-human experimental SCI, we retrieved five studies supporting the neuroprotective effect of VitD supplementation (51–55). The proposed mechanisms are multidimensional, involving promoting axonal survival (51, 52), reducing neuronal loss by attenuating oxidative stress (53, 55), suppressing neuroinflammation (54) and modulation of autophagy ((55), Table 5). Oral delivery of VitD for 4 months in a compression SCI model at T10 level improved respiratory adjustment to fatigue and normalized Hoffman reflex *via* increasing the number of axons crossing the lesion site (51). Later, the same research group replicated the findings in a hemisection SCI model at a higher level (C2) (52). Notably, the short-term administration of calcitriol immediately after SCI was also reported to attenuate the histological damage and neuron loss by reducing oxidative stress, inhibiting apoptosis, and promoting autophagy. In addition, pretreatment of calcitriol before SCI also exhibited a protective effect on the ischemia/reperfusion injury of the spinal cord, which was mediated by inhibiting neuronal apoptosis and suppressing regional and general oxidative stress (53). The immunomodulatory property of VitD raises the potential that it may alter the functional status of microglia/macrophages and astrocytes, the key players in the post-SCI neuroinflammation, to improve repairment. Indeed, Khajoueinejad et al. (54) observed that VitD had an immunomodulatory effect on the proliferative response of lymphocytes in the spleen and lymph nodes, which was associated with reduced secretion of proinflammatory cytokines (IFN-γ and IL-17A) and less leukocyte infiltration into the lesion center. However, our knowledge regarding the neuroprotective mechanism of VitD is still limited, and well-designed mechanistic research is needed to elucidate its underlying mechanism.

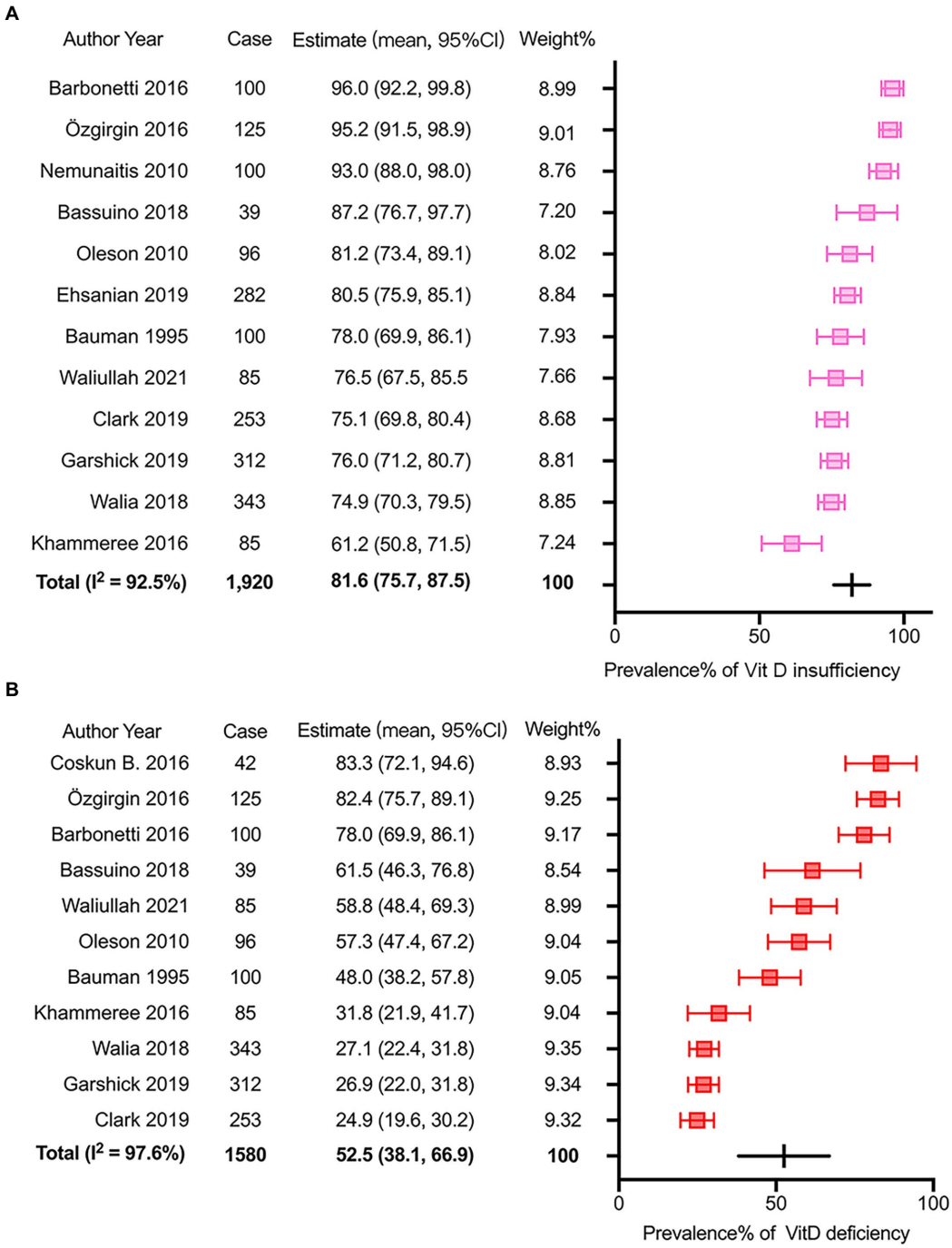

FIGURE 2
Meta-analysis of the prevalence of insufficient and deficient Vitamin D status after SCI. **(A)** the prevalence of Vitamin D insufficiency after SCI. **(B)** the prevalence of Vitamin D deficiency after SCI.

From the evidence of VitD's effect in both SCI and other neurological disease models, we speculated that the potential neuroprotective effect of Vitamin D might be mediated by its genomic and non-genomic effects (Figure 4). First of all, VitD diffuses through the cell membrane, binds to VDR, and dimerizes with RXR, which then translocates into the nucleus and binds to VitD response element (VDRE). VDRE locates on a large number of genes and its binding with VitD-VDR-RXR complex leads to the transcription of target genes, which could modulate inflammatory response (54, 76), attenuate oxidative stress *via* Nrf2 and Klotho pathway (53, 55, 77, 78), maintain intracellular calcium homeostasis (79), enhance the expression of multiple growth factors and neurotrophins (e.g., vascular endothelial growth factor [VEGF], brain-derived neurotrophic factor [BDNF], etc.) (80), promote axonal regeneration, angiogenesis and neurogenesis (51, 52) (81), modulation of autophagy (55), etc. (Figure 4). Besides, it was recognized that VitD could exert an immediate non-genomic effect through membrane VDR in CNS, which then modulated the functional status of calcium-and kinase-activated signaling pathways (82).

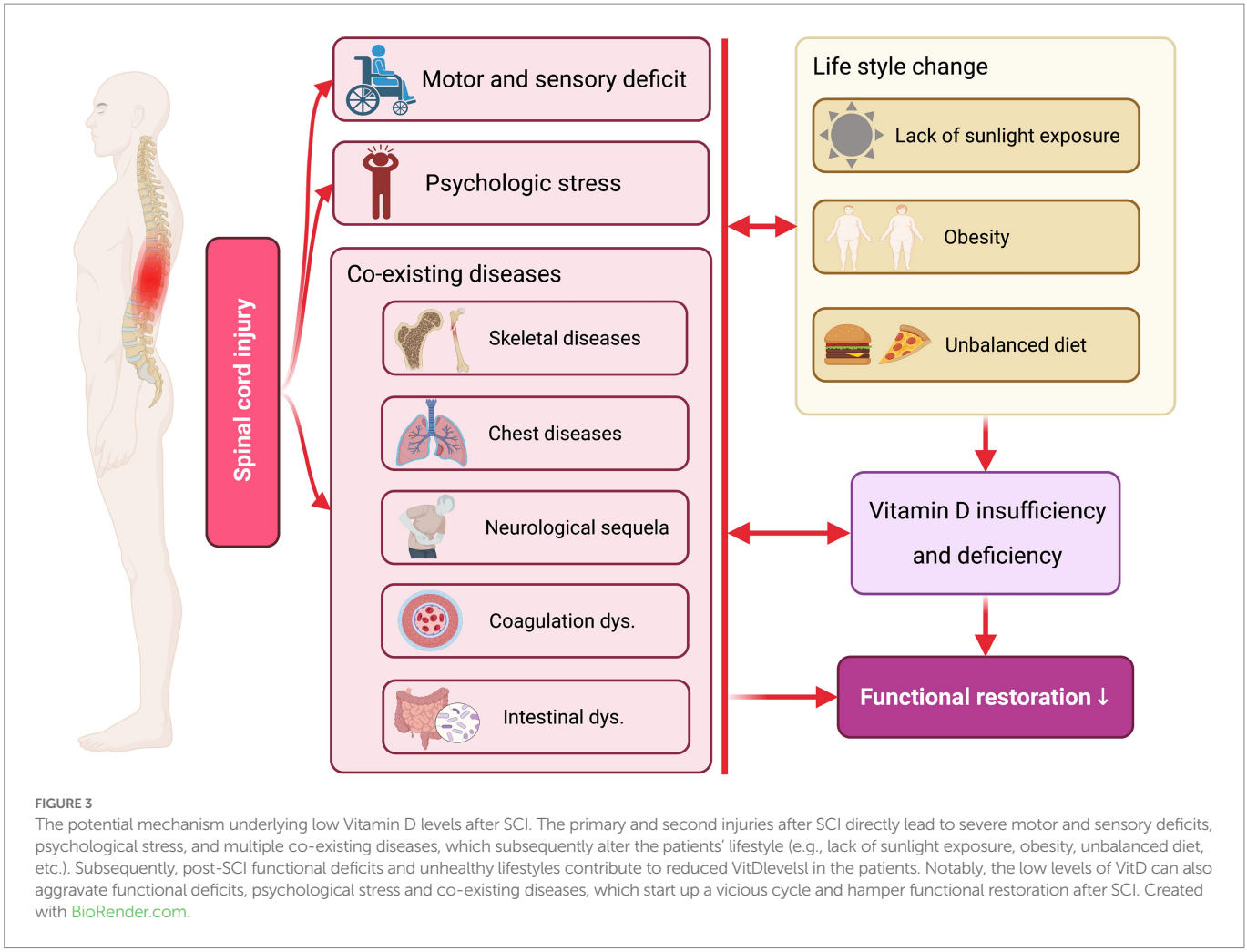

FIGURE 3
The potential mechanism underlying low Vitamin D levels after SCI. The primary and second injuries after SCI directly lead to severe motor and sensory deficits, psychological stress, and multiple co-existing diseases, which subsequently alter the patients' lifestyle (e.g., lack of sunlight exposure, obesity, unbalanced diet, etc.). Subsequently, post-SCI functional deficits and unhealthy lifestyles contribute to reduced VitDlevelsl in the patients. Notably, the low levels of VitD can also aggravate functional deficits, psychological stress and co-existing diseases, which start up a vicious cycle and hamper functional restoration after SCI. Created with BioRender.com.

4.5. Future direction

In this systematic review, we retrieved a multi-level of evidence supporting the beneficial effects of VitD on post-SCI rehabilitation. However, the neuroprotective mechanisms of VitD have not been elucidated by current experimental studies, and most clinical studies in the field are observational and small-scale, which limits its wide application.

In the experimental research, more in-depth *in vitro* and *in vivo* investigations are needed to explicate the precise molecular mechanism underlying the neuroprotective effect of VitD. In the adult brain, VitD has both genomic and non-genomic actions on various neurological functions (82). It remains to be seen whether VitD could exert neuroprotection *via* similar VDR-depended mechanisms in the context of SCI. In particular, VitD modulated inflammatory response in CNS and immune systems (83). Future researchers should elucidate the exact effect of VitD in the systematic inflammation and neuroinflammation after SCI (84). Furthermore, VitD can enhance neural stem cell proliferation and differentiation into neurons and oligodendrocytes (85, 86). It would be interesting to explore whether VitD could interact with neural stem cells to promote neurons and myelin regeneration after SCI *via* enhancing and modulating endogenous neurogenesis (87–89). Recent literature indicated that small molecules combined with collagen hydrogel directed neurogenesis and migration of neural stem cells after implantation in the lesioned spinal cord (90). As consecutive systematic administration of VitD is known to have poor delivery efficiency in the lesion site and lead to adverse effects in a large dose, topical application of VitD treatment *via* a combination of hydrogel, small molecules, cells, and other small-releasing systems is a promising therapeutic strategy towards SCI.

In future clinical studies, clinicians should design large-scale, double-blinded, and random-control trials to validate the therapeutic effect of VitD as an adjuvant treatment on the functional restoration following SCI. We should explore and standardize the administration protocol of VitD monitoring and supplementation (e.g., dose, therapeutic window, duration, etc.) in the acute and chronic stages of SCI. Another interesting direction is combining VitD treatment with other micronutrients and medicine (e.g., progesterone, Vitamin E, Vitamin C, etc.) and determining whether they have a synergic effect on the improvement of SCI rehabilitation (17, 91).

4.6. Limitations

Our systematic review with meta-analysis has several limitations. First, in the meta-analysis of the prevalence of VitD insufficiency and deficiency, there is high heterogeneity among included studies. To diminish its impact on the validity of the results, a single-arm meta-analysis with random effect was applied. We also performed the subgroups analysis to explore the source of heterogeneity and identified more substantial heterogeneity among studies with patients less than 200 and years of injury less than 10 years. However, the high

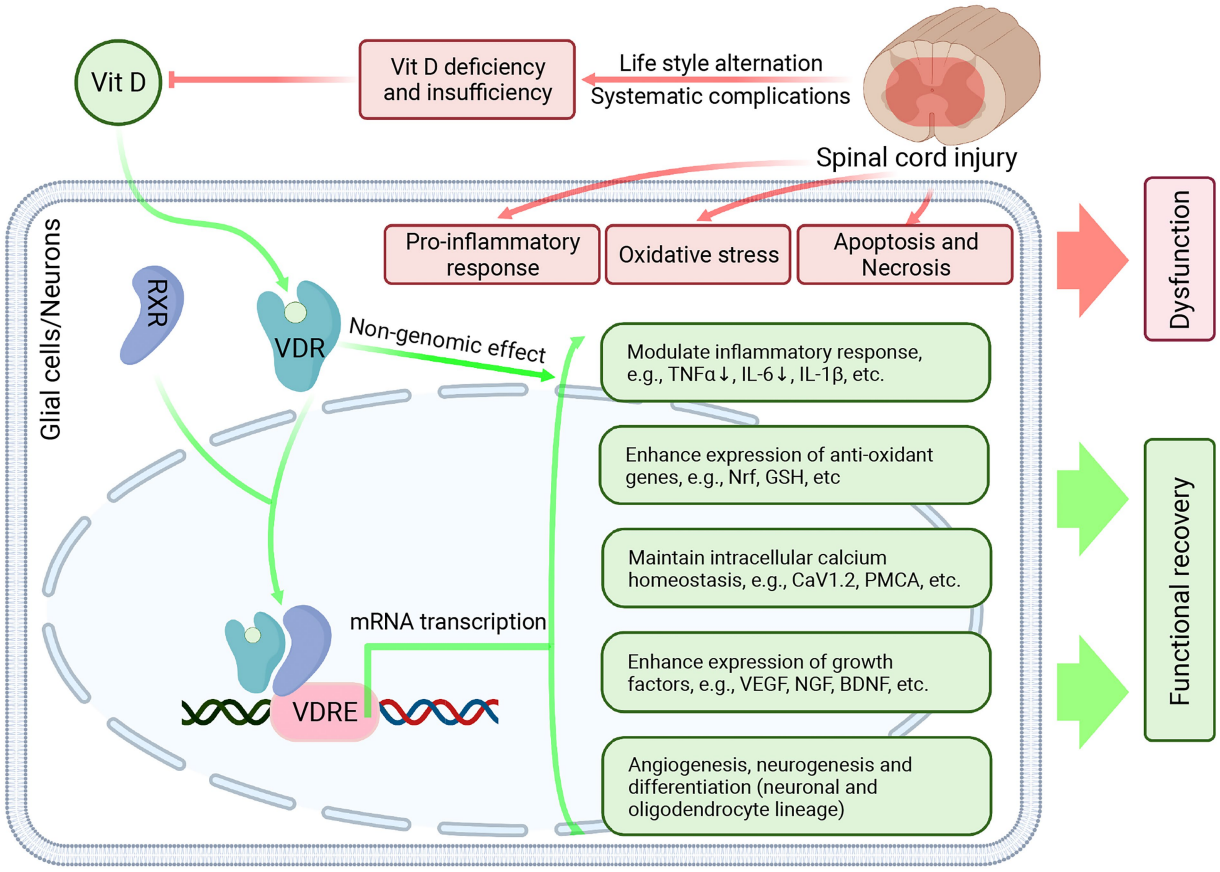

FIGURE 4
The potential neuroprotective effect of Vitamin D in SCI. After SCI, the primary impact and secondary injury lead to persistent inflammatory response, enhanced oxidative stress, neuronal apoptosis, and necrosis, which results in permanent functional deficits and delays neurofunctional restoration. Besides, due to lifestyle change and systematic complications, the VitD levels were significantly reduced in the SCI individuals. VitD treatment can combat secondary injury after SCI and exert a neuroprotective effect. In the glial cells and neurons, VitD treatment can facilitate its diffusion across the cell membrane. Then VitD binds to VDR and dimerizes with RXR. The VitD-VDR-RXR complex then translocates into the nucleus, binds to VDRE, and promotes the transcription of target genes, which can modulate inflammatory response, attenuate oxidative stress, maintain intracellular calcium homeostasis, enhance the expression of multiple growth factors and neurotrophins, promote angiogenesis and neurogenesis. Created with BioRender.com.

heterogeneity inevitably limits the outcomes of this meta-analysis. Second, in the systematic review, most of the included clinical papers are observational, hospital-based, and performed in different countries, which can introduce potential bias. Third, in the clinical studies, the inclusion and exclusion criteria vary, and the participants have different baseline levels of VitD, which may bias the results.

5. Conclusion

Based on the discovered protective and bioactive effect of VitD on neurological disorders, we hypothesize that VitD might play an essential role in post-SCI rehabilitation. Through this meta-analysis and systematic review, we retrieved multi-level evidence that supported this hypothesis, including (1) there was a high prevalence of VitD insufficiency and deficiency in the SCI population, (2) low-level of VitD was associated with several complications, which hampered the functional restoration, (3) the supplement treatment might have potential benefits to accelerate rehabilitation in mechanistically related processes after SCI. However, due to the limitation of the evidence, our results should be interpreted carefully, and more well-designed randomized controlled trials and mechanism experimental research are needed to validate its therapeutic effect, elucidate its neuroprotective mechanism, and develop novel treatments.

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

LW and DL: conceptualization, data curation, funding acquisition, project administration, supervision, writing—original draft, and writing—review and editing. LW, JG, JW, and YZ: formal analysis, investigation, methodology, resources, software, validation, and visualization. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by National Nature Science Foundation of China (grant number 81771334), Natural Science Foundation of Hubei Province of China (grant number 2017CFB706) and Free Innovation Fund of Wuhan Union Hospital (grant number 2021xhyn105). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.920998/full#supplementary-material

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frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 27 February 2023
DOI 10.3389/fnut.2023.1016243

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Nutrition, a section of the journal Frontiers in Nutrition

RECEIVED 11 August 2022
ACCEPTED 13 February 2023
PUBLISHED 27 February 2023

CITATION
Lu Y, Su H, Wang Y and Li H (2023) Micronutrients and risks of three main urologic cancers: A mendelian randomization study. *Front. Nutr.* 10:1016243. doi: 10.3389/fnut.2023.1016243

Micronutrients and risks of three main urologic cancers: A mendelian randomization study

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Background: The effect of micronutrients on urologic cancers has been explored in observational studies. We conducted the two-sample mendelian randomization (TSMR) study to investigate whether micronutrients could causally influence the risk of urologic cancers.

Methods: Summary statistics for four micronutrients and three main urologic cancers outcomes were obtained from genome-wide association studies (GWAS). MR analyses were applied to explore the potential causal association between them. Sensitivity analyses using multiple methods were also conducted.

Results: Genetically predicted one SD increase in serum copper and iron concentrations was causally associated with increased risks of renal cell carcinoma (RCC) (OR = 3.021, 95%CI = 2.204–4.687, $P < 0.001$, male; OR = 2.231, 95%CI = 1.524-3.953, $P < 0.001$, female; OR = 1.595, 95%CI = 1.310–1.758, $P = 0.0238$, male; OR = 1.484, 95%CI = 1.197–2.337, $P = 0.0210$, female, respectively) and per SD increase in serum zinc levels was related to decreased risks of RCC (OR = 0.131, 95%CI = 0.0159–0.208, $P < 0.001$, male; OR = 0.124, 95%CI = 0.0434–0.356, $P < 0.001$, female). No significant results were observed between micronutrients and the risk of bladder cancer after Bonferroni correction. Additionally, per SD increase in serum zinc level was associated with a 5.8% higher risk of prostate cancer (PCa) [OR = 1.058, 95%CI = 1.002–1.116, $P = 0.0403$, inverse-variance weight (IVW)].

Conclusions: Micronutrients play a vital role in the development of urological tumors. Future studies are required to replicate the findings, explore the underlying mechanisms, and examine the preventive or therapeutic role of micronutrients in clinical settings.

KEYWORDS

micronutrients, prostate cancer, renal cell carcinoma, bladder cancer, Mendelian randomization

1. Introduction

Urologic tumor refers to tumors that affect the organs and structures of the urinary system of both men and women and the reproductive system of men. Three most prevalent types of urologic tumors are: prostate cancer (PCa), renal cell carcinoma (RCC), and bladder cancer (BCa) (1). The incidence of kidney, bladder, and prostate cancers cases increased between 1990 and 2013 and mortality increased 1.6-fold during the same time period. Urologic cancer burden has increased globally amid population growth and aging (2). Efforts to expand the global oncologic workforce and reduce preventable factors may contribute to cancer management (3). Nowadays, several risk factors have been established, such as lipid composition, obesity, and cigarette, etc (4). However, the role of nutrition in urologic cancer development is still unclear.

Dietary trace metals, including zinc, copper, iron, and selenium, etc. have been shown to influence the risk of cancer through oxidative stress, DNA injury and repair, regulating cell cycle, and angiogenesis (5). Some observational studies using food frequency questionnaires (FFQs) indicated the anti-tumor role of nutrients or dietary intake of nutrients in urologic cancer, while these results are conflicting and concerns about potential biases from confounding factors can't be dispelled (6).

Mendelian randomization (MR) that uses genetic variants as instrumental variables is widely used in epidemiological studies to examine whether a potential factor could casually influence an outcome. Different from traditional observational studies, this method could dramatically lower the effect of confounders and reverse causation (7). Two-sample Mendelian randomization (TSMR), which belongs to MR methodology and uses two samples drawn from the same underlying population with no overlap of participants between the two samples, is a method to estimate the causal effect of an exposure on an outcome using only summary statistics from genome-wide association studies (GWAS). Some large-scale GWAS on micronutrients and urologic cancers have also been published, providing high-quality genetic instruments to conduct MR study (8, 9). These GWAS have been used and validated in several previous MR studies (10, 11). To fill in the gap, we conducted the TSMR study to identify the potential effect of microelement levels on urologic cancer risk.

2. Method

2.1. Study design

The MR analysis was designed to evaluate the associations between microelement levels and risks of urologic tumors (RCC, BCa, and PCa). Single nucleotide polymorphisms (SNPs) for common microelements (Cu, Zn, Fe, and Se) were selected as instrumental variables from previously published genome-wide association study (GWAS) analyses. Three key assumptions need to be satisfied: (a) the SNPs should have strong associations with microelement levels; (b) the chosen SNPs should be independent of confounders; (3) the SNPs should affect cancer only *via* microelement levels. The diagram of the TSMR was shown in Figure 1 (12).

2.2. Data sources

The study utilized summarized genetic data from the Genetics of Iron Status (GIS) consortium (13), Prostate Cancer Association Group to Investigate Cancer Associated Alterations in the Genome (PRATICAL) (8), International Academic & Research Consortium (IARC) (14), and UK Biobank (UKB) (9). Details about the sources were shown in Table 1. The original GWAS had been approved by corresponding ethics committee, and the approval of current study was obtained from the Medical Research Ethics Committee of Peking Union Medical College Hospital.

2.3. Instrumental variable selection

Instrumental variable selection for Cu, Zn, and Se levels (serum), iron levels, and blood and toenail Se levels were based on a GWAS with 2,603 adults from Australia and the UK., a GWAS involving 48,972 individuals of European ancestry (GIS Consortium), and the UK Biobank study, respectively (13, 15, 16). Instrumental variables for RCC, BCa, and PCa were

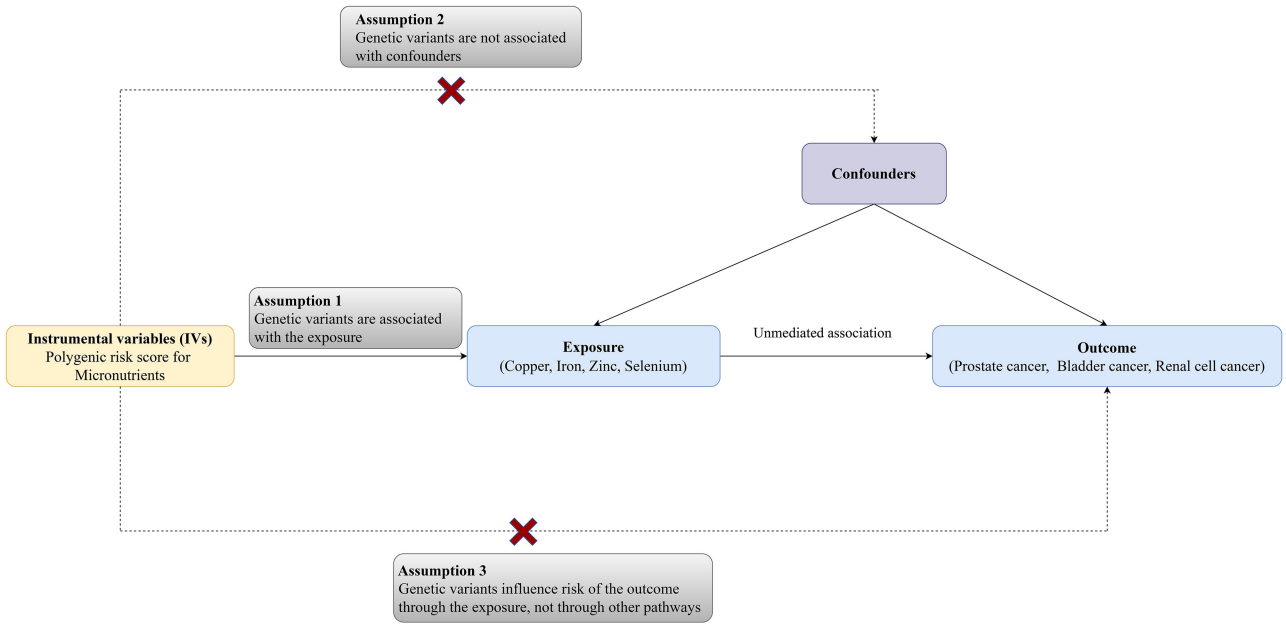

FIGURE 1
The diagram of the study.

TABLE 1 The characteristics of GWAS studies on the exposures and outcomes.

Exposure	Consortium	Total population	Cases/controls	Ethnicity	References
Copper	NA	2,603	NA	European	Genome-wide association study identifies loci affecting blood copper, selenium and zinc PubMed id: 23720494
Zinc	NA	2,603	NA	European	Genome-wide association study identifies loci affecting blood copper, selenium and zinc PubMed id: 23720494
Iron	GIS	23,986	NA	European	Novel loci affecting iron homeostasis and their effects in individuals at risk for hemochromatosis PubMed id: 25352340
Blood and toenail selenium	NA	4,162	NA	European	Genome-wide association Study of selenium concentrations. PubMed id: 25343990
Blood selenium	NA	2,603	NA	European	Genome-wide association study identifies loci affecting blood copper, selenium and zinc PubMed id: 23720494
Outcome	**Consortium**	**Total population**	**Cases/controls**	**Ethnicity**	**References**
Overall PCa	PRATICAL	140,254	79,148/61,106	European	Association analyses of more than 140,000 men identify 63 new prostate cancer susceptibility loci. PubMed id: 29892016
RCC in female	IARC	5,087	1,992/3,095	European	Sex-specific associations in genome-wide association analysis of renal cell carcinoma PubMed id: 31231134
RCC in male	IARC	8,143	3,227/4,916	European	Sex-specific associations in genome-wide association analysis of renal cell carcinoma PubMed id: 31231134
Bladder Cancer	UKB	462,933	1,101/ 461,832	European	UK Biobank: An Open Access Resource for Identifying the Causes of a Wide Range of Complex Diseases of Middle and Old Age PubMed id: 25826379

N.A., not available; GIS, Genetics of Iron Status; PRATICAL, Prostate Cancer Association Group to Investigate Cancer Associated Alterations in the Genome; IARC, International Academic and Research Consortium; UKB, UK Bioban.

obtained from IARC (5,219 RCC cases and 8,011 controls of European ancestry), UKB (1,101 BCa cases and 461,832 controls of European ancestry), and PRATICAL consortium (79,148 PCa cases and 61,106 controls of European ancestry), respectively (8, 9, 14). Single nucleotide polymorphisms (SNPs) that met the locus-wide significance level ($P < 10^{-5}$) and have genome-wide statistical significance ($P < 5\times10^{-8}$) were proposed as instrumental variables. Phenoscanner website was used to examine the pleiotropic effects of selected IVs and all used IVs were validated in previous studies (17, 18). All the SNPs selected in the study were shown in the Supplementary Table 1.

2.4. Study outcomes

RCC, BCa, and PCa were the outcomes. The latest GWAS involving the most complete available data on three types of cancers was selected. The sources were presented in Table 1.

2.5. Statistical analysis

Five different statistical methods were used to conduct the MR analyses. Firstly, the inverse-variance weight (IVW) approach was applied for the primary TSMR to quantify the causal associations between micronutrient (Cu, Fe, Se, and Zn) concentrations and the risk of three types of cancers (19). In the process, the ratio of coefficients was calculated to evaluate the causal effects. MR-Egger regression was used to examine the horizontal pleiotropy between IVs and three types of cancers, which adjusted micronutrients levels. Additionally, weighted median method (WM) only needs half of the effective SNPs was used as a supplement for the IVW approach (20). Finally, weighted mode and simple mode analyses were used to estimate the causal effect (21). https://shiny.cnsgenomics.com/mRnd/ was used for sample size test. This required four parts data: (a) Proportion of cases in the (intended) study; (b) Total sample size; (c) True odds ratio of the outcome variable per standard deviation of the exposure variable; (d) Proportion of variance in exposure variable explained by SNPs. Results indicated that with all the given sample size, analysis in each subgroup has strong statistical power (22). All traits related to screened SNPs were searched on the PhenoScanner website. Statistical analyses were performed repeatedly after removing confounder-related SNPs to improve the robustness and handle potential horizontal pleiotropy. Sensitivity analysis was also performed to assess whether some SNPs had a significantly independent influence on results *via* leave-one-out approach and the remaining estimate effect was shown when one SNP was excluded (23). The level of heterogeneity was estimated by using Cochran's Q statistics. All analyses were conducted in R software (version 4.1.2; http://www.rproject.org) with the "TwoSampleMR"

TABLE 2 Two-sample MR estimates of relationship between genetically predicted micronutrients and cancer.

Exposure	MR Method	Prostate cancer			Bladder cancer			Renal cell cancer (Female)			Renal cell cancer (Male)		
		No. of SNPs	OR (95% CI)	*P*-Value	No. of SNPs	OR (95% CI)	*P*-Value	No. of SNPs	OR (95% CI	*P*-Value	No. of SNPs	OR (95% CI	*P*-Value
Copper	IVW	2	1.005 (0.952–1.061)	0.867	2	NA	NA	2	2.231 (1.524–3.953)	<0.001	2	3.021 (2.204–4.687)	<0.001
Iron	IVW	3	0.951 (0.896–1.011)	0.106	2	1.000 (0.999–1.001)	0.794	2	1.484 (1.197–2.337)	0.0210	2	1.595 (1.310–1.758)	0.0238
	MR-Egger	3	0.917 (0.814–1.034)	0.392	NA	NA	NA	NA	NA	NA	NA	NA	NA
	WM	3	0.954 (0.892–1.020)	0.170	NA	NA	NA	NA	NA	NA	NA	NA	NA
Zinc	IVW	2	1.058 (1.002–1.116)	0.0403	NA	NA	NA	2	0.124 (0.0434–0.356)	<0.001	2	0.131 (0.0159–0.208)	<0.001
	Wald ratio	NA	NA	NA	1	1.001 (1.000–1.002)	0.0841	NA	NA	NA	NA	NA	NA
Blood selenium	IVW	22	0.996 (0.975–1.017)	0.676	9	0.998 (0.997–0.999)	0.0317	22	0.790 (0.0779–8.030)	0.842	22	0.702 (0.0712–6.919)	0.762
	MR-Egger	22	1.023 (0.943–1.110)	0.595	9	0.999 (0.997–1.000)	0.560	22	1.070 (0.0991–1.9652)	0.166	22	3.858 (0.584–8.340)	0.203
	WM	22	1.003 (0.976–1.031)	0.835	9	1.000 (0.999–1.000)	0.0612	22	1.851 (0.918–2.977)	0.201	22	2.135 (1.940–2.387)	0.073
Blood and toenail selenium	IVW	12	0.985 (0.946–1.025)	0.458	4	0.999 (0.998–1.002)	0.182	12	0.175 (0.004–8.244)	0.375	11	0.0960 (0.00173–5.319)	0.253
	MR-Egger	12	1.044 (0.912–1.196)	0.545	4	1.000 (0.997–1.003)	0.213	12	0.08715 (0.0052–0.1597)	0.0257	11	1.7553 (0.4133–2.9804)	0.0570
	WM	12	0.985 (0.946–1.025)	0.452	4	0.999 (0.998–1.000)	0.986	12	0.224 (0.085–0.397)	<0.001	11	0.283 (0.0173–0.537)	0.0408

OR, odds ratio; CI, confidential interval; IVW, inverse-variance weight; WM, weighted median; SNP, single nucleotide polymorphisms; N.A., not available.

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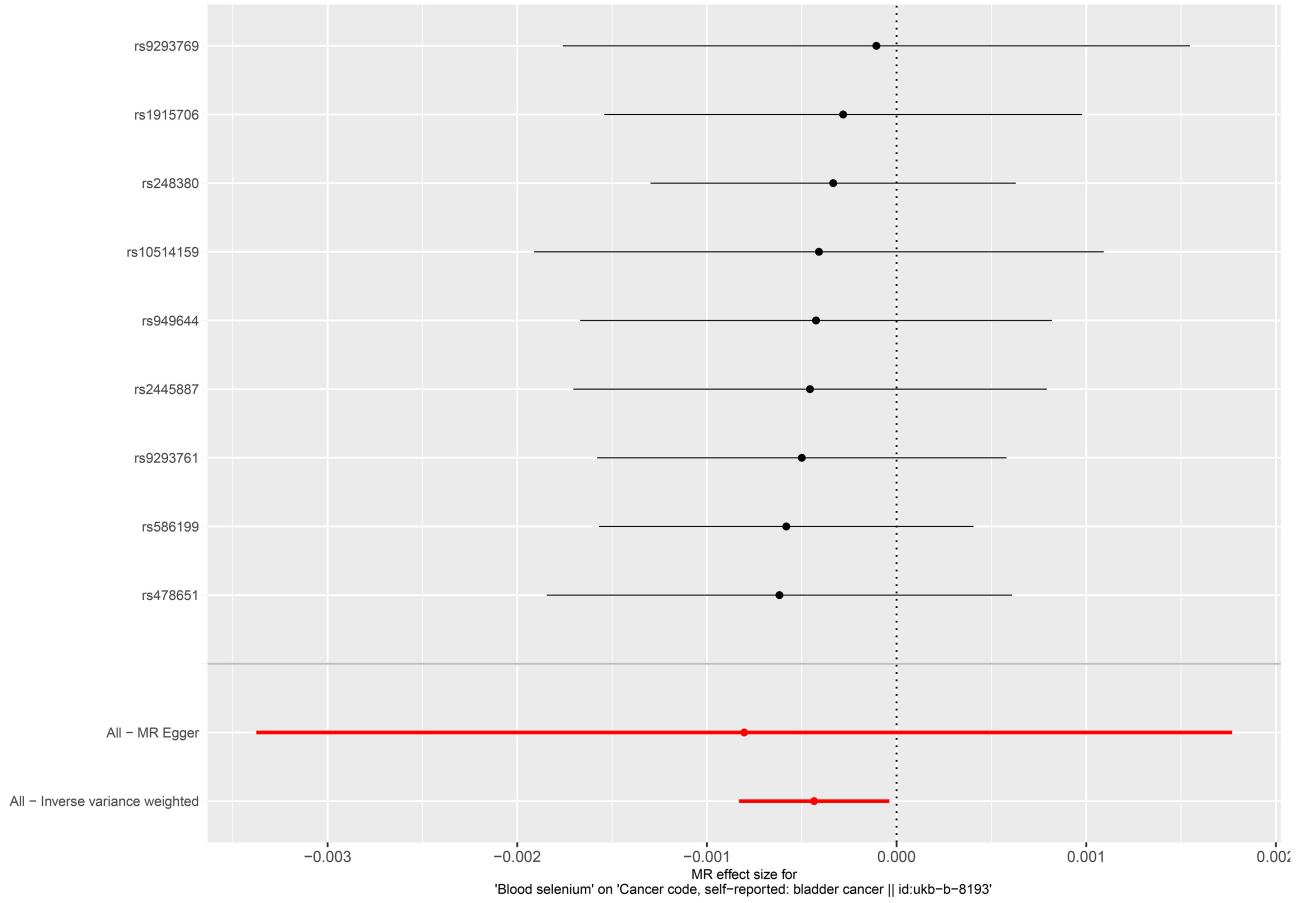

FIGURE 2
Forest plot for the association between circulating selenium and the risk of bladder cancer.

package (version 0.5.6). Associations were considered as strong between micronutrients levels and cancer risks if they surpassed a stringent Bonferroni-corrected *P*-value threshold of 1.67×10^{-3} (0.05/3 cancer outcomes). The reporting of the MR study followed the existed rule (24).

3. Results

3.1. Associations between micronutrients and risk of RCC

For the four micronutrients, the primary estimate by IVW indicated that genetically predicted one SD increase in serum copper and iron concentrations was causally associated with increased risks of RCC (OR = 3.021, 95%CI = 2.204–4.687, $P < 0.001$, male; OR = 2.231, 95%CI = 1.524–3.953, $P < 0.001$, female; OR = 1.595, 95%CI = 1.310–1.758, $P = 0.0238$, male; OR = 1.484, 95%CI = 1.197–2.337, $P = 0.0210$, female, respectively) and per SD increase in serum zinc levels was related to decreased risks of RCC (OR = 0.131, 95%CI = 0.0159–0.208, $P < 0.001$, male; OR = 0.124, 95%CI = 0.0434–0.356, $P < 0.001$, female). However, no causal effect was observed in serum selenium and serum and toenail selenium (Table 2). Not all sensitivity analysis supported the causation between these micronutrients and RCC risk (Table 2 and Supplementary Figures 1–4).

3.2. Associations between micronutrients and risk of BCa

No causal associations were observed between risks of BCa and serum iron level (OR = 1.000, 95%CI = 0.999–1.001, $P = 0.794$, IVW), zinc level (OR = 1.001, 95%CI = 1.000–1.002, $P = 0.0841$, Wald ratio), serum selenium (OR = 0.998, 95%CI = 0.997–0.999, $P = 0.0317$, IVW), and blood and toenail selenium (OR = 0.999, 95%CI = 0.998–1.002, $P = 0.182$, IVW) (Figures 2, 3). Sensitivity analyses revealed consistent results (Table 2 and Supplementary Figures 5–8).

3.3. Associations between micronutrients and risk of PCa

Per SD increase in serum zinc level was associated with a 5.8% higher risk of PCa (OR = 1.058, 95%CI = 1.002–1.116, $P = 0.0403$, IVW). No causal associations were observed between

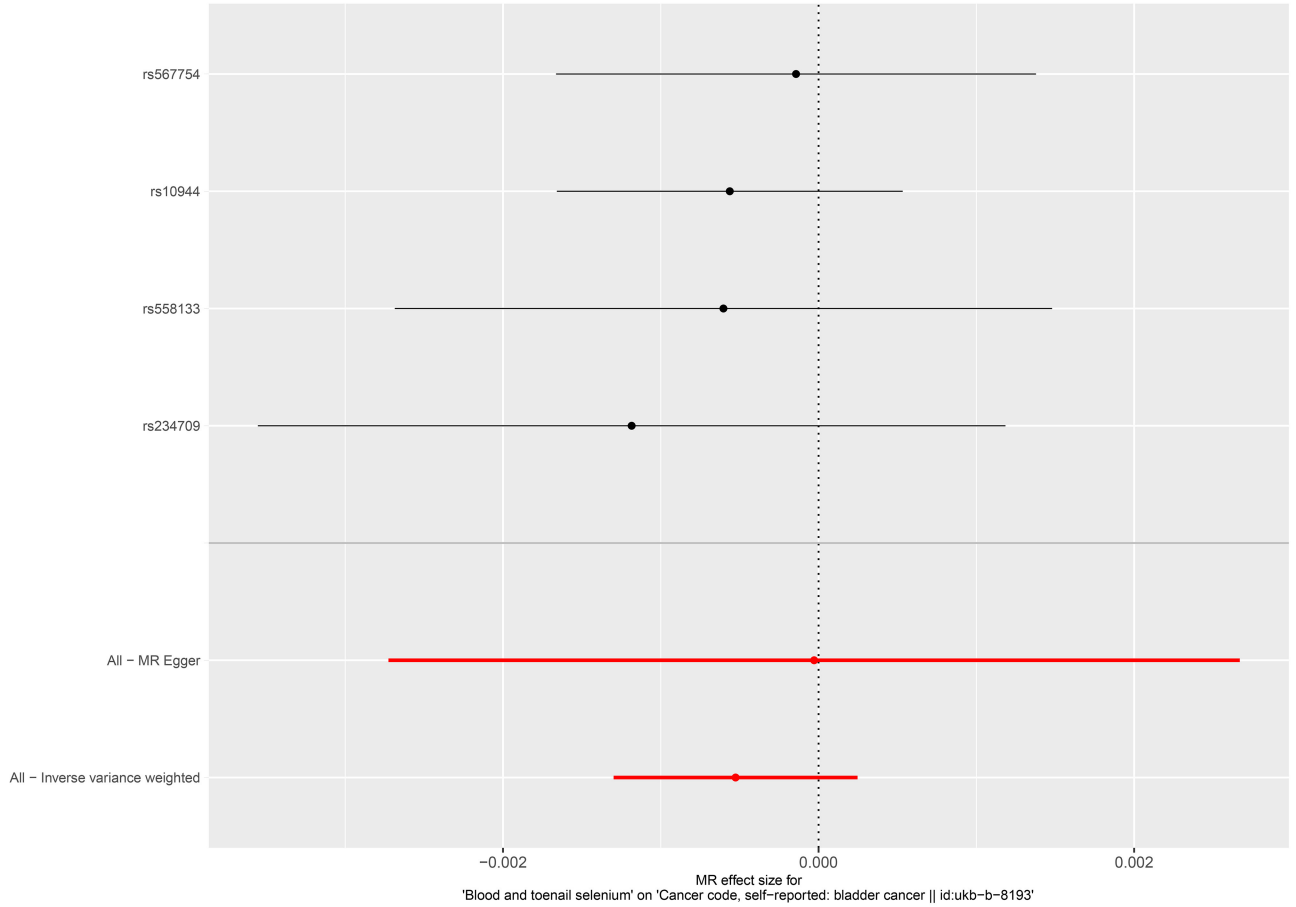

FIGURE 3
Forest plot for the association between blood and toenail selenium and the risk of bladder cancer.

risks of PCa and serum copper level (OR = 1.005, 95%CI = 0.952–1.061, $P = 0.867$, IVW), iron level (OR = 0.951, 95%CI = 0.896–1.011, $P = 0.106$, IVW), serum selenium (OR = 0.996, 95%CI = 0.975–1.017, $P = 0.676$), and blood and toenail selenium (OR = 0.985, 95%CI = 0.946–1.025, $P = 0.458$, IVW) (Figures 4–6). Consistent results were also achieved in sensitivity analysis (Table 2 and Supplementary Figures 9–14).

4. Discussion

In the current study, we investigated the causal effects of four micronutrients (Copper, Iron, Selenium, and Zinc) on risks of 3 urological tumors. The findings indicated that genetically increased Zinc levels were related to increased risk of PCa, while reduced risk of RCC. Furthermore, increased Copper and iron level were associated with increased risk of RCC. In terms of BCa, no causal effects were observed.

Prostate cancer is a common malignancy that affects millions of men globally (1). Prior observational and mendelian studies have indicated that serum calcium and selenium levels were not associated with the risk of PCa (10, 11), which was consistent with our results. A former study indicated that decreased zinc or increased copper level might play important role in the initiation of PCa, while no mendelian studies ever investigated the genetic causation between them (25). We found that increased serum zinc level was causatively related to increased risk of PCa, which was in accordance with a population-based study (26). A possible explanation for the phenomenon is the redistribution of zinc, leading to increased serum zinc and reduced intracellular zinc in prostate (27). However, no GWAS about intracellular zinc were available, the underlying mechanisms require further investigation.

RCC is another common urologic malignancy (1). No mendelian study ever investigated the effect of micronutrients on the risk of RCC. Previous studies focus on exploring the role of zinc-finger protein in RCC (28, 29), demonstrating that these zinc-finger proteins could suppress the proliferation, invasion and improve long-term prognosis. Only one study indicated that Zn in the medulla was significantly lower in RCC cases than in controls (30). The result is consistent with what we found. Greene et al. demonstrated that RCC development is commonly represented by accumulated iron and Wu et al. conducted an *in vivo* study that indicated that STEAP3 played a crucial role in the iron dysfunction in ccRCC (31, 32). Few clinical studies had showed the association between iron level and ccRCC risk in humans to date. Sridhar et al. indicated that a significantly higher copper concentration is noted in the blood and urine in RCC patients as compared to healthy controls (33). In accordance with this, we found that increased

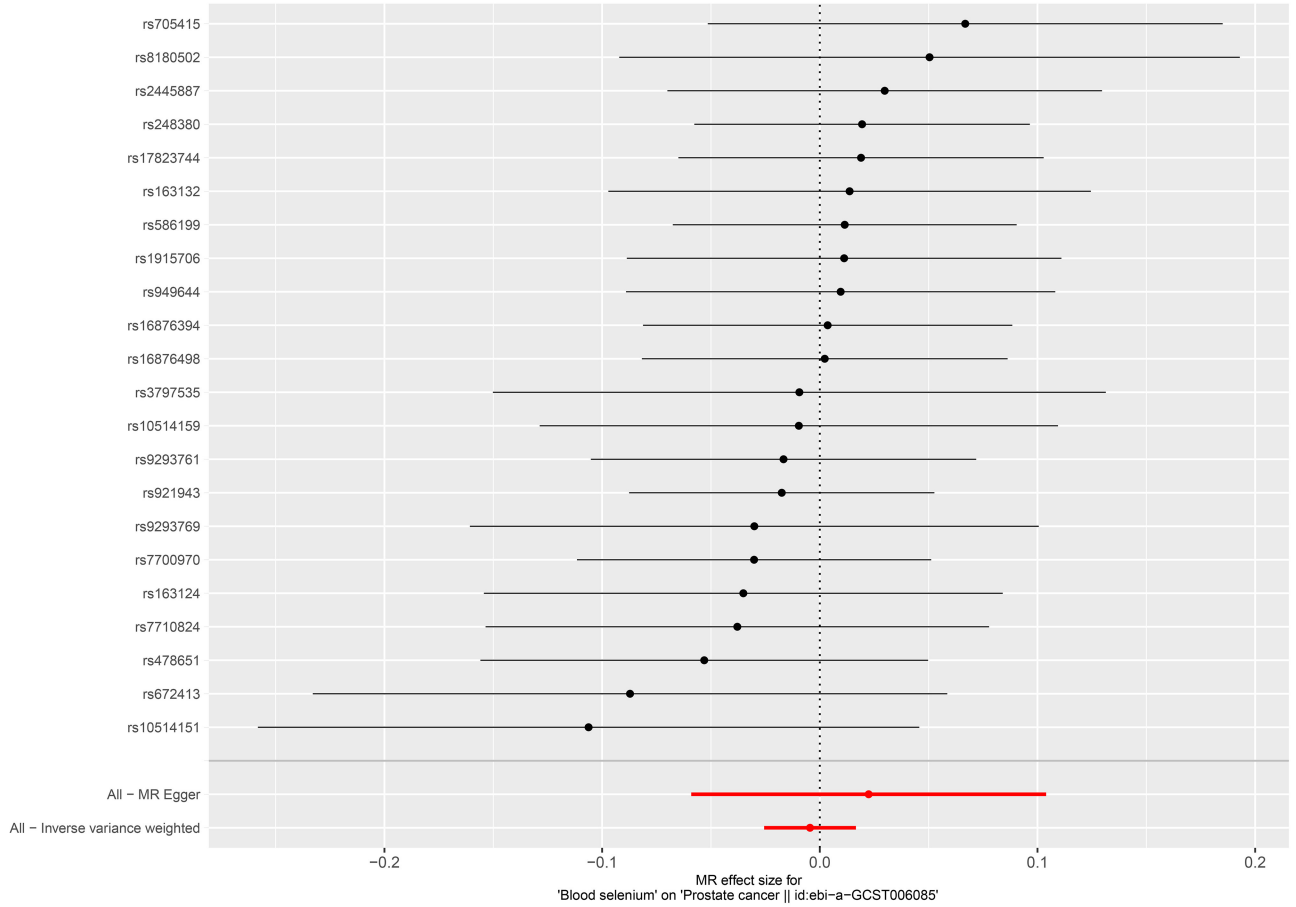

FIGURE 4
Forest plot for the association between circulating selenium and the risk of prostate cancer.

serum copper levels could genetically increase the risk of RCC. It might be attributed to oxidative stress responses to accumulation of heavy metals, while the exact underlying mechanism requires further studies.

For bladder cancer, former studies indicated that increased copper or zinc levels in the blood of patients were associated with angiogenesis in BCa and the risk of BCa (34, 35). However, we found no genetic associations between copper or zinc levels and BCa risk. Two reasons might explain the difference. Firstly, the small number of available SNPs may cause statistical biases. Secondly, the results of former observational studies were influenced by confounders. More GWAS and experimental studies are warranted.

Two uncommon diseases featured by the pathological accumulation of micronutrients should be mentioned. Wilson disease (WD) is an inherited disorder of copper metabolism, which is caused by homozygous or compound heterozygous mutations (the presence of two different mutant alleles) in *ATP7B* (36). Copper absorbed from the diet and copper released from hepatocytes with exhausted endogenous copper storage capacity progressively accumulate in other organs, most notably in the brain, eyes, kidneys, bones, and heart, exerting extrahepatic toxicity. Almost 90% of patients with WD has reduced level of serum copper and copper is mainly accumulated in organs. The most frequently reported cancer in WD patients is hepatocellular carcinoma (37). Few studies have reported WD patients complicated with urologic cancers, while the anti-copper therapy has been used and verified as a validated treatment in several cancers (38, 39). Haemochromatosis is a systemic iron overload of genetic origin, caused by a reduction in the concentration of the iron regulatory hormone hepcidin, or a reduction in hepcidin-ferroportin binding. Similar to WD, haemochromatosis featured by the accumulation of iron in the liver, is associated with elevated serum ferritin and increased serum transferrin saturation rather than serum iron level and it is mostly reported to have an association with hepatocellular carcinoma (40). The association between the two diseases and urologic tumors still requires further investigation.

Our findings have some clinical and research implications. Firstly, we firstly indicated the genetic associations between micronutrients and three main urologic tumors by using mendelian randomization. Some of the micronutrients we identified in this study can be used as cancer biomarkers for risk prediction. While the prerequisite to achieving this is the clear association between the serum micronutrient level and cancer risks (linear or U-shape or . . .). Based on this, we can further make a classification strategy, for example, using the median level as the cut-off. Given current evidence, there is still a long way to go. Secondly, appropriate therapy that could adjust micronutrient levels in the blood will contribute to the prevention of urologic tumors and, eventually, of the cancer-associated disease burden and mortality. However,

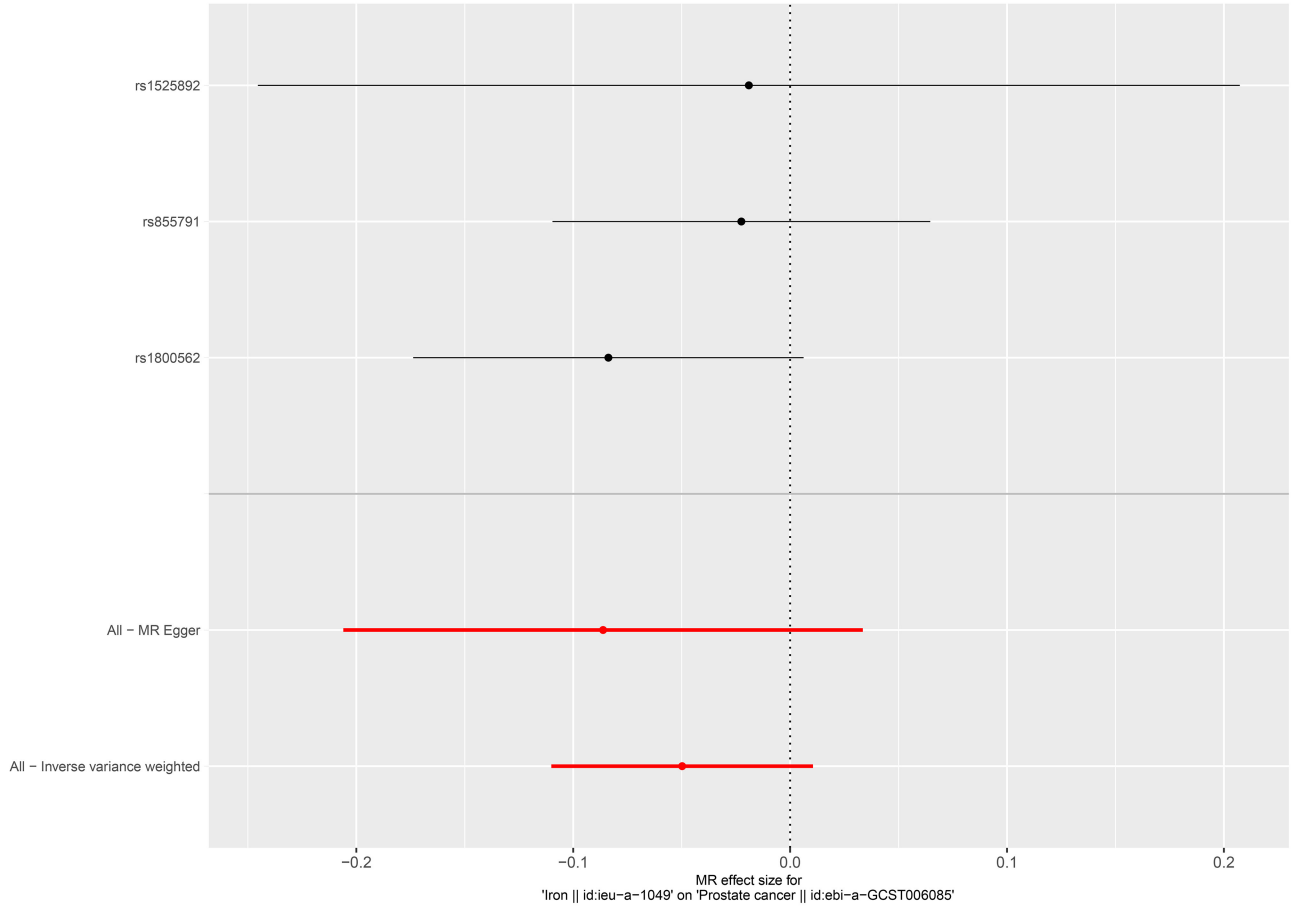

FIGURE 5
Forest plot for the association between circulating iron and the risk of prostate cancer.

it should be noted that no clear evidence (the number of RCTs is <20) about the micronutrient intervention and cancer risk or cancer progression can be found. While more research is needed to assess whether micronutrients may modify the risk of cancer in individuals with a specific genetic background or nutritional status, and to investigate possible differential effects of various forms of micronutrients. Thirdly, the bias caused by limited numbers of SNPs should be validated in experimental studies. Fourthly, the conflicting findings on the effect of Zinc on PCa and RCC should be examined in experimental studies. According to currently available literature, we supposed that different zinc-related protein expressions in the kidney and the prostate might play a role in the development of the two cancers. Moreover, the balance between the zinc influx protein family and zinc efflux transporters on different organs might make a difference (27).

Our study has some strengths. Firstly, the study was the first MR study to investigate the casual association between micronutrient levels and the risk of urologic tumors. Effects of confounders in observational studies are avoided. Secondly, all the included individuals were of European-descent, which could minimize the potential bias from population stratification. Additionally, four common micronutrients and three main urologic tumors were analyzed, which is comprehensive and informative. Finally, limitations should be pointed out. Firstly, findings achieved from the MR study consisted of European-descent population limited the generativity to other races. Secondly, serum micronutrients might have associations with nutrition status, intelligence, income, and education level, etc (41). All these factors might play as a confounder between micronutrients and urologic tumors, while a concrete role of these factors was not the aim of the study and it requires further research.

In conclusion, we found that genetically increased Zinc levels were related to increased risk of PCa, while reduced risk of RCC. Furthermore, increased Copper and iron level were associated with increased risk of RCC, no causal effects were observed in BCa. The results indicate that micronutrients play a vital role in urological tumors. Future studies are therefore warranted to validate our findings and examine whether micronutrient concentration surveillance or supplements could be potential interventions for urologic cancer prevention and treatment.

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.

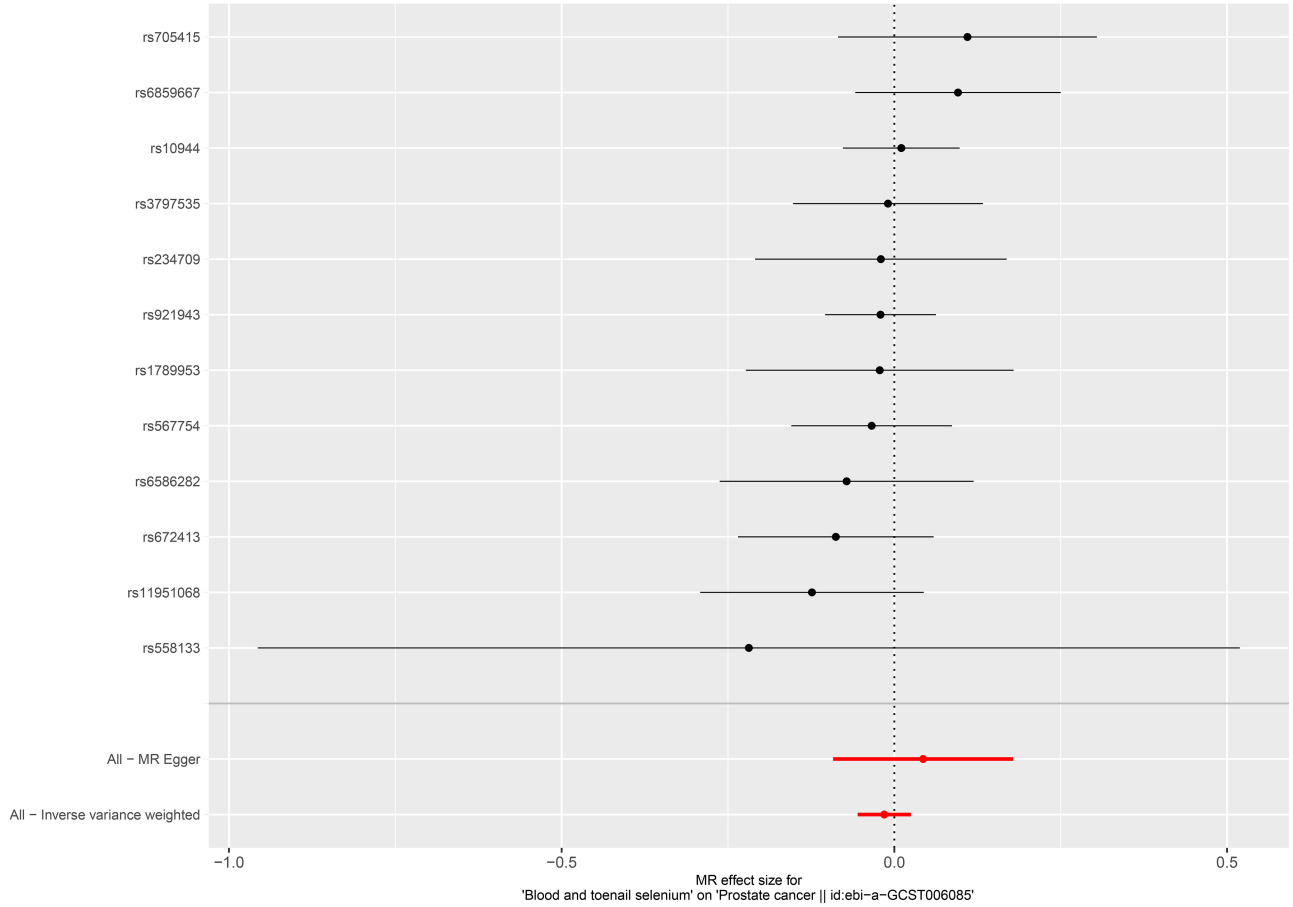

FIGURE 6
Forest plot for the association between blood and toenail selenium and the risk of prostate cancer.

Ethics statement

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

YL, YW, and HL: conception and design. HL: administrative support. YL, HS, and YW: collection and assembly of data. YW and HS: data analysis and interpretation. All author manuscript writing and final approval of manuscript.

Funding

This work was supported by the grant from National Population Health Science Data Sharing Service Platform Clinical Medical Science Data Center (NCMI-ABD02-201906) to HL.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1016243/full#supplementary-material

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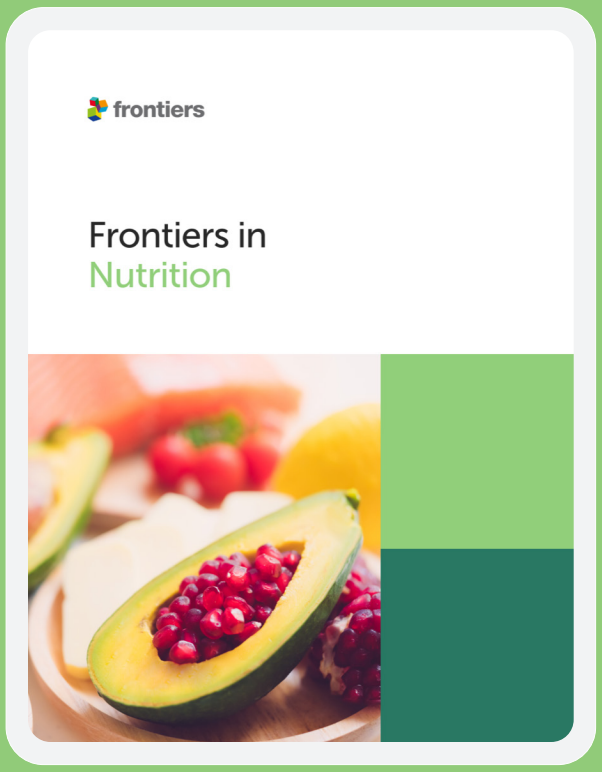

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