frontiers | Research Topics

Resistant starch: advances and applications in nutrition for disease prevention

Edited by
Hongliang Zeng, Xianyang Bao and Hongmin Dong

Published in
Frontiers in Nutrition

ISSN 1664-8714
ISBN 978-2-8325-6537-7
DOI 10.3389/978-2-8325-6537-7

Generative AI statement
Any alternative text (Alt text) provided alongside figures in the articles in this ebook has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Resistant starch: advances and applications in nutrition for disease prevention

Topic editors

Hongliang Zeng — Fujian Agriculture and Forestry University, China

Xianyang Bao — Harvard University, United States

Hongmin Dong — Cornell University, United States

Citation

Zeng, H., Bao, X., Dong, H., eds. (2025). *Resistant starch: advances and applications in nutrition for disease prevention*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-6537-7

Table of contents

frontiers | Frontiers in Nutrition

TYPE Editorial
PUBLISHED 19 June 2025
DOI 10.3389/fnut.2025.1636551

OPEN ACCESS

EDITED AND REVIEWED BY
Elena Ibañez,
Spanish National Research Council (CSIC), Spain

*CORRESPONDENCE
Hongmin Dong
✉ hongmin1@ualberta.ca

RECEIVED 28 May 2025
ACCEPTED 04 June 2025
PUBLISHED 19 June 2025

CITATION
Dong H, Bao X and Zeng H (2025) Editorial: Resistant starch: advances and applications in nutrition for disease prevention.
Front. Nutr. 12:1636551.
doi: 10.3389/fnut.2025.1636551

Editorial: Resistant starch: advances and applications in nutrition for disease prevention

Hongmin Dong[1]*, Xianyang Bao[2] and Hongliang Zeng[3]

[1]Department of Food Science, Cornell University, Ithaca, NY, United States, [2]John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, United States, [3]Department of Food Science, Fujian Agriculture and Forestry University, Fuzhou, China

KEYWORDS

resistant starch (RS), gut micobiome, glycemic control, food processing, metabolic health, chronic disease prevention, starch modification, short-chain fatty acid (SCFA)

Editorial on the Research Topic
Resistant starch: advances and applications in nutrition for disease prevention

With the escalating global burden of chronic diseases including type 2 diabetes, cardiovascular disease, obesity, and inflammatory conditions, there is increasing interest in dietary strategies that extend beyond simple caloric reduction. Among emerging bioactive components, resistant starch (RS) has gained considerable attention due to its unique physiological functions and broad applications in nutrition and health. This Research Topic, "*Resistant Starch: Advances and Applications in Nutrition for Disease Prevention*," brings together 10 peer-reviewed articles that collectively present a multidisciplinary and up-to-date exploration of RS. These contributions offer critical insights into its molecular characteristics, metabolic effects, food applications, and future directions in research and functional food innovation.

Molecular classification and structural characteristics

The foundation of understanding RS is rooted in its classification and molecular structure. RS is typically classified into five primary types: RS1 (physically inaccessible starch), RS2 (native granular starches such as high amylose maize), RS3 (retrograded starch formed by cooking and cooling), RS4 (chemically modified starch), and RS5 (amylose lipid complexes). Among these, RS5 has gained increasing interest due to its stability, resistance to digestion, and potential physiological benefits. Studies in this Research Topic highlight how preserving starch crystallinity and molecular architecture is crucial for digestion resistance and fermentation patterns in the colon (Baptista et al.). These structural insights guide the design of targeted modifications to starch, optimizing RS formation and enhancing its functional potential in food products (Zhang et al.; Warwate et al.). One study investigated the synergistic modification of rice starch using hot-melt extrusion and nobiletin, a citrus-derived polymethoxylated flavone (Zhang et al.). This combined approach resulted in enhanced molecular interactions and altered multi-scale

structures, resulting in increased thermal stability and reduced *in vitro* digestibility of starch. The application of such dual-modification techniques offers promising directions for tailoring the functional properties of starch-based ingredients, including increasing RS content and reducing glycemic impact in functional food formulations.

Physiological effects and gut microbiome interactions

Several studies in this Research Topic confirmed that intake of RS, particularly RS1 and RS2, improves glycemic control by lowering postprandial glucose and fasting insulin levels (Kaur et al.; Chauhan et al.). This effect is highly relevant for the management and prevention of type 2 diabetes and related metabolic conditions. Furthermore, RS acts as a prebiotic, selectively stimulating the growth of beneficial gut bacteria such as *Bifidobacterium*, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*. This microbial modulation enhances SCFA production, particularly butyrate, which has been linked to anti-inflammatory effects, improved gut barrier function, and even modulation of systemic immune responses. One clinical trial using a resistant starch blend from potato, banana, and apple fibers demonstrated improved gastrointestinal symptoms and favorable shifts in microbiome composition, demonstrating RS's potential in human health interventions (Hanes et al.). These findings highlight the role of individual microbiome variability in influencing RS fermentation and health outcomes, emphasizing the need for personalized nutrition approaches.

Food processing and technological applications

The translation from laboratory research to commercial application depends heavily on understanding how food processing affects RS content. Processing methods such as milling, heating, fermentation, and cooling induce changes in starch structure and consequently RS levels. Several studies in this Research Topic investigate how traditional and novel cooking and storage methods impact RS content in staple foods. For example, research on commonly consumed Indian wheat products demonstrates that cooking techniques like boiling and shallow frying increase RS levels, while deep frying reduces them. Storage conditions, especially refrigeration, promote starch retrogradation, thereby increasing RS content (Kaur et al.). Similarly, mung beans subjected to specific cooking and storage regimes showed increased RS content and favorable metabolic outcomes *in vivo* (Chauhan et al.). Such findings offer practical guidance for food manufacturers and consumers aiming to maximize RS intake through everyday foods. Food technologists also explore the creation of RS-enriched staples such as bread, pasta, and rice by incorporating high-amylose or modified starches. Challenges related to dough manipulation, sensory characteristics, and consumer acceptance are addressed, demonstrating that RS enrichment can lower the glycemic index of foods without compromising taste or texture (Warwate et al.). These innovations offer practical strategies for delivering RS's health benefits through everyday diets.

Broader metabolic and health implications

Beyond glucose regulation and gut health, RS impacts diverse metabolic and immunological pathways. It modulates bile acid metabolism, gut immune responses, and systemic inflammatory markers such as C-reactive protein and interleukins. Randomized controlled trials report reductions in LDL cholesterol and systemic inflammation with RS supplementation, suggesting benefits that extend to cardiovascular risk reduction and weight management (Wan et al.). The link between RS intake and weight regulation is particularly notable. Clinical data indicate that RS enhances satiety and appetite control, contributing to modest reductions in body weight and fat mass, especially in overweight or diabetic populations. These findings position RS as an adjunctive nutritional strategy in combating obesity and related metabolic disorders. Importantly, epidemiological evidence from large cohort studies associates higher RS intake with reduced all-cause and cancer-specific mortality, highlighting its potential role in long-term health and longevity (Wan et al.). These associations warrant further mechanistic and interventional studies to confirm causality and elucidate optimal intake levels.

Emerging frontiers and future directions

This Research Topic concludes with promising perspectives on novel RS complexes, such as starch-protein and starch-polyphenol conjugates. These innovative structures exhibit improved stability and enhanced physiological effects, broadening the functional repertoire of RS. One study highlights the synergistic effect of dietary amylose-to-amylopectin ratio on antioxidant status and amino acid metabolism in piglets, showing how starch structure interacts with nutrient metabolism in the liver (Yang et al.). Additionally, new research explores the role of mineral intake in brain health, including associations between manganese, zinc, magnesium, and cognitive performance, which may intersect with RS's influence on gut-brain axis and metabolic regulation (Chen et al.). Furthermore, a study from the Iranian Teachers Cohort reported that higher dietary glycemic index and load were significantly associated with increased odds of osteoporosis, independent of insulin-related dietary measures. These findings emphasize the importance of carbohydrate quality in bone health and suggest that RS, by lowering glycemic response and supporting SCFA production, could potentially contribute to the mitigation of osteoporosis risk (HoushiarRad et al.).

Future studies should continue to elucidate mechanistic pathways underlying RS's health effects and explore

personalized nutrition strategies that consider individual microbiome and metabolic profiles. Moreover, optimizing food processing technologies to maximize RS content while ensuring consumer acceptance will be crucial for translating research findings into impactful public health solutions.

Conclusion

This Research Topic provides a timely and comprehensive examination of resistant starch, highlighting its complex molecular characteristics, physiological benefits, interactions with gut microbiota, and practical food applications. As a versatile dietary component with the potential to improve metabolic health, reduce inflammation, and support chronic disease prevention, RS represents an important focus in contemporary nutrition science. The integration of fundamental research with food technology innovation offers a promising path toward leveraging resistant starch for enhanced public health worldwide.

Author contributions

HD: Writing – original draft, Writing – review & editing. XB: Writing – original draft, Writing – review & editing. HZ: Writing – review & editing, Writing – original draft.

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

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 29 September 2022
DOI 10.3389/fnut.2022.987216

OPEN ACCESS

EDITED BY
Alfonso Benítez-Páez,
Principe Felipe Research Center (CIPF), Spain

REVIEWED BY
Armando R. Tovar,
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ), Mexico
Jane Adair Mullaney,
AgResearch Ltd., New Zealand

*CORRESPONDENCE
Noelle Patno
npatno@stanfordalumni.org

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

RECEIVED 05 July 2022
ACCEPTED 22 August 2022
PUBLISHED 29 September 2022

CITATION
Hanes D, Nowinski B, Lamb JJ, Larson IA, McDonald D, Knight R, Song SJ and Patno N (2022) The gastrointestinal and microbiome impact of a resistant starch blend from potato, banana, and apple fibers: A randomized clinical trial using smart caps. *Front. Nutr.* 9:987216. doi: 10.3389/fnut.2022.987216

The gastrointestinal and microbiome impact of a resistant starch blend from potato, banana, and apple fibers: A randomized clinical trial using smart caps

Douglas Hanes[1], Brent Nowinski[2], Joseph J. Lamb[3], Ilona A. Larson[4], Daniel McDonald[5], Rob Knight[2,5,6,7], Se Jin Song[2] and Noelle Patno[4]*

[1]National University of Natural Medicine, Helfgott Research Institute, Portland, OR, United States, [2]Center for Microbiome Innovation, University of California, San Diego, San Diego, CA, United States, [3]Personalized Lifestyle Medicine Center, Gig Harbor, WA, United States, [4]Formerly Metagenics, Inc., Aliso Viejo, CA, United States, [5]Department of Pediatrics, University of California, San Diego, San Diego, CA, United States, [6]Department of Bioengineering, University of California, San Diego, San Diego, CA, United States, [7]Department of Computer Science and Engineering, University of California, San Diego, San Diego, CA, United States

The gastrointestinal (GI) impact of fibers including resistant starch (RS) consumption depends on various types and amounts of fibers, the initial microbiome states, and accurate intake measurements. A randomized clinical trial evaluated the GI impact of varying doses of a novel resistant starch blend (RSB) with smart cap monitoring. RSB contained at least 50% RS and was a proprietary mixture of a potato starch, green banana flour, and apple fiber powder (a source of apple pectin, not resistant starch). The study design randomized participants to one of four arms: 10 g/day of potato starch (0 RSB), 10 g/day of RSB, 10 to 20 to 20 g/day of RSB or 10 to 20 to 30 g/day RSB for two-week intervals over 6 weeks. Results confirmed that while resistant starch of approximately 5 g per day improves GI symptoms at 2, 4, and 6 weeks, it did not demonstrate a detectable effect on short chain fatty acids. Increasing doses of the blend (RSB) led to a decrease in the diarrhea score. Using an estimate of total consumption of RSB based on smart cap recordings of container openings and protocol-specified doses of RSB, a reduction in the sleep disturbance score was associated with higher RSB dose. The exploratory microbiome evaluation demonstrated that among the 16S rRNA gene sequences most associated with the consumption of the novel blend RSB, two belong to taxa of notable interest to human health: *Faecalibacterium* and *Akkermansia*.

KEYWORDS

prebiotic, monitoring, microbiome, *Faecalibacterium*, *Akkermansia*, compliance, fiber, sleep

Introduction

The recommended dose of total dietary fiber is currently set at 25–38 g/day in the US depending on the person's caloric needs, based on a 14 g/1,000 kcal adequate intake (1). This established intake is well-supported by epidemiological studies identifying the cardiovascular disease prevention benefit, with additional evidence for reducing risk of developing type II diabetes and colon cancer, improving gastrointestinal health and body weight control, and lowering risk of mortality (1–4). However, there is not a universally recommended daily dietary amount of resistant starch, nor is there an understanding of which sources of fiber or resistant starch need to comprise the 25–38 g/day (1). Most dietary fiber and resistant starch trials show benefits for a single ingredient above 15 g/day (1, 5, 6) yet it is unknown whether a blend of resistant starch and fibers may provide equivalent or improved benefits.

Of the four types of resistant starch, typically the retrograded starch (type III, example: cooked and cooled potatoes) and type II are the most common in the diet. Resistant starch type II, which is starch that escapes digestion in the small intestine due to its natural granular structure, can be found in high-amylose maize starch (HAMS), green banana starch, and raw potato starch (4). Resistant starch contains amylase-resistant glycans, resists digestion in the upper GI tract and has been shown to be metabolized by colonic amylolytic bacteria such as *Ruminococcus bromii* (7). It shows promise for controlling blood glucose and insulin levels, as well as acting as a prebiotic ("a substrate that is selectively utilized by host microorganisms conferring a health benefit") (8) by modulating the microbiome (4, 9). However, studies suggest that effects may be dependent on both dose and type of RS content (10–12).

High levels of resistant starch have been associated with health benefits and altering gut microbiota levels. The most recognized RS research supports the glucose-lowering benefit for reducing the risk for type 2 diabetes and used 15–40 g/day of HAMS (13–15). At the quantity of 159 g/day, HAMS (containing 66 g of RS) altered endogenous microbiota levels, including increasing the beneficial *Faecalibacterium* (10). Relative to stool samples from healthy individuals, *F. prausnitzii* has been found at lower levels in diseases including inflammatory bowel diseases (particularly Crohn's), irritable bowel syndrome, colorectal cancer (16), severity of coronavirus disease (COVID-19) (17) and cystic fibrosis (18). It has been shown to exhibit anti-inflammatory effects through butyrate production and immune cell modulation (19). As an obligate anaerobe, difficult to cultivate and not yet developed as a probiotic, *F. prausnitzii* has been a next-generation microbe of interest to target with prebiotics (20).

Interestingly, *F. prausnitzii* has also been found to be associated with individuals who consume a high diversity of plants in their diet (21), suggesting that this species may be influenced by components besides resistant starch. Consistent with this idea, *in vitro* studies have shown that specifically apple pectin (a fiber but not a resistant starch) supports the growth of multiple strains of *F. prausnitzii* (22, 23). Pectin also has greater specificity in stimulating specific microbes' growth and short-chain fatty acids when compared to fructooligosaccharides (FOS) and resistant starch type II (24). Extrapolating from these *in vitro* studies, apple pectin has the potential to modulate *F. prausnitzii* at lower doses than the quantities observed from RS intake studies.

Raw potatoes and green bananas provide alternative sources for resistant starch and also have been studied for glucose control benefits and microbiome impact. While the concentration in HAMS is 46%, raw potato starch contains a higher quantity (63%) and green banana flour contains similar quantities (44%), as described in the Association of Official Analytical Collaboration (AOAC) 2002.02 method publication (25). Although native potato starch has the highest content, its consumption at 48 g/day yielded variable responses in butyrate production which may have been due to differences in the initial microbiomes of the young men (26). Studies using 30 g raw potato starch/day for 12 weeks led to relative increases in *bifidobacteria* and improved glycemic responses in elderly people (27, 28). In other studies, 38.3–40 g of native banana starch improved postprandial glycemic responses and reduced supplemented meal consumption (29, 30). A few studies have shown daily consumption of various amounts and types of resistant starch (17–66 g) resulted in higher levels of SCFA (10, 12, 26, 31–33) in as little as 1–3 weeks. Yet it remains to be explored whether a diversity of fibers at a lower dose may obtain GI and glycemic benefits in a short period of time or whether the baseline microbiome or other characteristics impact a person's response to such an intervention.

Disparities in study outcomes may also be due to differences in participant consumption. Adherence to protocols can vary for reasons including anticipated negative effects of consuming large amounts, such as flatulence resulting from 39 g (12). Moreover, traditional methods of self-reporting can overestimate adherence by 17%, and pill count can overestimate by as much as 8%, which suggests that alternative methods may need to be developed to track consumption more accurately, such as electronic detection of package opening by the participant (34). Measuring methods such as tracking in a log, performing a pill count, or weighing the supplement remaining after being dispensed have been utilized, and adherence in a clinical trial has been observed to be as low as 46 or 55% (35). For this study, a unique smart cap designed to detect acceleration of the cap rotation, and subsequent flipping upside-down (36) was used to track opening of the supplement container.

The resistant starch blend of natural fibers was developed to deliver a high quantity of resistant starch (utilizing raw potato starch), to utilize a diversity of resistant starches (with the addition of green banana flour) and to deliver a positive impact to the microbiome (e.g., anticipated to be enhanced

with the addition of apple pectin). The primary aim was to evaluate the impact compared to a single-source RS from potato on short-chain fatty acids (SCFA), specifically butyrate. The study was designed to evaluate doses of the blend ranging between 10–30 g/day in 2–6 weeks for its impact on SCFA production, GI symptoms, well-being, and sleep measures, as well as explore the impact on the gut microbiome. With a diversity of ingredients, the increasing doses of the resistant starch blend (RSB) was anticipated to increase SCFA, improve GI symptoms, and possibly alter microbiota in ways associated with human health.

Materials and methods

Eligibility

The clinical trial ran from June 2019 through December 2020 at the Personalized Lifestyle Medicine Center (PLMC) in Gig Harbor, WA as a single-center, randomized, blinded, placebo-controlled parallel trial. Men and women ages 21–65 years, self-reporting on the presence of minor bloating, constipation, or irregular bowel movements, were recruited. Exclusion criteria were the following: unwillingness to follow study procedures; current or recent consumption of probiotics, resistant starch, prebiotic, or fiber supplements (14 days before first stool collection); current or recent (within last 28 days) use of antibiotic, antiparasitic, or antifungal drugs; current use of supplements or medications such as proton pump inhibitors (PPIs), opioids, or selective serotonin reuptake inhibitors (SSRIs) that may impact GI motility; current unstable or serious illnesses or infections or cardiovascular diseases; history of diabetes or hypoglycemia or prediabetes; personal history of mental illnesses; known allergy or intolerance to supplement ingredients; diseases affecting digestion and absorption of nutrients; GI/bariatric surgery within the last 5 years; current colostomy/ileostomy; genitourinary bacterial infections within the last 28 days; major hospitalizations within the preceding 3 months; skin or cervical cancer within the last 5 years; current or recent (past 30 days) use of nicotine or smoking; alcoholism or diagnosis within the last 12 months and during study; alcohol consumption that was more than 2 glasses at a time or more than 4 glasses within the prior two weeks; use of recreational drugs within 12 months prior and during the study; major changes to diet or exercise within 28 days of screening or during study, including dietary weight loss program; and current or recent (within 28 days) involvement in another interventional study. To minimize impact to the intestinal microbiota, alcohol usage was limited to 1–2 glasses of light beer or wine for 1–2 days after stool collection during the study for those who selected Track A, while the majority selected Track B, the no-alcohol consumption option during the study. Participants were primarily recruited locally via flyer and word of mouth, online via the Personalized Lifestyle Medicine Center's Facebook page, and then expanded to other states through multiple online ads during the COVID-19 pandemic-associated lockdown when the trial became a virtually conducted, remote study.

The study protocol was approved by Aspire Institutional Review Board (IRB) on 14 May 2019. The IRB tracking number is 520190117. All participants provided written informed consent. Trial registration number at ClinicalTrials.gov: NCT03983772.

Study design and randomization

The study design included four groups with one on a potato starch for 10 g a day for 6 weeks and three groups on RSB in a dose-ramping design as follows: group 1 on 10 g/day of RSB for 6 weeks, group 2 taking 10 g/day of RSB for 2 weeks and then increasing the dose from 10 g/day to 20 g/day of RSB for the next 4 weeks, and group 3 taking 10 g/day of RSB for 5 weeks, then 20 g/day of RSB for 2 weeks, and finally 30 g/day of RSB for 2 weeks (see Supplementary Figure S1). Participants were advised to consume their RSB cold or cool, mixed in liquid or food, and were asked to track how they took the RSB. All groups experienced a 2-week run-in period prior to the interventional supplement consumption for baseline measurements to evaluate physiological variability of the measured outcomes. Power calculations used data from previously published work from Phillips et al. (12), a randomized crossover design testing 5 v. 39 g/day resistant starch consumption that resulted in 7.2 mmol/L more butyrate following the high RS consumption after 3 weeks. We calculated that, with 10 participants per group, we would have 80% power to detect a mean change of 7.2 mmol/L in butyrate between two independent groups using an independent *t*-test design (calculations made using STATA v.14). The intended enrollment number was therefore determined to be 40 with 10 per group. PLMC study staff randomly assigned participants in a 1:1:1:1 ratio by using the program at https://www.randomizer.org to associate group assignments with the order of enrollment. Participants were randomized after they passed screening but prior to receiving the supplement. For any participants who withdrew prior to receiving supplement, corresponding group assignments were re-entered at the end of the randomization list for reassignment to new subjects entering the study. Participants and physician were blinded to the product assignment at the beginning of the study. For a few adverse events, the physician and participant, upon request of the study staff, were informed of the product assignment.

All participants were asked to maintain their current diet, exercise, and lifestyle habits and were provided with a list of foods to avoid and a list of foods to maintain similar consumption throughout the study (to minimize changes in the possible confounder of dietary fiber intake throughout the study) (list included in Supplementary Table S1). To monitor

dietary consumption throughout the study, a 24-h food recall was requested within 3 days of each visit. Participants were asked to provide the dietary recall immediately after stool collection as much as possible. Dietary intake data for 24-h recalls were collected and analyzed using the Automated Self-Administered 24-h (ASA24) Dietary Assessment Tool, version 2018 developed by the National Cancer Institute, Bethesda, MD (37). The data extracted from the ASA24 reports for subsequent analysis were total calories (kilocalories), fat (as a percentage of kilocalories), protein (in grams), carbohydrates (in grams), and fiber (in grams). The fiber intake reported on the ASA24 did not include the fiber in the intervention, as consumption of the intervention was recorded on the product dosing log, extrapolated from the product weight and the smart cap monitoring.

Material and smart caps

The material for the intervention was a proprietary RSB containing the clinically-studied (27, 28) native potato starch (MSPrebiotic®; Carberry, Manitoba, Canada), green banana flour (Nubana; Alsip, IL) and a source of apple pectin from apple fiber powder (Mayer Brothers; West Seneca, NY). The resistant starch content of the RSB was designed to be at least 50% per the AOAC 2002.02 method and was confirmed in prior batches. The comparison was a native potato starch called Potato Starch Superior from Emsland (Piscataway, NJ) referenced herein as PS for Potato Starch). The lot used for quality acceptance testing of the Emsland potato starch material resulted in <5% of resistant starch per the AOAC 2002.02 method. While the supplier's product specification stated that only traces of fiber were present, test results of product returned by participants confirmed amounts of overall RS equivalent to that in the RSB; RSB resistant starch content was 55.7% and the potato starch resistant starch content was 52.6% (AOAC 2002.02 method, Covance lab, Madison, WI). Repeating the testing on the potato starch resulted in 68.4% for the resistant starch, and 72.0% for the total fiber content, using the 2011.25 method. The RSB test results for fiber were 55.8% soluble fiber with 12.3% insoluble fiber while the potato starch had 59.0% soluble fiber and 12.9% insoluble fiber (AOAC 2009.01 and 2011.25 methods, Covance Lab, Madison, WI). Metagenics, Gig Harbor, WA, tested the RSB and Emsland Potato Starch for heavy metals and microbiological contamination prior to releasing the clinical test product for use. Material was packaged in jars with "smart" caps to track intake compliance using an internal monitor to detect each event that the cap had been twisted off and set upside down with a time and date stamp (US Patent 10,874,591 B2) (36). Smart caps were generously provided by Amway (Ada, MI).

Outcomes

The primary outcomes of the study were fecal butyrate, total SCFA, acetate, and propionate, as obtained from self-collected samples. The protocol requested stool sample collection to occur within 3 days of the protocol-scheduled visit based on the participant's ability and motility (see Supplementary Figure S1). Participants used the pink-top tube which contained preservative from the Genova Diagnostics' test kit, which includes a built-in scoop in the tube lid for participants to collect stool from the stool deposited into a container. Participants shipped tubes per the kit manufacturer's instructions (Federal Express) to Genova Diagnostics for analysis. Fecal SCFA test results were provided by Genova Diagnostics (Asheville, NC) using gas chromatography-mass spectrometry (GC-MS) as described by Lihong et al. (38).

Secondary outcomes included the Bristol Stool Form (Bristol Stool chart rating) and fecal frequency (using the average across 7 consecutive days reported within each two-week interval between visits) as well as GI symptoms from validated questionnaires (39). Patient Reported Outcomes Measurement Information Systems (PROMIS) Scale v1.0—GI Diarrhea 6a T-score, PROMIS Scale v1.0—GI Constipation T-score, and PROMIS Scale v1.0—GI Gas and Bloating 13a T-score collected at each visit. T-scores are also simply called PROMIS scores in this manuscript.

Exploratory outcomes included wellbeing as assessed by validated questionnaire Quality of Life in Neurological Disorders (Neuro-QoL) Item Bank v1.0—Positive Affect and Well-Being (40), PROMIS Short Form v1.0—Sleep Disturbance 8b (41), standard lipid panel (total cholesterol, LDL, triglycerides, and HDL); insulin (through the Comprehensive Metabolic Panel from QuestQuanum, Quest Diagnostics, (West Hills, CA and Seattle, WA); and evaluation of microbiome changes through the American Gut Project (DNA sequencing of the V4 region of the 16S rRNA gene) (21).

Fasted blood draws were performed at visits 1, 2, and 5 to evaluate physiological changes at run-in and the 6-week impact of the intervention on cholesterol and insulin as exploratory outcomes, as well as serving as a safety check. These safety measures involved the following: vital signs (blood pressure, respiration rate, pulse), height (first visit only), body weight, and blood draws (previously described). Compliance measures included the following: ASA24 (previously described), a supplement container using a "smart" cap that counted times rotated and turned upside down for container openings (36) and supplement weight measurements and participant logs of supplement consumption.

Vital signs and blood draws were suspended during the COVID-19 pandemic associated lockdown while telehealth rather than in-person visits were utilized.

Statistical analysis

The intention-to-treat (ITT) analysis included all participants enrolled in the study, regardless of adhering to restricted alcohol intake or medication use during the

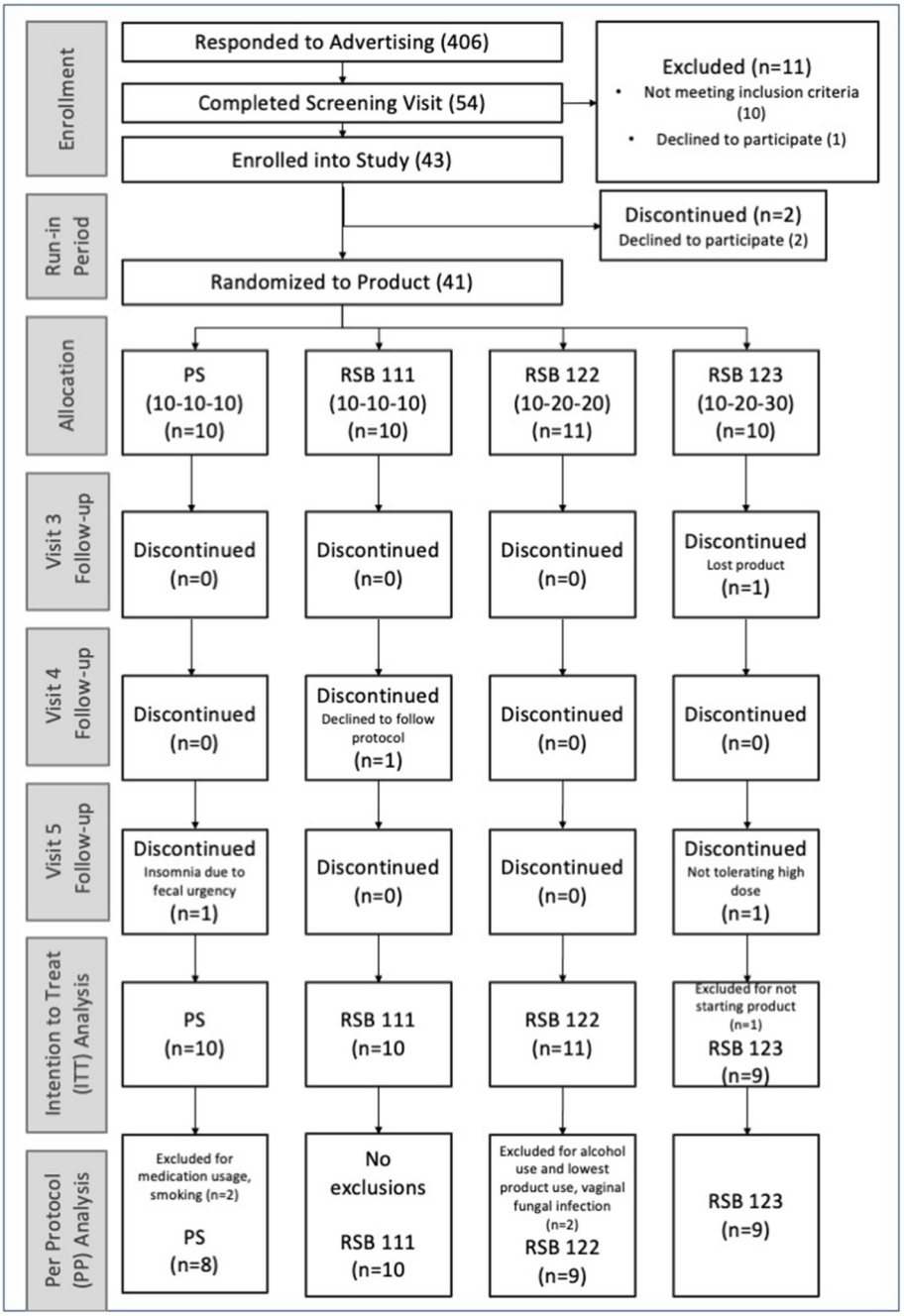


FIGURE 1
Consort diagram. Enrollment of the participants and numbers per group included in the ITT and PP analyses. Randomization to product occurred at visit 2.

study (refer to Figure 1). An evaluation of the protocol criteria was performed on the data collected in study visits with the physician as well as evaluating the consumption of supplement weight (using a minimum cut-off of 50% for the supplement weight and a minimum of 70% for the dosing log). The per-protocol (PP) analysis excluded four participants due to alcohol consumption prior to stool collection, smoking, excluded medications, an ongoing infection present at baseline; the PP analysis also excluded visit 5 data from two participants who discontinued supplementation (refer to the CONSORT diagram, Figure 1).

For missing data in the fecal SCFA category, we imputed values returned as below the detection level using random imputation between the reported lower detection limit and/or the lowest reported value (whichever was lower) and one-half that value. Data missing for all other outcome measures was not replaced. The PROMIS outcome measures were scored using the HealthMeasures Scoring Service, an online application powered by Assessment CenterSM. Means and confidence intervals were calculated for the primary and secondary outcome measures from both the ITT and PP analyses for each visit as recommended by CONSORT guidelines (42).

For comparison across time points and categories, the baseline was established as an average of the data from visit 1 and visit 2 (or the value at one of these visits, if the other was missing), minimizing data loss. Differences in outcomes between groups were assessed using linear mixed models with a random intercept and either a factor for randomized group or a measure of time-dependent dose as the main predictor. Significant effects of randomized group were followed by pairwise comparisons of treatment dose groups against PS. For smart cap analysis, the percentage of smart cap usage was calculated as the number of days having at least one event of using the smart cap per day during the time period between visits divided by the total number of days during the visits (during which consumption was expected). This smart cap percentage was multiplied by the expected delivered dose of RSB per day to determine the "smart cap dose" and was summed over the 6 weeks for the "total smart cap dose." The smart cap dose thus reflected the dose of g of RSB and was zero for all those in the PS group. Counting days tracked of supplement consumption using participant logs and using the dispensed supplement weight were alternative measures of estimating supplement consumption also used to explore effects on secondary and exploratory questionnaire outcome measures. Additionally, fiber intake from the ASA24 questionnaires was used to assess as a confounder. Statistical analysis of treatment effects was carried out using R v.4.0.2 (43).

Microbiome analysis

Stool samples were collected and processed using the American Gut Project protocols for analysis of the V4 region of the 16S rRNA gene (21). Briefly, DNA was extracted using the Qiagen MagAttract PowerSoil kit in three separate batches, the V4 region was amplified using the 515f/806r primer set as described in the Earth Microbiome Project [(http://www.earthmicrobiome.org/protocols-and-standards/16s), and the resulting pool was sequenced on an Illumina MiSeq instrument] in two separate runs. Raw reads were demultiplexed and quality-filtered using Qiita (44) keeping reads with a Phred score of 4 or higher, trimmed to 150 nt, and denoised to amplicon sequence variants (ASVs) using deblur v1.1.0 (45). A phylogenetic tree for diversity analyses was created through fragment insertion with the Greengenes v13_8 as a reference backbone (46, 47). Microbiome analyses with amplicon sequencing variants (ASVs) were run using QIIME2-2020.6 (48). Amplicon sequencing variants (ASVs) were classified using the SILVA 138 release (49), and taxa to bloom during the storage of samples prior to processing were removed as previously described (50). For alpha and beta diversity analyses, ASV tables were rarified to 1,400 sequences per sample, and metrics were calculated using the Q2 core-metrics-phylogenetic plugin. Prior to rarefying, 6 samples with fewer than 1,400 reads were discarded, and the remaining samples ranged from 1,410 to 46,684 reads (average: 19,161). Robust Aitchison PCA (RPCA) was also run on the unrarefied data to verify that results were not influenced by rarefaction. Longitudinal analyses and linear mixed effect models of beta diversity across timepoint were done using the QIIME longitudinal plugin. Ranked differential abundance of taxa associated with RSB vs. PS was done through the Q2 Songbird plugin (51) on the unrarefied data with the following parameters: batch_size = 14, summary_interval = 0.01, epochs = 40,000, num_random_test_examples = 11, and differential_prior = 0.5. Log-ratios of the top and bottom 10% of ranked ASVs associated with RSB or PS were visualized and extracted using Qurro (52), and Kruskal-Wallis rank sum test computed with the kruskal.test function in R version 4.1.1 (53).

Results

Characteristics of the participants

Of 406 advertisement respondents, 54 people completed the screening visit; of these, 10 were not eligible due to protocol criteria, and one elected not to participate. Of the remaining 43 enrolled into the study, two withdrew before starting the supplement. Participants were randomized to groups at the second visit, before receiving the supplement. Four participants dropped out after receiving the supplement. Two of these participants were not able to comply with the protocol during the study: one lost the supplement, and the other declined to follow instructions. The other two dropped out due to lack of tolerance: one did not want to take the 30 g of RSB and another stopped the 10 g/day PS. The latter reported to the physician insomnia associated with fecal urgency while taking the potato starch. As planned, 40 participants were enrolled and included in the Intention-To-Treat (ITT) analysis. While 37 participants completed the study, 36 qualified for consideration in the Per Protocol (PP) Analysis (Figure 1). Demographic characteristics are listed in Table 1. Measures of body mass index and vitals were only obtained from a subset of participants prior to the study's becoming a remotely conducted study and therefore not included in the final analysis.

10.3389/fnut.2022.987216

TABLE 1 Baseline characteristics of the study population.

	PS	RSB111	RSB122	RSB123	Entire Cohort
Age (years, range)	27–50	22–59	24–64	24–59	22–64
Age (years, mean ± SD)	39.4 ± 5.9	45.3 ± 12.5	45.9 ± 14.3	35.2 ± 11.2	41.7 ± 11.9
Sex (number, %)					31
Female	5 (50%)	10 (100%)	11 (100%)	5 (56%)	(77.5%)
Male	5 (50%)	0	0	4 (45%)	9 (22.5%)
Race (number, %)					32 (80%)
White	6 (60%)	8 (80%)	9 (81.8%)	9 (100%)	2 (5%)
Black	1 (10%)	1 (10%)			4 (10%)
Asian, Pacific Islander	2 (20%)	1 (10%)	1 (9.1%)		2 (5%)
N/A – did not disclose	1 10%)		1 (9.1%)		
ethnicity, (number, %)					14 (35%)
Not Hispanic	4 (40%)	3 (30%)	4 (36.4%)	3 (33.3%)	1 (2.5%)
Hispanic			1 (9.1%)		
N/A – did not disclose	6 (60%)	7 (70%)	6 (54.5%)	6 (66.7%)	25 (62.5%)

Data are means ± standard deviation (SD), or number (%).

The dose effect of the blend (RSB)

Table 2 presents the values of the SCFA and GI symptom outcomes, ITT analysis. Supplementary Table S2 presents the values of the exploratory outcomes for the ITT analysis and Supplementary Table S3 presents the values for all outcomes for the PP analysis.

Significant effects of the dose of the blend were found for the PROMIS Diarrhea T-Score and Bristol Stool Form Score. Using the time-dependent dose predictor variable in the linear mixed model, higher doses of RSB were associated with reductions in diarrhea when adjusted for baseline in the ITT analysis ($p = 0.021$, $p = 0.082$ in PP analysis). An increase in the Bristol Stool Form Score was associated with increasing doses of RSB at visit 5 ($p = 0.046$ for the ITT analysis and 0.0038 for the PP analysis).

Temporal effects observed across dosing groups

Over the course of the 6 week study, all groups saw improved GI symptoms with decreases in the average scores of PROMIS diarrhea, constipation, and gas and bloating (see Table 2). Significant decreases over time in all groups were observed at 2, 4, and 6 weeks after supplement initiation for the PROMIS T-score of gas and bloating. The Bristol Stool Form score means remained between 3 ("like a sausage but with cracks on its surface") and 5 ("soft blobs with clear-cut edges, passed easily") (54) throughout. In the PP analysis (see Supplementary Table S3), time had an effect on a short-chain fatty acid, with butyrate higher at visit 4 ($p = 0.03$). The average sleep disturbance score was lower at each subsequent visit for all groups consuming any quantity of RSB in both ITT and PP analyses.

Fiber and RSB consumption

The fiber intake from the ASA24 dietary recall data was not identified as a significant covariate in any analyses. Table 3 shows the dietary intake averages and standard deviations, which were reported in between visits. Table 4 shows the RSB consumption as determined by self-reported product dosing logs, product weight calculations, and smart cap usage. These three methods of RSB consumption estimates were used in calculating the dose effect of RSB for the reported outcomes and the significant findings are presented in section The effect of the dose of the blend as calculated by smart cap usage.

The effect of the dose of the blend as calculated by smart cap usage

Using the dose of RSB as calculated by smart cap openings (see Methods), associations of RSB dose with decreases in diarrhea was consistent with the ITT and PP analyses ($p = 0.017$). Using the cumulative dose over time, as determined by smart cap openings or the RSB weight or dosing log, a decrease in constipation and the Bristol Stool Form number at visit 5 were associated with increased intake of RSB (all $p < 0.05$).

Lower sleep disturbance score was associated with increasing cumulative doses of RSB as calculated by smart cap openings by V4 (4 weeks) with $p = 0.04$ (see Figure 2). The change in T-score corresponded to a mean decrease of 2.23 in sleep disturbance after 2 weeks.

TABLE 2 Changes in SCFA and GI symptom outcomes during the study, ITT analysis.

Outcome	Group	Baseline	Visit 3			Visit 4			Visit 5		
		Mean	Mean ± SD	Mean change (CI)	*p*-value	Mean ± SD	Mean change (CI)	*p*-value	Mean ± SD	Mean change (CI)	*p*-value
Acetate (micromole/g)	PS	26.34 ± 12.64	24.44 ± 16.73	0.03 (−7.04 to 7.09)	0.99	38.65 ± 26.96	7.93 (−12.49 to 28.36)	0.38	35.42 ± 26.66	7.42 (−14.21 to 29.05)	0.44
	RSB111	29.17 ± 11.43	17.76 ± 12.05	−11.41* (−21.82 to −1.01)	0.03	32.00 ± 22.92	4.75 (−12.90 to 22.40)	0.54	19.56 ± 11.95	−6.63 (−13.36 to 0.10)	0.05
	RSB122	26.54 ± 18.3	31.99 ± 15.95	5.45 (−4.25 to 15.16)	0.24	38.03 ± 29.3	13.32 (−2.68 to 29.32)	0.09	29.47 ± 11.96	2.93 (−9.72 to 15.59)	0.62
	RSB123	27.01 ± 14.7	33.96 ± 11.52	4.06 (−3.08 to 11.2)	0.22	40.23 ± 13.74	10.33 (−7.41 to 28.08)	0.21	40.34 ± 12.84	10.44 (−7.09 to 27.98)	0.20
Propionate (micromole/g)	PS	10.70 ± 6.66	7.50 ± 5.54	−2.04 (−4.46 to 0.39)	0.09	9.52 ± 6.36	−1.99 (−5.56 to 1.57)	0.22	11.64 ± 9.53	1.03 (−5.66 to 7.72)	0.73
	RSB111	11.80 ± 6.36	7.89 ± 3.53	−3.90 (−7.98 to 0.17)	0.06	11.19 ± 7.54	−0.03 (−6.01 to 5.95)	0.99	7.86 ± 4.18	−2.54* (−4.49 to −0.59)	0.02
	RSB122	11.37 ± 6.55	10.41 ± 5.01	−0.96 (−3.61 to 1.69)	0.44	13.45 ± 9.72	2.32 (−2.32 to 6.96)	0.28	12.45 ± 9.14	1.08 (−4.15 to 6.32)	0.65
	RSB123	11.93 ± 6.60	13.18 ± 5.01	−0.02 (−5.09 to 5.06)	0.99	15.17 ± 5.49	1.98 (−5.07 to 9.02)	0.53	13.75 ± 4.12	0.56 (−5.96 to 7.07)	0.85
n-Butyrate (micromole/g)	PS	10.97 ± 8.29	10.39 ± 5.77	1.12 (−3.11 to 5.35)	0.55	13.24 ± 8.59	0.34 (−6.05 to 6.72)	0.90	13.58 ± 10.31	1.74 (−4.96 to 8.45)	0.56
	RSB111	10.74 ± 4.37	7.97 ± 5.54	−2.78 (−5.48 to −0.07)	0.05	13.91 ± 8.41	3.64 (−1.91 to 9.19)	0.16	8.30 ± 5.34	−2.28 (−4.61 to 0.06)	0.05
	RSB122	12.48 ± 8.35	11.63 ± 7.35	−0.85 (−3.25 to 1.54)	0.44	12.89 ± 7.94	1.31 (−2.77 to 5.39)	0.48	12.39 ± 8.53	−0.09 (−8.26 to 8.08)	0.98
	RSB123	9.99 ± 3.94	11.25 ± 3.92	0.21 (−3.48 to 3.89)	0.90	15.64 ± 7.54	4.59 (−1.86 to 11.05)	0.14	11.31 ± 4.48	0.27 (−4.25 to 4.79)	0.89
Total SCFA (micromole/g)	PS	47.38 ± 26.88	40.82 ± 28.47	−1.61 (−13.53 to 10.32)	0.76	60.71 ± 41.46	5.91 (−19.40 to 31.23)	0.59	59.98 ± 45.77	9.83 (−24.47 to 44.13)	0.52
	RSB111	51.45 ± 18.95	32.46 ± 19.09	−18.99* (−35.46 to −2.52)	0.03	56.52 ± 38.17	8.03 (−20.37 to 36.43)	0.53	34.61 ± 21.8	−12.27* (−21.75 to −2.79)	0.02
	RSB122	50.14 ± 32.47	54.04 ± 27.02	3.90 (−10.09 to 17.88)	0.55	64.37 ± 46.23	17.25 (−6.93 to 41.43)	0.14	54.19 ± 27.46	4.05 (−20.61 to 28.71)	0.72
	RSB123	48.28 ± 25.45	58.38 ± 19.06	4.24 (−9.17 to 17.65)	0.48	71.05 ± 25.52	16.91 (−12.14 to 45.96)	0.21	65.41 ± 20.34	11.28 (−16.27 to 38.82)	0.37
Diarrhea (PROMIS score)	PS	50.11 ± 7.86	49.72 ± 9.95	−0.39 (−3.99 to 3.21)	0.81	48.18 ± 9.68	−1.93 (−4.47 to 0.60)	0.12	47.27 ± 7.81	−2.84 (−7.63 to 1.95)	0.21
	RSB111	45.70 ± 6.49	42.37 ± 5.23	−3.33 (−7.18 to 0.53)	0.08	43.62 ± 5.12	−1.32 (−4.54 to 1.91)	0.37	45.24 ± 6.21	0.31 (−4.34 to 4.95)	0.88
	RSB122	48.40 ± 8.27	45.32 ± 6.99	−3.08 (−6.10 to −0.06)	0.05	42.15 ± 5.22	−6.25* (−10.62 to −1.89)	0.01	43.40 ± 7.03	−5.00* (−9.77 to −0.23)	0.04
	RSB123	45.10 ± 5.01	43.53 ± 4.48	−1.57 (−4.29 to 1.15)	0.22	44.67 ± 6.63	−0.43 (−3.58 to 2.71)	0.76	41.46 ± 3.85	−3.64* (−7.04 to −0.25)	0.04
Constipation (PROMIS score)	PS	50.99 ± 5.55	47.18 ± 7.54	−3.60 (−10.71 to 3.51)	0.28	46.00 ± 8.44	−4.78 (−9.67 to 0.12)	0.05	48.28 ± 8.71	−2.71 (−8.06 to 2.64)	0.28
	RSB111	52.31 ± 7.94	48.77 ± 7.51	−3.54 (−9.35 to 2.27)	0.20	48.62 ± 7.53	−4.37 (−10.42 to 1.69)	0.13	47.20 ± 9.17	−5.79 (−12.75 to 1.18)	0.09
	RSB122	53.10 ± 8.73	48.45 ± 8.26	−4.65* (−8.51 to −0.79)	0.02	48.10 ± 6.10	−5.00* (−9.49 to −0.50)	0.03	47.12 ± 6.09	−5.98 (−12.16 to 0.20)	0.06
	RSB123	49.30 ± 5.88	42.56 ± 7.05	−6.74** (−10.54 to −2.95)	0.003	44.42 ± 9.52	−4.88 (−10.83 to 1.08)	0.10	43.70 ± 7.71	−5.60* (−10.74 to −0.46)	0.04
Gas and bloating (PROMIS score)	PS	58.10 ± 5.88	53.58 ± 7.94	−4.59* (−7.75 to −1.42)	0.01	50.18 ± 9.74	−7.99* (−13.89 to −2.09)	0.01	51.20 ± 7.86	−6.90* (−12.03 to −1.77)	0.01
	RSB111	59.28 ± 7.65	55.37 ± 8.25	−3.91 (−10.62 to 2.8)	0.22	54.04 ± 6.41	−5.43 (−12.99 to 2.12)	0.14	52.67 ± 8.29	−6.81* (−11.94 to −1.68)	0.02
	RSB122	56.29 ± 5.42	53.46 ± 8.81	−2.83 (−8.11 to 2.45)	0.26	52.05 ± 6.03	−4.24* (−7.71 to −0.77)	0.02	50.54 ± 6.07	−5.75* (−10.56 to −0.95)	0.02
	RSB123	56.34 ± 6.75	52.79 ± 6.52	−3.56 (−7.43 to 0.32)	0.07	51.81 ± 7.04	−4.53* (−7.66 to −1.41)	0.01	51.30 ± 7.76	−5.04 (−11.36 to 1.27)	0.10

Data are means ± SD. Mean of the change (size of effect) in subjects with available data from baseline to the respective visit are presented with the confidence interval (CI). Listed *p*-values are for the respective visit as evaluated against the baseline (average of visit 1 and 2 values) within the group assessed by paired comparison t-tests. *p-value < 0.05; **p-value < 0.01.

10.3389/fnut.2022.987216

10.3389/fnut.2022.987216

TABLE 3 Fiber intake per day, in grams, from self-reported ASA24 results, ITT analysis.

ASA24	PS	RSB111	RSB122	RSB123
1	16.38 ± 15.58	14.96 ± 7.35	21.57 ± 15.38	15.74 ± 12.46
2	20.20 ± 14.83	19.66 ± 14.7	18.62 ± 11.75	17.90 ± 9.96
3	22.81 ± 10.14	20.48 ± 8.34	24.89 ± 21.05	20.60 ± 12.96
4	19.06 ± 9.70	19.85 ± 12.14	20.37 ± 11.69	22.56 ± 14.56
5	23.29 ± 17.86	16.63 ± 6.30	21.56 ± 12.39	15.92 ± 8.90

Data are means ± standard deviation (SD).

TABLE 4 RSB intake presented as a percentage compliance to protocol-directed doses, ITT analysis.

	Dosing log				Product weight				Smart cap usage			
Visit	PS	RSB111	RSB122	RSB123	PS	RSB111	RSB122	RSB123	PS	RSB111	RSB122	RSB123
3	98 ± 5	98 ± 5	94 ± 10	90 ± 10	88 ± 14	92 ± 24	88 ± 15	86 ± 28	75 ± 27	78 ± 32	56 ± 23	67 ± 13
4	98 ± 3	92 ± 15	96 ± 5	95 ± 10	80 ± 11	83 ± 22	91 ± 15	89 ± 24	37 ± 41	59 ± 46	47 ± 27	42 ± 27
5	94 ± 9	93 ± 14	87 ± 33	88 ± 31	89 ± 12	89 ± 16	98 ± 37	87 ± 34	69 ± 51	58 ± 38	61 ± 36	36 ± 26

Data are means ± standard deviation (SD).

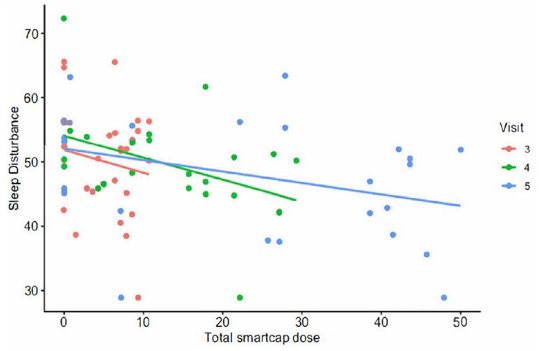


FIGURE 2
The PROMIS T-score for sleep disturbance plotted vs. total smart cap dose of RSB.

The magnitude of effect using 10 g of total smart cap dose was an increase in total SCFA of 9.60 mmol/g after 4 weeks. After 2 weeks the magnitude of effect consumption of 10 g of total smart cap dose corresponded to the following T-score changes: a 3.60-unit decrease in diarrhea, 4.63-unit decrease in gas and bloating, and a 2.36-unit decrease in constipation score after 2 weeks.

Safety and tolerability

No severe adverse events occurred. Most participants tolerated the supplement. Per person, the number of adverse events (AEs) possibly or probably related to the potato starch was 0.6, while those related to RSB was 0.5. These AEs included bloating, constipation, fatigue, flatus, cramping, diarrhea, or reflux symptoms. The participant who discontinued the study due to insomnia due to fecal urgency was in the PS group, and the physician determined that 75% of the adverse event was resolved upon discontinuation 2 weeks later. The participant who discontinued after one dose of 30 g of RSB due to severe bloating and constipation that was possibly related to RSB fully recovered from these symptoms prior to the follow-up visit 2 weeks later.

Exploratory microbiome findings

A permutational multivariate analysis of variance (PERMANOVA) of the pairwise distances [using unweighted, weighted UniFrac distance, and Robust Aitchison PCA (RPCA)] determined that there were no significant differences in the overall microbial composition based on batch (extraction plate, sequencing run), age, race, sex, alcohol consumption (as identified by the selected "track"), diet (calories, carbs, fat, protein, or fiber), or the supplement group at baseline or at the last visit, V5 (see Supplementary Figure S3). Longitudinal analyses of beta diversity across all timepoints also did not reveal significant shifts in the overall microbial composition (see Supplementary Figure S4). Moreover, linear mixed effects models incorporating RSB group did not explain the variance significantly in alpha diversity metrics.

The impact of RSB was further evaluated through Songbird differential abundance analysis at the last time point, Visit Five. This type of analysis allows for a more robust evaluation of

10.3389/fnut.2022.987216

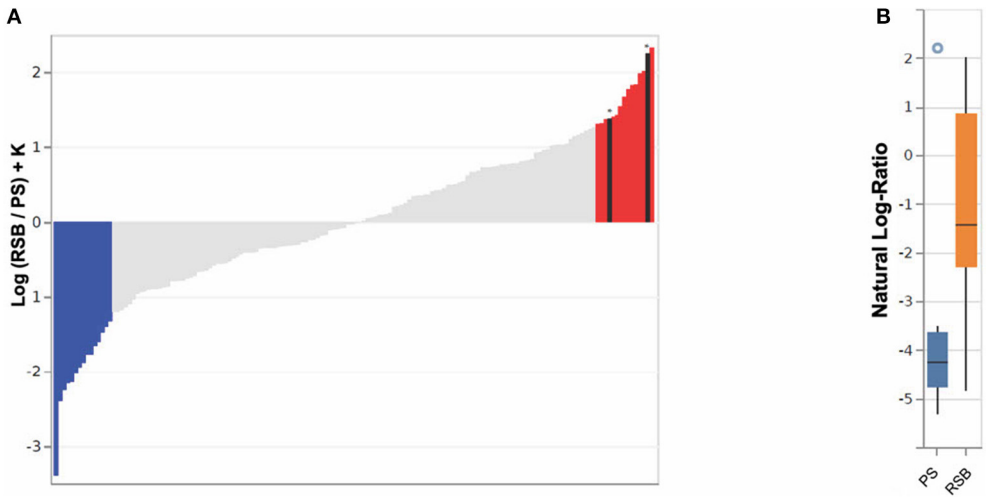


FIGURE 3
Bacterial ASVs associated with RSB or PS consumption at time point five, using 16S analysis. **(A)** A rank plot highlights the differentials for the top 10% of ASVs ($n = 15$) associated with RSB (colored in red) and the bottom 10% ($n = 15$) associated with PS (colored in blue). ASVs classified as *Akkermansia muciniphila* (second from right) and *Faecalibacterium prausnitzii* (twelfth from right), (see Table S4) are shown in black with an asterisk above. The y-axis shows the log-fold change known up to a bias constant K. **(B)** Boxplot of the log-ratio of the two ASVs assigned as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii* over the top 10% ($n = 15$) associated with PS.

differential abundance, accounting for the compositional nature of microbiome data by expressing abundance as log-ratios rather than simple relative abundance measures. The Songbird model utilizing information on whether a participant received any RSB resulted in a Q2 score of 0.02, indicating the model is more predictive of microbial composition when including RSB supplementation as a covariate than the null model. Meanwhile a model utilizing information on RSB amount (specific RSB and PS groups) resulted in a Q2 score of −0.02, suggesting low predictive value. Therefore, analysis of model output was continued only for all RSB groups combined compared to the PS group. The ASVs were ranked in order of association with RSB vs. PS based on a log-fold change in abundance between groups (see Figure 3A) (51). The log-ratios of the top vs. bottom 10% of ASVs associated with RSB were found to be significantly different between the RSB and PS groups at the last visit [Kruskal-Wallis H(1) = 4.69, $p = 0.030$], while log-ratios of these same taxa were not significantly different between the RSB and PS groups at baseline [Kruskal-Wallis H(1) = 2.43, p-value = 0.12].

The ASVs most associated with RSB or PS, along with their assigned taxonomies, for Figure 3A are listed in Supplementary Table S4. ASVs classified as the species *Akkermansia muciniphila*, *F. prausnitzii*, and *Alistipes onderdonkii* were among those most associated with RSB consumption. ASVs assigned as belonging to the families *Verrucomicrobiaceae*, *Rikenellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Christensenellaceae*, *Bacteroidaceae*, and *Mogibacteriaceae* were also identified in the top 10% ASVs associated with RSB. ASVs included in *Mogibacteriaceae*, *Blautia*, *Lachnospira*, and *Bacteroides* associated with both RSB and PS (i.e., appear in both lists). ASVs within *Oscillospira*, *Coprococcus*, *Anaerostipes*, and *Bifidobacterium adolescentis* associated with PS. It should be noted that while taxonomic classifications from 16S sequence data may only be reliable to the genus level, it is highly likely that *Akkermansia muciniphila* and *F. prausnitzii* are correctly identified, due to the low diversity of species described in both of those genera. Log ratios of these two ASVs over the top 10% of ASVs associated with PS were found to be significantly larger in individuals who received RSB (−1.26 ± 2.05) vs. those in the PS group (−3.46 ± 2.39) [Kruskal-Wallis H(1) = 4.70, $p = 0.030$] in Figure 3B. At baseline, these ASVs did not have different log-ratios in the RSB vs PS [Kruskal-Wallis H(1) = 0.28, $p = 0.60$].

Discussion

This trial evaluated increasing doses of RSB, a novel resistant starch blend of MSPrebiotic® potato starch, green banana flour and apple fiber powder, and included a different potato starch of similar resistant starch content as "0 RSB." In this study we showed that, consistent with previous studies, intake of RS, regardless of source, can provide improvement of GI symptoms. However, we also show that increasing the dose of the proprietary blend, which also included a non-resistant starch source of apple pectin, resulted in significant benefits beyond the benefits obtained from the potato starch in the analytical

model. The use of the smart cap monitoring device was essential in finding that increasing doses of RSB improved the sleep disturbance score. Additionally, the unique combination RSB significantly differed from the PS at the end of the consumption period in its associations with ASVs assigned to microbes linked to health.

This trial did not show that increasing doses of the RSB over the course of 6 weeks had a significant impact on SCFA, the primary outcome. By itself, the 10 g/day of the potato starch, which also contained resistant starch, did not show statistically significant shifts in SCFA over time. Possible reasons that we did not observe significant SCFA increases as compared to other studies include and are not limited to elevated baseline levels of SCFA, insufficient quantities of resistant starch or duration of consumption, variability in the diet, variability in participant self-collection, and differences between the test method used and those reported in the literature. The test kit (used for SCFA measurement in this study) from Genova Diagnostics is utilized by functional medicine practitioners and has been documented in studies on patients (55, 56), a probiotic tolerability study (57), as well as a randomized, controlled pilot trial (58). The latter trial did not see a statistically significant change in SCFA after a prebiotic intervention. The average total SCFA ranged from 47–51 micromol/g in the groups, while Genova's internal standard based on their population studies consider ≥23.3 micromol Total SCFA/g as "good." Similarly, this trial's butyrate levels started in Genova's green healthy zone, above 3.6 micromol/g (averages from 10–12 micromol/g). Thus, the participants did not need to increase butyrate in their feces according to Genova's standards. Additionally, in a study using 22 grams of RS/day over 4 weeks (59) (as compared to 5.5 g/day for 4 weeks in the PS group of this study) butyrate levels did increase for most participants, yet often decreased when baseline levels of butyrate was high, which may have occurred here as well. Although a recent review on resistant starch type II stated that an increase in SCFA is a consistent result (60), the studies reviewed used a higher dose of resistant starch and a different test kit and collection method. Specifically, they did not use the Genova Diagnostics' preservation fluid test kit and self-collection method, and these studies also contained a daily dose of at least 20 grams of resistant starch (compared to 5.5—16.5 g resistant starch in RSB or 6 g resistant starch from the PS in this trial). The previous trial reported on the MSPrebiotic® potato starch in the RSB resulted in significant increases in butyrate using 21 g of resistant starch (30 g raw potato starch)/day for 12 weeks in an elderly population, which shows dose, duration, host age and initial microbiome differences from our trial (28).

While fiber has been shown to be effective in addressing constipation and global IBS symptoms, systematic review results indicate that the benefits are marginal, and insoluble fibers may sometimes aggravate outcomes (61). Both the PS and RSB contained primarily soluble fiber, with similar levels of soluble and insoluble fiber, and both ameliorated constipation, gas and bloating, and diarrhea. We observed small effect sizes for 10 g/day of RSB (corresponding to about 5.5 g/day of resistant starch), and that increasing doses of RSB was associated with more benefit for diarrhea. This suggests that the estimated intake of resistant starch 3–9 g in the typical diets observed in the US, UK, and Australia (62–64) may need to be increased to obtain more benefits as well.

The two ingredients of green banana and apple pectin that make the RSB different from the PS group may be partially responsible for the result that increasing doses of RSB led to improvement in the diarrhea score. Studies in children with persistent diarrhea showed that green banana and apple pectin significantly reduced the duration of diarrhea, vomiting incidents, stool amount, amount of oral rehydration and intravenous fluids, as well as an improvement in intestinal permeability (65, 66).

Although there were no significant findings for wellbeing and sleep in the ITT analysis, the cumulative smart cap dose used for RSB consumption showed a significant association on sleep disturbance score in addition to the benefit to decreasing diarrhea. Sleep disruption is often reported in IBS and has been shown to predict next-day symptoms in women with IBS (67). Thus, in addition to resolution of GI disturbances, improving sleep is a logical target, as it behaves as a potential cause or leading indicator of GI symptoms. It is interesting to note that the cumulative dose of RSB as calculated by smart cap usage was associated with a reduction in sleep disturbance at the end of four weeks. Taken together with the observation that an ASV classified as *Bacteroides* was associated with RSB, a possible mechanism for sleep benefit is that the GABA production by *Bacteroides* members (68) may help with sleep. However, a separate ASV also classified as *Bacteroides* was also associated with PS, highlighting a need to better understand the potential role of specific species or strains of bacteria on such outcomes. Future studies with more smart cap or monitoring data as well as greater understanding the microbiome and metabolites and rigorous sleep monitoring via appropriate devices could further elucidate the actual impact on sleep.

The exploratory microbiome findings suggest RSB consumption is associated with ASVs that are beneficial to GI health. The association with an ASV classified as *F. prausnitzii* showed clinical support for results of *in vitro* studies of apple pectin supporting the growth of *F. prausnitzii* strains. Although the SCFA did not appear to be changed by RSB intake as compared to the PS intake, RSB consumption was associated with butyrate-producer *Faecalibacterium* and acetate-producer *Akkermansia*. The review on resistant starch II consumption using more than 20 g of resistant starch per day in most studies showed that *Ruminococcus bromii* and *F. prausnitzii* were associated with resistant starch intake, which is consistent with the finding reported here for RSB intake while the *Bifidobacterium adolescentis*, also previously reported to associated with resistant starch intake, associated with the

PS which also had resistant starch (Supplementary Table S4) (62). More research may need to be done to determine which of the bacteria prefer different types of resistant starch. According to a review of dietary fibers, Bacteroides is among the microbial genera where some species can ferment pectin (69), which supports the finding of *Bacteroides* association with RSB (containing the apple pectin) in this study. Finally, certain proportions of *Clostridiales* such as *Oscillospira* and *Lachnospiracea* were reduced frequently in other studies (62) and this trial showed association of the abundance of those members with intake from the PS or RSB which both contained RS.

Higher levels of *Akkermansia* are also found in healthy individuals' feces than in feces from individuals with inflammatory or metabolic diseases (70). The families to which ASVs associated with RSB are assigned, *Verrucomicrobiaceae*, *Rikenellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Christensenellaceae*, and *Mogibacteriaceae*, were also enriched in long-lived families in a recent study (71). Additionally, the family Christensenellaceae consistently has been associated with reduced visceral fat mass and leanness (72), and *Christenellaceae*, *Mogibacteriaceae*, and *Rikenellaceae* were associated with leanness, longevity, healthy aging, and protective of cardiovascular and metabolic disorders (73, 74).

Limitations

Major limitations included the heterogeneity and health of participants in the study's recruitment cohort, the circumstances of the COVID-19 pandemic-associated lockdown, significant participant dependence for self-reported measures and stool collection, sample size limitations, and missing samples. The small sample size, study design (RSB v. Emsland-sourced raw potato starch), and different microbiome analytical methods preclude comparison to prior research using MSPrebiotic® raw potato starch (RPS) (27, 28, 75, 76) or further subgroup analysis with baseline microbiome and smart cap data.

Heterogeneity of health status may have precluded differences in SCFA results as well. In a systematic review and meta-analysis of fecal SCFAs in patients with irritable bowel syndrome (IBS), those with constipation-dominant IBS had lower propionate and butyrate, while those with diarrhea-predominant IBS had higher fecal butyrate compared to healthy controls' fecal SCFAs (77).

The good health of the participants did not allow for much improvement in several outcomes, as can be observed by baseline scores. The study recruited for generally healthy people with at least one GI symptom of constipation, irregularity, or bloating; individuals with diarrhea were not specifically recruited, and specific Rome criteria or other standards were not used to characterize the population relative to specific subtypes of IBS. Some if not all participants would likely fall into the "healthy" description of controls in case-control studies observing differences with IBS (78). The range of "healthy" stool frequency is generally accepted to be between three bowel movements per day and three bowel movements per week (78), which is rather a wide window and fully encompasses the study cohort's stool frequency throughout the study.

Despite using random number assignments, there was a distribution of more women in groups consuming the blend which was an unfortunate limitation. Available sample size does not allow for subgroup analysis to identify the impact of this distribution.

Finally, the original choice for a control was a potato starch that had prior test results showing little to no RS; however, *post-hoc* test results showed that the potato starch had similar RS content to the RSB, which resulted in a study design lacking a true control without RS. However, it did allow for an evaluation of whether a multi-source RS blend delivered different results than a single source potato starch and whether different dosage effects could be detected. We anticipate that a follow-up study would further show that RSB results in even more dramatic improvement in GI symptoms and sleep, and more significant shifts in gut microbiota over using a supplement free of RS.

Conclusions

This study suggested that a novel resistant starch blend exhibited benefits beyond those of a standard resistant starch, which also improved GI symptoms. The novel RSB improved GI symptoms, particularly diarrhea, in 2 to 6 weeks with 10, 20, or 30 g doses tested. With the smart cap, this study confirmed expected outcomes such as increasing Bristol Stool Form, decreasing diarrhea and constipation scores, and showed that the cumulative RSB dose led to a decrease in the sleep disturbance after 4 weeks.

The exploratory microbiome analysis showed that a novel RSB is associated with *Akkermansia muciniphila*, *F. prausnitzii*, and other ASVs belonging to families of bacteria that have previously been associated with longevity and health.

For the field of resistant starch research, the trial suggests that a supplementary dose of 5–6 g of resistant starch consumed above normal dietary intake (estimated at 3–9 grams) per day, at least in the course of 2 to 6 weeks, is insufficient to move SCFA levels. While it may not be necessary to consume 20 or more grams of supplemental RS for four or more weeks as has been showed in previously reviewed studies to obtain SCFA increases, it is still unknown what a "good" level of fecal SCFA is. At the same time, this trial also points to the difficulties in resistant starch testing, which varied in its results and has been the subject of debate (Englyst or other methods).

This original research provokes the field to consider unique combinations of prebiotics and resistant starches rather than single sources while at the same time calling for more rigorous and standardized methods including adherence monitoring to evaluate the impact of dietary interventions on the GI.

Our multifaceted monitoring of supplement consumption indicated significant variation in the amount of the supplement consumed, even within the same dosing group. As a result, future research may require larger participant samples in order to account for differences in compliance. Since the smart cap monitoring suggested an impact on sleep disturbance that was otherwise not apparent, future studies in this area would benefit from this or similar technology. Future studies with enhanced control over the population, stricter protocol requirements, and larger sample sizes to account for lack of adherence to protocol-directed study product consumption may provide future insight into personalizing novel blends of prebiotics, RS, and fibers at appropriate doses to modulate the microbiome and promote health.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number (s) can be found at: https://www.ebi.ac.uk/ena, ERP134986; https://qiita.ucsd.edu, 13692.

Ethics statement

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

Author contributions

Conceptualization, validation, writing—original draft preparation, and funding acquisition: NP. Data curation: NP, PLMC staff, and CMI. Methodology, formal analysis, and visualization: NP, DH, and BN. Software: DH and BN. Supervision of PLMC staff and investigation: JL. Resources: all authors. Writing—review and editing: NP, DH, BN, and SS. Supervision: NP, SS, IL, RK, and DM. Project administration: NP, SS, and IL. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by Metagenics, Inc.

Acknowledgments

PLMC study staff: Kim Koch, CMA and Angela Jaeger, Brianne Simon, Nicole Ellsworth, Tawnya Strum, Ana Ahmad for data verification. NUNM: Jennifer Ryan, ND, MS for consultation/ideas. Metagenics: Annalouise O'Connor, Ph.D., Nikhat Contractor, Ph.D. for approving/funding work, consultation. Amway: Greg Hillebrand for consultation, Brandon Iker for consultation, facilitating usage of smart caps, Josh Taylor for facilitating usage of smart caps. Edgar Diaz and his team at CMI for supporting American Gut kit collection.

Conflict of interest

Authors IL and NP were previously employed through data analysis and initial drafting of the manuscript by Metagenics, Inc., the provider of the resistant starch blend and sponsor of the entire research. Author JL is the co-owner of Personalized Medicine, Inc., which is a consultant for Metagenics, Inc.

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.

The authors declare that this study received funding from Metagenics. The funder had the following involvement in the study: design and initial writing of the article.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.987216/full#supplementary-material

References

1. Joanne S. Fiber and prebiotics: mechanisms and health benefits. *Nutrients*. (2013) 5:1417–35. doi: 10.3390/nu5041417

2. Anderson JW, Baird P, Davis RH, Ferreri S, Knudtson M, Koraym A, et al. Health benefits of dietary fiber. *Nutr Rev*. (2009) 67:188–205. doi: 10.1111/j.1753-4887.2009.00189.x

3. Barber TM, Kabisch S, Pfeiffer AFH, Weickert MO. The health benefits of dietary fibre. *Nutrients*. (2020) 12:3209. doi: 10.3390/nu12103209

4. Birt DF, Boylston T, Hendrich S, Jane J-L, Hollis J, Li L, et al. Resistant starch: promise for improving human health. *Adv Nutrition Int Rev J*. (2013) 4:587–601. doi: 10.3945/an.113.004325

5. Amini S, Mansoori A, Maghsumi-Norouzabad L. The effect of acute consumption of resistant starch on appetite in healthy adults: a systematic review and meta-analysis of the controlled clinical trials. *Clin Nutr ESPEN*. (2021) 41:42–8. doi: 10.1016/j.clnesp.2020.12.006

6. Jia L, Dong X, Li X, Jia R, Zhang H-L. Benefits of resistant starch type 2 for patients with end-stage renal disease under maintenance hemodialysis: a systematic review and meta-analysis. *Int J Med Sci*. (2021) 18:811–20. doi: 10.7150/ijms.51484

7. Ze X, Duncan SH, Louis P, Flint HJ. Ruminococcus Bromii is a keystone species for the degradation of resistant starch in the human colon. *ISME J*. (2012) 6:1535–43. doi: 10.1038/ismej.2012.4

8. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroentero*. (2017) 14:491–502. doi: 10.1038/nrgastro.2017.75

9. Cione E, Fazio A, Curcio R, Tucci P, Lauria G, Cappello AR, et al. Resistant starches and non-communicable disease: a focus on Mediterranean diet. *Foods*. (2021) 10:2062. doi: 10.3390/foods10092062

10. Maier TV, Lucio M, Lee LH, VerBerkmoes NC, Brislawn CJ, Bernhardt J, et al. Impact of dietary resistant starch on the human gut microbiome, metaproteome, and metabolome. *Mbio*. (2017) 8:e01343-17. doi: 10.1128/mBio.01343-17

11. Muir JG, Lu ZX, Young GP, Cameron-Smith D, Collier GR, O'Dea K. Resistant starch in the diet increases breath hydrogen and serum acetate in human subjects. *Am J Clin Nutrition*. (1995) 61:792–9. doi: 10.1093/ajcn/61.4.792

12. Phillips J, Muir JG, Birkett A, Lu ZX, Jones GP, O'Dea K, et al. Effect of resistant starch on fecal bulk and fermentation-dependent events in humans. *Am J Clin Nutr*. (1995) 62:121–130. doi: 10.1093/ajcn/62.1.121

13. Maki KC, Pelkman CL, Finocchiaro ET, Kelley KM, Lawless AL, Schild AL, et al. Resistant starch from high-amylose maize increases insulin sensitivity in overweight and obese men. *J Nutr*. (2012) 142:717–23. doi: 10.3945/jn.111.152975

14. Dainty SA, Klingel SL, Pilkey SE, McDonald E, McKeown B, Emes MJ, et al. Resistant starch bagels reduce fasting and postprandial insulin in adults at risk of type 2 diabetes. *J Nutr*. (2016) 146:2252–9. doi: 10.3945/jn.116.239418

15. Johnston KL, Thomas EL, Bell JD, Frost GS, Robertson MD. Resistant starch improves insulin sensitivity in metabolic syndrome. *Diabetic Med*. (2010) 27:391–7. doi: 10.1111/j.1464-5491.2010.02923.x

16. Lopez-Siles M, Duncan SH, Garcia-Gil LJ, Martinez-Medina M. *Faecalibacterium prausnitzii*: from microbiology to diagnostics and prognostics. *ISME J*. (2017) 11:841–52. doi: 10.1038/ismej.2016.176

17. Zuo T, Zhang F, Lui GCY, Yeoh YK, Li AYL, Zhan H, et al. Alterations in gut microbiota of patients with COVID-19 during time of hospitalization. *Gastroenterology*. (2020) 159:944–55.e8. doi: 10.1053/j.gastro.2020.05.048

18. Debyser G, Mesuere B, Clement L, de Weygaert JV, Van Hecke P, Duytschaever G, et al. Faecal proteomics: a tool to investigate dysbiosis and inflammation in patients with cystic fibrosis. *J Cyst Fibros*. (2016) 15:242–50. doi: 10.1016/j.jcf.2015.08.003

19. Xiaoya H, Shuyang Z, Yan L. *Faecalibacterium prausnitzii*: a next-generation probiotic in gut disease improvement. *Can J Infect Dis Medical Microbiol*. (2021) 2021:e6666114. doi: 10.1155/2021/6666114

20. Rebeca M, Sylvie M, Leandro B, Chantal B, Véronique R, Sylvie H, et al. Functional characterization of novel faecalibacterium prausnitzii strains isolated from healthy volunteers: a step forward in the use of *F. Prausnitzii* as a next-generation probiotic. *Front Microbiol*. (2017) 8:1226. doi: 10.3389/fmicb.2017.01226

21. McDonald D, Hyde E, Debelius JW, Morton JT, Gonzalez A, Ackermann G, et al. American gut: an open platform for citizen science microbiome research. *mSystems*. (2018) 3:e00031-18. doi: 10.1128/mSystems.00031-18

22. Lopez-Siles M, Khan TM, Duncan SH, Harmsen HJM, Garcia-Gil LJ, Flint HJ. Cultured representatives of two major phylogroups of human colonic faecalibacterium prausnitzii can utilize pectin, uronic acids, and host-derived substrates for growth. *Appl Environ Microb*. (2012) 78:420–8. doi: 10.1128/AEM.06858-11

23. Chung WSF, Meijerink M, Zeuner B, Holck J, Louis P, Meyer AS, et al. Prebiotic potential of pectin and pectic oligosaccharides to promote anti-inflammatory commensal bacteria in the human colon. *FEMS Microbiol Ecol*. (2017) 93:1–9. doi: 10.1093/femsec/fix127

24. Cantu-Jungles TM, Bulut N, Chambry E, Ruthes A, Iacomini M, Keshavarzian A, et al. Dietary fiber hierarchical specificity: the missing link for predictable and strong shifts in gut bacterial communities. *Mbio*. (2021) 12:e01028-21. doi: 10.1128/mBio.01028-21

25. AOAC Official Method 2002.02. *Resistant Starch in Starch and Plant Materials-Methods of AOAC-International-Food Laws & Regulations-Documents-Global FoodMate*. Gaithersburg, MD: AOAC INTERNATIONAL (2003). p. 1103.

26. Venkataraman A, Sieber JR, Schmidt AW, Waldron C, Theis KR, Schmidt TM. Variable responses of human microbiomes to dietary supplementation with resistant starch. *Microbiome*. (2016) 4:33. doi: 10.1186/s40168-016-0178-x

27. Alfa MJ, Strang D, Tappia PS, Olson N, DeGagne P, Bray D, et al. A randomized placebo controlled clinical trial to determine the impact of digestion resistant starch MSPrebiotic® on glucose, insulin, and insulin resistance in elderly and mid-age adults. *Front Med*. (2018) 4:260. doi: 10.3389/fmed.2017.00260

28. Alfa MJ, Strang D, Tappia PS, Graham M, Domselaar GV, Forbes JD, et al. A randomized trial to determine the impact of a digestion resistant starch composition on the gut microbiome in older and mid-age adults. *Clin Nutr*. (2018) 37:797–807. doi: 10.1016/j.clnu.2017.03.025

29. Ble-Castillo JL, Juárez-Rojop IE, Tovilla-Zárate CA, García-Vázquez C, Servin-Cruz MZ, Rodríguez-Hernández A, et al. Acute consumption of resistant starch reduces food intake but has no effect on appetite ratings in healthy subjects. *Nutrients*. (2017) 9:696. doi: 10.3390/nu9070696

30. Jimenez-Dominguez G, Ble-Castillo JL, Aparicio-Trapala MA, Juarez-Rojop IE, Tovilla-Zarate CA, Ble-Castillo DJ, et al. Effects of acute ingestion of native banana starch on glycemic response evaluated by continuous glucose monitoring in obese and lean subjects. *Int J Environ Res Public Health*. (2015) 12:7491–7505. doi: 10.3390/ijerph120707491

31. Bergeron N, Williams PT, Lamendella R, Faghihnia N, Grube A, Li X, et al. Diets high in resistant starch increase plasma levels of trimethylamine-n-oxide, a gut microbiome metabolite associated with CVD Risk. *Brit J Nutr*. (2016) 116:2020–9. doi: 10.1017/S0007114516004165

32. Cummings JH, Beatty ER, Kingman SM, Bingham SA, Englyst HN. Digestion and physiological properties of resistant starch in the human large bowel. *Brit J Nutr*. (1996) 75:733–47. doi: 10.1079/BJN19960177

33. Silvester KR, Englyst HN, Cummings JH. Ileal recovery of starch from whole diets containing resistant starch measured in vitro and fermentation of ileal effluent. *Am J Clin Nutrition*. (1995) 62:403–11. doi: 10.1093/ajcn/62.2.403

34. Alili ME, Vrijens B, Demonceau J, Evers SM, Hiligsmann M. A scoping review of studies comparing the Medication Event Monitoring System (MEMS) with alternative methods for measuring medication adherence. *Brit J Clin Pharmaco*. (2016) 82:268–79. doi: 10.1111/bcp.12942

35. Hamilton GA. Measuring adherence in a hypertension clinical trial. *Eur J Cardiovasc Nur*. (2003) 2:219–28. doi: 10.1016/S1474-5151(03)00058-6

36. US10874591B2. *System and Method for Monitoring Intake Compliance - Google Patents*. Available online at: https://patents.google.com/patent/US10874591B2/en (accessed November 23, 2021).

37. Subar AF, Kirkpatrick SI, Mittl B, Zimmerman TP, Thompson FE, Bingley C, et al. The Automated Self-Administered 24-Hour Dietary Recall (ASA24): a resource for researchers, clinicians, and educators from the national cancer institute. *J Acad Nutr Diet*. (2012) 112:1134–7. doi: 10.1016/j.jand.2012.04.016

38. Lihong C, Courtney R, Robert D, Amy PB. Development of an index score for intestinal inflammation-associated dysbiosis using real-world stool test results. *Dig Dis Sci*. (2019) 65:1111–24. doi: 10.1007/s10620-019-05828-8

39. Spiegel BMR, Hays RD, Bolus R, Melmed GY, Chang L, Whitman C, et al. Development of the NIH Patient-Reported Outcomes Measurement Information

System (PROMIS) gastrointestinal symptom scales. *Am J Gastroenterol.* (2014) 109:1804–14. doi: 10.1038/ajg.2014.237

40. Salsman JM, Victorson D, Choi SW, Peterman AH, Heinemann AW, Nowinski C, et al. Development and validation of the positive affect and well-being scale for the Neurology Quality of Life (Neuro-QOL) measurement system. *Qual Life Res.* (2013) 22:2569–80. doi: 10.1007/s11136-013-0382-0

41. Yu L, Buysse DJ, Germain A, Moul DE, Stover A, Dodds NE, et al. Development of short forms from the PROMIS™ sleep disturbance and sleep-related impairment item banks. *Behav Sleep Med.* (2011) 10:6–24. doi: 10.1080/15402002.2012.636266

42. Moher D, Hopewell S, Schulz KF, Montori V, Gøtzsche PC, Devereaux PJ, et al. CONSORT 2010 explanation and elaboration: updated guidelines for reporting parallel group randomised trials. *BMJ.* (2010) 340:c869. doi: 10.1136/bmj.c869

43. Team RC. *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing (2020).

44. Gonzalez A, Navas-Molina JA, Kosciolek T, McDonald D, Vázquez-Baeza Y, Ackermann G, et al. Qiita: rapid, web-enabled microbiome meta-analysis. *Nat Methods.* (2018) 15:796–8. doi: 10.1038/s41592-018-0141-9

45. Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Xu ZZ, et al. Deblur rapidly resolves single-nucleotide community sequence patterns. *Msystems.* (2017) 2:e00191-16, doi: 10.1128/mSystems.00191-16

46. Janssen S, McDonald D, Gonzalez A, Navas-Molina JA, Jiang L, Xu ZZ, et al. Phylogenetic placement of exact amplicon sequences improves associations with clinical information. *Msystems.* (2018) 3:e00021-18. doi: 10.1128/mSystems.00021-18

47. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, et al. An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *Isme J.* (2012) 6:610–8. doi: 10.1038/ismej.2011.139

48. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol.* (2019) 37:852–7. doi: 10.1038/s41587-019-0209-9

49. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA Ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* (2013) 41:D590–6. doi: 10.1093/nar/gks1219

50. Amir A, McDonald D, Navas-Molina JA, Debelius J, Morton JT, Hyde E, et al. Correcting for microbial blooms in fecal samples during room-temperature shipping. *Msystems.* (2017) 2:e00199-16. doi: 10.1128/mSystems.00199-16

51. Morton JT, Marotz C, Washburne A, Silverman J, Zaramela LS, Edlund A, et al. Establishing microbial composition measurement standards with reference frames. *Nat Commun.* (2019) 10:2719. doi: 10.1038/s41467-019-10656-5

52. Fedarko MW, Martino C, Morton JT, González A, Rahman G, Marotz CA, et al. Visualizing'omic feature rankings and log-ratios using Qurro. *Nar Genom Bioinform.* (2020) 2:lqaa023. doi: 10.1093/nargab/lqaa023

53. Kaul A, Mandal S, Davidov O, Peddada SD. Analysis of microbiome data in the presence of excess zeros. *Front Microbiol.* (2017) 8:2114. doi: 10.3389/fmicb.2017.02114

54. Blake MR, Raker JM, Whelan K. Validity and reliability of the bristol stool form scale in healthy adults and patients with diarrhoea-predominant irritable bowel syndrome. *Aliment Pharm Therap.* (2016) 44:693–703. doi: 10.1111/apt.13746

55. Julie R, Yinglin X, Barbara J, Jun S. Gut inflammation and dysbiosis in human motor neuron disease. *Physiol Rep.* (2017) 5:e13443. doi: 10.14814/phy2.13443

56. O'Neil-Smith K, Barber MS. Treatment of chronic atopy and irritable bowel syndrome in a 7-year-old: a case report. *Integr Medicine Encinitas Calif.* (2017) 16:48–52.

57. Ryan JJ, Patno NM. Short-term tolerability, safety, and gut microbial composition responses to a multi-strain probiotic supplement: an open-label study in healthy adults. *Integr Med.* (2021) 19:24–34.

58. Rebello CJ, Burton J, Heiman M, Greenway FL. Gastrointestinal microbiome modulator improves glucose tolerance in overweight and obese subjects: a randomized controlled pilot trial. *J Diabetes Complications.* (2015) 29:1272–6. doi: 10.1016/j.jdiacomp.2015.08.023

59. McOrist AL, Miller RB, Bird AR, Keogh JB, Noakes M, Topping DL, et al. Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. *J Nutr.* (2011) 141:883–9. doi: 10.3945/jn.110.128504

60. Bendiks ZA, Knudsen KEB, Keenan MJ, Marco ML. Conserved and variable responses of the gut microbiome to resistant starch type 2. *Nutr Res.* (2020) 77:12–28. doi: 10.1016/j.nutres.2020.02.009

61. Bijkerk CJ, Muris JWM, Knottnerus JA, Hoes AW, Wit NJD. Systematic review: the role of different types of fibre in the treatment of irritable bowel syndrome. *Aliment Pharm Therap.* (2004) 19:245–51. doi: 10.1111/j.0269-2813.2004.01862.x

62. Murphy MM, Douglass JS, Birkett A. Resistant starch intakes in the United States. *J Am Diet Assoc.* (2008) 108:67–78. doi: 10.1016/j.jada.2007.10.012

63. Patterson MA, Maiya M, Stewart ML. Resistant starch content in foods commonly consumed in the united states: a narrative review. *J Acad Nutr Diet.* (2020) 120:230–44. doi: 10.1016/j.jand.2019.10.019

64. Tomlin J, Read NW. The effect of resistant starch on colon function in humans. *Brit J Nutr.* (1990) 64:589–95. doi: 10.1079/BJN19900058

65. Rabbani GH, Teka T, Zaman B, Majid N, Khatun M, Fuchs GJ. Clinical studies in persistent diarrhea: dietary management with green banana or pectin in Bangladeshi children. *Gastroenterology.* (2001) 121:554–60. doi: 10.1053/gast.2001.27178

66. Rabbani GH, Teka T, Saha SK, Zaman B, Majid N, Khatun M, et al. Green banana and pectin improve small intestinal permeability and reduce fluid loss in Bangladeshi children with persistent diarrhea. *Dig Dis Sci.* (2004) 49:475–84. doi: 10.1023/B:DDAS.0000020507.25910.cf

67. Buchanan DT, Cain K, Heitkemper M, Burr R, Vitiello MV Zia J, et al. Sleep measures predict next-day symptoms in women with irritable bowel syndrome. *J Clin Sleep Med.* (2014) 10:1003–9. doi: 10.5664/jcsm.4038

68. Nize O, Kun Y, Denisa M, Laura B, Florentin CA, Fabián C, et al. GABA production by human intestinal bacteroides spp.: prevalence, regulation, and role in acid stress tolerance. *Front Microbiol.* (2021) 12:656895. doi: 10.3389/fmicb.2021.656895

69. Heather A, Inderdeep M, Zhengxiao Z, David A, Eytan W. Not all fibers are born equal: variable response to dietary fiber subtypes in IBD. *Front Pediatr.* (2021) 8:620189. doi: 10.3389/fped.2020.620189

70. Derrien M, Belzer C, de Vos WM. Akkermansia muciniphila and its role in regulating host functions. *Microb Pathog.* (2017) 106:171–81. doi: 10.1016/j.micpath.2016.02.005

71. Ren M, Li H, Fu Z, Li Q. Succession analysis of gut microbiota structure of participants from long-lived families in Hechi, Guangxi, China. *Microorg.* (2021) 9:2524. doi: 10.3390/microorganisms9122524

72. Waters JL, Ley RE. The human gut bacteria christensenellaceae are widespread, heritable, and associated with health. *BMC Biol.* (2019) 17:83. doi: 10.1186/s12915-019-0699-4

73. Tavella T, Rampelli S, Guidarelli G, Bazzocchi A, Gasperini C, Pujos-Guillot E, et al. Elevated Gut microbiome abundance of christensenellaceae, porphyromonadaceae and rikenellaceae is associated with reduced visceral adipose tissue and healthier metabolic profile in italian elderly. *Gut Microbes.* (2021) 13:1880221. doi: 10.1080/19490976.2021.1880221

74. Oki K, Toyama M, Banno T, Chonan O, Benno Y, Watanabe K. Comprehensive analysis of the fecal microbiota of healthy japanese adults reveals a new bacterial lineage associated with a phenotype characterized by a high frequency of bowel movements and a lean body type. *BMC Microbiol.* (2016) 16:284. doi: 10.1186/s12866-016-0898-x

75. Bush JR, Alfa MJ. Decreasing levels of sporacetigenium correlate with improved diabetic parameters in healthy adults consuming msprebiotic® digestion resistant starch. *J Aging Res Clin Prac.* (2018) 7:176–80. doi: 10.14283/jarcp.2018.28

76. Bush JR, Alfa MJ. Increasing levels of parasutterella in the gut microbiome correlate with improving low-density lipoprotein levels in healthy adults consuming resistant potato starch during a randomised trial. *BMC Nutr.* (2020) 6:72. doi: 10.1186/s40795-020-00398-9

77. Sun Q, Jia Q, Song L, Duan L. Alterations in fecal short-chain fatty acids in patients with irritable bowel syndrome. *Medicine.* (2019) 98:e14513. doi: 10.1097/MD.0000000000014513

78. Ghorbani S, Nejad A, Law D, Chua KS, Amichai MM, Pimentel M. Healthy control subjects are poorly defined in case-control studies of irritable bowel syndrome. *Ann Gastroenterol.* (2015) 28:87–93.

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 08 December 2022
DOI 10.3389/fnut.2022.1004667

OPEN ACCESS

EDITED BY
Buyun Liu,
University of Science and Technology of China, China

REVIEWED BY
Yang Du,
The University of Iowa, United States
Emilia Ruggiero,
Mediterranean Neurological Institute Neuromed (IRCCS), Italy

*CORRESPONDENCE
Jichun Chen
chenjich@hotmail.com
Shaodong Ye
yeshaodong9999@163.com
Xiang Li
dawei1966@126.com

†These authors have contributed equally to this work

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

RECEIVED 27 July 2022
ACCEPTED 14 November 2022
PUBLISHED 08 December 2022

CITATION
Wan J, Li X, Gu M, Li Q, Wang C, Yuan R, Li L, Li X, Ye S and Chen J (2022) The association of dietary resistance starch intake with all-cause and cause-specific mortality. *Front. Nutr.* 9:1004667. doi: 10.3389/fnut.2022.1004667

The association of dietary resistance starch intake with all-cause and cause-specific mortality

Jiang Wan[1†], Xiaocong Li[2†], Ming Gu[3], Qi Li[4], Chuyun Wang[4], Run Yuan[4], Lin Li[5], Xiang Li[4*], Shaodong Ye[5*] and Jichun Chen[4*]

[1]Department of Epidemiology, Key Laboratory of Cardiovascular Epidemiology, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, [2]Medical Research and Biometrics Center, National Clinical Research Center for Cardiovascular Diseases, Fuwai Hospital, National Center for Cardiovascular Diseases, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China, [3]Department of Cardiac Surgery, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, [4]Department of Nutrition, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, [5]Department of Cardiology, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Background: Several studies have estimated daily intake of resistant starch (RS), but no studies have investigated the relationship of RS intake with mortality.

Objective: We aimed to examine associations between RS intake and all-cause and cause-specific mortality.

Methods: Data from US National Health and Nutrition Examination Survey (NHANES) from 1999 to 2018 with 24-h dietary recall data was used in current study. The main exposure in this study was RS intake, and the main outcome was the mortality status of participants until December 31, 2019. The multivariable Cox proportional hazards regression models were developed to evaluate the hazard ratios (HRs) and 95% confidence interval (95% CI) of cardiovascular disease (CVD), cancer, and all-cause mortality associated with RS intake.

Results: A total of 42,586 US adults [mean (SD) age, 46.91 (16.88) years; 22,328 (52.43%) female] were included in the present analysis. During the 454,252 person-years of follow-up, 7,043 all-cause deaths occurred, including 1,809 deaths from CVD and 1,574 deaths from cancer. The multivariable-adjusted HRs for CVD, cancer, and all-cause mortality per quintile increase in RS intake were 1 (95%CI, 0.97–1.04), 0.96 (95%CI, 0.93–1), and 0.96 (95%CI, 0.95–0.98), respectively. The associations remained similar in the subgroup and sensitivity analyses.

Conclusion: Higher RS intake is significantly associated with lower cancer and all-cause mortality, but not significantly with CVD mortality. Future studies focusing on other populations with different food sources of RS and RS subtypes are needed to access the dose–response relationship and to improve global dietary recommendations.

KEYWORDS

dietary, resistant starch, mortality, CVD, cardiovascular disease, cancer

1. Introduction

Diet plays a crucial role in people's overall health and well-being. Previous studies have identified dietary factors associated with mortality (1, 2). Suboptimal diet, an important preventable risk factor for non-communicable diseases (NCDs), is responsible for more deaths than any other risks worldwide (3), and improvement of diet could potentially prevent one in every five deaths globally (4).

Carbohydrates are the main source of energy for most of the world's population, providing 50% or more of daily energy (5). There are already evidences that high-carbohydrate diets increase the risk of mortality (6). Beyond the quantity, the quality, and food sources of carbohydrate have been proved to play a role in health consequences (7). Starch is the major source of carbohydrate in the human diet, and resistance starch (RS) is defined as the total amount of starch and its degradation products that resists digestion in the small intestine of healthy individuals. Meeting the three criteria for being a prebiotic (8): resistance to the upper gastrointestinal environment, fermentation of the gut microbiota, and selective stimulation of beneficial bacterial growth and/or activity, resistant starch (RS) seems to be a promising nutritional strategy to improve people's health. There is limited evidence that RS can benefit gut health (9), glucose homeostasis (10), insulin sensitivity (11), lipid profile (12), cancer (13), chronic kidney disease (14), and improve inflammation and oxidative stress (15).

To our knowledge, no studies have assessed the associations of RS intake with mortality. Given the potential benefits of RS and the uncertainty in the literature, we conducted the current research to examine associations between RS intake and all-cause and cause-specific mortality using the data of U.S. adults from the US National Health and Nutrition Examination Survey (NHANES).

Abbreviations: BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; DBP, diastolic blood pressure; HR, hazard ratio; PIR, poverty income ratio; RCS, restricted cubic spline; RS, resistant starch; SBP, systolic blood pressure; SCFA, short chain fatty acids; SD, standard deviation.

2. Materials and methods

2.1. Study design and population

The National Health and Nutrition Examination Survey (NHANES) is a stratified, multistage study designed by the National Center for Health Statistics (NCHS) to assess the health and nutritional status among a nationally representative sample. All data and materials used in this study from the NHANES database are free and directly downloadable from https://wwwn.cdc.gov/nchs/nhanes/Default.aspx. Mortality data are available from https://ftp.cdc.gov/pub/Health_Statistics/NCHS/datalinkage/linked_mortality/.

To maximize the sample size, datasets required for this study during the 10 cycles from 1999 to 2018 were downloaded. Finally, 42,586 participants (including 20,258 males and 22,328 females, aged ≥20 years) were enrolled with qualified follow-up data (without any missing information on mortality) and dietary data (without any missing information on any dietary intake, and total energy intake ≥5,000 or ≤500 kcal/d). A flow diagram for the inclusion and exclusion of participants in this study is presented in Figure 1. The institutional review board approval of the National Center for Health Statistics (NCHS) and written informed consent for each participant were obtained before data collection.

2.2. Main exposure and resistant starch assessment

The main exposure in this study was RS intake. The 24-h dietary recall was employed to collect the food intake data for two non-consecutive days. The first 24-h dietary recall was conducted manually, and the second dietary recall (added in 2002 and later) was collected by telephone and was scheduled 3- to 10-days later (16). To assess RS consumed by NHANES participants, the weighted average of RS for each food in the RS database was first matched with the unique eight-digit Food and Nutritious Database for Dietary Studies (FNDDS) food code (Supplementary 1) (17, 18), which defined food groups and subgroup. Next, the amount of RS in each food record was

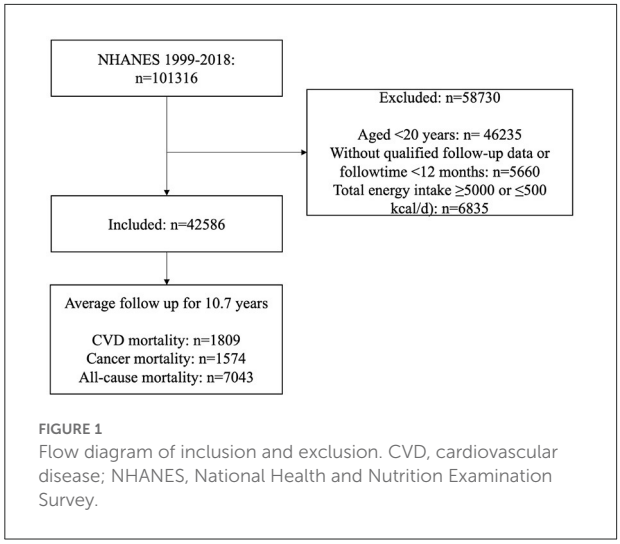


FIGURE 1
Flow diagram of inclusion and exclusion. CVD, cardiovascular disease; NHANES, National Health and Nutrition Examination Survey.

calculated by multiplying the weighted average by the grams of the food. Finally, the amount of RS in each food record was added up to get the total amount of RS consumed by each individual. Data are reported as total RS in g/(d * 1,000 kcal).

2.3. Main outcome

The main outcome of this study was the survival condition of participants, which has been updated with mortality follow-up data by the National Death Index (NDI) through December 31, 2019. As the most complete source of death information in the United States, the NDI has been used to determine the mortality status of participants in this study. The International Classification of Diseases, Tenth Edition (ICD-10) was used to determine the specific cause of death. The ICD-10 codes for CVD were I00–I09, I11, I13, and I20–I51. The ICD-10 codes for cancer were C00–C97. In the end, 7,043 people died, including 1,809 from cardiovascular disease (CVD) and 1,574 from cancer.

2.4. Covariates

Non-dietary covariates included age (years), sex (male/female), race/ethnicity (Mexican American/Other Hispanic/Non-Hispanic White/Non-Hispanic Black/Other Race—including Multi-Racial), educational level (less than high school/high school diploma—including General Educational Development/college or above), body mass index (kg/m^2), smoking status (never smoked/currently smoking/ex-smoking), drink status (yes/no), disease histories of hypertension, dyslipidemia, and diabetes mellitus (yes/no), marriage status (yes/no), and poverty income ratio (PIR) (<1.3/1.3–3.49/≥ 3.5). Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Participants who smoked at least 100 cigarettes during their lifetime were classified as smokers, and drinkers were defined as individuals who drank a minimum of 12 drinks in any given year. Participants could be defined as comorbid conditions (cancer, CVD, diabetes, hypertension, and dyslipidemia) if they reported that they had been told by a health care professional that they had these conditions and/or were taking prescription drugs for them and/or met the appropriate diagnostic criteria.

2.5. Statistics analysis

According to the NHANES analytic guidelines, sample weights, stratification, clustering were taken into consideration to account for the complex, multistage, probability sampling survey design. Data years were combined (using different sampling weights) to maximize sample sizes and evaluate for time trends. Demographic characteristics, dietary intakes, examination variables, and laboratory variables were presented as mean ± SD for continuous variables and as number (percentage) for categorical variables. $\chi 2$ tests and one-way analyses of variance were applied to compare the differences of baseline characteristics and mortality status by quantiles. The Bonferroni test was used for multiple comparisons.

The multivariable Cox proportional hazards regression models were developed to evaluate the hazard ratios (HRs) and 95% confidence interval (95% CI) of CVD, cancer, and all-cause mortality associated with RS intake. We first assessed the proportional hazards assumption by evaluating the weighted Schoenfeld residuals (19), and several violations were observed ($P < 0.05$). The violation of proportional risk assumption is addressed by adding its interaction with time to the model. Survival time was calculated as the number of months from the date of NHANES interview until death or the date of census (December 31, 2019). To control the potential confounders, age, sex, race/ethnicity were adjusted in model 1. We further adjusted for carbohydrate intake, educational level, smoking, drinking, history of relevant disease in model 2. In our final model 3, disease histories of diabetes mellitus, hypertension, and dyslipidemia, marriage status, and income were adjusted. Resistant starch intake was first fitted as an unweighted restricted cubic spline (RCS) with four knots at 5^{th}, 35^{th}, 65^{th}, and 95^{th} centiles and then divided into quintiles to flexibly model the association of RS intake with mortality. A 20-percentile increase was used to estimate the HRs for mortality from CVD, cancer, and all-cause. The trends were estimated by treating the quintiles as a continuous variable. Interaction between continuous linear quintiles of RS intake and covariates was tested by introducing a two-factor interaction term in the multivariable adjusted Cox regression model. Participants with missing values are not included in the corresponding model.

TABLE 1 Characteristics of study participants according to quintiles of resistant starch intake.

Characteristic[a]	Total	Quintiles of resistant starch intake					*P*-value[b]
		Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	
Participants, No.	42,586	8,518	8,516	8,515	8,516	8,521	NA
Age, years	46.91 ± 16.88	45.77 ± 16.24	47.13 ± 16.90	46.52 ± 16.87	47.22 ± 17.12	48.15 ± 17.25	<0.001
Female	22,328 (52.43)	4,425 (51.94)	4,472 (52.51)	4,314 (50.66)	4,479 (52.6)	4,678 (54.9)	<0.001
Followtime, years	11.00 ± 5.18	12.00 ± 5.10	11.29 ± 5.14	10.83 ± 5.13	10.55 ± 5.16	10.11 ± 5.18	<0.001
SBP, mm Hg[c]	122.32 ± 17.74	121.87 ± 17.42	122.42 ± 17.93	122.23 ± 17.09	122.34 ± 17.92	122.86 ± 18.42	<0.001
DBP, mm Hg[c]	70.73 ± 12.39	71.11 ± 12.45	70.91 ± 12.50	70.91 ± 12.53	70.41 ± 12.13	70.18 ± 12.30	<0.001
BMI, kg/m^2 [c]	28.67 ± 6.69	28.56 ± 6.69	28.73 ± 6.62	28.80 ± 6.83	28.77 ± 6.76	28.46 ± 6.48	0.001
PIR[c]							<0.001
<1.3	11,141 (26.16)	2,380 (27.94)	2,175 (25.53)	2,031 (23.86)	2,124 (24.94)	2,467 (28.96)	
1.3–3.49	14,381 (33.77)	2,925 (34.33)	2,864 (33.63)	2,867 (33.68)	2,904 (34.1)	2,810 (32.97)	
≥3.5	17,064 (40.07)	3,213 (37.72)	3,477 (40.83)	3,616 (42.47)	3,488 (40.96)	3,244 (38.07)	
Smoking							<0.001
Never smoked	22,337 (52.45)	3,889 (45.65)	4,307 (50.57)	4,520 (53.08)	4,749 (55.76)	4,995 (58.62)	
Currently smoking	9,234 (21.68)	2,578 (30.27)	1,990 (23.37)	1,747 (20.51)	1,500 (17.61)	1,286 (15.1)	
Ex-smoking	11,015 (25.86)	2,051 (24.08)	2,219 (26.06)	2,249 (26.41)	2,268 (26.63)	2,240 (26.28)	
Drinking	30,375 (71.33)	6,275 (73.67)	6,214 (72.97)	6,186 (72.64)	6,012 (70.6)	5,591 (65.62)	<0.001
Educational level							<0.001
Less than high school	7,522 (17.66)	1,579 (18.54)	1,335 (15.68)	1,384 (16.25)	1,458 (17.12)	1,824 (21.41)	
High school diploma or GED[c]	10,067 (23.64)	2,259 (26.52)	2,118 (24.87)	1,914 (22.48)	1,982 (23.28)	1,736 (20.37)	
College or above	24,997 (58.7)	4,680 (54.94)	5,063 (59.45)	5,217 (61.27)	5,076 (59.61)	4,961 (58.22)	
Married	23,923 (56.17)	4,416 (51.85)	4,788 (56.22)	4,886 (57.38)	4,929 (57.88)	4,944 (58.03)	<0.001
Race/ethnicity							<0.001
Mexican American	3,407 (8)	413 (4.85)	500 (5.87)	625 (7.35)	885 (10.39)	1,073 (12.59)	
Other Hispanic	2,299 (5.4)	383 (4.5)	367 (4.31)	429 (5.03)	436 (5.12)	735 (8.63)	
Non-Hispanic White	29,619 (69.55)	6,046 (70.98)	6,353 (74.6)	6,137 (72.08)	5,827 (68.43)	5,088 (59.72)	
Non-Hispanic Black	4,633 (10.88)	1,181 (13.87)	925 (10.86)	863 (10.14)	807 (9.48)	829 (9.73)	
Other Race	2,628 (6.17)	495 (5.81)	371 (4.35)	460 (5.41)	561 (6.59)	796 (9.34)	
Diabetes	5,160 (12.12)	839 (9.85)	961 (11.28)	982 (11.53)	1,047 (12.29)	1,331 (15.62)	<0.001
Hypertension	17,939 (42.12)	3,575 (41.97)	3,625 (42.56)	3,488 (40.96)	3,555 (41.74)	3,697 (43.39)	0.024
Dyslipidemia	13,166 (30.92)	2,412 (28.32)	2,684 (31.52)	2,575 (30.24)	2,677 (31.43)	2,818 (33.07)	<0.001
History of CVD[c]	4,583 (10.76)	927 (10.88)	989 (11.61)	828 (9.72)	899 (10.56)	940 (11.03)	0.002
History of cancer	3,888 (9.13)	758 (8.9)	867 (10.18)	759 (8.91)	799 (9.38)	705 (8.27)	<0.001

[a]Values are means ± SDs for continuous variables and numbers (%) for categorical variables.
[b]*P*-value for the comparisons between quintiles.
[c]RS, resistant starch; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; PIR, poverty income ratio; GED, General Educational Development; CVD, cardiovascular disease.

Based on previous evidence of possible effect modification, we conducted subgroup analyses for associations between RS intake and mortality according to several confounding factors at baseline. We conducted several sensitivity analyses to test the robustness of our findings. First, we excluded participants who were followed for less than 5 years or died within 5 years. Second, we excluded the participants with a history of CVD or cancer. Third, we conducted a competing risk model to evaluate and quantify the bias of competing risks. Fourth, due to the lack of some variables for calculating HEI-2015 (Healthy Eating Index) in NHANES from 1999 to 2004, another sensitivity analysis was carried out by incorporating HEI-2015 into the model using the data of NHANES from 2005 to 2018. Fifth, a newly defined CVD outcome with ICD-10 codes I00–I09, I11, I13, I20–I51, and I60–I69 was included in the analysis.

All statistical analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and R 4.1.1 (R Core Team, Vienna, Austria). The two-sided *P*-values <0.05 were considered statistically significant.

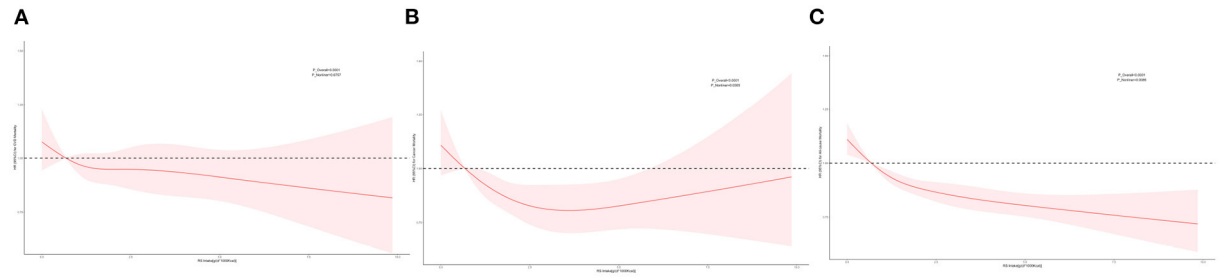


FIGURE 2
Restricted cubic spline (RCS). Multivariable-adjusted HRs (red lines) and 95%CI (pink areas) for risk of mortality in model 3. An intake of 0.68 g/(d * 1,000 kcal) was set as reference (dashed lines) (HR = 1.00). **(A)** RCS for CVD mortality. **(B)** RCS for cancer mortality. **(C)** RCS for all-cause mortality.

TABLE 2 Associations between resistant starch intake and mortality.

Characteristic	Quintiles of resistant starch intake					*P*-value	Per quintile increase
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5		
Participants, No.	8,518	8,516	8,515	8,516	8,521	NA	NA
Followtime, years	11.46 ± 5.07	10.99 ± 5.08	10.60 ± 5.08	10.34 ± 5.11	9.94 ± 5.08	NA	NA
RS intake, g/(d * 1,000 kcal)[d]	0.33 ± 0.22	0.99 ± 0.19	1.69 ± 0.22	2.57 ± 0.32	4.74 ± 1.64	NA	NA
CVD deaths, No.[d]	391	368	337	358	355	NA	NA
Cancer deaths, No.	367	346	298	280	283	NA	NA
All-cause deaths, No.	1,580	1,510	1,324	1,305	1,324	NA	NA
HRs (95% CI) of CVD mortality[d]							
Model 1[a]	1 (Reference)	0.82 (0.71–0.94)	0.81 (0.7–0.94)	0.87 (0.75–1)	0.87 (0.75–1)	0.171	0.98 (0.94–1.01)
Model 2[b]	1 (Reference)	0.89 (0.77–1.02)	0.89 (0.77–1.03)	0.97 (0.84–1.13)	0.96 (0.83–1.11)	0.938	1 (0.97–1.04)
Model 3[c]	1 (Reference)	0.88 (0.76–1.02)	0.9 (0.77–1.04)	0.99 (0.85–1.15)	0.96 (0.83–1.12)	0.799	1 (0.97–1.04)
HRs (95% CI) of cancer mortality[d]							
Model 1[a]	1 (Reference)	0.86 (0.74–1)	0.8 (0.69–0.94)	0.76 (0.65–0.89)	0.77 (0.65–0.9)	<0.001	0.93 (0.9–0.97)
Model 2[b]	1 (Reference)	0.9 (0.78–1.05)	0.88 (0.75–1.02)	0.83 (0.71–0.97)	0.85 (0.73–1)	0.024	0.96 (0.93–0.99)
Model 3[c]	1 (Reference)	0.91 (0.78–1.06)	0.91 (0.78–1.06)	0.84 (0.71–0.98)	0.86 (0.74–1.02)	0.04	0.96 (0.93–1)
HRs (95% CI) of All-cause mortality[d]							
Model 1[a]	1 (Reference)	0.85 (0.79–0.91)	0.8 (0.74–0.86)	0.79 (0.73–0.85)	0.8 (0.74–0.86)	<0.001	0.95 (0.93–0.96)
Model 2[b]	1 (Reference)	0.89 (0.83–0.96)	0.86 (0.8–0.93)	0.86 (0.8–0.93)	0.86 (0.8–0.93)	<0.001	0.97 (0.95–0.98)
Model 3[c]	1 (Reference)	0.89 (0.83–0.96)	0.87 (0.81–0.94)	0.85 (0.79–0.92)	0.85 (0.78–0.91)	<0.001	0.96 (0.95–0.98)

[a]Cox proportional hazard model adjusted for age, sex, race/ethnicity.
[b]Further adjusted for total carbohydrate intake, educational level, smoking, drinking, history of CVD or cancer.
[c]Further adjusted for disease histories of diabetes mellitus, hypertension and dyslipidemia, marriage status, and income.
[d]RS, resistant starch; CVD, cardiovascular disease; HR, hazard ratio; CI, confidence interval; NA, not applicable.

3. Results

3.1. Baseline characteristics

For the 42,586 US adults included in the present analysis, mean (SD) age at baseline was 46.91 (16.88) years and 22,328 (52.43%) of all participants were female. The mean ± SD and median (interquartile range) follow-up was 11.00 ± 5.18 and 10.25 (8.33) years, respectively. During the 454,252 person-years of follow-up, 7,043 all-cause deaths occurred, including 1,809 deaths from CVDs and 1,574 deaths from cancer. Table 1 shows the characteristics of the participants at baseline according to the quintiles of RS intake. Compared with participants with the lowest RS intake, participants with the highest RS intake were more likely to be older, female, married, non-drinkers, non-smokers, Mexican American and Hispanic; to have higher SBP, educational level; and to have lower DBP and BMI. And the proportion of participants with morbidity conditions

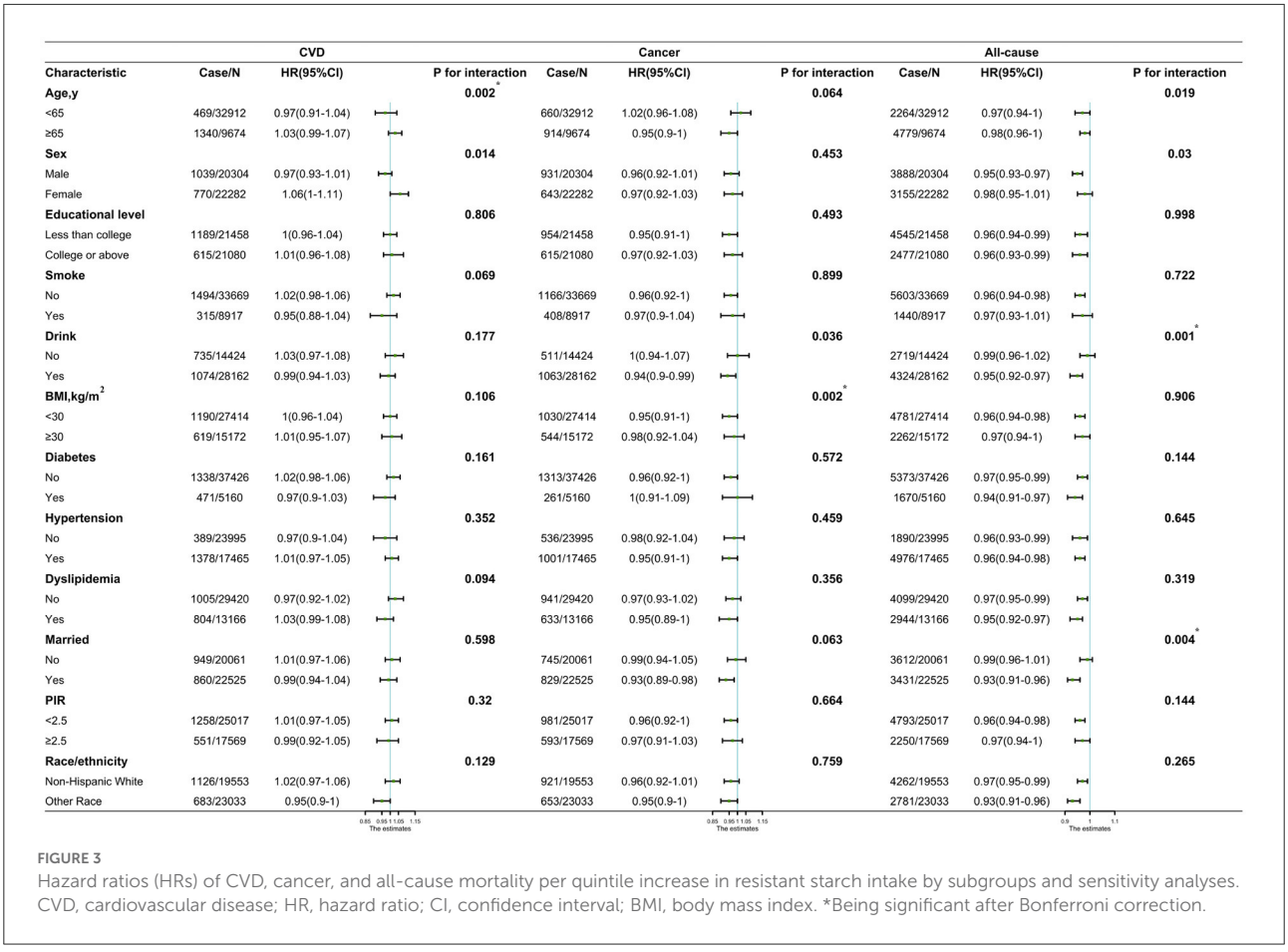


FIGURE 3
Hazard ratios (HRs) of CVD, cancer, and all-cause mortality per quintile increase in resistant starch intake by subgroups and sensitivity analyses. CVD, cardiovascular disease; HR, hazard ratio; CI, confidence interval; BMI, body mass index. *Being significant after Bonferroni correction.

(diabetes, hypertension, dyslipidemia, and CVD) increased and the proportion of participants with cancer decreased.

3.2. RS intake and mortality

We used unweighted RCS to flexibly model and visualize the relationship of predicted RS intake with mortality (Figure 2). Dose–response relationship between cancer mortality risk and RS intake approximates a U-shaped curve. The risk of all-cause mortality decreased with increased RS intake, and a significant protective effect was observed at higher RS intake. Restricted cubic splines revealed possible linear or non-liner relationships of RS intake with cancer and all-cause mortality (*P*-overall <0.001 and *P*-overall <0.001, respectively; *P*-non-liner = 0.03 and *P*-non-liner <0.01, respectively).

The RS intake was associated with cancer and all-cause deaths, but not associated with CVD deaths. The multivariable-adjusted HRs for CVD mortality from the lowest quintile to the highest quintile were 1 (reference), 0.88 (95%CI, 0.76–1.02), 0.9 (95%CI, 0.77–1.04), 0.99 (95%CI, 0.85–1.15), 0.96 (95%CI, 0.83–1.12) (*P* = 0.80 for trend); for cancer mortality, 1 (reference), 0.91 (95%CI, 0.78–1.06), 0.91 (95%CI, 0.78–1.06), 0.84 (95%CI, 0.71–0.98), 0.86 (95%CI, 0.74–1.02) (*P* = 0.04 for trend); and for all-cause mortality, 1 (reference), 0.89 (95%CI, 0.83–0.96), 0.87 (95%CI, 0.81–0.94), 0.85 (95%CI, 0.79–0.92), 0.85 (95%CI, 0.78–0.91) (*P* <0.001 for trend) (Table 2).

A per 20-percentile increase in RS intake was not significantly associated with the risk of CVD mortality (HR, 1; 95%CI, 0.97–1.04), whereas a per 20-percentile increase in RS intake was associated with an 4% lower risk of cancer mortality (HR, 0.96; 95%CI, 0.93–1) and a 4% lower risk of all-cause mortality (HR, 0.96; 95%CI, 0.95–0.98).

3.3. Subgroup and sensitivity analyses

In subgroup analyses, the associations between RS intake and cancer and all-cause deaths remained persistent in most subgroups (Figure 3). A statistically significant interaction between RS intake and BMI (*P* = 0.002 for interaction) for cancer. Significant interactions were found between RS intake and alcohol consumption and marital status (*P* = 0.001 and *P* = 0.004 for interaction, respectively). The HRs for cancer mortality

per 20-percentile RS increase were 0.95 (95%CI, 0.91–1) among participants with BMI <30 kg/m^2 vs. 0.98 (95%CI, 0.92–1.04) among participants with BMI ≥ 30 kg/m^2; and for all-cause mortality, 0.95 (95%CI, 0.92–0.97) among participants who drank alcohol vs. 0.99 (95%CI, 0.96–1.02) among participants who did not drink alcohol and 0.93 (95%CI, 0.91–0.96) among participants who were married vs. 0.99 (95%CI, 0.96–1.01) among unmarried participants.

When we further excluded participants with a follow-up of less than 5 years, these associations remained similar in sensitivity analyses. The results for the newly defined CVD outcome were consistent with those for CVD in the primary analysis. Statistically significant associations were not detected when we applied a Fine-Gray competing risk model or a model with HEI-2015 or excluded patients with CVD or cancer. Details of the sensitivity analysis are shown in Supplementary 2.

4. Discussion

4.1. Main findings

To the best of our knowledge, our research is the first cohort study to investigate the association of RS intake with overall and cause-specific mortality. The estimated intake of RS in our research [2.09 ± 1.76 g/(d * 1,000 kcal)] was similar to previous studies (17). The usual average intake of RS in American adults is approximately 4.2 g/d, much less than the 15–20 g/d of RS recommended for health benefits (20). We observed that higher RS intake was associated with lower cancer and all-cause mortality in a nationally representative sample of US adults. The risk of death from cancer and any causes were 14% and 15% lower in those reporting the highest RS intake, respectively. Results from RCSs showed that individuals in the general population with an intake of RS of approximately 3 g/(d * 1,000 kcal) had the lowest risk of cancer.

A growing body of literature demonstrates that the use of dietary fiber can manipulate the microbiota and greatly impact health. Resistant starch shares some characteristics with dietary fiber and may have similar health effects, and prebiotic-RS seems to be a promising nutritional strategy (9, 15, 21). Our results were consistent with previous observational studies reporting a positive association between dietary fiber intake and health outcomes (22–25). In contrast to these findings, no evidence that RS supplementation at 30 g/day has an effect on development of colorectal cancer in carriers of hereditary colorectal cancer was found in CAPP2 study with 937 participants who were followed for up to 4 years, a randomized trial to assess the effectiveness of RS supplement on carcinoma in human beings (13). The results of these studies may be controversial depending on the tumor type, region, or ethnicity studied.

In addition, although higher dietary fiber intake was reported to be associated with a significantly reduced risk of first stroke (26), we failed to find a significant association between RS intake and CVD mortality. The role of dietary fiber in the prevention of CVD remains controversial. We speculate that it may be the result of low RS intake and low adherence, since short-term high-RS diets do not improve markers of cardiometabolic health (12). Another possibility is that increased RS intake is accompanied by increased carbohydrate intake, which increases the risk of CVD (7). Perhaps the ratio of RS to starch affected the primary outcome, which requires further in-depth study.

4.2. Interpretations of our findings

Several possible mechanisms could be involved in the associations of RS intake with mortality. Obesity is associated with comorbidities such as diabetes, CVD, and cancer, which are among the leading causes of death in the Western world, and RS has many properties that could ameliorate the impact of these comorbidities by promoting weight loss and/or weight maintenance (20). Consumption of RS can not only increase intestinal satiety peptide release, reduce postprandial glucose and insulin (10), but also increase fat oxidation, reduce fat storage in adipocytes, and maintain lean body mass. In addition, total energy consumption increases due to the fiber-like properties of RS, which increases the thermal effect of the food (27). Outside of these properties, RS has other notable health benefits. It has been confirmed that RS positively regulates the gut microbiome, and significantly increases stool output and fecal moisture content, as well as the concentration of short-chain fatty acids (SCFA) (28, 29). The major SCFA are acetate, propionate, and butyrate, which are primarily derived from fermentation of dietary fibers and play key roles in host gut, metabolic, and immune function mainly due to their impact on gene regulation (30). Gut microbiota actively communicates with host cells through the production of SCFA and strongly modulate multiple cellular mechanisms (30), such as regulating cell proliferation and differentiation by inducing apoptosis in colorectal cancer cells while providing energy for normal colonocytes, a situation termed the "Butyrate Paradox" (31). In addition, RS has positive effects on other functions (inflammation, cholesterol, gut hormonal activity, etc.) through bacterial fermentation in the intestine (12, 15).

4.3. Strengths and limitations

The strengths of this study should be acknowledged. For this study, a nationally representative sample of U.S. adults with a longitudinal study design was used to collect dietary and health data using validated methods. We first conducted this study to explore the relationship of RS intake with mortality.

However, there are several limitations. First, the amount of RS in food varies depending on how it was handled and how long it was stored. Due to the limited capabilities to quantify the actual amount of RS in these foods, RS intake may be mis-estimated resulting in erroneous results. Second, RS has been categorized into four main types, but not all RSs behave the same. In the present study, we failed to further investigate the effect of RS subtypes on health. Perhaps one of the subtypes of RS has a major effect on health outcomes, or perhaps different subtypes of RS have a combined effect on health outcomes. Third, due to methodological limitations, we failed to consider weights in RCS. Although such results reflect the NHANES population, they may not reflect the actual situation in the U.S. population.

4.4. Clinical importance

Some guidelines recommend that adults consume 15–20 g of RS per day for health benefits (20), however, RS intake increases with total carbohydrate intake, which increases the risk of mortality (32). Our findings suggest that total energy intake or total carbohydrate intake should be considered when increasing RS intake. Resistant starch is found naturally in several foods, the best sources being whole grains and legumes. We suggest taking more foods rich in RS to increase daily RS intake without increasing total energy intake or total carbohydrate intake. In addition, the amount of RS varies greatly depending on how food is prepared, cooked, and whether it is reheated (33). Cooked legumes, peas, and cooked and cooled starchy foods are high in RS. It may be wise to prepare food this way often, or to eat food prepared this way often. This finding, if confirmed in more studies, will have important clinical and public health implications.

5. Conclusion

Based on a nationally representative sample of U.S. adults, our study provides evidence that higher RS intake is associated with lower cancer and all-cause mortality, but not with CVD mortality. Future studies focusing on other populations with different food sources of RS and RS subtypes are needed to access the dose-response relationship and to improve global dietary recommendations for different populations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://wwwn.cdc.gov/nchs/nhanes/Default.aspx.

Ethics statement

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

Author contributions

XianL, JC, SY, and JW contributed to conception and design of the study. JW, XiaoL, and QL prepared the data for analyzes. MG and QL validated the data for analyzes. JW and XiaoL performed the formal analyzes and wrote the original draft. LL, CW, and RY reviewed and edited the draft. XianL, SY, and JC modified and provided reviews for the draft. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by China National Key R&D (Program Nos. 2020YFC2009002 and 2017YFC0211702) and Special Fund for Scientific Research of the Nursing Department in Fuwai Hospital (HLB2020006).

Acknowledgments

We thank for the participants and staff members of US National Health and Nutrition Examination Survey for their valuable participation and contributions.

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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1004667/full#supplementary-material

References

1. Petermann-Rocha F, Parra-Soto S, Gray S, Anderson J, Welsh P, Gill J, et al. Vegetarians, fish, poultry, and meat-eaters: who has higher risk of cardiovascular disease incidence and mortality? A prospective study from UK Biobank. *Eur Heart J.* (2021) 42:1136–43. doi: 10.1093/eurheartj/ehaa939

2. Huang J, Liao LM, Weinstein SJ, Sinha R, Graubard BI, Albanes D. Association between plant and animal protein intake and overall and cause-specific mortality. *JAMA Intern Med.* (2020) 180:1173–84. doi: 10.1001/jamainternmed.2020.2790

3. GBD 2019 Risk Factors Collaborators. Global burden of 87 risk factors in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019 *Lancet.* (2020) 396:1223–49. doi: 10.1016/S0140-6736(20)30752-2

4. GBD 2017 Diet Collaborators. Health effects of dietary risks in 195 countries, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. (2019) 393:1958–72. doi: 10.1016/S0140-6736(19)30041-8

5. Ludwig DS, Hu FB, Tappy L, Brand-Miller J. Dietary carbohydrates: role of quality and quantity in chronic disease. *BMJ*. (2018) 361:k2340. doi: 10.1136/bmj.k2340

6. Akter S, Mizoue T, Nanri A, Goto A, Noda M, Sawada N, et al. Low carbohydrate diet and all cause and cause-specific mortality. *Clin Nutr.* (2021) 40:2016–24. doi: 10.1016/j.clnu.2020.09.022

7. Shan Z, Guo Y, Hu FB, Liu L, Qi Q. Association of low-carbohydrate and low-fat diets With mortality among US adults. *JAMA Intern Med.* (2020) 180:513–23. doi: 10.1001/jamainternmed.2019.6980

8. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol.* (2017) 14:491–502. doi: 10.1038/nrgastro.2017.75

9. DeMartino P, Cockburn DW. Resistant starch: impact on the gut microbiome and health. *Curr Opin Biotechnol.* (2020) 61:66–71. doi: 10.1016/j.copbio.2019.10.008

10. Gourineni V, Stewart ML, Wilcox ML, Maki KC. Nutritional bar with potato-based resistant starch attenuated post-prandial glucose and insulin response in healthy adults. *Foods.* (2020) 9:1679. doi: 10.3390/foods9111679

11. Stewart ML, Wilcox ML, Bell M, Buggia MA, Maki KC. Type-4 resistant starch in substitution for available carbohydrate reduces postprandial glycemic response and hunger in acute, randomized, double-blind, controlled study. *Nutrients.* (2018) 10:129. doi: 10.3390/nu10020129

12. Yuan HC, Meng Y, Bai H, Shen DQ, Wan BC, Chen LY. Meta-analysis indicates that resistant starch lowers serum total cholesterol and low-density cholesterol. *Nutr Res.* (2018) 54:1–11. doi: 10.1016/j.nutres.2018.02.008

13. Mathers JC, Movahedi M, Macrae F, Mecklin JP, Moeslein G, Olschwang S, et al. Long-term effect of resistant starch on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet Oncol.* (2012) 13:1242–9. doi: 10.1016/S1470-2045(12)70475-8

14. Snelson M, Kellow NJ, Coughlan MT. Modulation of the gut microbiota by resistant starch as a treatment of chronic kidney diseases: evidence of efficacy and mechanistic insights. *Adv Nutr.* (2019) 10:303–20. doi: 10.1093/advances/nmy068

15. Esgalhado M, Kemp JA, Azevedo R, Paiva BR, Stockler-Pinto MB, Dolenga CJ, et al. Could resistant starch supplementation improve inflammatory and oxidative stress biomarkers and uremic toxins levels in hemodialysis patients? A pilot randomized controlled trial. *Food Funct.* (2018) 9:6508–16. doi: 10.1039/C8FO01876F

16. Moshfegh AJ, Rhodes DG, Baer DJ, Murayi T, Clemens JC, Rumpler WV, et al. The US Department of Agriculture Automated Multiple-Pass Method reduces bias in the collection of energy intakes. *Am J Clin Nutr.* (2008) 88:324–32. doi: 10.1093/ajcn/88.2.324

17. Miketinas DC, Shankar K, Maiya M, Patterson MA. Usual dietary intake of resistant starch in US adults from NHANES 2015–2016. *J Nutr.* (2020) 150:2738–47. doi: 10.1093/jn/nxaa232

18. Patterson MA, Maiya M, Stewart ML. Resistant starch content in foods commonly consumed in the United States: a narrative review. *J Acad Nutr Diet.* (2020) 120:230–44. doi: 10.1016/j.jand.2019.10.019

19. Grambsch PM, Therneau TM. Proportional hazards tests and diagnostics based on weighted residuals. *Biometrika.* (1994) 81:515–26. doi: 10.1093/biomet/81.3.515

20. Birt DF, Boylston T, Hendrich S, Jane JL, Hollis J, Li L, et al. Resistant starch: promise for improving human health. *Adv Nutr.* (2013) 4:587–601. doi: 10.3945/an.113.004325

21. Esgalhado M, Kemp JA, Paiva BR, Brito JS, Cardozo L, Azevedo R, et al. Resistant starch type-2 enriched cookies modulate uremic toxins and inflammation in hemodialysis patients: a randomized, double-blind, crossover and placebo-controlled trial. *Food Funct.* (2020) 11:2617–25. doi: 10.1039/C9FO02939G

22. Zheng B, Shen H, Han H, Han T, Qin Y. Dietary fiber intake and reduced risk of ovarian cancer: a meta-analysis. *Nutr J.* (2018) 17:99. doi: 10.1186/s12937-018-0407-1

23. Aune D, Chan DS, Greenwood DC, Vieira AR, Rosenblatt DA, Vieira R, et al. Dietary fiber and breast cancer risk: a systematic review and meta-analysis of prospective studies. *Ann Oncol.* (2012) 23:1394–402. doi: 10.1093/annonc/mdr589

24. Sun L, Zhang Z, Xu J, Xu G, Liu X. Dietary fiber intake reduces risk for Barrett's esophagus and esophageal cancer. *Crit Rev Food Sci Nutr.* (2017) 57:2749–57. doi: 10.1080/10408398.2015.1067596

25. Kim Y, Je Y. Dietary fiber intake and total mortality: a meta-analysis of prospective cohort studies. *Am J Epidemiol.* (2014) 180:565–73. doi: 10.1093/aje/kwu174

26. Threapleton DE, Greenwood DC, Evans CE, Cleghorn CL, Nykjaer C, Woodhead C, et al. Dietary fiber intake and risk of first stroke: a systematic review and meta-analysis. *Stroke.* (2013) 44:1360–8. doi: 10.1161/STROKEAHA.111.000151

27. Higgins JA. Resistant starch and energy balance: impact on weight loss and maintenance. *Crit Rev Food Sci Nutr.* (2014) 54:1158–66. doi: 10.1080/10408398.2011.629352

28. Le Leu RK, Winter JM, Christophersen CT, Young GP, Humphreys KJ, Hu Y, et al. Butyrylated starch intake can prevent red meat-induced O6-methyl-2-deoxyguanosine adducts in human rectal tissue: a randomised clinical trial. *Br J Nutr.* (2015) 114:220–30. doi: 10.1017/S0007114515001750

29. Shen D, Bai H, Li Z, Yu Y, Zhang H, Chen L. Positive effects of resistant starch supplementation on bowel function in healthy adults: a systematic review and meta-analysis of randomized controlled trials. *Int J Food Sci Nutr.* (2017) 68:149–57. doi: 10.1080/09637486.2016.1226275

30. Martin-Gallausiaux C, Marinelli L, Blottière HM, Larraufie P, Lapaque N. SCFA: mechanisms and functional importance in the gut. *Proc Nutr Soc.* (2021) 80:37–49. doi: 10.1017/S0029665120006916

31. Donohoe DR, Collins LB, Wali A, Bigler R, Sun W, Bultman SJ. The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. *Mol Cell.* (2012) 48:612–26. doi: 10.1016/j.molcel.2012.08.033

32. Dehghan M, Mente A, Zhang X, Swaminathan S, Li W, Mohan V, et al. Associations of fats and carbohydrate intake with cardiovascular disease and mortality in 18 countries from five continents (PURE): a prospective cohort study. *Lancet.* (2017) 390:2050–62. doi: 10.1016/S0140-6736(17)32252-3

33. Raigond P, Ezekiel R, Raigond B. Resistant starch in food: a review. *J Sci Food Agric*. (2015) 95:1968–78. doi: 10.1002/jsfa.6966

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 28 November 2023
DOI 10.3389/fnut.2023.1284487

OPEN ACCESS

EDITED BY
Hongmin Dong,
Cornell University, United States

REVIEWED BY
Elisa Julianti,
University of North Sumatra, Indonesia
Zhang Yayuan,
Guangxi Academy of Agricultural Science, China

*CORRESPONDENCE
Prabhjot Kaur
✉ prabhjotchahal72@yahoo.in

RECEIVED 28 August 2023
ACCEPTED 02 November 2023
PUBLISHED 28 November 2023

CITATION
Kaur P, Kaur H, Aggarwal R, Bains K, Mahal AK, Gupta OP, Singla LD and Singh K (2023) Effect of cooking and storage temperature on resistant starch in commonly consumed Indian wheat products and its effect upon blood glucose level.
Front. Nutr. 10:1284487.
doi: 10.3389/fnut.2023.1284487

Effect of cooking and storage temperature on resistant starch in commonly consumed Indian wheat products and its effect upon blood glucose level

Prabhjot Kaur[1]*, Harpreet Kaur[1], Renuka Aggarwal[1], Kiran Bains[1], Amrit Kaur Mahal[2], O. P Gupta[3], Lachhman Das Singla[4] and Kulvinder Singh[5]

[1]Department of Food and Nutrition, Punjab Agricultural University, Ludhiana, India, [2]Department of Mathematics, Statistics and Physics, Punjab Agricultural University, Ludhiana, India, [3]ICAR-Indian Institute of Wheat and Barley Research, Karnal, India, [4]Department of Parasitology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India, [5]Department of Livestock Production Management, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

Background/objectives: The health benefits provided by resistant starch have been well documented; however, few studies are available on the resistant starch content of wheat products in India. Moreover, few studies have examined the *in vivo* efficacy of resistant starch in wheat products in improving glucose levels. This study was conducted to evaluate the effect of cooking and storage temperature on the formation of resistant starch in Indian wheat products and its effect on blood glucose levels in humans and rats.

Methods: Wheat products were prepared by common cooking methods including roasting (*Chapati*), boiling (*Dalia*), Shallow frying (*Paratha*), and Deep frying (*Poori*). They were then stored at different temperatures including freshly prepared within 1h (T1), stored for 24h at room temperature (20-22°C) (T2), kept at 4°C for 24h (T3) and reheated after storing at 4°C for 24h (T4). The products were then analyzed for proximate composition (moisture, crude protein, crude fat, ash crude fibre, and carbohydrates). The effect of different cooking methods and storage temperatures on Resistant, non-resistant and total starch, total dietary fibre (soluble and insoluble), *in vitro* starch digestion rate (rapidly and slowly digestible starch), amylose and amylopectin content were analysed using standard operating procedures. The effect of products found to have higher resistant starch was studied on the post prandial blood glucose response of 10 healthy individuals using change in by analysing their glycemic index and glycemic load of wheat products. Further, the effect of resistant starch rich *chapati* on the blood glucose level of rats was also studied. Tukey's test in factorial CRD was used to assess the effect of cooking and temperature on various parameters.

Results: The amount of resistant starch was found to be high in *dalia* (boiling, 7.74%), followed by *parantha* (shallow frying, 4.94%), *chapati* (roasting, 2.77%) and *poori* (deep frying 2.47%). Under different storage temperatures, it was found high in products stored at 4°C (T3), followed by products stored at room temperature (T2), reheated products (T4) and lesser in freshly prepared products (T1). The glycemic index and glycemic load were found low in *chapati* (43, 32.3) and *dalia* (41.1, 28.6) stored at 4°C (T3) compared to others. The resistant starch content found in *chapati* stored at T3 was found to be more effective at reducing blood

glucose levels in rats from 291.0mg/100mL to 225.2mg/100mL in 28days of study compared to freshly prepared *chapati* (T1) and stored at room temperature (T2).

Conclusion: Cooking methods including boiling, roasting and shallow frying increased the amount of resistant starch in foods, but cooking methods such as deep frying decreased the amount of resistant starch in food. Products stored at 4°C and at room temperature for 24h increased the amount of resistant starch whereas the products that were freshly cooked and reheated decreased the amount of resistant starch in foods. At 4°C the stored products have a high amount of insoluble dietary fibre, slowly digestible starch, high amylose and low glycemic index. They take time to digest, meaning that they slowly increase blood glucose levels. The effect of insoluble dietary fibre and resistant starch in the inhibition of glucose diffusion in the small intestine is suggested to be due to the absorption or inclusion of the smaller sugar molecules. *In vivo* research showed that fibre and resistant starch in the digestive system of rats acts as the main factors in slowing glucose absorption and reducing a rise in blood glucose levels by promoting glycogen synthesis and inhibition of gluconeogenesis.

KEYWORDS

resistant starch, glycemic index, dietary fibre, amylose, amylopectin, wheat products, cooking methods, storage temperature

1 Introduction

Starch is a form of polysaccharide that occurs in plants with ample storage and is considered to be the most important part of a human diet. The starch present in the food is indigestible and the duration of cooking improves the digestibility of starch. Starch is made up of amylose and amylopectin chains. Amylose contains α-(1–4)-linked glucan in a straight chain, while Amylopectin contains α-(1–4) and α-(1–6)-glycosidic linkages, which results in a highly branched structure. Starch is also classified into 3 forms depending on their digestibility: rapidly digesting starch (RDS), slowly digesting starch (SDS), and resistant starch (RS). RDS is defined as a type of starch that is rapidly (within 20 min) converted into glucose molecules by enzymatic digestion. SDS is defined as a type of starch that is converted into glucose after 120 min of enzymatic digestion. (1)Whereas RS is not digested even after 120 min and it directly goes into the large intestine where it is fermented into short chain fatty acids by gut microflora (2). This can be because the digestibility of starch fraction is affected by its structure, and digestive enzymes do not hydrolyze different forms of starch structure equally (3, 4). Processing techniques may change granular starch to non granular forms (5).

Resistant starch skips digestion in the small intestine and enters directly into the large intestine. It is then fermented into short chain fatty acids (SCFAs) (6), like acetate, propionate and butyrate along with gases like H2, CO2, and CH4 by probiotic bacteria present in the large intestine (17).

There are five forms of resistant starch present in foods. Resistant starch 1 (RS1) is starch that is not accessible to digestion owing to the presence of complete, undamaged cell walls in the grains, tubers and seeds (7). Resistant starch (RS2) is a native uncooked starch granule poorly influenced by hydrolysis due to its crystalline nature. The third form of resistant starch (RS3) is retrograded starch, formed during cooking and then kept under room or low temperature (8). The fourth type of resistant starches (RS4) are those that are customized chemically to acquire resistance from digestion enzymatically such as esters and ethers of starch, and cross-linked starches (9). Fifth, resistant starch (RS5) is formed when amylose comes in contact with lipids known as amylose-lipid complexes. In many plant sources which contain a high amylose content, amylose chains are perforated by lipids and form amylose-lipid complexes (10).

The third type of starch (RS3), retrograded starch, is affected by the cooking technique used. Retrogradation is the process that causes the recrystallization of the starch chains after the gelatinized paste cools. Starch structures that are molecular or crystalline are affected by storage conditions like duration, temperature and water content, which determine the retrogradation rate and its extent. Wheat starches show higher retrogradation rates due to longer amylopectin chains and high amylose (21.7%) content compared to rice (17.55%) (11). A high amylose content leads to low digestibility in food products (12). Maize starches with high amylose content and longer chains structured themselves into double helices which resists digestion (13). Due to internal structure and B-type crystallinity, high amylose starch resists digestion by enzymes (14).

The process of starch degradation leads to the formation of starch and its by-products, which are not assimilated and absorbed in the small intestine of healthy individuals (15). Both the rate and extent of hydrolysis of starch in the small intestine determine the formation of starch by-products that play a crucial role in the body. The metabolism of resistant starch happens 5–7 h after eating as compared to typically cooked starch, which is immediately digested. Reduction in insulinemia and postprandial blood glucose occur due to the 5–7 h delay in digestion, meaning it has the potential to increase the satiety period (16). This outstanding nutritional activity, compared to dietary fibre, is mainly associated with its physiological effects. Gut-related microbiota and immune modulation, which lead to the significant production of short chain fatty acids (SCFAs), occur with normal consumption of subclasses of fermentable dietary fibre sources in the daily diet (10).

The ability of any food to raise the blood glucose level after being consumed depends on its glycemic response or glycemic index. Food that has a high glycemic index raises blood glucose levels quickly compared to low glycemic food, which slowly increases the blood glucose level. Hence, low glycemic foods are beneficial for controlling glycemic responses. Wheat, rice and maize food products have a high glycemic index and can easily raise blood glucose levels. The overconsumption of high glycemic food for longer periods can cause several metabolic disorders such as obesity and type-2 diabetes. Insulin resistance and insulin insensitivity in muscles leads to hyperinsulinemia caused by obesity (18).

Some studies have suggested that retrograded starch may reduce serum cholesterol concentration through numerous mechanisms, along with an increase in faecal bile acid excretion (19). It has been suggested that resistant starch has properties like an ability to reduce insulinemic response, postprandial glycemic responses, enhance whole body insulin sensitivity, extend satiety, and limit fat storage, and the fact that it lowers plasma cholesterol and triglyceride concentrations are exhibited by resistant starch. Thus, it could be used to prevent illnesses associated with dyslipidemia, the development of weight loss diets, and insulin resistance, and could be a dietary treatment for type 2 diabetes and coronary heart diseases (20).

The main challenge of using resistant starch in the food industry is the process of manufacturing consumer-friendly foods that contain enough resistant starch to result in the significant enhancement of public health. In response to the potential health benefits of resistant starch, the present study was undertaken to determine the effect of cooking and storage temperature on the resistant starch of wheat products that are part of the staple diet of North Indian people. We also studied the *in vivo* efficacy of resistant starch in wheat products to improve blood glucose levels.

2 Materials and methods

2.1 Procurement and the cooking process

The most commonly consumed Indian wheat variety (HD3086) was procured from the Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The grains were cleaned and ground using a sample milled with 60 mesh size for making flour and 22 mesh size for making *dalia* (Figure 1). We chose four common cooking methods: roasting, boiling, shallow frying and deep frying, which are commonly used in North Indian cuisine. We then prepared commonly consumed food products using these methods ie. *Chapati*

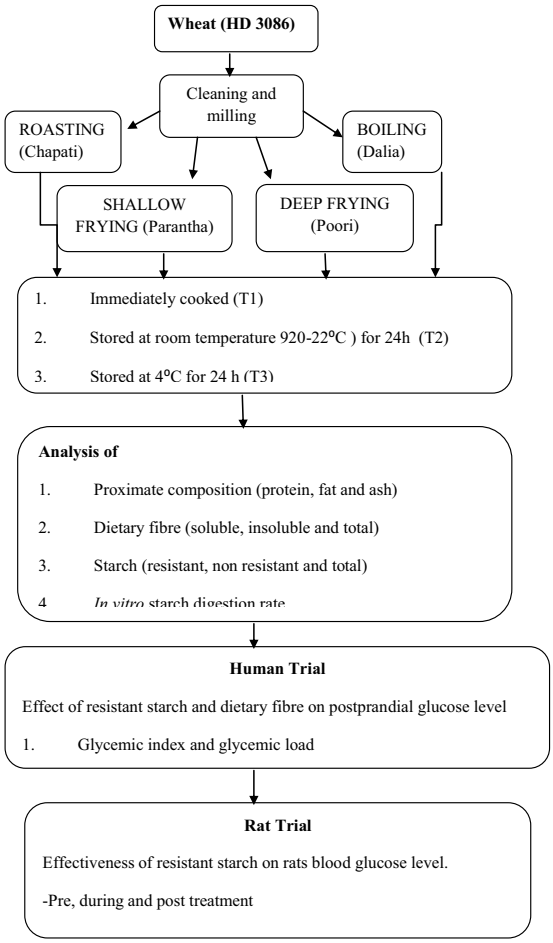


FIGURE 1
Methodology of the study.

TABLE 1 Preparation of commonly consumed wheat products in India.

Cereal product	Ratio of ingredients	Method
Chapati (flattened bread, roasting)	Whole wheat flour to water (2·5: 1, w/v)	Whole wheat flour was kneaded with the addition of water and made into a soft dough. About 25 g of dough was taken and flattened into a *chapati* by using a wooden rolling pin and board to 10–12 cm diameter and then toasted for 2 min directly on a hot iron tawa without smearing oil on both sides till golden brown.
Dalia (broken wheat, boiling)	Broken wheat and water (1–7.5 w/v)	*Dalia* was prepared by mixing broken wheat and water and then boiling it for 10 min in an open pan.
Parantha (shallow fried flattened bread)	Whole wheat flour and water (2·5: 1, w/v)	Whole wheat flour was kneaded by the addition of water and made into a soft dough. About 30 g of dough was taken and flattened using a wooden rolling pin and board to 10–12 cm diameter and then about 1.5 mL of oil was smeared and the *parantha* was folded 4 times and again flattened and put on hot tawa and toasted for 2 min on both sides with smeared oil until golden brown in color.
Poori (fried bread, deep frying)	Whole wheat flour and water (2.5:1, w/v)	The whole wheat flour, a pinch of salt and water were mixed and left to settle for 10 min. Approximately 20 g of dough was taken and flattened and then deep fried in mustard oil (preheated at a temperature of 200–220°C to prevent the food from sticking) for 35–40 s until golden-brown in colour, then removed from the oil.

(flattened bread, roasting), *dalia* (broken wheat, boiling), *parantha* (shallow fried flattened bread), and *poori* (fried bread, deep frying) (Table 1). These four food products were analysed at four different conditions of storage, which were considered to be four different treatments, including freshly prepared within 1 h (T_1) stored for 24 h at room temperature (20-22°C and 45–50% RH) (T_2), stored at 4°C for 24 h (T_3), and finally, reheated after being stored at 4°C for 24 h (T_4) (Figure 1). For the study, out of five sets of each wheat product (in triplicate), one set was kept as a control (raw samples of wheat flour without any treatment) in triplicate. The other four sets (each in triplicate) were kept for cooking. After the treatments, the samples were dried and used for nutritional analysis.

2.2 Nutritional analysis

Nutritional analyses of raw and cooked samples were undertaken, examining crude protein, crude Fat and ash using standardized methods. The macro-Kjeldahl method was used for the determination of crude protein. Crude fat and ash content were also measured by using (AOAC 2000) method (21).

2.2.1 Dietary fibre

The total dietary fibre was determined using a megazyme total dietary fibre (K-TDFR-200A) kit. The soluble and insoluble dietary fibre contents were also analyzed using the standard protocol given by (21). The dietary fibre was calculated using the formula:

$$\text{Dietary fiber (\%)} = \frac{R_1 + R_2 \quad p \quad A \quad B}{2} \times 100$$

$$\frac{m_1 + m_2}{2}$$

Where: R_1 = residue weight 1 from m1, R_2 = residue weight 2 from m_2, m_1 = sample weight 1, m_2 = sample weight 2, A = ash weight from R_1, p = protein weight from R_2 and B = blank Where: BR = blank residue, BP = blank protein from BR1, BA = blank ash from BR2.

2.2.2 Total starch and resistant starch

The total starch and resistant starch were determined using a megazyme K-RSTAR assay, as outlined previosuly (22). Resistant starch and non-resistant (solubilized) starch were added to determine the total amount of starch.

2.2.3 *In vitro* starch digestion rate

The *in vitro* starch digestion rate was determined using the procedure given in another study (23). In total, 500 mg of the sample was exposed for 15–20 s to 250 U porcine amylase in 1 mL of synthetic saliva (carbonate buffer; Sigma A-3176 Type VI-B). Then, 5 mL of pepsin (1 mL per ml of 0.02 M aq. HCl; from gastric porcine mucosa; Sigma P-6887) was added and incubated for 30 min in a water bath at 37°C. The digesta was neutralized by adding 0.02 M aq. Sodium hydroxide (5 mL) before adjusting the pH 6. (25 mL of 0.2 M C2H3NaO2 buffer) 5 mL of amyloglucosidase (Sigma A-7420 from Aspergillus niger; 28 U per mL of acetate buffer) and pancreatin (2 mg per mL of acetate buffer; Sigma P1750 from porcine pancreas) were added. Then the solution was incubated for 4 h, and at various times during that period, an Accucheck glucometer was used to monitor the digesta's glucose concentration.

2.2.4 Rapidly digestible starch and slowly digestible starch

The glucometer reading at 15 min was converted to the percentage of starch digested using the following equation

$$DS = \frac{0.9 \times G_G \times 180 \times V}{W \times S[100 - M]}$$

Where:

GG = Reading of the glucometer (mM/L). V = Digest volume (mL), 180 = glucose's molecular weight W = sample weight (g).

S = sample's starch content (g per 100 g dry sample). M = moisture percentage in the sample (g per 100 g sample). 0.9 = starch stoichiometric constant from glucose concentrations. RDS% = percentage of starch digested at 15 min. SDS% = percentage of starch digested at 120 min – percentage of starch digested at 15 min.

2.2.5 Amylose and amylopectin

The Amylose Content was measured by colorimetric estimation of the amylose-iodine complex (24). The defatted sample weighing 100 mg was taken in a boiling tube and mixed with 1 mL of distilled ethanol. Then, 9 mL of 1 N sodium hydroxide was added and the tube was placed in a boiling water bath for 10 min. The volume was made up to 100 mL, out of which 5 mL were transferred to a 100 mL volumetric flask, mixed with 1 mL 1 N acetic acid (MP Biomedicals) and 2 mL iodine solution (1 g iodine and 10 g KI/500 mL distilled water) and kept in darkness for 20 min. Finally, the volume was made to 100 mL and the absorbance was measured at 620 nm using a blank 5 mL 0.09 N NaOH, to which acetic acid (1 mL) and iodine solution (2 mL) were added in 100 mL total volume.

Amylopectin = 100-amylose.

2.3 Impact of resistant starch and soluble fibre components on postprandial glucose response by measuring glycemic index

The glycemic index was calculated using the method given by Goni (25). Ten healthy individuals were selected for measurement of blood glucose levels. The food was given in the morning after 12 h of fasting and the food was eaten within 15 min. Blood samples were taken using a finger-prick using a Glucometer (Dr. Morphine). Blood glucose levels were measured at fasting 0, 15, 30, 45, 60, 90, and 120 min after taking 50 g of carbohydrates in the form of cooked cereal products. To compare the effect of cooked food on blood glucose, the control sample was also given in the form of 50 g of glucose. Volunteers were allowed to drink 150–300 mL of water depending on the food consumed during the study. Then, the glycemic index was calculated by applying the formula,

$$GI = \frac{Area\ under\ the\ curve\ for\ 50\,gm\ carbohydrate\ for\ test\ sample}{Area\ under\ the\ curve\ for\ 50\,gm\ carbohydrates\ from\ control\ (glucose)} \text{X } 100$$

Glycemic load was calculated as:

$$Glycemic\ load = \frac{GI \times Available\ carbohydrates}{100}$$

2.4 Effectiveness of resistant starch on blood glucose level in rats

Previous human supplementation research has indicated that cooked wheat products that had been subject to different treatments had a low glycemic index. We hypothesized that lower glycemic index foods have a positive effect on treating diabetes maybe through enhanced insulin secretion or by reducing its sensitivity. For this, we conducted a rat experiment to have authentic, real and unbiased data. Moreover, rats are very similar to humans genetically, and biologically and their behavioral characteristics closely resemble that of humans.

2.4.1 Animal collection

35 Wistar albino rats aged 2–3 months with weights 180-220 g were obtained from the animal house and breeding centre (AHBC) at Akal College of Pharmacy and Technical Education Mastuana Sahib, Sangrur (Registered breeder of CCSEA). The experiment was conducted as per the permission provided by the Institutional Animal Ethics Committee (IAEC no.:- GADVASU/2023/1AEC/68/12). The animals were housed in cages, fed with commercial pellets and had access to water *ad libitum*.

2.4.2 Induction of diabetes

The Wistar albino rats were given an intraperitoneal injection of freshly prepared 230 mg/kg Nicotinamide (NA) with buffer saline NaCl 0.9%. After 15 min, the rats were again given intraperitoneal injections of Streptozotocin (STZ) at about 60 mg/kg. Rats were provided with 5% of glucose water after injection to prevent hypoglycaemia. After 5 days of induction, their blood samples were taken and used to measure blood glucose and insulin levels. A blood glucose level of more than 200 mg/kg was an indicator of diabetic rats. Rats were treated for 28 days and blood glucose levels were checked first, third, and last week of the experiment.

2.4.3 Treatment protocol

1. Group-I: (Normal control) consists of normal rats given a normal diet for 28 days.
2. Group-II: (Diabetic control) after induction of diabetes were given a normal diet for 28 days.
3. Group-III: (Treatment group) Diabetic rats with supplement FWC (freshly prepared wheat *chapati*, T1) orally for 28 days.
4. Group-IV: (Treatment group) Diabetic rats with supplement 24WC (24 h stored at room temperature wheat *chapati,* T2) orally for 28 days.
5. Group-V: (Treatment group) Diabetic rats with the supplement RehWC (reheated wheat *chapati* after storing 4°C for 24 h, T4) orally for 28 days.

Rats did not eat the diet in Treatment 3 as it was not accepted by the subjects due to its cold temperature. Moreover, as it is not the habit of people in North India to consume this product at this temperature, it was not fed to the rats.

3 Results

3.1 Proximate composition

The crude protein content was found to be highest in *Chapati* (10.79%), which was prepared using roasting followed by *dalia* (boiling,9.53%), *parantha* (shallow frying, 9.46%), and *poori* (deep frying, 9.16%) (Table 2). A significant difference (≤0.001*) was also observed in the protein content of the products stored at different temperatures with the highest content in T2. The protein content of

TABLE 2 Effect of different cooking methods and storage temperatures on crude protein, crude fat, and ash content of wheat products (per 100g).

Wheat products	Treatments				
	T1	T2	T3	T4	Treatment Mean
Crude Protein (raw/flour)	11.36 ± 0.56				
Chapati	10.59 ± 0.09^{b}	11.02 ± 0.54^{b}	11.06 ± 0.07^{b}	10.52 ± 0.20^{b}	10.79^{A}
Parantha	8.01 ± 0.22^{ef}	9 ± 0.10^{cd}	11.72 ± 0.25^{a}	8.13 ± 0.33^{de}	9.46^{BC}
Poori	9.03 ± 0.17^{cd}	10.98 ± 0.27^{b}	9.39 ± 0.16^{ef}	8.26 ± 0.46^{c}	9.16^{C}
Dalia	9.16 ± 0.66^{c}	8.84 ± 0.37^{b}	9.22 ± 0.17^{f}	8.91 ± 0.32^{b}	9.53^{B}
Storage mean	9.21^{C}	10.45^{A}	9.58^{B}	9.70^{B}	
Crude fat (raw/flour)	1.62 ± 0.29				
Chapati	1.39 ± 0.14^{efg}	1.30 ± 0.07^{fg}	1.48 ± 0.03^{defg}	1.08 ± 0.11^{g}	1.31^{C}
Parantha	2.42 ± 0.13^{d}	2.35 ± 0.17^{def}	2.45 ± 0.01^{defg}	2.41 ± 0.08^{de}	2.27^{B}
Poori	12.05 ± 0.68^{a}	11.56 ± 0.94^{b}	12.23 ± 0.36^{c}	11.26 ± 0.56^{c}	13.02^{A}
Dalia	1.86 ± 0.05^{defg}	1.70 ± 0.06^{defg}	2.30 ± 0.18^{defg}	1.99 ± 0.01^{defg}	1.89^{B}
Storage Mean	5.43^{A}	4.80^{B}	4.07^{C}	4.18^{C}	
Ash (raw/flour)	0.83 ± 0.16				
Chapati	1.36 ± 0.05^{bcd}	1.60 ± 0.05^{abc}	1.87 ± 0.03^{a}	1.21 ± 0.12^{d}	1.51^{AB}
Parantha	1.65 ± 0.14^{ab}	1.58 ± 0.06^{abc}	1.82 ± 0.06^{a}	1.29 ± 0.06^{cd}	1.58^{A}
Poori	1.20 ± 0.20^{d}	1.12 ± 0.07^{d}	1.24 ± 0.24^{d}	1.41 ± 0.08^{bcd}	1.24^{C}
Dalia	1.43 ± 0.13^{bcd}	1.44 ± 0.09^{bcd}	1.59 ± 0.06^{abc}	1.33 ± 0.10^{bcd}	1.44^{B}
Storage mean	1.40^{BC}	1.43^{B}	1.62^{A}	1.31^{C}	

Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. T1 - freshly prepared within 1 h, T2 - stored at room temperature (20-22°C for 24 h), T3 - Kept at 4°C for 24 h, T4 – samples reheated after being stored at 4°C for 24 h.
Wheat chapati (roasting); Parantha (shallow-frying); Poori (deep-frying); Dalia (boiling).

chapatti (11.06%) *parantha* (11.72%) and *dalia* (9.22%) was found highest in T3, i.e., when stored at 4°C for 24 h. This was followed by T2, T1, and T4 in *chapatti* and T2, T4, and T1 in *parantha* and T4, T1, and T2. P*oori* stored for 24 h at room temperature (20-22°C and 45–50% RH) showed the highest protein content of 10.98% compared to other storage temperatures. All methods of cooking led to a decline in the protein content of the products as compared to the raw uncooked sample of wheat flour (11.3%).

Contrary to protein, the crude fat content was observed to be higher in *poori* (deep frying, 13.02%) followed by *parantha* (shallow frying, 2.27%) than the raw and other cooked products because of the addition of extra oil. Among different storage temperatures, crude fat content was high in T1 with 5.43%, followed by T2 (4.80%), T4 (4.18%), and T3 (4.07%). Raw wheat samples contained 0.83% ash, which was increased after cooking. The ash content was found to be high in *parantha* (shallow frying, 1.58%), followed by *chapati* (roasting, 1.51%), *dalia* (boiling, 1.44%), and *poori* (deep frying, 1.24%). Keeping the products at 4°C for 24 h (i.e., treatment T3) resulted in higher ash content, followed by the products stored at room temperature (T2).

3.2 Dietary fibre

The soluble (2.05%), insoluble (10.78%) and total dietary fibre (12.84%) were found to be maximum in *dalia*, which was prepared by boiling. Storing of the prepared food products at different temperatures, affected the dietary fibre content. The insoluble and total dietary fibre content increased with an increase in storage period at low temperatures and was observed to be highest in products stored at 4°C (T3) (10.43, 12.20%) for 24 h while the soluble fibre content was higher in the fresh food samples (T1) (2%) (Table 3).

3.3 Resistant starch and total starch

The resistant starch content of the raw samples (0.52%) increased after cooking and the amount of resistant starch was found to be highest in *dalia* (boiling, 5.45%) followed by *parantha* (shallow frying, 3.46%), *chapati* (roasting, 2.37%), and *poori* (deep frying, 2.04%). Wheat products stored at T3 were found to have a higher amount of resistant starch content (4.47%) followed by T2 (3.32%), T4 (2.97%) and a lesser amount in freshly prepared products T1 (2.57%) (Table 4). On the other hand, non-resistant starch content in raw samples (70.57%) was higher, which was reduced after cooking. The content was highest in *chapati* (roasted, 68.45%) and *poori* (deep frying, 68.42%), respectively and during storage it was found to be high during T1 (68.8%) compared to T3 (66.1%) (Table 5). No significant difference was seen in the total starch content of raw and cooked samples after cooking. Only the type of starch (resistant and non significant starch) was affected. Results showed that the total starch content in wheat products lies between (69.9 to 72.93%) with the highest level of resistant starch at 72.93% in *dalia* with T1 and the lowest level at 69.9% in T3 *chapati* (Table 6).

TABLE 3 Effect of different cooking methods and storage temperatures on dietary fibre (Soluble, Insoluble, and Total) content of cereal products (per 100g).

Wheat products	Treatments				
	T1	T2	T3	T4	Treatment mean
Raw/flour (soluble dietary fibre)	1.41 ± 0.02				
Chapati	1.97 ± 0.06^{bcde}	1.87 ± 0.06^{defg}	1.77 ± 0.06^{fgh}	1.90 ± 0.00^{cdef}	1.87^{B}
Parantha	1.64 ± 0.06^{hi}	1.33 ± 0.06^{jk}	1.03 ± 0.06^{l}	1.23 ± 0.06^{k}	1.31^{D}
Poori	2.10 ± 0.10^{b}	1.83 ± 0.06^{efgh}	1.47 ± 0.06^{ij}	1.67 ± 0.08^{gh}	1.76^{C}
Dalia	2.30 ± 0.10^{a}	2.03 ± 0.06^{bcd}	1.83 ± 0.06^{efgh}	2.07 ± 0.06^{bc}	2.05^{A}
Storage Mean	2.0^{A}	1.76^{B}	1.52^{C}	1.71^{B}	
Raw/flour (Insoluble dietary fibre)	9.48 ± 0.13				
Chapati	9.45 ± 0.09^{i}	9.83 ± 0.06^{h}	10.17 ± 0.06^{fg}	9.27 ± 0.12^{ij}	9.68^{D}
Parantha	10.43 ± 0.12^{de}	10.83 ± 0.06^{b}	11.10 ± 0.10^{a}	10.37 ± 0.06^{def}	10.68^{B}
Poori	9.10 ± 0.10^{j}	10.23 ± 0.06^{efg}	10.80 ± 0.10^{bc}	10.07 ± 0.06^{gh}	10.05^{C}
Dalia	10.41 ± 0.08^{de}	10.83 ± 0.06^{b}	11.33 ± 0.06^{a}	10.57 ± 0.06^{cd}	10.78^{A}
Storage Mean	9.85^{D}	10.43^{B}	10.85^{A}	10.06^{C}	
Raw/flour (Total dietary fibre)	10.89 ± 0.12				
Chapati	11.42 ± 0.03^{gh}	11.70 ± 0.00^{efg}	11.93 ± 0.06^{de}	11.17 ± 0.12^{h}	11.55^{D}
Parantha	12.08 ± 0.17^{cd}	12.17 ± 0.06^{cd}	12.13 ± 0.15^{cd}	11.60 ± 0.00^{fg}	11.99^{B}
Poori	11.20 ± 0.17^{h}	12.07 ± 0.06^{cd}	12.27 ± 0.12^{c}	11.74 ± 0.10^{ef}	11.81^{C}
Dalia	12.71 ± 0.15^{b}	12.87 ± 0.06^{ab}	13.17 ± 0.06^{a}	12.63 ± 0.06^{b}	12.84^{A}
Storage Mean	11.85^{C}	12.20^{B}	12.37^{A}	11.78^{C}	

Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. T1-freshly prepared within 1 h, T2-stored at room temperature (20-22°C for 24 h), T3-Kept at 4°C for 24 h, T4-samples reheated after being stored at 4°C for 24 h.
Wheat chapati (roasting); Parantha (shallow-frying); Poori (deep-frying); Dalia (boiling).

TABLE 4 Effect of different cooking methods and storage temperatures on the resistant starch content of cereal products (per 100g).

Wheat products	Treatments				
	T1	T2	T3	T4	Treatment Mean
Raw/ flour	0.523 ± 0.02				
Chapati	2.13 ± 0.02^{ij}	2.39 ± 0.01^{hi}	2.77 ± 0.02^{fg}	2.21 ± 0.02^{hij}	2.37^{C}
Parantha	2.30 ± 0.10^{hi}	3.60 ± 0.01^{e}	4.94 ± 0.04^{bc}	3.03 ± 0.15^{f}	3.46^{B}
Poori	1.73 ± 0.06^{k}	2.07 ± 0.06^{ij}	2.47 ± 0.06^{gh}	1.90 ± 0.00^{jk}	2.04^{D}
Dalia	4.13 ± 0.12^{d}	5.23 ± 0.21^{b}	7.74 ± 0.06^{a}	4.73 ± 0.29^{c}	5.45^{A}
Storage Mean	2.57^{D}	3.32^{B}	4.47^{A}	2.97^{C}	

Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. T1-freshly prepared within 1 h, T2-stored at room temperature (20-22°C for 24 h), T3-Kept at 4°C for 24 h, T4-samples reheated after being stored at 4°C for 24 h.
Wheat chapati (roasting); Parantha (shallow-frying); Poori (deep-frying); Dalia (boiling).

3.4 *In vitro* starch digestion rate

The *in vitro* starch digestion rate is important in determining the potential of a food product to raise the blood glucose levels of an individual. The *in vitro* starch digestion rate of wheat products (*chapati, dalia, paratha and poori*) was affected by different storage temperatures. and was determined at 120 min after completion of the digestion of the food sample (Figures 2–5). The wheat *chapati* stored with T3 and T2 had a slower digestion rate of 42.5 and 50% at 120 min compared to T1 and T4 treated *chapati* which had a completed digestion rate of 56 and 53% at 90 min. In wheat *dalia*, the rate of starch digestion was lower in T3 and T2 with 26 and 29% at 120 min as compared to T1 (36%) and T4(33%). The starch digestion rate of wheat *paratha* was high in T1 (41%) followed by T4 (38%), T2(33.5%) and T3 (32%) at 60 min. In *poori*, the starch digestion rate was also found high in T1 (56%) and T4 (53%) compared to products stored at low temperatures for 24 h (T2 and T3).

TABLE 5 Effect of different cooking methods and storage temperatures on the non resistant starch content of cereal products (per 100g).

Wheat products	Treatments				
	T1	T2	T3	T4	Treatment Mean
Raw/flour	70.57 ± 0.40				
Chapati	69.60 ± 0.53^{a}	68.13 ± 0.12^{abcd}	67.13 ± 0.12^{cdef}	68.93 ± 0.95^{ab}	68.45^{A}
Parantha	68 ± 0.85^{bcde}	67 ± 0.80^{def}	66.47 ± 0.67^{ef}	67.03 ± 0.06^{def}	67.12^{B}
Poori	69.07 ± 0.67^{ab}	68.63 ± 0.38^{abc}	67.53 ± 0.35^{bcde}	68.47 ± 0.29^{abcd}	68.42^{A}
Dalia	68.80 ± 0.17^{ab}	65.60 ± 0.35^{f}	63.47 ± 0.40^{g}	66.47 ± 0.58^{ef}	66.0^{C}
Stoarge Mean	68.8^{A}	67.3^{B}	66.1^{C}	67.2^{B}	

Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. T1-freshly prepared within 1 h, T2-stored at room temperature (20-22°C for 24 h), T3-Kept at 4°C for 24 h, T4-Samples reheated after being stored at 4°C for 24 h.
Wheat chapati (roasting); Parantha (shallow-frying); Poori (deep-frying); Dalia (boiling).

TABLE 6 Effect of different cooking methods and storage temperatures on the total starch content of cereal products (per 100g).

Wheat products	Treatments				
	T1	T2	T3	T4	Treatment Mean
Raw/flour	71.09 ± 0.41				
Chapati	71.73 ± 0.52^{ab}	70.52 ± 0.10^{bc}	69.9 ± 0.14^{c}	71.15 ± 0.95^{bc}	70.82^{B}
Parantha	70.30 ± 0.92^{bc}	70.60 ± 0.81^{bc}	71.40 ± 0.71^{c}	70.07 ± 0.21^{c}	70.59^{B}
Poori	70.80 ± 0.66^{bc}	70.70 ± 0.35^{bc}	70 ± 0.30^{c}	70.37 ± 0.29^{bc}	70.46^{B}
Dalia	72.93 ± 0.06^{a}	70.83 ± 0.25^{bc}	71.20 ± 0.46^{bc}	71.2 ± 0.50^{bc}	71.5^{A}
Storage Mean	71.4^{A}	70.66^{B}	70.62^{B}	70.69^{B}	

Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. T1-freshly prepared within 1 h, T2-stored at room temperature (20-22°C for 24 h), T3-Kept at 4°C for 24 h, T4-samples reheated after being stored at 4°C for 24 h.
Wheat chapati (roasting); Parantha (shallow-frying); Poori (deep-frying); Dalia (boiling).

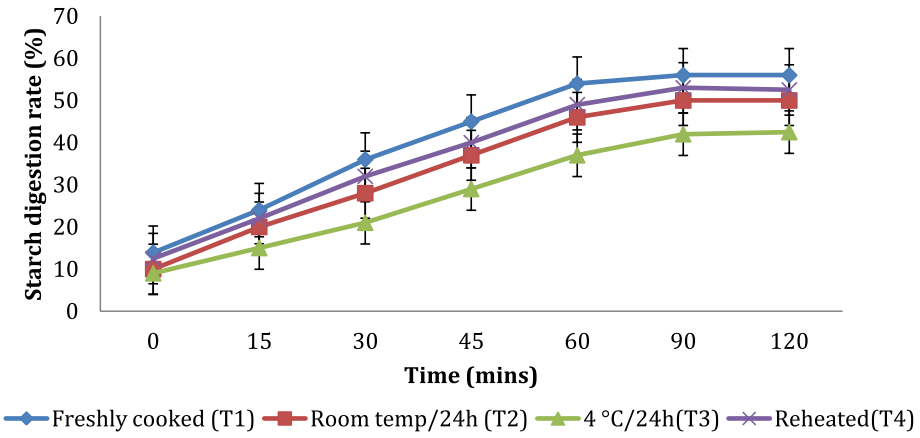


FIGURE 2
Effect of different storage temperatures on *in vitro* starch digestion rate of wheat chapati.

3.5 Rapidly digestible starch (RDS) and slowly digestible starch (SDS)

Similar to the *in vitro* starch digestion rate, the amount of SDS in *dalia* (boiling, 38.76%) was highest followed *parantha* (shallow frying, 34.07%), *chapati* (roasting, (29.23%) and *poori* (deep frying, 23.01%). Treatment 3(T3) increased the level of SDS to the maximum followed by T2, T4 and T1. Due to higher SDS content (Figures 6, 7). *Dalia* (boiling, 14.63%) was observed to have the lowest RDS followed by *chapati* (roasting, 16.98%), *parantha* (shallow frying, 18.06%), and *poori* (deep frying, 23.47%) when stored with treatment 3.

3.6 Amylose and amylopectin

The amylose content was found to be highest in *dalia* (boiling, 34.97%), followed by *parantha* (shallow frying, 30.16%), *chapati* (roasting, 29.64%) and *poori* (deep frying, 21.76%). Treatment 3 resulted in increased amylose content of the products followed by T2,

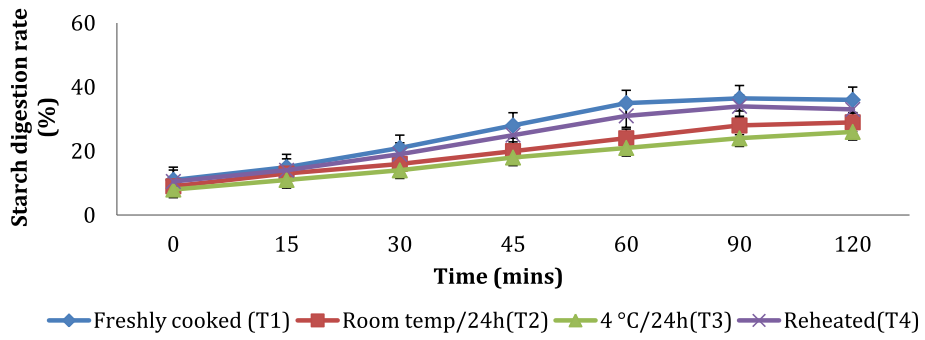


FIGURE 3
Effect of different storage temperatures on *in vitro* starch digestion rate of wheat dalia.

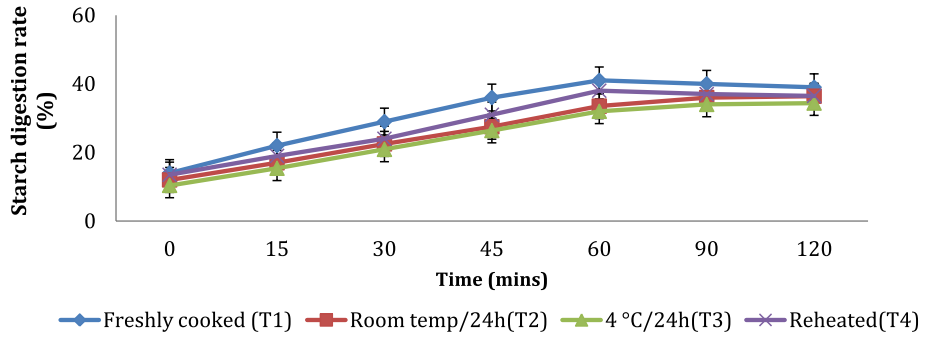


FIGURE 4
Effect of different storage temperatures on the *in vitro* starch digestion rate of wheat paratha.

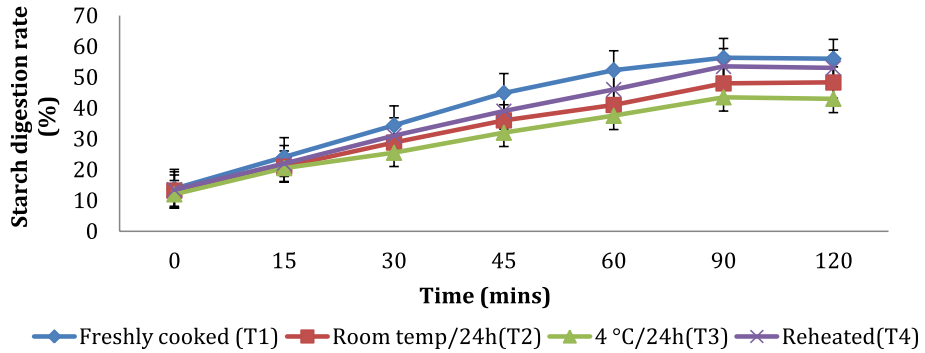


FIGURE 5
Effect of different storage temperatures on the *in vitro* starch digestion rate of wheat poori.

T4 and T1 (Figure 8). On the other hand, the amylopectin content was found highest in *poori* (deep frying, 86.61%), followed by *parantha* (shallow frying, 81.06%), *chapati* (roasting, 76.99%) and *dalia* (boiling, 75.73%) respectively and under storage conditions, it was found highest with T1 followed by T4, T2, and T3 (Figure 9).

3.7 Impact of resistant starch and soluble fibre components on postprandial glucose response

Wheat products like *chapati* and *dalia* having high RS content with all the treatments were selected for feeding to 10 healthy human subjec to evaluate glycemic response. However, chapattis having treatment 3 was not accepted by the subjects due to its cold temperature so was denied by them for its consumption. Moreover, in the north of India people tend not to consume this product at this particular temperature. Therefore, treatment 3 was not considered for the evaluation of the glycemic index of the chapattis. Similarly, for the wheat dalia, we had to discard the product, having treatment 2 as storing at this temperature, led to microbial growth in the product. The lowest glycemic index was observed after the consumption of d*alia* (41.12%) with T3 followed by *chapati* (45.2%) and treatment 2 with a glycemic load of 28.6 and 35.5%, respectively. Treatments 2 and 3 were found to be the best storage conditions for lowering the glycemic index and may prove beneficial for iabetic individuals (Table 7).

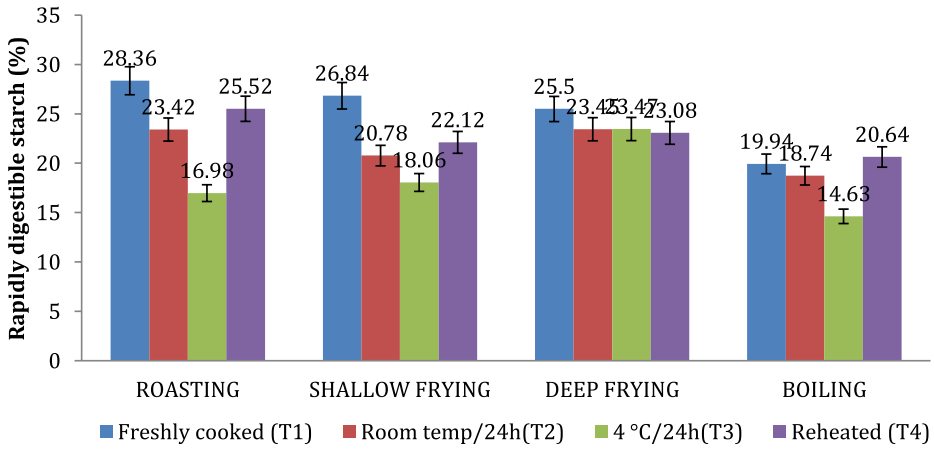


FIGURE 6
Effect of different cooking methods and storage temperatures on rapidly digestible starch in wheat products.

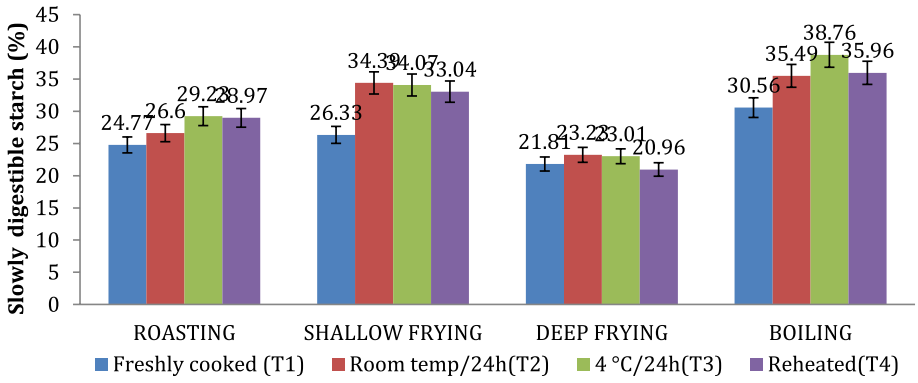


FIGURE 7
Effect of different cooking methods and storage temperatures on slowly digestible starch in wheat products.

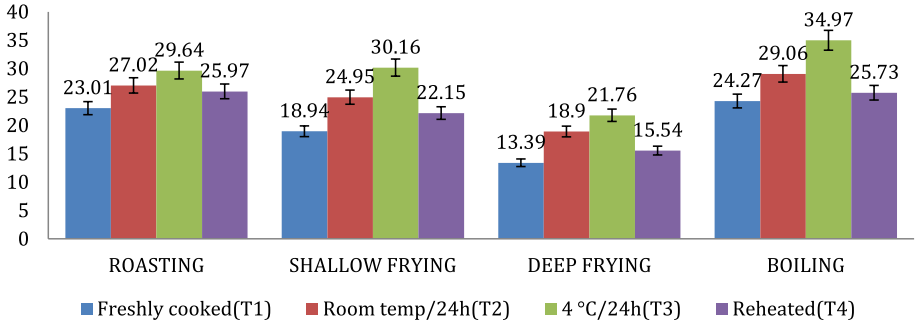


FIGURE 8
Effect of different cooking methods and storage temperatures on the amylose content in wheat products.

3.8 Effectiveness of resistant starch on blood glucose level in rats

Similar to human experiments, treatment 3 for *chapatis* was not considered for the rat trial. *Dalia* was not given to rats due to the limited number available to us and because *dalia* is consumed once or twice a week whereas *chapatis* tend to be consumed thrice a day in north India. The results indicated that resistant starch from different diet groups had a decreasing tendency in blood glucose concentrations, but the mean values were not different from either control or diabetic control groups. Blood glucose levels were significantly increased from 114.3 ± 6.9 in normal rats to 286.5 ± 16.5 mg/ 100 mL in diabetic rats. However, significantly, the levels in pre-treatment groups returned to near normal range from each treatment group (G3-G5) after giving

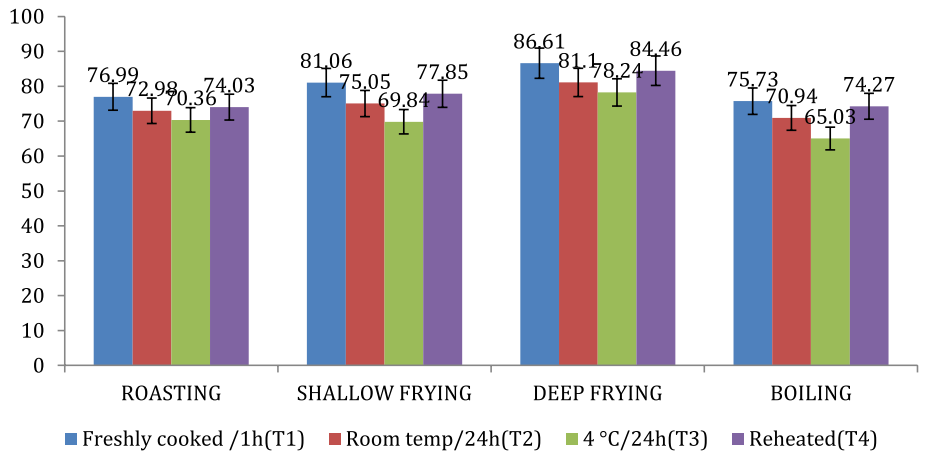


FIGURE 9
Effect of different cooking methods and storage temperatures on amylopectin content in wheat products.

TABLE 7 Effectiveness of wheat products stored at different temperatures on the glycemic Index and glycemic load of human subjects.

Wheat products	Treatments				
Glycemic Index	T1	T2	T3	T4	*p*-Value
Wheat chapati	54.18 ± 1.45^{a}	45.2 ± 2.2^{c}	-	51.0 ± 1.19^{b}	≤0.001*
Dalia	50.3 ± 1.5^{a}	-	41.1 ± 1.3^{c}	47.5 ± 1.3^{b}	≤0.001*
Glycemic Load					
Wheat chapati	39.7 ± 1.06^{a}	$35.5 \pm .66^{b}$	-	$38.4 \pm .90^{a}$	≤0.001*
Dalia	34.9 ± 1.07^{a}	-	$28.6 \pm .96^{c}$	$32.5 \pm .93^{b}$	≤0.001*

Values are mean ± SD of 10 subjects; Different small letters in different rows show significant differences at 5%; *significant at 5%; NS, non-significant. T1-freshly prepared within 1 h, T2-stored at room temperature (20-22°C for 24 h), T3-Kept at 4°C for 24 h, T4-samples reheated after being stored at 4°C for 24 h.

TABLE 8 Effectiveness of resistant starch on blood glucose level in rats.

Diet group	Pre treatment	During treatment	Post treatment	Treatment Mean
G1	114.3 ± 6.86^{g}	115 ± 5.4^{g}	112.6 ± 4.32^{g}	114.0^{E}
G2	286.5 ± 16.5^{a}	285.6 ± 13.8^{a}	283.3 ± 8.35^{a}	285.1^{A}
G3	275.8 ± 29^{abc}	259.3 ± 28.5^{abcde}	225.5 ± 20.0^{ef}	256.8^{BC}
G4	291.0 ± 9.8^{a}	269.3 ± 14.0^{abcd}	225.2 ± 15.0^{f}	255.1^{BC}
G5	292.0 ± 12.1^{a}	279.17 ± 14.3^{ab}	256.3 ± 12.4^{abcde}	275.8^{AB}

Each value is the mean of six observations, Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. Table shows the standard of the mean of diet groups; 1. Control group (normal diet fed rats) (G1); 2. Diabetic control (Normal diet fed rats) (G2); 3. Freshly prepared within 1 h wheat chapati fed rats (G3); 4. Wheat chapati stored at room temperature (20-22°C) for 24 h fed rats (G4); 5. Samples reheated after being stored at 4°C for 24 h storage fed rats.

the treatment Diet. In G3, *chapati* stored at T1 was given and blood glucose level reduced from 275.8 ± 29 to 225.5 ± 20.0. In G4, *chapati* stored at T2 was given and found blood glucose level reduced from 291.0 ± 9.8 to 225.1 ± 15.0. In G5 *chapati* stored at T4 was given and found blood glucose levels reduced from (292.0 ± 12.1 to 256.3 ± 12.4), respectively (Table 8). The diet results in G3, G4, and G5 found 81.7, 77.3, and 87.7% decreases in the blood glucose levels in the rats.

4 Discussion

The present study aimed to find the best storage conditions and cooking methods for increasing the resistant starch content of wheat products commonly consumed in India. The protein content was found to be higher in raw flour (without any treatment) compared to cooked examples, as cooking leads to denaturation of the protein structure. The milling method and the mesh size used in the current study might have also had an effect on the nutritional composition of the flour samples. The protein content of all wheat products was found to be low in freshly prepared products, i.e., within 1 h (T_1) and the products were reheated after being stored at 4°C for 24 h (T_4).

Food stored at low temperatures and room temperature had a higher amount of moisture content compared to freshly prepared and reheated products. This might be because protein content increased with the increase in the moisture content of food (26). In a study, protein content was found to decrease with increasing temperature from 4 to 25°C and the greatest increase in the protein content of quinoa (Titicaca) was found to happen in storage

conditions of 4°C (3.35% increase) and 10°C (3.71% increase) respectively (27). Similarly in another study, the soluble protein content in steamed bread was increased up to 2.03 mg/g when frozen at-12°C. During the freezing process, the soluble protein contents increased from 1.57 mg/g to 1.75 mg/g at-24°C. This might be due to the fact that storing food at low temperatures redistributes water and ice recrystallization, which destroys the internal structure of gluten proteins and leads to depolymerization and shedding of gluten and an increase in protein solubility (28).

Fat is the prerequisite of every food as it increases the palatability of the product. In the roasting and boiling techniques used for the preparation of products, fat content was not changed significantly compared to raw uncooked samples, but in shallow and deep frying methods, additional fat was used, which led to more absorption and retention of fat in the cooked product. Ash content increased in all cooked products compared to raw samples. Ash content is an indication of the mineral content and organic constituents of food like protein, fat and fibre. The storage temperature which is suitable to increase the level of organic constituents of food, will lead to an increase in the ash content of that food. Therefore, in the current investigation, ash content was found high in T3. Compared to different cooking methods, roasted samples contained a higher amount of ash compared to deep-fried samples. This could be because roasting causes less reduction in organic constituents such as protein, fat, and fibre compared to deep frying.

The results obtained in the present study are in agreement with reports outlining that roasting increases the crude fibre and ash content in food (29). Another study observed that the high temperature used when roasting foods can increase the ash content in bar Salak (a species of palm tree). On the contrary, in deep frying, the ash content was reduced because of the reduction of fibre content and other nutrients like protein and fibre (30).

Dietary fibre is resistant to digestion by human digestive enzymes. It manages large intestine functions and has significant physiological effects on mineral bioavailability, lipid, and glucose metabolism (31). The soluble and insoluble nature of dietary fibre mainly determines its solubility in water. Soluble dietary fibre draws bodily water into the digestive tract like a magnet (32). In the gut, soluble fibre makes a gel-like structure (water soluble) and helps the food to move easily (33). Cellulose, hemicellulose, resistant starch, and lignin are the type of water-insoluble fibre, which do not dissolve in water. Insoluble fibre increases intestinal pressure, assisting in the evacuation of faeces (34). In the present study, the amount of soluble, insoluble and total dietary fibre was increased in cooked samples compared to raw samples. The increase was found to be dependent on the amount and duration of cooking temperature, as heat treatments of wheat and barley flours at 100°C convert the insoluble dietary fibre into a soluble form by increasing the water extract viscosities (35). This is the main reason for the high amount of soluble fibre content in T1 compared to T3 in the study. During thermal treatments, Maillard's reaction increases the amount of insoluble dietary fibre content (36).

In another previous study, cooking cereals into *chapatti* had an impact on the total and insoluble dietary fibre content except from ragi, in which total dietary fibre (30 per cent) and insoluble dietary (36 per cent) increased after cooking. This increase in the total dietary fibre of ragi may be due to the formation of resistant starch (37). Hence, an increase in cellulose content led to an increased amount of TDF and IDF. Storing products at low temperatures also led to an increase in resistant starch, which is responsible for the higher amount of insoluble dietary fibre and total dietary fibre content. *In-vitro* rapidly digestible starch was reduced during boiling and frying because of a significant increase in both the resistant starch (RS) and water-insoluble dietary fibre (IDF). The total dietary fibre content increased after cooking because of the increase in cellulose, lignin and pectin content during soaking and cooking (38). In the present study, boiled *dalia* had the highest amount of total dietary fibre which may be because during boiling there is the formation of fibre-protein complexes that are resistant to heat and digestion, leading to the production of increased dietary fibre content (39). Treatment 3 led to a maximum increase in the dietary fibre content, which could be attributed to increased insoluble dietary fibre and resistant starch content.

Whole wheat flour contained 0.50 g and refined wheat flour contained 0.65 g of resistant starch, respectively, (40). Retrogradation of resistant starch rate depends on starch properties like its type of structure, cooking and storage duration, time and temperature (11). In the current investigation, we found that *dalia* followed by *parantha*, *chapatti* and *poori,* stored at 4°C for 24 h (T3) followed by stored at room temperature (T2) had higher RS content than their counterparts. The storage and reheating of products in treatment 1 (i.e., freshly prepared and treatment 4 reheating) caused a reduction of RS content due to the degeneration of the crystalline structure of starch granules as compared to T3, meaning it led to retrogradation and recrystallization of starch granules, resulting in higher RS. Compared to the food products prepared using different cooking methods, we observed that *dalia* prepared by boiling had higher RS content because, during boiling, food comes into contact with both heat and water, leading to the swelling of starch cells and its gelatinization, which results in the release of amylose in the solution (41, 42). A higher level of gelatinization occurs with an increase in the duration of cooking, leading to a higher RS content of the product.

Chapati prepared by roasting also had an RS content of 2.37 per 100 g. This was due to the damaging of starch cells and its partial gelatinization, which affected the formation of resistant starch, i.e., less than the boiled food product (43). Similarly, this occurred in *Parantha* (which are cooked by shallow frying) due to the formation of lipid-amylose complex. This form of RS5 led to there being a higher amount of resistant starch than *Poori*, which was prepared by deep-frying. More water was evaporated during deep-frying, which led to less formation of crystalline structure and, hence, lower resistant starch formation (25, 44). All the results revealed that the resistant starch content in wheat is inversely proportional to the non-resistant starch content and starch digested. RS is directly proportional to the total starch content, insoluble dietary fibre, and protein content (45). Hence, non-resistant starch was found high in freshly prepared and reheated wheat products compared to others. One study indicated that cereals contained less resistant starch content (25.07–31.59%) and the highest amount of non-resistant starch (30.12–56.67%) (46). The foregoing results indicated that the cooking method, like boiling and the storage temperature (stored at 4°C for 24 h),

affected the formation of the RS content in wheat products by retrogradation of starch after boiling and cooling.

No significant difference was seen in the total starch content of raw and cooked samples after cooking. Only the type of starch (resistant and non-significant starch) was affected. Results showed that the total starch content in wheat products was between 70.46 to 71.5%. Freshly prepared products had the highest level of total starch content present in *Dalia* (boiling) and lowest in *chapati* (roasting). During roasting, when food came into contact with heat, the total starch was broken down into sugar. The total starch content of Pearl millet was found to be reduced after the action of the amylase enzyme when total starch was converted into sugar (47). On the contrary, the effect of different storage temperatures on total starch content indicated that freshly prepared products had high content, which decreased with increasing duration of storage. The content was reduced when exposed to high temperatures (25°C) for 6 months of storage studied (48, 49).

Plant genotype can also affect the digestibility of starch, with the amylose/amylopectin ratio being a crucial determinant of the rate of starch hydrolysis in both peas and wheat (50). The starch digestion rate was high in freshly prepared and reheated wheat products because of the presence of more non-resistant starch and less amylose content. The starch digestion rate was low in wheat products stored at 4°C and at room temperature because of retrogradation which occurs at low temperatures leading to the formation of resistant starch, which escapes digestion and absorption in the small intestine. It therefore provides satiety for a longer period of time.

The frequency and quantity of starch digestion are also affected by the structure, composition, processing, and cooking of starch granules associated with other nutrients like lipids, protein, fibre, minerals, and antinutritional factors (50, 51). Compared to different cooking methods, *dalia* prepared by boiling had the lowest starch digestion rate compared to *parantha* (shallow frying), *chapati* (roasting), and *poori* (deep frying). This variation in the digestibility of wheat products occurred due to the heat–moisture remedy proving that these treatments alter the structure of starch and increase the quantities of SDS and RS at the same time as decreasing the proportion of RDS. Differences in the physical and morphological properties of cereal starches cause lower digestibility.

Cooking cereal starches brings about modifications, for example in the physical and chemical disruption and gelatinization of starch granules. The extent of gelatinization in turn is dependent on the amount of water present, the cooking time and the temperature (52), which is the reason for the low digestibility of wheat products prepared by boiling. Studies have indicated that wheat starch may swell more slowly than other starches, which may also restrict the amount of starch gelatinization (53). The presence of protein bodies around starch granules might also limit granule swelling and starch gelatinization and as a result, reduce the susceptibility to enzymatic attack. This could be partly accountable for its low digestibility. The nature of starch also influences how it is digested. When there is a higher material content of amylose there is a decrease in the starch digestibility. Obvious variations in the digestibility of amylose and amylopectin are attributed to the larger surface area of amylopectin and the distinctly prepared and insoluble aggregates, which might lower the accessibility of cleavage sites to enzyme attack. As a result, the nature and source of starch in cereals might also have an impact on their digestibility (54).

The amylose content in raw samples increased after cooking because during cooking and cooling the amylose aligned themselves and associated with each other during retrogradation (55). *Dalia* (boiling) had a high amount of amylose content compared to *parantha* (shallow frying), *chapati* (roasting), and *poori* (deep frying) and was found to be highest at storage temperature T3 (4°C for 24 h). Wheat starches stored at T3 showed higher retrogradation rates due to longer amylopectin chains and high amylose content (56). Hence, the high amylose content is directly proportional to the high resistant starch content. Due to the presence of water in *Dalia,* the leaching out of amylose in water during boiling (57) led to a greater amount of amylose content in *dalia*, which has been linked to a greater retrogradation tendency in starches (58). Amylopectin and intermediate materials also play an important role in starch retrogradation during refrigerated storage (59). Better transition temperatures for cereal products might also result from there being a greater number of rigid granular structures and, due to the presence of lipids (60), might be the cause of resistant starch forming in *parantha*. Extra energy is needed to start melting due to the fact that amylopectin plays a chief function in starch granule crystallinity, and because amylose lowers the melting factor of crystalline regions as well as the power created by starting gelatinization in the absence of amylose-rich amorphous regions. This correlation suggests that starch with higher amylose content has extra amorphous regions and much less crystalline structures, which lower the gelatinization temperatures (61).

Amylopectin content was found to be high in freshly prepared products because after cooking and cooling (T3) the formation of amylose increased, resulting in low amylopectin content and a high amount of resistant starch. The formation of amylopectin content was inversely proportional to the amylose content. The starch from grains such as maize, wheat, rice and low amylose maize, tubers such as potatoes and sweet potatoes, and legumes such as kidney beans, has the highest starch content (8.51%), while kidney bean starch has the higher amylose content (49.50%). Large-size starches (including potato, sweet potato, and kidney bean starches) showed longer amylopectin chains high amylose content and resistant starch confirming that they had the most slowly digestible starch content (62).

The glycemic index determines the rate of rise of blood glucose by a specific food. It was found low in *dalia* compared to *chapati* when stored at 4°C (T3) compared to freshly prepared (T1). Because of the presence of retrograded starch at low temperatures. It takes time to digest and hence, slowly raises blood glucose levels. Low glycemic index diets improve both diabetic and healthy people's risk of coronary heart disease. Low-glycemic index meals promote satiety and help limit food consumption in obese or overweight people. A healthy person's post-prandial glucose and lipid metabolism is improved by choosing foods with a low glycemic index (63). The GL of a normal serving of food is made from the quantity of available carbohydrates and the GI of the type of food. The higher the GL, the higher the chances of elevation in blood glucose level and the insulinogenic impact of the food. The consumption of a diet with a particularly high GL for a long period

of time is associated with an increased chance of type 2 diabetes and heart disease (64). Food which has a low glycemic index is also found to have a low glycemic load.

The effect of high RS foods on the blood glucose level of rats was determined in a rat trial. After consumption of *chapati* stored at T2 (room temperature for 24 h) for 28 days, it was found to be more effective in reducing blood glucose levels than consumption of products stored at T1 and T4. This happens because wheat products stored at room temperature comprise excessive quantities of nutritional fibre and resistant starch. These products release sugar slowly in the blood and also diminish glucose absorption. The gradual digestion of resistant starch (RS) has implications for the application of managed glucose release (2). The metabolism of resistant starch takes place 5–7 h after intake, compared to starch that is cooked using more conventional approaches. As previously mentioned, digestion takes 5-7 h, and slowly increases blood glucose levels resulting in reduced postprandial glycaemia and insulinemia and providing satiety for longer (65). The effect of insoluble nutritional fibre in the inhibition of glucose diffusion in the small gut is usually recommended because of the absorption or inclusion of smaller sugar molecules (66). In the current investigation, we observed that GLP-1 and peptide YY production were increased by the consumption of products containing resistant starch and dietary fibre. This stimulates insulin secretion and reduces glucagon secretion (67). *In vivo* research showed that fibre and resistant starch in the digestive system of rats act as the main factors that slow glucose absorption and reduce the rise of blood glucose levels by promoting glycogen synthesis (68) and inhibition of gluconeogenesis (69).

5 Conclusion

Cooking methods including boiling, roasting and shallow frying increased while deep frying decreased the amount of resistant starch in foods. Products stored at 4°C (T3) and at room temperature for 24 h (T2) showed an increased amount of resistant starch whereas freshly cooking (T1) and reheating (T4) decreased the amount of resistant starch of foods. Products stored at 4°C (T3) have a high amount of insoluble dietary fibre, slowly digestible starch and amylose content. The glycemic index and glycemic load were also found to be low in T3 wheat products. These contained a high amount of resistant starch, which reduced the blood glucose level by regulating the promotion of glycogen synthesis and inhibition of gluconeogenesis. Consumption of *chapati* (T3) in a rat study was found to be more beneficial in controlling the rise of blood glucose levels. Thus, it leads to a slower rise in blood glucose levels, providing longer satiety. In India, people consume a large variety of starchy preparations, meaning that modifications to the food cooking methods and storage temperatures used for starchy foods may lead to several health benefits. Greater awareness should be fostered regarding the nutritional and health benefits of resistant starch consumption to maintain blood levels. People also need to be educated about the correct ways of cooking and storing food products to increase the amount of resistant starch in food at the domestic level.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Institutional ethics committee, PAU. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because there was no chance to harm the human. In the study only their blood glucose levels was measured. 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

PK: Writing – original draft. HK: Methodology, Writing – review & editing. RA: Writing – review & editing. KB: Visualization, Writing – review & editing. AM: Data curation, Writing – review & editing. OG: Project administration, Writing – review & editing. LS: Visualization, Writing – review & editing. KS: Visualization, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank all the study participants for their valuable contributions. This paper was developed from a thesis submitted for the fulfilment of a PhD degree.

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

References

1. Englyst HN, Kingman SM, Cummings JH. Classification and measurement of nutritionally important starch fractions. *Eur J Clin Nutr*. (1992) 46:33–50.

2. Nugent AP. Health properties of resistant starch. *Nutr Bull*. (2005) 30:27–54. doi: 10.1111/j.1467-3010.2005.00481.x

3. Patil SK. Resistant starches as low-carb ingredients - current applications and issues. *Cereal Foods World*. (2004) 49:292–4.

4. Tapsell LC. Diet and metabolic syndrome: where does resistant starch fit in? *J Assoc Official Analyt Chem*. (2004) 87:756–60. doi: 10.1093/jaoac/87.3.756

5. Murphy MM, Douglass JS, Birkett A. Resistant starch intakes in the United States. *J American Dietetic Assoc*. (2008) 108:67–78. doi: 10.1016/j.jada.2007.10.012

6. Raigond P, Ezekiel R, Raigond B. Resistant starch in food: a review. *J Sci Food Agric*. (2015) 95:1968–78. doi: 10.1002/jsfa.6966

7. Sajilata MG, Singhal RS, Kulkarni PR. Resistant starch–a review. *Compr Rev Food Sci Food Saf*. (2006) 5:1–17. doi: 10.1111/j.1541-4337.2006.tb00076.x

8. Hernández O, Emaldi U, Tovar J. In vitro digestibility of edible films from various starch sources. *Carboh Polym*. (2008) 71:648–55. doi: 10.1016/j.carbpol.2007.07.016

9. Sanz-Penella JM, Tamayo-Ramos JA, Sanz J, Haros M. Phytate reduction in bran-enriched bread by phytase producing bifidobacteria. *J Agric Food Chem*. (2009) 57:10239–44. doi: 10.1021/jf9023678

10. Fuentes-Zaragoza E, Sánchez-Zapata E, Sendra E, Sayas E, Navarro C, Fernández-López J, et al. Resistant starch as prebiotic: a review. *Starch - Stärke*. (2011) 63:406–15. doi: 10.1002/star.201000099

11. Sakac N, Karnaš DJ, Jozanović HM, Gvozdić V, Kovač-Andrić E, Sakač M, et al. Application of spectrophotometric fingerprint in cluster analysis for starch origin determination. *Food Technol Biotechnol*. (2020) 58:5–11. doi: 10.17113/ftb.58.01.20.6239

12. Chung H, Hoover R, Liu Q. The impact of single and dual hydrothermal modifications on the molecular structure and physicochemical properties of normal corn starch. *Int J of Biolo Macro*. (2009) 44:203–10. doi: 10.1016/j.ijbiomac.2008.12.007

13. Ozturk S, Koksel H, Perry KWN. Characterization of resistant starch samples prepared from two highAmylose maize starches through debranching and heat treatments. *Cereal Chem*. (2011) 86:503–10. doi: 10.1094/CCHEM-86-5-0503

14. Margareta Leeman A, Karlsson ME, Eliasson AC, Bjorck IME. Resistant starch formation in temperature treated potato starches varying in amylose/ amylopectin ratio. *Carbohydr Polym*. (2006) 65:306–13. doi: 10.1016/j.carbpol.2006.01.019

15. Muir JG, O'dea K, Cameron-Smith D, Brown YL, Collier GR. Resistant starch-the neglected dietary fiber? Implication for health. A review of the effects of dietary fiber and resistant starch on small and large bowel physiology. *Dietary Fibre*. (1993) 1:33–47.

16. Reader D, Johnson ML, Hollander P, Franz M. Response of resistant starch in a food bar vs. two commercially available bars in persons with type II diabetes mellitus. *Diabetes*. (1997) 46:254.

17. Cummings JH, Englyst HN. Measurement of starch fermentation in the human large intestine. *Can J Physiol Pharmacol*. (1991) 69:121–9. doi: 10.1139/y91-018

18. Brand-Miller J, Hayne S, Petocz P, Colagiuri S. Low-glycemic index diets in the management of diabetes: a meta-analysis of randomized controlled trials. *Diabetes Care*. (2003) 26:2261–7. doi: 10.2337/diacare.26.8.2261

19. Levrat MA, Moundras C, Younes H, Morand C, Demigne C, Remesy C. Effectiveness of resistant starch, compared to guar gum, in depressing plasma cholesterol and enhancing fecal steroid excretion. *Lipids*. (1996) 31:1069–75. doi: 10.1007/BF02522464

20. Higgins JA, Higbee DR, Donahoo WT, Brown IL, Bell ML, Bessesen DH. Resistant starch consumption promotes lipid oxidation. *Nutr Meta*. (2004) 1:8. doi: 10.1186/1743-7075-1-8

21. AOAC. *Official methods of analysis. 17th* ed. Washington, DC: Association of Official Analytical Chemists (2000).

22. AOAC. *Official methods of analysis. 16th* ed. Washington, DC: Association of Official Analytical Chemists (2002).

23. Sopade PA, Gidley MJ. A rapid in-vitro digestibility assay based on glucometry for. Investigating the kinetics of starch digestion. *Starch-Stärke*. (2009) 61:245–55. doi: 10.1002/star.200800102

24. Juliano BO, Perez CM, Blakeney AB, Castillo T, Kongseree N, Laignelet B, et al. International cooperative testing on the amylose content of milled Rice. *Starch Stärke*. (1981) 33:157–62. doi: 10.1002/star.19810330504

25. Goni I, Garia-Alonso A, Saura-Calixto F. A starch hydrolysis procedure to estimate glycemic index. *Nutr Res*. (1997) 17:427–37. doi: 10.1016/S0271-5317(97)00010-9

26. McLean LA, Sosulski FW, Youngs CG. Effects of nitrogen and moisture on yield and protein in field peas. *Can J Plant Sci*. (1974) 54:301–5. doi: 10.4141/cjps74-047

27. Kibar H, Sonmez F, Temel S. Effect of storage conditions on nutritional quality and color characteristics of quinoa varieties. *J Stored Prod Res*. (2021) 91:101761. doi: 10.1016/j.jspr.2020.101761

28. Zhang K, Shi Y, Zeng J, Gao H, Wang M. Effect of frozen storage temperature on the protein properties of steamed bread. Food. *Sci Technol*. (2022) 42:42. doi: 10.1590/fst.68622

29. Adegunwa MO, Adebowale AA, Solano EO. Effect of thermal processing on the biochemical composition, antinutritional factors and functional properties of beniseed flour. *Am J Biochem Mol Biol*. (2012) 2:175–82. doi: 10.3923/ajbmb.2012.175.182

30. Siregar NS, Julianti E, Silalahi J. The effect of roasting temperature on proximate and dietary fiber of food bar salak (Sidimpuan cultivar) fruit. *E3S Web Conf*. (2021) 332:03001. doi: 10.1051/e3sconf/202133203001

31. Ötles S, Ozgoz S. Health effects of dietary fiber. *Acta Sci PolonorTechnol Aliment*. (2014) 13:191–202. doi: 10.17306/J.AFS.2014.2.8

32. Everything Kate F. *You need to know about soluble Fiber* (2020). Available at: http://www.katefarms.com.

33. Cheng L, Zhang X, Hong Y, Li Z, Li C, Gu Z. Characterisation of physicochemical and functional properties of soluble dietary fibre from potato pulp obtained by enzyme-assisted extraction. *Int J Bio Macromol*. (2017) 101:1004–11. doi: 10.1016/j.ijbiomac.2017.03.156

34. Akinola OO, Raji TJ, Olawoye B. Lignocellulose, dietary fibre, inulin and their potential application in food. *Heliyon*. (2022) 29:104–59. doi: 10.1016/j.heliyon.2022.e10459

35. Caprita A, Caprita R, Simulescu VO, Drehe RM. The effect of temperature on soluble dietary fiber fraction in cereals. *J Agro aliment Processes Technol*. (2011) 17:214–7.

36. Chang MC, Morris WC. Effect of heat treatments on chemical analysis of dietary fiber. *J Food Sci*. (1990) 55:1647–50. doi: 10.1111/j.1365-2621.1990.tb03591.x

37. Ramulu O, Rao U. Effects of processing on dietary fiber content of cereals and pulses. *Plant Foods Hum Nutr*. (1997) 50:249–57. doi: 10.1007/BF02436061

38. Vasishtha Hina Srivastava RP. Dietary fiber, protein and lectin contents of lentils (*Lens culinaris*) with soaking and cooking. *Current Adv Agri Sci*. (2013) 5:238–41.

39. Caprez A, Arrigoni E, Amado R, Neucom H. Influence of different types of thermal treatment on the chemical composition and physical properties of wheat bran. *J Cereal Sci*. (1986) 4:233–9. doi: 10.1016/S0733-5210(86)80025-X

40. Nigudkar MR. Estimation of resistant starch content of selected routinely consumed indian food preparations. *Curr Res Nutr Food Sci*. (2014) 2:73–83. doi: 10.12944/CRNFSJ.2.2.03

41. Ek KL, Brand-Miller J, Copelan L. Glycemic effect of potatoes. *Food Chem*. (2012) 133:1230–40. doi: 10.1016/j.foodchem.2011.09.004

42. Kolaric L, Minarovičová L, Lauková M, Karovičováand J, Kohajdová Z. Pasta enriched with sweet potato starch: impact on quality parameters and resistant starch content. *J Text Stud*. (2020) 51:464–74. doi: 10.1111/jtxs.12489

43. Kumar and Prasad. Changes in the characteristics of indica rice in the process of flaking. *J Food Eng*. (2018) 6:2310–7.

44. Mangala SL, Vidyasankar K, Tharanathan RN. Resistant starch from processed cereals. The influence of amylopectin and non-carbohydrate constituents on its. Formation. *Food Chem*. (1999) 64:391–6. doi: 10.1016/S0308-8146(98)00142-3

45. Gopinath V, Saravanan S, Al-Maleki AR, Ramesh M, Vadivelu J. A review of natural polysaccharides for drug delivery applications: special focus on cellulose, starch and glycogen. *Biomed Pharmacother*. (2010) 107:96–108. doi: 10.1016/j.biopha.2018.07.136

46. Shewry PR, Hey SJ. The contribution of wheat to human diet and health. *Food Energy Secur*. (2015) 4:178–202. doi: 10.1002/fes3.64

47. Sharma A, Kapoor AC. Effect of processing on the nutritional quality of pearl millet. *J Food Sci Technol*. (1997) 34:50–3.

48. Rehman ZU, Habib S, Zafar SI. Nutritional changes in maize (*Zea mays*) during storage at three temperatures. *Food Chem*. (2002) 77:197–201. doi: 10.1016/S0308-8146(01)00337-5

49. Liu K, Liu Q. Enzymatic determination of total starch and degree of starch gelatinization in various products. *Food Hydrocoll*. (2020) 103:105639. doi: 10.1016/j.foodhyd.2019.105639

50. Regmi PR, Metzler-Zebeli BU, Ganzle MG, Van Kempen TATG, Zijlstra R. Starch with high amylose content and low in vitro digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes Bifidobacteria in pigs. *J Nutr*. (2011) 141:1273–80. doi: 10.3945/jn.111.140509

51. Al-Rabadi GJS, Gilbert RG, Gidley MJ. Effect of particle size on kinetics of starch digestion in milled barley and sorghum grains by porcine alpha-amylase. *J Cereal Sci*. (2009) 50:198–204. doi: 10.1016/j.jcs.2009.05.001

52. Williams MR, Bowler P. Starch gelatinization – a morphological study of Triticeae and other starches. *Starch Stärke*. (1982) 34:221–3. doi: 10.1002/star.19820340703

53. Davis EA. Wheat Starch. *Cereals Food World*. (1994) 36:34–9. doi: 10.1080/10261133.1994.9673935

54. Goddard MS, Young G, Marcus R. The effect of amylase content on insulin and glucose responses to ingested rice. *Am J Clin Nutr*. (1984) 39:388–92. doi: 10.1093/ajcn/39.3.388

55. Sagum R, Arcot J. Effect of domestic processing methods on the starch, non starch polysaccharides and in vitro starch and protein digestibility of three varieties of rice with varying levels of amylose. *Food Chem*. (2000) 70:107–11. doi: 10.1016/S0308-8146(00)00041-8

56. Singh N, Kaur A, Shevkani K. *Maize: grain structure, composition, milling, and starch characteristics nutrition dynamics and novel uses* Springer India (2014).

57. Chung H, Liu Q, Hoover R. Impact of annealing and heat-moisture treatment on rapidly digestible, slowly digestible and resistant starch levels in native and gelatinized corn, pea and lentil starches. *Carbohydr Polym*. (2011) 75:436–47. doi: 10.1016/j.carbpol.2008.08.006

58. Whistler RL, BeMiller JN. Carbohydrate chemistry for food scientists. *Starch*. (1996):117–51.

59. Yamin FF, Lee M, Pollak LM, White PJ. Thermal properties of starch in corn variants isolated after chemical mutagenesis of inbred line B73. *Cereal Chem*. (1999) 76:175–81. doi: 10.1094/CCHEM.1999.76.2.175

60. Singh J, Singh N. Studies on the morphological and rheological properties of granular cold water soluble corn and potato starches. *Food Hydrocoll*. (2003) 17:63–72. doi: 10.1016/S0268-005X(02)00036-X

61. Sasaki T, Yasui T, Matsuki J. Effect of amylose content on gelatinization, retrogradation and pasting properties of starches from waxy and non-waxy wheat and their F1 seeds. *Cereal Chem*. (2000) 77:58–63. doi: 10.1094/CCHEM.2000.77.1.58

62. Bajaj R, Singh N, Kaur A, Inouchi N. Structural, morphological, functional and digestibility properties of starches from cereals, tubers and legumes: a comparative study. *J Food Sci Technol*. (2018) 55:3799–808. doi: 10.1007/s13197-018-3342-4

63. Rizkalla SW, Bellisle F, Slama G. Health benefits of low glycemic index foods, such as pulses, in diabetic patients and health individuals. *BrJ Nutr*. (2002) 88:255–62. doi: 10.1079/BJN2002715

64. Liu Q, Zhang Y, Laskowski JS. The adsorption of polysaccharides onto mineral surfaces: an acid/base interaction. *Int J Min Process*. (2000) 60:229–45. doi: 10.1016/S0301-7516(00)00018-1

65. Raben A, Tagliabue A, Christensen N, Madsen J, Holst J, Astrup A. Resistant starch: the effect on postprandial glycemia, hormonal response, and satiety. *Am J Clin Nutr*. (1994) 60:544–51. doi: 10.1093/ajcn/60.4.544

66. Lopez HW, Levrat-Verny MA, Coudray C, Besson C, Krespine V, Messager A. Class 2 resistant starches lower plasma and liver lipids and improve mineral retention in rats. *J Nutr*. (2001) 131:1283–9. doi: 10.1093/jn/131.4.1283

67. Zhou J, Matrin RJ, Tulley RT, Raggio AM, McCutcheon KL, Shen L, et al. Dietary resistant starch upregulates total GLP-1 and PYY in a sustained day-long manner through fermentation in rodents. *Am J Physiol Endocrinol Metab*. (2008) 295:E1160–6. doi: 10.1152/ajpendo.90637.2008

68. Ou S, Kwok KC, Li Y, Fu L. In vitro study of possible role of dietary fiber in lowering postprandial serum glucose. *J Agric Food Chem*. (2011) 49:1026–9. doi: 10.1021/jf000574n

69. Wood L, Wilbourne J, Kyne-Grzebalski D. Administration of insulin by injection. *Pract Diabet Int*. (2002) 19:S1–4. doi: 10.1002/pdi.330

frontiers | Frontiers in Nutrition

TYPE Review
PUBLISHED 20 March 2024
DOI 10.3389/fnut.2024.1369950

OPEN ACCESS

EDITED BY
Xianyang Bao,
Harvard University, United States

REVIEWED BY
Hai He,
Hainan Medical University, China
Chengdeng Chi,
Fujian Normal University, China

*CORRESPONDENCE
Mickaël Durand-Dubief
✉ m.durand-dubief@lesaffre.com;
✉ mickael.duranddubief@gmail.com

†These authors have contributed equally to this work

RECEIVED 13 January 2024
ACCEPTED 05 March 2024
PUBLISHED 20 March 2024

CITATION
Baptista NT, Dessalles R, Illner A-K, Ville P, Ribet L, Anton PM and Durand-Dubief M (2024) Harnessing the power of resistant starch: a narrative review of its health impact and processing challenges.
Front. Nutr. 11:1369950.
doi: 10.3389/fnut.2024.1369950

Harnessing the power of resistant starch: a narrative review of its health impact and processing challenges

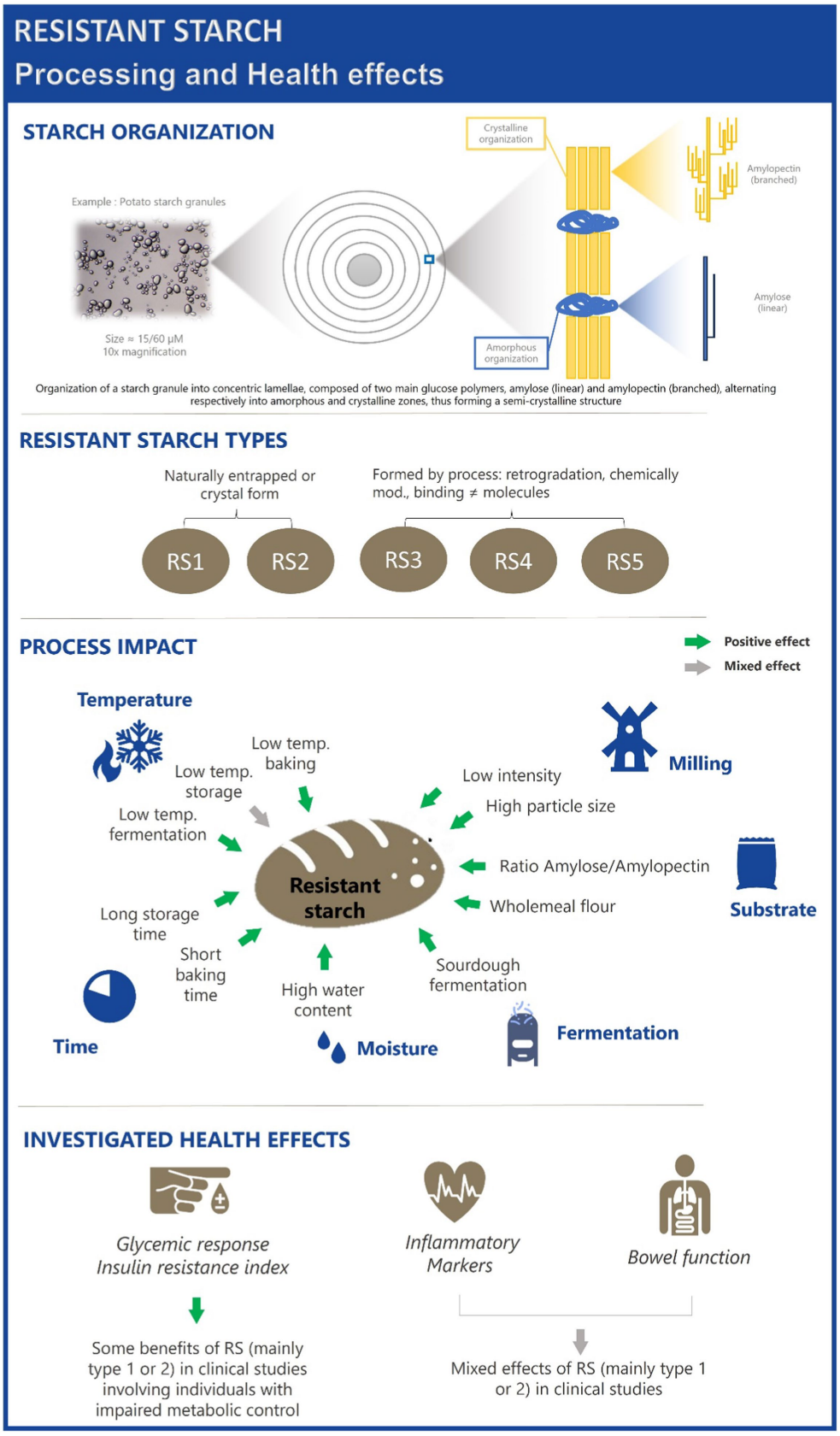
Nathália Trunckle Baptista[1†], Robin Dessalles[2†], Anne-Kathrin Illner[3], Patrice Ville[4], Léa Ribet[3], Pauline M. Anton[3] and Mickaël Durand-Dubief[5*]

[1]Baking Science and Nutrition, Lesaffre Institute of Science and Technology, Lille, France, [2]Robin Dessalles EI, Lyon, France, [3]Transformations and Agroressources, Institut Polytechnique UniLaSalle, Université d'Artois, Beauvais, France, [4]Department of Regulatory Department, University of Lesaffre International, Marcq-en-Baroeul, France, [5]Discovery and Front-End Innovation, Lesaffre Institute of Science and Technology, Lille, France

Starch is a primary energy storage for plants, making it an essential component of many plant-based foods consumed today. Resistant starch (RS) refers to those starch fractions that escape digestion in the small intestine and reach the colon where they are fermented by the microflora. RS has been repeatedly reported as having benefits on health, but ensuring that its content remains in food processing may be challenging. The present work focuses on the impact RS on health and explores the different processes that may influence its presence in foods, thus potentially interfering with these effects. Clinical evidence published from 2010 to 2023 and studying the effect of RS on health parameters in adult populations, were identified, using PUBMED/Medline and Cochrane databases. The search focused as well on observational studies related to the effect of food processes on RS content. While processes such as milling, fermentation, cooking and heating seem to have a deleterious influence on RS content, other processes, such as cooling, cooking time, storage time, or water content, may positively impact its presence. Regarding the influence on health parameters, there is a body of evidence suggesting an overall significant beneficial effect of RS, especially type 1 and 2, on several health parameters such as glycemic response, insulin resistance index, bowel function or inflammatory markers. Effects are more substantiated in individuals suffering from metabolic diseases. The effects of RS may however be exerted differently depending on the type. A better understanding of the influence of food processes on RS can guide the development of dietary intake recommendations and contribute to the development of food products rich in RS.

KEYWORDS

resistant starch, human health, food processing, nutritional properties, fibers



GRAPHICAL ABSTRACT

Introduction

Carbohydrates (CHO) are fundamental components of human nutrition, serving as the primary source of energy in most diets. Additionally, they play a significant role in human health through their influence on glucose homeostasis via their metabolism in the gastrointestinal tract. Carbohydrates are abundantly found in cereals, fruits, vegetables and legumes. Among them, starch is the most abundant digestible polysaccharide in human diets, playing a crucial role in numerous plant-based foods such as wheat, maize, rice, rye, potato or peas (1, 2). Starches are classified based on their digestion rates into three major categories: rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). RDS and SDS are both fully digested in the small intestine, though at different rates: within 20 min for the former versus 120 min for the latter (3–5). In contrast, RS mostly escapes digestion in the small intestine and is fermented in the colon by microorganisms, producing short-chain fatty acids (SCFA) (3, 4, 6). This metabolic pathway looks like that of dietary fibers, plant components, originally defined as "*portion of food which is derived from cellular walls of plants which are digested very poorly by human beings*." This led to an expansion of the definition to encompass RS along with other compounds such as resistant oligosaccharides or hydrocolloids (7). Dietary fibers have long been recognized as an important component of a healthy diet and are emphasized in dietary guidelines worldwide. For example, a daily intake of at least 25 grams per day is recommended in Europe to reduce the risk of chronic diseases (1). However, actual intake of dietary fibers in Europe varies significantly across countries, and often falls short of these recommendations. Reports indicate an average intake of 12.5 g/d in Spain, 17 g/d per day in Belgium, 20 g/d in France, Sweden, and the Netherlands, 21 g/d in Austria and Finland, with only Norway and Germany approaching the recommended 25 g/d (8). In the United States, it was estimated that only 7.4% of adults reached the recommended intake of 14 g/1000 kcal (9). In Australia, a typical diet provides 4 times less than the 15–20 g/day recommended for supporting bowel health (10). More limited data are available regarding RS consumption. Global intake is estimated to be between 3 and 10 g per day. In Europe, intake ranges from approximately 3 to 6 g/d, while in China, it is about 15 g/d, mainly from wheat and rice products. In Africa, intake can be as high as 20–30 g/d (11).

Apart from its contribution to fiber intake, increasing the RS content in foods may be an opportunity to promote health since starch microstructure manipulation may modulate health parameters such as those related to metabolic response. However, the influence of food processing on RS content appears as a key determinant in this respect.

In the present work, we aim to describe the influence of various food processes on RS content, and to review clinical evidence related to the effects of RS on health, in order to highlight the barriers and knowledge gaps that remains to be addressed before developing public health strategies.

Methods

To compile this narrative review, a comprehensive literature search was conducted on 3 scientific databases: Medline, COCHRANE, and The Lens. The focus was on articles examining the health benefits of RS and the dietary processes that may influence its content in foods were searched. The keyword "resistant starch" was combined with others, related to health conditions, as follows: "resistant starch" AND ("glycemic response" OR "glycaemic response" OR diabetes OR diabetics OR metabolic OR overweight OR obese OR glucose OR insulin OR lipids OR inflammation OR microbiota OR microflora OR gastrointestinal OR oxidative OR antioxidant). Searches with keywords related to food processes were performed in parallel: "resistant starch" AND (cooking OR cooling OR milling OR storage OR heating OR breeding OR microwave). The retrieved records were screened by reviewers, and studies were selected based on title, abstract and keywords, according to the following eligibility criteria: peer-review publications in English, published in the last 20 years, either randomized controlled trials, systematic reviews, meta-analyses or observational trials, conducted in the general adult population. Studies conducted on tertiary-care patients, such as those suffering from cancer or undergoing hemodialysis were excluded. Cross-reference searches were performed during the selection and reviewing processes. Following selection by the authors, full-text articles were retrieved and analyzed.

Types of resistant starch and their properties

RS is usually listed in five categories (see Table 1). Resistant starch type 1 (RS1) is physically entrapped, non-accessible, in a non-digestible matrix such as whole grains (intact cells) due to wall barrier. This starch is heat-stable and does not break during cooking, but it does during milling. It is commonly found in products such as whole bread, seeds or legumes. Resistant starch type 2 (RS2), is a native granular RS found in raw potatoes or green bananas. Its crystalline organization protects it from digestive enzymes. RS2 has been widely investigated in clinical trials, notably through the use of HI-MAIZE® 260 (Ingredion United Kingdom, Ltd) as an additive to the test products. Resistant starch type 3 (RS3) is also known "retrograded starch" which results from the formation of double helixes by long-branch chains of amylopectin following the cooling of foods cooked in the presence of moisture. It cannot be hydrolysed by digestive enzymes. Resistant starch type 4 (RS4) is a chemically-modified starch engineered to resist to enzymatic digestion. As well as RS2, it can be added to foods as an ingredient. Products containing RS4 might be derived from sources such as potato, high-amylose maize, or tapioca starches (VERSAFIBE™ 1,490, VERSAFIBE™ 2,470 or NOVELOSE®3,490,

Abbreviations: BMI, Body Mass Index; CHO, Carbohydrates; CRP, CI, C-reactive protein confidence intervals; HS, CRP, High sensitivity C-reactive protein; EFSA, European Food Safety Agency; FPG, Fasting Plasma Glucose; GIP, Glucose-dependent insulinotropic polypeptide; GLP-1, Glucagon-Like Peptide-1; GT, gelatinization temperature; HbA1c, Glycated hemoglobin; HDL-C, High-Density Lipoprotein Cholesterol; HMT, heat-moisture treatment; IL-6, Interleukin 6; LDL-C, Low-density lipoprotein cholesterol; NDA, EFSA Panel on Dietetic Products, Nutrition, and Allergies; RCT, Randomized controlled trial; RMD, Resistant maltodextrin; RS, Resistant starch; RDS, Rapidly digestible starch; SCFA, Short-chain fatty acids; SDS, slowly digestible starch; SMD, Standardized mean difference; TAC, Total antioxidant capacity; TNF-α, Tumor necrosis Factor; T2DM, type-2 diabetes mellitus.

TABLE 1 Characteristics of resistant starch fractions (5, 12, 13).

Type of RS	Description	Food sources	Digestion in small intestine
RS 1	Non-accessible, physically entrapped	Whole or partly milled grains, seeds or legumes	Slow rate; partial degree; totally digested if properly milled
RS 2	Ungelatinized resistant granules	Raw potatoes, green bananas, some legumes, high-amylose corn, specific ingredients (e.g., HI-MAIZE® 260)	Very slow rate; little degree; totally digested when freshly cooked
RS 3	Retrograded starch	Cooked and cooled potatoes, bread, cornflakes, food products with repeated moist heat treatment	Slow rate; partial degree; reversible digestion; digestibility improved by reheating
RS 4	Chemically modified starches due to cross-linking with chemical reagents	Foods in which modified starches have been used (e.g., breads, cakes). Example of ingredients: VERSAFIBE™ 1,490, VERSAFIBE™ 2,470, NOVELOSE®3,490	Result of chemical modification; can resist hydrolysis
RS 5	Amylose-lipid complexes	Foods with high amylose content	Can resist enzymatic digestion

respectively) (6). Initially, a fifth class of resistant starch (RS5) was established to characterize cases where RS is modified and form starch-lipid complexes with resistant properties. However, recent evidence has described the generation of starch complexes that involve other molecules, such as amino acids, peptides, polysaccharides or polyphenols, and have a similar structure to starch-lipid complexes. Therefore, an update of the "RS5" category has been suggested to include these new complexes as well (14, 15).

Effects of food processing on resistant starch content

The preservation of starch's crystalline structure is crucial for maintaining low digestibility to maximize their nutritional properties (low glycaemia index, satietogenic properties ….). Some foods, such as unriped bananas and uncooked oats, are naturally rich in RS. However, various factors may alter this content in one direction or another (16). These factors may be encountered at an early stage (e.g., arising from varietal selection), or later as a result of the physical treatments occurring in various steps of food processing, from milling to food storage (17).

Varietal differences and breeding techniques

Developing crops with a modified amylose-to-amylopectin ratio can enhance RS levels in foods. Higher amylose content promotes the retrogradation after cooking, thus regenerating RS. This is because amylose has a smaller and more flexible structure due to its linearity, making it easier to regenerate compared to amylopectin (17–20). Several studies have demonstrated that using a wheat cultivar with elevated amylose content significantly increases the final RS content in bread, compared to a conventional cultivar (18, 21–23). This elevated content seems to translate into health benefits, as the production of high-amylose rice (24), noodles (25), or wheat (26) positively impacted postprandial glycemia in several randomized controlled trials. However, it is worth noting that, using specific high-amylose cultivars may negatively impact the characteristics of dough and bread (27).

Milling

Milling disrupts the crystalline structure of starch, increasing its exposure to enzymatic degradation and leading to a significant loss of RS. Compared to whole grains, cereal flours, especially wheat, are typically lower in RS (12, 28, 29). Coarse milling or selecting larger particles after fractionation could attenuate this loss (19), though other production steps also significantly affect RS content (18). Additionally, whole grain products are more likely to contain RS1, as starch is encapsulated within a plant structure (28).

Cooking/heating

Cooking has a major influence on both starch organization and its level in foods. At or above a defined temperature called the "gelatinization temperature" (GT), and in the presence of sufficient water, the crystalline structure of the starch granule is disrupted, making starch resistant (both RS1 and RS2) more digestible. However, during cooling, starch polymers (mostly amylose), tend to reassociate, forming packed structures which remain unavailable for enzymatic hydrolysis. This process is called retrogradation (29, 30).

This new fraction, retrograded starch (RS3), is the main form of RS in processed foods (22) and the major contributor to RS intake (13).

Microwave cooking, through dielectric heating and electromagnetic effects, generally leads to retrogradation (31). Microwave reheating of rice, regardless of water content, increase RS while reducing digestible starch fractions (32, 33). This effect seems to extend to other foods such as potatoes (34). Cooking wheat noodles in a microwave was found more effective than boiling or steaming in preserving RS and lowering glycemic index (35). In a meta-analysis of 31 articles investigating the effect of microwave treatment on starch content of high-carbohydrate foods, Isra et al. highlighted that this method of cooking significantly increased the level of RS, whatever the food matrix [2.755% (95% CI: 2.106 to 3.403); $p < 0.001$]. Moreover, RS enrichment resulted in enhanced prebiotic properties, based on several parameters, namely starch composition, amylose interaction, lactic acid bacteria viability, and enteropathogenic *Escherichia coli* viability (36).

Control over starch-to-moisture ratio, temperature, and heating time can significantly alter the resulting starch levels (37). For example, heat-moisture treatment (HMT) and annealing are two hydrothermal treatments commonly used to alter starch properties (38). HMT consists in heating the starch granule at high temperature (ranging from 84 to 140°C) while maintaining low moisture (10 to 35%) for specific period of time, in order to prevent gelatinization. Studies assessing the effects of different HMT treatments on starch characteristics have been extensively reviewed (38). Recently, progressive increase in temperature during HMT has been shown to reduce the digestibility of sweet potato starch by

decreasing RDS and increasing RS fractions, with optimum conditions at 110°C, 25% moisture for 4 h (39). Similar results were observed in rice starch (40, 41), and starch from other foods under various conditions (38). Subjecting barley to HMT has been reported to improve glycemic response and to promote the growth of SCFA-producing bacteria in rats (42). Overall, the ability to modify starch swelling capacity, crystallinity, gelatinization or retrogradation, digestibility through HMT gives an indication of its potential to impact metabolic response to foods and thus, its benefits on health.

Modifications of RS crystallinity and organization may also be obtained through the treatment of starch with excess water and limited temperature, using a process called annealing. Typical treatment conditions are around 50°C temperature for 24 h or more (38). However, the ability of annealing to modify the characteristics of starch seems dependent on the botanical source: Zheng et al. recently evidenced increased crystallinity of maize and potato starch with increasing time of annealing treatment, while the effect on pea starch was limited. An increase in RS together with a decrease in RDS were noted after potato starch treatment, while no such changes were observed in the case of maize or pea starch (43). In another study, a decreased in proso millet starch digestibility was observed after annealing without modifications of the crystallinity (44). Annealing was found to raise RS content of cornstarch by almost 9-fold, and was shown to be more effective in this respect than HMT, autoclaving or microwave treatments (45). Therefore, the use of annealing to enhance starch functional properties needs cautious selection of the appropriate conditions and substrate.

Autoclaving (i.e., high temperature, high-pressure treatment) is also known to induce RS3 formation, especially when coupled to cooling (4). Based on a meta-analysis of 10 studies, Faridah et al. highlighted that the effect of autoclaving-cooling treatment was dependent on the food source, the water ratio, as well as treatment time and temperature. Thus, using corn, oat or rice as food source, having a starch-to-water ratio of 1:4 and performing two cycles of autoclaving-cooling for 30 min of autoclaving at 121°C were the conditions allowing maximization of the RS content (46).

Long and low-temperature bread baking (e.g., pumpernickel conditions: 20 h, 120°C), significantly increases RS content compared to standard baking (45 min at 200°C) (18, 21–23). For instance, white wheat bread baked at 120°C for 4 h, and another at 150°C for 3 h, showed, respectively, a 24% (1.46 g/100 g) and 15% (1.36 g/100 g) increase in RS content, compared to the same bread baked for 30 min at 200°C (1.18 g/100 g) (30).

Cooling/storage

Storage conditions, particularly cooling after cooking, play key role for the RS content in foods. Cooling after cooking is a crucial step during which starch retrogrades, partially restoring its crystallinity (17, 28). In a study, freshly baked bread was stored at ambient (20°C), frozen (− 17°C) or refrigerated (3.5°C) temperatures for 7 days; authors reported that the RS content was significantly increased in the refrigerated bread compared to the other storage conditions (47). Cooling white rice for 24 h at 4°C before reheating resulted in a higher RS content of 1.65 g/100 g compared to the rice cooled at ambient temperature for 10 h. In addition, the glycemic response was significantly reduced compared to freshly cooked white rice in healthy individuals (48). Similar preparation conditions also showed a significant reduction of postprandial glycemia in type 1 diabetics compared to rice served immediately after cooking (49). Another study noted that the impact of refrigeration on RS content differs depending on the rice variety. Maximization of RS content was attained when long-grain rice was prepared using a rice cooker and then refrigerated for 3 days at 4°C (2.55 g RS per 100 g). In contrast, short-grain rice prepared in a pressure cooker and similarly refrigerated showed the lowest RS dose among the tested combinations (0.20 g RS/100 g rice) (50). The influence of storage time and temperature on RS content appears to be food-specific: RS content in noodles was maximized when microwave-heated and stored for 48 h at room temperature (51).

One study highlighted a steady increase in RS content in sourdough teff breads over 5 days of storage, with retrogradation was evidenced by a concomitant decrease of RDS (52). The effect of storage time on RS content, regardless of temperature, has been observed in wheat bread as well (30). Interestingly, the rate of starch recrystallization during storage varies among cereals. For instance, rye sourdough bread exhibits a slower crystallization rate than wheat bread (53).

Fermentation conditions

Several studies reported an effect of fermentation conditions on the RS content of foods.

One study found that sourdough bread, regardless of flour type (whole or white wheat), had a significantly higher RS content compared to yeast bread. Furthermore, at the same fermentation temperature, using type-2 sourdough fermentation using indigenous strains (*Lactobacillus brevis* ELB99, *Lactiplantibacillus plantarum* ELB75, and *Saccharomyces cerevisiae* TGM55) yielded higher RS content in bread compared to type-1 (spontaneous) fermentation. In white wheat bread, lower fermentation temperature (25°C vs. 30°C) also increased the RS content of bread, whatever the fermentation type. In whole wheat bread, this was the case only for type-1 fermentation (54). In contrast, another study did not find any significant impact of sourdough addition on the RS in white flour (30). In the case of Teff bread, Shumoy et al. reported that the RS content increased with the proportion of incorporated sourdough (52).

In experiments involving barley malt production, Teixeira et al. highlighted that the increase in RS was correlated to the barley variety used, particularly its amylose content. Specifically, steeping with 0.4% lactic acid in Tipple, regardless of the temperature used, was shown to enhance RS content (55).

Impact of resistant starch on health based on a clinical perspective

The effects of RS on health have been widely investigated in the past decades. For this review, a total of 14 meta-analyses compiling data from these studies were retrieved (56–70). These meta-analyses vary in their focus on different types of RS and target populations. The following sections will review and summarize the impact of RS on different health parameters. Characteristics of the meta-analyses are detailed in Table 2.

TABLE 2 Characteristics of included meta-analyses.

Studies characteristics												Results
Reference	Nb of studies	Type	Design	Total nb of subjects	Target population	Intervention	Control	RS dose range (g/d)	Study duration range (weeks)	RS type	Outcomes	
(59)	14	RCT	// or CO	515	T2DM or obesity w/o T2DM	RS supplementation	non-RS supplementation	4–6	4–52	Any	fasting insulin, fasting glucose, BMI and HOMA-IR;	↓ FPG in T2DM + OB; ↓ FPI in T2DM + OB; = FPI in T2DM; = BMI in T2DM; ↓ HOMA-IR in T2DM + OB; = HOMA-IR in T2DM
(61)	19	RCT	// or CO	1,014	Any	RS supp or intervention	intake of digestible starches or other CHOs	8–34	3–48	Any (mostly RS2)	glycemic status, serum lipoproteins and inflammatory markers	↓ FPG; ↓ FPI; = HOMA-IR; ↓ HbA1c; ↓TC; ↓ LDL; = HDL; = TAG; = IL-6; ↓ TNF-α; = CRP
(64)	31	RCT	Any	982	T2DM or prediabetes; no other chronic conditions	Resistant starch type 1–5, starch-containing foods	Starch ot other carbohydrates	1–45	0–52	Any	Markers of glycemia	↓ PPG; = PPI; ↓ FPG; ↓ FPI
(66)	22	RCT	Any	670	Healthy or MetS or T2DM	Resistant starch type 2	Any	8–66	1–12	RS2 (mostly high amylose maize starch)	fasting blood glucose, glycated hemoglobin (HbA1c), insulin resistance, appetite/ satiety levels, lipid levels or body weight	= FPG, ↓ body weight; = HOMA-IR; = HbA1c; = TC; = LDL; = HDL; = TAG
(70)	15	RCT	Any	503	Any	resistant starch supplementation (excluding type 1)	Any	5,1–66	2–12	Any (mostly RS2)	blood glucose or insulin	↓ FPG; = FPI; = HbA1c;
(68)	13	RCT	Any	428	Overweight / Obesity	RS	Any	10–45	2–12	Any	fasting glucose or fasting insulin or plasma lipid or insulin sensitivity or insulin resistance	↓ FPG; ↓ FPI; = HOMA-IR; ↓ HbA1c; = TC; ↓ LDL; = HDL; = TAG
(71)	14	RCT	Any	820	Adults, healthy or not,no lipid-lowering medication	RS	placebo without the functions of DF or diets low in resistant starch	10–66	2–52	Any (mostly RS2)	TC, LDL-C, TGs, HDL	↓ TC; ↓ LDL; = HDL; =TAG

(Continued)

10.3389/fnut.2024.1369950

TABLE 2 (Continued)

Studies characteristics												Results
Reference	Nb of studies	Type	Design	Total nb of subjects	Target population	Intervention	Control	RS dose range (g/d)	Study duration range (weeks)	RS type	Outcomes	
(60)	8	RCT	Any	308	Any but inflammatory diseases	RS2	Placebo	10–45	4–12	RS2 (Hi-maize 260)	IL-6, hs-CRP, TNF-a as outcomes	= IL-6; = TNF-α; = CRP
(63)	16	RCT	Any	706	Any	RS alone	Any	6–27	4–12	RS2	Inflammatory or oxidative stress biomarkers	↓ IL-6 in non-T2DM; ↓ TNF-α in non-T2DM; ↓ CRP in T2DM; = uric acid; ↑ TAC; ↑ SOD; ↓ MDA
(67)	13	RCT	Any	672	Adults, healthy or not	Resistant Starch ("resistant maltodextrin," "resistant dextrin," "indigestible starch," "high amylose starch")	Any but RS	10–45	4–14	Any (mostly RS2)	IL-6, CRP, hs-CRP and TNF-α	↓ IL-6; ↓ TNF-α; = CRP
(69)	16	RCT	Any	739	Adults	RS	Any	Any	2–12	Any	IL-6, CRP, hs-CRP and TNF-α	↓ IL-6; ↓ TNF-α; = CRP; ↑ TAC; = SOD; = MDA
(65)	9	RCT	Any	193	Healthy adults	RS	Any	22–45 (avg 33)	1–4	RS2, RS3 and RS4	butyrate concentration, defecate frequency, fecal wet weight, fecal PH	↑ stool volume; = stool frequency; ↑ butyrate levels; ↓ fecal pH
(57)	7	Any	Any	248	Any	RS	Baseline	Any	NA	Any	microbial diversity, bacterial counts	↓ gut microbial α-diversity; ↑ counts of *Ruminococcus, Agathobacter, Faecalibacterium and Bifidobacterium*
(72)	29	RCT	SB or DB	1,208	Healthy adults	RMD	Any w/o RMD	3,8-13,5	1–3	RMD	stool frequency and stool volume	↑ stool volume; ↑ stool frequency
(73)	20	RCT	Any		Colorectal cancer, familial adenomatous polyposis (FAP), Lynch syndrome (LS), sporadic adenoma (SA) and healthy subjects	RS/inulin with or without other drugs	Digestible carbohydrate or low-dose NDC	10–60	4–116	Any	SCFA production	= SCFAs; = butyrate

BMI, body mass index; CRP, c-reactive protein; FPG, fasting plasma glucose; FPI, fasting plasma insulin; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide 1; HbA1c, glycated hemoglobin; HDL, high-density lipoproteins cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; IL-6, interleukin 6; LDL, low-density lipoprotein cholesterol; MDA, malondialdehyde; PPG, postprandial glucose; PPI, postprandial insulin; SOD, superoxide dismutase; TAC, total antioxidant capacity; TAG, triacylglycerol; TC, total cholesterol; TNF-α, tumor necrosis factor alpha; ↓: significantly decreased vs. control; ↑ significantly increased vs control.

10.3389/fnut.2024.1369950

Resistant starch and gastrointestinal outcomes (stools characteristics, microbiota and short-chain fatty acids)

RS has been reported to impact gastrointestinal outcomes, from microbiota population counts and activity, to stools characteristics.

A meta-analysis included 9 randomized controlled trials administering RS at doses varying from 22 g/day to 45 g/day (average 33 g/day) to healthy subjects. Control groups typically followed low-RS diets. The pooled analysis revealed significant improvements in fecal weight, butyrate levels, and fecal pH following RS supplementation, compared to the control. Conversely, defecation frequency was not significantly changed (65).

In another meta-analysis (72) a significant increase in stool volume and frequency compared to the control was found based on a pooled analysis of 29 randomized controlled trials administering resistant maltodextrin (RMD) from 3.8 to 13.5 g daily to healthy individuals.

Regarding studies focusing on bacterial counts in human gut microbiota, pooled data from 7 studies involving 248 individuals revealed an association between RS consumption and an increased abundance of *Ruminococcus*, *Agathobacter*, *Faecalibacterium* and *Bifidobacterium*. RS appeared to impact mechanisms related to carbohydrate and lipid metabolism. Notably, different RS types were found to alter differently microbiome responses (57).

Focusing on gut microbiota activity, one meta-analysis reported no significant changes in total SCFAs or butyrate concentration following RS intervention, based on data from 4 and 3 studies, respectively. Similarly, total SCFAs and butyrate excretion were unchanged in the intervention group (RS2 or inulin) compared to control (placebo or digestible CHO), based on data from 5 and 3 studies, respectively. The same study also assessed the potential of non-digestible fibers in reducing the risk of colorectal cancer, but the evidence reviewed did not support this hypothesis (73).

Resistant starch and metabolic response (glucose, insulin)

RS may dampen glycemic and insulin responses by delaying the absorption of food boluses. This topic has been the subject of extensive research in recent years, as evidenced by 7 meta-analyses published between 2018 and 2023.

Most recently, RS was found to significantly reduce postprandial glucose following both acute [−0.65 (95% CI: −0.98, −0.32); $p < 0.0001$; 14 studies] and chronic [−0.31 (95% CI: −0.50, −0.13), $p = 0.001$; 7 studies] intake. Chronic intake of RS also led to a significant reduction in fasting blood glucose [−0.31 (95% CI: −0.51, −0.11); $p = 0.002$; 14 studies], with doses used in studies ranging from 6 to 40 g/d. Interestingly, both RS1 and RS2, but not RS3 achieved this reduction. For fasting blood glucose, significant results were observed only with chronic intake of RS2. Focusing on the target population, the authors noted that the acute postprandial glycemic response was significantly reduced in both prediabetics and type-2 diabetics, but the chronic response was effective only in diabetics subjects. Other metabolic markers such as glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), and insulin sensitivity (HOMA-IR) remained unaffected by RS (64).

In another paper (70), results from a pooled analysis showed significant improvements in fasting plasma glucose (FPG) [−0.09 (95% CI −0.13, −0.04) mmol/l; $p = 0.001$; 16 trials] and insulin resistance (HOMA-IR) [−0.33 (95% CI −0.51, −0.14); $p = 0.001$; 3 trials], in 503 individuals, healthy or not, receiving RS (except type 1) compared to unsupplemented control food. However, no significant effects of RS were observed on other parameters such as fasting plasma insulin, glycated hemoglobin (HbA1c) or insulin sensitivity; of note, high heterogeneity between studies was observed for these outcomes. Higher doses of RS (≥ 26 g/d) compared to a lower dose (< 26 g/d) appeared more effective on FPG, as well as longer intervention duration (≥ 8 weeks vs. < 8 weeks). The effect was more pronounced in overweight subjects or those at risk of having diabetes, compared to healthy or diabetic subjects.

Aggregating data from 19 randomized controlled trials involving 1,014 individuals at risk of metabolic diseases (e.g., type 2 diabetics, prediabetics, overweight or dyslipidemic subjects) resulted in significant reductions in fasting plasma glucose [−4.28 (95% CI: −7.01, −1.55); $p = 0.000$; 14 studies], insulin [−1.95 (95% CI: −3.22, −0.68); $p = 0.000$; 12 studies] and HbA1c [−0.60 (95% CI: −0.95, −0.24); $p = 0.000$; 8 studies] due to RS interventions compared to digestible starches, other carbohydrates or other fibers. However, insulin resistance (HOMA-IR) was not significantly impacted (10 studies). Fasting plasma glucose or insulin resistance were significantly impacted in subjects with metabolic or renal diseases but not in at-risk subjects. Of note, using maize as a RS source appeared to not significantly affect FPG (61).

Gao et al. included 14 articles with a total of 515 subjects in their meta-analysis. Individuals presented obesity (6 studies) or had type-2 diabetes mellitus (T2DM) (6 studies) or without (2 studies) obesity. The studies included all forms of RS, and control typically consuming unfermented digestible carbohydrates. No significant effects of RS supplementation on either body mass index (BMI) or fasting plasma glucose were found after pooling 8 and 11 studies, respectively. However, a significant reduction was noted in patients with T2DM and obesity [−0.19 (95% CI: −0.29, −0.10); $p < 0.0001$; 5 studies]. Fasting plasma insulin was significantly lower in the RS supplementation group compared to the controls [−2.07 (95% CI: −3.25, −0.89); $p < 0.0006$; 8 studies], albeit with high heterogeneity ($I^2 = 84\%$). A more significant reduction was observed in the subgroup receiving 10 g/d of RS versus 30–40 g/d. Insulin sensitivity (HOMA-IR) was significantly reduced versus control only in the subgroup with T2DM [−0.71 (95% CI: −1.23, −0.20); $p < 0.007$; 4 studies], and that of T2DM with obesity [−0.91 (95% CI: −1.36, −0.45); $p < 0.0001$; 8 studies] (59).

In another meta-analysis, the investigators focused on 20 studies (670 participants) administering RS from high amylose maize starch (type 2) at doses ≥8 g per day (66). Participants included healthy individuals, as well as those overweight/obese, or with metabolic syndrome or type-2 diabetes mellitus. No significant effect of RS against placebo was reported on fasting plasma glucose, based on 15 studies. Subgroup analyses by health status did not reveal any significant changes. Similarly, HbA1c and HOMA-IR appeared unaffected by RS compared to placebo. However, body weight was significantly reduced compared to control [−1.19 (95% CI: −2.27, −0.12); $p < 0.03$; 6 studies], with subgroup analysis indicating this effect was heavily influenced by studies focusing on type-2 diabetics.

Finally, Wang et al. reported that RS administered at doses ranging from 10 to 45 g per day to 428 overweight or obese participants from 13 randomized controlled trials significantly reduced fasting plasma glucose [−0.26 (95% CI: −0.5, −0.02); $p = 0.035$; 12 trials], insulin [−0.72 (95% CI: −1.13 to −0.31); $p = 0.001$; 10 trials], and glycated

hemoglobin [−0.43 (95% CI: −0.74, −0.13); $p = 0.005$; 4 trials] compared to control, in both diabetic and non-diabetic subjects. However, no significant changes were reported for HOMA-IR (68).

Resistant starch and lipoproteins response

A meta-analysis specifically examined the effects of RS on blood lipids through randomized controlled trials. This pooled analysis included 14 studies with a total of 820 participants, both healthy or others, who were administered RS (mostly of type 2) at a dose ranging from 10 to 66 g per day. The authors reported significant decreases serum total cholesterol [−7.33 mg/dL (95% CI: −12.15, −2.52 mg/dL); 19 trials] and Low-Density Lipoprotein Cholesterol (LDL-C) [−3.40 mg/dL (95% CI: −6.74, −0.07 mg/dL); 16 trials] compared to control. Serum triglycerides and High-Density Lipoprotein Cholesterol (HDL-C), however, remained unaffected. The authors noted that longer RS supplementation periods (>4 weeks) had a more significant impact on total cholesterol and LDL-C levels. A higher dose (>20 g/d) of RS also appeared to lower triglyceride levels (71).

Other meta-analyses, initially focusing on glycemic response, also reported findings on lipoproteins. One such meta-analysis reported that RS significantly reduced serum total cholesterol [−8.19 (95% CI: −15.38, −1.00); 13 trials] and LDL-C [−8.57 (95% CI: −13.48, −3.66); 10 trials] while HDL-cholesterol and triglycerides remained unaffected compared to control (61). Snelson and colleagues, however, did not observe a significant effect of RS on blood lipoproteins (66), while another meta-analysis reported a significant reduction LDL-C levels [−0.35 (95% CI: −0.61 to −0.09; $p = 0.008$; 6 trials)] (68).

Resistant starch and inflammatory response

A total of 3 papers reporting the impact of RS on inflammatory mediators were retrieved by the present review.

One pooled result from 16 trials, primarily using RS2 (13 out of the 16 studies). The RS dose ranged from 6 g/d to 27 g/d, with controls being digestible cornstarch, manioc or maltodextrin. Subjects included those with type-2 diabetes, end-stage renal disease, chronic kidney disease, or at risk of having diabetes, along with two trials involving healthy participants. Significant improvements were observed in total antioxidant capacity [2.64 (95% CI: 0.34, 4.94); $p = 0.03$; 3 trials], and blood malondialdehyde [−0.55 (95% CI: −0.94, −0.17); $p = 0.01$;6 trials] in the intervention groups vs. control. Regarding inflammatory biomarkers, a significant reduction in blood C-reactive protein was observed in individuals with T2DM [−0.35 (95% CI: −0.65, −0.05); $p = 0.02$; 3 trials], but not in other subjects. Similarly, interleukin-6 [−0.90 (95% CI: −1.36, −0.45); $p < 0.01$; 3 trials] and tumor necrosis factor alpha (TNF-α) [−0.55 (95% CI: −1.02, −0.09); $p = 0.02$; 4 trials] levels were significantly reduced compared to control. Others parameters were not significantly altered between intervention and control groups (63).

Haghighatdoost et al. performed a meta-analysis of 8 randomized controlled trials (RCTs) including a total of 308 individuals. Four studies included individuals with renal disease, 3 with diabetes, prediabetes, or diabetes risk factors, and one study focused on overweight or obese individuals. Included studies investigated RS2, more specifically high amylose maize RS ("Hi-maize® 260"). Control food was mostly waxy corn starch, manioc or regular wheat flour. No significant effect of RS2 on hs-CRP or IL-6 could be observed based on a meta-analysis of 7 and 4 studies, respectively. Finally, a small but significant decrease in TNF-α levels following RS consumption was noted compared to control, [−0.003 pg./mL (95% CI: −0.004, −0.001); $p < 0.0001$; 4 trials], though with significant heterogeneity ($I^2 = 98.0\%$). The authors noted that the effect of RS on CRP was significantly more pronounced when considering studies using an intervention dose above 20 g/d compared to less than 20 g/d. Similar observations were made for overweight compared to normal-weight individuals and for studies with an intervention period of 6 weeks or more, compared to a shorter period. Overall, the authors concluded that RS failed to significantly reduce inflammatory mediators (60).

According to another meta-analysis, only TNF-α was significantly reduced following RS administration compared to control [−2.02 (95% CI: −3.14, −0.90); 5 trials] (61).

Pooling results from a total of 13 randomized controlled trial involving 672 subjects overall, Vahdat et al. evidenced that RS administered to individuals at doses ranging from 10 to 45 g per day did reduce significantly inflammatory biomarkers such as IL-6 [−1.11 (95% CI: −1.72, −0.50); 7 trials] or TNF-α [−2.19 (95% CI: −3.49, −0.90); 8 trials], though not CRP, whatever the subjects' conditions (healthy, healthy overweight, type-2 diabetics, hemodialytics) (67).

Finally, it was reported in a pooled analysis of 16 randomized controlled trials, including a total of 739 individuals, that RS intake decreased significantly TNF-α [−0.711 (95% CI: −1.227, −0.194); $p = 0.007$] and IL-6 [SMD: −0.609 (95% CI: −0.924, −0.294); $p < 0.001$]. A significant increase in total antioxidant capacity (TAC) was also reported in the RS intervention group versus control [2.543 (95% CI: 0.069: 5.017); $p = 0.044$)]. No significant changes on other parameters were noted, except for CRP levels that were significantly reduced, though only in the subgroup of diseased subjects (69).

Discussion

The available evidence, as summarized in this narrative review, suggests that RS may have a positive impact on health parameters related to glycemic control in individuals with impaired metabolic control. However, consensus has not been firmly established for the general population or for other outcomes such as inflammatory response or variations in lipoproteins. Notably, the meta-analyses included in this review mostly considered RS as a whole, and did not allow comparisons of RS types. Nonetheless, Pugh et al., in their subgroup analyses, highlighted that significant effects on glycemic response were primarily observed with RS1 and RS2 but not with RS3. This observation can be attributed to the fact that RS1 and RS2 received more extensive research attention across the scientific literature, whereas the analysis of RS3 was based on only two studies (64). A limited number of clinical trials also focused on RS4. For instance, in a randomized controlled trial involving 38 healthy adults, Gourineni et al. reported that a RS4 nutritional bar significantly decreased postprandial glucose compared with a control bar (74). Similar conclusions were drawn in another study comparing a RS4 muffin with a control muffin in 28 healthy individuals (75), while mixed results were reported in another study (76). Additionally, increased short-term satiety was reported after consumption of a RS4 scone compared to a standard scone (77). Interestingly, Cai et al. highlighted that factors such as a high amylose content, less-gelatinized starch, the presence of retrograded starch, and the maintenance of a larger particle size were significant determinants of an attenuated glycemic and insulinemic response in healthy individuals (78).

Overall, the benefits of the different types of RS are likely exerted through different pathways (64), as demonstrated by the varying microbiota responses to different types of RS. For instance, after 12 weeks of consuming 12 g of RS4 daily, a significant decrease in bile acid in stools were reported compared to control (79). In an *in vitro* study, both RS2 and RS4 in rice sticks led to significantly higher counts of *Bifidobacterium* and *Lactobacillus* compared to the control food under pH-controlled batch culture conditions. While the bifidogenic effect of RS2 seemed stronger with RS2, *Lactobacillus* counts were maintained longer with RS4. Of note, selective suppression of *Clostridium* was shown with RS4, while RS2 seemed to target *Bacteroides*. Moreover, both RS2 and RS4 significantly increased production of total SCFA and butyric acid compared to the control, though the concentration was highest with RS2 after 24 h fermentation (80). Another study by Li et al. also investigated the human microbiota response to RS2 (Hi Maize® 260), RS3 (Novelose® 330) and RS4 (Fibersym® RW) through *in vitro* culture and metaproteomic, and reported that responses seemed highly dependent on the individual microbiome characteristics. Nonetheless, a shift in microbiome response was highlighted following RS2 and RS3 culture. Both types had similar abilities to increase butyrate-producing bacteria, possibly because both products originated from high-amylose maize, according to the authors. RS3 significantly increased protein production of *Bifidobacteriaceae* and *Ruminococcaceae* (81). It is worth noting that this significant inter-individual variation in microbiota response to RS has already been reported (82). Among the different types of RS, RS5 has evolved and is still under investigation. Complexation of RS with lipids has the potential to present high resistance to enzymatic hydrolysis, and more stability than RS3 (83). RS5 has also potential as a functional ingredient as replacement of fat in white pan bread was shown to reduce bread energy value and delay retrogradation while maintaining acceptable bread characteristics (40). Accordingly, rice starch-lipid complex has been shown to improve body weight, dyslipidemia and SCFAs production in obese rats (84), and to dampen glycemic response in mice (85). Furthermore, the recent developments related to the classification of RS5, and the extension of possible complexes with other components than lipids raises the potential of RS5 has a health promoting ingredient. Owing to their V-type crystalline structure, most RS5 complexes presents high resistance to digestion, though to various extents depending on the non-starch component (14). Wheat starch complexation with gluten or with pea protein hydrolysates was reported to hinder α-amylase activity (86, 87). A complex of rice starch with xanthan gum and locust bean gum undergoing HMT was shown to significantly raise RS content (88). Research continues on the identification of starch complex components that would present the best characteristics to constitute a health-promoting ingredient.

Overall, the complexity of establishing whether RS confers health benefits arise from both the variability in inter-individual response and the different metabolic pathways affected by the different RS types. Furthermore, different modifications of starch structures during food processing or physical treatments also impact physiological response differently. From most of the studies presented in this review, none really correlated RS effects on health to the food process used.

Despite all these variables, the documented benefits of RS on health cannot be dismissed. The European Food Safety Authority (EFSA) established that "health claims related to the benefits of RS on postprandial glycemic response may be made when digestible starch in a given food has been replaced by RS so that the final content of RS is at least 14% of total starch" (89). While much of the evidence belong to RS2 from high amylose maize, the EFSA considered that all sources of RS would achieve a similar effect, as it would consist in replacing digestible CHO by indigestible CHO.

In conclusion, according to the current state of the literature, the relationship between RS and health appears to be multifaceted, with different RS types exerting distinct effects. Further research is needed to comprehensively characterize the specific properties and mechanisms of action of various RS forms.

Author contributions

NTB: Visualization, Writing – review & editing. RD: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Data curation, Methodology. A-KI: Writing – review & editing, Methodology. PV: Writing – review & editing. LR: Writing – review & editing. PA: Writing – review & editing. MD-D: Conceptualization, Funding acquisition, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported partly by Lesaffre Institute of Research and Technology at Lesaffre International (Marcq-en-Baroeul, France).

Acknowledgments

We acknowledge Jean-Michel Lecerf (Pasteur Institute of Lille) for comments that improved this manuscript.

Conflict of interest

RD has temporary consulting activities for Lesaffre. NTB, LR, PV, and MD-D were employed by Lesaffre International (Marcq-en-Baroeul, France).

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.

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

References

1. EFSA Panel on Dietetic Products, Nutrition, and Allergies. Scientific opinion on dietary reference values for carbohydrates and dietary fibre. *EFSA J*. (2010) 8:1462. doi: 10.2903/j.efsa.2010.1462

2. Stylianopoulos C. *Encyclopedia of human nutrition. Carbohydrates: Chemistry and classification*. *3rd* ed. Cambridge, MA: Academic Press, Elsevier (2013).

3. Englyst HN, Kingman SM, Cummings JH. Classification and measurement of nutritionally important starch fractions. *Eur J Clin Nutr*. (1992) 46 Suppl 2:S33–50.

4. Ma Z, Boye JI. Research advances on structural characterization of resistant starch and its structure-physiological function relationship: a review. *Crit Rev Food Sci Nutr*. (2018) 58:1059–83. doi: 10.1080/10408398.2016.1230537

5. Raigond P, Ezekiel R, Raigond B. Resistant starch in food: a review. *J Sci Food Agric*. (2015) 95:1968–78. doi: 10.1002/jsfa.6966

6. Bojarczuk A, Skąpska S, Mousavi Khaneghah A, Marszałek K. Health benefits of resistant starch: a review of the literature. *J Funct Foods*. (2022) 93:105094:105094. doi: 10.1016/j.jff.2022.105094

7. Tsitsou S, Athanasaki C, Dimitriadis G, Papakonstantinou E. Acute effects of dietary Fiber in starchy foods on glycemic and Insulinemic responses: a systematic review of randomized controlled crossover trials. *Nutrients*. (2023) 15:2383. doi: 10.3390/nu15102383

8. European Commission. *Overview of dietary fibre intake across European countries. Avec la collaboration de I. Elmadfa et a. Meyer. University of Vienna*. European Commission website, Consulté le September 2023. (2021).

9. Derek M, Wesley T, Mindy P, Crystal D. Usual dietary Fiber intake in US adults with diabetes: NHANES 2013–2018. *Curr Dev Nutr*. (2021) 5:1061. doi: 10.1093/cdn/nzab053_054

10. Yan R, Andrew L, Marlow E, Kunaratnam K, Devine A, Dunican IC, et al. Dietary fibre intervention for gut microbiota, sleep, and mental health in adults with irritable bowel syndrome: a scoping review. *Nutrients*. (2021) 13:159. doi: 10.3390/nu13072159

11. Chen L, Liu R, Qin C, Meng Y, Zhang J, Wang Y, et al. Sources and intake of resistant starch in the Chinese diet. *Asia Pac J Clin Nutr*. (2010) 19:274–82.

12. Fuentes-Zaragoza E, Riquelme-Navarrete MJ, Sánchez-Zapata E, Pérez-Álvarez JA. Resistant starch as functional ingredient: a review. *Food Res Int*. (2010) 43:931–42. doi: 10.1016/j.foodres.2010.02.004

13. Laurentin A, Edwards C. *Encyclopedia of human nutrition. Carbohydrates | resistant starch and oligosaccharides*. San Diego: Academic Press (1999).

14. Gutiérrez TJ, Tovar J. Update of the concept of type 5 resistant starch (RS5): self-assembled starch V-type complexes. *Trends Food Sci Technol*. (2021) 109:711–24. doi: 10.1016/j.tifs.2021.01.078

15. Zhanggui W, Shuli W, Qinhong X, Qi K, Fei L, Lin L, et al. Synthesis and functions of resistant starch. *Adv Nutr (Bethesda, Md)*. (2023) 14:5, 1131–1144. doi: 10.1016/j.advnut.2023.06.001

16. Patterson MA, Maiya M, Stewart ML. Resistant starch content in foods commonly consumed in the United States: a narrative review. *J Acad Nutr Diet*. (2020) 120:230–44. doi: 10.1016/j.jand.2019.10.019

17. Lisha S, Jiayang L, Yunhai L. Resistant starch formation in rice: genetic regulation and beyond. *Plant Commun*. (2022) 3:100329. doi: 10.1016/j.xplc.2022.100329

18. Maina Ndegwa H, Anne R, De BY, Noora M-S, Stefan S, Mattila O, et al. Process-induced changes in the quantity and characteristics of grain dietary Fiber. *Foods (Basel, Switzerland)*. (2021) 10:2566. doi: 10.3390/foods10112566

19. Roman L, Martinez MM. Structural basis of resistant starch (RS) in bread: natural and commercial alternatives. *Foods*. (2019) 8:267. doi: 10.3390/foods8070267

20. Tian S, Sun Y. Influencing factor of resistant starch formation and application in cereal products: a review. *Int J Biol Macromol*. (2020) 149:424–31. doi: 10.1016/j.ijbiomac.2020.01.264

21. Åkerberg A, Liljeberg H, Björck I. Effects of amylose/amylopectin ratio and baking conditions on resistant starch formation and Glycaemic indices. *J Cereal Sci*. (1998) 28:71–80. doi: 10.1006/jcrs.1997.0173

22. Hallström ElinorSestili F, Lafiandra D, Björck I, Östman E. A novel wheat variety with elevated content of amylose increases resistant starch formation and may beneficially influence glycaemia in healthy subjects. *Food Nutr Res*. (2011) 55:7074. doi: 10.3402/fnr.v55i0.7074

23. Liljeberg HG, Björck IM. Delayed gastric emptying rate as a potential mechanism for lowered glycemia after eating sourdough bread: studies in humans and rats using test products with added organic acids or an organic salt. *Am J Clin Nutr*. (1996) 64:886–93. doi: 10.1093/ajcn/64.6.886

24. Zenel AM, Stewart ML. High amylose white Rice reduces post-prandial glycemic response but not appetite in humans. *Nutrients*. (2015) 7:5362–74. doi: 10.3390/nu7075225

25. Ang K, Bourgy C, Fenton H, Regina A, Newberry M, Diepeveen D, et al. Noodles made from high amylose wheat flour attenuate postprandial glycaemia in healthy adults. *Nutrients*. (2020) 12:2171. doi: 10.3390/nu12082171

26. Belobrajdic DP, Regina A, Klingner B, Zajac I, Chapron S, Berbezy P, et al. High-amylose wheat lowers the postprandial glycemic response to bread in healthy adults: a randomized controlled crossover trial. *J Nutr*. (2019) 149:1335–45. doi: 10.1093/jn/nxz067

27. Lenka Š, Jana B, Tibor S, Marie H, Vlasta F, Václav D, et al. Influence of technological processing of wheat grain on starch digestibility and resistant starch content. *Starch Stärke*. (2016) 68:593–602. doi: 10.1002/star.201500162

28. Alsaffar AA. Effect of food processing on the resistant starch content of cereals and cereal products–a review. *Int J Food Sci Technol*. (2011) 46:455–62. doi: 10.1111/j.1365-2621.2010.02529.x

29. Delcour JA, Bruneel C, Derde LJ, Gomand SV, Pareyt B, Putseys JA, et al. Fate of starch in food processing: from raw materials to final food products. *Annu Rev Food Sci Technol*. (2010) 1:87–111. doi: 10.1146/annurev.food.102308.124211

30. Amaral O, Guerreiro CS, Gomes A, Cravo M. Resistant starch production in wheat bread: effect of ingredients, baking conditions and storage. *Eur Food Res Technol*. (2016) 242:1747–53. doi: 10.1007/s00217-016-2674-4

31. Bilbao-Sáinz C, Butler M, Weaver T, Bent J. Wheat starch gelatinization under microwave irradiation and conduction heating. *Carbohydr Polym*. (2007) 69:224–32. doi: 10.1016/j.carbpol.2006.09.026

32. Cheng Z, Li J, Qiao D, Wang L, Zhao S, Zhang B. Microwave reheating enriches resistant starch in cold-chain cooked rice: a view of structural alterations during digestion. *Int J Biol Macromol*. (2022) 208:80–7. doi: 10.1016/j.ijbiomac.2022.03.034

33. Liu T, Zhang B, Wang L, Zhao S, Qiao D, Zhang L, et al. Microwave reheating increases the resistant starch content in cooked rice with high water contents. *Int J Biol Macromol*. (2021) 184:804–11. doi: 10.1016/j.ijbiomac.2021.06.136

34. Peng Z, Cheng L, Meng K, Shen Y, Wu D, Shu X. Retaining a large amount of resistant starch in cooked potato through microwave heating after freeze-drying. *Curr Res Food Sci*. (2022) 5:1660–7. doi: 10.1016/j.crfs.2022.09.023

35. Yu T, Ming L, Aoxing T, Jay-Lin J, Sushil D, Boli G. RS content and eGI value of cooked noodles (I): effect of cooking methods. *Foods (Basel, Switzerland)*. (2020) 9:328. doi: 10.3390/foods9030328

36. Isra M, Andrianto D, Setiarto RHB. Effect of microwave heat treatment for resistant starch levels and prebiotic properties of high carbohydrate foods: Meta-analysis study. *Chiang Mai Univ J Natl Sci*. (2022) 21:32. doi: 10.12982/CMUJNS.2022.032

37. Da Zavareze E, Dias ARG. Impact of heat-moisture treatment and annealing in starches: a review. *Carbohydr Polym*. (2011) 83:317–28. doi: 10.1016/j.carbpol.2010.08.064

38. Fonseca LM, Halal SLM, Dias ARG, Zavareze EDR. Physical modification of starch by heat-moisture treatment and annealing and their applications: a review. *Carbohydr Polym*. (2021) 274:118665. doi: 10.1016/j.carbpol.2021.118665

39. Sun Y, Qin R, Zeng J, Li G. Effect of heat-moisture treatment on the structure and digestibility of sweet potato starch. *Foods*. (2023) 12:76. doi: 10.3390/foods12163076

40. Lee C-S, Chung H-J. Enhancing resistant starch content of high amylose Rice starch through heat-moisture treatment for industrial application. *Molecules*. (2022) 27:375. doi: 10.3390/molecules27196375

41. Li ZhiyuanGuo D, Li X, Tang Z, Ling X, Zhou T, et al. Heat-moisture treatment further reduces in vitro digestibility and enhances resistant starch content of a high-resistant starch and low-Glutelin Rice. *Foods (Basel, Switzerland)*. (2021) 10. doi: 10.3390/foods10112562

42. Zheng B, Zhong S, Tang Y, Chen L. Understanding the nutritional functions of thermally-processed whole grain highland barley in vitro and in vivo. *Food Chem*. (2020) 310:125979. doi: 10.1016/j.foodchem.2019.125979

43. Zheng Y, Chai Z, Kong X, Chen S, Ye X, Tian J. Effect of annealing treatment on the physicochemical properties and enzymatic hydrolysis of different types of starch. *Food Chem*. (2023) 403:134153. doi: 10.1016/j.foodchem.2022.134153

44. Wang J, Ye Y, Chai Z, Liu H, Wei X, Ye X, et al. Physicochemical properties and in vitro digestibility of proso millet starch modified by heat-moisture treatment and annealing processing. *Int J Biol Macromol*. (2023) 235:123829. doi: 10.1016/j.ijbiomac.2023.123829

45. Bimo Setiarto RHIsra M, Andrianto D, Widhyastuti N, Masrukhin M. Improvement of prebiotic properties and resistant starch content of corn flour (*Zea mays* L.) Momala Gorontalo using physical, chemical and enzymatic modification. *Trop Life Sci Res*. (2023) 34:255–78. doi: 10.21315/tlsr2023.34.2.13

46. Faridah DN, Silitonga RF, Indrasti D, Afandi FA, Jayanegara A, Anugerah MP. Verification of autoclaving-cooling treatment to increase the resistant starch contents in food starches based on meta-analysis result. *Front Nutr*. (2022) 9:904700. doi: 10.3389/fnut.2022.904700

47. Sullivan WR, Hughes JG, Cockman RW, Small DM. The effects of temperature on the crystalline properties and resistant starch during storage of white bread. *Food Chem*. (2017) 228:57–61. doi: 10.1016/j.foodchem.2017.01.140

48. Sonia S, Witjaksono F, Ridwan R. Effect of cooling of cooked white rice on resistant starch content and glycemic response. *Asia Pac J Clin Nutr*. (2015) 24:620–5. doi: 10.6133/apjcn.2015.24.4.13

49. Sylwia S, Anita R-F, Stanislaw P, Joanna LT-B, Anna K, Dorota Z-Z. Influence of resistant starch resulting from the cooling of rice on postprandial glycemia in type 1 diabetes. *Nutr Diabetes*. (2022) 12:21. doi: 10.1038/s41387-022-00196-1

50. Chiu Y-T, Stewart ML. Effect of variety and cooking method on resistant starch content of white rice and subsequent postprandial glucose response and appetite in humans. *Asia Pac J Clin Nutr*. (2013) 22:372–9. doi: 10.6133/apjcn.2013.22.3.08

51. Tian Y, Li M, Liu X, Jane J-L, Guo B, Dhital S. Storage temperature and time affect the enzyme resistance starch and glycemic response of cooked noodles. *Food Chem*. (2021) 344:128702. doi: 10.1016/j.foodchem.2020.128702

52. Shumoy H, van Bockstaele F, Devecioglu D, Raes K. Effect of sourdough addition and storage time on in vitro starch digestibility and estimated glycemic index of tef bread. *Food Chem*. (2018) 264:34–40. doi: 10.1016/j.foodchem.2018.05.019

53. Anna M, Ivo H, Rainer T, Tõnis P, Arvo M, Toomas P. Structural changes of starch during baking and staling of rye bread. *J Agric Food Chem*. (2012) 60:8492–500. doi: 10.1021/jf3021877

54. Demirkesen-Bica H, Muhammet A, Mustafa Y, Salih K, Osman S. Effect of Different Fermentation Condition on Estimated Glycemic Index, In Vitro Starch Digestibility, and Textural and Sensory Properties of Sourdough Bread. *Foods*. (2021) 10:514. doi: 10.3390/foods10030514

55. Teixeira C, Nyman M, Andersson R, Alminger M. Effects of variety and steeping conditions on some barley components associated with colonic health. *J Sci Food Agric*. (2016) 96:4821–7. doi: 10.1002/jsfa.7923

56. Amini S, Mansoori A, Maghsumi-Norouzabad L. The effect of acute consumption of resistant starch on appetite in healthy adults; a systematic review and meta-analysis of the controlled clinical trials. *Clin Nutr ESPEN*. (2021) 41:42–8. doi: 10.1016/j.clnesp.2020.12.006

57. Ruimin C, Zhang C, Xu F, Yu L, Tian F, Chen W, et al. Meta-analysis reveals gut microbiome and functional pathway alterations in response to resistant starch. *Food Funct*. (2023) 14:5251–63. doi: 10.1039/d3fo00845b

58. X. DuWu J, Gao C, Tan Q, Xu Y. Effects of resistant starch on patients with chronic kidney disease: a systematic review and Meta-analysis. *J Diabetes Res*. (2022) 2022:1–11. doi: 10.1155/2022/1861009

59. Gao C, Rao M, Huang W, Wan Q, Yan P, Long Y, et al. Resistant starch ameliorated insulin resistant in patients of type 2 diabetes with obesity: a systematic review and meta-analysis. *Lipids Health Dis*. (2019) 18:205. doi: 10.1186/s12944-019-1127-z

60. Haghighatdoost F, Gholami A, Hariri M. Effect of resistant starch type 2 on inflammatory mediators: a systematic review and meta-analysis of randomized controlled trials. *Complement Ther Med*. (2021) 56:102597. doi: 10.1016/j.ctim.2020.102597

61. Halajzadeh J, Milajerdi A, Reiner Ž, Amirani E, Kolahdooz F, Barekat M, et al. Effects of resistant starch on glycemic control, serum lipoproteins and systemic inflammation in patients with metabolic syndrome and related disorders: a systematic review and meta-analysis of randomized controlled clinical trials. *Crit Rev Food Sci Nutr*. (2020) 60:3172–84. doi: 10.1080/10408398.2019.1680950

62. Kingra K, Curtis S, Mollard RC, Shamloo M, Askin N, Tangri N, et al. The effects of resistant starch consumption in adult patients with chronic kidney disease: a systematic review. *Can J Kidney Health Dis*. (2022) 9:205435812211000. doi: 10.1177/20543581221100023

63. Lu J, Ma B, Qiu X, Sun Z, Xiong K. Effects of resistant starch supplementation on oxidative stress and inflammation biomarkers: a systematic review and meta-analysis of randomized controlled trials. *Asia Pac J Clin Nutr*. (2021) 30:614–23. doi: 10.6133/apjcn.202112_30(4).0008

64. Pugh JE, Cai M, Altieri N, Frost G. A comparison of the effects of resistant starch types on glycemic response in individuals with type 2 diabetes or prediabetes: a systematic review and meta-analysis. *Front Nutr*. (2023) 10:1118229. doi: 10.3389/fnut.2023.1118229

65. Deqiang S, Hao B, Zhaoping L, Yue Y, Huanhuan Z, Liyong C. Positive effects of resistant starch supplementation on bowel function in healthy adults: a systematic review and meta-analysis of randomized controlled trials. *Int J Food Sci Nutr*. (2017) 68:149–57. doi: 10.1080/09637486.2016.1226275

66. Snelson M, Jong J, Manolas D, Kok S, Louise A, Stern R, et al. Metabolic effects of resistant starch type 2: a systematic literature review and Meta-analysis of randomized controlled trials. *Nutrients*. (2019) 11:1833. doi: 10.3390/nu11081833

67. Vahdat M, Ahmad HS, Golsa KM, Javad H, Mehran R. Effects of resistant starch interventions on circulating inflammatory biomarkers: a systematic review and meta-analysis of randomized controlled trials. *Nutr J*. (2020) 19:33. doi: 10.1186/s12937-020-00548-6

68. Wang Y, Chen J, Song Y-H, Zhao R, Xia L, Chen Y, et al. Effects of the resistant starch on glucose, insulin, insulin resistance, and lipid parameters in overweight or obese adults: a systematic review and meta-analysis. *Nutr Diabetes*. (2019) 9:19. doi: 10.1038/s41387-019-0086-9

69. Yali W, Xiyu Z, Yan M, Qian W, Hongzhao X, Liyong C. The effects of resistant starch on biomarkers of inflammation and oxidative stress: a systematic review and Meta-analysis. *Nutr Cancer*. (2022) 74:2337–50. doi: 10.1080/01635581.2021.2019284

70. Xiong K, Wang J, Kang T, Xu F, Ma A. Effects of resistant starch on glycaemic control: a systematic review and meta-analysis. *Br J Nutr*. (2021) 125:1260–9. doi: 10.1017/S0007114520003700

71. Yuan HC, Meng Y, Bai H, Shen DQ, Wan BC, Chen LY. Meta-analysis indicates that resistant starch lowers serum total cholesterol and low-density cholesterol. *Nutr Res*. (2018) 54:1–11. doi: 10.1016/j.nutres.2018.02.008

72. Watanabe N, Suzuki M, Yamaguchi Y, Egashira Y. Effects of resistant maltodextrin on bowel movements: a systematic review and meta-analysis. *Clin Exp Gastroenterol*. (2018) 11:85–96. doi: 10.2147/CEG.S153924

73. Rao M, Gao C, Hou J, Gu J, Betty L, Kwan Y, et al. Non-digestible carbohydrate and the risk of colorectal neoplasia: a systematic review. *Nutr Cancer*. (2021) 73:31–44. doi: 10.1080/01635581.2020.1742360

74. Gourineni V, Stewart ML, Wilcox ML, Maki KC. Nutritional Bar with potato-based resistant starch attenuated post-prandial glucose and insulin response in healthy adults. *Foods*. (2020) 9:679. doi: 10.3390/foods9111679

75. Stewart Maria L, Paul ZJ. Postprandial glucose and insulin response to a high-fiber muffin top containing resistant starch type 4 in healthy adults: a double-blind, randomized, controlled trial. *Nutrition (Burbank, Los Angeles County, Calif)*. (2018) 53:59–63. doi: 10.1016/j.nut.2018.01.002

76. Du Y, Wu Y, Xiao D, Guzman G, Stewart ML, Gourineni V, et al. Food prototype containing resistant starch type 4 on postprandial glycemic response in healthy adults. *Food Funct*. (2020) 11:2231–7. doi: 10.1039/c9fo02674f

77. Stewart ML, Wilcox ML, Bell M, Buggia MA, Maki KC. Type-4 resistant starch in substitution for available carbohydrate reduces postprandial glycemic response and hunger in acute, randomized, double-blind, controlled study. *Nutrients*. (2018) 10:129. doi: 10.3390/nu10020129

78. Cai M, Dou B, Pugh JE, Lett AM, Frost GS. The impact of starchy food structure on postprandial glycemic response and appetite: a systematic review with meta-analysis of randomized crossover trials. *Am J Clin Nutr*. (2021) 114:472–87. doi: 10.1093/ajcn/nqab098

79. Dhakal S, Dey M. Resistant starch type-4 intake alters circulating bile acids in human subjects. *Front Nutr*. (2022) 9:930414. doi: 10.3389/fnut.2022.930414

80. Alfilasari N, Sirivongpaisal P, Wichienchot S. Gut health function of instant dehydrated Rice sticks substituted with resistant starch types 2 and 4. *Curr Microbiol*. (2021) 78:3010–9. doi: 10.1007/s00284-021-02564-z

81. Li L, Ryan J, Ning Z, Zhang X, Mayne J, Lavallée-Adam M, et al. A functional ecological network based on metaproteomics responses of individual gut microbiomes to resistant starches. *Comput Struct Biotechnol J*. (2020) 18:3833–42. doi: 10.1016/j.csbj.2020.10.042

82. DeMartino P, Cockburn DW. Resistant starch: impact on the gut microbiome and health. *Curr Opin Biotechnol*. (2020) 61:66–71. doi: 10.1016/j.copbio.2019.10.008

83. Thakur M, Rai AK, Singh SP. Structural characteristics, physicochemical properties, and digestibility analysis of resistant starch type-V prepared from Debranched corn starch and fatty acid complexation. *ACS Omega*. (2023) 8:25799–807. doi: 10.1021/acsomega.3c01093

84. Zheng B, Wang T, Wang H, Chen L, Zhou Z. Studies on nutritional intervention of rice starch-oleic acid complex (resistant starch type V) in rats fed by high-fat diet. *Carbohydr Polym*. (2020) 246:116637. doi: 10.1016/j.carbpol.2020.116637

85. Guo J, Ellis A, Zhang Y, Kong L, Tan L. Starch-ascorbyl palmitate inclusion complex, a type 5 resistant starch, reduced in vitro digestibility and improved in vivo glycemic response in mice. *Carbohydr Polym*. (2023) 321:121289. doi: 10.1016/j.carbpol.2023.121289

86. López-Barón N, Sagnelli D, Blennow A, Holse M, Gao J, Saaby L, et al. Hydrolysed pea proteins mitigate in vitro wheat starch digestibility. *Food Hydrocoll*. (2018) 79:117–26. doi: 10.1016/j.foodhyd.2017.12.009

87. Xu H, Zhou J, Yu J, Wang S, Wang S. Mechanisms underlying the effect of gluten and its hydrolysates on in vitro enzymatic digestibility of wheat starch. *Food Hydrocoll*. (2021) 113:106507. doi: 10.1016/j.foodhyd.2020.106507

88. Zhang Y, Dou B, Jia J, Liu Y, Zhang N. A study on the structural and digestive properties of Rice starch-hydrocolloid complexes treated with heat-moisture treatment. *Foods*. (2023) 12:241. doi: 10.3390/foods12234241

89. EFSA Panel on Dietetic Products, Nutrition and Allergies. Scientific opinion on the substantiation of health claims related to resistant starch and reduction of post-prandial glycaemic responses (ID 681), "digestive health benefits" (ID 682) and "favours a normal colon metabolism" (ID 783) pursuant to article 13(1) of regulation (EC) no 1924/2006. *EFSA J*. (2011) 9:2024. doi: 10.2903/j.efsa.2011.2024

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 15 May 2024
DOI 10.3389/fnut.2024.1398380

OPEN ACCESS

EDITED BY
Xianyang Bao,
Harvard University, United States

REVIEWED BY
Yiping Zhang,
Henan Normal University, China
Yidan Zhang,
Guangdong Provincial People's Hospital, China

*CORRESPONDENCE
Hai He
✉ h.hai@hainmc.edu.cn

RECEIVED 09 March 2024
ACCEPTED 16 April 2024
PUBLISHED 15 May 2024

CITATION
Zhang Z, Feng Y, Wang H and He H (2024) Synergistic modification of hot-melt extrusion and nobiletin on the multi-scale structures, interactions, thermal properties, and *in vitro* digestibility of rice starch.
Front. Nutr. 11:1398380.
doi: 10.3389/fnut.2024.1398380

Synergistic modification of hot-melt extrusion and nobiletin on the multi-scale structures, interactions, thermal properties, and *in vitro* digestibility of rice starch

Zhihong Zhang[1], Ying Feng[1], Honglan Wang[1] and Hai He[1,2]*

[1]Department of Nutrition and Food Hygiene, School of Public Health, Heinz Mehlhorn Academician Workstation, Hainan Medical University, Haikou, Hainan, China, [2]Department of Endocrinology and Metabolism, Shunde Hospital, Southern Medical University (The First People's Hospital of Shunde), Foshan, Guangdong, China

Background: Rice starch has high digestibility due to its large carbohydrate content. Synergistic modification of hot-melt extrusion (HME) and additives such as flavonoids, hydrocolloids, proteins, lipids, and other additives has the tendency to retard the rate of starch hydrolysis. Hence, the current investigation aimed to study the combined effect of the HME-assisted addition of nobiletin (NOB, 0, 2, 4, and 6%) on the multi-scale structures, interactions, thermal, and digestibility characteristics of rice starch.

Methods: The study employed density functional theory calculations and an infrared second derivative of an Fourier-transform infrared (FTIR) spectrometer to analyze the interactions between NOB and starch. The physicochemical properties of the starch extrudates were characterized by FTIR, ^{13}C nuclear magnetic resonance, X-ray diffraction, and differential scanning calorimetry, while the digestibility was evaluated using an *in vitro* digestion model.

Results: HME was found to disrupt the crystalline structure, helix structure, short-ordered structure, and thermal properties of starch. The interaction between NOB and starch involved hydrophobic interactions and hydrogen bonds, effectively preventing the molecular chains of starch from interacting with each other and disrupting their double helix structure. The addition of NOB led to the formation of a highly single-helical V-type crystalline structure, along with the formation of ordered structural domains. Consequently, the combined treatment significantly enhanced the ordered structure and thermal stability of starch, thus effectively leading to an increase in resistant starch and slowly digestion starch.

Discussion: The study underscores that synergistic modification of HME and NOB holds promise for enhancing both the nutritional value and functional properties of rice starch. These findings offer valuable insights for developing high-quality rice starch products with broader applications.

KEYWORDS

rice starch–nobiletin complex, *in vitro* starch digestibility, thermal properties, multi-scale structure, hot-melt extrusion, molecular interaction

1 Introduction

Rice is one of the most important staple foods in Asia, and the main component of rice, starch, provides most of the carbohydrates consumed by humans (1). Nonetheless, prolonged ingestion of rice starch-based goods has been strongly linked to elevated blood glucose levels after meals, which may lead to type 2 diabetes and further metabolic disorders (2). Therefore, it is important to find an appropriate modification to improve the digestibility of rice starch to meet the requirements of low glycemic index (GI) value rice foods. The emphasis on modifying starch digestibility has increased recently. These methods include processing food to increase the amount of resistant starch (RS), physically changing the starch's molecular structure, or adding non-starch ingredients like proteins, lipids, flavonoids, and others to interact molecularly with starch and change its physicochemical and digestibility properties (3, 4).

Hot-melt extrusion (HME) technology could provide a lead in the restructuring of heterogeneous food matrix at the molecular level because of the processing capabilities of extruders. These capabilities encompass simultaneous processes such as flavor generation, encapsulation, heating, cooling, shaping, venting, mixing, shearing, and conveying. The versatility of HME technology allows for efficient and comprehensive transformations of diverse dietary matrices (5). Additionally, HME may result in improved water hydration capabilities, degree of soluble fiber content from cell wall components, digestibility, shelf-stability, and expansion ratio (6). During the HME process, food ingredients such as proteins, lipids, hydrocolloids, bioactive compounds, fibers, and others may be added to the extruded food to enhance its quality (7). Utilizing extruders as a bioreactor, starch molecules are esterified with organic acids to create cross-linked starch with a higher RS content (8). During processing, food ingredients undergo physicochemical and structural changes that are often intended to enhance the product's digestibility, nutritional bioavailability, textural, organoleptic, sensory requirements, and storage stability (9).

Phenolic compounds like flavonoids and tannins can form non-covalent bonds with starch. These bonds affect starch's structural and functional characteristics, including hydrogen bonds, hydrophobic interactions, and van der Waals forces (10–12). Previous studies indicate that inclusion (V-type) and non-inclusion complexes may be formed by interacting starches with various phenolic chemicals (10–12). The V-type inclusion complex was effectively embedded within the internal hydrophobic helix of the starch, achieving successful encapsulation. For example, A classic V-type diffraction peak was seen at $2\theta = 13.0°$ and 19.8° in the inclusion complex created by gallic acid and rice starch (13). The starch-lipid complex (RS_5), which belongs to the RS_5 family, exhibits a similar organizational mechanism to that of the inclusion complex formed by starch-polyphenol compounds (14). As previously indicated, non-covalent connections between polyphenol chemicals and starch facilitate direct interactions, which are specifically referred to as the non-inclusion complex. However, this is not the same as the V-type inclusion complicated development (15). For instance, in a study conducted by Huang et al. (16), it was found that lotus seed starch and tea polyphenol formed a non-inclusion complex following treatment with high hydrostatic pressing. This was confirmed by inspection using confocal laser scanning microscopy and scanning electron microscopy.

Nobiletin (NOB) is an O-methylated flavonoid widely found in citrus peels, and is structurally characterized by the presence of a benzopyran moiety and multiple methoxyl groups on the aromatic ring (17). This combination of hydroxyl and methoxy groups modifies the structural and functional characteristics of starch and suppresses the activity of amylase (18). In our current study, we employed physical modification through HME to craft the final product with altered characteristics. However, the study on the production of starch–NOB complex with high enzymatic digestion resistance under HME has been limited, and the starch–NOB complex interaction has not been fully explained. Therefore, the objective of this study was to investigate the effect of HME with different NOB concentrations on the multi-scale structures, interactions, and thermal and digestibility properties of rice starch. This work could guide industrially HME production of digestion-resistant starches derived from the starch–flavonoids complex.

2 Materials and methods

2.1 Reagents

The rice starch (Remy-DR) with 64.10% amylopectin, 22.45% amylose, 10.25% (d.b) moisture, 0.48% (d.b) protein, 0.20% (d.b) lipid, and 0.24% (d.b) ash was supplied by Beneo-orafti (Oreye, Belgium). Shanghai, China-based Yuanye Bio-Technology Co., Ltd. provided the NOB. Sigma-Aldrich Co., Ltd. (Sigma, St Louis, MO, United States) provided the amyloglucosidase (A3306, activity 318 U/mL) and porcine pancreatic α-amylase (P7545, activity 8 × USP). Megazyme (Wicklow, Ireland) provided the glucose oxidase-peroxidase (GOPOD) test kit. Analytical reagents were all other substances.

2.2 Twin-screw extruder-based in-situ complexation of rice starch with NOB

Based on rice starch, various NOB concentrations (0, 2, 4, and 6% w/w) were added to 40% moisture-content rice starch. The mixture was homogeneously blended using a mixing agitator at 100 r/min for 5 min. Next, the reaction mixtures were introduced into a co-rotating twin-screw extruder with a 52 length-to-diameter ratio (HK-36 model, Nanjing KY Chemical Machinery CO., Ltd., Nanjing, China). The extruder operated at a screw speed of 90 r/min and maintained a heating temperature of 85°C. This procedure facilitated the homogenization and gelatinization of the mixtures, producing a uniform and consistent product. The gelatinization of the mixture is further facilitated by the high shear stress of the extruder (19). Following a 48-h drying procedure at 45°C, the extrudates were processed in a room-temperature mill (BJ-800A, Baijie, Huzhou, China). After passing through a 100 mesh sieve, the powder samples were stored at room temperature with a moisture content of less than 8% and kept out of direct sunlight.

2.3 Analysis of the starch digestibility *in vitro*

The *in vitro* digestibility of the NRS and HMERS samples was evaluated following with the previous studies (20, 21). 1 g of dry-based starch was placed in sodium acetate buffer (20 mL, 0. 1 M, pH = 5. 2), and the starch samples were hydrolyzed with an

enzyme mixture (0. 5 mL, 190 r/min, 37°C) containing porcine pancreatic α-amylase (300 U/mL) and amyloglucosidase (20 U/mL). When the *in vitro* digestion process was underway, 0.5 mL of digestible fluid was taken at 0, 20, 60, 120, and 180 min. Each sample received 25 mL of 70% ethanol to fully inactivate the enzyme. The samples were then centrifuged (4,000 r/min, 5 min), and the supernatant (0.1 mL) was added to GOPOD (3 mL). Following that, the samples were incubated for 20 min at 45°C. Eqs. 1–4 were used to compute the quantities of rapidly digested starch (RDS), slowly digested starch (SDS), and RS, taking into account the values of G_{20} and G_{120}.

$$C\ (\%) = \frac{G_t - G_0}{TS} \times 0.9 \times 100 \quad (1)$$

$$RDS\ (\%) = \frac{G_{20} - G_0}{TS} \times 0.9 \times 100 \quad (2)$$

$$SDS\ (\%) = \frac{G_{120} - G_0}{TS} \times 0.9 \times 100 \quad (3)$$

$$RS\ (\%) = \frac{TS - (RDS + SDS)}{TS} \times 100 \quad (4)$$

t stands for the digestion time points, while C (%) is the starch digestibility rate. G_t represents the amount of glucose present at time t, whereas G_0, G_{20}, and G_{120} stand for the amounts present at 0, 20, and 120 min, respectively. TS stands for total starch samples.

To evaluate starch samples' *in vitro* digestibility and forecast their physiological reaction, we used the logarithm of the slope (LOS) (22, 23). First-order kinetics adapts to digestibility in the following way Eq. 5:

$$C_t = C_\infty \left(1 - e^{-kt}\right) \quad (5)$$

C_t represents the starch sample's digested ratio at time t (min) and C_∞ indicates the digested ratio at the reaction's ending. K stands for the first-order kinetics rate constant. We generated a LOS plot by differentiating the first-order equation and then representing it in a logarithmic format (Eq. 6):

$$\ln\left(\frac{dC}{dt}\right) = -kt + \ln\left(C_\infty k\right) \quad (6)$$

2.4 Predicted glycemic index (pGI)

To assess the hydrolysis index (HI), the starch digestion curve was obtained at different time intervals (0, 20, 60, 120, and 180 min). The HI was determined by calculating the proportion of total glucose released within 180 min compared to the glucose released from white bread within the same time period. To estimate the pGI, the formula (pGI = 44.78 + 0.3797 × HI) established by Goni et al. (24) with slight modifications was utilized.

2.5 Differential scanning calorimetry (DSC)

To examine the thermal properties of the NRS and HME samples, the DSC (DSC-1, Mettler Toledo, Zurich, Switzerland) was used. Weigh the sample in an aluminum pot (keeping an empty pot as a reference) to a weight of about 6 mg. Before measurement, the temperature was calibrated using indium at the same scanning rate. Dry nitrogen (50 mL/min) was used to scan the samples at 30–120°C (10°C/min).

2.6 Analysis of the complex's multi-scale structure

The molecular interactions of the NRS and HMERS samples were investigated using an attenuated total reflectance (ATR) Fourier-transform infrared spectrometer (FTIR) equipped with a deuterated triglycine sulfate detector (Nicolet iN10, Thermo Fisher Scientific, United States). Sixty-four scans were carried out at a resolution of 4 cm^{-1} in the 4,000–400 cm^{-1} range. The content of the ordered structure with a short range was indicated by the absorption ratios ($R_{1047/1022}$) at 1,047 and 1,022 cm^{-1} (25).

^{13}C CP/MAS NMR (cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance) spectroscopy and a magnet 400 (Bruker, Karlsruhe, Germany) were used to examine the helical structures of the NRS and HMERS samples. Before testing, the moisture concentrations of the complex were adjusted. A 4-mm MAS solid-state probe was used for the ^{13}C CP/MAS NMR test, which was conducted at a frequency of 150.9 MHz. 10,000 kHz was the speed at which the test was carried out, along with 90° (5 μs) pulse width, 1 ms exposure period, 5 s delay, and at least 2,400 total frequency cycles. The temperature during the test was maintained at 25°C. The spectra were assessed, and the contents of the helix structure were ascertained using PeakFit v4.12 (26).

The NRS and HMERS samples' X-ray diffraction (XRD) patterns were examined using an advanced wide-angle XRD (D8, Bruker, Germany), according to a previously defined procedure (26). The diffraction strengths (2θ) of NRS and HMERS samples were scanned from 5° to 60° (40 kV, 30 mA, scan speed = 2°/min, step size = 0.013°). The proportions of A-type (%) and V-type (%) were calculated by an MDI Jade program (Materials Data, Livermore, CA, United States, V6.5) in the range of 5°–40° (2θ), and the total crystalline (X_{Total}, %) was computed as = A-type (X_A, %) + V-type (X_V, %) (26).

2.7 Computational method for the interactions of starch and NOB

Gaussian View software was used to generate the initial structures of the rice starch–NOB complex, rice starch, and NOB for density functional theory (DFT) computations. The optimized molecular structures and corresponding vibrational assignments of rice starch,

NOB, and the complex have been investigated by using DFT B3lyp/6-31 g(d) (27, 28). Atoms-in-molecule theory (AIM) analyses were performed using the Multiwfn program (29) to provide more information on the interactions of the complex. Gaussian 09 software was used for all other computations (30).

2.8 Analytical statistics

The mean ± the standard deviation (SD) was used to represent the experimental data. ANOVA and Duncan's multiple range test were used to find any differences in the data analysis, which was done with SPSS software (V22.0, Inc. Chicago, IL, United States). Significant was defined as $p < 0.05$.

3 Results and discussion

3.1 *In vitro* digestibility

NRS had 1.44% RS, 3.52% SDS, and 95.04% RDS, as shown in Table 1. The increase in the quantities of SDS and RS in HMERS ($p < 0.05$), coupled with a decrease in the quantity of RDS in the sample ($p < 0.05$), suggests that the HME method employed in this study attenuated the digestion of NRS. Shear-induced starch molecule fragmentation that leads to increased retrogradation during storage is the cause of this decline (31). Additionally, there was a significant increase in the content of NOB and a considerable rise in the content of SDS and RS. An increase in SDS content from 6.62 to 8.33% ($p < 0.05$), and the RS content rose from 11.16 to 19.85% ($p < 0.05$). Together, these findings indicate that the binding of NOB leads to an increase in RS content. The strong crystalline structure that results from the interaction of NOB and rice starch is partly responsible for the increase in resistance to enzymatic hydrolysis. Furthermore, the resistance to enzymatic hydrolysis is further enhanced by NOB via hydrophobic contacts and hydrogen bonding with the active region of α-amylase (32). In line with the findings of Lemlioglu-Austin et al. (33), tannin was observed to decrease starch digestibility when combined with starch, resulting in a reduced pGI. Our experiments similarly showed that interactions with NOB led to a significant decrease in the pGI of HMERS ($p < 0.05$). In addition, the pGI of HMERS decreased gradually with increasing NOB content (Table 1).

3.2 Analysis of the first-order kinetics

Supplementary Figure 1 displays the digestibility plots and LOS curves for the NRS and HMERS samples. All graphs clearly show an exponential growth shape, characterized by a rapid increase period, followed by a slow digestion phase until the maximum digestion range of starch was reached and digested after 120 min. According to this result, first-order kinetics were likely followed in the digestion of the NRS and HMERS samples. The k and C_∞ values that were taken from the samples' LOS curves throughout the digestion process are shown in Table 1. The results showed that a single digestion phase ($R^2 > 0.99$) effectively replicated the entire digestive process, indicating comparable α-amylase contents in both NRS and HMERS samples (34). In this regard, the substantial variations in the k and C_∞ values between the NRS and HMERS samples were corroborated by our findings. The values of k and C_∞ for NRS were 0.1765 min^{-1} and 97.536%, while HMERS had values of 0.1447 min^{-1} and 91.768% ($p < 0.05$). In the HMERS/NOB samples, the presence of NOB led to a reduction in glucose release rate. Overall, these findings reflect a consistent pattern with the digestibility profile of the starch samples outlined in Table 1.

3.3 Thermal properties

The DSC data (Supplementary Figure 2 and Table 2) reveals that the HME treatment significantly reduced the onset temperature (T_o), peak temperature (T_p), and conclusion temperature (T_c) of NRS compared to its pre-HME treatment state. The low gelatinization state of NRS after HME treatment indicated the structural instability of starch granules due to the low ordering and crystallinity (35). In addition, the NRS after HME treatment showed a significant reduction in enthalpy (ΔHg) compared to the NRS before ($p < 0.05$). This can be attributed to the decreased double helix structure observed in the HME samples. Table 2 show that T_o, T_p, T_c, and ΔHg increased with the addition of NOB ($p < 0.05$). The increase in these values indicates an improvement in the crystalline homogeneity and crystalline structure of the starch. As the crystalline structure increased, the energy required to destroy the crystalline structure increased (36), resulting in a higher gelatinization ΔHg of the starch after NOB addition.

3.4 Short-range order structure, helical structure, and crystalline structure

The vibrational absorption peaks at 1022 and 997 cm^{-1} correspond to C-O-H bonds. The former is associated with the chain structure of the starch molecule, while the latter is linked to the intramolecular hydrogen bonding of the hydroxyl group on the dehydrated glucose unit C_6 of the starch molecule (37). Since it reflected more chemical structure details and had a better resolution, the second-order infrared spectrum and the starch samples' infrared spectra were analyzed using the second-order mode, as Figure 1A illustrates. When comparing the NRS spectra, the low-frequency peaks in HMERS were found to be moving at 1022 and 997 cm^{-1} (Table 3). This change may be ascribed to the mechanical contact between heat energy and water molecules during the HME process, which breaks the starch molecular chain and causes it to lose its double-helical structure. Consequently, there is an increase in the amount of amorphous structure, which makes the starch molecule chain easier to move and reassemble. Ultimately, this causes the starch molecules to form hydrogen bonds with each other (38). The addition of NOB shifted towards a higher frequency of the infrared absorption maxima located at 1022 and 997 cm^{-1} (Table 3). The absorption bands observed at 1022 and 997 cm^{-1}, primarily attributed to C-O-H bending vibrations, exhibit heightened sensitivity to variations in water content. These vibrations, such as hydrogen bonding, are likely influenced by interactions between water and starch molecules, consequently affecting the C-O-H bending modes. Alterations in this spectral region have been attributed to fluctuations in the molecular environment surrounding the primary hydroxyl group in V-type amylose, stemming from shifts

TABLE 1 Evaluation of RDS, SDS and RS content, digestibility parameters of LOS plots, and pGI for NRS and HMERS samples.*

Samples	k (min^{-1})	C_∞(%)	RDS (%)	SDS (%)	RS (%)	pGI
NRS	0.1765 ± 0.0032^e	97.536 ± 0.680^e	95.04 ± 0.19^e	3.52 ± 0.20^a	1.44 ± 0.55^a	91.87 ± 1.02^d
HMERS	0.1447 ± 0.0021^d	91.768 ± 0.392^d	86.74 ± 0.15^d	5.33 ± 0.34^b	7.93 ± 0.43^b	81.25 ± 1.05^c
HMERS/NOB-2%	0.1348 ± 0.0030^c	88.283 ± 0.645^c	82.22 ± 0.32^c	6.62 ± 0.26^b	11.16 ± 0.22^c	80.19 ± 0.93^b
HMERS/NOB-4%	0.1248 ± 0.0021^b	87.495 ± 0.301^b	80.51 ± 0.22^b	7.43 ± 0.26^c	12.06 ± 0.32^d	79.39 ± 0.80^b
HMERS/NOB-6%	0.1158 ± 0.0020^a	79.515 ± 0.754^a	71.82 ± 0.25^a	8.33 ± 0.30^d	19.85 ± 0.36^e	76.71 ± 1.10^a

*Results are means ± standard deviation of three replicate tests ($n = 3$); values indicated by an additional letter are regarded as $p < 0.05$.
C_∞, the equivalent value at the endpoint; k (min^{-1}), the digestion rate constant; RDS, rapidly digestion starch; SDS, slowly digestion starch; RS, resistant starch; pGI, predicted glycemic index; NRS, native rice starch; LOS, the logarithm of the slope; HMERS, hot-melt extruded rice starch; HMERS/NOB, hot-melt extruded rice starch–nobiletin complex.

TABLE 2 Thermal properties for NRS and HMERS samples.*

Samples	T_o (°C)	T_p (°C)	T_c (°C)	ΔHg (J/g)
NRS	59.1 ± 0.3^e	82.2 ± 0.1^d	95.4 ± 0.2^e	1.912 ± 0.012^e
HMERS	46.5 ± 0.2^a	62.1 ± 0.2^a	73.3 ± 0.3^a	0.582 ± 0.015^b
HMERS/NOB-2%	48.3 ± 0.1^b	62.5 ± 0.2^a	74.5 ± 0.1^b	0.472 ± 0.015^a
HMERS/NOB-4%	49.6 ± 0.2^c	63.4 ± 0.1^b	75.7 ± 0.3^c	1.001 ± 0.013^c
HMERS/NOB-6%	53.6 ± 0.3^d	65.1 ± 0.2^c	78.9 ± 0.1^d	1.371 ± 0.013^d

*Results are means ± standard deviation of three replicate tests ($n = 3$); values indicated by an additional letter are regarded as $p < 0.05$.
T_o, T_p, T_c, and ΔHg are the onset temperature, peak temperature, conclusion temperature, and enthalpy. NRS, native rice starch; HMERS, hot-melt extruded rice starch; HMERS/NOB, hot-melt extruded rice starch–nobiletin complex.

in intramolecular hydrogen bonding dynamics (39). Hydrogen bonding between starch and NOB and between starch molecules was revealed by the HME treatment. This resulted in the formation of a spatially ordered aggregate, which decreased the amount of amorphous HMERS structure.

The short-range ordered structure of starch is visible in the infrared spectrum at an absorption ratio of 1047/1022 cm^{-1} ($R_{1047/1022}$) (40, 41). Figure 1B displays the starch samples' deconvoluted FTIR spectra in the 1,200–800 cm^{-1} range, and the $R_{1047/1022}$ values are compiled in Table 3. Before the HME procedure, the $R_{1047/1022}$ value was 0.455, but after the HME treatment, the NRS value climbed to 0.566 ($p < 0.05$). Comparable results were obtained with the HMERS/NOB samples. NOB addition could effectively improve $R_{1047/1022}$ as indicated by increased short-range order degree of NOB-treated rice starch. The degree of order in the starch molecules was enhanced by creating strong hydrogen bonds between the –O–H proton and the –C–H proton of the benzene ring on NOB (42). Additionally, the $R_{1047/1022}$ of HMERS/NOB samples were slightly increased with NOB addition because NOB was easily inserted into the single helical cavity of starch and formed a V-type inclusion complex (42). Meanwhile, NOB formed a non-inclusion complex with starch molecules through hydrogen bonding (43), producing relatively dense local-order structures.

^{13}C CP/MAS NMR spectroscopy was used to evaluate the starch samples' helical structures. According to prior research, changes in starch's helical structures may affect chemical shifts in starch and glucose caused by C_1 and C_4 (Figure 1C) (44). The contents of single and double helical structures found in starch samples are shown in Table 3. Following the HME process, HMERS's single helix structure content rose from 3.18% to 2.33% ($p < 0.05$), while the percentage of its double helix structure content dropped from 42.46% to 18.21% ($p < 0.05$). Thermal processing breaks the α-1,4 and α-1,6 glycosidic bonds, increasing the amount of straight-chain starch and creating more single helix structures (45, 46). The susceptibility to shear degradation and branch density increase with shorter starch branch lengths (47). After NOB addition, we observed an increase in the single helical structures (9.55% to 17.58%, $p < 0.05$) and a decrease in the double helical structure content (16.26% to 11.69%, $p < 0.05$) in the HMERS/NOB samples. The strong hydrogen bonding between starch and NOB may be responsible for the slower binding of the helical structures, which is one of the reasons for the reduction of the double helix structures (48).

The XRD patterns displaying the broad angles for the starch samples are depicted in Figure 1D. At 15.3°, 17.3°, 18.3°, and 23.2° (2θ), the NRS exhibited characteristic peaks that are indicative of a particular A-type crystalline structure (49). Because of the differences in the XRD spectrum of HMERS and NRS, as well as the larger peak shapes and lower peak intensities, it was concluded that the HME treatment had a negative impact on the A-type crystalline structure of NRS. Furthermore, the HME treatment resulted in a considerable reduction in the relative degree of crystallinity and a reduction in the double helix structure content found in rice starch (Table 3). Furthermore, a notable diffraction peak at 20.2° (2θ) was seen by HMERS, encouraging the development of a crystalline structure in the V-type. The crystalline structure of the HMERS/NOB samples, characterized by an A + V-type arrangement, is evident from the peaks observed in the spectra at 15.3°, 17.3°, 18.3°, 20.2° and 23.2°(2θ) (50). The addition of NOB to rice starch increased the intensity of the V-type crystallization peaks and decreased the intensity of the A-type crystallization peaks. Table 3 shows that the A-type content decreases from 8.13% to 6.64% with the addition of NOB ($p < 0.05$), while the V-type content increases from 3.33% to 6.54% ($p < 0.05$). The A-type and V-type crystalline structures are derived from the double and single helical structures of starch (42). These results matched the ^{13}C NMR-characterized helical structures rather well (Table 3).

3.5 Interactions between NOB and rice starch

The findings above showed that the dynamic interactions between starch molecules and NOB during HME were responsible for the multi-scale structural alterations in HMERS/NOB. The intermolecular interaction location and intensity of the HMERS/NOB samples were investigated using further DFT computations. We first perform a geometric structure optimization to simulate the physical molecular

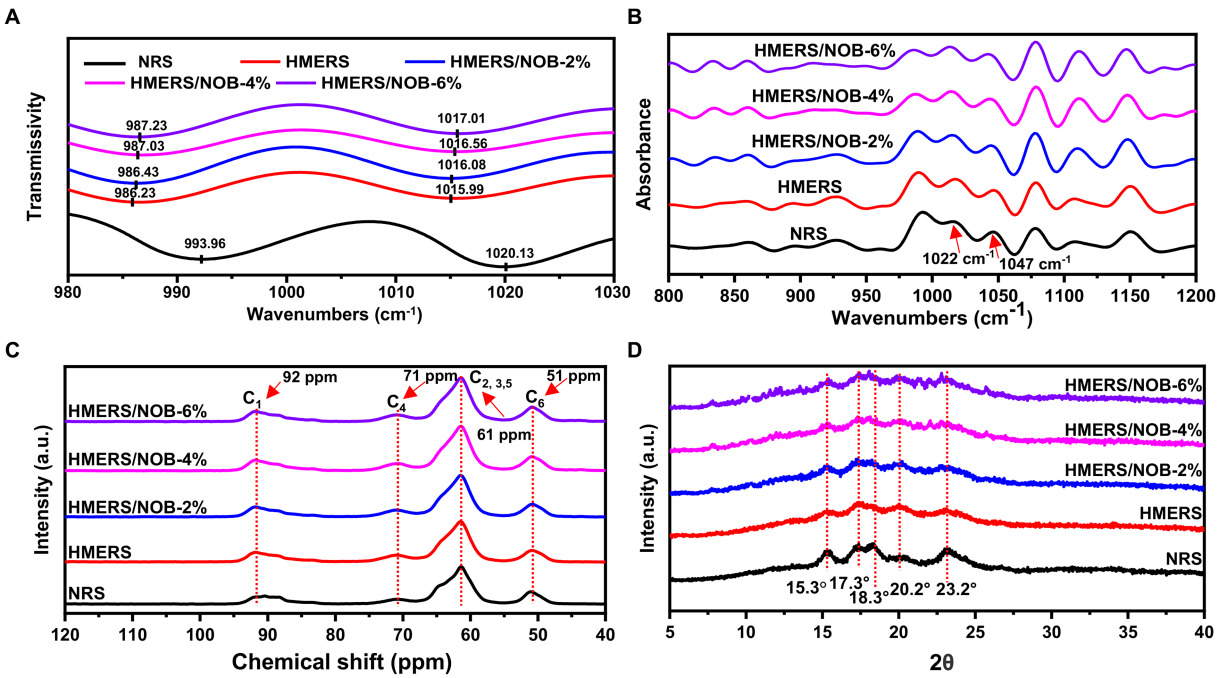


FIGURE 1
(A) Infrared spectroscopy with second derivative, **(B)** spectra of deconvoluted ATR-FITR, **(C)** ^{13}C NMR spectra, and **(D)** XRD spectra for NRS and HMERS samples. NRS, native rice starch; HMERS, hot-melt extruded rice starch; HMERS/NOB, hot-melt extruded rice starch–nobiletin complex.

TABLE 3 The parameters being analyzed for NRS and HMERS samples include helical structures, total crystallinity, A-type crystallinity, V-type crystallinity, short-range ordered degree, C-O-H def., and CH_2.*

Samples	Amorphous	Single helix	Double helix	X_{Total} (%)	X_A (%)	X_V (%)	$R_{1047/1022}$	C-O-H def., CH_2	
NRS	55.21 ± 0.13^a	2.33 ± 0.17^a	42.46 ± 0.16^e	25.43 ± 0.15^d	24.91 ± 0.14^e	0.52 ± 0.10^a	0.455 ± 0.013^a	1020.13	993.96
HMERS	78.61 ± 0.12^e	3.18 ± 0.19^b	18.21 ± 0.25^d	11.18 ± 0.18^a	9.49 ± 0.13^d	1.69 ± 0.05^b	0.566 ± 0.006^b	1015.99	986.23
HMERS/NOB-2%	74.19 ± 0.14^d	9.55 ± 0.23^c	16.26 ± 0.23^c	11.46 ± 0.20^a	8.13 ± 0.11^c	3.33 ± 0.09^c	0.579 ± 0.003^c	1016.08	986.43
HMERS/NOB-4%	72.32 ± 0.20^c	14.33 ± 0.32^d	13.35 ± 0.23^b	12.26 ± 0.22^b	7.78 ± 0.12^b	4.48 ± 0.10^d	0.591 ± 0.002^d	1016.56	987.03
HMERS/NOB-6%	70.53 ± 0.30^b	17.58 ± 0.36^e	11.69 ± 0.15^a	13.18 ± 0.10^c	6.64 ± 0.10^a	6.54 ± 0.09^e	0.623 ± 0.005^e	1017.01	987.23

*Results are means ± standard deviation of three replicate tests ($n = 3$); values indicated by an additional letter are regarded as $p < 0.05$.
X_A, A-type crystallinity; X_V, V-type crystallinity; X_{Total}, total crystallinity; $R_{1047/1022}$, short-range ordered degree; C-O-H def., CH_2, the wavenumbers of C-O-H bonds; NRS, native rice starch; HMERS, hot-melt extruded rice starch; HMERS/NOB, hot-melt extruded rice starch–nobiletin complex.

interactions between starch and NOB using DFT calculations. Figure 2 shows the optimized structures of glucose dimer (starch) (51), NOB, and rice starch–NOB complex formed from glucose dimer and NOB. Based on previous studies, we calculated the typical configuration of glucose dimer–NOB complex (52, 53). As shown in Figure 2C and Table 4, the H···O bond lengths in the system are in the range of 1.7353–3.4223 Å, which can be assigned to hydrogen bond interactions between glucose dimer and flavones (54). The number of hydrogen bonds was seven for the glucose dimer and NOB system. Therefore, hydrogen bonding exists between glucose dimer and NOB. Specifically, for glucose dimer to form hydrogen bonds, the active sites as donors were oxygen atoms of hydroxyl oxygen on the 59(O) and 79(O), and hydrogen atoms as acceptors are hydrogen atoms of 64(H), 93(H), and 94(H) (Figure 2C). For NOB, the active sites as donors were oxygen atoms of hydroxyl oxygen on the 11(O) and 13(C), and hydrogen atoms as acceptors are hydrogen atoms of 22(H), 23(H), 24(H), and 27(H) (Figure 2C).

The AIM provides a general tool for classifying binding interactions (55). According to AIM topology analysis, charge density (ρ_{BCP}) is used to characterize the bond strength. In general, the higher the value of ρ_{BCP}, the stronger the bonding. The value of Laplacian ($\nabla^2\rho_{BCP}$) indicates the type of interaction between atoms. A negative value ($\nabla^2\rho_{BCP} < 0$) suggests the presence of covalent bonds, while a positive value ($\nabla^2\rho_{BCP} > 0$) indicates that the interaction between atoms is primarily governed by electrostatic interactions (55). Furthermore, the binding interactions may be more accurately described by the energy density (H_{BCP}) of electrons at the bond critical point (BCP) (56, 57). When $H_{BCP} > 0$, then $\nabla^2\rho_{BCP} > 0 > 0$, indicating that the interaction between the two atoms is dominated by electrostatic interactions; if $H_{BCP} < 0$, then two scenarios occur: (1) $\nabla^2\rho_{BCP} < 0$, indicating that the interaction is dominated by covalent bonding interactions; and (2) $\nabla^2\rho_{BCP} > 0$, indicating that the interaction is dominated by electrostatic interactions, but already contains some covalent bonding components. The values of the ρ_{BCP} and $\nabla^2\rho_{BCP}$ are distributed in the range of 0.0015–0.0432 a.u.

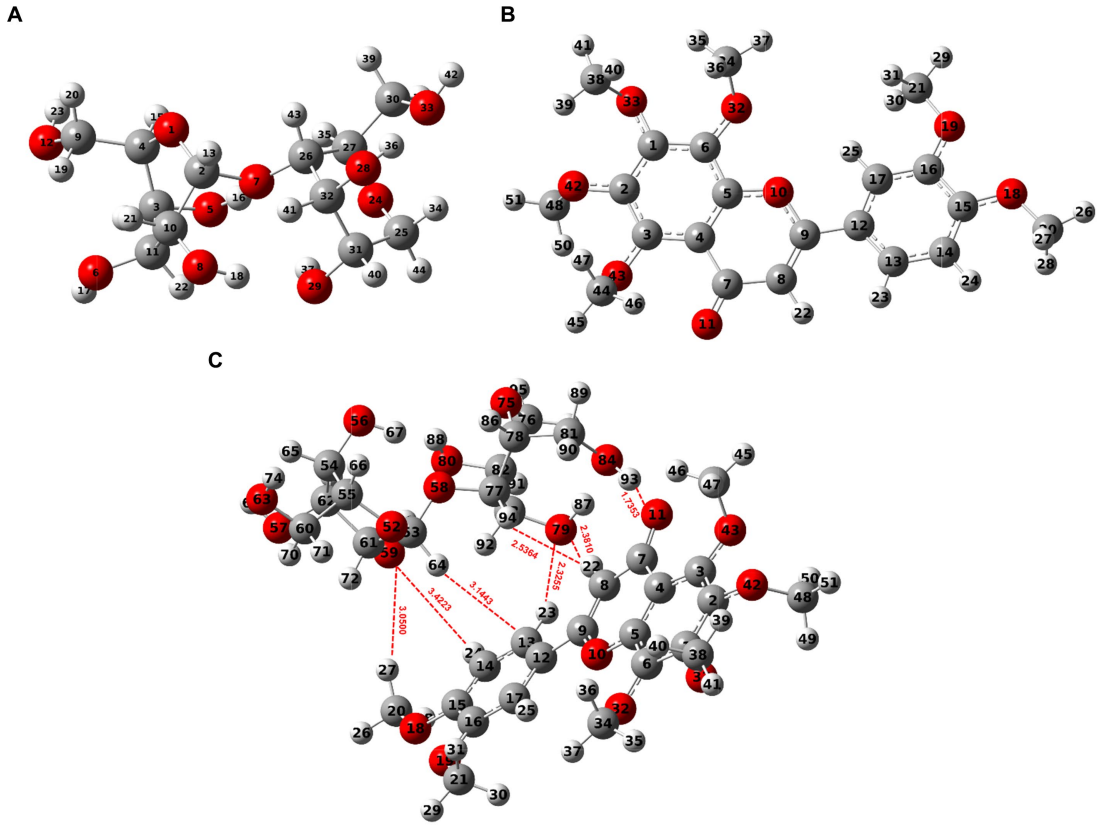


FIGURE 2
Optimized stable conformer of glucose dimer-NOB system [DFT B3lyp/6-31 g(d)]. **(A)** Glucose dimer (Starch). **(B)** NOB. **(C)** Glucose dimer + NOB. The O atom is represented by the red sphere, the C atom by the grey sphere, and the H atom by the white ball. A dotted red line indicates hydrogen bonds. NOB, nobiletin.

and 0.0106–0.1299 a.u., with H_{BCP} >0 [except for 93(H)-11(O)] (Table 4), indicating a predominant reliance on hydrogen bonding to evaluate the complex interactions (58). By employing the empirical hydrogen bond energy formula, it is possible to calculate the binding energy of an intermolecular hydrogen bond: $\Delta E(H) \approx -223.08 \times \rho_{BCP} + 0.7423$ (58). The sum energies of the hydrogen bonds were found to be 14.1026 kcal/mol for the complex of glucose dimer–NOB. These findings indicate the strength of hydrogen bond interactions between NOB and glucose dimers.

3.6 Regulation mechanism of HME with NOB on digestibility of rice starch

Based on the digestibility, thermal properties, multi-scale structure, and theoretical calculations of the above HMERS/NOB samples, Figure 3 depicts the molecular mechanism of NOB in regulating rice starch digestibility during the HME process. When heat energy, shear stress, and cooperative water molecules worked together, they disrupted the α-1, 4 and α-1, 6-glycosidic bonds in rice starch, lowering the molecular weight (38). Hydrogen bond interactions between rice starch molecules and water molecules also resulted in molecular chain depolymerization, the dissolution of the crystalline structures, and the disintegration of the double helix structures (Table 3). Conversely, HME promoted the rearrangement, agglomeration, and synthesis of the starch molecular chain, creating a novel single helical structure, a V-type crystal structure including the lipid within the starch, and a short-range ordered structure (Table 3). These dense domains with a local order hindered the migration of α-amylase in starch molecules and obstructed α-amylase interaction sites. This in turn reduces the RDS content and increases the SDS and RS content in rice starch, thereby enhancing the slow digestibility and resistance to digestion of rice starch (Table 1).

Under HME, the occurrence of intramolecular hydrogen bonding leads to the rotation of the starch chain, resulting in the formation of a left-handed spiral hollow structure (59). When NOB became available, V-type crystalline was formed via hydrophobic interactions in helical cavities. The complex was simultaneously stabilized by hydrogen bonding between the –O–H protons on the starch glucose unit and the –C–H protons of the benzene ring on the NOB (Figure 3). In addition to the common –C–H donor groups found in alkyl and aromatic compounds, various chemicals form unique bonds with specific starch molecules through two or three weak CH-π and hydrogen interactions (Figure 2 and Table 4) (60). The above effects strengthen the structural order of the complex in terms of both short-term and long-term structures (Table 3). Overall, the multi-scale structure of the HMERS/NOB had a better degree of order than that of the HMERS. In addition, there was a slight increase in ΔHg, representing the shear resistance of starch particles at high temperatures, and this trend was more pronounced with increasing NOB contents (Table 2). The order degree of the rice starch–NOB complex was fundamentally improved under HME, which repressed

10.3389/fnut.2024.1398380

TABLE 4 The characteristics of the BCP (a.u.), the lengths of the hydrogen bonds (Å), and the energies of the hydrogen bond interactions for the glucose dimer + NOB.*

Glucose dimer + NOB					
Interaction modles	HB length (Å)	ρ_{BCP}	$\nabla^2\rho_{BCP}$	H_{BCP}	|ΔE(H)| (kcal/mol)
93(H)-11(O)	1.7353	0.0432	0.1299	−0.0018	8.8948
94(H)-22(H)	2.5364	0.0041	0.0147	0.0010	0.1723
79(O)-22(H)	2.3810	0.0125	0.0379	0.0002	2.0462
79(O)-23(H)	2.3255	0.0137	0.0438	0.0002	2.3139
64(H)-13(C)	3.1443	0.0037	0.0106	0.0006	0.0831
59(O)-24(H)	3.4223	0.0015	0.0071	0.0005	0.4077
59(O)-27(H)	3.0500	0.0025	0.0106	0.0007	0.1846

*ρ_{BCP}, electron density; $\nabla^2\rho_{BCP}$, the laplacian value of electron density; H_{BCP}, the energy density of electrons at the BCP point; |ΔE(H)|, the energies of the hydrogen bond interactions; NOB, nobiletin.

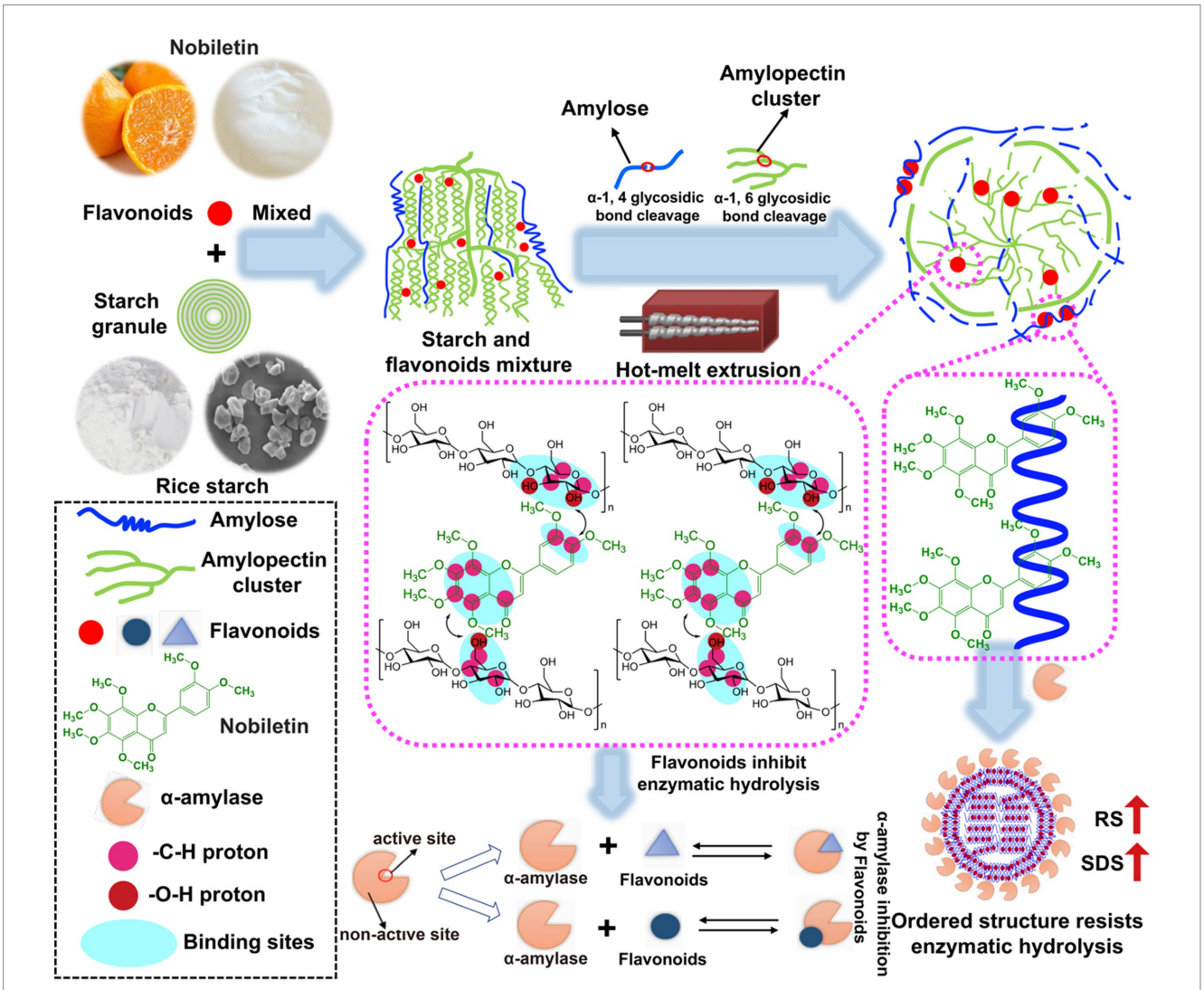


FIGURE 3
Schematic diagram of a model of synergistic modification of HME and NOB to regulate the digestibility and multi-scale structure of rice starch. Magenta circle, -C-H proton; Red circle, -O-H proton; Cyan circle, hydrogen bond position; Large circle, strong correlation of binding sites; Small circle, weak binding site correlation. HME, hot-melt extrusion; NOB, nobiletin.

the limiting of rice starch with α-amylase, and subsequently decreased the C_∞ of rice starch. Even though NOB forms specific ordered domains that resist enzymatic hydrolysis by interacting non-covalently with starch molecules, it also separates during digestion and acts as an enzyme inhibitor. These two synergistic effects significantly enhance the anti-digestibility of rice starch.

4 Conclusion

The study investigated the combined effects of HME and NOB on the *in vitro* digestibility, thermal characteristics, multi-scale structures, and interactions of rice starch. HME was found to decrease both the anti-digestibility and thermal stability of rice starch. However, there are hydrophobic interactions and hydrogen bonding between NOB and starch, and the formation of complexes effectively hinders the digestion of NOB. This interaction disrupted the double helix structures of starch, favoring the formation of single helix structures. The synergistic modification of HME and NOB, rather than HME alone, resulted in an enhanced ordered structure of starch and a slight improvement in ΔHg. These findings highlight the combined influence of HME and NOB on the multi-scale structures and properties of rice starch. Overall, the study suggests that synergistic modification of HME and NOB could enhance the nutritional value and functional properties of rice starch, potentially broadening its high-value applications. However, the anti-digestibility of the complex raises questions about its prebiotic activity. Further research is needed to investigate the fermentation process and prebiotic potential of the complex systematically.

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

ZZ: Data curation, Investigation, Methodology, Software, Writing – original draft. YF: Data curation, Investigation, Methodology, Software, Writing – review & editing. HW: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. HH: Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by the National Natural Science Foundation of China (No. 82304137), Hainan Provincial Natural Science Foundation of China (No. 824MS066), Guangdong Basic and Applied Basic Research Foundation (No. 2022A1515110478), and Talent Project of Hainan Medical University (No. RZ2300002106 and RZ2300005977).

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.2024.1398380/full#supplementary-material

References

1. Guan C, Long X, Long Z, Lin Q, Liu C. Legumes flour: a review of the nutritional properties, physiological functions and application in extruded rice products. *Int J Food Sci*. (2023) 58:300–14. doi: 10.1111/ijfs.16005

2. Cheng Z, Qiao D, Zhao S, Zhang B, Lin Q, Xie F. Whole grain rice: updated understanding of starch digestibility and the regulation of glucose and lipid metabolism. *Compr Rev Food Sci F*. (2022) 21:3244–73. doi: 10.1111/1541-4337.12985

3. Yi X, Li C. Main controllers for improving the resistant starch content in cooked white rice. *Food Hydrocoll*. (2022) 122:107083. doi: 10.1016/j.foodhyd.2021.107083

4. Zhang Z, Bao J. Recent advances in modification approaches, health benefits, and food applications of resistant starch. *Starch Stärke*. (2023) 75:2100141. doi: 10.1002/star.202100141

5. Lazou AE. Food extrusion: an advanced process for innovation and novel product development. *Crit Rev Food Sci*. (2022) 1-29:1–29. doi: 10.1080/10408398.2022.2143474

6. Dendegh TA, Enefola OS, Akpapunam SO, Yelmi BM, Abdullahi MJB. Extrusion technology and its application in food processing-an overview. *Trop J Eng Sci Technol*. (2022) 1:25–53.

7. Bamidele OP, Emmambux MN. Encapsulation of bioactive compounds by "extrusion" technologies: a review. *Crit Rev Food Sci*. (2021) 61:3100–18. doi: 10.1080/10408398.2020.1793724

8. Mohamed IO. Interaction of starch with some food macromolecules during the extrusion process and its effect on modulating physicochemical and digestible properties. A review. *Carbohyd Polym Technol Appl*. (2023) 5:100294. doi: 10.1016/j.carpta.2023.100294

9. Mohamed IO. Effects of processing and additives on starch physicochemical and digestibility properties. *Carbohyd Polym Technol Appl*. (2021) 2:100039. doi: 10.1016/j.carpta.2021.100039

10. Zhang Q, Fan S, Xie H, Zhang Y, Fu L. Polyphenols from pigmented quinoa as potential modulators of maize starch digestion: role of the starch-polyphenol inclusion and non-inclusion complexes. *Food Hydrocoll*. (2023) 144:108975. doi: 10.1016/j.foodhyd.2023.108975

11. Zhang W, Roy S, Ezati P, Yang D, Rhim J. Tannic acid: a green crosslinker for biopolymer-based food packaging films. *Trends Food Sci Tech*. (2023) 136:11–23. doi: 10.1016/j.tifs.2023.04.004

12. Zhang W, Zhu H, Rong L, Chen Y, Yu Q, Shen M, et al. Purple red rice bran anthocyanins reduce the digestibility of rice starch by forming V-type inclusion complexes. *Food Res Int*. (2023) 166:112578. doi: 10.1016/j.foodres.2023.112578

13. Nag S, Majumder S. Starch, gallic acid, their inclusion complex and their effects in diabetes and other diseases—a review. *Food Sci Nutr*. (2023) 11:1612–21. doi: 10.1002/fsn3.3208

14. Raza H, Xu H, Zhou Q, He J, Zhu B-W, Li S, et al. A review of green methods used in starch-polyphenol interactions: physicochemical and digestion aspects. *Food Funct*. (2023) 14:8071–100. doi: 10.1039/D3FO01729J

15. Kan L, Capuano E, Oliviero T, Renzetti S. Wheat starch-tannic acid complexes modulate physicochemical and rheological properties of wheat starch and its digestibility. *Food Hydrocoll*. (2022) 126:107459. doi: 10.1016/j.foodhyd.2021.107459

16. Huang Y, Liu Q, Yu J, Zhang Q, He C, Han L, et al. Electron beam irradiation pretreatment enhances the formation of granular starch-phenolics complexes. *Food Res Int*. (2023) 163:112288. doi: 10.1016/j.foodres.2022.112288

17. Peng Z, Zhang H, Li W, Yuan Z, Xie Z, Zhang H, et al. Comparative profiling and natural variation of polymethoxylated flavones in various citrus germplasms. *Food Chem*. (2021) 354:129499. doi: 10.1016/j.foodchem.2021.129499

18. Zhao Y, Wang M, Huang G. Structure-activity relationship and interaction mechanism of nine structurally similar flavonoids and α-amylase. *J Funct Foods*. (2021) 86:104739. doi: 10.1016/j.jff.2021.104739

19. Arora B, Yoon A, Sriram M, Singha P, Rizvi SSH. Reactive extrusion: a review of the physicochemical changes in food systems. *Innov Food Sci Emerg*. (2020) 64:102429. doi: 10.1016/j.ifset.2020.102429

20. Cummings JH, Beatty ER, Kingman SM, Bingham SA, Englyst HN. Digestion and physiological properties of resistant starch in the human large bowel. *Brit J Nutr*. (1996) 75:733–47. doi: 10.1079/BJN19960177

21. Englyst HN, Kingman SM, Hudson GJ, Cummings JH. Measurement of resistant starch *in vitro* and in vivo. *Brit J Nutr*. (1996) 75:749–55. doi: 10.1079/BJN19960178

22. Butterworth PJ, Warren FJ, Grassby T, Patel H, Ellis PR. Analysis of starch amylolysis using plots for first-order kinetics. *Carbohyd Polym*. (2012) 87:2189–97. doi: 10.1016/j.carbpol.2011.10.048

23. Marta H, Cahyana Y, Djali M. Densely packed-matrices of heat moisture treated-starch determine the digestion rate constant as revealed by logarithm of slope plots. *J Food Sci Tech*. (2020) 58:2237–45. doi: 10.1007/s13197-020-04734-6

24. Goñi I, Garcia-Alonso A, Saura-Calixto F. A starch hydrolysis procedure to estimate glycemic index. *Nutr Res*. (1997) 17:427–37. doi: 10.1016/S0271-5317(97)00010-9

25. Zhu B, Zhan J, Chen L, Tian Y. Amylose crystal seeds: preparation and their effect on starch retrogradation. *Food Hydrocoll*. (2020) 105:105805. doi: 10.1016/j.foodhyd.2020.105805

26. He H, Bian H, Xie F, Chen L. Different effects of pectin and κ-carrageenan on the multiscale structures and *in vitro* digestibility of extruded rice starch. *Food Hydrocoll*. (2021) 111:106216. doi: 10.1016/j.foodhyd.2020.106216

27. Clark T, Chandrasekhar J, Spitznagel GW, Schleyer PVR. Efficient diffuse function-augmented basis sets for anion calculations. III. The 3-21+G basis set for first-row elements, Li–F. *J Comput Chem*. (1983) 4:294–301. doi: 10.1002/jcc.540040303

28. Ditchfield R, Hehre WJ, Pople JA. Self-consistent molecular-orbital methods. IX. An extended Gaussian-type basis for molecular-orbital studies of organic molecules. *J Chem Phys*. (1971) 54:724–8. doi: 10.1063/1.1674902

29. Lu T, Chen F. Multiwfn: a multifunctional wavefunction analyzer. *J Comput Chem*. (2012) 33:580–92. doi: 10.1002/jcc.22885

30. Frisch M., Trucks G., Schlegel H., Scuseria G., Robb M., Cheeseman J., et al. Gaussian 09, Revision B. 04. *Wallingford CT, Pittsburgh, PA: Gaussian Inc*. (2009). Available at: https://gaussian.com/

31. Roman L, Campanella O, Martinez MM. Shear-induced molecular fragmentation decreases the bioaccessibility of fully gelatinized starch and its gelling capacity. *Carbohyd Polym*. (2019) 215:198–206. doi: 10.1016/j.carbpol.2019.03.076

32. Ding Z, Bourven I, Guibaud G, van Hullebusch ED, Panico A, Pirozzi F, et al. Role of extracellular polymeric substances (EPS) production in bioaggregation: application to wastewater treatment. *Appl Microbiol Biot*. (2015) 99:9883–905. doi: 10.1007/s00253-015-6964-8

33. Lemlioglu-Austin D, Turner ND, McDonough CM, Rooney LW. Effects of Sorghum [Sorghum bicolor (L.) Moench] crude extracts on starch digestibility, estimated glycemic index (EGI), and resistant starch (RS) contents of porridges. *Molecules*. (2012) 17:11124–38. doi: 10.3390/molecules170911124

34. Cheng L, Hu Y. Combination of parallel and sequential digestion kinetics reveals the nature of digestive characteristics of short-term retrograded rice starches. *Food Hydrocoll*. (2020) 108:106071. doi: 10.1016/j.foodhyd.2020.106071

35. Li H, Gui Y, Li J, Zhu Y, Cui B, Guo L. Modification of rice starch using a combination of autoclaving and triple enzyme treatment: structural, physicochemical and digestibility properties. *Int J Biol Macromol*. (2020) 144:500–8. doi: 10.1016/j.ijbiomac.2019.12.112

36. Ge X, Shen H, Sun X, Liang W, Zhang X, Sun Z, et al. Insight into the improving effect on multi-scale structure, physicochemical and rheology properties of granular cold water soluble rice starch by dielectric barrier discharge cold plasma processing. *Food Hydrocoll*. (2022) 130:107732. doi: 10.1016/j.foodhyd.2022.107732

37. van Soest JJG, Tournois H, de Wit D, Vliegenthart JFG. Short-range structure in (partially) crystalline potato starch determined with attenuated total reflectance Fourier-transform IR spectroscopy. *Carbohydr Res*. (1995) 279:201–14. doi: 10.1016/0008-6215(95)00270-7

38. He H, Chi C, Xie F, Li X, Liang Y, Chen L. Improving the *in vitro* digestibility of rice starch by thermomechanically assisted complexation with guar gum. *Food Hydrocoll*. (2020) 102:105637. doi: 10.1016/j.foodhyd.2019.105637

39. Tanner SF, Ring SG, Whittam MA, Belton PS. High resolution solid state 13C nmr study of some α (1–4) linked glucans: the influence of water on structure and spectra. *Int J Biol Macromol*. (1987) 9:219–24. doi: 10.1016/0141-8130(87)90056-0

40. Cai J, Man J, Huang J, Liu Q, Wei W, Wei C. Relationship between structure and functional properties of normal rice starches with different amylose contents. *Carbohyd Polym*. (2015) 125:35–44. doi: 10.1016/j.carbpol.2015.02.067

41. Yong H, Wang X, Sun J, Fang Y, Liu J, Jin C. Comparison of the structural characterization and physicochemical properties of starches from seven purple sweet potato varieties cultivated in China. *Int J Biol Macromol*. (2018) 120:1632–8. doi: 10.1016/j.ijbiomac.2018.09.182

42. Chi C, Li X, Zhang Y, Chen L, Li L, Wang Z. Digestibility and supramolecular structural changes of maize starch by non-covalent interactions with gallic acid. *Food Funct*. (2017) 8:720–30. doi: 10.1039/C6FO01468B

43. Bordenave N, Hamaker B, Ferruzzi M. Nature and consequences of non-covalent interactions between flavonoids and macronutrients in foods. *Food Funct*. (2014) 5:18–34. doi: 10.1039/C3FO60263J

44. Tan I, Flanagan BM, Halley PJ, Whittaker AK, Gidley MJ. A method for estimating the nature and relative proportions of amorphous, single, and double-helical components in starch granules by 13C CP/MAS NMR. *Biomacromolecules*. (2007) 8:885–91. doi: 10.1021/bm060988a

45. Liu Y, Chen J, Luo S, Li C, Ye J, Liu C, et al. Physicochemical and structural properties of pregelatinized starch prepared by improved extrusion cooking technology. *Carbohyd Polym*. (2017) 175:265–72. doi: 10.1016/j.carbpol.2017.07.084

46. Van Soest JJG, Hulleman SHD, De Wit D, Vliegenthart JFG. Crystallinity in starch bioplastics. *Ind Crop Prod*. (1996) 5:11–22. doi: 10.1016/0926-6690(95)00048-8

47. Liu W, Halley PJ, Gilbert RG. Mechanism of degradation of starch, a highly branched polymer, during extrusion. *Macromolecules*. (2010) 43:2855–64. doi: 10.1021/ma100067x

48. Campos CA, Gerschenson LN, Flores SK. Development of edible films and coatings with antimicrobial activity. *Food Bioprocess Tech*. (2011) 4:849–75. doi: 10.1007/s11947-010-0434-1

49. Si H, Zhao X, Zhang Z, Liao Q, Zhang Y. Low-temperature electron-transporting materials for perovskite solar cells: fundamentals, progress, and outlook. *Coordin Chem Rev*. (2024) 500:215502. doi: 10.1016/j.ccr.2023.215502

50. Lopez-Rubio A, Flanagan BM, Gilbert EP, Gidley MJ. A novel approach for calculating starch crystallinity and its correlation with double helix content: a combined XRD and NMR study. *Biopolymers*. (2008) 89:761–8. doi: 10.1002/bip.21005

51. Akman F, Kazachenko AS, Vasilyeva NY, Malyar YN. Synthesis and characterization of starch sulfates obtained by the sulfamic acid-urea complex. *J Mol Struct*. (2020) 1208:127899. doi: 10.1016/j.molstruc.2020.127899

52. Kromann JC, Christensen AS, Steinmann C, Korth M, Jensen JH. A third-generation dispersion and third-generation hydrogen bonding corrected PM6 method: PM6-D3H+. *Peer J*. (2015) 2:e449. doi: 10.7287/peerj.preprints.353v1

53. Lu T. (2019). *Molclus program, version 1.8*. Beijing Kein Research Center for Natural Science, 2205–2211. Available at: http://www.keinsci.com/research/molclus.html

54. Deshmukh MM, Gadre SR. Molecular tailoring approach for the estimation of intramolecular hydrogen bond energy. *Molecules*. (2021) 26:2928. doi: 10.3390/molecules26102928

55. Kutzelnigg W. Atoms in molecules. A quantum theory.(Reihe: international series of monographs on chemistry, Vol. 22.) Von RFW Bader. Clarendon press, Oxford, 1990. XVIII, 438 S., geb.£ 50.00.–ISBN 0-19-855168-1. Angew. *Chem*. (1992) 104:1423–3. doi: 10.1002/ange.19921041040

56. Bader RFW, Keith TA, Gough KM, Laidig KE. Properties of atoms in molecules: additivity and transferability of group polarizabilities. *Mol Phys*. (1992) 75:1167–89. doi: 10.1080/00268979200100901

57. Wang Y, Li H, Han S. The chemical nature of the C–H⊕···X– (X=cl or Br) interaction in imidazolium halide ionic liquids. *J Chem Phys*. (2006) 124:044504. doi: 10.1063/1.2161174

58. Zhang Y, Hao J, Wang X, Zhou W, Tang T. A theoretical study of some pseudo-π hydrogen-bonded complexes: cyclopropane·HCl and tetrahedrane·HCl. *J Mol Struct*. (1998) 455:85–99. doi: 10.1016/S0166-1280(98)00247-4

59. Obiro WC, Ray SS, Emmambux MN. V-amylose structural characteristics, methods of preparation, significance, and potential applications. *Food Rev Int*. (2012) 28:412–38. doi: 10.1080/87559129.2012.660718

60. Nishio M, Umezawa Y, Fantini J, Weiss MS, Rti CB, Chakrabarti P. CH–π hydrogen bonds in biological macromolecules. *Phys Chem Chem Phys*. (2014) 16:12648–83. doi: 10.1039/C4CP00099D

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 25 September 2024
DOI 10.3389/fnut.2024.1424112

OPEN ACCESS

EDITED BY
Xianyang Bao,
Harvard University, United States

REVIEWED BY
Elisa Julianti,
Department of Food Science Universitas Sumatera Utara, Indonesia
Nirmal Mazumder,
Manipal Academy of Higher Education, India

*CORRESPONDENCE
Prabhjot Kaur
✉ prabhjotchahal72@yahoo.in

RECEIVED 27 April 2024
ACCEPTED 09 September 2024
PUBLISHED 25 September 2024

CITATION
Chauhan S, Kaur H, Aggarwal R, Kaur P and Bains K (2024) Exploring the impact of cooking techniques and storage conditions on resistant starch levels in mung beans and its effect upon blood glucose level and lipid profile *in vivo*. *Front. Nutr.* 11:1424112. doi: 10.3389/fnut.2024.1424112

Exploring the impact of cooking techniques and storage conditions on resistant starch levels in mung beans and its effect upon blood glucose level and lipid profile *in vivo*

Saloni Chauhan, Harpreet Kaur, Renuka Aggarwal, Prabhjot Kaur* and Kiran Bains

Department of Food and Nutrition, Punjab Agricultural University, Ludhiana, Punjab, India

Introduction: Mung beans contain various antinutritional components. Processing and cooking methods can reduce these antinutritional factors and increase the availability and digestibility of nutrients. Resistant starch is also known as dietary fiber, which helps to reduce the cholesterol and glucose level in blood. It is formed during cooking and storage of food at low temperature.

Objectives: This study aimed to assess the effects of cooking and storage temperature on the formation of resistant starch in processed mung bean, as well as its effect on blood glucose levels and lipid profile in humans and rats.

Methods: The common cooking methods namely boiling, steaming after germination, roasting, and pressure cooking were chosen. The cooked samples were stored at different temperatures including freshly prepared within 1 h (T1), stored for 24 h at room temperature (20–22°C) (T2), kept at 4°C for 24 h (T3), and reheated after storing at 4°C for 24 h (T4).

Results: The study revealed that germinated-steamed mung beans had significantly higher levels of resistant starch (27.63 ± 0.76), and lower level of glycemic index (26.28 ± 3.08) and amylose (40.91 ± 0.06) when stored at 4°C for 24 h (T3) followed by (T2), (T4), and (T1) as compared to other cooking methods (boiling, pressure cooking, and roasting). The germinated-steamed mung beans (T1) resulted in 96% decline in blood glucose parameters of rats (36 Wistar albino rats aged 2 to 3 months were selected) than the control group as observed in 28 days diet intervention (100 mg/kg resistant starch orally).

Conclusion: There is a need to make people aware about the selection of appropriate cooking (steamed after germination) and storage methods (T3) to increase the RS content and to lower the glycemic index of food at domestic level.

KEYWORDS

cooking methods, dietary fiber, glycemic index, processed mung bean, resistant starch, storage temperature

1 Introduction

Worldwide, Legumes are recognized as the second most significant human food crop, after cereals. Mung bean (*Vigna radiata* L.) is a significant edible legume in several Asian nations, such as India, and is an excellent source of proteins (20–24%), carbohydrates (50–60%), crude fiber (3.8–6.2%), and lipids (0.7–1.9%) and substantial quantities

of micronutrients, Mung beans contain various antinutritional components, such as hemagglutinins, phytic acid, phenolics, trypsin inhibitors, tannins, oligosaccharides, saponins, and phytic acid, in addition to their nutritional value. Processing and cooking methods can reduce these anti-nutritional factors and increase the availability and digestibility of nutrients, according to various studies. However, in addition to their antioxidant, antimicrobial, anti-inflammatory, anti-diabetic, antihypertensive, and anti-cancerous properties, these antinutritional factors also possess potent health benefit (20, 30). Mung bean seeds are predominately composed of starch, constituting a substantial proportion of the dried matter, ranging from 37 to 58% and it has been reported that mung bean starch contains a substantial proportion of resistant starch and amylose (30–45%) (7, 12, 21).

Resistant starch (RS) is comprised of alpha-linked glucose molecules unaffected by digestive enzymes in the small intestine, passing straight to the large intestine, where the gut microbiota ferments it. Based on properties that permit it to resist digestion, resistant starch is classified into four groups. RS type 1 (RS 1) is present in whole cereals and legumes and is unapproachable to enzymes that help digestion because a defensive matrix encloses it. RS type 2 (RS 2) is found in bananas (unripe), potatoes (uncooked), and maize. It has starch with high amylose and solid starch particles that are ungelatinized. RS type 3 (RS 3) are retrograded starches formed when starchy foods are cooled after cooking. RS type 4 (RS 4) is found in processed foods and is formed through cross-linking of starch chemically by adding ether and ester groups. Elongated and unbranched starch chains combine with free fatty acids to form the final type, known as RS 5. This combination forms a helical structure that makes it difficult to digest. Additionally, RS 5 includes resistant maltodextrin, a novel dietary fiber that is non-viscous and produced by deliberately rearranging starch molecules (33, 41, 42).

Resistant starch undergoes a high degree of fermentation anaerobically by resident microbiota into hydrogen, carbon dioxide, methane, and short-chain fatty acids, i.e., acetate, propionate, and butyrate, upon entering the colon. The foremost short-chain fatty acid produced from resistant starch is butyrate, which is involved in maintaining the homeostasis of the intestine. Pulses and potatoes are the best natural sources of resistant starch. The intake of resistant starch increases satiety and whole-body insulin sensitivity, reducing storage of fat, postprandial glycaemic and insulinemic responses, plasma cholesterol, and triglyceride concentrations. Resistant starch also seems to function as a prebiotic by supporting the growth of probiotic microorganisms. Regular consumption of pulses is linked with better glycemic control and lipid metabolism indicators and lower body weight (23, 35, 39, 49). Due to urbanization and exposure to social media, consumers are becoming more aware of the relationship between diet and disease. Consequently, the food industry is making efforts to produce functional foods based on different cereals, wholegrain flour, and low-glycemic-index foods.

The resistant starch content of pulses is affected by cooking and storage time and temperature; however, cooking methods, including steaming, baking, and boiling, are especially recommended to increase the amount of resistant starch in food (32). In this way, the prebiotic content of food is also improved artificially, leading to positive outcomes on health (15, 45). The amount of resistant starch in food can be increased by changing the processing parameters, such as the number of heating and cooling cycles, pH, moisture, pressure, temperature, time, freezing, and drying (18).

In the Indian diet, different pulses and legumes are used as whole grains, in the form of *dhal* (decorticated split legumes) and legume flour, in various preparations. Considering the regular intake of mung bean among Indians and many other Asians, the present study was conducted to investigate the amount of RS formed in mung bean after cooking with different techniques. Also, in today's busy world, people cook the food and store it for their convenience, the study evaluated the effect of different storage conditions including the variations in temperature and its effect on the RS content of the dhal. The effect of RS was also studied *in vivo* to investigate the efficacy of resistant starch to improve blood glucose levels.

2 Materials and methods

2.1 Procurement and cooking

The commonly consumed Indian mung bean variety (ML 2056) was procured from the department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The grains were cleaned and ground using a sample mill with a 60-mesh size for making flour. Four common cooking methods used by North Indians, i.e., roasting for 10 min at 100°C, boiling for 30 min at 100°C, germination (48h) then steamed for 5 min, and pressure cooking (with 6 h soaking in tap water) at 100°C and at 15 lbs pressure for 8 min, were selected. These four cooking methods applied to mung bean seeds and these seeds was stored at different conditions of storage, which were considered four treatments, i.e., freshly prepared within hour (h; T1), stored for 24 h at room temperature (20–22°C; T2), stored at 4°C for 24 h (T3), and lastly reheated after storing at 4°C for 24 h (T4). After the treatments, seeds were dried and milled for making flour and used for nutritional analysis (Figure 1).

2.2 Nutritional analysis

The nutritional analysis of raw and cooked samples was conducted using standardized protocols to determine the levels of crude protein and crude fat (Code for crude protein AOAC 2001.11 and crude fat AOAC 945.16) (2). The Macro-Kjeldahl method was employed to calculate the nitrogen content and estimate the crude protein levels. Subsequently, nitrogen was converted into crude protein using a conversion factor of 6.25. Crude fat was estimated using soxhlet assembly. Thimbles were used to be moisture-free. The fat was extracted using petroleum ether as a solvent. The apparatus was set to a temperature of 150°C for a period of 30 min to extract fat. The beakers were placed on a heated plate to evaporate the ether, and after cooling, the beakers were weighed for fat content.

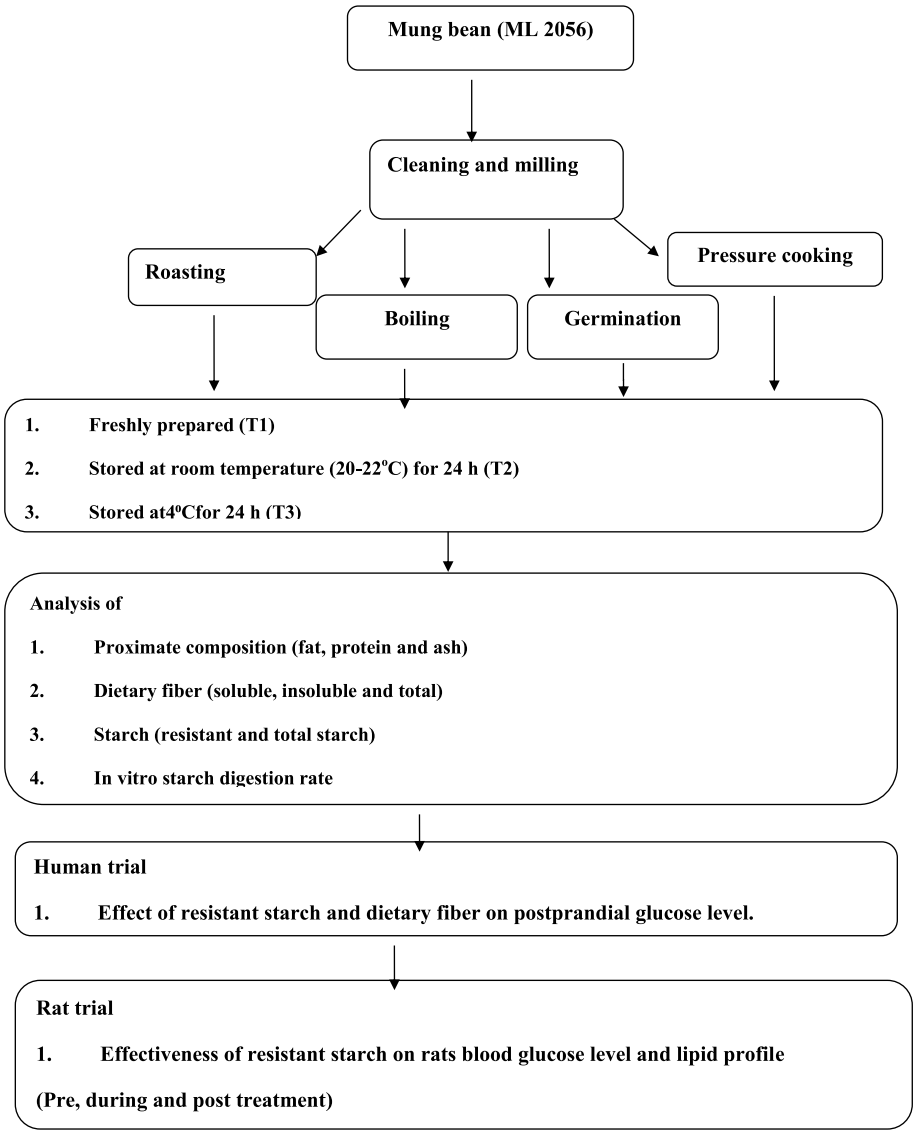


FIGURE 1
Methodology of the study.

2.3 Dietary fiber

The total amount of dietary fiber was determined using a Megazyme total dietary fiber (K-TDFR-200A) kit. The standard method provided by AOAC (2) was also employed to examine the composition of both soluble and insoluble dietary fiber. The dietary fiber was determined using the following formula:

$$\text{Dietary fiber (\%)} = \frac{\frac{R_1+R_2}{2} - p - A - B}{\frac{m_1+m_2}{2}} \times 100$$

Where:

R_1 = residue weight 1 from mL, R_2 = residue weight 2 from m_2, m_1 = sample weight 1, m_2 = sample weight 2, A = ash weight from R_1, p = protein weight from R_2 and

$$B = \text{blank} = \frac{BR_1 + BR_2}{2} - BP - BA$$

Where:

BR = blank residue, BP = blank protein from BR1, and BA = blank ash from BR2.

2.4 Total starch and resistant starch

Using a megazyme K-RSTAR test kit given by AOAC (3). A calculation of both total and resistant starches was done. In order to determine the total amount of starch, both solubilized (non-resistant) starch and resistant starch were added.

2.5 *In vitro* starch digestion rate

Using the methodology outlined in reference (43), *in vitro* starch digestion rate was determined. A volume of 1 mL of synthetic saliva (carbonate buffer "Sigma A-3176" Type VI-B) was used to expose 500 mg of the sample to 250 U (Unit) porcine amylase for 15.2–20 s. The solution was incubated at 37°C for 30 min after 5 mL of pepsin (breaks down proteins into smaller peptides and amino acids, it helps digest the proteins in food; 1 mL per mL of 0.02 M aq. (aqueous) HCl; obtained from gastric porcine mucosa; Sigma P-6887) was added. Prior to the pH-6 adjustment, the digest was neutralized with 5 mL of 0.02 M aq. sodium hydroxide (52.5 mL of $C_2H_3NaO_2$ buffer at 0.2 M). The addition was made of 2 mg/mL of pancreatin (Sigma P1750 derived from porcine pancreas) and 5 mL of amyloglucosidase (It is an enzyme that can break down the α-1,4 glycosidic bonds in starch, specifically at the non-reducing ends, resulting in the production of glucose; Sigma A-7420 from *Aspergillus niger*; 28 U per mL of acetate buffer). After incubating the solution for 4 h, the glucose concentration of the digest was monitored at various intervals with an Accucheck glucometer.

2.6 Rapidly digestible starch and slowly digestible starch

The glucometer value taken at 15 min was used to calculate the percentage of starch digested using a specific equation (43),

Where:

$$DS = \frac{0.9 \times G_G \times 180 \times V}{W \times S[100 - W]}$$

GG = Reading of Glucometer (mM/L).

180 = molecular weight of glucose.

W = sample weight (g).

V = Digest volume (mL).

S = starch content (dry sample g/100 g).

M = %age of moisture (g/100 g).

0.9 = starch stoichiometric constant from glucose concentrations.

RDS% = %age of starch digested at 15 min.

SDS% = subtracting the %age of starch digested at 15 min from the %age of starch digested at 120 min.

2.7 Amylopectin and amylose

By using a colorimetric estimation of the amylose-iodine complex, the amylose content was determined (22). A defatted sample weighing 100 mg was combined with 1 mL of distilled ethanol in a boiling tube. Following the addition of 9 mL of 1 N sodium hydroxide to the tube, it was submerged in a water bath set to simmer for 10 min. Following the preparation of a 100-mL volume, 5 mL was transferred to a 100-ML volumetric vial. After combining this with 2 mL of iodine solution (1 g KI/500 ml distilled water) and 1 mL of 1 N acetic acid (MP Biomedicals), the mixture was left in the dark for 20 min. At 620 nm, the absorbance was measured using a blank solution that was made up of 5 mL of 0.09 N NaOH, 1 mL of acetic acid, and 2 mL of iodine solution. The volume was then set to 100 mL. The formula for amylopectin is 100 minus amylose.

TABLE 1 Sample size: total 10 females were given different treatment diet for 28 days.

Group	Treatment diet	Number (*n*)
Group-I- (G1)	Steamed after Germinated *mung bean*	10
Group-II-(G2)	Boiled *mung bean* sample after reheating	10
Group-III-(G3)	Boiled *mung bean* stored at 4°C	10
Group-IV-(G4)	Boiled *mung bean* sample after freshly prepared	10
Group-V-(G5)	Pressure cooked *mung bean* stored at 4°C	10
Group-VI-(G6)	Pressure cooked *mung bean* after reheating	10
Group-VII-(G7)	Pressure cooked *mung bean* after freshly prepared	10

2.8 Effect of resistant starch and soluble fiber components on postprandial glucose response by glycemic index measurement

The human supplementation was done to study the impact of processed mung beans with different treatments on the glycemic index of females. The human study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The research was carried out in adherence to the guidelines and permission provided by the Institutional Ethics Committee of Punjab Agricultural University, Ludhiana, Punjab, India. All the procedures were performed in compliance with the relevant laws. The participants were made aware of the study protocols, and informed consent was obtained prior to the study. The glycemic index of the subjects was determined using the method given by Goni et al. (19).

2.8.1 Research participants and data collection

Ten female individuals aged 24–28 years were selected randomly from the girl's hostel (because of the convenience to implement study and to get unbiased results from the same participants till the end of research) at the Punjab Agricultural University in Ludhiana for measurement of blood glucose levels (Table 1). The food (containing 50 gm of carbohydrate) cooked and stored with different treatments was given in the morning after 12 h of fasting to seven different experimental groups (details below at 2.8.2), and 15 min were given to finish the meal. Blood samples were obtained using a finger-prick using a glucometer (Dr. Morphine). Blood glucose levels were assessed at specific time intervals (0, 15, 30, 45, 60, 90, and 120 min) following the consumption of 50 grams of carbohydrates from cooked Mung beans (boiled, steamed after germination, and pressure-cooked). To assess the impact of the prepared meal on blood glucose levels, 50 g of glucose was administered to a separate group of females serving as the control group. Volunteers were permitted to have 150–300 mL of water

based on the food they had throughout the trial. The glycemic index was determined using a specific formula given by Wolever (49).

$$GI\ (Glycemic\ index) = \frac{Area\ under\ the\ curve\ for\ 50gm\ carbohydrate\ for\ test\ sample}{Area\ under\ the\ curve\ for\ 50gm\ carbohydrates\ from\ control\ (glucose)} \times 100$$

2.8.2 Ethical measure

The research was conducted with the approval of the Institutional Ethic Review Committee of the Punjab Agricultural University.

2.8.3 Data analysis and outcome measures

Blood glucose level was assessed to calculate the glycemic index. The data was analyzed using SAS/STAT software. Mean ± Standard Deviation (S.D) for various parameters were analyzed. The data was analyzed by using analysis of Variance (ANOVA) for glycemic Index. Values were considered statistically significant at $p < 0.01$.

2.9 Effect of resistant starch on blood glucose level and lipid profile in *albino* male rats

The human supplementation study showed that mung beans treated with various methods had a low glycemic index. We hypothesized that foods with a lower glycemic index may positively impact diabetes treatment by potentially increasing insulin secretion or decreasing insulin sensitivity, and resistant starch as a form of dietary fiber may help to regulate lipid levels. For this, we conducted an animal trial using wistar rats to obtain authentic, real, and unbiased data. Moreover, rats are also biologically and genetically like humans, and their behavioral characteristics are strikingly similar.

2.9.1 Inclusion and exclusion criteria of rats

No need of including extra rats in any group and excluding animals during the experiment and data points during analysis.

2.9.2 Randomization

From the animal house and breeding center (AHBC) of Akal College of Pharmacy and Technical Education Mastuana Sahib, Sangrur (a registered breeder of CCSEA), 36 Wistar albino rats weighing between 180 and 220 g and aged 2–3 months were obtained. These rats were then randomly divided into six groups, with each group containing six rats. The research was carried out in adherence to the guidelines and permission provided by the Institutional Animal Ethics Committee (IAEC No. GADVASU/2023/1AEC/68/15). The animals were confined to enclosures, provided with water *ad libitum*, and fed commercial pellets. During the duration of the experiment, the animals exhibited a high degree of adaptability to the standard environmental conditions, which included temperature (22 ± 5°C), humidity (55 ± 5%), and 12-h light-dark cycles.

TABLE 2 Sample size: total 36 albino male rats with six rats in each group.

Group	Treatment diet	Number (*n*)
Group-I- Normal control (G1)	Standard diet	6
Group-II- Diabetic control (G2)	Standard diet	6
Group-III- Treatment group (G3)	Whole germinated steamed *mung bean* diet (MSG)	6
Group-IV- Treatment group (G4)	Pressure cooked *mung bean* after freshly prepared diet (MPI)	6
Group-V- Treatment group (G5)	Pressure cooked *mung bean* after stored at 4°C (MPRF)	6
Group-VI- Treatment group (G6)	Pressure cooked *mung bean* after reheat (MPR)	6

2.9.3 Blinding/masking

The investigators' roles were as follows: the first investigator gave the treatment according to the randomization table. This investigator was the sole individual informed about the treatment group throughout the allocation and execution of the experiment. A second investigator was responsible for the outcome assessment whereas a third investigator (also unaware of treatment) assessed data.

2.9.4 Study design

Thirty-six Wistar albino male rats aged 2–3 months with a weight of 180–220 g was divided into six groups. Group I was the control and was given AIN96M (American Institute of Nutrition Rodent Diets), i.e., the standard diet (42). Five different experimental groups, from Group II to Group VI, Mung beans processed with different treatments through oral feeding in the form of pellets were given. Foods were prepared according AIN96M (American Institute of Nutrition Rodent Diets) (Table 2). Different diets, such as normal or standard diet (STTD), whole germinated steamed mung bean diet (MSG), pressure cooked mung bean after freshly prepared diet (MPI), pressure cooked mung bean after storage at 4°C (MPRF), and pressure-cooked mung bean after reheating (MPR; detailed at 2.9.2). Rats were made diabetic by injecting them intraperitoneally with 230 mg/kg Nicotinamide (NA) in buffered saline NaCl 0.9%. After a 15-min interval, rats received a second injection of 60 mg/kg of streptozocin (STZ). Rats were given a 5% glucose solution in water for 24 h after receiving the injection to prevent hypoglycemia in groups II to VI. A window of 5 days was taken and considered as the rest period for rats. To ensure hyperglycemia, the blood glucose level, insulin level, and lipid level of the rats were measured. Blood glucose levels >200 mg/dL were considered the cutoff value for hyperglycemia. Rats were treated with a treatment diet for 28 days, and blood glucose levels were measured in the 1st, 3rd, and 4th weeks, while serum insulin and lipid profiles were measured at the beginning and end of the last week of the experiment (1, 17, 36, 37, 45, 47). Following

the completion of the experiment and blood sample collection, the animals were not left untreated after the experimental procedures. The rats were euthanized by administering anesthesia (CO_2, Isoflurane, Ketamine) method given by AVMA Guidelines for Euthanasia of animals. In accordance with university protocol, the carcasses were disposed of in a manner that ensured compliance with ethical and safety guidelines.

2.9.5 Statistical methods

The data was analyzed using SAS/STAT software. Mean ± Standard Deviation (S.D) for various parameters were analyzed. The change in blood glucose was analyzed by using Tukey's test in factorial CRD while the *t*-test was used to assess lipid profile and body weight. Values were considered statistically significant at $p < 0.01$ (Tables 6, 7A, B).

3 Results

3.1 Crude protein and fat

The crude protein content was found to be highest in germinated mung bean, where a higher value was observed in the sample having T3 (kept at 4°C for 24 h) 32.13, followed by T2 (stored for 24 h at room temperature) 31.86, T4 (reheated after stored for 24 h at room temperature) 30.46, and T1 (freshly prepared) 31.07. It was observed that all the mung bean samples cooked with boiling, roasting, and pressure cooking also had higher protein content with T3, while all the cooked mung bean samples with T1 were found to have the lowest protein content. Contrary to the protein content, all the germinated mung bean samples had a lower fat content with different treatments. The highest fat content of 1.44 g was observed in the roasted sample with T3. However, it was also seen that T3 raised the protein and fat content of the mung bean cooked with different cooking techniques (Table 3).

3.2 Dietary fiber

The soluble dietary fiber content was found to be highest in boiled mung bean (6.25%), while insoluble (28.47%) and total dietary fiber (30.48%) were highest in pressure-cooked mung bean. Storing processed mung beans at various temperatures affected the dietary fiber content. The insoluble and total dietary fiber content increased with storage and was observed to be highest in products stored at 4°C (T3; 28.47, 30.48%), while the soluble fiber content was higher in freshly prepared samples (T1; 6.25%; Table 4).

3.3 Resistant starch

The resistant starch content of raw samples (7.1%) increased after cooking except roasting, and the amount of resistant starch was found to be highest in germinated mung beans (17.5%), followed by boiling (12.58%), pressure cooking (8.36%), and roasting (4.28%). Processed mung bean stored at T3 had a higher amount of resistant starch content (27.63%), followed by T2 (23.44%), T4 (25.76%), and a lesser amount in freshly prepared products at T1 (17.05%; Figure 2). It was observed that all the treatments resulted in an increase in the RS content, while T3 resulted in the maximum percent increase in resistant starch content of all the differently cooked mung bean samples (Figure 3). The RS content increased from 4.28 to 84.69% with cooking and storage temperature and duration, indicating that time and temperature are two important factors in changing the RS content in a food sample.

3.4 *In vitro* starch digestion rate

The *in vitro* starch digestion rate is important in assessing a food product's ability to impact an individual's blood glucose levels. Differences in the rate and degree of starch hydrolysis affect the metabolic response to numerous starch-rich meals (13).

The *in vitro* starch digestion rate of mung bean products (boiled, steamed, germinated mung bean and pressure-cooked) affected by different storage temperatures has been shown in Figures 4A, B. The digestion rate of starch was determined at 120 min after the digestion of the food products. The germinated and boiled mung bean stored at 4°C(T3) and reheated after being stored at 4°C for 24 h (T4) had a slower digestion rate of 16.8, 27.7%, and 29.7% at 120 min compared to the freshly prepared that had completed digestion rate of 33.8% at 120 min. In pressure-cooked mung bean, the rate of starch digestion was lower in refrigerated and reheated products after being stored at 4°C for 24 h products (27.7 and 30.6%) at 120 min as compared to freshly prepared products (38.0%) at 120 min. It was observed that the starch digestion rate of pressure cooked and boiled mung bean was higher in freshly prepared products as compared to those stored at 4°C and reheated products.

3.5 Total starch, rapidly digestible starch (RDS %) and slowly digestible starch (SDS %)

The total starch content was observed maximum in the germinated mung bean with the highest value found in T3. On the contrary, the mean percent of rapidly digested starch and slowly digestible starch was significantly (<0.001*) higher in T1 pressure-cooked mung bean (22.19 and 13.94%), followed by boiled (17.10 and 6.41%), and germinated (11.65 and 1.28%). Whereas, in mung bean, the results observed that the mean percentage of slowly digested starch, when different processing methods were compared, found that the maximum value was found in pressure-cooked samples (13.94%), which was significantly (0.001*) higher than boiled (6.41%) and germinated samples (1.28%).

3.6 Amylose and amylopectin

The amount of amylose in boiled mung bean samples with T3 (38.84%) was found to be significantly (≤0.001) greater than that of T4 (37.97%), T1 (36.43%), and T2 (37.33%). Higher amylose content was also observed with T4 and cooked with different techniques i.e., roasting and pressure cooking. Contrary to this, higher amylopectin content was observed in all the samples receiving treatment T1 (Table 5).

TABLE 3 Crude protein and fat content of mung bean (g/100 gm, dry weight basis) statistical analysis was conducted both by rows or columns and factorial CRD (completely randomized design) was applied.

Mung bean	Treatment	T1	T2	T3	T4	LS mean
	Boiled	24.56 ± 0.02	25.04 ± 0.02	26.04 ± 0.02	25.43 ± 0.01	25.27^{C}
	Roasted	24.05 ± 0.03	24.45 ± 0.02	24.83 ± 0.08	24.12 ± 0.02	24.36^{D}
	Germinated	31.07 ± 0.01	31.86 ± 0.02	32.13 ± 0.03	30.46 ± 0.02	31.38^{A}
	Pressure cooked	24.87 ± 0.04	25.54 ± 0.02	26.27 ± 0.01	25.37 ± 0.01	25.51^{B}
LS mean		26.13^{D}	26.72^{B}	27.31^{A}	26.35^{C}	
Fat						
	Boiled	1.05 ± 0.03	1.17 ± 0.01	1.27 ± 0.01	1.05 ± 0.01	1.14^{C}
	Roasted	0.97 ± 0.01	1.24 ± 0.02	1.44 ± 0.02	1.12 ± 0.02	1.19^{A}
	Germinated	0.65 ± 0.01	0.81 ± 0.01	0.87 ± 0.01	0.74 ± 0.03	0.77^{D}
	Pressure cooked	1.07 ± 0.01	1.16 ± 0.02	1.27 ± 0.01	1.13 ± 0.01	1.16^{B}
LS mean		0.94^{D}	1.09^{B}	1.21^{A}	1.01^{C}	

Effect of different cooking methods and storage temperature in the formation of protein and fat content in mung bean products. Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. T1—freshly prepared sample, T2—sample stored at room temperature for 24 h, T3—sample stored at 4°C for 24 h after cooking, and T4—reheating sample after stored at 4°C for 24 h.

TABLE 4 Dietary fiber content (soluble, insoluble, and total dietary fiber) of mung bean (g/100 gm, dry weight basis).

Mung bean	Treatment	T1	T2	T3	T4	LS mean
Soluble dietary fiber						
	Boiled	6.25 ± 0.05	6.02 ± 0.02	5.84 ± 0.02	6.22 ± 0.01	6.08^{A}
	Roasted	0.15 ± 0.03	1.10 ± 0.00	0.93 ± 0.04	1.16 ± 0.02	0.84^{D}
	Germinated	3.45 ± 0.04	3.20 ± 0.01	3.00 ± 0.01	3.41 ± 0.01	3.27^{B}
	Pressure cooked	2.61 ± 0.01	2.21 ± 0.01	2.01 ± 0.01	3.41 ± 0.01	2.56^{C}
	LS mean	3.12^{B}	3.13^{B}	2.95^{C}	3.55^{A}	
Insoluble dietary fiber						
	Boiled	19.85 ± 0.03	21.90 ± 0.05	23.31 ± 0.12	15.90 ± 0.22	20.24^{D}
	Roasted	21.10 ± 0.06	22.85 ± 0.04	24.39 ± 0.26	20.19 ± 0.08	22.13^{C}
	Germinated	22.80 ± 0.10	23.71 ± 0.12	25.26 ± 0.47	20.16 ± 0.13	22.98^{B}
	Pressure cooked	26.00 ± 0.01	27.01 ± 0.01	28.47 ± 0.21	25.10 ± 0.09	26.66^{A}
	LS Mean	22.44^{C}	23.87^{B}	25.35^{A}	20.34^{D}	
Total dietary fiber						
	Boiled	26.11 ± 0.03	27.92 ± 0.07	29.15 ± 0.14	22.11 ± 0.22	26.32^{B}
	Roasted	21.25 ± 0.08	23.95 ± 0.04	25.32 ± 0.24	21.35 ± 0.07	22.97^{C}
	Germinated	26.25 ± 0.14	26.91 ± 0.12	28.26 ± 0.47	23.56 ± 0.13	26.25^{B}
	Pressure cooked	28.62 ± 0.02	29.22 ± 0.01	30.48 ± 0.21	28.50 ± 0.09	29.20^{A}
LS mean		25.55^{C}	26.99^{B}	28.30^{A}	23.88^{D}	

Effect of different cooking methods and storage temperature in the formation of soluble, insoluble, and total dietary fiber in mung bean products. Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. T1—freshly prepared sample, T2—sample stored at room temperature for 24 h, T3—sample stored at 4°C for 24 h after cooking, and T4—reheating sample after stored at 4°C for 24 h.

3.7 Effect of resistant starch and soluble fiber on blood glucose levels in human subjects

The group I supplemented with germinated and steamed mung bean depicted the lowest levels of glycemic index (26.24) after the intake of the product (a sample contains 50 g of carbohydrate). The boiled and pressure-cooked mung bean samples prepared with different treatments when ingested by the experimental groups (G2–G7) showed a GI ranging from 40.17 to 49.74, with the lowest GI in the boiled T4, while in the pressure-cooked sample G5, who received food with treatment T4, the GI was 45.57. The results showed that all the samples had low GI content, which resulted in a slower rise in blood glucose levels in the participants (Figure 5).

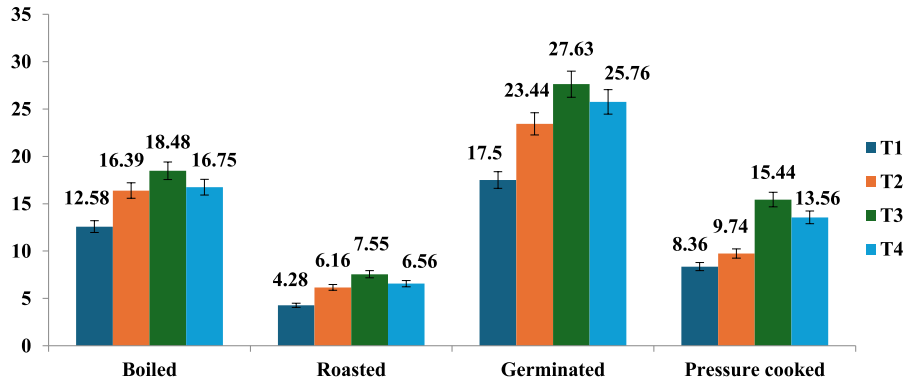


FIGURE 2
Resistant starch content (value) of processed mung bean (g/100 gm, dry weight basis). Showed different storage temperature: T1—freshly prepared sample, T2—sample stored at room temperature for 24 h, T3—sample stored at 4°C for 24 h after cooking, and T4—reheating sample after stored at 4°C for 24 h.

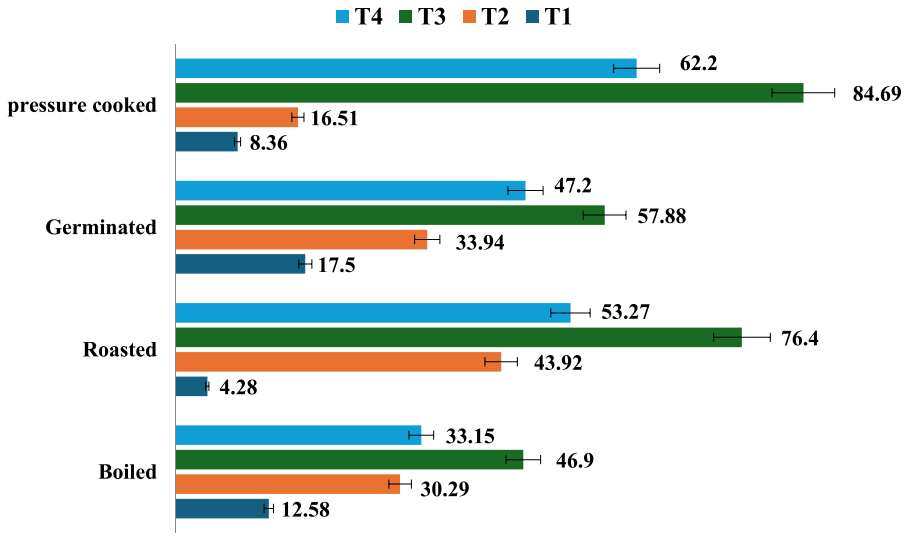


FIGURE 3
Percentage (%) change in resistant starch content among different storage condition of processed mung bean (g/100 gm, dry weight basis). Showed percentage change in resistant starch content of processed *mung bean* as compared to T1 sample as a control—T1—freshly prepared sample, T2—sample stored at room temperature for 24 h, T3—sample stored at 4°C for 24 h after cooking, and T4—reheating sample after stored at 4°C for 24 h.

3.8 Effectiveness of resistant starch of processed mung bean on blood glucose level in rats

In a 28-day trial, the effect of RS present in the treated mung bean products on the blood glucose level of rats were assessed three times, i.e., before, during, and after the completion of the diet intervention in albino male rats (Table 6). The results showed that resistant starch from various diet groups led to a decrease in blood glucose levels, although the average values did not differ significantly from either the control or diabetic control groups. In the control group (G1) and diabetic control group (G2), in all three blood assessments, no significant difference was recorded in the blood glucose level of rats. While the results of the experimental groups indicated that the maximum reduction in the blood glucose level was found in the G3 group fed on a germinated steamed mung bean diet, where their blood glucose values reduced from 278 to 144 mg/dl, The blood glucose levels of the rats belonging to G4, G5, and G6 fed on pressure-cooked mung bean with T1, pressure-cooked mung bean with T4, and pressure-cooked mung bean with T3, respectively, also reduced significantly ($\leq$0.001) after the experiment, proving a strong effect of RS on the blood glucose levels.

3.9 Effectiveness of resistant starch of processed mung bean on lipid profile in rats

The effect of RS on the body weight and lipid profile of the rats was also studied (Tables 7A, B). A change in body weight,

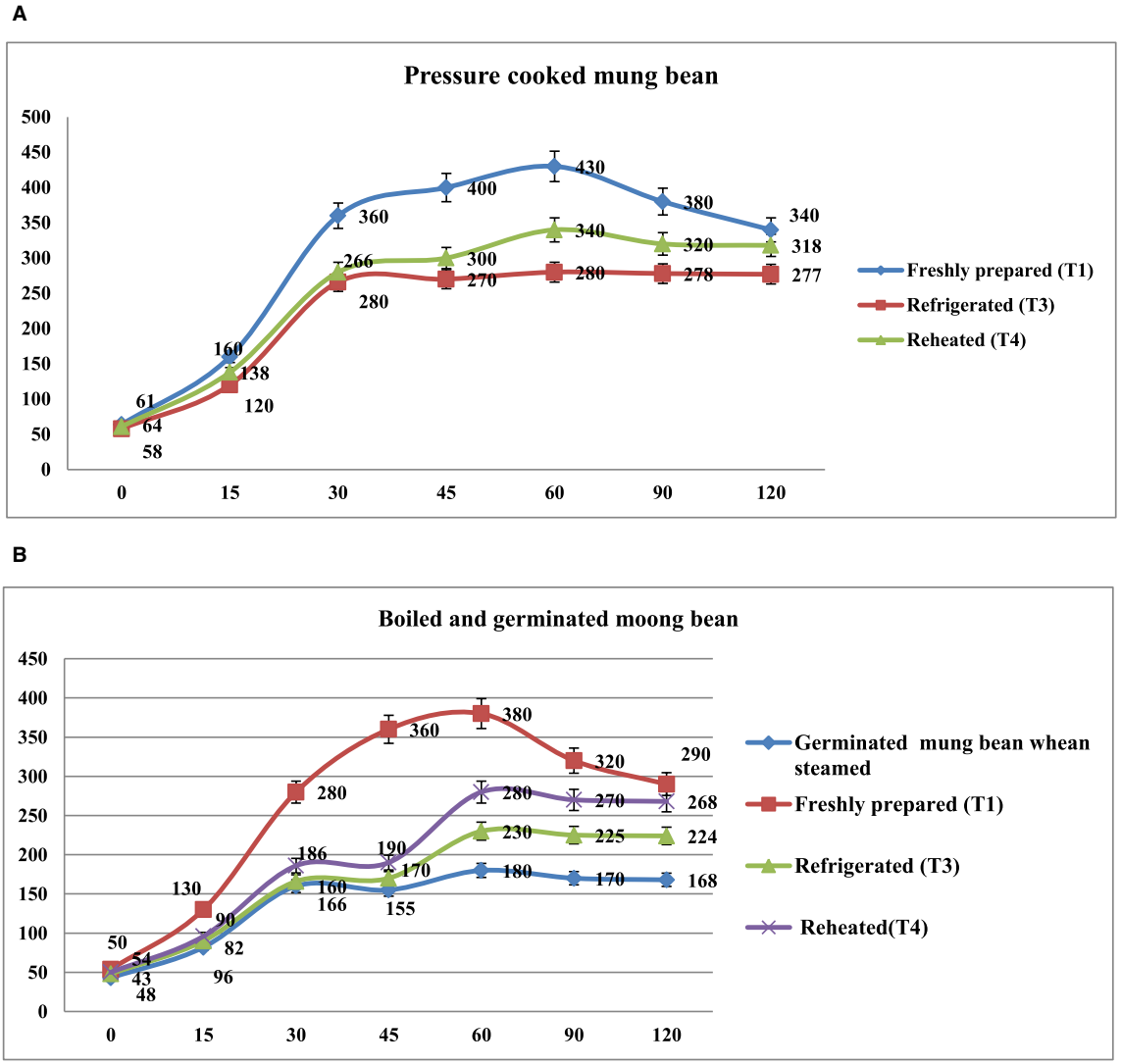


FIGURE 4
(A) *In vitro* starch digestion rate of pressure cooked mung bean. Showed different storage temperature: T1—freshly prepared sample, T3—sample stored at 4°C for 24 h after cooking, and T4—reheating sample after stored at 4°C for 24 h. **(B)** *In vitro* starch digestion rate of boiled and germinated mung bean. Showed different storage temperature: Germinated *mung bean* when steamed (freshly prepared), T1—immediately cooked sample, T3—sample stored at 4°C for 24 h after cooking, and T4—reheating sample after stored at 4°C for 24 h.

plasma insulin, and lipid profile levels was observed in both control groups, i.e., G1 and G2. The change in these parameters can be attributed to the basic metabolic changes taking place in the animals due to the controlled environmental conditions. However, in all the experiment groups (G3, G4, G5, and G6), a significant ($\leq$0.001) reduction in body weight, triglycerides, total cholesterol, and LDL levels was observed at the completion of the trial. The RS-rich diets resulted in a significant ($\leq$0.001) increase in plasma insulin and HDL levels. The maximum increase in the plasma insulin level was found in G3, followed by G5, G6, and G4. As the plasma insulin levels increased, a similar decreasing trend in blood glucose levels was seen in G3, G6, G5, and G4. As the intervention plan was conducted under fully controlled conditions, the results clearly indicate that the decrease in blood glucose levels is purely due to the RS content of diets.

4 Discussion

The present study was conducted with the objective of finding the optimal storage conditions and cooking method for increasing the resistant starch content of the commonly consumed mung bean in India and its effectiveness on blood glucose levels. The protein content was found to be higher in the germinated mung bean after steaming compared to other cooked samples. This could be due to the fact that germination increases the amount of protein and also improves protein quality by increasing the availability of amino acids while methods like, roasting decrease the protein content due to thermal degradation and oxidation of amino acids (29, 40). The soluble protein content leaches out during the cooking techniques in which water is used, as in the current study, pressure cooking and boiling resulted in a decrease in the protein content. Moreover, denaturation and aggregation of proteins during these processes

10.3389/fnut.2024.1424112

TABLE 5 Amylose and amylopectin of mung bean (g/100 gm, dry weight basis).

Mung bean	Treatment	T1	T2	T3	T4	LS mean
Amylose						
	Boiled	36.43 ± 0.01	37.33 ± 0.18	38.84 ± 0.16	37.97 ± 0.01	37.64^{B}
	Roasted	30.22 ± 0.02	31.46 ± 0.02	33.62 ± 0.02	32.04 ± 0.02	31.83^{D}
	Germinated	38.83 ± 0.04	39.88 ± 0.02	40.91 ± 0.06	39.02 ± 0.01	39.66^{A}
	Pressure cooked	32.81 ± 0.01	33.96 ± 0.02	35.23 ± 0.01	34.02 ± 0.01	34.01^{C}
	LS mean	34.57^{D}	35.66^{C}	37.15^{A}	35.76^{B}	
Amylopectin						
	Boiled	63.57 ± 0.01	62.67 ± 0.18	61.16 ± 0.16	62.03 ± 0.01	62.36^{C}
	Roasted	69.78 ± 0.02	68.54 ± 0.02	66.38 ± 0.02	67.96 ± 0.02	68.17^{A}
	Germinated	61.17 ± 0.04	60.12 ± 0.02	59.09 ± 0.06	60.98 ± 0.01	60.34^{D}
	Pressure cooked	67.19 ± 0.01	66.04 ± 0.02	64.77 ± 0.01	65.98 ± 0.01	65.99^{B}
LS mean		65.43^{A}	64.34^{B}	62.85^{D}	64.24^{C}	

Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. This table showed different storage temperature: T1—freshly prepared sample, T2—sample stored at room temperature for 24 h, T3—sample stored at 4°C for 24 h after cooking, and T4—reheating sample after stored at 4°C for 24 h.

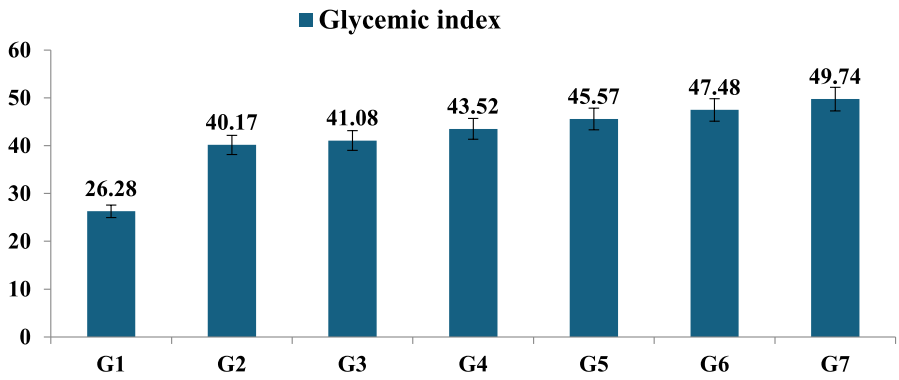


FIGURE 5
Glycemic index of processed mung bean. Showed the standard the mean glycemic index of seven *mung bean* products; 1. Steamed after Germinated mung bean (G1); 2. Boiled *mung bean* sample after reheating (G2); 3. Boiled *mung bean* stored at 4°C (G3); 4. Boiled *mung bean* sample after immediately cooked (G4); 5. Pressure cooked *mung bean* stored at 4°C (G5). 6. Pressure cooked *mung bean* after reheating (G6); 7. Pressure cooked *mung bean* after immediately cooked (G7).

TABLE 6 Effect of processed mung bean on blood glucose of rats before, during and after the experiments.

Diet group	Pre-treatment	During-treatment (after 10 days of diet intervention)	Post-treatment (after 28 days of diet intervention)	LS mean
G1	114.33 ± 6.86	115.00 ± 5.44	112.67 ± 4.32	114.00^{F}
G2	286.50 ± 16.54	285.50 ± 13.75	283.33 ± 8.36	285.11^{A}
G3	278.33 ± 11.69	175.00 ± 15.17	144.33 ± 4.63	199.22^{E}
G4	294.33 ± 13.65	252.67 ± 13.95	216.33 ± 4.63	254.44^{B}
G5	293.00 ± 26.25	242.00 ± 35.55	194.00 ± 4.73	243.0^{C}
G6	258.50 ± 25.09	185.50 ± 8.71	169.33 ± 5.89	204.44^{D}
LS Mean	256.13^{A}	212.85^{B}	188.28^{C}	

Values are mean ± SD; Mean values with different superscripts are significantly ($p \leq 0.05$) different. This table showed the standard the mean of diet group; 1. Control group (G1); 2. Diabetic control (G2); 3. Germinated mung bean fed rats (G3); 4. Pressure cooked mung bean after freshly prepared fed rats (G4); 5. Pressure cooked mung bean after reheating fed rats (G5); 6. Pressure cooked mung bean after stored at 4°C (G6) fed rats.

TABLE 7A Effects of processed mung bean stored at different temperature on lipids profile of normal and diabetic rats.

Groups	Body weight (grams)			Plasma insulin (u/ml)			Total cholesterol (mg/dl)		
	Initial (pre)	Final (post)	*P*-value	Initial (pre)	Final (post)	*P*-value	Initial (pre)	Final (post)	*P*-value
G1	175.67 ± 7.17	178.33 ± 7.31	0.02*	24.33 ± 0.82	24.83 ± 1.17	0.36NS	85.17 ± 3.97	85.50 ± 2.59	0.81NS
G2	171.00 ± 5.06	152.17 ± 9.02	≤0.001*	12.67 ± 1.63	10.00 ± 0.89	≤0.001*	137.33 ± 11.06	165.50 ± 5.58	0.003*
G3	169.67 ± 6.02	153.17 ± 4.49	≤0.001*	12.00 ± 1.26	31.17 ± 1.17	≤0.001*	134.83 ± 7.22	56.33 ± 2.34	≤0.001*
G4	172.50 ± 7.74	158.83 ± 7.00	≤0.001*	13.00 ± 1.78	17.67 ± 1.51	≤0.001*	138.00 ± 10.52	82.00 ± 1.41	≤0.001*
G5	170.33 ± 1.96	159.83 ± 5.08	≤0.001*	12.33 ± 1.03	23.83 ± 1.17	≤0.001*	134.00 ± 8.31	73.50 ± 1.87	≤0.001*
G6	176.83 ± 4.12	161.17 ± 5.98	≤0.001*	13.83 ± 1.83	23.33 ± 1.97	≤0.001*	134.50 ± 2.51	62.67 ± 1.75	≤0.001*

Values are mean ± SD; *Significant at 5%; NS, non-significant. This table showed the standard the mean of diet group; 1. Control group (normal diet fed rats) (G1); 2. Diabetic control (normal diet fed rats) (G2); 3. Germinated mung bean fed rats (G3); 4. Pressure cooked mung bean after freshly prepared fed rats (G4); 5. Pressure cooked mung bean after reheating fed rats (G5); 6. Pressure cooked mung bean after stored at 4°C (G6) fed rats.

TABLE 7B Effects of processed mung bean stored at different temperature on lipids profile of normal and diabetic rats.

Groups	Triglyceride (mg/dl)			HDL [high density lipoprotein (mg/dl)]			LDL [low density lipoprotein (mg/dl)]		
	Initial (pre)	Final (post)	*P*-value	Initial (pre)	Final (post)	*P*-value	Initial (pre)	Final (post)	*P*-value
G1	72.67 ± 5.57	73.83 ± 6.15	0.22 NS	54.17 ± 4.58	56.17 ± 1.17	0.36 NS	23.50 ± 1.05	22.33 ± 1.03	0.20 NS
G2	126.83 ± 1.47	124.50 ± 2.66	0.084 NS	27.33 ± 1.21	27.33 ± 1.21	0.034*	74.33 ± 1.63	77.50 ± 3.78	0.141 NS
G3	129.67 ± 3.67	56.67 ± 2.07	≤0.001*	23.83 ± 2.31	67.83 ± 1.60	≤0.001*	70.50 ± 2.88	38.33 ± 1.86	≤0.001*
G4	128.17 ± 1.72	81.33 ± 1.03	≤0.001*	24.00 ± 1.55	53.17 ± 1.47	≤0.001*	68.50 ± 2.25	47.17 ± 1.47	≤0.001*
G5	125.83 ± 3.76	73.17 ± 1.47	≤0.001*	23.83 ± 1.17	56.33 ± 1.03	≤0.001*	68.67 ± 2.65	43.17 ± 1.17	≤0.001*
G6	128.67 ± 2.16	63.50 ± 1.38	≤0.001*	23.33 ± 1.96	62.33 ± 1.63	≤0.001*	66.83 ± 2.23	38.50 ± 1.52	≤0.001*

Each value is the mean of six observation, Values are mean ± SD. *Significant at 5%; NS, non-significant. This table showed the standard the mean of diet group; 1. Control group (normal diet fed rats) (G1); 2. Diabetic control (normal diet fed rats) (G2); 3. Germinated mung bean fed rats (G3); 4. Pressure cooked mung bean after freshly prepared fed rats (G4); 5. Pressure cooked mung bean after reheating fed rats (G5); 6. Pressure cooked mung bean after stored at 4°C fed rats (G6).

TABLE 8 Total starch content, rapid starch digestion rate (%RDS) and slowly digestion starch rate (%SDS) in processed mung bean.

Mung bean	Treatment	T1	T2	T3	T4	LS mean
Total starch						
	Boiled	52.75 ± 0.22	55.49 ± 0.23	56.50 ± 0.13	56.20 ± 0.22	55.24^{B}
	Roasted	48.44 ± 0.23	48.31 ± 0.11	47.69 ± 0.28	48.41 ± 0.19	48.21^{D}
	Germinated	56.34 ± 0.14	61.06 ± 0.45	63.26 ± 0.70	62.50 ± 0.03	60.79^{A}
	Pressure cooked	52.48 ± 0.12	51.79 ± 0.19	55.49 ± 0.04	56.79 ± 0.27	54.14^{C}
LS mean		55.51^{C}	54.16^{B}	55.73^{A}	55.97^{A}	
Rapid starch digestion rate (RDS %)						
	Boiled	17.10 ± 0.09	13.24 ± 0.19	14.20 ± 0.14	≤0.001*	
	Pressure cooked	22.19 ± 0.08	17.94 ± 0.08	19.95 ± 0.07	≤0.001*	
	Germinated	11.65 ± 0.05	-	-	-	
Slowly starch digestion rate (SDS %)						
	Boiled	6.41 ± 0.19	2.29 ± 0.08	3.86 ± 0.02	≤0.001*	
	Pressure cooked	13.94 ± 0.29	9.54 ± 0.02	10.53 ± 0.20	≤0.001*	
	Germinated	1.28 ± 0.25	-	-	-	

Effect of different cooking methods and storage temperature in the formation of total starch, slowly starch digestion rate (SDS %), rapidly starch digestion rate (RDS %) in mung bean products. T1—freshly prepared sample, T2—sample stored at room temperature for 24 h, T3—sample stored at 4°C for 24 h after cooking, and T4—reheating sample after stored at 4°C for 24 h. *Significant at 5%. Mean values with different superscripts are significantly ($p \leq 0.05$) different.

also lead to a loss of protein content (9, 40). The different storage treatments also led to change in the protein content as during storage of food products at refrigeration temperature results in increasing or preserving the protein content due to slowing down the growth of microorganism (6). This can be clearly seen in the results found in the study as the germinated mungbean receiving T3 (kept at 4°C for 24 h) had highest protein content.

The total amount of soluble fiber is highly dependent on the cooking temperature. The high temperature breaks the linkage of glycosidic bonds in polysaccharides, which can lead to the release of

oligosaccharides, increasing the percentage of soluble dietary fiber in the food. In the present study, soluble fiber was higher in freshly cooked boiled products (6.25%) than other storage treatments (4, 27, 48). The insoluble dietary fiber is >70% of TDF in the raw legumes, further increasing with different processing. Hence, processed legumes are effective in reducing glycemic responses (46). It has been reported that due to the biochemical changes occurring during cooking, the starch breaks down into soluble and insoluble dietary fiber (26). According to the present research, the pressure-cooked mungbean had a higher content of insoluble dietary fiber which could be due to the breakdown of the cell wall of the grains, which led to a higher content of insoluble fiber. Further, the pressure-cooked mungbean sample with T3 (kept at 4°C for 24 h) had the maximum insoluble fiber content, which could be due to the slowdown of the enzymatic activity and the loss of water content, resulting in an increase in the insoluble dietary fiber (31).

The highest total starch content was observed in the germinated mung bean because of biosynthesis process of starch, involving the conversion of sugars and glucose into starch molecules, mainly amylose and amylopectin (28). Resistant starch contents in different pulses and legumes that are stored at different temperature ranged from 31.60 ± 4.12 to 41.94 ± 0.43 (%w/w; mean ± SD) of the samples, this result indicates that legume starch maybe slowly digested (16). In this study, we found that resistant starch was highest in germinated mung bean stored at 4°C for 24 h. During the sprouting process, starch is rapidly hydrolyzed by the action of α- and β-amylases and α-glucosidase into dextrins and simple sugars, resulting in improved starch digestibility (15) but, the indigestible part of starch (resistant starch) remains mainly intact during germination. Moreover, during the cooling process of pulses when stored at refrigeration temperature, the de-crystalline structure of starch starts recrystallization process to become resistant to digest i.e., resistant starch. So, the rise in the RS content of the mung bean sample with T3 (kept at 4°C for 24 h) resulted in slower *in vitro* starch digestion, making it an ideal food for diabetic patients. The retrogradation process in T3 also resulted in high amylose content, as when cooked pulses and legumes are kept in the refrigerator, retrogradation occurs at a slower rate compared to room temperature, immediate cooking, and reheating. Slower retrogradation leads to the formation of a higher proportion of amylose in the starch structure (8, 48).

It was also observed that pressure-cooked mung beans with T1 had a higher starch digestion rate, which may be due to gelatinization, where the digestibility of starch is increased due to the breaking down of starch granules and making them more accessible to digestive enzymes (11, 34). In Table 8 results showed that germinated mung bean had slow starch digestion rate due to high insoluble fiber, resistant starch content, which further slows down the digestion process (5, 25). On the other hand, the total starch content in raw mung bean samples (49.23%) increased after cooking. Similarly, due to the high amount of resistant starch, insoluble fiber, and protein content, germinated mung bean when steamed had the lowest glycemic index (26.28) and glycemic load (18.14). The low glycemic index of germinated mung beans can be due to their high content of resistant starch, insoluble fiber, and protein (10). Starch is almost completely digested, but resistant starch can be digested anywhere from 5 to 7 h after a meal. The digestion process takes 5–7 h and gradually increases blood sugar levels, lowers blood sugar and insulin levels, and provides satiety for a longer period. Insoluble dietary fiber absorbs glucose molecules and prevents glucose from passing through the small intestine (25). In the human digestive system, fiber slows the increase in blood glucose and reduces glucose absorption. When it is hydrated, fiber works more efficiently to lower blood glucose levels (50).

The intervention trial results indicated a maximum reduction in blood glucose levels in the group fed on germinated mung beans. The hypoglycemic effect of germinated mung beans can be due to their higher alpha-amylase and alpha-glucosidase inhibitory activity, high resistant starch content and dietary fiber content (14, 24, 44, 49). The diet also resulted in enhanced plasma insulin and HDL (high-density lipoprotein) levels. Insulin interferes with lipolysis because lipolysis involves the synthesis of fatty acids (fats) and triglycerides (triglycerides) in adipose tissue. In diabetic rats, insulin decreased the body's ability to utilize fat for energy production through lipolysis (20) leading to an increase in the production of acetyl-CoA, which in turn increased the levels of ketones and cholesterol (17).

The values for triglyceride, total cholesterol, and LDL (low lipoprotein) were also found to be decreased (26). Resistant starch and insoluble dietary fibers also reduce lipolysis and increase the levels of Glucagon-like peptide 1 (GLP-1), peptide YY, and insulin secretion (38). GLP-1 stimulates insulin secretion and decreases glucagon secretion. Pancreatic peptide (YY) reduces appetite and increases feeling of fullness. This helps to regulate blood glucose and lipids.

5 Conclusion

Boiling and germination enhanced the level of resistant starch in mung beans, whereas roasting and pressure cooking reduced it. Products stored at 4°C for 24 h (T3) and at room temperature (T2) showed an increase in resistant starch content, while freshly cooked (T1) and reheated products (T4) exhibited a decrease in resistant starch. Products stored at 4°C (T3) exhibited elevated levels of insoluble dietary fiber, slowly digested starch, amylose content, and a low glycemic index and glycemic load. Research on rats showed that consuming steam-germinated mung beans is more effective in controlling the increase in blood glucose levels. Consequently, it resulted in a gradual increase in blood glucose levels, leading to extended feelings of fullness. Indians use a wide range of starchy meals. Making changes in cooking techniques and storage temperatures can result in increased content of RS in the starchy diets leading to various health advantages to the consumers.

5.1 Limitations

During research, weather conditions (summer/high temperature) were unsuitable for research. Because it causes the growth of microorganisms in some cooked food samples,

which were stored at room temperature for 24 h and this food was unsuitable to give to the participants to consume, because it might lead to harmful health issues.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethics Committee of Punjab Agricultural University, Ludhiana. No harm was given to the subjects. Only blood glucose levels were checked. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Institutional Animal Ethics Committee (IAEC No. GADVASU/2023/1AEC/68/15). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SC: Writing – original draft. HK: Writing – original draft. RA: Writing – review & editing. PK: Writing – review & editing. KB: Visualization, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors express gratitude to all the study participants for their excellent contributions. This document originated from a thesis written to fulfill the requirements for receiving a Ph. D. degree.

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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Anderson L, Dinesen B, Jorgonsen PN, Poulsen F, Roder ME. Enzyme immune assay for intact human insulin in serum or plasma. *Clin Chem*. (1993) 39:578–82. doi: 10.1093/clinchem/39.4.578

2. AOAC. *Official Methods of Analysis*. 16th ed. Washington, DC: Association of Official Analytical Chemists (2000).

3. AOAC. *Official Methods of Analysis*. 16th ed. Washington, DC: Association of Official Analytical Chemists (2002).

4. Bader H, Saeed F, Khan MA, Niaz B, Rohi M, Nasir MA, et al. Modification of barley dietary fiber through thermal treatments. *Food Sci Nutri*. (2019) 7:1816–20. doi: 10.1002/fsn3.1026

5. Bodinham CL, Frost GS, Robertson MD. Acute ingestion of resistant starch reduces food intake in healthy adults. *Brit J Nutra*. (2010) 103:917–22. doi: 10.1017/S0007114509992534

6. Boye P, Zare F, Pletch A. Pulse proteins: processing, characterization, functional properties and applications in food and feed. *Food Res Int*. (2010) 43:414–31. doi: 10.1016/j.foodres.2009.09.003

7. Calles T, Xipsiti M, Del CR. Legacy of the international year of pulses. *Environ Earth Sci*. (2019) 78:124. doi: 10.1007/s12665-019-8106-6

8. Chakraborty I, Govindaraju I, Kunnel S, Managuli V, Mazumder N. Effect of storage time and temperature on digestibility, thermal, and rheological properties of retrograded rice. *Gels*. (2023) 9:142. doi: 10.3390/gels9020142

9. Dent T, Campanella O, Maleky F. Enzymatic hydrolysis of soy and chickpea protein with Alcalase and Flavourzyme and formation of hydrogen bond mediated insoluble aggregates. *CRFS*. (2023) 6:100487. doi: 10.1016/j.crfs.2023.100487

10. Dianzhi H, Qingyu Z, Laraib Y, Yong X, Qun S. *In vitro* starch digestibility and estimated glycemic index of mung bean (*Vignaradiata L.*) as affected by endogenous proteins and lipids, and exogenous heat-processing methods. *Plant Foods for Hum Nutr*. (2020) 75:547–52. doi: 10.1007/s11130-020-00845-9

11. Ding Y, Yang L, Xia Y, Wu Y, Zhou Y, Wang H. Effects of frying on starch structure and digestibility of glutinous rice cakes. *J Cereal Sci*. (2018) 83:196–203. doi: 10.1016/j.jcs.2018.08.014

12. Eashwarage LS, Herath HMT, Gunathilake KGT. Dietary fiber, resistant starch and *in vitro* starch digestibility of selectd eleven commonly consumed legumes (mung bean, cow pea, soyabean and horse gram) in Sri Lanka. *Res J Chem Sci*. (2017) 7:27–33.

13. Edwards CH, Cochetel N, Setterfield L, Perez-Moral N, Warren FJ. A single-enzyme system for starch digestibility screening and its relevance to understanding and predicting the glycaemic index of food products. *Food Funct*. (2019) 10:4751–60. doi: 10.1039/C9FO00603F

14. Erba D, Angelino D, Marti A, Manini F, Faoro F, Morreale F, et al. Effect of sprouting on nutritional quality of pulses. *Int J Food Sci Nutr*. (2019) 70:30–40. doi: 10.1080/09637486.2018.1478393

15. Ikram A, Saeed F, Afzaal M, Imran A, Niaz B, Tufail T, et al. Nutritional and end-use perspectives of sprouted grains: a comprehensive review. *Food Sci Nutr*. (2021) 9:4617–28. doi: 10.1002/fsn3.2408

16. Ezugwu EC, Nwosu E, Omeje KO, Ubani CS. Estimation of resistant starch, non-resistant starch and total starch of unprocessed foods sourced within Nsukka Town. *EAS J Nutr Food Sci*. (2020) 2:159–60. doi: 10.36349/easjnfs.2020.v02i04.001

17. Friedwald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low density lipoprotein cholesterol in plasma, without the use of preparative centrifuge. *Clin Chem*. (1972) 18:499–502. doi: 10.1093/clinchem/18.6.499

18. Fuentes-Zaragoza E, Riquelme-Navarrete MJ, Sanchez-Zapata E, Perez-Alvarez JA. Resistant starch as functional ingredient: a review. *Food Res Inter*. (2010) 43:931–42. doi: 10.1016/j.foodres.2010.02.004

19. Goni I, Garcia A, Saura-Calixto F. A starch hydrolysis procedure to estimate glycemic index. *Nutr Res*. (1997) 17:427–37. doi: 10.1016/S0271-5317(97)00010-9

20. Grewal A, Jood S. Effect of processing treatments on nutritional and antinutritional contents of green gram. *J Food Chem.* (2006) 30:535–46. doi: 10.1111/j.1745-4514.2006.00080.x

21. Hall C, Hillen C, Robinson JG. Composition, nutritional value, and health benefits of pulses. *Cereal Chem.* (2017) 94:11–31. doi: 10.1094/CCHEM-03-16-0069-FI

22. Juliano BO, Perez CM, Blakeney AB, Castillo DT, Kongseree N, Laignelet B, et al. International cooperative testing on the amylose content of milled rice. *Starch-Starke.* (1981) 33:157–62. doi: 10.1002/star.19810330504

23. Kendall CWC, Emam A, Augustin LSA, Jenkins DJA. Resistant starches and health. *J AOAC Inter.* (2004) 87:769–74. doi: 10.1093/jaoac/87.3.769

24. Liyanage R, Chathurang K, Visvanathan R, Jayathilake C. Hypolipidemic and hypoglycemic potential of raw, boiled, and sprouted Mung beans (*Vignaradiata* L. Wilczek) in rats. *J Food Biochem.* (2022) 42:12457. doi: 10.1111/jfbc.12457

25. Lopez HW, Levrat-Verny MA, Coudray C, Besson C, Krespine V, Messager A. Class 2 resistant starches lower plasma and liver lipids and improve mineral retention in rats. *J Nutr.* (2001) 131:1283–89. doi: 10.1093/jn/131.4.1283

26. Lopez-Exposito I, Ciruelos A. Dietary fiber composition of legumes and nuts: effect of processing. *Food Res Inter.* (2012) 48:777–85.

27. Lou Yi T, Wang K, Zhuang Z, Pan S, Huang X. Comparative analysis of dietary fibre extract isolated from citrus juice by-products using water extraction, fermentation and enzymatic treatment methods. *Advance J Food Sci Tech.* (2014) 6:1058–66. doi: 10.19026/ajfst.6.160

28. Megat RMR, Azrina A, Norhaizan ME. Effect of germination on total dietary fiber and total sugar in selected legumes. *Inter Food Res J.* (2016) 23:257–61.

29. Mubarak AE. Nutritional composition and antinutritional factors of mung bean seed (*Phaseolus aureus*) as affected by some home traditional processes. *Food Chem.* (2005) 89:489–95. doi: 10.1016/j.foodchem.2004.01.007

30. Omenna EC, Olanipekun OT, Kolade RO. Effect of boiling, pressure cooking and germination on the nutritional and antinutrients content of cowpea (*Vignaunguiculata*). *J Food Agric Sci.* (2016) 6:1–8. doi: 10.23880/FSNT-16000104

31. Pereira EJ, Carvalho LM, Dellamora GM, Cardoso FS, Carvalho JL, Viana DS, et al. Effects of cooking methods on the iron and zinc contents in cowpea (*Vignaunguiculata*) to combat nutritional deficiencies in Brazil. *Food Nutr Res.* (2013) 58:212–25. doi: 10.3402/fnr.v58.20694

32. Perera A, Meda V, Tyler RT. Resistant starch, a review of analytical protocols for determining resistant starch and of factors affecting the resistant starch content of foods. *Food Res Inte.* (2010) 43:1959–74. doi: 10.1016/j.foodres.2010.06.003

33. Raigond P, Ezekiel R, Raigond B. Resistant starch in food: a review. *J Sci Food Agric.* (2015) 95:1968–78. doi: 10.1002/jsfa.6966

34. Ratnaningsih N, Suparmo A, Harmayani E, Marsono Y. Physicochemical properties, *in vitro* starch digestibility, and estimated glycemic index of resistant starch from cowpea (*Vignaunguiculata*) starch by autoclaving-cooling cycles. *Int J Biol Macromol.* (2020) 142:191–200. doi: 10.1016/j.ijbiomac.2019.09.092

35. Reynolds A, Mann J, Cummings J, Winter N, Mete E, TeMorenga L. Carbohydrate quality and human health: a series of systematic reviews and meta analyses. *Lancet.* (2019) 393:434–45. doi: 10.1016/S0140-6736(18)31809-9

36. Rice EW, Roderick P, Mac DRP. Determination of triglycerides. Standard method. *Clin Chem.* (1970) 6:215–22. doi: 10.1016/B978-0-12-609106-9.50027-0

37. Richmond W. Preparation and properties of a cholesterol oxidase from *Nocardia* sp. and its application to the enzymatic assay of total cholesterol in serum. *Clin Chem.* (1973) 19:1350–6. doi: 10.1093/clinchem/19.12.1350

38. Rimm EB. Vegetable, fruit, and cereal fiber intake and risk of coronary heart disease among men. *J Am Med Assoc.* (1996) 275:447. doi: 10.1001/jama.1996.03530300031036

39. Robillard N. *Resistant Starch Friend or Foe?* (2020). Available at: https://digestivehealthinstitute.org/2013/05/10/resistant-starch-friend-or-foe/ (accessed April 9, 2020).

40. Sánchez-Velázquez OA, Ribéreau S, Mondor M, Cuevas-Rodríguez EO, Arcand Y, Hernández Alvarez AJ. Impact of processing on the *in vitro* protein quality, bioactive compounds, and antioxidant potential of 10 selected pulses. *Legume Sci.* (2021) 88:1–18. doi: 10.1002/leg3.88

41. Santana ANL, Meireles MAA. New starches are the trend for industry applications: a review. *Food Publ Health.* (2014) 4:229–41. doi: 10.5923/j.fph.20140405.04

42. Snelson M, Kellow N, Coughlan MT. Modulation of the gut microbiota by resistant starch as a treatment of chronic kidney diseases: evidence of efficacy and mechanistic insights. *Adv Nutr.* (2019) 10:303–20. doi: 10.1093/advances/nmy068

43. Sopade PA, Gidley MJ. A rapid *in-vitro* digestibility assay based on glucometry for investigating kinetics of starch digestion. *Starch-Stärke.* (2009) 61:245–55. doi: 10.1002/star.200800102

44. Thilagavathi T, Kanchana S. A study on the effect of millet and pulse based pasta on blood glucose and lipid profile in alloxan-induced diabetic rats. *IJPCBS.* (2017) 7:112–21.

45. Trinder P. Determination of blood glucose using 4-amino phenazone as oxygen acceptor. *J Clin Pathol.* (1969) 22:246. doi: 10.1136/jcp.22.2.246-b

46. Veena A, Urooj A, Puttaraj S. Effect of processing on the composition of dietary fiber& starch in some legumes. *Die nahrung.* (1995) 39:132–8. doi: 10.1002/food.19950390206

47. Wahjuningsih SB, Haslina H, Marsona M. Hypolipidamic effect of high resistant starch sago and Red Bean flour-based Analog Rice on Diabetic rats. *Mater Sociomed.* (2018) 30:232–9. doi: 10.5455/msm.2018.30.232-239

48. Wang S, Li C, Copeland L, Niu Q, Wang S. Starch retrogradation: comprehensive review. *Compr Rev Food Sci Food Saf.* (2015) 14:568–85. doi: 10.1111/1541-4337.12143

49. Wolever A, Jenkins D. The use of the glycemic index in predicting the blood glucose response to mixed meals. *Am J Clin Nutr.* (1986) 43:167–72. doi: 10.1093/ajcn/43.1.167

50. Wood L, Wilbourne J, Kyne-Grzebalski D. Administration of insulin by injection. *Pract Diabet Int.* (2002) 19:S1–4. doi: 10.1002/pdi.330

frontiers | Frontiers in Nutrition

TYPE Correction
PUBLISHED 12 June 2025
DOI 10.3389/fnut.2025.1605700

OPEN ACCESS

EDITED AND REVIEWED BY
Barbara R. Cardoso,
Monash University, Australia

*CORRESPONDENCE
Prabhjot Kaur
✉ prabhjotchahal72@yahoo.in

RECEIVED 03 April 2025
ACCEPTED 29 May 2025
PUBLISHED 12 June 2025

CITATION
Chauhan S, Kaur H, Aggarwal R, Kaur P and Bains K (2025) Corrigendum: Exploring the impact of cooking techniques and storage conditions on resistant starch levels in mung beans and its effect upon blood glucose level and lipid profile *in vivo*.
Front. Nutr. 12:1605700.
doi: 10.3389/fnut.2025.1605700

Corrigendum: Exploring the impact of cooking techniques and storage conditions on resistant starch levels in mung beans and its effect upon blood glucose level and lipid profile *in vivo*

Saloni Chauhan, Harpreet Kaur, Renuka Aggarwal, Prabhjot Kaur* and Kiran Bains

Department of Food and Nutrition, Punjab Agricultural University, Ludhiana, Punjab, India

KEYWORDS

cooking methods, dietary fiber, glycemic index, processed mung bean, resistant starch, storage temperature

A Corrigendum on
Exploring the impact of cooking techniques and storage conditions on resistant starch levels in mung beans and its effect upon blood glucose level and lipid profile *in vivo*

by Chauhan, S., Kaur, H., Aggarwal, R., Kaur, P., and Bains, K. (2024). *Front. Nutr.* 11:1424112. doi: 10.3389/fnut.2024.1424112

In the published article, there was an error. A correction has been made to **Materials and Methods**, *Effect of resistant starch on blood glucose level and lipid profile in albino male rats*, paragraph five 2.94 Study design. After induction of diabetes, the rats were left untreated and was not included at the end of the paragraph. The sentence should have been written as "Rats were treated with a treatment diet for 28 days, and blood glucose levels were measured in the 1st, 3rd, and 4th weeks, while serum insulin and lipid profiles were measured at the beginning and end of the last week of the experiment (1, 17, 36, 37, 45, 47). Following the completion of the experiment and blood sample collection, the animals were not left untreated after the experimental procedures. The rats were euthanized by administering anesthesia (CO_2, Isoflurane, Ketamine) method given by AVMA Guidelines for Euthanasia of animals. In accordance with university protocol, the carcasses were disposed of in a manner that ensured compliance with ethical and safety guidelines."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

Publisher's note

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 23 October 2024
DOI 10.3389/fnut.2024.1448450

OPEN ACCESS

EDITED BY
Jinsong Bao,
Zhejiang University, China

REVIEWED BY
Niroshan Siva,
The Pennsylvania State University (PSU), United States
Yuyue Zhong,
Max Planck Society, Germany
Bin Teng,
Anhui Academy of Agricultural Sciences (CAAS), China

*CORRESPONDENCE
Archana Singh
✉ sarchana19@gmail.com

RECEIVED 14 June 2024
ACCEPTED 08 October 2024
PUBLISHED 23 October 2024

CITATION
Warwate SI, Awana M, Thakare SS, Krishnan V, Kumar S, Bollinedi H, Arora A, Sevanthi AM, Ray M, Praveen S and Singh A (2024) Exploring the synergy of enzymes, nutrients, and gene networks in rice starch granule biogenesis. *Front. Nutr.* 11:1448450. doi: 10.3389/fnut.2024.1448450

Exploring the synergy of enzymes, nutrients, and gene networks in rice starch granule biogenesis

Sunil Indrajit Warwate[1], Monika Awana[1], Swapnil S. Thakare[1], Veda Krishnan[1], Suresh Kumar[1], Haritha Bollinedi[2], Ajay Arora[3], Amitha Mithra Sevanthi[4], Mrinmoy Ray[5], Shelly Praveen[1] and Archana Singh[1]*

[1]Division of Biochemistry, ICAR-Indian Agricultural Research Institute, New Delhi, India, [2]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India, [3]Division of Plant Physiology, ICAR-Indian Agricultural Research Institute, New Delhi, India, [4]ICAR-National Institute for Plant Biotechnology, New Delhi, India, [5]Division of Forecasting and Agricultural Systems Modeling, ICAR-Indian Agricultural Statistics Research Institute, New Delhi, India

Introduction: Rice is a primary food source almost for more than 50% of the total world's population. Glycemic index (GI) is high in most of the rice varieties, limiting their consumption by diabetic and obese people. As a result, developing new rice varieties with low GI necessitates a thorough understanding of starch biogenesis gene expression and its interrelationship.

Methods: A total 200 rice genotypes were analyzed for total starch content (TSC), amylopectin content (APC), and amylose content (AC). The clustering of these rice genotypes was done based on their AC. Further, these genotypes were categorized into three groups up to 10% amylose-low, 10–26% amylose-medium, and more than 26% amylose-high. Among them, six genotypes 1 from low AC (NJ-72), 2 from medium AC (UPRI-2003-18, PRR-126), and 3 from high AC (RNRM-7, Urvashi and Ananga) were selected. The genotypes selected from the medium and high AC groups were having 2% amylose variation among themselves respectively and they were further used to study the level of RS, protein content (PC), fatty acid (FA) profiles, and granule morphology along with low group sample.

Results: Resistant starch (RS) content ranged from 0.33–2.75%, and fatty acid profiling revealed high levels of palmitic, linoleic, and oleic acids. The degree of crystallinity and APC% were found to be positively correlated. Ananga, the genotype with the highest RS, displayed compact starch granules. Further, NJ-72 showing low RS and Ananga with high RS were selected for investigation of enzymatic activities of starch biosynthesis, metabolites accumulation, and expressions of 20 starch biogenesis genes in developing endosperm. Starch branching enzymes (SBE) and starch synthase (SS) activities peaked at 13 days after anthesis (DAA), while starch debranching enzymes (DBE) were most active at 18 DAA. In Ananga, TSC, AC, APC, and RS levels progressively increased from 3 to 23 DAA. Ananga showed 1.25-fold upregulation of *granule-bound starch synthase I (GBSSI)* at 18DAA. Higher expressions of *SSI* and *SBEIIb* were observed in NJ-72 at 13DAA. *PUL2* was predominantly expressed followed by *ISA1*. GBSSI was positively correlated with both AC and RS while *SS*, *SBE*, and *DBE* were positively related to APC.

Conclusion: This research could lead to the development of rice varieties with improved nutritional qualities, such as higher RS content, which is beneficial for human health due to its role in lowering glycemic response and promoting gut

health. Additionally, the study provides insights into how the modulation of key genes and enzymes can affect starch composition, offering strategies to breed rice varieties tailored for specific dietary needs or industrial applications.

KEYWORDS

rice endosperm, grain morphology, fatty acids, resistant starch, starch biogenesis, gene expression

1 Introduction

Globally, 537 million adults (20–79 years) are living with diabetes. By 2030, this number is expected to reach 643 million, and by 2045, it is expected to reach 783 million. In 2021, diabetes was responsible for 6.7 million deaths, and it caused at least 966 billion dollars in health expenditure (1). Starchy foods have a high glycemic index (GI). Rice is consumed globally by about 4 billion people. White rice provides 23% of the world's calorie supply (2). It is a rich source of carbohydrates, and most of the rice varieties have high GI thus resulting in high blood glucose levels (3). Long-term consumption of such food leads to type II diabetes, fatigue, obesity, etc. Starch is the primary storage component in cereals, accounting for up to 80% of all calories taken by humans. Amylose and amylopectin are two major components of starch. Starch's nutritional qualities are determined by the amount of its principal components, ratio of amylose to amylopectin, crystallinity degree, granule morphology, and some minor components. Based on digestibility, starch is categorized into rapidly digestible, slowly digestible, and resistant starch (RS) (4). RS is a component of starch that remains undigestible in the small intestine by amylases and ferments in the large intestine by enzymes in healthy individuals. Generally, RS is divided into five types: RS1, RS2, RS3, RS4, and RS5. These are determined by the inaccessibility, structure, retrogradation, or chemical modification of starch, be it naturally occurring or added to foods (5). The level of RS is also contributed by the starch granule morphology and the crystallinity of amylopectin. Starch granules are heterogeneous in morphological structure, and hence their compactness, shape, etc. may determine the rate of starch hydrolysis. Protein is the second major component of rice grain next to the starch; therefore, its content may help for RS. Because RS gets digested incompletely in the small intestine, it has many health benefits, as it acts as soluble fiber (6), improves insulin sensitivity, lowers blood glucose levels, improves short-chain volatile fatty acid levels, and prevents inflammatory bowel disease (7). Developing cereal crops with a high RS content is increasingly necessary to tackle the rapidly growing challenges of nutrition as a public health issue.

Starch biosynthesis involves starch synthesizing, branching (BE), and debranching (DBE) enzymes that play a crucial role in controlling amylose and amylopectin ratio to regulate starch biosynthesis (8). Starch with high amylose content (AC) contributed to the formation of RS that reduces glycemic response (9). Starch synthesis enzymes are encoded by at least 7 groups of rice genome genes: two are for *granule-bound starch synthase (GBSSI)*, 8 for *starch synthase (SS)*, 6 for *ADP-glucose pyrophosphorylase (AGPase)*, 3 for *BE*, 2 for *phosphorylase (Pho)*, 4 for *DBE*, and 2 for *Disproportionating enzyme* (10). Individual isoforms of each class of enzyme have distinct relative activities that are tissue- and species-specific (11). AGPase mRNA and protein levels are relatively low during the initial stages of endosperm development, maximum at the middle, and again lower at maturation (12). In higher plants, SS has five subfamilies: GBSS, SSI, SSII, SSIII, and SSIV. There are two isoforms of GBSS: GBSSI and GBSSII. GBSSI, also referred to as WAXY protein is strongly linked to starch granules, catalyzes the amylose elongation in storage starch, and also accounts for a major proportion of overall GBSS activity (13, 14), whereas GBSSII is engaged in amylose biosynthesis in tissues not suitable for storage, such as leaves (15). In the opinion of Tetlow and Emes (16), as compared to SSI and SSII, SSIII generates longer and cluster-spanning chains (i.e., cluster-filling chains). SSIV, on the other hand, is involved in the initiation of starch granules rather than determining the amylopectin structure (17). In the rice genome, there is one *SSI* gene, three *SSII* genes (*SSIIa, SSIIb*, and *SSIIc*), two SSIII genes (*SSIIIa* and *SSIIIb*), and two *SSIV* genes (*SSIVa* and *SSIVb*). GBSSI and GBSSII each have a single gene (18). Studies showed that in rice, *OsSSIVa* exhibited lower and *OsSSIVb* exhibited higher expression levels in leaves and endosperm (10).

There are at least 3 SBE isoforms in rice endosperm (*OsSBEI, OsSBEIIa*, and *OsSBEIIb*). It was observed that a reduction in *OsSBEIIb* activity increases the AC in rice (19). DBEs in addition to SS and SBE play a crucial role in the production of semi-crystalline amylopectin, as DBE mutants showed lower amylopectin content (20). To date, two types of DBE have been identified in rice: isoamylase (ISA) and pullulanase (PUL). ISA has three copies, whereas the latter has only one. These enzymes' substrate specificities differed, with ISA debranching amylopectin and glycogen and PUL debranching pullulan and amylopectin (21). In compliance with Dinges et al. (22) in maize knockout mutants, endosperm and leaves require PUL to degrade starch normally. GBSS1 synthesizes amylose (23) while the combined activity of AGPase, SSs, SBEs, DBEs, PUL, and Phos are required

Abbreviations: AC, amylose content; AGPS, ADP-glucose pyrophosphorylase; APC, amylopectin content; BE, branching enzymes; DAA, days after anthesis; DAF, days after flowering; DBE, debranching enzymes; FAME, fatty acid methyl ester; GBSS; granule bound starch synthase; GCMS, gas chromatography mass spectrometry; GI, glycemic index; GOPOD, glucose oxidase peroxidase reagent; HP-5 MS, [(5%-phenyl)-methylpolysiloxane phase]; ISA, isoamylase; PERs, protein efficiency ratios; Pho, phosphorylase; PUFA, polyunsaturated fatty acid; PUL, pullulanase; RDS, rapidly digestible starch; RS, resistant starch; SBE, starch branching enzymes; SDS, slowly digestible starch; SEM, scanning electron microscope; SS, starch synthase; TSC, total starch content; Wx, waxy.

for amylopectin biosynthesis (24). Basic experiments on alteration in the activities of important enzymes involved in starch, sucrose, and intermediary carbohydrate metabolism during endosperm development in the cereals are critical for identifying the role of essential enzymes concerning the synthesis of starch and also to unravel how their synthesis can be regulated throughout the endosperm development.

To understand the characteristics of grains in different developmental stages, we need to study and analyze the expression patterns of individual genes involved in starch synthesis. To our knowledge, scanty literature is available on starch granule biogenesis and expression analysis of genes involved in the developing endosperm of rice grains. The present study was carried out to unravel the dynamics of starch granule biogenesis and enzyme profiles for improved starch quality in rice. To this, we studied how the matrix components, starch granule morphology, and degree of amylopectin crystallinity may contribute to the production of RS. Further, we have investigated the expression profile of 20 starch biogenesis genes, their enzymatic activities, and metabolites in contrasting rice genotypes at different developing stages of rice endosperm. In the future, the current study might be helpful for people with diabetes or at risk of developing it, choosing the right genotype of rice can be an important first step toward diet-based diabetes prevention.

2 Material and methods

In the present study, grains of 200 mature rice genotypes were obtained from Division of Genetics, ICAR-Indian Agricultural Research Institute (IARI), New Delhi and total starch content (TSC), AC, and amylopectin content (APC) were carried out in the grains of these rice genotypes. Clustering of these rice genotypes was done using "cluster" package of R software into 3 clusters denoted as 1-blue colored, 2- yellow colored and 3-red colored (Supplementary Figure S1) based on their AC. Further, these genotypes were categorized into three groups as up to 10% amylose-low, 10%−26% amylose-medium, and more than 26% amylose–high. Among them six genotypes, 1 from low AC (NJ-72), 2 from medium AC (UPRI-2003-18, PRR-126), and 3 from high AC (RNRM-7, Urvashi and Ananga) were selected. The genotypes selected from medium and high AC group were having 2% amylose variation among themselves respectively and were further used to study the level of RS, protein content (PC), fatty acid (FA) profiles, and granule morphology along with low group sample.

Of these six genotypes, two contrasting RS genotypes (NJ-72; low RS and Ananga; High RS) were selected to study the gene expression levels, enzymatic activities, as well as levels of metabolites at different stages of endosperm development. Therefore, developing grains (2 biological and 3 technical replicates) of two contrasting rice genotypes, NJ-72 (low amylose), and Ananga (high amylose) were collected at five different endosperm developing stages [3 days after anthesis (DAA), 8DAA, 13DAA, 18DAA, and 23DAA] from the fields of Division of Genetics, ICAR-IARI), New Delhi, and stored at −80°C for further analysis. HiMedia (Delhi, India) and Sigma Aldrich (St. Louis Street, MO, USA) provided analytical-grade chemicals.

2.1 Estimation of metabolites

2.1.1 Total starch

To estimate total starch content (TSC), Clegg's method (25) was used. One hundred mg of samples were finely ground using liquid nitrogen. The starch was extracted twice with hot 80% ethanol. The residue was treated with distilled water and perchloric acid (52%). At 25°C, centrifugation was performed for 10 min. A total of 100 mL of distilled water was added to the supernatant after it was pooled. Anthrone (0.2%) was added to a suitable aliquot, and absorbance at 620 nm was recorded. A glucose standard curve was used to calculate TSC and was expressed in percentage (%).

2.1.2 Amylose content

Amylose content (AC) was estimated by Juliano's colorimetric method (26). One hundred mg of samples were finely grounded in liquid nitrogen, then 1 mL of 95% ethanol and 9 mL of 1 N sodium hydroxide were added. For 15 min, the tubes were placed in a boiling water bath. The volume was made to 100 mL with distilled water. In 100 mL volumetric flask, 5 mL of aliquot, 1 mL of 1 N acetic acid, and 2 mL of 0.2% iodine solution was added and kept in the dark for 20 min, and absorbance was noted down at 620 nm. A standard curve made from potato amylose (Sigma) was used to calculate the AC in samples and expressed in %.

2.1.3 Amylopectin content

An Amylose/Amylopectin Megazyme assay kit was used to measure amylopectin content (APC). DMSO was used for defatting the 20 mg sample, and 95% ethanol was used to precipitate starch from the defatted sample. Then, 100 mM acetate-salt solution (pH 4.5) was added to it. Amylopectin was precipitated by centrifugation with a 4 mL solution of concanavalin A lectin. The amylose-containing supernatant was then hydrolyzed enzymatically into D-glucose and measured using glucose oxidase-peroxidase (GOPOD). As previously stated, the total starch in the acetate-salt solution was calculated separately. At 510 nm, the amount of amylopectin/amylose in the starch sample was calculated as a ratio of glucose-GOPOD absorbance in the concanavalin A precipitated sample supernatant.

2.1.4 Resistant starch

The resistant starch (RS) was estimated using RS assay Kit (Megazyme International Ireland, Ltd., Bray, Ireland). One hundred mg powdered samples were digested with pancreatic α-amylase (10 mg/mL) containing amyloglucosidase (3 U/mL) for 16 h at 37°C with continuous shaking at 200 rpm. Centrifuged at 3,000 rpm for 10 min. RS pellet was washed twice with 50% ethanol, then suspended in 2 mL of 2 M KOH and kept on stirring in an ice-water bath for 10 min. Eight mL of sodium acetate buffer (1.2 M, pH 3.8), and 0.1 mL of amyloglucosidase (3 U/mL) were added. The tubes were vortexed and incubated at 50°C for 30 min. Centrifuged at 3,000 rpm for 10 min. 0.1 mL of supernatant was mixed with 3 mL of GOPOD and incubated at 50°C for 20 min. The absorbance was measured at 510 nm. RS was calculated as per the formula given in the kit itself and expressed in %.

2.1.5 X-ray diffraction

Rice-powdered samples (1.2 g) were scanned with a Phillips X-ray diffractometer (PW 1710 diffractometer control, PW 1729 X-ray generator) with Automated Powder Diffractometer (APD) software using Ni-filtered Cu-Kα radiation (λ, 0.1542 nm) at a scanning speed of 1.5°2θ min^{-1} over a range of 4 to 50 °2θ. Using intensity (counts) and diffraction angle (°2θ) the crystallinity degree was calculated.

2.1.6 Fatty acid profiling

Rice powdered samples weighing 1 g were dried overnight in an oven. Five mL of methanol and two drops of concentrated H_2SO_4 were added. The solution was incubated in a water bath at 65°C, cooled, and then mixed with 3 mL hexane. Fatty acid methyl esters (FAMEs) separated from the hexane layer. A hexane layer of 2 μL was injected into the GC system (DSQ-II model, Thermo Fisher Scientific, USA) at a flow rate of 10 mL/min. The stationary phase is the HP-5 MS capillary column, whereas the mobile phase is helium gas. Helium gas was injected at a rate of 1 mL/min. At 70 eV, the mass spectrometer was in electron impact mode. The composition of FA is represented as a proportion of total FA and expressed in %.

2.1.7 Protein estimation

The protein content was estimated using Kjeldahl's method (27). In order to digest 500 mg of powered rice, sulfuric acid was used at a temperature of 350–380°C. Potassium sulfate and catalyst are added to improve digestion rates and efficiency. The sample is then cooled at 25°C, diluted with water, and transferred to a distillation unit after digestion. With the addition of NaOH during the distillation process, NH_4^+ is converted to NH_3, which is captured by 2%—4% of aqueous boric acid in the receiver vessel. Titration is performed with sulfuric acid to determine nitrogen concentration. A conversion factor of 5.95 is applied to determine protein percentage.

2.1.8 Granule morphology

Scanning electron microscope (Zeiss EVOMA10, Germany) was used to examine the granule morphology of six rice genotypes. Surgical blades were used to cut mature rice grains in half transversely. Specimens were gold coated for 30 s at 35 mA and then adhered on a circular aluminum stub with the cracks facing up using adhesive tape. After coating, the samples were photographed at an accelerator potential of 5 kV using SEM.

2.2 Activity assays of starch biogenesis enzymes in developing endosperm

2.2.1 Starch synthase

Starch synthase (SS) activity was measured according to Leloir et al. (28). One g tissue was ground in 10 mL ice-cold extraction medium [0.05M *N*-2-hydroxyethyl piperazine-*N*′-ethanesulfonic acid pH 7, 10 mM EDTA, 5 mM dithiothreitol (DTT), and 1% polyvinylpyrrolidone]. The supernatant was collected after centrifugation for 20 min at 4°C at 15,000 g. The reaction mixture for the SS assay consisted of glycine buffer (0.08 M, pH 8.3), EDTA (4 mM), amylopectin (50 mg/mL), glutathione (40 mg/mL), and ADP-glucose (6 mM). An enzyme extract of 0.1 mL was added to tubes and incubated for 4 h in a shaking water bath at 37°C. A solution of phosphoenolpyruvate and pyruvate kinase (10 U) was added along with 0.2 mL dinitrophenyl hydrazine reagent, and it was incubated at 37°C for 15 min. A mixture of 0.2 mL NaOH (10 N) and 2 mL ethanol (95%) was added, and a 520 nm wavelength was used to measure the absorbance of brown color.

2.2.2 Starch branching enzyme

Based on Hawker et al. method (29), starch branching enzyme (SBE) activity was measured. A sample of 0.5 g was ground in 2 mL of extraction buffer (50 mM 3-N-morpholino propane sulfonic acid pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, and 2 mM DTT). The supernatant was collected after centrifugation at 15,000 g for 20 min at 4°C. The reaction mixture containing sodium citrate buffer (100 μM), amylase (300 μg), and 0.1 mL enzyme extract was incubated at 30°C for 15 min. The reaction was stopped by adding 0.5 mL of 2 N HCl followed by 1 mL iodine reagent, and using distilled water, the final volume was made to 5 mL. A wavelength of 590 nm was used to measure the absorbance.

2.2.3 Starch debranching enzyme

The Fujita et al. method (30) was used to test the isoamylase (ISA) activity. One g of sample was homogenized in 3 mL of grinding solution (50 mM imidazole-HCl pH 7.4, 8 mM $MgCl_2$, 50 mM 2-mercaptoethanol, and 12.5% glycerol). The supernatant was obtained after being centrifuged at 10,000 g for 2 min at 4°C. The reaction mixture containing MES-NaOH (50 mM, pH 6.5), $CaCl_2$ (20 mM), rabbit liver glycogen (3 mg), and for 20 min, 0.1 mL of enzyme was incubated at 30°C. Tubes were placed in a boiling water bath for 1 min to terminate the reaction, and absorbance was measured at 520 nm.

Pullulanase (PUL) activity was determined following the protocol of Pullulanase/Limit-Dextrinase Assay Kit. The rice sample (250 mg) was ground in 12.5 mL of Buffer B (sodium acetate buffer, 100 mM, pH 5) and gently the slurry was stirred over about 15 min until the sample was completely dispersed. The solution was centrifuged (1,000 g, 10 min) and 0.1 mL aliquots of PULLG6 reagent solution were dispensed into test tubes and pre-incubated at 40°C for 5 min. PUL extract was also pre-incubated at 40°C for 5 min. To each tube containing PULLG6 reagent solution (0.1 mL), 0.1 mL of pre-equilibrated PUL extract was added directly to the bottom of the tube. Incubation was carried out at 40°C for 10 min, and the reaction was stopped by the addition of 3 mL of stopping reagent (2% Tris buffer solution, pH 9.0). Absorbance was read at 400 nm against distilled water, and PUL activity was calculated as per the formula provided by the manufacturer's protocol and expressed in PULLG6 unit/g.

By utilizing BSA as a standard and the Bradford method to assess total protein content, the specific activity (U/mg protein) for each enzyme was determined.

2.3 Expression profiling of starch biosynthesis genes

Total RNA was isolated from developing endosperms of both the genotypes (NJ-72 and Ananga) by the TRIzol method

and cDNA was synthesized using a Verso cDNA synthesis kit (Fermentas). The coding sequences for all the starch biosynthesis-related genes were retrieved from NCBI and primers were designed for all the genes using Primer3Plus software (Supplementary Table S1). A quantitative real-time PCR mixture (20 μL) with 10 picomoles of forward and reverse primers was made using 1 μL of cDNA. The program includes initial denaturation (95°C) for 3 min followed by 39 cycles each of denaturation (94°C) for 20 s, annealing (52–65°C; depending on the Tm of specific primers) for 20 s, and extension (72°C) for 40 s. *Actin* and *18S rRNA* were used as reference genes. The mean of the replications was used to determine the Ct values for the reference genes and genes of interest to assess relative gene expression. The Pfaffl formula (Ratio = $2^{-\Delta\Delta Ct}$) was used to calculate the fold change of the selected genes.

2.4 Statistical analysis

The correlation between 20 starch biogenesis genes with 4 metabolites (total starch, amylose, amylopectin, and RS) was developed by the "qgraph" package of R software. Phylogeny of starch biogenesis genes and their corresponding enzymes were studied by using Mega-X software. At a significance level of $p < 0.05$, Fisher's least significant difference was applied to assess the significant difference between the means. The information is given as mean standard deviation (SD).

3 Results

3.1 Matured rice genotypes showed different levels of metabolites

Rice starch and more precisely its principal components contribute to its nutritional quality, hence, we have estimated the percentage of TSC, AC, and APC in a total of 200 diversified mature rice genotypes. The variation in TSC depends on the growing location and varieties. In the current study, the TSC ranged from 67.31%—91.34% (Supplementary Table S2), where the Gull baber rice genotype had the highest TSC, while NJ-72 had the lowest. AC varied from 7.63%—33.68% in 200 matured rice genotypes (Supplementary Table S2). In NJ-72, the AC was lowest, while in Ananga the content was highest. The APC of 200 matured rice genotypes ranged from 39.57%—75.30% (Supplementary Table S2).

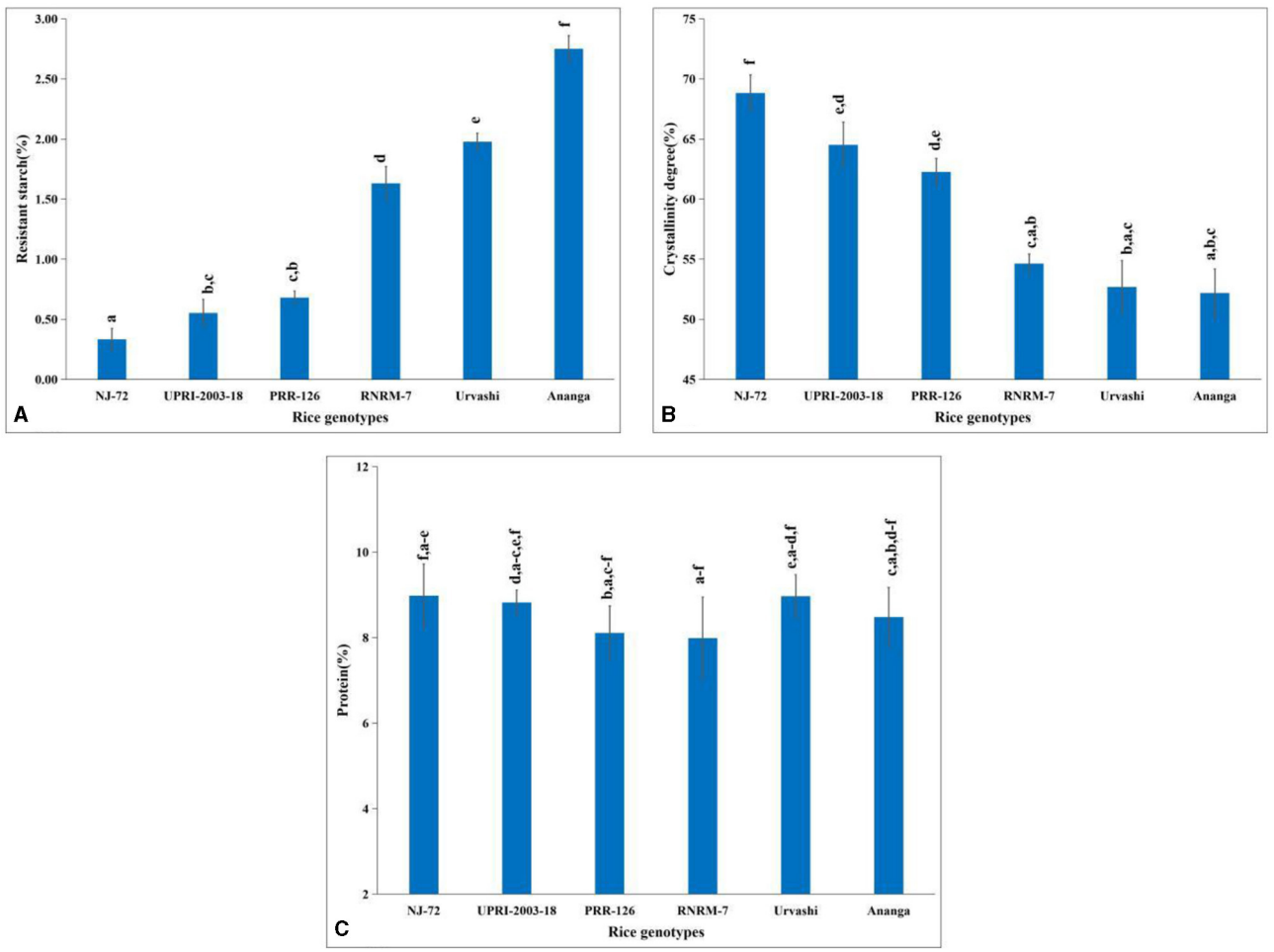


FIGURE 1
Change in metabolites concentration in contrasting six rice genotypes **(A)** resistant starch **(B)** amylopectin crystallinity and **(C)** protein content. Bar indicates ± SD. Different lowercase letters indicate a significant difference between mean at $p < 0.05$ [Fishers' least significant difference (LSD) test].

Urvashi possessed the lowest amount of amylopectin, while Pant dhan 15 rice genotypes had the highest content. In the current study, the lowest RS was found in NJ-72 (0.33%), whereas Ananga (2.75%) has the highest RS (Figure 1A). RS content is 0.55, 0.68, 1.63, and 1.98% in UPRI-2003-18, PRR-126, RNRM-7, and Urvashi respectively.

3.2 Crystallinity degree

An amylopectin double helix is packed in a unit cell to ensure starch crystallinity. In the current study, genotypes with high AC have a lower percentage of crystallinity, and those with lower AC have a higher percentage of crystallinity. Amylopectin has a more crystalline structure than amylose. The percentage of crystallinity was 68.82, 64.50, 62.27, 54.63, 52.68, and 52.18 in NJ-72, UPRI-2003-18, PRR-126, RNRM-7, Urvashi, and Ananga respectively (Figure 1B). Compared with Urvashi and Ananga, the NJ-72 with a high crystallinity percentage might be due to its high APC.

3.3 Variation in protein content and fatty acid composition

The highest protein percentage was found in NJ-72 (8.98%) and the lowest was found in RNRM-7 (7.99%) (Figure 1C). The protein content is 8.82, 8.11, 8.97, and 8.48 percent in UPRI-2003-18, PRR-126, Urvashi, and Ananga, respectively.

The composition of different fatty acids (FA), including essential and non-essential FAs determines their nutritional importance. In the current study, FA profiling using GC-MS showed a total of 10 FAs in contrasting 6 rice genotypes (Table 1). These are myristic acid, palmitic acid, linoleic acid, oleic acid, linolenic acid, stearic acid, 11- eicosenoic acid, arachidonic acid, behenic acid, and lignoceric acid. Some of the FAs are not found in some genotypes. 11- Eicosenoic acid and linolenic acid were not detected in NJ-72 and RNRM-7 respectively. Behenic acid and lignoceric acid were not detected in UPRI-2003-18. Among the 10 FAs, palmitic acid, linoleic acid, and oleic acid were present in high amounts compared to the remaining 7 FAs. In the current study, palmitic acid and linoleic acid were found high in the high amylose genotypes of Ananga, Urvashi, and RNRM-7 followed by intermediate amylose genotypes, UPRI-2003-18 and PRR-126. The palmitic acid content varies from 33.69%−37.18%. Ananga has a 37.18% maximum palmitic acid while NJ-72 has a 33.69% minimum. Linoleic acid content varies from 28.42%−30.90%. Ananga has 30.90% of maximum linoleic acid while NJ-72 has a 28.42% minimum. Oleic acid was highest in the low amylose NJ-72 genotype, followed by intermediate amylose genotypes. Oleic acid content varies from 23.72%−34.96%. NJ-72 has a 34.96% maximum oleic acid, while Ananga has a 23.72% minimum. Although the content of myristic acid is low compared to the three major FAs (palmitic acid, linoleic acid, and oleic acid), it is found high in the high amylose rice genotypes, followed by genotypes with intermediate and low amylose. Myristic acid content varies from 1.07%−2.81%. Ananga has a 2.81% of maximum stearic acid while NJ-72 has a 1.07% minimum.

3.4 Starch granule morphology

Starch granules have differences in their compactness and shape, and hence these characteristics may influence starch hydrolysis. In the current study, the SEM micrograph of six rice genotypes (NJ-72, UPRI-2003-18, PRR-26, RNRM 7, Urvashi, and Ananga) having varying RS content is shown at 2, 10, 100, and 200 μM in Figure 2. The structural differences between genotypes were primarily observed at 2 μM, followed by 10 μM. With genotypes, the shape of starch granules varies significantly. NJ-72, a genotype with low amylose, exhibits loosely packed small, angular, or cube-shaped starch granules, while UPRI-2003-18 and PRR-126, genotypes with intermediate amylose, have loosely packed cube-shaped starch granules. A comparative analysis of the high amylose genotypes reveals that RNRM-7 and Urvashi have compound starch granules that are typically cubical, whereas Ananga has granules that are spherical with smooth surfaces.

3.5 Variation in metabolites at developing stages of endosperm

TSC in five developing stages of contrasting rice genotypes ranged between 18.58%−64.88%. TSC was maximum at 23DAA and minimum at 3DAA in both genotypes. Both the genotypes accumulated nearly equal amounts of TSC up to 13DAA, but beyond that Ananga showed more TSC in comparison to NJ-72. NJ-72 showed 50% and Ananga showed 64.88% TSC at 23DAA (Figure 3A). Amylose content varied from 2.33% to 25.05% in different developing stages of NJ-72 and Ananga (Figure 3B). It increased from 8DAA to 23DAA in both genotypes and was more in Ananga in all stages as compared to NJ-72. APC was found higher in NJ-72 in comparison to Ananga throughout the developing stages. NJ-72 showed 28.30%, 33.97%, and 45.19% while Ananga showed 19.85%, 25.28%, and 39.44% APC at 13, 18, and 23DAA respectively (Figure 3C). RS content in developing endosperms of NJ-72 and Ananga varied between 0.016%−0.380%. It increased from 3DAA to 23DAA in both the genotypes (Figure 3D) and was significantly higher in Ananga as compared to NJ-72 in all the stages. Ananga showed 0.4% while NJ-72 showed 0.1% RS content at 23DAA respectively.

3.6 Alteration in activities of starch biogenesis enzymes

SS activity in developing endosperms of NJ-72 and Ananga spans from 0.040–0.188 U/mg protein. NJ-72 showed more SS activity in all developing stages in comparison to Ananga. The maximum activity was observed at 13DAA in both genotypes. NJ-72 and Ananga exhibited 0.18 and 0.16 U/mg protein SS activity at 13DAA respectively (Figure 4A). SBE activity ranged from 0.676–2.703 U/mg protein in NJ-72 and Ananga. The activity was increased from 3DAA to 13DAA and after that decreased in both genotypes (Figure 4B). The maximum ISA and PUL activities were observed at 18DAA in both genotypes. The ISA activity ranged from 0.091–0.201 U/mg protein (Figure 4C) and

10.3389/fnut.2024.1448450

TABLE 1 The composition of individual fatty acids (FA) identified in contrasting six rice genotypes.

S. No.	Fatty acids	NJ-72	UPRI-2003-18	PRR-126	RNRM-7	Urvashi	Ananga
1	Myristic acid	0.92 ± 0.21	1.35 ± 0.18	2.32 ± 0.35	0.94 ± 0.30	1.42 ± 0.22	0.69 ± 0.19
2	Palmitic acid	28.20 ± 1.30	35.82 ±2.10	35.23 ± 1.80	31,881 ± 0.46	36.61 ± 2.01	37.68 ± 1.69
3	Linoleic acid	34.96 ± 0.99	18.52 ± 1.30	30.54 ± 1.20	27.24 ± 0.97	25.24 ± 0.58	22.72 ± 1.17
4	Oleic acid	33.96 ± 1.54	39.97 ± 1.02	25.95 ± 0.57	36.06 ± 1.79	26.81 ± 1.07	28.60 ± 0.89
5	Linolenic acid	0.55 ± 0.12	0.06 ± 0.01	0.14 ± 0.08	ND	4.56 ± 0.51	0.11 ± 0.07
6	Stearic acid	1.87 ± 0.09	3.31 ± 0.88	3.75 ± 0.71	1.46 ± 0.59	4.23 ± 1.04	5.81 ± 1.00
7	11- Eicosenoic acid	ND	0.28 ± 0.05	0.17 ± 0.02	0.17 ± 0.07	0.14 ± 0.11	1.07 ± 0.47
8	Arachidonic acid	0.12 ± 0.08	0.69 ± 0.32	0.46 ± 0.18	0.33 ± 0.05	0.52 ± 0.26	1.33 ± 0.42
9	Behenic acid	0.04 ± 0.02	ND	0.11 ± 0.04	0.05 ± 0.20	0.14 ± 0.08	0.74 ± 0.49
10	Lignoceric acid	0.05 ± 0.03	ND	0.2 ± 0.11	0.45 ± 0.33	0.33 ± 0.15	1.25 ± 0.93

ND represents not detected.

PUL activity varied from 0.025–0.057 U/mg protein. At 18DAA, Ananga showed 0.057 and NJ-72 showed 0.043 U/mg protein PUL activity (Figure 4D). Among the DBEs, ISA activity was found to be significantly higher as compared to PUL in both the genotypes, which showed that ISA is a main DBE in starch biogenesis.

3.7 Differential expression of starch biogenesis genes in the developing endosperm

3.7.1 ADP glucose pyrophosphorylase and granule-bound starch synthase

The *AGPS1* expression level varied from 0.30–2.67 in NJ-72 and 0.43–2.76 in Ananga at different developing stages (Figure 5A). At 3DAA and 8DAA, *AGPL4* expression level was low, while at 13DAA it rapidly increased and then decreased toward 18DAA and 23DAA in both the genotypes (Figure 5B). At 13DAA, NJ-72 showed a 1.25-fold higher expression level of *AGPL4* in comparison to Ananga.

GBSSI expression was found maximum at 18DAA. It was more in Ananga in comparison to NJ-72 at all the stages. Ananga showed 1.25 and 1.63-fold more expression as compared to NJ-72 at 18 and 13DAA respectively (Figure 5C). *GBSSII* expression was downregulated at 3DAA in both the genotypes while upregulated in rest of the stages (Figure 5D). Ananga showed 2.3, 4, and 1.9-fold while NJ-72 showed 1.63, 2.3, and 1.52-fold more expression of *GBSSII* at 13, 18, and 23DAA respectively in comparison to their 8DAA stage.

3.7.2 Starch synthases

The relative expression of *SSI* in the endosperm developmental stages ranged from 1.19–4.62 in NJ-72 and 0.71–3.19 in Ananga. The maximum expression was observed at 13DAA in both genotypes. NJ-72 showed a 1.44-fold upregulation of *SSI* in comparison to Ananga at 13DAA (Figure 5E). In the middle stage, the higher expression of *SSI* may be attributed to its role in elongating shorter chains (DP 6–10) of starch. The *SSIIa* expression was lower at the initial (3DAA) and final (23DAA) stages. At the middle stages, NJ-72 showed 1.8, 1.32, and 2.3-fold upregulation of *SSIIa* expression at 8, 13, and 18DAA respectively, in comparison to Ananga (Figure 5F). In all the developing endosperm stages, Ananga showed downregulation of *SSIIb* except at 8DAA (Figure 5G). NJ-72 showed upregulation of *SSIIb* at 8 and 13DAA while at other stages it was downregulated. NJ-72 showed 2.6-fold upregulation of *SSIIb* at 8DAA as compared to Ananga. The relative expression of *SSIIc* in NJ-72 ranged from 0.31–1.07, while in Ananga it ranged from 0.1–0.59 (Figure 5H). *SSIIc* was highly expressed at 8DAA in both genotypes. NJ-72 showed more upregulation at all stages in comparison to Ananga. The *SSIIIa* expression increases initially from 3DAA to 13DAA and then decreases at later stages in both genotypes (Figure 5I). NJ-72 showed 1.64, 1.71, 1.66, 1.73, and 2.58-fold upregulation of *SSIIIa* expression as compared to Ananga at 3, 8, 13, 18, and 23DAA respectively. NJ-72 and Ananga showed upregulation of *SSIIIb* expression at 3 and 8DAA while downregulation at later stages except NJ-72 at 13DAA (Figure 5J). The expression level of *SSIIIb* was less in Ananga as compared to NJ-72 at all developing stages. The *SSIVa* expression was downregulated at 3DAA in both genotypes. The *SSIVa* expression level ranged from 0.61–1.19 in NJ-72 and 0.15–0.66 in Ananga from 8DAA to 23DAA (Figure 5K). The expression level of *SSIVb* was found upregulated at all the stages in NJ-72, whereas in Ananga downregulation was observed at 3DAA and upregulation at the rest of the stages. At 23DAA, the *SSIVb* gene was not detected in Ananga (Figure 5L). NJ-72 showed 2.6, 3.1, and 2.3-fold upregulation of *SSIVb* gene at 8, 13, and 18DAA respectively in comparison to Ananga.

3.7.3 Starch branching genes

The expression level of *SBEI* and *SBEIIa* was found maximum at 8DAA, while *SBEIIb* showed maximum expression at 13DAA. *SBEI* expression in NJ-72 showed 2.07, 1.91, and 1.88-fold variation at 8, 13, and 18DAA respectively as compared to Ananga (Figure 6A). *SBEIIa* expression in NJ-72 and Ananga showed 1.3-fold upregulation at 8DAA in comparison to 13DAA (Figure 6B).

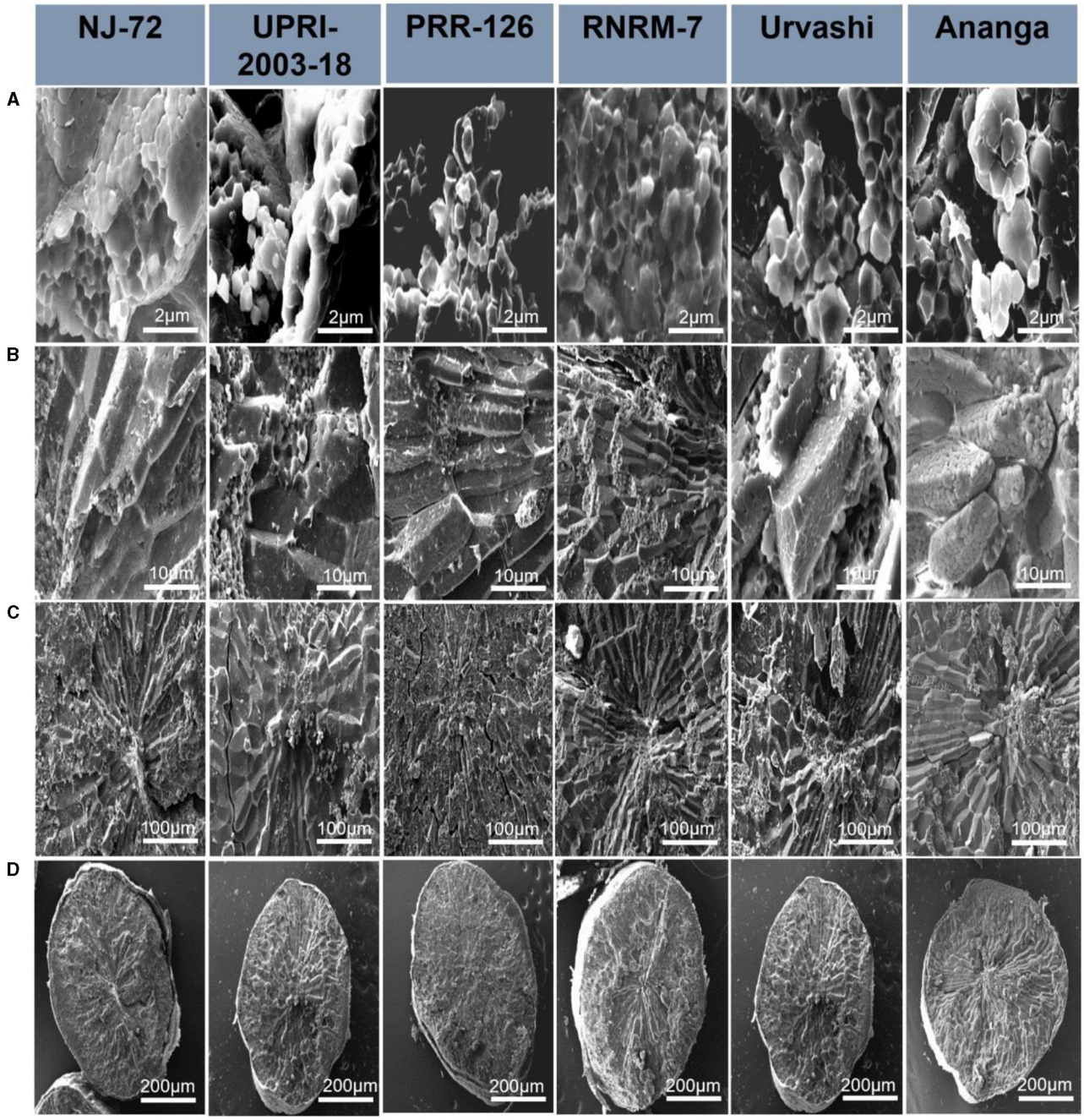


FIGURE 2
Scanning electron microscopy (SEM) of six rice genotypes having varying resistant starch content: NJ-72, UPRI-2003-18, PRR-126, RNRM-7, Urvashi and Ananga. Magnification obtained at **(A)** 2 μm, **(B)** 10 μm, **(C)** 100 μm, and **(D)** 200 μm.

Ananga showed 2.57, 1.52, 2.06, 2.27, and 2.93-fold downregulation in *SBEIIb* gene level at 3, 8, 13, 18, and 23DAA respectively, as compared to NJ-72 at respective stages (Figure 6C).

3.7.4 Starch debranching genes

Starch DBE, *ISA* (*ISA1, ISA2,* and *ISA3*) was increased from 3 to 18DAA and then decreased at 23DAA. NJ-72 showed more expression of *ISA* isoforms as compared to Ananga. The relative expression of *ISA1* was 2.2-fold higher in NJ-72 as compared to Ananga at 18DAA (Figure 6D). Ananga showed less expression of *ISA2* at all developing stages in comparison to NJ-72 (Figure 6E). The relative expression of *ISA2* ranged from 0.05–1.26 in Ananga and 0.34–2.28 in NJ-72 at all developing stages. The relative expression of *ISA3* was downregulated at 3DAA while upregulated at the rest of the stages in both genotypes (Figure 6F). At 13DAA, NJ-72 showed 2.04 upregulation of *ISA3* in comparison to Ananga.

The *PUL* gene expression was maximum at 18DAA. *PUL1* expression was low at the initial stages. NJ-72 showed more expression in comparison to Ananga. NJ-72 showed 2, 4, and

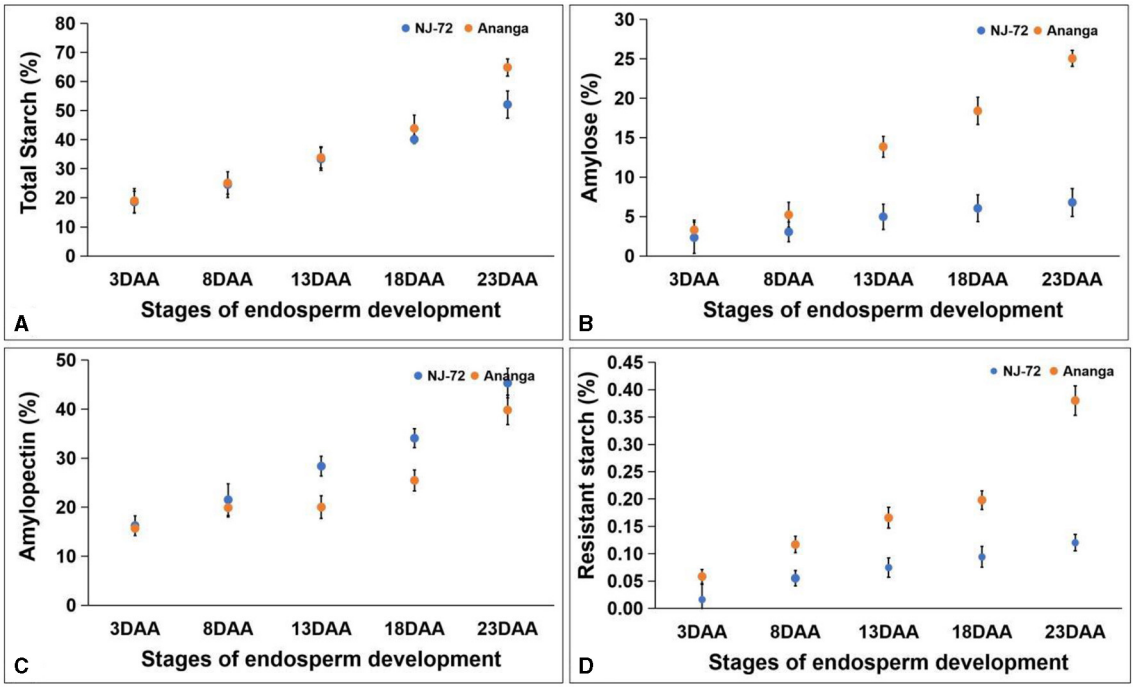


FIGURE 3
Change in metabolites with developing endosperm **(A)** total starch, **(B)** amylose, **(C)** amylopectin, and **(D)** resistant starch. DAA- days after anthesis. Bar indicates ± SD.

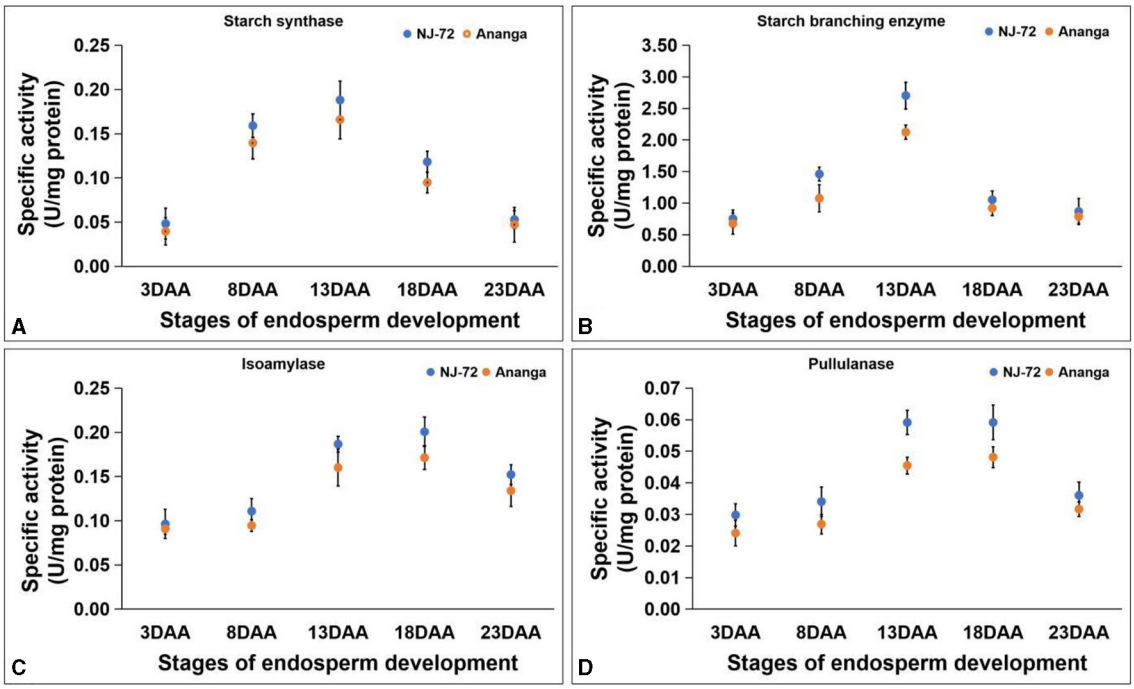


FIGURE 4
Change in activities of starch biogenesis enzymes with developing endosperm **(A)** starch synthase, **(B)** starch branching enzyme, **(C)** isoamylase and **(D)** pullulanase. Bar indicates ± SD.

1.2-fold more upregulation of the *PUL1* gene at 13, 18, and 23DAA respectively, in comparison to its expression at 8DAA (Figure 6G). The relative expression of *PUL2* was higher in NJ-72 at all developing stages as compared to Ananga but the difference between the two genotypes concerning *PUL2* expression was much less (Figure 6H). At 18DAA, NJ-72 showed a 1-fold upregulation of the *PUL2* gene in comparison to Ananga.

10.3389/fnut.2024.1448450

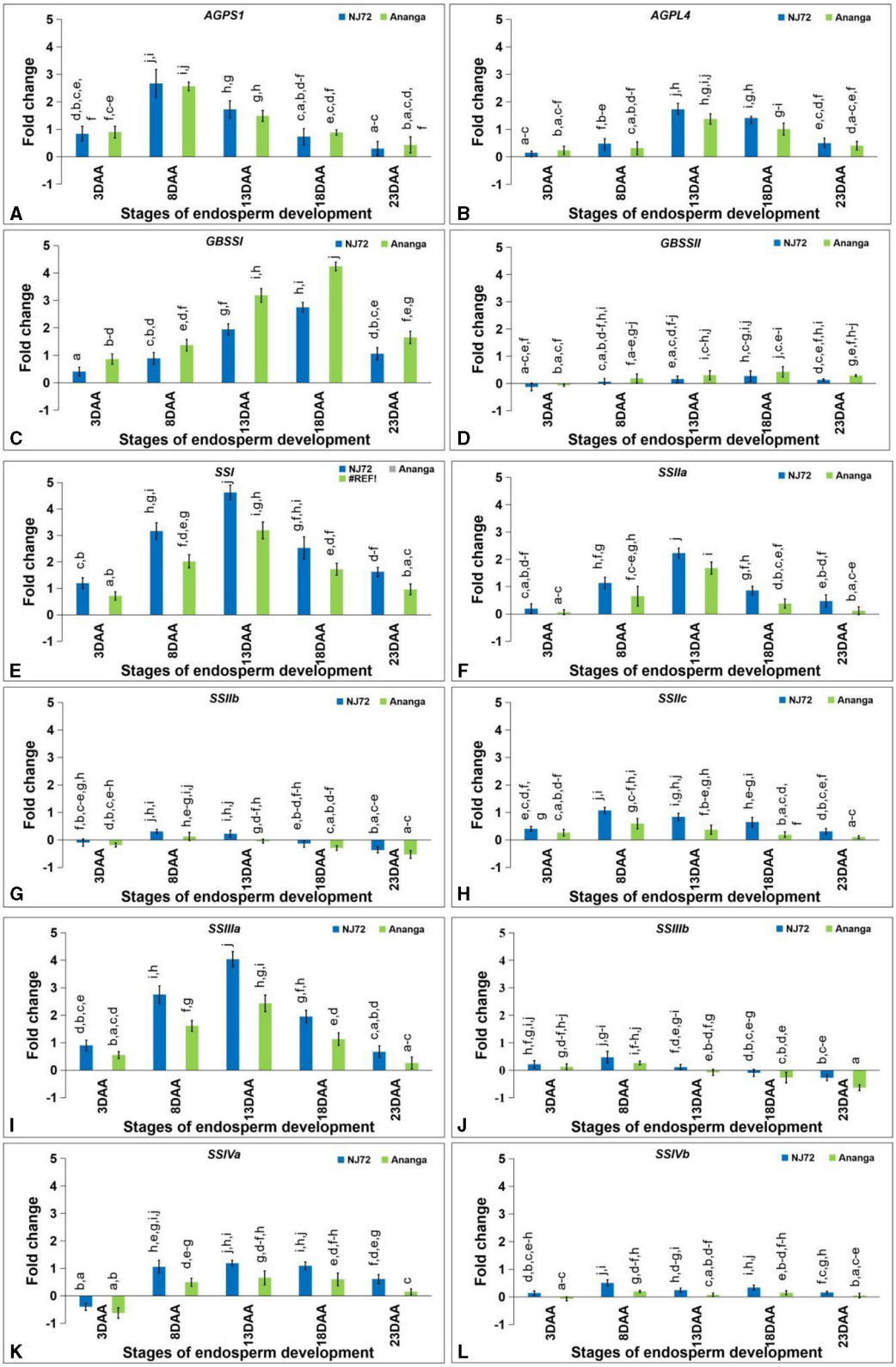


FIGURE 5
Differential expression of starch biogenesis genes in the developing endosperm of contrasting rice genotypes (NJ72 and Ananga) **(A)** *AGPS1*, **(B)** *AGPL4*, **(C)** *GBSSI*, **(D)** *GBSSII*, **(E)** *SSI*, **(F)** *SSIIa*, **(G)** *SSIIb*, **(H)** *SSIIc*, **(I)** *SSIIIa*, **(J)** *SSIIIb*, **(K)** *SSIVa*, and **(L)** *SSIVb*. Bar indicates ± SD. Different lowercase letters indicate significant difference between mean at $p < 0.05$ [Fishers' least significant difference (LSD) test].

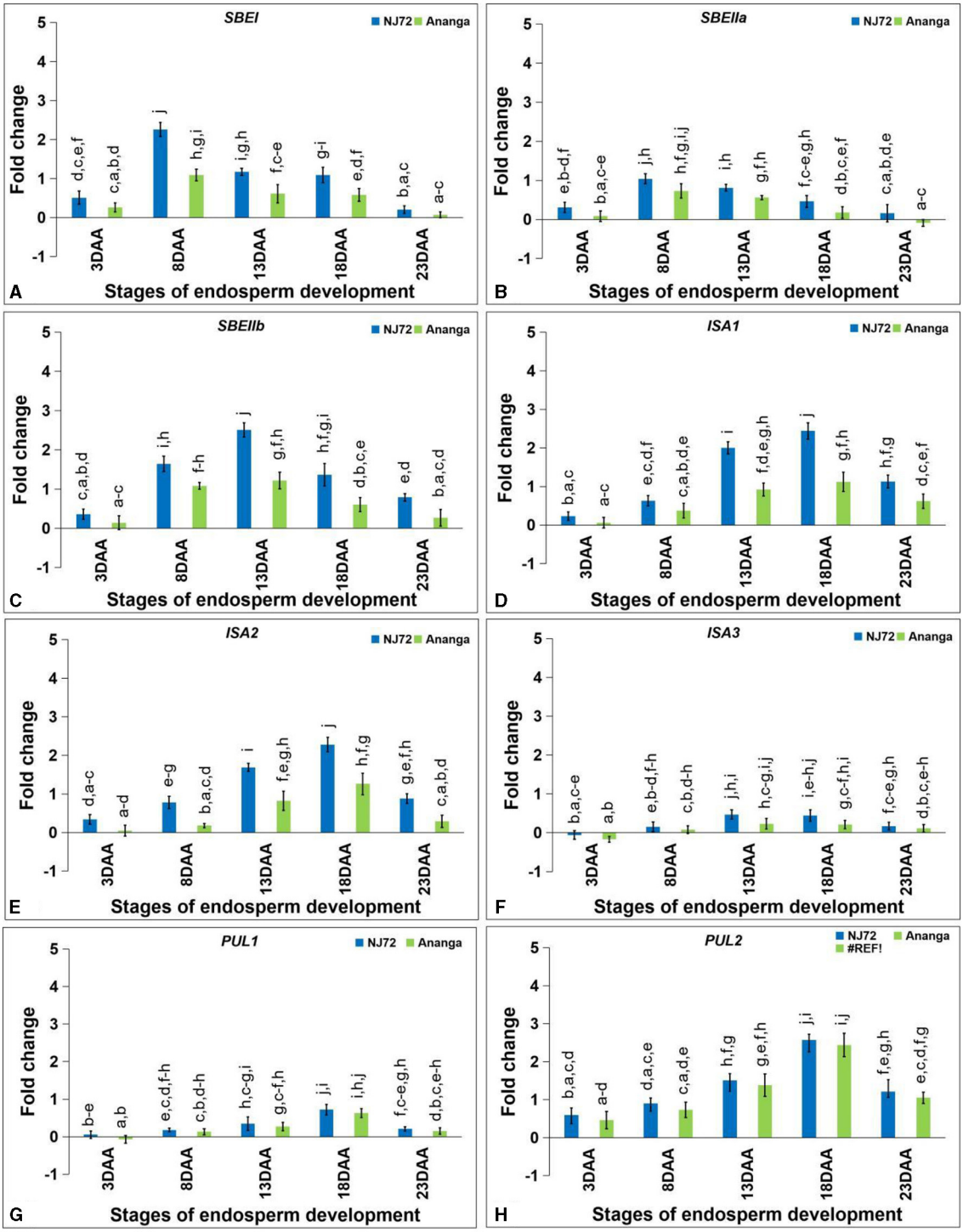
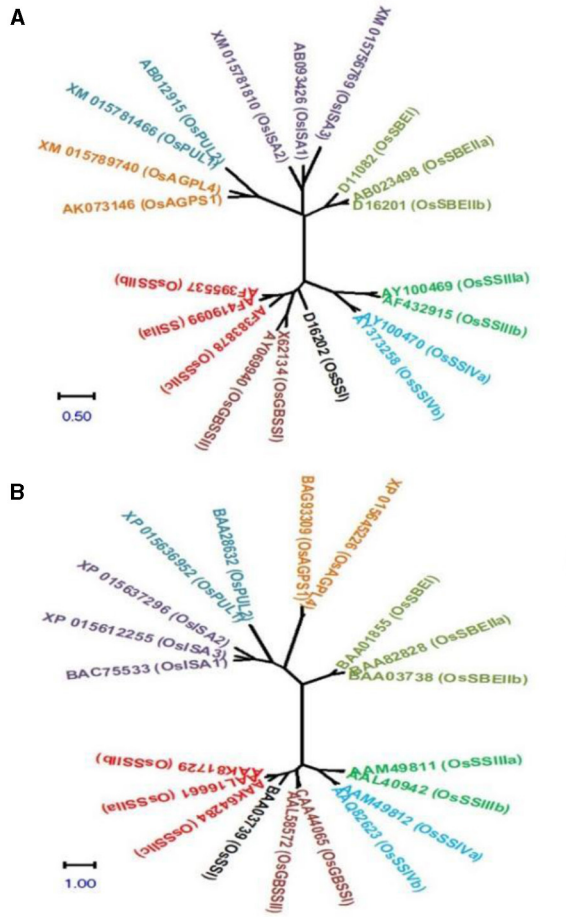


FIGURE 6
Differential expression of starch branching **(A)** *SBEI*, **(B)** *SBEIIa* and **(C)** *SBEIIb* and debranching **(D)** *ISA1*, **(E)** *ISA2*, **(F)** *ISA3*, **(G)** *PUL1*, and **(H)** *PUL2* genes in the developing endosperm of contrasting rice genotypes (NJ-72 and Ananga). Bar indicates ± SD. Different lowercase letters indicate significant difference between mean at $p < 0.05$ [Fishers' least significant difference (LSD) test].

3.8 Gene to metabolite correlation and phylogeny

The phylogenetic relationship of rice starch biogenesis genes and their corresponding protein sequences is shown in Figures 7A, B respectively. The gene ID, protein ID, and their functions are shown in Figure 7C. The rice starch biogenesis gene and protein family comprised 20 isoforms that are grouped into 9 clades: 2 AGP, 2 GBSS, 1 SSI, 3 SSII, 2 SSIII, 2 SSIV, 3 SBE, 3 ISA, and 2 PUL. Genes and proteins in the same clade conferred sequence similarities.

To simplify our understanding of the relative expression of genes and the accumulation of metabolites concerning five



Gene ID	Protein ID	Function
AK073146	BAG93309	Catalyses rate limiting step
XM_015789740	XP_015645226	Regulatory function
X62134	CAA44065	Amylose synthesis in storage tissues
AY069940	AAL58572	Amylose synthesis in leaves
D16202	BAA03739	Synthesis of short chains of amylopectin in seed
AF419099	AAL16661	Synthesis of medium chains of amylopectin in seed
AF395537	AAK81729	Amylopectin synthesis in leaves
AF383878	AAK64284	Amylopectin synthesis
AY100469	AAM49811	Formation of B1 and B2 chains of amylopectin in seed
AF432915	AAL40942	Amylopectin synthesis in leaves
AY100470	AAM49812	Priming of starch granule formation
AY373258	AAQ82623	Function unknown
D11082	BAA01855	Branching long chains in amylopectin
AB023498	BAA82828	Branching short chains
D16201	BAA03738	Branching shorts chains in of endosperm
AB093426	BAC75533	Trimming of improper branches
XM_015781810	XP_015637296	Debranch amylopectin
XM_015756769	XP_015612255	Starch decomposition
XM_015781466	XP_015636952	Debranch amylopectin
AB012915	BAA28632	Debranch amylopectin chains

FIGURE 7
Phylogeny of starch biogenesis genes **(A)** and corresponding proteins **(B)** Gene ID, protein ID, and their catalytic functions **(C),** and the correlation network between genes and metabolites **(D).** Positive and negative correlations are highlighted with green and red colored edges. Color thickness is proportional to the correlation coefficient values. NJ7- NJ-72, Ang- Ananga, D3- 3DAA, D8- 8DAA, D13- 13DAA, D18- 18DAA, D23- 23DAA, RS- resistant starch, TS- total starch, Amyls- amylose, Amylp- amylopectin, AGPS- AGPS1, and AGPL- AGPL4.

endosperm developing stages, the correlation network between 20 isoforms of starch biosynthesis genes and 4 metabolites (total starch, amylose, amylopectin, and RS) was developed. NJ-72 and Ananga are contrasting genotypes based on their AC and RS content, and therefore they have a strong negative correlation. The total accumulation of RS, total starch, amylose, and amylopectin was high at 23DAA and the expression level for *GBSSI, ISA1, ISA2*, and *PUL2* was high at 18DAA (Figure 7D). At 8DAA strong positive correlation was found between *AGPS1, SSIIc*, and *SBEI*. Some of the genes such as *SSIIb, SSIIIb, SSIVb, GBSSII, SBEIIa, ISA3*, and *PUL1* are not predominantly expressed in the endosperm tissue therefore their correlation was not found significant.

4 Discussion

4.1 Matrix components, microstructure, and starch quality are determinants of nutritional traits in rice

The total starch content (TSC) of 200 diverse rice genotypes in this study aligns with the findings of Omar et al. (31), who reported TSC ranging from 81.23% to 92.73% in rice. The average TSC in rice typically falls between 70% and 90%, with variations attributable to factors such as genotypic and varietal differences, and differing methods of starch estimation, including variations in the instruments used to measure absorbance. The functionality of rice starch is determined by its components, such as APC, AC, and RS, as well as the ratios between them. RS content is positively correlated with AC, with higher amylose cultivars generally exhibiting higher RS levels. Deepa et al. (32) conducted a comparative study on the RS content of pigmented ("Njavara" and "Jyothi") and non-pigmented ("IR 64") rice varieties, reporting RS levels between 0.6% and 1%, which is consistent with the present study's findings. Similarly, this study showed a positive correlation between RS content and AC, in agreement with the findings of Hu et al. (33), who observed that RS content increased with rising AC across three different rice cultivars. It is hypothesized that genotypes with high RS content may lower blood glucose levels by reducing the glycemic index (GI), a conclusion supported by Kumar et al. (34).

A significant positive correlation was found between the degree of crystallinity and the percentage of amylopectin. Our findings are consistent with that of Martens et al. (35) who studied starch digestion kinetics and concluded that the type of crystalline structure and amylopectin chain length distribution of starch significantly correlate with the digestion kinetics of starches across botanic sources in an *in vitro* pig model. The XRD results from this study indicate that rice genotypes with differing AC also show variations in the degree of crystallinity, which may influence starch digestibility.

Among the 10 FAs analyzed, palmitic acid, linoleic acid, and oleic acid were found in higher concentrations. Our results are consistent with Al-Bahrany (36), who examined the FAs composition in two Hassawi rice genotypes and concluded that palmitic, oleic, and linoleic acids were the most abundant. Notable, linoleic acid is the predominant polyunsaturated fatty acid (PUFA) among the studied rice genotypes. In japonica, ssIIIa mutants, linoleic acid (C18:2) was complexed with amylose to form RS5 which significantly contributes to the enhanced RS content (37). Furthermore, there is a direct correlation between the percentage of amylose and FA composition, particularly with palmitic acid, linoleic acid, and stearic acid. This amylose-lipid complex is thought to render starch more resistant to digestion. These findings are consistent with Taylor et al. (38), who reported that lipids can form complexes with amylose, subsequently reducing starch digestibility.

Protein content was highest in NJ-72 (8.98%) and lowest in RNRM-7 (7.99%). Khatun et al. (39) indicated that rice protein may affect starch digestibility by building a protective barrier around rice starch or by modifying starch characteristics, which contrast with our findings. Furthermore, Zhu et al. (40) discovered that the protein level of rice flour was inversely connected with RDS and SDS, but positively correlated with RS. Our findings matched with Li et al. (41), who studied twelve milled rice grain samples and observed that the crude protein content varies from 6.5% to 9.4%. Based on these findings, we concluded that the protein level may not significantly affect the digestibility of genotypes with varying RS levels, as there was minimal variation in protein percentage across the different genotypes.

By examining starch granule morphology, we hypothesized that high RS genotypes might be more resistant to amylase hydrolysis due to their well-packaged, complex structures and larger granule sizes. This finding agrees with Zaman and Sarbini (42), who found that smaller granules are more sensitive to enzyme digestion, attributed to their larger specific surface area, which increases enzyme binding rate. Corgneau et al. (43), reported that high RS potato starch was composed of large rounded granules having smooth surfaces. In contrast, waxy rice starch was rich in amylopectin and displayed small diameters and angular shapes. The degree of amylose, which is directly proportional to the RS, influences the size and shape of starch granules. This conclusion is supported by Krishnan et al. (44), who looked at the morphology of rice starch granules and found that genotypes with high inherent RS exhibited tight starch packing within the granules. This tight packing could contribute to the observed enzyme resistance, potentially linked to the structural complexity of amylose and amylopectin. Thus, starch granule morphology illustrates how variations in amylose percentage can influence the shape of starch granules, ultimately affecting starch digestibility and blood sugar levels.

4.2 Variation in metabolites at developing stages of endosperm

The maximum rate of accumulation of the carbohydrates and RS occurred between 8DAA to 23DAA. In accordance with our results Zi et al. (45) found that the rate of TSC, AC, and APC was significantly increased between 10DAA and 25DAA in developing stages of waxy and non-waxy wheat genotypes. Verma et al. (46) reported that in rice TSC was maximum at 21DAA as compared to other developing stages. Similarly, Asai et al. (47) studied the developmental changes in rice endosperm and found a rapid increase in AC up to 20 days after fertilization (DAF).

4.3 Alteration in activities of starch biogenesis enzymes

The activity of all the studied starch biogenesis enzymes was found higher in NJ-72 in comparison to Ananga throughout

the developing stages. This difference suggests that the genes responsible for amylopectin synthesis are predominantly expressed in low amylose genotypes, such as NJ-72. At 3DAA activity of all the enzymes was very low because of the high hexose to sucrose ratio that leads to cell division rather than supplying photosynthetic carbon to endosperm development. The increased activities of SS and BE at 13 DAA in NJ-72 highlight the critical period for starch accumulation, where enzyme activity correlates with amylopectin synthesis. Furthermore, the elevated activity of pullulanase (PUL) and isoamylase (ISA) at 18 DAA suggests their roles in starch remodeling and debranching become prominent after initial primer synthesis and branching, facilitating further starch granule development. Similarly, Wang et al. (48) found maximum SS activity at 12 and SBE activity at 15 days after pollination in low and high starch-content wheat genotypes. Fujita et al. (21) studied PUL-deficient mutant rice and concluded that the function of PUL partially overlaps with that of ISA1, and the absence of PUL has a much smaller impact on the production of amylopectin than the absence of ISA1. These findings will enhance the understanding of starch biogenesis, particularly in low amylose rice genotypes, highlighting critical developmental stages where enzyme activities peak, which could inform breeding strategies for improved starch accumulation. Further, to elaborate on the differentiation in activity assays of these starch biogenesis enzymes (SS, SBE, and DBE) in developing stages of rice endosperm, genes in their respective isoforms encoding these enzymes were also evaluated.

4.4 Differential expression of starch biogenesis genes in the developing endosperm

Mutations in genes encoding GBSS, soluble SS, and SBE have been shown to affect RS content in rice (19, 49). The expression level of *AGPS1* in NJ-72 was slightly higher at 8DAA and 13DAA than in Ananga while in rest of the stages, *AGPS1* expression was higher in Ananga. These observations indicated that this isoform is needed for both low and high-amylose genotypes. Nagai et al. (50) studied transgenic rice expressing upregulated *cytoplasmic AGPase* in developing seeds and concluded that this gene catalyzes the rate-limiting step of the starch biosynthetic pathway. Slattery et al. (51) explored how increasing amylose levels through *AGPase* activity leads to greater RS formation and reduced starch digestibility. This suggests that manipulating *AGPS1* expression could optimize starch biosynthesis across different rice varieties and enhancing *AGPase* function could be a viable strategy for increasing RS. In contrast to our finding, Jabeen et al. (52) observed that *GBSS* protein was highly accumulated at 10DAA in contrasting GI rice lines. Downregulation of *GBSSII* at the initial stage and very low expression at later stages compared to *GBSSI* indicated that *GBSSII* may be expressed more in non-storage tissues. In addition, the expression of *GBSSI* was more at all stages compared to *GBSSII*, so it is presumed that *GBSSI* is mainly responsible for amylose synthesis and is unique to endosperm whereas *GBSSII* plays a minor role. These results are in agreement with Vrinten and Nakamura (53) who studied *GBSSI* and *GBSSII* transcripts in wheat and observed that *GBSSI* was highly expressed in the endosperm while *GBSSII* was expressed in leaf, culm, and pericarp, but not in endosperm tissue. This reinforces the notion that *GBSSII* may not be essential for starch storage in the endosperm but might play a role in other tissues, suggesting a tissue-specific regulatory mechanism. Overexpression of *GBSSI* has been also been linked to enhanced RS content in rice (54). The association of *GBSSI* overexpression with increased RS content further underscores the significance of amylose in starch digestibility and its potential benefits for developing rice varieties with favorable nutritional profiles.

Among the soluble SS, at 13DAA *SSI* showed maximum relative expression followed by *SSIIIa, SSIIa, SSIVa,* and *SSIIc*. Our findings suggest that these genes are endosperm-specific and they contribute primarily to the production of amylopectin in the NJ-72. In accordance with this, Fujita et al. (55) characterized starch biosynthesis-related enzymes in *SSI-deficient* rice mutant lines and summarized that the coordinated actions of starch synthases such as *SSI, SSIIa*, and *SSIIIa* isoforms generate amylopectin chains. Also, Hayashi et al. (56) generated the mutants of rice seeds (*ss1/ss2a/ss3a*) and concluded that mutated plants retained their capability to synthesize starch and were able to accumulate amylose but less amylopectin. Rice varieties with deleterious variants in the *SSI* gene exhibit higher RS levels, indicating the critical role of *SSI* in RS formation (57). Downregulation of *SSI* in both *japonica* and *indica* rice cultivars significantly increases AC while negatively impacting eating and cooking quality (58). In the rice cultivar Nipponbare, suppression of *SSI* alters amylopectin chain distribution, increases GBSS activity, and elevates AC (59). These findings suggest that *SSI* influences RS formation by modulating AC and amylopectin structure. Although the expression of *SSIIb, SSIIIb,* and *SSIVb* were slightly high in NJ-72 as compared to Ananga the fold variation was much less throughout the developing endosperms. In addition, their expression was minute in comparison to the above-mentioned SS genes. It can be assumed that *SSIIb, SSIIIb,* and *SSIVb* are not endosperm-specific genes (for rice) and their expression might be high in non-storage tissues.

The consistently higher expression levels of *SBE* isoforms (*SBEI*, *SBEIIa*, and *SBEIIb*) in NJ-72 across all developmental stages indicate a robust starch branching capability in this low amylose genotype. Wang et al. (60) used TNG82 japonica rice cultivar to mutate the *OsSBEIIb* gene through CRISPR/Cas9. The total activity of SBE at 20 days was 1.75 times higher than SBE at 25 DAF in both wild type as well as in mutant lines. The observation that *SBEIIb* had the highest expression, followed by *SBEI* and *SBEIIa*, implies a hierarchy in the functional roles of these enzymes during starch biosynthesis. *SBEIIa*'s limited role suggests that it may not be as critical for starch branching in endosperm development, which may have implications for future research targeting specific isoforms for genetic improvement. The findings are consistent with previous studies, such as that of Miura et al. (61), indicating that overexpression of *SBEI* can lead to increased RS content. This correlation suggests that manipulating the expression of *SBEI* could be a potential strategy for enhancing RS levels in rice, which is beneficial for health-related attributes such as improved glycemic response.

The overall expression of *PUL2* is higher as compared to *PUL1* in both genotypes. Therefore, it was observed that *PUL2* is more important than *PUL1* as a DBE. Among the 5 DBEs at 18DAA, *PUL2* has the highest expression followed by *ISA1* and *ISA2*. Similarly, Yamakawa et al. (62) studied the expression of grain-filling-related genes in rice at 10DAF under high temperatures and based on microarray and RT-PCR results reported that *ISA1, ISA3*, and *PUL* showed 0.94, 0.93, and 0.83-fold change respectively. In contrast to their findings, our results indicated that *PUL2* was predominantly expressed followed by *ISA1*, while *ISA3* having minor role as a DBE. Ohdan et al. (10) reported that all the DBEs had low levels of transcripts at the early stages of seed development (1-3 DAF), which was maintained by both *ISA2* and *ISA3* up to the later stages. But *PUL* and *ISA1* showed 12 and 56-fold increases in transcript level from 3 to 7 DAF respectively advising that *ISA1* and *PUL* play crucial roles in the starch accumulation process throughout the endosperm. In addition to their findings, the current results also indicated that the 18DAA stage is the most favorable stage for studying the expression of DBEs and at this stage, structural winding and unwinding of starch molecules occurs the most. This knowledge can guide future research to optimize the timing of interventions aimed at improving starch characteristics. *PUL* plays a role in RS formation by releasing linear amylose-like chains that promote starch retrogradation, resulting in the synthesis of type 3 RS and reduced starch digestibility (63). This aspect is crucial for developing rice varieties with enhanced nutritional benefits, especially for consumers seeking low GI foods.

4.5 Gene to metabolite correlation and phylogeny

Among the metabolites, RS and AC have a strong positive correlation ($r = 0.96$). Krishnan et al. (44) in rice varieties showed that RS is moderately dependent on the percentage of amylose while weakly dependent on the percentage of amylopectin. This reinforces the importance of focusing on amylose synthesis and regulation to influence RS content in rice varieties. In comparison to other starch biogenesis genes, GBSS has a slightly positive correlation with AC ($r = 0.52$) and RS ($r = 0.40$), while with amylopectin it has a weak positive correlation ($r = 0.28$). Similarly, Zi et al. (45) reported that AC was found to be significantly and positively correlated with GBSS activity ($r = 0.80$) in waxy and non-waxy wheat cultivars. SS, DBE, AGP, and BE were found to be correlated with the RS content to some extent in the rice-developing grains and their weaker relationships suggest that *GBSS* may be more critical for enhancing RS. This could mean that manipulating GBSS expression may yield more significant improvements in RS content compared to other genes. At 13DAA there is a strong positive correlation between *AGPL4, SSI, SSIIa, SSIIIa, SSIVa*, and *SBEIIb*. Overall results showed strong networking of starch biogenesis genes with metabolites in starch accumulation and structural partitioning in the form of AC, APC, and RS at differential levels during the developmental stages of rice endosperm.

5 Conclusion

Rice starch digestibility depends on several factors, including the amount of amylose, amylopectin, RS, compositions of different FAs, starch granule morphology, crystallinity degree percentage, protein percentage, the enzymatic activity of starch biogenesis, etc. Amylose has a significant positive correlation with that of RS and compactness of starch, but it has a negative correlation found with the percentage of amylopectin, crystallinity degree percentage, activity of SS, SBE, and ISA, etc. As the amount of amylose increases, it causes tight packaging of starch granules and a low crystallinity degree percentage that might limit the availability of starch to the digestive enzymes and hence lowers the energy density. Among the metabolites, RS and AC have a strong positive correlation with their corresponding encoding genes. The present study also revealed that a positive correlation exists between starch biosynthetic enzyme activity and AC and TSC accumulation in two contrasting rice genotypes. The genes exhibiting high expression either at early or late or across all the endosperm developmental stages can be used as a candidate gene for developing molecular markers through association or linkage analysis, which further can be used for starch quality improvement in rice.

Therefore, these findings suggest that eating rice that has high amylose and/or RS may lower blood glucose levels and hence reduce the risk of diseases resulting from high blood sugar. It can be utilized to modify the starch biogenesis enzymes and genes to meet the requirement of quality starch for increasing population, and expanding food industries while keeping in mind to protect the environment from non-degradable biopolymers. Further investigations are required to reveal how the alterations in the enzymatic activities related to starch biosynthesis govern the partitioning of AC, APC, and RS affecting structural modulations toward the starch granule formation and accumulation of quality starch contents (RS).

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

SW: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. MA: Data curation, Methodology, Validation, Writing – review & editing. ST: Writing – review & editing. VK: Conceptualization, Validation, Writing – review & editing. SK: Supervision, Writing – review & editing. HB: Resources, Writing – review & editing. AA: Supervision, Writing – review & editing. AMS: Supervision, Writing – review & editing. MR: Software, Writing – review & editing. SP: Supervision, Writing – review & editing. AS: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The financial assistance was provided by DST-SERB (project number-EMR/2016/005722), Department of Science and Technology, Government of India and ICAR-Indian Agricultural Research Institute (IARI), Ministry of Agriculture, Government of India for this research.

Acknowledgments

We are thankful to our team members for their guidance, assistance, providing laboratory facilities, designing the manuscript, etc.

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

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1448450/full#supplementary-material

References

1. Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, et al. IDF Diabetes Atlas: global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes Res Clin Pract.* (2022) 183:109119. doi: 10.1016/j.diabres.2021.109119

2. Brar DS, Khush GS. 14 transferring genes from wild species into rice. *Qnt Genet Genom Plant Breed.* (2002) 24:197. doi: 10.1079/9780851996011.0197

3. Salari-Moghaddam A, Keshteli AH, Haghighatdoost F, Esmaillzadeh A, Adibi P. Dietary glycemic index and glycemic load in relation to general obesity and central adiposity among adults. *Clin Nutr.* (2019) 38:2936–42. doi: 10.1016/j.clnu.2018.12.036

4. Bello-Perez LA, Flores-Silva PC, Agama-Acevedo E, Tovar J. Starch digestibility: past, present, and future. *J Sci Food Agric.* (2020) 100:5009–16. doi: 10.1002/jsfa.8955

5. Maziarz MP, Preisendanz S, Juma S, Imrhan V, Prasad C, Vijayagopal P. Resistant starch lowers postprandial glucose and leptin in overweight adults consuming a moderate-to-high-fat diet: a randomized-controlled trial. *Nutr J.* (2017) 16:1–10. doi: 10.1186/s12937-017-0235-8

6. Matsuki J. Resistant starch. *J Jap Soc Food Sci.* (2010) 57:224. doi: 10.3136/nskkk.57.224

7. Chena Z, Liang N, Zhang H, Li H, Guo J, Zhang Y, et al. Resistant starch and the gut microbiome: Exploring beneficial interactions and dietary impacts. *Food Chem.* (2024) 10:101118. doi: 10.1016/j.fochx.2024.101118

8. Pandey MK, Rani NS, Madhav MS, Sundaram RM, Varaprasad GS, Sivaranjani AK, et al. Different isoforms of starch-synthesizing enzymes controlling amylose and amylopectin content in rice (*Oryza sativa* L.). *Biotechnol Adv.* (2012) 30:1697–1706. doi: 10.1016/j.biotechadv.2012.08.011

9. Zhao X, Andersson M, Andersson R. Resistant starch and other dietary fiber components in tubers from a high-amylose potato. *Food Chem.* (2018) 251:58–63. doi: 10.1016/j.foodchem.2018.01.028

10. Ohdan T, Francisco Jr PB, Sawada T, Hirose T, Terao T, Satoh H, et al. Expression profiling of genes involved in starch synthesis in sink and source organs of rice. *J Exp Bot.* (2005) 56:3229–44. doi: 10.1093/jxb/eri292

11. Nakamura Y, Fujita N, Kubo A, Rahman S, Morell M, Satoh H. Engineering of amylopectin biosynthesis in rice endosperm. *J Appl Glycosci.* (2003) 50:197–200. doi: 10.5458/jag.50.197

12. Reeves CD, Krishnan HB, Okita TW. Gene expression in developing wheat endosperm: accumulation of gliadin and ADPglucose pyrophosphorylase messenger RNAs and polypeptides. *Plant Physiol.* (1986) 82:34–40. doi: 10.1104/pp.82.1.34

13. Rongine de Fekete MA, Leloir LF, Cardini CE. Mechanism of starch biosynthesis. *Nature.* (1960) 187:918–9. doi: 10.1038/187918a0

14. Dry I, Smith A, Edwards A, Bhattacharyya M, Dunn P, Martin C. Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. *Plant J.* (1992) 2:193–202. doi: 10.1046/j.1365-313X.1992.t01-42-00999.x

15. Nakamura Y. Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. *Plant Cell Physiol.* (2002) 43:718–25. doi: 10.1093/pcp/pcf091

16. Tetlow IJ, Emes MJ. *Comprehensive Biotechnology*. Amsterdam: Elsevier (2011).

17. Hawkins E, Chen J, Watson-Lazowski A, Ahn-Jarvis J, Barclay JE, Fahy B, et al. Starch synthase 4 is required for normal starch granule initiation in amyloplasts of wheat endosperm. *New Phytol.* (2021) 230:2371–86. doi: 10.1111/nph.17342

18. Hirose T, Terao T. A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). *Planta.* (2004) 220:9–16. doi: 10.1007/s00425-004-1314-6

19. Baysal C, He W, Drapal M, Villorbina G, Medina V, Capell T, et al. Inactivation of rice starch branching enzyme IIb triggers broad and unexpected changes in metabolism by transcriptional reprogramming. *Proc Natl Acad Sci.* (2020) 117:26503–12. doi: 10.1073/pnas.2014860117

20. Martin C, Smith AM. Starch biosynthesis. *Plant Cell.* (1995) 7:971. doi: 10.2307/3870051

21. Fujita N, Toyosawa Y, Utsumi Y, Higuchi T, Hanashiro I, Ikegami A, et al. Characterization of pullulanase (PUL)-deficient mutants of rice (*Oryza sativa* L.) and the function of PUL on starch biosynthesis in the developing rice endosperm. *J Exp Bot.* (2009) 60:1009–1023. doi: 10.1093/jxb/ern349

22. Dinges JR, Colleoni C, James MG, Myers AM. Mutational analysis of the pullulanase-type debranching enzyme of maize indicates multiple functions in starch metabolism. *Plant Cell.* (2003) 15:666–80. doi: 10.1105/tpc.007575

23. Adegoke TV, Wang Y, Chen L, Wang H, Liu W, Liu X, et al. Posttranslational modification of waxy to genetically improve starch quality in rice grain. *Int J Mol Sci.* (2021) 22:4845. doi: 10.3390/ijms22094845

24. Qu J, Xu S, Zhang Z, Chen G, Zhong Y, Liu L, et al. Evolutionary, structural and expression analysis of core genes involved in starch synthesis. *Sci Rep.* (2018) 8:12736. doi: 10.1038/s41598-018-30411-y

25. Clegg KM. The application of the anthrone reagent to the estimation of starch in cereals. *J Sci Food Agric.* (1956) 7:40–4. doi: 10.1002/jsfa.2740070108

26. Juliano BO. A simplified assay for milled rice amylose. *Cereal Sci.* (1971) 12:334–60.

27. Kirk PL. Kjeldahl method for total nitrogen. *Anal Chem.* (1950) 22:354–8. doi: 10.1021/ac60038a038

28. Leloir LF, Rongine de Fekete MA, Cardini CE. Starch and oligosaccharide synthesis from uridine diphosphate glucose. *J Biol Chem.* (1961) 236:636–41. doi: 10.1016/S0021-9258(18)64280-2

29. Hawker JS, Ozbun JL, Ozaki H, Greenberg E, Preiss J. Interaction of spinach leaf adenosine diphosphate glucose α-1, 4-glucan α-4-glucosyl transferase and α-1, 4-glucan, α-1, 4-glucan-6-glycosyl transferase in synthesis of branched α-glucan. *Arch Biochem Biophys.* (1974) 160:530–51. doi: 10.1016/0003-9861(74)90430-5

30. Fujita N, Kubo A, Francisco PB, Nakakita M, Harada K, Minaka N, et al. Purification, characterization, and cDNA structure of isoamylase from developing endosperm of rice. *Planta.* (1999) 208:283–93. doi: 10.1007/s004250050560

31. Omar KA, Salih BM, Abdulla NY, Hussin BH, Rassul SM. Evaluation of starch and sugar content of different rice samples and study their physical properties. *Indian J Nat Sci.* (2016) 6:11084–93.

32. Deepa G, Singh V, Naidu KA. A comparative study on starch digestibility, glycemic index and resistant starch of pigmented ('Njavara' and 'Jyothi') and a non-pigmented ('IR 64′) rice varieties. *J Food Sci Technol.* (2010) 47:644–9. doi: 10.1007/s13197-010-0106-1

33. Hu P, Zhao H, Duan Z, Linlin Z, Wu D. Starch digestibility and the estimated glycemic score of different types of rice differing in amylose contents. *J Cereal Sci.* (2004) 4:231–7. doi: 10.1016/j.jcs.2004.06.001

34. Kumar A, Sahoo U, Baisakha B, Okpani OA, Ngangkham U, Parameswaran C, et al. Resistant starch could be decisive in determining the glycemic index of rice cultivars. *J Cereal Sci.* (2018) 79:348–53. doi: 10.1016/j.jcs.2017.11.013

35. Martens BM, Gerrits WJ, Bruininx EM, Schols HA. Amylopectin structure and crystallinity explains variation in digestion kinetics of starches across botanic sources in an in vitro pig model. *J Anim Sci Biotechnol.* (2018) 9:1–13. doi: 10.1186/s40104-018-0303-8

36. Al-Bahrany AM. Chemical composition and fatty acid analysis of Saudi Hassawi rice *Oryza sativa* L. *Pak J Biol Sci.* (2002) 5:212–4. doi: 10.3923/pjbs.2002.212.214

37. Zhou Y, Cheng Z, Jiang S, Cen J, Yuan S, Yu C, et al. Inactivation of SSIIIa enhances the RS content through altering starch structure and accumulating C18: 2 in japonica rice. *Carbohydr Polym.* (2023) 318:121141. doi: 10.1016/j.carbpol.2023.121141

38. Taylor JR, Emmambux MN, Kruger J. Developments in modulating glycaemic response in starchy cereal foods. *Starke.* (2015) 67:79–89. doi: 10.1002/star.201400192

39. Khatun A, Waters DL, Liu L. A review of rice starch digestibility: effect of composition and heat-moisture processing. *Starch-Starke.* (2019) 71:9–10. doi: 10.1002/star.201900090

40. Zhu LJ, Liu QQ, Wilson JD, Gu MH, Shi YC. Digestibility and physicochemical properties of rice (*Oryza sativa* L.) flours and starches differing in amylose content. *Carbohydr Polym.* (2011) 86:1751–9. doi: 10.1016/j.carbpol.2011.07.017

41. Li H, Prakash S, Nicholson TM, Fitzgerald MA, Gilbert RG. The importance of amylose and amylopectin fine structure for textural properties of cooked rice grains. *Food Chem.* (2016) 196:702–11. doi: 10.1016/j.foodchem.2015.09.112

42. Zaman SA, Sarbini SR. The potential of resistant starch as a prebiotic. *Crit Rev Biotechnol.* (2016) 36:578–84. doi: 10.3109/07388551.2014.993590

43. Corgneau M, Gaiani C, Petit J, Nikolova Y, Banon S, Ritie-Pertusa L, et al. Digestibility of common native starches with reference to starch granule size, shape and surface features towards guidelines for starch-containing food products. *Int J Food Sci Technol.* (2019) 54:2132–40. doi: 10.1111/ijfs.14120

44. Krishnan V, Awana M, Samota MK, Warwate SI, Kulshreshtha A, Ray M, et al. Pullulanase activity: a novel indicator of inherent resistant starch in rice (*Oryza sativa* L). *Int J Biol Macromol.* (2020) 152:1213–23. doi: 10.1016/j.ijbiomac.2019.10.218

45. Zi Y, Ding J, Song J, Humphreys G, Peng Y, Li C, et al. Grain yield, starch content and activities of key enzymes of waxy and non-waxy wheat (*Triticum aestivum* L.). *Sci Rep*. (2018) 8:4548. doi: 10.1038/s41598-018-22587-0

46. Verma VC, Agrawal S, Kumar A, Jaiswal JP. Starch content and activities of starch biosynthetic enzymes in wheat, rice and millets. *J Pharmacogn Phytochem.* (2020) 9:1211–8. doi: 10.22271/phyto.2020.v9.i4q.11883

47. Asai H, Abe N, Matsushima R, Crofts N, Oitome NF, Nakamura Y, et al. Deficiencies in both starch synthase IIIa and branching enzyme IIb lead to a significant increase in amylose in SSIIa-inactive japonica rice seeds. *J Exp Bot.* (2014) 65:5497–507. doi: 10.1093/jxb/eru310

48. Wang Z, Li W, Qi J, Shi P, Yin Y. Starch accumulation, activities of key enzyme and gene expression in starch synthesis of wheat endosperm with different starch contents. *J Food Sci Technol.* (2014) 51:419–29. doi: 10.1007/s13197-011-0520-z

49. Zhou H, Wang L, Liu G, Meng X, Jing Y, Shu X, et al. Critical roles of soluble starch synthase SSIIIa and granule-bound starch synthase Waxy in synthesizing resistant starch in rice. *Proc Natl Acad Sci.* (2016) 113:12844–9. doi: 10.1073/pnas.1615104113

50. Nagai YS, Sakulsingharoj C, Edwards GE, Satoh H, Greene TW, Blakeslee B, et al. Control of starch synthesis in cereals: metabolite analysis of transgenic rice expressing an up-regulated cytoplasmic ADP-glucose pyrophosphorylase in developing seeds. *Plant Cell Physiol.* (2009) 50:635–43. doi: 10.1093/pcp/pcp021

51. Slattery CJ, Kavakli IH, Okita TW. Engineering starch for increased quantity and quality. *Trends Plant Sci.* (2000) 5:291–8. doi: 10.1016/S1360-1385(00)01657-5

52. Jabeen I, Hafiz M, Ratnam W, Karupaiah T, Dipti S, Fujita N, et al. Differential expression of three key starch biosynthetic genes in developing grains of rice differing in glycemic index. *J Cereal Sci.* (2021) 99:103187. doi: 10.1016/j.jcs.2021.103187

53. Vrinten PL, Nakamura T. Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. *Plant Physiol.* (2000) 122:255–64. doi: 10.1104/pp.122.1.255

54. You H, Liang C, Zhang O, Xu H, Xu L, Chen Y, et al. Variation of resistant starch content in different processing types and their starch granules properties in rice. *Carbohydr Polym.* (2022) 276:118742. doi: 10.1016/j.carbpol.2021.118742

55. Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, Hirochika H, et al. Function and characterization of starch synthase I using mutants in rice. *Plant Physiol.* (2006) 140:1070–84. doi: 10.1104/pp.105.071845

56. Hayashi M, Crofts N, Oitome NF, Fujita N. Analyses of starch biosynthetic protein complexes and starch properties from developing mutant rice seeds with minimal starch synthase activities. *BMC Plant Biol.* (2018) 18:1–15. doi: 10.1186/s12870-018-1270-0

57. Raja RB, Agasimani S, Jaiswal S, Thiruvengadam V, Sabariappan R, Chibbar RN, et al. EcoTILLING by sequencing reveals polymorphisms in genes encoding starch synthases that are associated with low glycemic response in rice. *BMC Plant Biol.* (2017) 17:1–13. doi: 10.1186/s12870-016-0968-0

58. Li Q, Liu X, Zhang C, Jiang L, Jiang M, Zhong M, et al. Rice soluble starch synthase I: allelic variation, expression, function, and interaction with waxy. *Front Plant Sci.* (2018) 9:1591. doi: 10.3389/fpls.2018.01591

59. Zhao Q, Du X, Han Z, Ye Y, Pan G, Zhou Q, et al. Suppression of starch synthase I (SSI) by RNA interference alters starch biosynthesis and amylopectin chain distribution in rice plants subjected to high temperature. *Crop J.* (2019) 7:573–86. doi: 10.1016/j.cj.2019.03.009

60. Wang HC, Hsu YC, Wu YP, Yeh SY, Ku MS. Production of high amylose and resistant starch rice through targeted mutagenesis of starch branching enzyme IIb by Crispr/cas9. *Res Sq.* (2021) 10:1–29. doi: 10.21203/rs.3.rs-585478/v1

61. Miura S, Koyama N, Crofts N, Hosaka Y, Abe M, Fujita N. Generation and starch characterization of non-transgenic BEI and BEIIb double mutant rice (*Oryza sativa*) with ultra-high level of resistant starch. *Rice.* (2021) 14:1–16. doi: 10.1186/s12284-020-00441-0

62. Yamakawa H, Hirose T, Kuroda M, Yamaguchi T. Comprehensive expression profiling of rice grain filling-related genes under high temperature using DNA microarray. *Plant Physiol.* (2007) 144:258–77. doi: 10.1104/pp.107.098665

63. Kumar A, Sahoo U, Lal MK, Tiwari RK, Lenka SK, Singh NR, et al. Biochemical markers for low glycemic index and approaches to alter starch digestibility in rice. *J Cereal Sci.* (2022) 106:103501. doi: 10.1016/j.jcs.2022.103501

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 04 December 2024
DOI 10.3389/fnut.2024.1424614

OPEN ACCESS

EDITED BY
Qinghua He,
Southwest University, China

REVIEWED BY
Claudia Perez-Cruz,
National Polytechnic Institute of Mexico (CINVESTAV), Mexico
Steven E. Riechman,
Texas A&M University, United States

*CORRESPONDENCE
Qi Xu
✉ isuxuqi@163.com
Jiajie Zang
✉ zangjiajie@scdc.sh.cn

†These authors have contributed equally to this work

RECEIVED 28 April 2024
ACCEPTED 13 November 2024
PUBLISHED 04 December 2024

CITATION
Chen Y, Sun Z, Zhang Y, Zhou R, Lin X, Du Y, Xu J, Xu Q and Zang J (2024) The associations of dietary manganese, iron, copper, zinc, selenium and magnesium with cognitive outcomes in Chinese adults: a cross sectional study in Shanghai.
Front. Nutr. 11:1424614.
doi: 10.3389/fnut.2024.1424614

The associations of dietary manganese, iron, copper, zinc, selenium and magnesium with cognitive outcomes in Chinese adults: a cross sectional study in Shanghai

Yujie Chen[1†], Zhuo Sun[2†], Yali Zhang[1†], Ren Zhou[3], Xingyu Lin[1], Yuewen Du[1], Jiayi Xu[1], Qi Xu[1*] and Jiajie Zang[2*]

[1]School of Public Health, Shanghai University of Traditional Chinese Medicine, Shanghai, China, [2]Division of Health Risk Factors Monitoring and Control, Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China, [3]Department of Anesthesiology, The Ninth People's Hospital of Shanghai, Jiao Tong University School of Medicine, Shanghai, China

Introduction: The role of individual nutrients including vitamins and minerals in cognitive function gained increasing attention in recent years. With regard to the association between dietary minerals and cognitive function, the results of human studies are inconclusive. The objective of this study was to explore the association between mineral intake and cognitive function using the data from Shanghai Health and Nutrition Survey (SHNS) in 2018.

Methods: In total, 835 adults were included in a crosssectional study, and completed a three-day dietary record to estimate their average daily intake of minerals. Mini-Mental State Examination (MMSE) was used for the assessment of cognitive function, and logistic regression analyses were performed on participants to examine the association between dietary mineral intake and cognitive performance. The participants were divided into tertiles according to their mineral intake.

Results: Participants in the second and third tertile of the dietary copper intake had lower rates of low MMSE scores compared to those in the lowest tertile. We found the adjusted OR and 95%CI values were 0.44 (0.21–0.89) and 0.40 (0.16–0.94), respectively. Participants in the second tertile of dietary magnesium intake showed a trend of lower rates of low MMSE score compared to those in the lowest tertile (p = 0.06). The adjusted OR and 95%CI values were 0.35 (0.16–0.72). No significant association were observed between any of the other minerals including iron, zinc, selenium and manganese and cognitive function.

Discussion: Our findings suggest that dietary intake of copper and magnesium may have a protective effect on cognitive performance in elderly over 60 years old. To prevent cognitive decline, elderly should get recommended amounts of copper and magnesium from diet or supplements.

KEYWORDS

dietary minerals, low MMSE scores, cognitive function, dietary copper, dietary magnesium

Introduction

With the elderly population increasing dramatically, cognitive impairment has become a public health challenge. It is estimated that around 55 million people suffer from dementia globally and about 10 million new cases are identified yearly (1). Moreover, its global prevalence is projected to increase to 132 million by 2050 (2). Mild cognitive impairment (MCI), characterized by a decline in cognitive function that occurs with typical aging, is the early stage of cognitive impairment between the aging and dementia (3). Since there is no effective treatment to slow down or reverse the dementia (4), delaying the transition from mild cognitive impairment to dementia is imminent.

In recent decades, there is growing evidence supporting the key role of diet and nutrition in the onset and severity of age-related cognitive deterioration (5). Among these, minerals have gained great attention. On the one hand, minerals, such as iron, manganese, copper, zinc, and magnesium serve as essential components of thousands of enzymes and proteins, involving DNA repair and brain development (6). They also play an important role in regulating cellular function and nerve transmission, which can thus decelerate or prevent the process of cognitive decline (7). On the other hands, it is well established that amyloid plaques contain high concentrations of copper, iron, and zinc, indicating the crucial role of these trace elements in the pathogenesis of neurological diseases such as Alzheimer's disease (AD).

Human studies with regard to the association between dietary intake of minerals and cognitive performance are also controversial and limited. For example, a cross-sectional data analysis from National Health and Nutrition Examination Surveys 2011–2014 (NHANES 2011–2014) suggested the copper intake below the inflection point was positively and independently associated with cognitive function (8). Another study from 10,269 participants of the Atherosclerosis Risks in Communities Study found that high dietary copper intake combined with a high intake of saturated fat may increase the risk of cognitive impairment (9). A 15-year longitudinal study suggested that higher iron intake is associated with poorer cognition in older Chinese individuals (10). However, the study from NHANES 2011–2014 found that raised iron levels in the serum were linked to a decreased risk of cognitive impairment (11). Moreover, Shanghai is the city with the highest level of aging in China. This study aimed to examine the relationship between dietary minerals including manganese, iron, copper, zinc, selenium and magnesium and cognition performance tested by Mini-Mental State Examination (MMSE) an well-known instrument for cognitive function, using the data from Shanghai Health and Nutrition Survey (SHNS) in 2018. We hope our study will help provide evidence for nutritional intervention to prevent or delay age related cognitive decline and maintain a good quality of life among elderly adults.

Methods

Study design and participants

The current study was a cross sectional analysis based on data from Shanghai Health and Nutrition Survey (SHNS) in the cycle of 2018 with a total of 1,516 participants. SHNS was carried out by the Shanghai Municipal Center for Disease Control and Prevention every 3 years. It is designed to examine the effects of the health, nutrition, and family planning policies and programs implemented by national and local governments. The study was approved by the Ethical Review Committee of the Shanghai Municipal Centers for Disease Control and Prevention. The written informed consents were obtained from all participants enrolled in the study.

Our analyses were limited to participants who completed the MMSE scale and had recorded intake of at least one mineral (n = 912). Those who did not complete the general questionnaire or had missing information on age, gender, educational level, smoking status or drinking status were excluded (n = 68). In addition, participants with extreme values of total dietary energy intake (>5,000 kcal/d or <800 kcal/d) were also excluded (n = 9). Finally, 835 participants (342 individuals less than 60 years old, 493 individuals aged over 60 years old were included in the analyses). The flow chart of participants was shown in Figure 1.

Dietary assessment

Dietary intake was obtained from the participants for three consecutive days on a 24-h recall basis during 2018. Individuals were asked about the exact type and weight of foods they consumed in 3 days, including 2 weekdays and 1 weekend day. The field interviewers had received three days of training about the collection of dietary data and had prior experience in both nutrition work and national surveys. The intake of each food group (not including dietary supplements) was converted from categorical responses to numeric variables, and the dietary intake of energy and certain nutrients were calculated using Chinese food composition table containing detailed lists of food types and nutrient values for all kinds of foods. We also calculated the contribution of various foods to copper and magnesium intake based on dietary assessment. The food categories include grains, vegetables, legumes, sea foods, meats and poultry, fruits, cookies and snacks, nuts, eggs, fungi and algae.

Cognitive performance outcomes

The Mini-Mental State Examination (MMSE) was used for assessing cognition in all participants. The participants were face-to-face interviewed for 30 min to evaluate cognitive function through five domains: orientation (10 points), recall (3 points), registration (3 points), attention/computation (5 points), and language (9 points). MMSE score ranges from 0 to 30. A higher MMSE score indicates the better cognitive function. In addition, the threshold for the MMSE score was set at 27, with a score of less than 27 considered as low MMSE score at greater risk of being diagnosed with dementia (12).

Covariates

To control for the effect of confounders on the results, the statistical model was adjusted for covariates to minimize the potential confounding bias. Age, gender, educational level were obtained from in-person household interviews. Educational level was classified into three categories: less than high school, high school (including

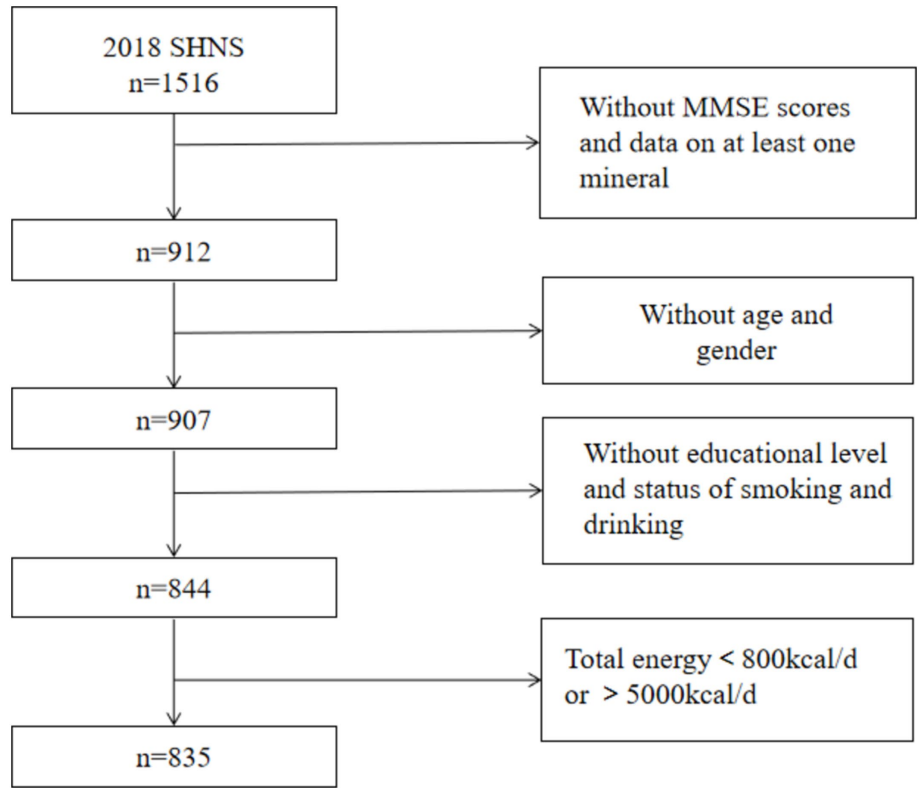


FIGURE 1
Flow chart of the selection process.

secondary technical school and vocational school), higher than high school. Total energy intake was obtained from the 3 consecutive 24-h recalls. Smoking status was divided into two groups (never smoked, smoked) based on the self-report of the participants to the question "Ever smoked cigarettes?." Participants were defined as alcohol drinkers if they had ever drank beer/alcohol in the previous year. Chinese version of the 14-item Perceived Stress Scale (PSS-14) was used to assess levels, and the threshold for the PSS-14 score was set at 28, with a score of more than 28 considered to have some sense of stress.

Statistical analysis

Medians and percentiles was used for describing the characteristics of all variables between the different groups. Dietary manganese, iron, copper, zinc and magnesium intakes were categorized into low moderate and high intake tertiles for each subject. Logistic regression analyses were conducted to examine the associations between manganese, iron, copper, zinc and magnesium intakes and the level of MMSE scores. The model 1 was adjusted for age (years), gender and total daily energy intake (kcal/d). Model 2 was further adjusted for educational level (less than high school, high school, higher than high school), smoking status (yes, or no) and alcohol drinking (yes, or no), and model 3 was built on model 2 plus the level of perceived stress score. Stratified analyses by two age groups were also conducted to further address the concern on the confounding by age. All statistical analyses were performed with R (4.0.5) and $p < 0.05$ was considered statistically significant.

Results

Characteristics of the participants

Characteristics of the participants by different age groups were summarized in Table 1. A total of 835 participants were included in the study, among whom 49.3% were male and 20.4% had education beyond high school. The percentage of smokers and drinkers was 75.0 and 79.3%, respectively. The median age of participants was 62 years old. The median total dietary energy intake was 1865 kcal and the median level of dietary mineral intake was 4.62 [3.45, 5.99] mg/d for manganese, 18.25 [14.12, 24.63] mg/d for iron, 1.51 [1.13, 2.04] mg/d for copper, 10.14 [8.26, 12.77] mg/d for zinc, 49.61 [38.32, 63.54] μg/d for selenium and 256.88 [198.13, 333.99] mg/d for magnesium. Among all participants, there were 73 of them with low MMSE (score less than 27), accounting for 8.7% of the total.

According to the median age of 62 years, compared with the latest (2023) Dietary Reference Intakes (DRIs) for Chinese residents, the median total dietary energy intake basically met the required estimated energy requirement (EER), and the median dietary mineral intake was higher than the recommended intake (RNI) for manganese (4 mg/d), iron (12 mg/d for males, and 10 mg/d for females) and copper (0.8 mg/d). While zinc intake met the RNI (12 mg/d for males

TABLE 1 Characteristics of the participants by age groups.

Characteristic	Total	Age < 60y	Age ≥ 60y	*p*
N	835	342	493	
Age in years at screening, median	62.00 [55.00, 68.00]	52.00 [46.00, 56.00]	67.00 [63.00, 71.00]	<0.001
Gender, n (%)				
Male	412 (49.3)	165 (48.2)	247 (50.1)	0.623
Female	423 (50.7)	177 (51.8)	246 (49.9)	
Education, n (%)				
<High school	310 (37.1)	91 (26.6)	219 (44.4)	<0.001
High school	355 (42.5)	153 (44.7)	202 (41.0)	
>High school	170 (20.4)	98 (28.7)	72 (14.6)	
Smoking, n (%)				
Yes	626 (75.0)	254 (74.3)	372 (75.5)	0.745
No	209 (25.0)	88 (25.7)	121 (24.5)	
Drinking, n (%)				
Yes	662 (79.3)	265 (77.5)	397 (80.5)	0.298
No	173 (20.7)	77 (22.5)	96 (19.5)	
Total energy (kcal), median [IQR]	1864.54 [1565.92, 2248.22]	1990.35 [1615.85, 2334.14]	1781.07 [1533.02, 2164.08]	<0.001
Dietary manganese (mg/d), median [IQR]	4.62 [3.45, 5.99]	4.68 [3.23, 6.13]	4.56 [3.51, 5.96]	0.897
Dietary iron (mg/d), median [IQR]	18.25 [14.12, 24.63]	19.18 [14.52, 24.91]	17.67 [13.95, 23.98]	0.119
Dietary copper (mg/d), median [IQR]	1.51 [1.13, 2.04]	1.52 [1.12, 2.09]	1.51 [1.13, 2.02]	0.790
Dietary zinc (mg/d), median [IQR]	10.14 [8.26, 12.77]	10.60 [8.81, 13.41]	9.91 [8.04, 12.18]	0.001
Dietary selenium (μg/d), median [IQR]	49.61 [38.32, 63.54]	50.91 [39.58, 66.69]	48.84 [37.38, 62.22]	0.028
Dietary magnesium (mg/d), median [IQR]	256.88 [198.13, 333.99]	263.19 [196.65, 337.83]	251.57 [198.87, 328.12]	0.612
Low MMSE score, *n* (%)	73 (8.7)	11 (3.2)	62 (12.6)	<0.001

and 8.5 mg/d for females) while the selenium intake failed to meet the dietary requirement (60 μg/d).

Participants over the age of 60 had a lower percentage of tertiary education and a higher percentage of low MMSE, smoking and drinking compared to participants under the age of 60. In addition, participants over 60 years of age had lower intakes of total dietary energy and each of the dietary minerals than those under 60 years of age. There were significant differences between people those under 60 and those over 60 years old in the distribution of education, MMSE score, total energy intake ($p < 0.001$), dietary selenium and zinc intake ($p < 0.05$). People over the age of 60 had lower education level, lower MMSE score, less total energy intake, and less dietary zinc and selenium intake than those under the age of 60.

Characteristics of the participants by cognitive performance were summarized in Table 2. Among participants under 60 years of age, there were no significant differences across all characteristics. Among the group over 60 years old, participants with low MMSE were older ($p < 0.001$) and had higher rates of alcohol drinking ($p < 0.05$), lower education level ($p < 0.05$), less total energy intake ($p < 0.05$), less copper intake ($p < 0.001$) and tended to have less magnesium intake ($p = 0.054$).

The association between mineral intake and cognitive function

Logistic regression analyses were performed on participants over 60 years of age to examine the association between dietary intake of manganese, iron, copper, zinc, and magnesium and the prevalence of low cognitive performance, with the lowest tertile of intake as the referent category. The associations between different dietary mineral intake and low MMSE of participants over 60 years of age were presented in Table 3. The OR with 95% CIs of model 1 indicated that dietary copper intake and magnesium intake had significant inverse associations with low MMSE score ($p < 0.05$). After adjustment for other potential confounders, participants in the second (≥1.24 to<1.78 mg/d) and third tertile (≥1.78 mg/d) of the dietary copper intake had lower rates of low MMSE scores compared to those in the lowest tertile ($p < 0.05$). The adjusted OR and 95%CI values were 0.44 (0.21–0.89) and 0.40 (0.16–0.94), respectively. In addition, participants in the second tertile of dietary magnesium (≥217.86 to<297.56 mg/d) intake showed a trend of lower rates of low MMSE score compared to those in the lowest tertile ($p = 0.06$). The adjusted OR and 95%CI values were 0.35 (0.16–0.72). Additionally, further adjustment for iron as a possible confounder of MMSE scores did not have an effect on the results (Supplementary Table S1).

To further elucidate the relationship between dietary copper and magnesium intake and cognitive performance, we performed an restricted cubic spline (RCS) analysis (Figure 2). We found the prevalence of low cognitive performance in MMSE decreased with increasing intakes of dietary magnesium intake, and showed a nonlinear L-shaped relationship.

Since our results suggested that dietary intake of copper and magnesium may have a protective effect on cognitive performance in elderly over 60 years old, we further calculated the contribution of various foods to copper and magnesium intake. The results showed that grains were the primary contributor to the copper and magnesium intake (32%), followed by vegetables (11.9%), legumes (10.7%), sea food (10.4%), meats and poultry (8.5%), fruits (5%), fast food (4.3%), cookies and snacks (3.3%), eggs (2.6%), fungi and alage (2.4%), and nuts (2.2%) (the data was shown in Supplementary Figure S1).

Discussion

With the increased aging population, cognitive impairment has become a global public health problem. A recent meta-analysis based on 96 studies reported an overall dementia prevalence of 5.3% for

TABLE 2 Characteristics of the participants by cognitive performance in different age groups.

Characteristic	Age < 60y				Age ≥ 60y			
	Total	Normal MMSE	Low MMSE	*p*	Total	Normal	Low MMSE	*p*
N	342	331	11		493	431	62	
Age in years at screening, median [IQR]	52.00 [46.00, 56.00]	52.00 [46.00, 56.00]	55.00 [53.00, 56.00]	0.247	67.00 [63.00, 71.00]	66.00 [63.00, 70.00]	70.00 [67.00, 77.00]	<0.001
Gender, n (%)								
Male	165 (48.2)	160 (48.3)	5 (45.5)	1.000	247 (50.1)	218 (50.6)	29 (46.8)	0.590
Female	177 (51.8)	171 (51.7)	6 (54.5)		246 (49.9)	213 (49.4)	33 (53.2)	
Education, n (%)								
<High school	91 (26.6)	85 (25.7)	6 (54.5)	0.105	219 (44.4)	185 (42.9)	34 (54.8)	0.010
High school	153 (44.7)	149 (45.0)	4 (36.4)		202 (41.0)	187 (43.4)	15 (24.2)	
>High school	98 (28.7)	97 (29.3)	1 (9.1)		72 (14.6)	59 (13.7)	13 (21.0)	
Smoking, n (%)								
Yes	254 (74.3)	247 (74.6)	7 (63.6)	0.483	372 (75.5)	321 (74.5)	51 (82.3)	0.209
No	88 (25.7)	84 (25.4)	4 (36.4)		121 (24.5)	110 (25.5)	11 (17.7)	
Drinking, n (%)								
Yes	265 (77.5)	256 (77.3)	9 (81.8)	1.000	397 (80.5)	341 (79.1)	56 (90.3)	0.039
No	77 (22.5)	75 (22.7)	2 (18.2)		96 (19.5)	90 (20.9)	6 (9.7)	
Total energy (kcal), median [IQR]	1990.35 [1615.85, 2334.14]	1983.59 [1607.23, 2343.77]	2218.05 [1895.64, 2284.55]	0.280	1781.07 [1533.02, 2164.08]	1797.74 [1563.36, 2162.76]	1716.83 [1358.56, 2182.11]	0.067
Dietary manganese (mg/d), median [IQR]	4.68 [3.23, 6.13]	4.70 [3.24, 6.13]	4.14 [3.42, 5.73]	0.937	4.56 [3.51, 5.96]	4.64 [3.55, 5.97]	4.14 [3.35, 5.46]	0.159
Dietary iron (mg/d), median [IQR]	19.18 [14.52, 24.91]	19.22 [14.63, 24.91]	16.42 [13.39, 23.67]	0.650	17.67 [13.95, 23.98]	17.80 [14.10, 23.98]	16.84 [13.23, 23.26]	0.462
Dietary copper (mg/d), median [IQR]	1.52 [1.12, 2.09]	1.51 [1.12, 2.09]	1.85 [1.04, 2.17]	0.644	1.51 [1.13, 2.02]	1.56 [1.16, 2.04]	1.23 [0.98, 1.67]	0.001
Dietary zinc (mg/d), median [IQR]	10.60 [8.81, 13.41]	10.62 [8.82, 13.42]	10.41 [7.84, 12.54]	0.746	9.91 [8.04, 12.18]	9.91 [8.09, 12.36]	9.61 [7.36, 11.79]	0.208
Dietary selenium (μg/d), median [IQR]	50.91 [39.58, 66.69]	50.83 [39.55, 66.53]	61.73 [46.14, 69.94]	0.187	48.84 [37.38, 62.22]	49.09 [38.29, 62.66]	47.13 [32.59, 59.04]	0.115
Dietary magnesium (mg/d), median [IQR]	263.19 [196.65, 337.83]	263.19 [196.75, 338.57]	263.19 [196.07, 288.83]	0.640	251.57 [198.87, 328.12]	256.88 [201.15, 332.47]	220.19 [192.59, 301.01]	0.054

Chinese population over 60 years old (13). The overall prevalence of MCI in the Chinese population over 60 is estimated to be 15.5%, with 11.1% in those aged 60–69 years, and the prevalence increases with age (14, 15). Since cognitive impairment imposes a heavy burden on the public and health care systems, it is necessary to discover the influencing factors of cognitive dysfunction and take early intervention to prevent the development from MCI to dementia.

Diet is a modifiable lifestyle behavior that can affect the quality of life and the prevalence of non-communicable diseases (NCDs) including dementia (16). For example, it has been demonstrated the beneficial effects of healthy dietary patterns such as the Mediterranean diet, or the Dietary Approach to Stop Hypertension (DASH) diet on cognition (17, 18). The role of individual nutrients including vitamins and minerals in cognitive function is also an area getting increasing attention in recent years. With regard to the association between dietary minerals and cognitive function, the results of human studies are inconclusive.

In the current study, we used data from SHNS 2018 including 835 participants and assessed the relationship between dietary intake of manganese, iron, copper, zinc, and magnesium and cognitive performance. Firstly, we found the positive association between higher copper dietary intake and lower rates of low MMSE score among the adults above 60 years old. These results are consistent with a previous perspective cohort study in Chinese elderly (aged over or equal to 55 years old) showing that the cognitive decline decreased significantly with the increment of dietary copper intake (19). In that study, the decrease reached a plateau when the intake of dietary copper exceeded about 1.3 mg/day. Our results also observed the threshold effects of

10.3389/fnut.2024.1424614

TABLE 3 Associations between dietary mineral intakes and low MMSE in participants over 60 years old.

	Model 1		Model 2		Model 3	
Dietary manganese (mg/d)	OR (95%CI)	*p* trend	OR (95%CI)	*p* trend	OR (95%CI)	*p* trend
T1 (<3.82)	Reference	0.069	Reference	0.072	Reference	0.097
T2 (≥3.82 to <5.25)	0.58 (0.28–1.16)		0.59 (0.29–1.18)		0.57 (0.28–1.17)	
T3 (≥5.25)	0.46 (0.19–1.07)		0.46 (0.19–1.08)		0.49 (0.20–1.16)	
Liner	0.79 (0.32–1.90)		0.83 (0.33–2.02)		0.88 (0.35–2.16)	
Dietary iron (mg/d)						
T1 (<14.99)	Reference	0.540	Reference	0.597	Reference	0.621
T2 (≥14.99 to <21.13)	0.83 (0.41–1.69)		0.85 (0.42–1.74)		0.85 (0.41–1.73)	
T3 (≥21.13)	0.77 (0.33–1.78)		0.80 (0.34–1.85)		0.81 (0.34–1.89)	
Liner	0.74 (0.29–1.79)		0.79 (0.30–1.95)		0.82 (0.31–2.04)	
Dietary copper (mg/d)						
T1 (<1.24)	Reference	0.027	Reference	0.030	Reference	0.028
T2 (≥1.24 to <1.78)	0.44 (0.21–0.88)		0.43 (0.21–0.88)		0.44 (0.21–0.89)	
T3 (≥1.78)	0.40 (0.17–0.93)		0.41 (0.17–0.95)		0.40 (0.16–0.94)	
Liner	0.45 (0.18–1.07)		0.46 (0.18–1.11)		0.46 (0.18–1.12)	
Dietary zinc (mg/d)						
T1 (<8.68)	Reference	0.691	Reference	0.643	Reference	0.641
T2 (≥8.68 to <11.24)	0.74 (0.35–1.55)		0.76 (0.36–1.60)		0.80 (0.37–1.70)	
T3 (≥11.24)	1.25 (0.51–3.08)		1.29 (0.52–3.20)		1.28 (0.51–3.17)	
Liner	1.16 (0.26–5.29)		1.34 (0.29–6.15)		1.29 (0.29–5.87)	
Dietary selenium (μg/d)						
T1 (<41.54)	Reference	0.872	Reference	0.951	Reference	0.957
T2 (≥41.54 to <56.65)	0.54 (0.26–1.12)		0.56 (0.26–1.17)		0.57 (0.27–1.21)	
T3 (≥56.65)	0.96 (0.45–2.03)		1.04 (0.47–2.28)		1.04 (0.47–2.28)	
Liner	0.55 (0.25–1.19)		0.6 (0.27–1.33)		0.59 (0.19–1.31)	
Dietary magnesium (mg/d)						
T1 (<217.86)	Reference	0.045	Reference	0.060	Reference	0.061
T2 (≥217.86 to <297.56)	0.37 (0.17–0.76)		0.36 (0.17–0.75)		0.35 (0.16–0.72)	
T3 (≥297.56)	0.45 (0.19–1.03)		0.48 (0.20–1.11)		0.48 (0.20–1.12)	
Liner	0.49 (0.17–1.41)		0.56 (0.18–1.67)		0.58 (0.19–1.73)	

copper intake on cognitive function. Moreover, the median dietary intake of copper in our current study is around 1.51 mg/day, which is much higher than RNI of 0.8 mg/day. The sufficient dietary copper intake may be due to participants' traditional "Southern River -style diet," which include large amounts of grains, legumes and marine fish (20). Although animal studies have demonstrated that brain copper overload is involved in the pathogenesis of neurodegeneration by enhancing oxidative damage and neuroinflammation, copper is also a co-factor of key enzymes and a signaling and regulatory molecule for brain development and function (21). Our study found the that the dietary copper intake was lower in elderly adults over 60 years old, compared to adults under 60 years old. Even the dietary copper intake meets RNI, higher dietary intake of copper may prevent cognitive decline in elderly population over 60 years old.

Secondly, we found the trend of relationship between higher magnesium intake and lower rates of low MMSE score. Our findings are consistent with another cross-sectional study from China observing the inverse association between high magnesium concentration and the incidence of mild cognitive impairment (MCI) in participants over the age of 60 (22). The beneficial effects of magnesium may be due to its ability to suppress inflammation by modulating inflammatory mediators such as interleukin-α, tumor necrosis factor-α and nitric oxide, and inhibit the excessive production of amyloid β-protein that are involved in the process of neurodegeneration (23, 24). In addition, magnesium is also important for optimal nerve transmission and neuromuscular coordination (25). It is worth mentioning that the median dietary intake of magnesium in our present study is 251.57 mg/day, which is below RNI for

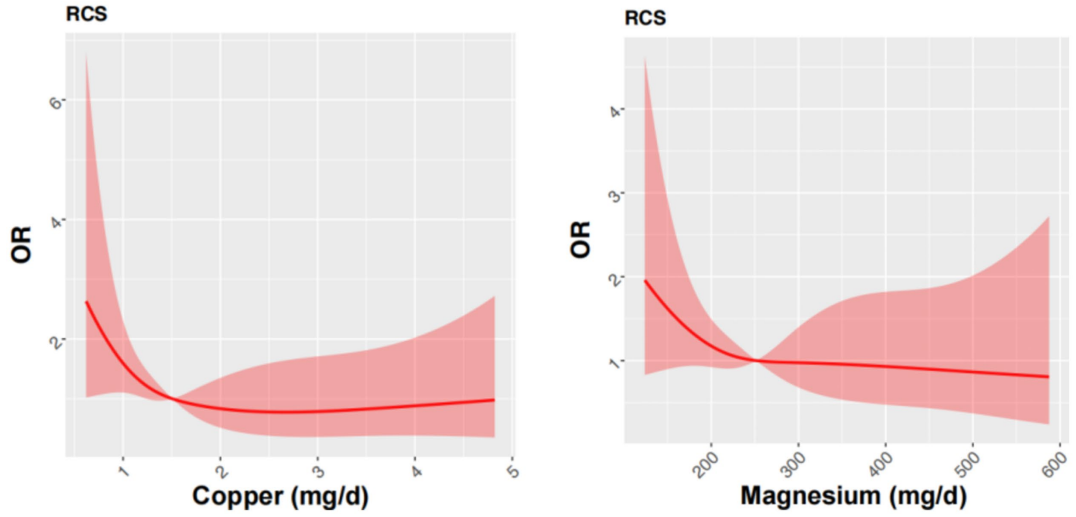


FIGURE 2
Restricted cubic spline models for the relationship between dietary copper and magnesium intake and the risk of low MMSE after matching. Restricted cubic spline model of the ORs of low cognitive performance with dietary copper and magnesium intakes. The model was adjusted for sex, age, educational level, smoking and alcohol intake. The 95% CIs of the adjusted ORs were represented by the red-shaded area. OR, odds ratio.

individuals in China. It is necessary for elderly adults over 60 years old to eat magnesium rich food to meet RNI and prevent cognitive decline.

In the present study, we found that elderly ate fewer calories with higher dietary intake of copper and magnesium than young adults. Other studies also supported the findings that the elderly had increased demand for healthy food rich in nutrients and bioactive compounds as they were more concerned about health (26). One study found that there was a remarkably energy excess derived from fat in younger group, if compared with the recommended allowances (27). Another study found that older outpatients reported higher frequency of consumption of fresh fruit and vegetables, and lower daily consumption of sweet high-fat foods among obese and overweight outpatients (28).

Regarding other minerals including iron, manganese and zinc, we did not observe the association of the dietary intake and cognitive function. Previous studies also showed no significant association of the dietary intake of zinc, selenium and manganese with cognition (29, 30). However, some other studies observed the relationship between high iron intake and poor cognition (31, 32). The different results could be explained by the assessment methods for dietary mineral intake and cognitive function. Thus studies with larger dataset and more participants are needed to further examine the association between the dietary mineral intake and cognitive outcomes. Moreover, we found that the median dietary selenium intake was lower than RNI, while the median dietary zinc intake just met the RNI of female and was lower than the RNI of male. We also found that individuals over 60 years old had lower dietary zinc and selenium intake compared to individuals under 60 years old. Even our study shows no association between zinc or selenium intake and cognitive function, adequate zinc and selenium intake play important roles in health maintenance in elderly population. Zinc deficiency is reported to affect immune function, cognitive ability, taste and many other aspects of health problems (32). Selenium is considered as longevity indicator and inadequate selenium status might accelerate the aging process or increase risk of various diseases including immunity dysfunction, neurodegeneration and cancers (33). Our study suggested local residents in Shanghai to eat a diet rich in zinc and selenium, especially for the elderly over 60 years old.

We recognized several limitations of the current study. First, our study is a cross-sectional study with a number of unmeasured confounders that may affect the dietary intake. So it is difficult to establish a causal relationship between dietary intake and cognitive performance. It is possible that other factors such as health status, emotional stress and medications may temporarily affect the cognitive test results, and thus further prospective long term cohort studies are needed to confirm the association. Second, our study did not provide a good indication of metabolism and absorption of the minerals since data on serum levels of minerals were not included in the analysis. Thirdly, our dietary intake data were derived from the 24-h dietary recall, which may be subject to recall bias and subjective dietary assessment. In addition, cognitive dysfunction may be associated with complex metal dis-regulation in the brain, which cannot be assessed through diet alone. Thus the underlying mechanisms of action of these minerals in the brain needs deeper exploration.

In conclusion, our findings suggest that dietary intake of copper and magnesium may have a protective effect on cognitive performance. It is necessary for Chinese individuals over 60 years old to get recommended amounts of copper and magnesium from diet or supplements. However, our current study is a preliminary screening of the community population. Thus further high quality prospective cohort studies to characterize the stage of dementia are necessary to confirm these findings and reveal the underlying mechanisms.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Medical Ethics Committee, Institute of Nutrition and Health, Chinese Center for Disease Control and Prevention, China. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YC: Data curation, Writing – original draft, Formal analysis, Methodology. ZS: Project administration, Writing – original draft, Methodology, Validation. YZ: Data curation, Formal analysis, Writing – original draft, Validation. RZ: Formal analysis, Software, Visualization, Writing – original draft. XL: Visualization, Writing – original draft. YD: Methodology, Writing – original draft. JX: Writing – original draft, Methodology. QX: Supervision, Writing – review & editing, Conceptualization, Investigation. JZ: Funding acquisition, Resources, Supervision, Writing – review & editing, Conceptualization, Investigation.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. The current study was supported by the Shanghai municipality Strengthening Public Health System Construction Three-Year Action Plan (2023–2025), Key Disciplines in the three-year plan of the Shanghai Municipal Public Health System (GWVI-11.1-42), the Rising-Star Program of Shanghai "Science and Technology Innovation Action Plan" (Yangfan Special Project) (23YF1437000), the Shanghai municipality Strengthening Public Health System Construction Three-Year Action Plan (2023–2025), Discipline Leader Program (GWVI-11.2-XD21), Shanghai Public Health System Construction Three-Year Action Plan Project (2023–2025) (GWVI-4), Shanghai Undergraduate Training Program on Innovation and Entrepreneurship (SUTPIE) grant (202410268156) and National Natural Science Foundation of China (82373563).

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.2024.1424614/full#supplementary-material

References

1. Beishon LC, Hosford P, Gurung D, Brassard P, Minhas JS, Robinson TG, et al. The role of the autonomic nervous system in cerebral blood flow regulation in dementia: a review. *Auton Neurosci*. (2022) 240:102985. doi: 10.1016/j.autneu.2022.102985

2. Anderson ND. State of the science on mild cognitive impairment (MCI). *CNS Spectr*. (2019) 24:78–87. doi: 10.1017/S1092852918001347

3. Petersen RC. Clinical practice. Mild cognitive impairment. *N Engl J Med*. (2011) 364:2227–34. doi: 10.1056/NEJMcp0910237

4. Cummings J, Lee G, Ritter A, Sabbagh M, Zhong K. Alzheimer's disease drug development pipeline: 2019. *Alzheimers Dement (N Y)*. (2019) 5:272–93. doi: 10.1016/j.trci.2019.05.008

5. Dominguez LJ, Veronese N, Vernuccio L, Catanese G, Inzerillo F, Salemi G, et al. Nutrition, physical activity, and other lifestyle factors in the prevention of cognitive decline and dementia. *Nutrients*. (2021) 13:4080. doi: 10.3390/nu13114080

6. Meramat A, Rajab NF, Shahar S, Sharif R. Cognitive impairment, genomic instability and trace elements. *J Nutr Health Aging*. (2015) 19:48–57. doi: 10.1007/s12603-014-0489-1

7. Nooyens AC, Milder IE, van Gelder BM, Bueno-de-Mesquita HB, van Boxtel MP, Verschuren WM. Diet and cognitive decline at middle age: the role of antioxidants. *Br J Nutr*. (2015) 113:1410–7. doi: 10.1017/S0007114515000720

8. Wang X, Li X, Xing Y, Wang W, Li S, Zhang D, et al. Threshold effects of Total copper intake on cognitive function in US older adults and the moderating effect of fat and saturated fatty acid intake. *J Acad Nutr Diet*. (2021) 121:2429–42. doi: 10.1016/j.jand.2021.06.002

9. Wei J, Gianattasio KZ, Bennett EE, Stewart JD, Xu X, Park ES, et al. The associations of dietary copper with cognitive outcomes. *Am J Epidemiol*. (2022) 191:1202–11. doi: 10.1093/aje/kwac040

10. Shi Z, Li M, Wang Y, Liu J, El-Obeid T. High iron intake is associated with poor cognition among Chinese old adults and varied by weight status-a 15-y longitudinal study in 4852 adults. *Am J Clin Nutr*. (2019) 109:109–16. doi: 10.1093/ajcn/nqy254

11. Lu K, Liu T, Wu X, Zhong J, Ou Z, Wu W. Association between serum iron, blood lead, cadmium, mercury, selenium, manganese and low cognitive performance in old adults from National Health and nutrition examination survey (NHANES): a cross-sectional study. *Br J Nutr*. (2023) 130:1743–53. doi: 10.1017/S0007114523000740

12. O'Bryant SE, Humphreys JD, Smith GE, Ivnik RJ, Graff-Radford NR, Petersen RC, et al. Detecting dementia with the mini-mental state examination in highly educated individuals. *Arch Neurol*. (2008) 65:963–7. doi: 10.1001/archneur.65.7.963

13. Qi S, Yin P, Zhang H, Zhang Q, Xiao Y, Deng Y, et al. Prevalence of dementia in China in 2015: a Nationwide Community-based study. *Front Public Health*. (2021) 9:733314. doi: 10.3389/fpubh.2021.733314

14. Jia L, Du Y, Chu L, Zhang Z, Li F, Lyu D, et al. Prevalence, risk factors, and management of dementia and mild cognitive impairment in adults aged 60 years or older in China: a cross-sectional study. *Lancet Public Health*. (2020) 5:e661–71. doi: 10.1016/S2468-2667(20)30185-7

15. Deng Y, Zhao S, Cheng G, Yang J, Li B, Xu K, et al. The prevalence of mild cognitive impairment among Chinese people: a Meta-analysis. *Neuroepidemiology*. (2021) 55:79–91. doi: 10.1159/000512597

16. Zang J, Yu H, Zhu Z, Lu Y, Liu C, Yao C, et al. Does the dietary pattern of Shanghai residents change across seasons and area of residence: assessing dietary quality using the Chinese diet balance index (DBI). *Nutrients*. (2017) 9:251. doi: 10.3390/nu9030251

17. Lorenzo-Mora AM, Lopez-Sobaler AM, Bermejo LM, Gonzalez-Rodriguez LG, Cuadrado-Soto E, Peral-Suarez A, et al. Association between mineral intake and cognition evaluated by Montreal cognitive assessment (MoCA): a cross-sectional study. *Nutrients*. (2023) 15:4505. doi: 10.3390/nu15214505

18. Scarmeas N, Anastasiou CA, Yannakoulia M. Nutrition and prevention of cognitive impairment. *Lancet Neurol*. (2018) 17:1006–15. doi: 10.1016/S1474-4422(18)30338-7

19. Meng Q, Liu C, Zu C, Wei Y, Su X, Gan X, et al. Association between dietary copper intake and cognitive decline: a perspective cohort study in Chinese elderly. *Am J Geriatr Psychiatry*. (2023) 31:753–63. doi: 10.1016/j.jagp.2023.05.001

20. Wang J, Lin X, Bloomgarden ZT, Ning G. The Jiangnan diet, a healthy diet pattern for Chinese. *J Diabetes*. (2020) 12:365–71. doi: 10.1111/1753-0407.13015

21. Lutsenko S, Washington-Hughes C, Ralle M, Schmidt K. Copper and the brain noradrenergic system. *J Biol Inorg Chem*. (2019) 24:1179–88. doi: 10.1007/s00775-019-01737-3

22. Lu Z, He R, Zhang Y, Li B, Li F, Fu Y, et al. Relationship between whole-blood magnesium and cognitive performance among Chinese adults. *Nutrients*. (2023) 15:2706. doi: 10.3390/nu15122706

23. Nielsen FH. Magnesium deficiency and increased inflammation: current perspectives. *J Inflamm Res*. (2018) 11:25–34. doi: 10.2147/JIR.S136742

24. Yu X, Guan PP, Zhu D, Liang YY, Wang T, Wang ZY, et al. Magnesium ions inhibit the expression of tumor necrosis factor alpha and the activity of gamma-secretase in a beta-amyloid protein-dependent mechanism in APP/PS1 transgenic mice. *Front Mol Neurosci*. (2018) 11:172. doi: 10.3389/fnmol.2018.00172

25. Grober U, Schmidt J, Kisters K. Magnesium in prevention and therapy. *Nutrients*. (2015) 7:8199–226. doi: 10.3390/nu7095388

26. Jedrusek-Golinska A, Gorecka D, Buchowski M, Wieczorowska-Tobis K, Gramza-Michalowska A, Szymandera-Buszka K. Recent progress in the use of functional foods for older adults: a narrative review. *Compr Rev Food Sci Food Saf*. (2020) 19:835–56. doi: 10.1111/1541-4337.12530

27. Fernandez-Ballart J, Gordillo B, Arija V, Marti-Henneberg C. Nutrition of the elderly in a Mediterranean city in Spain: effects of life-style patterns. *Int J Vitam Nutr Res*. (1989) 59:8–13.

28. Inelmen EM, Toffanello ED, Enzi G, Sergi G, Coin A, Busetto L, et al. Differences in dietary patterns between older and younger obese and overweight outpatients. *J Nutr Health Aging*. (2008) 12:3–8. doi: 10.1007/BF02982157

29. Zhao D, Huang Y, Wang B, Chen H, Pan W, Yang M, et al. Dietary intake levels of Iron, copper, zinc, and manganese in relation to cognitive function: a cross-sectional study. *Nutrients*. (2023) 15:704. doi: 10.3390/nu15030704

30. Ferdous KA, Knol LL, Park HA. Association between selenium intake and cognitive function among older adults in the US: National Health and nutrition examination surveys 2011-2014. *J Nutr Sci*. (2023) 12:e57. doi: 10.1017/jns.2023.43

31. Shi Z, El-Obeid T, Li M, Xu X, Liu J. Iron-related dietary pattern increases the risk of poor cognition. *Nutr J*. (2019) 18:48. doi: 10.1186/s12937-019-0476-9

32. Lu J, Hu Y, Li M, Liu X, Wang R, Mao D, et al. Zinc nutritional status and risk factors of elderly in the China adult chronic disease and nutrition surveillance 2015. *Nutrients*. (2021) 13:3086. doi: 10.3390/nu13093086

33. Bjorklund G, Shanaida M, Lysiuk R, Antonyak H, Klishch I, Shanaida V, et al. Selenium: an antioxidant with a critical role in anti-aging. *Molecules*. (2022) 27:27. doi: 10.3390/molecules27196613

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 06 January 2025
DOI 10.3389/fnut.2024.1435051

OPEN ACCESS

EDITED BY
Hongliang Zeng,
Fujian Agriculture and Forestry University, China

REVIEWED BY
Yang Li,
Shandong Agricultural University, China
Lei Wang,
Wuhan Polytechnic University, China
Xiaokang Lv,
Anhui Science and Technology University, China

*CORRESPONDENCE
Can Yang
yangcansky@163.com
Yulong Yin
yinyulong@isa.ac.cn

†These authors have contributed equally to this work

RECEIVED 19 May 2024
ACCEPTED 12 December 2024
PUBLISHED 06 January 2025

CITATION
Yang C, Tang X, Wang M, Yang H, Yang H, Wang Y and Yin Y (2025) Effects of dietary amylose/amylopectin ratio on antioxidant ability and amino metabolism in the liver of weaned piglets undergoing feed transition and challenged with lipopolysaccharide. *Front. Nutr.* 11:1435051. doi: 10.3389/fnut.2024.1435051

Effects of dietary amylose/amylopectin ratio on antioxidant ability and amino metabolism in the liver of weaned piglets undergoing feed transition and challenged with lipopolysaccharide

Can Yang[1,2*†], Xiaowu Tang[3†], Min Wang[2], Han Yang[1], Huansheng Yang[2], Yancan Wang[2] and Yulong Yin[2,4*]

[1]College of Life Sciences, Hunan Provincial Key Laboratory of Biological Resources Protection and Utilization in Nanyue Mountain Area, Hengyang Normal University, Hengyang, Hunan, China, [2]Hunan International Joint Laboratory of Animal Intestinal Ecology and Health, Hunan Normal University, Changsha, Hunan, China, [3]College of Bioengineering, Hunan Vocational Technical College of Environment and Biology, Hengyang, Hunan, China, [4]Key Laboratory of Agro-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Science, Changsha, Hunan, China

To find out whether dietary amylose/ amylopectin ratio (DAR) could attenuate injury in lipopolysaccharide (LPS)-challenged piglets, sixty male weaned piglets (Duroc × Landrace × Yorkshire, 21 days old, 6.51 ± 0.64 kg) were allotted to 5 dietary treatments with 12 cages per treatment, and fed ad libitum with diets different in DAR (0.00, 0.20, 0.40, 0.60 and 0.80). Feed transformation occurred from D15 to D21. On day 28, 12 h before slaughter, pigs were intraperitoneal injected with 100 μg/kg body weight LPS or sterile saline. Results showed that LPS stress caused an increase in serum urea nitrogen (UREA) and triglyceride (TG), but a decrease in alanine aminotransferase (ALT) activity and glucose (GLU) concentration ($p < 0.05$). Serum immunoglobulin G (IgG) concentration increased in DAR 0.80 but decreased in other groups after LPS stress ($p < 0.05$). Compared with the control group, concentrations of Ile, Leu, Phe, Val, Thr, Arg decreased in serum but increased in liver after LPS stress ($p < 0.05$). Serum Arg, Tyr, Sar, Ans, Orn increased linearly with increasing DAR ($p < 0.05$). Piglets in diet DAR 0.00 had highest superoxide dismutase (SOD1) and glutathione peroxidase 1 (*GPX1*) mRNA expression in liver than those in other groups ($p < 0.05$). There was significant effect of LPS stress * dietary DAR on total SOD activity and SOD1 mRNA gene expression ($p < 0.05$), LPS stress caused an increase in those two indices for pigs in groups 0.00 and 0.80. Piglets in diet 0.80 had the highest hepatic Cu, Fe, Mn, Zn concentrations than those in other groups ($p < 0.05$). Cecal indol(e) concentration was higher in diet 0.00 than that in diet 0.80 ($p < 0.05$). After LPS stress, colonic skatole concentration increased in DAR 0.40, 0.80 but decreased in other groups ($p < 0.05$). In conclusion, adverse effects of the LPS challenge could be reversed by feeding weaned piglets with low or high DAR diet through regulating amino metabolism and antioxidant function.

KEYWORDS

dietary amylose/amylopectin ratio, LPS stress, amino acid, antioxidant activity, weaned piglet

1 Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria such as *Escherichia coli*. It triggers a systemic inflammatory process by releasing pro-inflammatory mediators, nitric oxide, and reactive oxygen species (ROS), which are associated with oxidative stress (1). Weaned piglets with weakened mucosal barrier resistance to pathogens are particularly vulnerable to infections by enterotoxigenic *E. coli*. This strain of *E. coli* produces and secretes LPS, leading to intestinal dysfunction (2), oxidative stress (3), and hepatic damage, especially at doses like 80 μg/kg body weight of LPS (4). Pigs treated with LPS exhibited hepatocyte nuclear lysis, fibroblast proliferation, and karyopyknosis (5). Furthermore, LPS-induced immune stimulation decreases plasma flux for amino acids such as lysine (Lys), phenylalanine (Phe), and isoleucine (Ile) (6). However, stress biomarkers of inflammation in LPS-challenged piglets can be partially mitigated through supplementation with a 0.3% amino acid mixture containing arginine (Arg), leucine (Leu), valine (Val), and isoleucine (Ile), and cysteine (Cys) (7). Supplementation with tryptophan (Try) has been shown to ameliorate liver damage by reducing activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), maintaining the barrier function of the liver and alleviating hepatic oxidative stress in LPS-challenged piglets (5). Additionally, citrulline (Cit) and arginine deiminase (ADI-PEG20) may exert protective effects against inflammation by reducing CD45+ infiltrates in the liver and lowering plasma levels of pro-inflammatory cytokines in the piglets infused with LPS infusion (8).

Starch is composed of amylose and amylopectin. Waxy maize starches exhibit an A-type X-ray diffraction pattern, whereas high-amylose maize shows a B-type pattern (9) and a lower degree of crystallinity than waxy maize starches (10). Compared with waxy maize starch (DAR 0.07) and non-waxy maize starch (DAR 0.19), feeding finishing pigs a pea starch (DAR 0.28) diet downregulated gluconeogenesis, resulting in less fat and more protein deposition in the liver (11). Compared with normal corn starch, supplementing mice with 10% high-amylose maize enhanced the intestinal absorption of calcium, iron, and magnesium (12). Indigestible starch can be converted into short-chain volatile fatty acids (SCFAs) by intestinal microorganisms in the hindgut (13). Increasing carbohydrate availability by cecal corn starch infusion decreases hindgut aromatic amino acid (AAA) metabolism while increasing systemic AAA availability, thereby promoting hypothalamic neurotransmitter synthesis (14). Additionally, the degree of oxidative stress and inflammation was alleviated in chronic kidney disease (CKD) rats after the consumption of 59% high-amylose maize-resistant starch (HAMRS2) (15).

In commercial pig farms, post-weaning diarrhea usually occurs when weaned piglets are exposed to enterotoxigenic *E. coli* after undergoing the transitional weaning periods, which include changes in feed, environment, and separation from the mother. A dose of 100 μg/kg of LPS secreted by *E. coli* has been shown to cause both hepatic damage and gut injury (4). However, whether different dietary amylose/amylopectin ratios (DAR), achieved by blending high-amylose maize starch and waxy maize starch, can differently improve piglets' resilience under such challenges by regulating amino acid metabolism and liver antioxidant function remains unclear. Thus, this study examined the effect of dietary amylose/amylopectin ratio on the amino acid concentration in serum and liver, liver mineral element content, and liver antioxidant function in weaned piglets under LPS stress. We hypothesized that both high and low DAR diets could improve piglets' health by modulating amino acid metabolism and antioxidant function during stress.

2 Materials and methods

The experimental procedure in this study was reviewed and approved by the Animal Care and Use Committee of Hunan Normal University (ISA-2017-058).

2.1 Animals and diets

A total of 60 castrated male pigs (Landrace × Yorkshire, initial average body weight (BW) 6.51 ± 0.64 kg, 21 days old) were selected, blocked by BW, and assigned to five dietary treatments with 12 replicate cages per treatment and one pig per metabolic cage. "Pre-care period" and "late-care period" diets were formulated as starch-soybean meal-based diets that met the nutrient requirements established by the NRC (16) for pigs weighing 7–11 kg and 11–25 kg, respectively.

High-amylose maize starch (High-Maize 1043, National Starch Industry, Shanghai, China) and waxy maize starch (food market, Hengyang, China) were blended to create diets with differing dietary amylose/amylopectin ratios (DAR) of 0.00, 0.20, 0.40, 0.60, or 0.80, respectively (Supplementary Table 1). According to the manufacturer's instructions, the amylose and amylopectin contents were determined using their commercial assay kits (l-AMYL, Megazyme International Ireland Ltd., Wicklow, Ireland).

Pigs were fed and provided food *ad libitum*. Starting on day 15, the feed was gradually transitioned from the "pre-care period" diet to the "late-care period" diet over seven consecutive days, with ratios adjusted as follows: 80:20, 60:40, 50:50, 40:60, 30:70, 20:80, and 0:100. The experiment lasted 28 days.

On day 28, 12 h before slaughter, six pigs from each treatment group received intraperitoneal injections of 100 μg/kg BW lipopolysaccharides (LPS, from *Escherichia coli* O55:B5, Sigma Chemical Inc., St Louis, MO, USA, L2880), whereas another six pigs were administered an equivalent amount of sterile saline.

2.2 Sample collection

On day 15 before feed transition and on day 29, after LPS stress, blood samples were collected from the jugular vein and centrifuged at 3,000×*g* at 4°C for 10 min to separate serum. Piglets were euthanized via an intravenous injection of 40 mg/kg BW sodium pentobarbital solution into the jugular vein. The middle right lobe of the liver and chyme from the cecal and colon were collected using sterile scissors and tweezers and immediately stored at −80°C for further study.

2.3 Analysis of serum biochemical variables

Serum samples were thawed, and the concentration of total protein (TP), albumin (ALB), alanine transaminase (ALT), aspartate

aminotransferase (AST), blood urea nitrogen (BUN), creatinine (CREA), glucose (GLU), triglyceride (TG), total cholesterol (CHOL), amylase (AMS), hepatic lipase (LIPC) was measured using commercial kits in accordance with the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China) at the TBA-120FR Automatic Biochemistry Radiometer (Hitachi Co., Tokyo, Japan). Serum immunoglobulin G and M (IgG, IgM) concentrations were determined using a commercial ELISA kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.4 Analysis of AA contents in serum and liver

Samples were prepared for amino acid analysis as follows:

For serum, 600 μL of serum was mixed with an equivalent volume of 8% sulfosalicylic acid and incubated for 1 h at 4°C. Then, the mixture was then centrifuged at 10,000 rpm for 10 min at 4°C to collect supernatants.

For liver samples, approximately 0.1 g of ground, freeze-dried liver tissue was hydrolyzed in 10 mL of 6 mol/L hydrochloric acid solution at 110°C for 24 h. The hydrolyzed solution was diluted with distilled water to a final volume of 100 mL, and 1 mL of the supernatant was used for further analysis.

Amino acid concentrations were measured using an amino acid analyzer (L-8900, Hitachi, Japan) after both the serum supernatant and hepatic diluent solution were filtered through a 0.45-μm membrane.

2.5 Assay of hepatic antioxidant enzyme activities

Hepatic tissue samples were ground in liquid nitrogen, homogenized in saline, and centrifuged at 3,000×*g* 4°C for 10 min. The concentrations of malonaldehyde (MDA) and the activities of superoxide dismutase (SOD), including coper-zinc SOD (CuZn-SOD) and manganese SOD (Mn-SOD), glutathione peroxidase (GSH-PX/GPx), and total antioxidant capacity (T-AOC), were analyzed using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.6 RNA extraction and real-time quantitative PCR

Total RNA was isolated from the liver using RNAiso Plus (TaKaRa), and then reverse transcription reactions were performed using an RT reagent kit (TaKaRa). All the procedures were carried out as described by the manufacturer's protocol. The quantity and quality of RNA were determined using the NanoDrop ND-2000 spectrophotometer system (ThermoFisher Scientific). Primers were designed to assay genes related to antioxidant function (Supplementary Table 2). The β-actin was used as a reference gene. Real-time RT-PCR for target genes was performed on the MyIQ instrument (Bio-Rad, Hercules, California) using *SYBR* Green quantitative PCR mix (TaKaRa).

2.7 Analysis of mineral element contents in the liver

Macro and microelements (Ca, S, Mg, Cu, Fe, Mn, Zn) were analyzed using an analytical method described earlier (17). Briefly, liver samples (5.00 ± 0.20 g) were weighed in triplicate and digested using a mixture of acid (25 mL of HNO_3: $HClO_4$ in a volume ratio of 4:1) following heating (80°C for 60 min; 120°C for 30 min; and 180°C for 30 min). Samples were dried at 260°C and redissolved in 5 mL of 1% HNO_3. The solution was then transferred to a 25-mL volumetric flask and filled with 1% HNO_3. Finally, samples' target elements (Cu, Fe, Mn, and Zn) were determined on ICP-OES (Varian 720ES Agilent, Santa Clara, CA, USA) for confirmation with standard references.

2.8 Analysis of indole and skatole contents in chyme

Indole and skatole (3-methylindole) of luminal contents in the cecal and colon were analyzed as described previously on high-performance liquid chromatography (HPLC, Agilent 1260, Agilent Technologies Inc., USA) (18). Briefly, luminal contents from the cecum and colon were weighed (1.000 g) and extracted overnight using 8 mL of 0.4 mol/L perchloric acid. Then, the suspension was obtained by centrifugation at 8,000 r/min for 10 min at 4°C. Subsequently, 1 mL of the supernatant was mixed with 1 mL of sodium hydroxide (2 mol/L, pH 10.6) saturated sodium bicarbonate buffer solution. Then, 1 mL of dansulfonyl chloride (10 mg/mL acetone solution) was added to the mixture. The solution was incubated in a water bath at 40°C in a dark environment for 30 min, followed by the addition of 1 mL of 5% ammonia water. Subsequently, 3 mL of anhydrous ether was used to extract the solution. The anhydrous ether layer was dried, dissolved in 1 mL of methanol, filtered through a 0.22-μm syringe filter membrane, and analyzed using the appropriate analytical instrument.

2.9 Statistics analysis

Gene expression data from replicate measurements within the same RNA extraction were averaged and analyzed using the Livak (19) method. One-way ANOVA followed by Duncan's multiple range test was conducted using SAS 8.0 to evaluate significant differences in serum indices based on DAR on Day 15. Data from D29 were analyzed using two-way ANOVA, with "stress" (saline or LPS), dietary DAR, and their interaction as factors.

Duncan's multiple range test was used to compare differences among the various DAR groups. SAS's PROC REG procedure (with stepwise selection at a p-value of <0.15) was used to analyze potential linear or quadratic regressions between dietary DAR and serum or hepatic indices in non-stressed piglets. Data were expressed as least squares mean (Lsmeans) ± SEM. A p-value of <0.05 was considered statistically significant, while $0.05 < p < 0.10$ indicated a statistically significant trend.

3 Results

3.1 Serum biochemical variables at D15

DAR did not affect serum ALT, AST, LIPC, TP, ALB, UREA, CREA, or TC on D15 (Table 1). However, piglets in the DAR 0.00 and 0.60 groups exhibited higher serum AMS activity than those in the DAR 0.40 group ($p < 0.05$). Serum GLU levels were significantly higher in the DAR 0.80 group than in the other groups ($p < 0.05$). Serum TG levels were higher in the DAR 0.20 group compared to DAR 0.00 and 0.40 ($p < 0.05$). Furthermore, serum GLU levels increased quadratically with increasing DAR ($p < 0.05$).

3.2 Serum-free amino acids at D15

Serum essential amino acids on D15 were not significantly affected by DAR ($p > 0.05$), except for serum Arg concentrations, which were higher in the DAR 0.40 and 0.60 groups compared to DAR 0.00 ($p < 0.05$) (Table 2). Serum non-essential amino acids, such as tyrosine (Tyr), glutamate (Glu), glycine (Gly), serine (Ser), cysteine (Cys), taurine (Tau), urea, hydroxylysine (Hylys), ornithine (Orn), β-alanine (β-Ala), β-aminoisobutyric acid (β-AiBA), 1-methylhistidine (1Mehis), 3-methylhistidine (3Mehis), and hydroxy-L-proline (Hypro), were not significantly affected by DAR ($p > 0.05$).

Piglets in the DAR 0.80 group exhibited significantly higher serum alanine (Ala) and citrulline (Cit) concentrations compared to those in the DAR 0.00, 0.40, and 0.60 groups ($p < 0.05$), but these levels were not significantly different from those in the DAR 0.20 group ($p > 0.05$). Serum aspartate (Asp) concentrations were significantly lower in the DAR 0.80 and 0.60 groups compared to DAR 0.00 and 0.20 ($p < 0.05$). Piglets in the DAR 0.40 and 0.60 groups exhibited significantly higher serum carnosine (Car) concentrations than those in other groups ($p < 0.05$).

Serum α-amino-butyric acid (α-ABA) concentrations were significantly higher in the DAR 0.80 group compared to DAR 0.00 and 0.40 ($p < 0.05$). Serum proline (Pro) levels were significantly lower in the DAR 0.00 group than in all other groups ($p < 0.05$). Serum ethanolamine (EOHNH2) levels were significantly higher in the DAR 0.20 group compared to all other groups ($p < 0.05$). Piglets in the DAR 0.40 group had the highest serum sarcosine (Sar) concentrations among all groups ($p < 0.05$), while those in the DAR 0.60 group had the highest serum γ-aminobutyric acid (γ-ABA) concentrations ($p < 0.05$). Serum α-aminoadipic acid (a-AAA) concentrations in the DAR 0.80 group were significantly higher than in DAR 0.20, 0.40, and 0.60 ($p < 0.05$).

REG analysis showed that serum Phe tended to decrease linearly with increasing DAR ($p = 0.097$), while serum Tyr ($p = 0.085$) and Car ($p = 0.074$) tended to increase linearly with increasing DAR. Serum Ala, Pro, Cysthi, α-ABA, β-AiBA increased linearly with increasing DAR ($p < 0.05$), whereas serum Asp, Tau, Ans, EOHNH2, and Hypro decreased linearly with increasing DAR ($p < 0.05$).

3.3 Serum biochemical variables at D29

Different DAR diets showed no significant differences in serum ALT, AST, AMS, TP, ALB, UREA, CREA, GLU, TG, and IgM levels at D29 (Table 3, $p > 0.05$). Serum LIPC was significantly higher in the DAR 0.60 group compared to the DAR 0.00 and 0.20 groups ($p < 0.05$). Piglets in the DAR 0.60 and 0.80 groups exhibited higher serum TC levels than those in the DAR 0.40 group ($p < 0.05$). Serum IgG concentration was significantly higher in the DAR 0.80 group than in the other groups ($p < 0.05$). Serum AST decreased progressively with increasing DAR ($p < 0.05$). Serum TP decreased, whereas IgG increased linearly with increasing DAR ($p < 0.05$). Serum TC tended to increase linearly with increasing DAR ($p = 0.066$).

TABLE 1 Effects of dietary amylose/amylopectin ratio on serum biochemical index on day 15 in weaned piglets.

Items[1]	0.00	0.20	0.40	0.60	0.80	SEM	*p* value	P_L	P_Q
ALT, U/L	34.48	36.59	42.79	44.38	39.13	1.58	0.264	0.142	NS
AST, U/L	55.73	62.25	55.67	53.67	51.00	2.50	0.693	NS	NS
LIPC, U/L	4.22	4.56	3.93	4.55	4.13	0.12	0.421	NS	NS
AMS, U/L	2,642^{A}	2,230^{AB}	2,022^{B}	2,493^{A}	2,226^{AB}	63.79	0.031	NS	NS
TP, g/L	47.22	49.51	45.60	45.97	45.12	0.68	0.260	−0.108	−0.102
ALB, g/L	25.59	26.73	24.53	26.29	25.70	0.52	0.725	NS	NS
UREA, mmol/L	4.54	5.13	4.83	4.48	4.73	0.22	0.895	NS	NS
CREA, umol/L	60.00	63.92	66.92	63.75	63.00	1.60	0.762	NS	NS
GLU, mmol/L	4.68^{B}	4.73^{B}	4.53^{B}	4.58^{B}	5.32^{A}	0.08	0.027	0.070	0.017
TG, mmol/L	0.45^{B}	0.60^{A}	0.40^{B}	0.47^{AB}	0.53^{AB}	0.02	0.023	NS	NS
TC, mmol/L	1.87	2.07	1.87	2.02	2.05	0.05	0.405	NS	NS

[1]ALT, alanine transaminase; AST, aspartate aminotransferase, LIPC, hepatic lipase; AMS, amylase; TP, total protein; ALB, albumin; UREA, urea nitrogen; CREA, creatinine; GLU, glucose; TG, triglyceride; TC, total cholesterol; SEM, standard error of the mean; L, Q represent linear, quadratic response to increasing dietary amylose/amylopectin ratio. $^{A\text{-}B}$Values within a row with different superscripts differ significantly at $p < 0.05$. NS means a *p*-value of > 0.15.

10.3389/fnut.2024.1435051

TABLE 2 Effects of dietary amylose/amylopectin ratio on serum free amino acid concentration on day 15 in weaned piglets, ng/20 uL.

Items, full name	Items, abbreviation	0.00	0.20	0.40	0.60	0.80	SEM	*p* value	P_L	P_Q
Essential amino acid	–									
Lysine	Lys	418.65	488.86	441.79	413.45	432.75	19.48	0.752	NS	NS
Methionine	Met	118.90	131.43	122.62	134.92	147.40	4.92	0.403	NS	NS
Histidine	His	137.54	154.34	126.72	140.73	144.51	3.44	0.154	NS	NS
Isoleucine	Ile	200.15	227.27	203.17	206.78	212.50	5.90	0.623	NS	NS
Leucine	Leu	272.47	305.75	255.92	261.08	252.83	7.08	0.126	NS	NS
Phenylalanine	Phe	206.62	219.99	197.31	202.84	203.49	4.55	0.590	−0.097	−0.087
Valine	Val	368.39	402.46	347.66	370.04	365.57	8.80	0.400	NS	NS
Threonine	Thr	131.98	174.13	138.68	140.79	160.39	8.79	0.537	NS	NS
Arginine	Arg	407.26^{B}	471.80^{AB}	540.05^{A}	528.63^{A}	434.36^{AB}	15.44	0.037	NS	NS
Non-essential amino acid	–									
Tyrosine	Tyr	156.28	177.26	152.29	166.32	187.46	5.92	0.314	0.085	0.072
Alanine	Ala	648.46^{B}	781.34^{AB}	710.91^{B}	710.52^{B}	860.09^{A}	21.02	0.029	0.028	0.023
Aspartate	Asp	100.80^{A}	102.90^{A}	91.02^{AB}	80.77^{B}	82.94^{B}	2.51	0.019	−0.002	−0.003
Glutamate	Glu	734.33	747.36	586.50	639.88	820.27	29.13	0.101	NS	NS
Glycine	Gly	810.43	773.85	811.29	758.24	875.34	30.42	0.773	NS	NS
Serine	Ser	173.97	188.12	183.17	158.89	195.41	6.19	0.390	NS	NS
Cysteine	Cys	93.28	119.84	101.66	105.77	109.84	2.94	0.082	NS	NS
Proline	Pro	290.28^{B}	370.19^{A}	412.60^{A}	400.86^{A}	453.46^{A}	12.43	0.003	0.000	0.001
Taurine	Tau	237.35	242.97	214.22	191.53	216.51	5.98	0.065	−0.035	−0.078
Urea	Urea	1,971	2,219	1,948	1,891	1,967	82.46	0.750	NS	NS
Sarcosine	Sar	5.21^{B}	9.82^{B}	31.15^{A}	14.98^{B}	10.27^{B}	2.30	0.008	NS	NS
Citrulline	Cit	132.51^{C}	161.98^{AB}	132.65^{C}	141.87^{BC}	170.41^{A}	4.01	0.009	0.078	0.046
Anserine	Ans	20.60^{A}	1.61^{B}	2.51^{B}	3.19^{B}	1.80^{B}	1.83	0.008	−0.014	−0.079
Carnosine	Car	40.20^{B}	48.23^{B}	94.83^{A}	110.27^{A}	52.58^{B}	5.76	0.001	0.074	NS
Cystathionine	Cysthi	13.07^{B}	16.78^{AB}	15.94^{AB}	15.59^{AB}	18.45^{A}	0.55	0.054	0.021	0.031
Hydroxylysine	Hylys	14.74	17.63	16.17	14.23	17.52	0.67	0.383	NS	NS
Ornithine	Orn	118.98	136.44	118.74	117.85	140.36	5.55	0.537	NS	NS
a-Aminoadipic acid	a-AAA	118.00^{AB}	91.46^{B}	33.50^{C}	49.56^{C}	133.74^{A}	5.34	<0.0001	NS	NS
a-Amino-n-butyric acid	a-ABA	12.61^{C}	20.14^{AB}	16.63^{BC}	17.72^{ABC}	23.40^{A}	0.94	0.012	0.009	0.010
β-Alanine	β-Ala	37.47	33.59	36.23	36.23	35.88	1.82	0.975	NS	NS
β-Aminoisobutyric acid	β-AiBA	3.49	6.82	6.70	8.78	7.22	0.57	0.085	0.029	0.100
γ-Aminobutyric acid	γ-ABA	0.97^{B}	1.133^{B}	1.11^{B}	1.88^{A}	0.96^{B}	0.11	0.043	NS	NS
Ethanolamine	EOHNH2	10.25^{B}	12.49^{A}	9.16^{B}	9.32^{B}	9.18^{B}	0.23	<0.0001	−0.005	−0.004
1-Methylhistidine	1Mehis	61.51	57.55	55.54	49.90	56.91	2.41	0.666	NS	NS
3-Methylhistidine	3Mehis	33.20	32.72	30.37	33.34	34.29	1.71	0.963	NS	NS
Hydroxy-L-proline	Hypro	181.21	156.19	137.98	132.13	142.29	6.13	0.112	−0.025	−0.092
Sum	Sum	167.40	181.49	161.25	157.77	168.82	4.86	0.586	NS	NS

[A-C]Values within a row with different superscripts differ significantly at $p > 0.05$. NS means a $p < 0.15$. L, Q represents a linear, quadratic response to increasing dietary amylose/amylopectin ratio (DAR).

TABLE 3 Effects of dietary amylose/amylopectin ratio on serum biochemical index on day 29 in weaned piglets under LPS stress.

Items[1]	0.00		0.20		0.40		0.60		0.80		SEM	*p* value			P_L	P_Q
	LPS[2]	SAL	LPS	SAL	LPS	SAL	LPS	SAL	LPS	SAL		P_{DAR}	P_{stress}	P_{D*S}		
ALT, U/L	33.98	41.86	38.83	39.78	42.48	44.20	39.88	46.48	31.42	41.98	1.28	0.324	0.031	0.701	NS	NS
AST, U/L	67.83	72.80	89.50	69.00	80.17	77.17	110.67	56.33	99.67	54.50	3.72	0.853	0.002	0.055	−0.065	−0.045
LIPC, U/L	3.55^{C}	3.80	3.75^{BC}	4.63	4.30^{AB}	5.22	4.58^{A}	6.02	4.15^{ABC}	4.65	0.16	0.027	0.014	0.798	0.137	NS
AMS, U/L	2,487	2,248	2,153	2,454	1,950	2,429	2,585	2,370	2,406	2,593	81.08	0.712	0.519	0.528	NS	NS
TP, g/L	43.63	55.16	44.58	52.50	43.13	43.27	44.82	48.22	45.82	43.40	0.94	0.192	0.030	0.131	−0.021	−0.046
ALB, g/L	23.72	21.46	21.92	25.22	21.68	21.35	25.65	23.98	23.33	22.60	0.60	0.483	0.774	0.605	NS	NS
UREA, mmol/L	6.17	5.38	6.57	5.58	9.03	5.65	5.77	5.02	6.63	5.18	0.36	0.460	0.039	0.728	NS	NS
CREA, umol/L	91.33	57.20	96.33	63.83	87.50	64.83	107.00	60.33	100.67	61.83	2.26	0.670	0.000	0.521	NS	NS
GLU, mmol/L	4.43	4.90	4.17	5.23	3.83	5.22	3.88	4.97	3.37	5.33	0.12	0.869	0.000	0.419	NS	NS
TG, mmol/L	0.73	0.46	0.66	0.43	0.56	0.48	0.71	0.46	0.71	0.50	0.02	0.664	0.000	0.638	NS	NS
TC, mmol/L	2.14^{AB}	2.01	2.12^{AB}	2.21	1.78^{B}	2.03	2.11^{A}	2.30	2.28^{A}	2.37	0.04	0.014	0.238	0.675	0.066	0.061
IgG, g/L	0.37^{E}	0.87	1.09^{D}	6.99	5.57^{C}	7.52	6.74^{B}	17.19	16.41^{A}	12.85	0.26	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
IgM, g/L	0.66	0.85	0.51	0.51	0.54	0.55	0.47	0.77	0.68	0.54	0.03	0.146	0.259	0.171	NS	NS

[1]ALT, alanine transaminase; AST, aspartate aminotransferase, LIPC, hepatic lipase; AMS, amylase; TP, total protein; ALB, albumin; UREA, urea nitrogen; CREA, creatinine; GLU, glucose; TG, triglyceride; TC, total cholesterol; IgG, immunoglobulin G; IgM, immunoglobulin M.

[2]LPS, lipopolysaccharide, SAL, saline, SEM, standard error of the mean, DAR, dietary amylose/amylopectin ratio. L, Q represents a linear, quadratic response to increasing dietary amylose/amylopectin ratio. Only data from non-stress piglets were analyzed in this REG procedure.

[A-E]Values within a row with different superscripts differ significantly at $p < 0.05$. NS means a p-value of > 0.15.

As shown in Table 3, LPS stimulation significantly increased serum AST, UREA, CREA, and TG levels but decreased serum ALT, LIPC, TP, and GLU concentrations compared to controls ($p < 0.05$). Serum AMS, ALB, TC, and IgM concentrations were not significantly affected by LPS administration ($p > 0.05$).

A significant interaction between LPS and DAR was found for serum IgG concentrations ($p < 0.01$). In LPS-exposed animals, serum IgG concentration significantly increased in the DAR 0.80 group, whereas it decreased in the other groups after LPS administration. Additionally, a two-way interaction between LPS stress and DAR was observed for serum AST concentrations ($p = 0.055$). Serum AST levels tended to decrease in the DAR 0.00 group after the LPS challenge compared to the control group. In contrast, serum AST levels increased following the LPS challenge in the other DAR groups after the LPS challenge in other groups.

3.4 Serum-free amino acids at D29

Serum essential amino acid concentrations were not significantly affected by DAR ($p > 0.05$), except for serum Arg, which was higher in the DAR 0.80 group compared to the DAR 0.00, 0.20, and 0.40 groups (Table 4, $p < 0.05$). Similarly, serum non-essential amino acids were not affected by DAR ($p > 0.05$), except for Pro, Sar, Cit, Ans, Hylys, β-AiBA, and Hypro ($p < 0.05$). Serum Pro was significantly higher in the DAR 0.60 group than in the DAR 0.80, 0.40, and 0.00 groups ($p < 0.05$). Serum concentrations of Sar, Cit, Ans, and Hylys in the DAR 0.80 group were significantly higher than in the DAR 0.00 group ($p < 0.05$).

Pigs from the DAR 0.00, 0.60, and 0.80 groups exhibited significantly higher serum β-AiBA levels than those from the DAR 0.20 and 0.40 groups ($p < 0.05$). Serum Hypro concentrations were significantly higher in the DAR 0.20 and 0.60 groups than in the DAR 0.00 group ($p < 0.05$). Serum essential amino acid concentrations decreased after LPS stress ($p < 0.05$), except for His, which was not affected by LPS stress ($p > 0.05$).

Serum concentrations of Tyr, Ala, Asp, Glu, Cys, Pro, urea, Cit, Car, Orn, α-ABA, β-Ala, and Hypro were significantly affected by LPS stress ($p < 0.05$).

A significant interaction effect between DAR and LPS was observed for serum Met, Phe, Val, Arg, Tyr, Ser, and Hypro ($p < 0.05$). Serum Phe levels increased after LPS stress in the DAR 0.40 group but decreased in the other groups ($p < 0.05$). Serum Met and Val concentrations remained stable after LPS stress in the DAR 0.00 and 0.40 groups but decreased in the other groups ($p < 0.05$). Serum Arg and Tyr levels were stable after LPS stress in the DAR 0.00 group but decreased in the other groups ($p < 0.05$). Serum Ser concentrations decreased after LPS stress in the DAR 0.20 and 0.80 groups but increased in the other groups

TABLE 4 Effects of dietary amylose/amylopectin ratio on serum-free AA concentration on day 29 in weaned piglets under LPS stress, ng/20 uL.

Items[1]	0.00		0.20		0.40		0.60		0.80		SEM	*p* value			P_L	P_Q
	LPS[2]	SAL	LPS	SAL	LPS	SAL	LPS	SAL	LPS	SAL		P_{DAR}	P_{stress}	P_D*_S		
Lys	290.83	461.73	299.34	439.69	344.25	383.30	289.37	517.96	362.64	529.83	14.10	0.332	<0.0001	0.310	NS	0.139
Met	88.53	109.98	71.44	145.46	80.73	101.33	67.04	168.53	63.65	148.75	3.99	0.290	<0.0001	0.004	0.092	0.104
His	111.83	114.56	111.21	124.90	129.58	116.57	97.37	126.11	118.01	128.32	3.08	0.662	0.175	0.301	NS	NS
Ile	189.06	241.42	148.19	298.79	172.41	234.18	148.91	281.43	130.05	280.38	6.91	0.883	<0.0001	0.060	NS	NS
Leu	209.84	248.70	186.51	270.96	222.90	213.75	180.88	240.34	179.25	247.79	5.85	0.789	0.000	0.119	NS	NS
Phe	190.14	191.68	158.86	228.87	205.48	185.08	159.12	232.94	176.52	242.75	5.15	0.811	0.001	0.011	0.085	0.069
Val	321.02	375.51	292.89	450.46	350.52	357.90	279.32	407.13	273.61	427.02	9.16	0.886	<0.0001	0.045	NS	NS
Thr	88.26	93.72	85.80	126.89	96.80	96.43	86.83	121.27	83.86	122.10	3.80	0.715	0.003	0.272	NS	NS
Arg	328.56^{B}	365.26	300.14^{B}	430.07	310.30^{B}	417.24	275.51^{AB}	537.74	347.81^{A}	606.33	12.69	0.015	<0.0001	0.028	0.001	0.001
NEAA																
Tyr	175.17	181.98	149.26	224.12	157.65	170.40	131.81	229.11	133.36	256.84	5.27	0.437	<0.0001	0.003	0.022	0.011
Ala	817.28	732.17	881.35	834.48	961.51	669.89	1,219.58	817.30	903.89	703.50	27.71	0.060	0.001	0.248	NS	NS
Asp	64.50	78.11	59.85	92.65	73.18	78.19	54.64	83.10	60.92	80.89	2.61	0.872	0.000	0.456	NS	NS
Glu	616.87	639.59	526.48	793.71	584.36	642.12	495.57	735.61	553.77	661.64	23.35	0.955	0.005	0.375	NS	NS
Gly	785.89	620.18	722.03	948.18	874.09	789.05	1,016	795.10	668.99	775.45	27.50	0.128	0.614	0.066	NS	NS
Ser	169.54	137.75	150.71	207.84	179.11	156.57	206.91	180.88	150.52	165.47	4.97	0.089	0.869	0.033	NS	NS
Cys	104.91	81.56	99.86	76.33	113.40	94.96	119.98	93.51	131.90	94.06	3.12	0.092	0.000	0.894	0.141	NS
Pro	287.69^{C}	325.81	350.11^{AB}	477.26	350.40^{BC}	355.05	411.45^{A}	503.95	333.15^{BC}	405.19	10.57	0.001	0.003	0.408	NS	NS
Other AA																
Tau	170.19	207.61	169.97	192.20	181.97	162.60	156.89	163.98	172.31	186.49	4.70	0.405	0.197	0.427	NS	NS
Urea	2,985	2,251	2,747	2,300	2,445	2,480	2,459	2,121	2,912	2,307	65.11	0.502	0.002	0.410	NS	NS
Sar	5.03^{C}	6.57	23.50^{A}	27.01	14.50^{B}	14.04	31.95^{A}	34.50	29.80^{A}	30.71	1.06	<0.0001	0.531	0.992	0.003	0.010
Cit	166.38^{B}	136.89	186.49^{AB}	164.00	190.54^{AB}	154.22	182.22^{AB}	174.65	221.01^{A}	190.20	4.85	0.024	0.012	0.900	0.052	0.037
Ans	2.94^{BC}	2.37	0.52^{C}	0.00	4.11^{ABC}	5.22	5.44^{AB}	7.60	11.54^{A}	7.16	0.73	0.003	0.766	0.677	0.033	0.054
Car	47.58	30.79	37.03	38.89	44.61	39.59	53.22	26.42	43.69	32.99	1.57	0.926	0.001	0.057	NS	NS
Cysthi	20.40	14.14	17.15	20.00	17.05	15.95	18.66	17.41	17.04	14.88	0.74	0.787	0.289	0.450	NS	NS
Hylys	12.04^{B}	13.48	9.06^{B}	11.64	11.63^{B}	13.00	11.80^{B}	13.03	18.29^{A}	18.69	0.67	0.005	0.302	0.991	NS	0.079
Orn	155.05	232.42	173.61	243.92	171.88	211.13	165.96	256.64	197.85	276.09	5.67	0.100	<0.0001	0.674	0.031	0.028
a-AAA	149.35	109.72	131.54	164.00	151.77	137.55	164.84	157.76	170.94	158.45	5.77	0.357	0.490	0.420	NS	NS

(Continued)

10.3389/fnut.2024.1435051

TABLE 4 (Continued)

Items[1]	0.00		0.20		0.40		0.60		0.80		SEM	*p* value			P_L	P_Q
	LPS[2]	SAL	LPS	SAL	LPS	SAL	LPS	SAL	LPS	SAL		P_{DAR}	P_{stress}	P_D*$_S$		
a-ABA	16.66	21.25	17.80	27.98	17.62	17.92	12.13	24.97	14.23	26.31	1.06	0.582	0.001	0.288	NS	NS
β-Ala	57.39	31.15	34.28	30.19	41.16	26.63	45.87	39.06	53.16	35.74	1.89	0.115	0.001	0.389	NS	NS
β-AiBA	5.80^{A}	7.28	0.92^{B}	1.72	3.10^{B}	1.59	4.18^{A}	8.38	8.11^{A}	4.98	0.42	0.000	0.676	0.117	NS	NS
γ-ABA	2.09	1.69	1.50	1.66	2.78	1.25	1.45	3.92	3.11	3.25	0.26	0.304	0.748	0.193	0.137	0.149
EOHNH2	9.86	10.81	10.18	9.17	10.02	7.62	10.76	9.66	13.45	9.24	0.40	0.368	0.057	0.361	NS	NS
1Mehis	71.09	42.86	47.79	62.23	65.33	55.25	59.19	64.74	56.45	58.50	2.37	0.891	0.495	0.068	NS	NS
3Mehis	13.65	33.01	12.85	17.08	24.80	19.76	13.94	16.75	20.60	18.02	1.32	0.167	0.160	0.055	NS	NS
Hypro	161.69^{B}	85.02	155.94^{A}	161.40	153.41^{AB}	155.79	199.73^{A}	138.12	142.28^{AB}	146.45	4.20	0.019	0.004	0.003	NS	NS

[1]EAA, Essential amino acid; NEAA, Non-essential amino acid; Lys, Lysine; Met, Methionine; His, Histidine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Val, Valine; Thr, Threonine; Arg, Arginine; Tyr, Tyrosine; Ala, Alanine; Asp, Aspartate; Glu, Glutamate; Gly, Glycine; Ser, Serine; Cys, Cysteine; Pro, Proline; Tau, Taurine; Sar, Sarcosine; Cit, Citrulline; Ans, Anserine; Car, Carnosine; Cysthi, Cystathionine; Hylys, Hydroxylysine; Orn, Ornithine; a-AAA, a-Aminoadipic acid; a-ABA, a-Amino-butyric acid; b-Ala, β-Alanine; b-AiBa, β-Aminoisobutyric acid; γ-ABA, γ-Aminobutyric acid; EOHNH2, Ethanolamine; 1Mehis, 1-Methylhistidine; 3-Mehis, 3-Methylhistidine; Hypro, Hydroxy-L-proline.

[2]LPS, lipopolysaccharide, SAL, saline, SEM, standard error of the mean, L, Q represents a linear, quadratic response to increasing dietary amylose/amylopectin ratio. Only data from non-stress piglets were analyzed in this REG procedure.

[A–C]Values within a row with different superscripts differ significantly at $p < 0.05$. NS means a p-value of > 0.15.

($p < 0.05$). Serum Hypro levels increased after LPS stress in the DAR 0.00 and 0.60 groups but remained stable in the other groups ($p < 0.05$).

Serum Arg ($p < 0.01$), Tyr ($p < 0.05$), Sar ($p < 0.01$), Ans ($p < 0.05$), and Orn ($p < 0.05$) increased linearly with increasing DAR, while serum Cit exhibited a progressive increase with increasing DAR ($p < 0.05$).

3.5 Amino acid contents in liver

There was no significant effect of DAR on all tested hepatic amino acid concentrations ($p > 0.05$), except for hepatic Cys, which was significantly lower in the DAR 0.80 group compared to the DAR 0.00 and 0.20 groups ($p < 0.05$) (Table 5). The LPS challenge resulted in increased concentrations of all tested amino acid concentrations in the liver compared to the control group ($p < 0.05$).

A significant interaction between LPS and DAR was observed for hepatic Ile, Leu, Phe, Thr, Asp, Ser, Glu, and Gly concentrations ($p < 0.05$). In animals fed the DAP 0.20, these amino acid concentrations decreased 12 h after LPS exposure. However, their concentrations increased in animals from other dietary groups following LPS stress.

3.6 Antioxidant function of liver

The concentration of hepatic MDA was significantly higher in the DAR 0.20 group compared to the DAR 0.00, 0.40, and 0.80 groups ($p < 0.05$) (Table 6). The activity of T-AOC was not affected by DAR or LPS stress ($p > 0.05$). The activity of GSH-PX in the DAR 0.20 group was higher than in the DAR 0.40, 0.60, and 0.80 groups ($p < 0.05$). It increased after LPS stress ($p < 0.05$). T-SOD activity in DAR 0.00 was higher than that in DAR 0.20, 0.40, and 0.80 ($p < 0.05$). After LPS stress, hepatic T-SOD activity decreased in the DAR 0.40 group but increased in the DAR 0.00 and 0.80 groups ($p < 0.05$). The activity of Cu-SOD was lower in the DAR 0.20 group compared to the other groups ($p < 0.05$) and increased linearly with rising DAR ($p < 0.05$).

Gene expression related to antioxidant function, such as *SOD1*, nuclear translocation factors (*Nrf2*), and *GPX1* mRNA expressions, was affected by DAR, which was higher in DAR 0.00 than in other groups ($p < 0.05$). LPS stress caused an increase in the abundance of gene *GPX1* ($p < 0.05$). mRNA expression of *SOD1* decreased in DAR 0.40 and 0.60 after LPS stress but increased in other groups ($p < 0.05$). *Nrf2* mRNA expression decreased in the DAR 0.40, 0.60, and 0.80 groups after LPS stress but increased in the DAR 0.00 and 0.20 groups ($p < 0.05$). Gene of glutamate-cysteine ligase catalytic subunit (*GCLC*) expression in the DAR 0.40, 0.60, and 0.80 groups decreased after LPS stress but increased in the DAR 0.00 and 0.20 groups ($p < 0.05$).

3.7 Mineral element contents in liver

Mineral element concentrations such as Ca, Mg, and S were not affected by DAR ($p > 0.05$) (Table 7). The DAR 0.80 had higher Cu, Fe, Mn, and Zn concentrations than DAR 0.00, 0.20, and 0.40 ($p < 0.05$).

TABLE 5 Effects of dietary amylose/amylopectin ratio on amino acid composition in the liver of weaned piglets under LPS stress, μg/100 mg.

Items[1]	0.00		0.20		0.40		0.60		0.80		SEM	*p* value			P_L	P_Q
	LPS[2]	SAL	LPS	SAL	LPS	SAL	LPS	SAL	LPS	SAL		P_{DAR}	P_{stress}	P_D*_S		
Lys	3.20	2.60	2.79	2.97	3.11	2.52	3.18	2.32	2.87	2.42	0.05	0.526	<0.0001	0.057	−0.025	−0.023
Met	0.91	0.70	0.83	0.89	0.90	0.73	0.90	0.67	0.82	0.65	0.01	0.181	<0.0001	0.083	−0.045	−0.019
His	1.07	0.90	0.97	1.03	1.04	0.87	1.03	0.82	0.95	0.83	0.01	0.133	<0.0001	0.120	−0.024	−0.019
Ile	1.84	1.54	1.72	1.80	1.82	1.51	1.86	1.38	1.70	1.48	0.02	0.242	<0.0001	0.034	−0.036	−0.034
Leu	3.86	3.23	3.57	3.79	3.87	3.22	3.91	3.00	3.64	3.17	0.05	0.562	<0.0001	0.049	−0.078	−0.065
Phe	2.04	1.76	1.90	2.03	2.06	1.71	2.04	1.57	1.90	1.61	0.03	0.148	<0.0001	0.042	−0.012	−0.009
Val	2.36	2.01	2.21	2.31	2.32	1.91	2.36	1.78	2.15	1.87	0.03	0.129	<0.0001	0.062	−0.015	−0.017
Thr	1.98	1.66	1.81	1.93	1.99	1.61	1.99	1.51	1.85	1.55	0.03	0.317	<0.0001	0.031	−0.019	−0.015
Arg	2.77	2.29	2.57	2.67	2.78	2.22	2.80	2.05	2.59	2.14	0.04	0.320	<0.0001	0.059	−0.023	−0.021
Asp	4.00	3.36	3.69	3.88	4.03	3.29	4.04	3.08	3.76	3.18	0.05	0.414	<0.0001	0.046	−0.028	−0.023
Ser	2.11	1.79	1.92	2.05	2.10	1.72	2.12	1.61	1.96	1.66	0.03	0.311	<0.0001	0.037	−0.014	−0.012
Glu	6.11	5.14	5.65	5.87	6.21	5.06	6.19	4.75	5.79	4.88	0.07	0.419	<0.0001	0.041	−0.020	−0.015
Gly	2.48	2.14	2.28	2.42	2.47	2.08	2.52	1.891	2.29	2.02	0.03	0.378	<0.0001	0.047	−0.027	−0.030
Ala	2.76	2.48	2.68	2.82	2.86	2.40	2.94	2.23	2.71	2.33	0.04	0.464	<0.0001	0.056	−0.020	−0.020
Cys	0.32^{A}	0.28	0.33^{A}	0.32	0.33^{AB}	0.22	0.36^{AB}	0.19	0.29^{B}	0.20	0.01	0.034	<0.0001	0.126	−0.002	−0.005
Tyr	1.39	1.10	1.34	1.46	1.52	1.25	1.51	1.15	1.451	1.17	0.02	0.265	<0.0001	0.051	NS	NS
NH3	0.68	0.57	0.64	0.62	0.67	0.53	0.67	0.51	0.60	0.50	0.01	0.065	<0.0001	0.406	−0.005	−0.005
Pro	2.01	1.71	1.85	1.99	2.03	1.68	2.08	1.58	1.93	1.64	0.03	0.675	<0.0001	0.052	−0.052	−0.044

[1]Lys, Lysine; Met, Methionine; His, Histidine; Ile, Isoleucine; Leu, Leucine; Phe, Phenylalanine; Val, Valine; Thr, Threonine; Arg, Arginine; Asp, Aspartate; Ser, Serine; Glu, Glutamate; Gly, Glycine; Ala, Alanine; Cys, Cysteine; Tyr, Tyrosine; Pro, Proline.

[2]LPS, lipopolysaccharide, SAL, saline, SEM, standard error of the mean, DAR, dietary amylose/amylopectin ratio. L, Q represents a linear, quadratic response to increasing dietary amylose/amylopectin ratio. Only data from non-stress piglets were analyzed in this REG procedure.

[A-B]Values within a row with different superscripts differ significantly at $p < 0.05$. NS means a p-value of > 0.15.

The LPS stress caused an increase in Mg and S concentrations ($p < 0.05$). Cu concentration ($p < 0.01$) increased after LPS stress in DAR 0.00 and 0.80 but decreased in other groups after LPS stress. Hepatic Cu, Fe, Mn, and Zn concentrations increased linearly with increasing DAR ($p < 0.01$).

3.8 Indole and skatole contents in chyme

Cecal indole concentration in the DAR 0.00 group was higher than that in the DAR 0.80 group ($p < 0.05$). It was not affected by LPS stress ($p > 0.05$) (Table 8). Cecal skatole and colonic indole were not affected by DAR or LPS stress ($p > 0.05$). Piglets that had DAR 0.00 had lower colonic skatole than those from the DAR 0.40, 0.60, and 0.80 groups ($p < 0.05$). Colonic skatole concentration increased after LPS stress in DAR 0.40 and 0.80 but decreased in other groups ($p < 0.05$). No variables were selected in the REG procedure at a p-value of <0.15.

4 Discussion

When weaned piglets experienced LPS stress, serum AST activity and urea nitrogen levels increased, while serum glucose and glutamate concentrations decreased. In contrast, hepatic concentrations of Cys, Glu, and Gly increased. Serum AST activity is a key biochemical marker of liver health and function. The observed elevation of serum AST levels following LPS stress (20) indicates hepatic injury occurred under these situations.

In response to LPS stress, piglets may redirect AA from protein retention toward AA utilization in the immune response. The increase in serum urea nitrogen in this study can be associated with elevated AA catabolism, particularly in amino acids in excess. Specific AAs are utilized in the synthesis of immune system metabolites, such as immunoglobulins and glutathione, during LPS stress (6). This was confirmed by the elevated GSH-PX activity, upregulated *GPX1* gene expression in the liver, and increased plasma Cys flux in pigs challenged with LPS in this study.

Compared to the diet 0.00, piglets consuming a diet high in amylose (0.80) exhibited higher serum Arg, Cit, and glucose concentrations. Both hepatic T-SOD activity and SOD1 mRNA expression were also elevated in the 0.80 group after LPS stress. Additionally, serum IgG levels increased in piglets on a diet of 0.80 after LPS stress. Copper, zinc-superoxide dismutase (SOD1), a major intracellular antioxidant enzyme in mammals, plays a crucial role in mitigating LPS-induced hepatic protein nitration (21). The fermentation of raw potato starch (RS2) in the cecum enhances the intestinal absorption of minerals such as Ca, Mg, Fe, Zn, and Cu (22). Given the higher amylose content in diet 0.80, piglets from this group had significantly higher hepatic Cu concentrations after LPS stress compared to other groups. While Prates et al. (7) reported that plasma IgG and IgM concentrations in weaned piglets were reduced 3 days after an LPS challenge, the increased IgG observed in the 0.80 group in this study suggests

TABLE 6 Effects of dietary amylose/amylopectin ratio on antioxidant function in the liver of weaned piglets under LPS stress.[1]

Items[1]	0.00		0.20		0.40		0.60		0.80		SEM	*p* value			P_L	P_Q
	LPS[2]	SAL	LPS	SAL	LPS	SAL	LPS	SAL	LPS	SAL		P_{DAR}	P_{stress}	P_D*$_S$		
Activities/concentration																
MDA, nmol/mg pro	0.83^{C}	1.00	4.57^{A}	3.60	2.03^{BC}	2.01	2.91^{AB}	3.39	1.69^{BC}	2.15	0.20	0.000	0.958	0.797	NS	NS
T-AOC, mmol/mg pro	61.30	71.64	71.57	108.91	73.33	89.47	121.65	74.43	111.92	73.52	5.12	0.379	0.685	0.074	NS	NS
GSH-PX, U/mg pro	1,385^{AB}	576.11	1,700^{A}	805.25	585.06^{C}	187.56	468.24^{C}	172.90	748.88^{BC}	422.90	73.75	0.001	0.001	0.557	−0.107	NS
T-SOD, U/mg pro	1,102^{A}	358.90	54.75^{C}	55.93	172.82^{BC}	347.69	527.17^{AB}	513.93	401.35^{BC}	278.50	46.51	0.000	0.133	0.029	0.115	NS
Cu-SOD, U/mg pro	497.18^{A}	212.97	39.01^{B}	40.47	181.45^{A}	365.42	468.21^{A}	459.46	321.30^{A}	225.84	37.94	0.001	0.500	0.184	0.010	0.063
mRNA expression																
SOD1	2.51^{A}	1.06	1.05^{B}	0.77	0.71^{B}	0.89	1.08^{B}	1.27	0.82^{B}	0.69	0.07	0.000	0.044	0.005	–	–
Nrf2	2.40^{A}	1.05	1.34^{B}	1.01	0.94^{B}	1.27	1.30^{AB}	1.37	0.83^{B}	1.04	0.07	0.016	0.148	0.004	–	–
GPX1	2.95^{A}	1.10	1.73^{B}	0.50	0.92^{B}	0.64	1.27^{B}	1.05	1.48^{B}	0.67	0.10	0.006	0.000	0.083	–	–
GCLC	1.80	1.05	1.11	0.82	1.04	1.45	1.03	2.02	0.90	1.23	0.08	0.167	0.396	0.014	–	–

[1]MDA, malonaldehyde; T-SOD, total superoxide dismutase; CuZnSOD, cooper-zinc; Cu-SOD, cooper-zinc superoxide dismutase; GSH-PX, glutathione peroxidase; T-AOC, total antioxidant capacity; *GCLC,* glutamate-cysteine ligase catalytic subunit; *Nrf2,* nuclear factor, erythroid 2 like 2; *SOD1,* superoxide dismutase; *GPX1,* and glutathione peroxidase 1.

[2]LPS, lipopolysaccharide, SAL, saline, SEM, standard error of the mean, DAR, dietary amylose/amylopectin ratio. L, Q represents a linear, quadratic response to increasing dietary amylose/amylopectin ratio. Only data from non-stress piglets were analyzed in this REG procedure.

[A-C]Values within a row with different superscripts differ significantly at $p < 0.05$. NS means a p-value of > 0.15. – means not applicable.

10.3389/fnut.2024.1435051

10.3389/fnut.2024.1435051

TABLE 7 Effects of dietary amylose/amylopectin ratio on mineral element concentrations in the liver of weaned piglets under LPS stress.[1]

Items, mg/g	0.00	0.20		0.40		0.60		0.80		SEM	*p* value			P_L	P_Q
	LPS[1]	SAL	LPS	SAL	LPS	SAL	LPS	SAL	LPS	SAL		P_{DAR}	P_{stress}	P_D*$_S$		
Ca	886.14	548.05	757.36	444.94	501.06	808.66	676.50	433.16	523.10	438.81	38.16	0.379	0.095	0.088	NS	NS
S	7,964	5,326	7,390	3,577	5,095	5,229	5,351	5,235	7,050	5,380	368.17	0.684	0.038	0.434	NS	NS
Mg	169.61	128.46	176.96	108.27	145.03	165.15	151.72	95.68	118.25	99.10	6.99	0.249	0.027	0.357	−0.131	−0.090
Cu	18.11^{C}	7.47	9.10^{C}	11.66	7.41^{C}	8.13	6.86^{B}	44.77	58.99^{A}	47.34	1.67	<0.0001	0.279	0.000	<0.0001	<0.0001
Fe	173.35^{B}	126.28	98.46^{B}	60.56	85.90^{B}	125.01	261.27^{A}	12,102	8,588^{A}	14,655	863.02	0.000	0.052	0.147	0.000	<0.0001
Mn	6.59^{B}	1.29	1.66^{B}	1.22	1.35^{B}	1.95	5.98^{B}	43.89	31.97^{A}	86.69	5.09	0.003	0.102	0.270	0.000	<0.0001
Zn	147.72^{BC}	57.51	81.37^{C}	79.64	63.50^{C}	79.85	117.30^{B}	174.49	257.85^{A}	241.75	7.84	<0.0001	0.672	0.072	<0.0001	<0.0001

[1]LPS, lipopolysaccharide, SAL, saline, SEM, standard error of the mean, DAR, dietary amylose/amylopectin ratio. L, Q represents a linear, quadratic response to increasing dietary amylose/amylopectin ratio. Only data from non-stress piglets were analyzed in this REG procedure.

[A–C]Values within a row with different superscripts differ significantly at $p < 0.05$. NS means a p-value of > 0.15.

enhanced protection against oxidative stress and inflammation. Indoxyl sulfate and p-cresol sulfate, major microbial-derived pro-inflammatory and pro-oxidant uremic toxins, decrease under lower pH conditions (23). The lower pH associated with the diet 0.80 (24) likely contributed to the observed decrease in cecal indoles in this study. However, colonic skatole concentrations increased in the diet 0.80 group after LPS stress. A diet high in amylose may lead to the reorganization of the intestinal mucosa, thereby increasing gut cell debris availability for microbial skatole production from tryptophan (25). We speculate that piglets consuming the diet 0.80 were better protected against LPS stress due to enhanced antioxidant capacity and immune response.

Although average feed intake was the same across all groups (24), serum-free AAs such as Arg, Ala, Asp, Pro, Cit, Cysthi, a-ABA, and EOHNH2 were either higher or at least not lower in DAR 0.20 compared to other groups at D15. However, by day 29, only serum Pro, Sar, Cit, and Hypro concentrations remained elevated in DAR 0.20 relative to the other groups.

After 24 h of incubation with cecal and colonic digesta, the production of ammonia-nitrogen and branched-chain fatty acids (BCFAs), markers of protein fermentation, decreased as corn-resistant starch levels increased (26). In the DAR 0.20 group, protein fermentation in the large intestine was evident after feed transition, as indicated by significant increases in isobutyrate and isovalerate concentrations (24). Protein fermentation products have been associated with toxic and pro-inflammatory effects on the intestinal epithelium (27), potentially explaining the highest hepatic MDA concentration observed in the diet 0.20. Normally, liver protein synthesis increases when animals undergo immune challenges. However, the higher protein fermentation in DAR 0.20 likely contributed to reductions in hepatic Ile and Leu levels after LPS stress, as BCFAs such as iso-butyrate, 2-methyl-butyrate, and iso-valerate are products of valine, isoleucine, and leucine deamination. Notably, serum Ser concentrations decreased after LPS stress in the DAR 0.20 and 0.80 groups while increasing in the other groups. Serine plays a critical role in metabolic networks interlinking the folate and methionine cycles, supporting cell proliferation (28).

It also serves as a precursor for Gly and Cys, which contribute to the synthesis of the antioxidant glutathione (GSH). Conversely, hepatic amino acids such as Phe, Thr, Asp, Ser, Glu, and Gly were likely mobilized to counteract oxidative stress, as indicated by elevated hepatic MDA levels. Increasing the availability of amino acids entering the portal vein has been suggested to enhance tissue protein synthesis (29). Higher hepatic Cys levels in the DAR 0.20 group suggest active Glu synthesis in this group. Additionally, DAR 0.20 exhibited the highest GSH-PX activity in the liver among all groups, further underscoring its role in combating oxidative stress.

5 Conclusion

In conclusion, feeding weaned piglets a diet high in amylose (DAR 0.80) enhances their ability to cope with LPS stress by mobilizing amino acids for IgG synthesis and improving antioxidant function. This finding provides a new strategy to

TABLE 8 Effects of dietary amylose/amylopectin ratio on indole and skatole concentrations in cecal and colonic chyme of weaned piglets under LPS stress.[1]

Items	0.00		0.20		0.40		0.60		*0.80*		*SEM*	*p* value			P_L	P_Q
	LPS[1]	SAL	LPS	SAL	LPS	SAL	LPS	SAL	LPS	SAL		P_{DAR}	P_{stress}	P_D*_S	SAL	
Indol(e), μg/g																
Cecal chyme	11.79^{A}	9.71	19.24^{AB}	3.62	9.84^{AB}	6.54	3.47^{AB}	2.86	0.00^{B}	4.07	1.01	0.031	0.123	0.091	NS	NS
Colonic chyme	2.73	3.21	5.47	14.37	10.05	7.43	4.00	8.97	5.28	4.90	0.94	0.246	0.271	0.402	NS	NS
Skatole, μg/g																
Cecal chyme	11.01	8.93	23.64	13.57	20.69	6.59	14.17	5.44	0.35	8.78	1.46	0.102	0.108	0.214	NS	NS
Colonic chyme	3.59^{C}	8.18	8.81^{BC}	28.18	40.14^{AB}	23.62	30.56^{A}	44.94	39.81^{AB}	15.88	2.20	0.002	0.930	0.019	NS	NS

[1]LPS, lipopolysaccharide, SAL, saline, SEM, standard error of the mean, DAR, dietary amylose/amylopectin ratio. L, Q represents a linear, quadratic response to increasing dietary amylose/amylopectin ratio. Only data from non-stress piglets were analyzed in this REG procedure.
$^{A-C}$Values within a row with different superscripts differ significantly at $p < 0.05$. NS means a p-value of > 0.15.

protect piglets from LPS-induced stress by regulating dietary starch structure.

Data availability statement

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

Ethics statement

The animal studies were approved by Animal Care and Use Committee of Hunan Normal University (ISA-2017-058). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

CY: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. XT: Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing. MW: Investigation, Methodology, Writing – review & editing. HaY: Investigation, Methodology, Project administration, Writing – review & editing. HuY: Conceptualization, Investigation, Writing – review & editing. YW: Investigation, Methodology, Writing – review & editing. YY: Conceptualization, Investigation, Supervision, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by key projects of Hunan provincial department of education (22A0504); Research foundation of the education of Hunan province (21C1122); China postdoctoral science foundation funded project (2016M600630).

Acknowledgments

The authors gratefully thank the pig farm of the Institute of Subtropical Agriculture, Chinese Academy of Science, for using the experimental farm, the provision of experimental animals, and their assistance in animal handling throughout the experimental period.

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 reviewer XL declared a shared affiliation with the author YY to the handling editor at the time of review.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2024.1435051/full#supplementary-material

References

1. Qiao Y, Bai XF, Du YG. Chitosan oligosaccharides protect mice from LPS challenge by attenuation of inflammation and oxidative stress. *Int Immunopharmacol*. (2011) 11:121–7. doi: 10.1016/j.intimp.2010.10.016

2. Xiao D, Wang Y, Liu G, He J, Qiu W, Hu X, et al. Effects of chitosan on intestinal inflammation in weaned pigs challenged by enterotoxigenic *Escherichia coli*. *PLoS One*, 8(2014-8-4). (2014) 9:e104192. doi: 10.1371/journal.pone.0104192

3. Yuan S, Chen D, Zhang K, Yu B. Effects of oxidative stress on growth performance, nutrient digestibilities and activities of antioxidative enzymes of weanling pigs. *Asian Australas J Anim Sci*. (2007) 20:1600–5. doi: 10.5713/ajas.2007.1600

4. Xia B, Meng Q, Feng X, Tang X, Jia Z. Probing the molecular regulation of lipopolysaccharide stress in piglet liver by comparative proteomics analysis. *Electrophoresis*. (2018) 39:2321–31. doi: 10.1002/elps.201700467

5. Gu K, Wang F, Sun W, Liu G, Jia G, Zhao H, et al. Tryptophan alleviates lipopolysaccharide-induced liver injury and inflammation by modulating piglets' necroptosis and pyroptosis signaling pathways. *Anim Biotechnol*. (2023) 34:4069–80. doi: 10.1080/10495398.2023.2255064

6. McGilvray WD, Klein D, Wooten H, Dawson JA, Hewitt D, Rakhshandeh AR, et al. Immune system stimulation induced by *Escherichia coli* lipopolysaccharide alters plasma free amino acid flux and dietary nitrogen utilization in growing pigs. *J Anim Sci*. (2019) 97:315–26. doi: 10.1093/jas/sky401

7. Prates JAM, Freire JPB, de Almeida AM, Martins C, Ribeiro DM, Osório H, et al. Influence of dietary supplementation with an amino acid mixture on inflammatory markers, immune status and serum proteome in LPS-challenged weaned piglets. *Animals*. (2021) 11:1143. doi: 10.3390/ani11041143

8. Vonderohe C, Stoll B, Didelija I, Nguyen T, Mohammad M, Jones-Hall Y, et al. Citrulline and ADI-PEG20 reduce inflammation in a juvenile porcine model of acute endotoxemia. *Front Immunol*. (2024) 15:1400574. doi: 10.3389/fimmu.2024.1400574

9. Luo ZG, Shi YC. Preparation of acetylated waxy, normal, and high-amylose maize starches with intermediate degrees of substitution in aqueous solution and their properties. *J Agric Food Chem*. (2012) 60:9468–75. doi: 10.1021/jf301178c

10. Lopez-Rubio A, Flanagan BM, Gilbert EP, Gidley MJ. A novel approach for calculating starch crystallinity and its correlation with double helix content: a combined XRD and NMR study. *Biopolymers*. (2008) 89:761–8. doi: 10.1002/bip.21005

11. Xie C, Li Y, Li J, Zhang L, Zhou G, Gao F. Dietary starch types affect liver nutrient metabolism of finishing pigs. *Br J Nutr*. (2017) 118:353–9. doi: 10.1017/s0007114517002252

12. Zeng H, Huang C, Lin S, Zheng M, Chen C, Zheng B, et al. Lotus seed-resistant starch regulates gut microbiota and increases short-chain fatty acid production and mineral absorption in mice. *J Agric Food Chem*. (2017) 65:9217–25. doi: 10.1021/acs.jafc.7b02860

13. Yang C, He J, Yu B, Yu J, Mao XB, Chen DW, et al. The effect of dietary amylose/amylopectin ratio on serum and hepatic lipid content and its molecular mechanisms in growing-finishing pigs. *J Anim Physiol Anim Nutr*. (2018) 102:1657–65. doi: 10.1111/jpn.12884

14. Gao K, Pi Y, Mu CL, Farzi A, Liu Z, Zhu WY. Increasing carbohydrate availability in the hindgut promotes hypothalamic neurotransmitter synthesis: aromatic amino acids linking the microbiota-brain axis. *J Neurochem*. (2019) 149:641–59. doi: 10.1111/jnc.14709

15. Vaziri ND, Liu SM, Lau WL, Khazaeli M, Nazertehrani S, Farzaneh SH, et al. High amylose resistant starch diet ameliorates oxidative stress, inflammation, and progression of chronic kidney disease. *PLoS One*. (2014) 9:e114881. doi: 10.1371/journal.pone.0114881

16. Council NR. Nutrient requirements of swine. Washington, DC: National Academy Press (2012).

17. Zhang Y, Wan D, Zhou X, Long C, Wu X, Li L, et al. Diurnal variations in iron concentrations and expression of genes involved in iron absorption and metabolism in pigs. *Biochem Biophys Res Commun*. (2017) 490:1210–4. doi: 10.1016/j.bbrc.2017.06.187

18. Xu L, Geng M, Zhang L, Yuan H, Kong X, Wang J. Determination of biomines in colonic contents of piglets by pre-column column derivatization RP-HPLC. *Acta Nutr Sin*. (2014) 36:78–83.

19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. *Methods (San Diego, Calif)*. (2001) 25:402–8. doi: 10.1006/meth.2001.1262

20. Jing M, Munyaka P, Tactacan G, Rodriguez-Lecompte J, O K, House J. Performance, serum biochemical responses, and gene expression of intestinal folate transporters of young and older laying hens in response to dietary folic acid supplementation and challenge with *Escherichia coli* lipopolysaccharide. *Poult Sci*. (2014) 93:122–31. doi: 10.3382/ps.2013-03384

21. Zhu JH, Zhang X, Roneker CA, Mcclung JP, Zhang S, Thannhauser TW, et al. Role of copper, zinc-superoxide dismutase in catalyzing nitrotyrosine formation in murine liver. *Free Radic Biol Med*. (2008) 45:611–8. doi: 10.1016/j.freeradbiomed.2008.05.018

22. Lopez H, Levrat-Verny M, Coudray C, Besson C, Krespine V, Messager V, et al. Class 2 resistant starches lower plasma and liver lipids and improve mineral retention in rats. *J Nutr*. (2001) 131:1283–9. doi: 10.1093/jn/131.4.1283

23. Smith EA, Macfarlane G. Formation of phenolic and indolic compounds by anaerobic bacteria in the human large intestine. *Microb Ecol*. (1997) 33:180–8. doi: 10.1007/s002489900020

24. Yang C, Wang M, Tang X, Yang H, Li F, Wang Y, et al. Effect of dietary amylose/amylopectin ratio on intestinal health and cecal microbes' profiles of weaned pigs undergoing feed transition or challenged with *Escherichia coli* lipopolysaccharide. *Front Microbiol*. (2021) 12:693839. doi: 10.3389/fmicb.2021.693839

25. Wesoly R, Weiler U. Nutritional influences on skatole formation and skatole metabolism in the pig. *Animals*. (2012) 2:221–42. doi: 10.3390/ani2020221

26. He X, Sun W, Ge T, Mu C, Zhu W. An increase in corn-resistant starch decreases protein fermentation and modulates gut microbiota during the in vitro cultivation of large intestinal inocula in pigs. *Anim Nutr*. (2017) 3:219–24. doi: 10.1016/j.aninu.2017.06.004

27. Blaut M, Clavel T. Metabolic diversity of the intestinal microbiota: implications for health and disease. *J Nutr*. (2007) 137:751S–5S. doi: 10.1093/jn/137.3.751S

28. Ducker G, Rabinowitz J. One-carbon metabolism in health and disease. *Cell Metab*. (2016) 25:27–42. doi: 10.1016/j.cmet.2016.08.009

29. Liu G, Yu L, Yordan M, Ren W, Ni H, Naif AD, et al. Dietary *saccharomyces cerevisiae* cell wall extract supplementation alleviates oxidative stress and modulates serum amino acid profiles in weaned piglets. *Oxidative Med Cell Longev*. (2017) 2017:1–7. doi: 10.1155/2017/3967439

frontiers | Frontiers in Nutrition

TYPE Original Research
PUBLISHED 07 January 2025
DOI 10.3389/fnut.2024.1415817

OPEN ACCESS

EDITED BY
Flavia Prodam,
University of Eastern Piedmont, Italy

REVIEWED BY
Marco Barale,
University of Turin, Italy
Daniele Spadaccini,
University of Eastern Piedmont, Italy
Stella Pigni,
Humanitas Research Hospital, Italy

*CORRESPONDENCE
Morteza Abdollahi
✉ m.abdollahi@sbmu.ac.ir

RECEIVED 11 April 2024
ACCEPTED 04 December 2024
PUBLISHED 07 January 2025

CITATION
HoushiarRad A, Fotros D, Esmaili M, Sohouli MH, Ajami M, Abdollahi M and Hatami Marbini M (2025) Dietary glycemic and insulin indices with the risk of osteoporosis: results from the Iranian teachers cohort study. *Front. Nutr.* 11:1415817. doi: 10.3389/fnut.2024.1415817

Dietary glycemic and insulin indices with the risk of osteoporosis: results from the Iranian teachers cohort study

Anahita HoushiarRad[1], Danial Fotros[1], Mina Esmaili[1], Mohammad Hassan Sohouli[2], Marjan Ajami[1], Morteza Abdollahi[1]* and Motahare Hatami Marbini[1]

[1]Department of Clinical Nutrition and Dietetics, Faculty of Nutrition and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2]Student Research Committee, Department of Clinical Nutrition and Dietetics, Faculty of Nutrition and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Background: Osteoporosis is a chronic condition characterized by reduced bone strength and an elevated risk of fractures. The influence of diet and glucose metabolism on bone health and the development of osteoporosis has been an area of interest. This study aimed to investigate the potential association between dietary glycemic index (DGI), dietary glycemic load (DGL), dietary insulin index (DII), dietary insulin load (DIL), and the odds of osteoporosis among Iranian adults.

Methods: Data from 12,696 Iranian teachers (35–50 years) in a cross-sectional study on diet, nutrition, physical activity, and diseases were analyzed. The participants had no history of diabetes, cardiovascular diseases, stroke, thrombosis, or cancer and consumed between 800 and 4,200 kcal/day. We estimated DGI, DGL, DII, and DIL from a validated semi-quantitative food-frequency questionnaire (FFQ). We also diagnosed osteoporosis using dual-energy X-ray absorptiometry.

Results: In the fully adjusted model, higher DGI and DGL were significantly associated with increased odds of osteoporosis (OR = 1.78 and 1.46 for the highest vs. the lowest tertile; *P* trend <0.05). Nonetheless, no significant association was found between DII or DIL and osteoporosis prevalence. Moreover, higher DIL and DGL were associated with a higher intake of calorie-dense/nutrient-poor foods and a lower intake of antioxidant-rich foods.

Conclusion: Although our study showed that high DGI/DGL increased osteoporosis risk in Iranian teachers, no association was found between DII/DIL and osteoporosis prevalence. More research is needed to confirm these results and understand the mechanisms involved.

KEYWORDS

osteoporosis, glycemic index, insulin index, glycemic load, insulin load

Introduction

Osteoporosis is a chronic and prevalent disease that harms bone strength, leading to increased susceptibility to fractures, impaired physical mobility, and a diminished quality of life (1, 2). More than 200 million people around the world suffer from this disease (3), and its prevalence in Iran, according to the latest statistics, is ~17% (4).

Various factors, such as aging, genetics, certain diseases, some medications, and physical activity and lifestyle, are involved in increasing the risk of osteoporosis (5). Diet is one of the factors that has recently been considered because it can be modified and can play an important role in the prevention, management, and support of drug treatment in these patients (6). As an example, diets and some nutrients such as calcium, magnesium, vitamin D, and vitamin K have been shown to play an important role in bone health and reduce the risk of osteoporosis (7, 8). Moreover, there is a growing body of evidence indicating that diabetes, whether controlled or uncontrolled, may negatively influence bone mineral density (BMD) (9, 10). There are several factors involved in the pathophysiology of bone health regarding hyperglycemia (9–11). In fact, high blood sugar may cause an increase in bone resorption (12). Indeed, the risk of fragility fractures is increased in both patients with type 1 (T1DM) and type 2 (T2DM) diabetes, characterized by chronic hyperglycemia (13–15). However, fragility fractures may occur even in the presence of normal or even slightly elevated BMD in T2DM patients, and the pathophysiological mechanisms of DM-induced skeletal fragility are much more complex, including for instance increased oxidative stress, chronic inflammation, adipokine alterations, and accumulation of advanced glycation end products (13–15). Diets that have a high dietary glycemic index (DGI) and load (DGL), as well as a high dietary insulin index (DII), and load (DIL) cause significant increases in blood glucose and insulin levels (16, 17). Lower glycemic and insulin indexes can reduce inflammation, which may underlie osteoporosis progression (18–20). In general, DGI and DGL are dietary concepts that have been considered to reflect abnormal glucose metabolism and hyperglycemia (21), and DII is a new food ranking algorithm based on the insulin response to the use of isoenergetic reference food in healthy individuals (22). Several studies have demonstrated the existence of a positive correlation between osteoporosis and glycemic and insulin indices in specific populations (23, 24). However, it is crucial to acknowledge the scarcity of research about the connection between DGI, DGL, DII, and DIL and the risk of osteoporosis. This scarcity may hinder the ability to draw definitive conclusions.

Thus, we aimed to investigate the association of glycemic and insulin indices with the odds of osteoporosis in a large sample of the Iranian adult population.

Methods

Study participants

The participants investigated in the present study include all male and female teachers aged 35–50 in Iran who are willing to cooperate with the project and have completed the study questionnaires, including the consent questionnaire. The reason for choosing teachers as the investigated sample is as follows: 1. Since the questionnaire is self-administered, the participants must be literate. 2. Due to their job, teachers are fully familiar with how to complete a questionnaire and can understand the concept of questions and filling in the blanks or marking specific places. 3. Teachers are hired and organized by the Ministry of Education, so information about them can be obtained through that ministry. 4. All teachers are insured by the Health Services Insurance Organization and in case of suffering from a serious chronic disease, they will use their insurance. Therefore, if a participant gets sick and does not report it himself, it is possible to get information about his illness by using the database of the health service organization. This cross-sectional study was started in 2001 to determine the relationship between food intake, nutritional status, and physical activity with the incidence of non-communicable diseases. The details related to this study have been reported previously (25). Among the 14,058 participants who entered the present study as primary data, 1,362 people were excluded from the present study due to diabetes, cardiovascular diseases (CVDs), stroke, thrombosis, and cancer, as well as people receiving daily energy outside the range of 800–4,200 and pregnant and lactating women. The data from 12,696 participants were analyzed. In order to determine the sample size in a prospective study to estimate the relative risk, the minimum sample size for estimating with 95% confidence and estimating the relative risk is 1.5, while our estimate is at most 20% away from the actual value of the relative risk and the annual incidence of the desired disease in the non-exposed group is not less than one thousandth (1% during 10 years of study), we need 12,705 samples in each group for comparison. The physical activity levels of the participants were estimated by using a validated short form of the International Physical Activity Questionnaire (Short IPAQ) and reported as the metabolic equivalent of task (MET)-minutes/week (26). Data collection was performed by self-administered questionnaires (23).

Definition of osteoporosis

The researchers used dual-energy x-ray absorptiometry (GE Healthcare, Madison, WI, USA) with Hardware: Expert and Software: 1.91 to assess the BMD in the study participants. In postmenopausal women and men over 50 years of age in this study, osteoporosis was operationally defined as femoral BMD values that fell 2.5 standard deviations (T-scores of -2.5) or below the mean BMD values of Iranian individuals of the same sex within the age range of 20–29 years. The selection of this age group as the reference group is based on the fact that bone mass often reaches its maximum level within this particular range of ages (27). In pre-menopausal women and men under 50 years of age, BMD was expressed as Z-score, and individuals with Z-score $\leq$-2.0 SD were defined as having BMD "below the expected range for age" (28). For the study purpose, patients with osteoporosis or BMD "below the expected range for age" were classified together as having "low BMD/osteoporosis" (29). Osteopenia is operationally defined as T scores of -1.0 or lower (2, 30). Furthermore, in this study, all secondary causes of osteoporosis were adjusted based on statistical analysis. In fact, all comorbidities, including chronic diseases as well as diseases related to the malabsorption of micronutrients that can affect osteoporosis, have been adjusted based on statistical analysis and their effects have been removed.

Dietary assessment

The dietary intake over the previous year was obtained using a semi-quantitative food-frequency questionnaire (FFQ) which was

specifically developed for this study by experienced experts on food consumption in Iran (25, 31). The FFQ consisted of a list of usual Iranian dietary items with standard serving sizes. For each food item, the average portion size consumed and the frequency of intake were obtained from self-reports on the FFQ. The frequency of intake for each food item included: never, 2–3 times/month, 1 time/week, 2–4 times/week, 5–6 times/week, and daily. The portion sizes were reported in grams using standard Iranian household measures (32). The daily nutrient consumptions for each person were estimated by applying the United States Department of Agriculture's (USDA) national nutrient databank. The Nutritionist IV software (First Databank, San Bruno, CA, USA—modified for Iranian foods) was used to calculate the daily energy and nutrient intake for each participant.

Calculation of dietary insulin index and load (DII and DIL)

DII for foods containing calories refers to the incremental insulin area under the curve over 2 h in response to the consumption of a 1,000-kJ portion of the test food divided by the area under the curve after ingestion of a 1,000-kJ portion of the reference food. The DII for each calorie-containing food was obtained from FFQ data using data published by Professor Jennie Brand-Miller of the University of Sydney, Australia (33). For each study participant, the total DIL (DILoveral) over the past year, for each calorie-reported food in the FFQ, was determined by calculating its index DII, the calorie content of that food (kcal per portion of that nutrient intake), and its frequency of use (daily portion) and then the sum of the amounts. So, DIL is equal to the summation of (the insulin index of each food x energy content of a serving x number of servings/day of that food). The overall DII (DIIoveral) was also calculated by dividing the DILoveral by total energy intake (kcal/day).

Glycemic index and glycemic load measurement

The total DGI was calculated using the following formula:

$\sum$ (GI × available carbohydrate)/total available carbohydrate, where the available carbohydrate was calculated as the total carbohydrate minus fiber (34).

The total carbohydrate and fiber contents of the foods were derived from the United States Department of Agriculture food composition table. Of the food and beverage items included in the FFQ, 30 items (17.8%) contained no available carbohydrate. The calculation of the DGL and DGI was thus based on the remaining 138 items, with DGI values ranging from 10 to 123. We used several international (35) and Iranian DGI tables (36) that were previously published. All derived DGI values were relative to glucose as the reference food. The DGI of composite mixed meals was estimated based on the DGI of the individual food components (34). The DGL was calculated as (total GI × total available carbohydrate)/100 (34) and expressed as g/d. The r value for the correlation between carbohydrate intakes derived from the FFQ compared with the average of 3-day dietary records was 0.81, which indicated that the FFQ provides a reasonable measure of total carbohydrate intake over a long period of time (34).

Anthropometric assessment

The anthropometric measurements were obtained via self-report. The body mass index (BMI) was calculated as weight (kg) divided by the square of the height (m^2).

Statistical analyses

All statistical analyses were performed using SPSS software (version 19.0; SPSS Inc, Chicago IL). The normality of variables was evaluated by Kolmogorov–Smirnov and histogram tests. In addition, non-parametric statistics, including the Mann–Whitney *U*-test or Kruskal–Wallis test, were used for variables that were not normally distributed.

The mean values of more than two groups were assessed using analysis of variance (ANOVA) for normal distribution variables. Moreover, for comparing categorical variables, the chi-square test was used. Furthermore, the linear regression analysis method was used to analyze (Table 4). Binary logistic regression was used to estimate ORs and 95% confidence intervals (CIs) adjusted for multiple covariates in a different model. In the first model, adjustments were made for age, sex, and BMI. The second model underwent additional modifications to account for education, supplement intake of multivitamin–minerals (vitamins A, D, C, 89, calcium, and omega-3), physical activity, smoking, comorbidity, menopausal status, use of drugs or hormone therapy, and a special diet. The final model additionally incorporated the intake of energy, protein, fiber, calcium, vitamins (D, C, and B9), saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). In all models, participants in the lowest tertiles of DIL and DII were designated as the reference group. In adjusted models, confounders were used from statistical and conceptual approaches, respectively. In this way, the variables with a *P*-value of <0.2 were considered as possible confounders and were entered into the logistic regression, and the odds of getting osteoporosis/low BMD were investigated. Furthermore, in the conceptual approach of adjusting confounders in model 3, possible confounders were selected based on clinical concepts and based on past articles and added to other confounders. The data were presented as mean ± SD and OR with 95% CI, and in all results, the significance level was determined as a *p*-value of <0.05.

Results

The mean (± SD) age of the study population (33% men) was 43.81 ± 6.97 years. The mean (± SD) BMI was 26.45 ± 3.95 kg/m^2. Furthermore, the baseline mean ± SD of dietary indices including DII, DIL, DGI, and DGL were 56.51 ± 4.581, 312.94 ± 30.82, 70.2 ± 5.8, and 216.72 ± 28.12, respectively, among all participants in the study. The mean T-score and *Z*-score BMD in total participants were −1.15 and −1.27, respectively.

TABLE 1 Baseline characteristics among 12,696 participants of Study based on tertiles of dietary insulin load.

Variables		Total population	Insulin load			
			T1 ($n = 4,232$)	T2 ($n = 4,232$)	T3 ($n = 4,232$)	*P*-value[a]
Age (years)		43.81 (6.97)	43.84 (7.18)	43.61 (6.78)	43.98 (6.93)	0.059
Male, *n* (%)		4,199 (33.1)	874 (20.7)	1231 (29.1)	2094 (49.75)	**<0.001**
Weight (kg)		72.16 (12.31)	70.41 (11.74)	71.41 (12.27)	74.65 (12.50)	**<0.001**
BMI (kg/m^2)		26.45 (3.95)	26.45 (3.96)	26.39 (3.96)	26.51 (3.93)	0.411
BMD femoral (*T*-score)		−1.15 (0.75)	−1.35 (0.99)	−1.18 (0.80)	−0.97 (0.71)	**<0.001**
BMD femoral (*Z*-score)		−1.27 (0.71)	−1.39 (0.96)	−1.11 (0.83)	−0.93 (0.69)	**<0.001**
Physical activity (Met/min/week)		902.71 (905.03)	934.57 (918.99)	913.95 (913.80)	883.74 (913.06)	**0.036**
Osteoporosis, *n* (%)		611 (4.7)	168 (28.0)	204 (34.0)	228 (38.0)	**0.008**
Under a special diet, *n* (%)		2,168 (16.7)	826 (19.5)	718 (17.0)	578 (13.7)	**<0.001**
Menopausal status (postmenopausal), *n* (%)		1,274 (15.3)	508 (15.5)	467 (15.8)	299 (14.3)	**0.323**
Education	Under diploma	234 (1.8)	73 (1.7)	69 (1.6)	76 (1.8)	**0.008**
	Diploma	521 (4.0)	152 (3.6)	162 (3.8)	194 (4.6)	
	Bachelor's degree	1,758 (13.6)	621 (14.7)	559 (13.2)	536 (12.7)	
	Master's degree	8,324 (64.2)	2,751 (65.0)	2,709 (64.0)	2,720 (64.3)	
	Doctorate and above	2,121 (16.4)	635 (15.0)	733 (17.3)	706 (16.7)	
Current smoker, *n* (%)		799 (6.29)	310 (7.32)	198 (4.67)	291 (6.87)	0.091
Multivitamin intake, *n* (%)		916 (7.1)	330 (7.8)	315 (7.4)	255 (6.0)	**<0.001**
Vitamin D supplement intake, *n* (%)		2,603 (20.1)	897 (21.2)	892 (21.1)	777 (18.4)	**<0.001**
Vitamin A supplement intake, *n* (%)		394 (3.0)	130 (3.1)	132 (3.1)	119 (2.8)	0.168
Vitamin C supplement intake, *n* (%)		603 (4.7)	183 (4.3)	227 (5.4)	179 (4.2)	0.065
Vitamin B supplement intake, *n* (%)		573 (4.4)	220 (5.2)	199 (4.7)	145 (3.4)	**<0.001**
Folate supplement intake, *n* (%)		291 (2.2)	95 (2.2)	109 (2.6)	81 (1.9)	**<0.001**
Calcium supplement intake, *n* (%)		1,446 (11.2)	495 (11.7)	507 (12.0)	425 (10.0)	**<0.001**
Iron supplement intake, *n* (%)		2,092 (16.1)	772 (18.2)	732 (17.3)	554 (13.1)	**<0.001**
Omega 3 supplement intake, *n* (%)		656 (5.1)	243 (5.7)	244 (5.8)	158 (3.7)	**<0.001**
Dietary intakes						
Fruits (g/d)		394.85 (274.58)	441.30 (309.98) 2)	406.47 (267.16)	336.78 (230.4)	**<0.001**
Vegetables (g/d)		426.32 (339.71)	455.22 (351.60)	425.98 (335.37)	397.77 (329.41)	**<0.001**
Processed meat (g/d)		17.03 (16.96)	15.50 (14.79)	17.21 (16.43)	18.37 (19.24)	**<0.001**
Total dairy (g/d)		232.28 (200.67)	268.81 (232.60)	236.13 (195.92)	191.89 (159.23)	**<0.001**
Legumes (g/d)		14.65 (15.07)	17.82 (18.58)	14.22 (13.54)	11.91 (11.60)	**<0.001**
Nuts (g/d)		8.18 (10.80)	8.93 (12.28)	8.62 (10.87)	6.98 (8.88)	**<0.001**
Whole grains (g/d)		99.95 (75.69)	179.11 (73.14)	82.12 (31.26)	38.62 (22.70)	**<0.001**
Refined grains (g/d)		354.44 (176.10)	208.81 (84.43)	329.11 (99.94)	525.41 (157.63)	**<0.001**
Fish (g/d)		22.59 (32.78)	26.31 (42.07)	22.68 (29.64)	18.77 (23.38	**<0.001**
Energy (Kcal/d)		2,017.00 (728.79)	1,353.10 (278.37)	1,884.03 (304.74)	2,813.87 (574.13)	**<0.001**
Protein (g/d)		61.15 (23.75)	84.19 (21.01)	57.73 (13.69)	41.54 (11.66)	**<0.001**
Fat (g/d)		51.96 (25.56)	44.40 (21.35)	50.31 (22.71)	61.17 (29.04)	**<0.001**
Carbohydrate (mg/d)		311.06 (127.94)	189.02 (39.41)	286.77 (39.62)	457.39 (95.32)	**<0.001**
Cholesterol (mg/d)		159.34 (92.26)	129.13 (67.49)	158.66 (82.19)	190.22 (111.18)	**<0.001**

(Continued)

TABLE 1 (Continued)

Variables	Total population	Insulin load			
		T1 ($n = 4,232$)	T2 ($n = 4,232$)	T3 ($n = 4,232$)	*P*-value[a]
PUFA (g/d)	17.23 (11.02)	15.37 (10.36)	16.48 (10.13)	19.85 (11.99)	**<0.001**
MUFA (g/d)	14.41 (7.33)	12.11 (5.77)	13.96 (6.47)	17.17 (8.55)	**<0.001**
SFA (g/d)	14.42 (6.72)	12.10 (4.91)	14.19 (5.89)	16.96 (8.07)	**<0.001**
Calcium (mg/d)	677.41 (355.44)	830.68 (395.83)	674.10 (323.71)	527.44 (267.58)	**<0.001**
Iron (mg/d)	14.22 (5.91)	8.77 (1.98)	13.04 (2.11)	20.85 (4.64)	**<0.001**
Phosphorus (mg/d)	1,048.13 (410.37)	751.99 (248.74)	1,009.33 (294.35)	1,383.07 (394.12)	**<0.001**
Magnasium (mg/d)	326.96 (131.36)	458.96 (109.48)	305.13 (72.43)	216.80 (67.06)	**<0.001**
Zinc (mg/d)	9.63 (3.74)	13.51 (3.11)	9.02 (1.86)	6.36 (1.68)	**<0.001**
Sodium (mg/d)	2,103.45 (1,020.77)	1,358.89 (589.54)	1,940.25 (685.35)	3,011.20 (950.67)	**<0.001**
Vitamin A (mcg/d)	456.49 (315.29)	523.68 (407.96)	453.11 (262.21)	392.68 (233.42)	**<0.001**
Folate (mcg/d)	269.73 (99.07)	319.66 (112.61)	271.32 (83.91)	218.22 (67.66)	**<0.001**
Vitamin D (mcg/d)	1.43 (1.87)	1.56 (2.39)	1.42 (1.95)	1.36 (1.69)	**0.006**
Vitamin C (mg/d)	83.97 (54.61)	93.44 (60.34)	85.52 (53.16)	72.95 (47.62)	**<0.001**
Vitamin B6 (mg/d)	1.50 (0.61)	1.84 (0.67)	1.48 (0.52)	1.18 (0.44)	**<0.001**
Vitamin B12 (mg/d)	3.35 (2.90)	3.96 (4.07)	3.33 (2.23)	2.76 (1.74)	**<0.001**
Dietary fibre (g/d)	24.60 (10.74)	35.07 (9.28)	22.76 (6.33)	15.98 (5.71)	**<0.001**

BMI, body mass index; MET, metabolic equivalent.
Data are presented as mean [standard deviation (SD)] or number (percent).
[a]Obtained from ANOVA or Chi-square test, where appropriate.
Bolded numbers indicate a p-value < 0.05.

The baseline characteristics and dietary intakes of the study population based on the tertiles of dietary IL are shown in Table 1. Across tertiles of DIL, the weight, male present, and dietary intakes of energy, carbohydrate, fat, SFA, MUFA, PUFA, cholesterol, phosphorus, iron, sodium, red and processed meat, and refined grains were increased. However, BMD (*T*- and *Z*-scores), physical activity, the percentage of people receiving supplements (multivitamins, vitamin D, B, folate, calcium, iron, and omega-3), and dietary intakes of fruits, vegetables, total dairy, whole grains, nuts and legumes, fish, protein, and antioxidant nutrients and vitamins were decreased across tertiles of DIL. For other variables, there were no significant differences across tertiles of DIL.

General characteristics and dietary intake of subjects across tertiles of DGL are presented in Table 2. Compared with those in the lowest tertiles of DGL, subjects in the highest tertile had a higher male present, age, and weight, as well as lower BMD (*T*- and *Z*-scores) and physical activity. In addition, there was a significant difference between the tertiles of DGL in terms of following a special diet, education level, and receiving supplements (multivitamins, vitamin D, B, folate, calcium, iron, and omega-3). No other significant difference was found in other general characteristics across the tertiles of DGL. Furthermore, individuals with the highest tertiles of DGI had a higher intake of energy, carbohydrate, fat, SFA, MUFA, PUFA, cholesterol, phosphorus, iron, sodium, red and processed meat, and refined grains, as well as a lower intake of protein, fiber, calcium, magnesium, zinc, vitamin A, folate, vitamin C, B6, B12, total dairy, whole grains, fish, nuts, legumes, fruits, and vegetables compared with those in the bottom tertiles.

The ORs and 95% CIs for osteoporosis subjects based on the tertiles of available glycemic and insulin indices are reported in Table 3.

In the crude model, the DGI and DGL were directly associated with the odds of osteoporosis, with an OR of 2.10 and 1.36 for the highest tertile, respectively, as compared to the lowest tertile ($P < 0.05$ for trend). Furthermore, after we adjusted for age, sex, BMI, education, supplement intake of multivitamin–mineral, vitamin A, D, C, B9, calcium, omega-3, physical activity, smoking, comorbidity, menopausal status, use of drug or hormone therapy, under a special diet, as well as intake of energy, protein, fiber, phosphorus, calcium, vitamin D, C, B9, SFA, MUFA, and PUFA (in the fully adjusted model), in the highest vs. lowest tertile of GI and GL, the increase odds of osteoporosis remained significant (OR = 1.78, 95% CI: 1.90–3.52; *P* trend = 0.001 for trend and OR = 1.46, 95% CI: 1.17–2.02; *P* trend = 0.035 for trend, respectively).

On the other hand, in the first tertile compared to the last tertile, although an increase in the DIL score was associated with higher odds of osteoporosis in both the crude and first models (OR = 1.46, 95% CI: 1.17–1.82; *P* trend = 0.001 for trend and OR = 1.39, 95% CI: 1.05–1.77; *P* trend = 0.037 for trend, respectively), no significant relationship between the DII and the odds of this disease was observed in these two models (OR = 1.10, 95% CI: 0.89–1.36; *P* trend = 0.371 for trend and OR = 1.03, 95% CI: 0.88–1.28; *P* trend = 0.942 for trend, respectively). In addition, after adjusting

TABLE 2 Baseline characteristics among 12,696 participants of study based on tertiles of dietary glycemic load.

Variables		Total population	Glycemic load			*P*-value
			T1 ($n = 4,232$)	T2 ($n = 4,232$)	T3 ($n = 4,232$)	
Age (years)		43.81 (6.97)	43.92 (7.11)	43.58 (6.84)	43.93 (6.94)	**0.040**
Male, *n* (%)		4,199 (33.1)	937 (22.1)	1,242 (29.3)	2,020 (47.7)	**<0.001**
Weight (kg)		72.16 (12.31)	70.60 (11.80)	71.41 (12.32)	74.47 (12.46)	**<0.001**
BMI (kg/m^2)		26.45 (3.95)	26.46 (3.96)	26.39 (3.99)	26.50 (3.90)	0.456
BMD femoral (*T*-score or *Z*-score)		−1.15 (0.75)	−1.30 (0.90)	−1.12 (0.84)	−0.99 (0.75)	**<0.001**
BMD femoral (*Z*-score)		−1.27 (0.71)	−1.25 (0.92)	−1.13 (0.85)	−0.90 (0.68)	**<0.001**
Physical activity (Met/min/week)		902.71 (905.03)	941.08 (909.23)	909.15 (901.21)	871.09 (891.12)	**0.042**
Osteoporosis, *n* (%)		611 (4.7)	180 (30.0)	192 (32.0)	228 (38.0)	**0.038**
Under a special diet, *n* (%)		2,168 (16.7)	812 (19.2)	722 (17.1)	588 (13.9)	**<0.001**
Menopausal status (postmenopausal), *n* (%)		1,274 (15.3)	497 (15.4)	452 (15.4)	325 (15.0)	**0.896**
Education	Under diploma	234 (1.8)	73 (1.6)	64 (1.5)	81 (1.9)	**0.002**
	Diploma	521 (4.0)	172 (4.1)	150 (3.5)	186 (4.4)	
	Bachelor's degree	1,758 (13.6)	604 (14.3)	557 (13.2)	555 (13.1)	
	Master's degree	8,324 (64.2)	2,771 (65.5)	2,710 (64.0)	2,699 (63.8)	
	Doctorate and above	2,121 (16.4)	612 (14.5)	751 (17.7)	711 (16.8)	
Current smoker, *n* (%)		799 (6.29)	298 (7.04)	305 (7.20)	196 (4.63%)	0.091
Multivitamin intake, *n* (%)		916 (7.1)	320 (7.6)	320 (7.6)	260 (6.1)	**<0.001**
Vitamin D supplement intake, *n* (%)		2,603 (20.1)	879 (20.8)	889 (21.0)	798 (18.9)	**<0.001**
Vitamin A supplement intake, *n* (%)		394 (3.0)	133 (3.1)	121 (2.9)	127 (3.0)	0.335
Vitamin C supplement intake, *n* (%)		603 (4.7)	184 (4.3)	221 (5.2)	184 (4.3)	0.201
Vitamin B supplement intake, *n* (%)		573 (4.4)	230 (5.4)	191 (4.5)	143 (3.4)	**<0.001**
Folate supplement intake, *n* (%)		291 (2.2)	95 (2.2)	102 (2.4)	88 (2.1)	**<0.001**
Calcium supplement intake, *n* (%)		1,446 (11.2)	471 (11.1)	520 (12.3)	436 (10.3)	**<0.001**
Iron supplement intake, *n* (%)		2,092 (16.1)	739 (17.5)	728 (17.2)	591 (14.0)	**<0.001**
Omega 3 supplement intake, *n* (%)		656 (5.1)	241 (5.7)	237 (5.6)	167 (3.9)	**<0.001**
Dietary intakes						
Fruits (g/d)		394.85 (274.58)	483.85 (327.30)	404.44 (251.89)	296.25 (194.69	**<0.001**
Vegetables (g/d)		426.32 (339.71)	475.30 (360.36)	424.62 (332.27)	379.05 (318.37)	**<0.001**
Processed meat (g/d)		17.03 (16.96)	15.56 (14.97)	17.03 (16.16)	18.49 (19.33)	**<0.001**
Total dairy (g/d)		232.28 (200.67)	282.58 (241.38)	232.77 (189.71)	181.48 (146.49)	**<0.001**
Legumes (g/d)		14.65 (15.07)	18.25 (18.53)	14.11 (13.59)	11.59 (11.43)	**<0.001**
Nuts (g/d)		8.18 (10.80)	9.47 (12.66)	8.60 (10.81)	6.47 (8.24)	**<0.001**
Whole grains (g/d)		99.95 (75.69)	172.17 (79.51)	84.00 (38.79)	43.67 (26.94)	**<0.001**
Refined grains (g/d)		354.44 (176.10)	211.82 (82.51)	329.72 (98.98)	521.78 (165.78)	**<0.001**
Fish (g/d)		22.59 (32.78)	25.60 (38.89)	22.48 (31.24)	19.69 (26.79)	**<0.001**
Energy (Kcal/d)		2,017.00 (728.79)	1,356.55 (282.10)	1,882.94 (306.05)	2,811.50 (578.61)	**<0.001**
Protein (g/d)		61.15 (23.75)	83.73 (21.17)	57.65 (14.22)	42.08 (12.45)	**<0.001**
Fat (g/d)		51.96 (25.56)	44.96 (21.87)	49.95 (22.75)	60.98 (28.80)	**<0.001**
Carbohydrate (mg/d)		311.06 (127.94)	187.78 (37.25)	287.23 (36.23)	458.17 (94.86)	**<0.001**
Cholesterol (mg/d)		159.34 (92.26)	131.16 (70.43)	156.73 (81.54)	190.13 (110.36)	**<0.001**

(Continued)

TABLE 2 (Continued)

Variables	Total population	Glycemic load			
		T1 ($n = 4,232$)	T2 ($n = 4,232$)	T3 ($n = 4,232$)	*P*-value
PUFA (g/d)	17.23 (11.02)	15.65 (10.56)	16.38 (10.13)	19.67 (11.88)	**<0.001**
MUFA (g/d)	14.41 (7.33)	12.28 (5.91)	13.82 (6.44)	17.14 (8.52)	**<0.001**
SFA (g/d)	14.42 (6.72)	12.19 (5.11)	14.08 (5.89)	16.97 (7.96)	**<0.001**
Calcium (mg/d)	677.41 (355.44)	848.96 (394.00)	667.68 (309.99)	515.57 (268.35)	**<0.001**
Iron (mg/d)	14.22 (5.91)	8.86 (2.06)	13.08 (2.29)	20.72 (4.82)	**<0.001**
Phosphorus (mg/d)	1,048.13 (410.37)	746.25 (248.61)	1,004.12 (282.95)	1,394.01 (388.16)	**<0.001**
Magnasium (mg/d)	326.96 (131.36)	460.09 (109.86)	305.70 (69.76)	215.09 (64.35)	**<0.001**
Zinc (mg/d)	9.63 (3.74)	13.42 (3.20)	9.02 (1.96)	6.46 (1.77)	**<0.001**
Sodium (mg/d)	2,103.45 (1,020.77)	1,354.50 (528.43)	1,947.57 (681.28)	3,008.27 (989.32)	**<0.001**
Vitamin A (mcg/d)	456.49 (315.29)	540.59 (410.64)	450.50 (254.59)	378.39 (227.20)	**<0.001**
Folate (mcg/d)	269.73 (99.07)	330.87 (110.56)	268.39 (76.88)	209.94 (63.23)	**<0.001**
Vitamin D (mcg/d)	1.43 (1.87)	1.48 (2.00)	1.50 (2.25)	1.43 (1.84)	0.089
Vitamin C (mg/d)	83.97 (54.61)	100.44 (63.51)	85.18 (51.99)	66.29 (40.34)	**<0.001**
Vitamin B6 (mg/d)	1.50 (0.61)	1.90 (0.66)	1.47 (0.48)	1.13 (0.39)	**<0.001**
Vitamin B12 (mg/d)	3.35 (2.90)	3.97 (4.03)	3.29 (2.20)	2.79 (1.89)	**<0.001**
Dietary fibre (g/d)	24.60 (10.74)	35.55 (9.12)	22.85 (5.64)	15.41 (4.88)	**<0.001**

Bolded numbers indicate a p-value < 0.05.

for possible confounders in the final adjusted model, no significant relationship between dietary insulin indices (DII and DIL) and the odds of osteoporosis was observed (OR = 1.16, 95% CI: 0.93–1.27; *P* trend = 0.642 for trend and OR = 1.55, 95% CI: 1.09–2.31; *P* trend = 0.104 for trend, respectively) (Table 3).

The linear relationship between the T-score and the scores examined in the study is also shown in Table 4. However, no significant linear relationship between *T*-score and different scores was observed.

Discussion

This study indicates a significant association between high DGI/DGL and an increased odds of osteoporosis. According to our findings, being in the last tertile of the GI and GL is associated with 78 and 46% higher odds of osteoporosis, respectively.

Our findings are consistent with some previous studies, including one that demonstrated a link between high DGI and DGL with an elevated risk of fracture in an elderly Mediterranean population (23) and another study that found an association between BMD abnormality and DGI in postmenopausal women (24). In addition, one study has shown that increased glycemic variability in patients with T2DM is associated with osteoporosis (37).

Evidence shows that high DGI and DGL diets may increase oxidative stress and inflammation (38–40), which can impair bone health by stimulating osteoclastogenesis and bone resorption (41) and inhibiting osteoblast function (42). Moreover, several cytokines may contribute to osteoporosis pathogenesis, notably interleukin (IL)-6 (43), tumor necrosis factor (TNF)-α (44), and IL-1 (45). The direct and indirect effects of IL-6 on osteoclastic processes have been extensively studied (46–48). It has been shown that the inhibition of IL-6 receptor signaling inhibits the formation of osteoclasts both *in vitro* and *in vivo* (49). IL-6 can also inhibit osteoclast differentiation directly by acting on osteoclast progenitors (47). Furthermore, evidence suggests that TNF-α promotes bone resorption *in vitro* (44). TNF-α acts directly on surface receptors and induces the differentiation of osteoclasts (50). It also inhibits osteoblast function and bone formation by inhibiting insulin-like growth factor (IGF)-I expression and RUNX2 expression in osteoblast precursor cells (51, 52). Osteoclastogenesis may be directly stimulated by IL-1, specifically IL-1b, or indirectly induced by TNF-α (53). In fact, IL-1 stimulates osteoclast differentiation in bone marrow-derived macrophages by signaling through IL-1/IL-1R1 (54). Furthermore, high-glycemic foods tend to be more refined and processed than low-glycemic foods, which can lead to nutritional deficiencies (55). Similarly, our study found that individuals with a high GL tended to consume more processed meat and refined grains while consuming fewer fruits, vegetables, nuts, fish, whole grains, and dairy products. Hence, consuming a diet predominantly composed of high-glycemic index foods may result in inadequate intake of key nutrients required for optimal bone health, such as calcium, vitamin D, magnesium, and vitamin K. These nutrients play critical roles in bone formation, mineralization, and maintaining bone density (56). In addition, meat and grains typically impart a high dietary acid load, whereas fruits and vegetables provide an alkaline load (57–59). In the presence of a slight drop in the pH of the extracellular fluid, osteoblast activity will be suppressed,

TABLE 3 Odds ratio (OR) and 95% confidence interval (CI) for osteoporosis based on dietary insulin and glycemic scores among participants.

	Tertiles of scores			*P* for trend
	T1 ($n = 4,232$)	T2 ($n = 4,232$)	T3 ($n = 4,232$)	
Glycemic Index				
Mean (SD) score	64.70 (3.36)	69.72 (1.07)	76.18 (4.75)	
Crude model	1.00 (Ref)	2.28 (1.23–3.21)	2.10 (1.14–2.90)	**0.021**
Model 1*	1.00 (Ref)	2.18 (0.95–3.99)	1.74 (0.76–2.95)	**0.019**
Model 2‡	1.00 (Ref)	1.85 (0.37–3.08)	1.72 (0.17–2.81)	**0.043**
Model 3¥	1.00 (Ref)	1.82 (0.66–3.65)	1.78 (1.90–3.52)	**0.001**
Glycemic load				
Mean (SD) score	129.56 (25.14)	202.09 (21.25)	321.72 (39.05)	
Crude model	1.00 (Ref)	1.31 (1.06–1.62)	1.36 (1.10–1.68)	**0.004**
Model 1*	1.00 (Ref)	1.19 (0.96–1.48)	0.95 (0.76–1.19)	0.842
Model 2‡	1.00 (Ref)	1.22 (0.98–1.52)	0.97 (0.78–1.22)	0.998
Model 3¥	1.00 (Ref)	1.37 (1.05–1.79)	1.46 (1.17–2.02)	**0.035**
Insulin index				
Mean (SD) score	48.59 (4.93)	57.19 (1.65)	63.77 (3.69)	
Crude model	1.00 (Ref)	1.06 (0.86–1.31)	1.10 (0.89–1.36)	0.371
Model 1*	1.00 (Ref)	1.00 (0.81–1.25)	1.03 (0.88–1.28)	0.942
Model 2‡	1.00 (Ref)	1.01 (0.81–1.26)	0.98 (0.79–1.11)	0.250
Model 3¥	1.00 (Ref)	1.01 (0.78–1.31)	1.16 (0.93–1.27)	0.642
Insulin load				
Mean (SD) score	281.33 (35.22)	298.45 (26.93)	359.06 (23.25)	
Crude model	1.00 (Ref)	1.17 (0.95–1.44)	1.46 (1.17–1.82)	**0.001**
Model 1*	1.00 (Ref)	1.11 (0.89–1.32)	1.39 (1.05–1.77)	**0.037**
Model 2‡	1.00 (Ref)	1.06 (0.85–1.31)	0.98 (0.78–1.24)	0.512
Model 3¥	1.00 (Ref)	1.22 (0.91–1.59)	1.55 (1.09–2.31)	0.104

Binary logistic regression was used to obtain OR and 95% CI.

*Model 1: adjusted for age; sex; and BMI.

‡Model 2: Model 1 + education; supplement intake of multivitamin-mineral, vitamin A, D, C, B9, calcium, omega 3; physical activity; smoking; Comorbidity; menopausal status, use of drug or hormone therapy, under a special diet.

¥Model 3: Model 2 + intake of energy; protein; fiber; phosphorus, calcium; vitamin D, C, and B9; SFA; MUFA; PUFA.

Bolded numbers indicate a p-value < 0.05.

and matrix protein gene expression and alkaline phosphatase activity will be decreased (60). Furthermore, low-grade metabolic acidosis decreases bone calcium deposits by increasing urinary calcium excretion (61, 62). Finally, hyperglycemia promotes the production of advanced glycation end products (AGEs), which increase the cross-linking of collagens and the fragility of human bones (63).

Intriguingly, our study revealed a lack of significant correlation between DII and DIL and the risk of osteoporosis. This finding is consistent with that of Nouri et al., who found no correlation between DII and DIL and lumbar/femoral BMD in postmenopausal women (24). Previous studies have reported conflicting results regarding the impact of hyperinsulinemia on BMD. Some studies suggest that hyperinsulinemia increases BMD (9, 64, 65), while others observe a decrease (66, 67). In a recent study, a significant relationship between decreased BMD or osteoporosis with increased homeostatic model assessment for insulin resistance (HOMA-IR) was reported (68). By contrast, in a study by Napoli et al. (69), greater insulin resistance (IR) was associated with higher BMD in non-diabetic elderly subjects, suggesting that IR may affect fracture risk possibly through effects on bone quality. In line with this observation, patients with T2DM have an increased fracture risk despite normal or even slightly elevated BMD and frequently show impaired bone microstructure. However, consistent evidence that IR is associated with increased fracture risk after adjusting for BMI, and BMD was not observed in the aforementioned study. The lack of significant association in our study may be due to variations in study populations, methodologies, or other confounding factors that were not taken into account. So, it seems that the role of the dietary insulin response in osteoporosis development requires further investigation. In addition, the low prevalence of postmenopausal women (who have a low risk of osteoporosis) in the study population can also justify the lack of significant results and differences with other studies.

TABLE 4 Linear relationship between *T*-score and dietary insulin and glycemic scores among 12,696 participants.

Scores	Total *T*-score		*P*-Value
	SE	β	
Glycemic index*	0	−0.001	0.055
Glycemic load*	0.02	−0.018	0.39
Insulin index*	0.012	−0.018	0.153
Insulin load*	0.676	0.185	0.787

*Model 1: adjusted for age; sex; and BMI, education; supplement intake of multivitamin-mineral, vitamin A, D, C, B9, calcium, omega 3; physical activity; smoking; Comorbidity; menopausal status, use of drug or hormone therapy, under a special diet, intake of energy; protein; fiber; calcium; vitamin D, C, and B9; SFA; MUFA; PUFA.

This cross-sectional study provides valuable insight into the association between high DGI/DGL and osteoporosis risk. Our study's strengths include its relatively large sample size, which enhances the statistical power and generalizability of the findings to the target population. Additionally, using validated assessment tools for dietary intake and osteoporosis risk factors adds credibility to our data. Including diverse participants from different ethnographic and geographic backgrounds also strengthens the external validity of our findings.

However, we must acknowledge that this study has some limitations. First of all, due to the cross-sectional design, we are unable to establish causality between high DGI/DGL and osteoporosis risk. The temporal sequence of events cannot be determined, and the possibility of reverse causation remains. Longitudinal studies would be valuable in elucidating the cause-and-effect relationship. The second limitation is that dietary assessments in cross-sectional studies are subject to recall bias and rely heavily on self-reported information. Participants' memory, perception, and social desirability bias may influence dietary information accuracy. Despite efforts to minimize these biases through validated questionnaires and recruiting teachers as participants, the potential for measurement error cannot be eliminated entirely. Failure to report weather-related data as one of the influencing factors in the incidence of osteoporosis was one of the limitations of our study. Additionally, as with any observational study, confounding variables can influence the association between high DGI/DGL/DII/DIL and osteoporosis risk. While we adjusted for a number of potential confounders, residual confounding may arise from unmeasured or unknown factors. As a final point, our findings may be confined to the particular population under study.

In conclusion, our study provides evidence of a significant association between high DGI and DGL and increased osteoporosis prevalence. However, due to the limited literature available and the complexity of the underlying mechanisms, further research is needed to validate these findings and elucidate the precise pathways involved. Additionally, the lack of a significant relationship between DII/DIL and osteoporosis prevalence in our study warrants further exploration. By addressing these knowledge gaps, we can better understand the impact of dietary factors on osteoporosis and potentially develop targeted interventions to mitigate the risk.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Shahid Beheshti University of Medical Sciences. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

AH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. DF: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. ME: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft. MS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MAj: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. MAb: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MH: Investigation, Writing – original draft, Conceptualization, Data curation, Formal analysis.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The inception of this longitudinal study took place in 2001 under the guidance of Professor Walter Willett from the Department of Epidemiology at Harvard University in the United States. We express our gratitude to him and all the volunteers involved in the current study.

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

References

1. aaHernlund E, Svedbom A, Ivergard M, Compston J, Cooper C, Stenmark J, et al. Osteoporosis in the European Union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch Osteoporos.* (2013) 8:136. doi: 10.1007/s11657-013-0136-1

2. Kanis JA, Melton LJ 3rd, Christiansen C, Johnston CC, Khaltaev N. The diagnosis of osteoporosis. *J Bone Miner Res.* (1994) 9:1137–41. doi: 10.1002/jbmr.5650090802

3. Salari N, Ghasemi H, Mohammadi L, Behzadi MH, Rabieenia E, Shohaimi S, et al. The global prevalence of osteoporosis in the world: a comprehensive systematic review and meta-analysis. *J Orthop Surg Res.* (2021) 16:609. doi: 10.1186/s13018-021-02772-0

4. Doosti-Irani A GM, Cheraghi Z. The high prevalence of osteoporosis as a preventable disease: the need for greater attention to prevention programs in Iran. *Iran J Public Health.* (2018) 47:1220–1.

5. Pouresmaeili F, Kamalidehghan B, Kamarehei M, Goh YM. A comprehensive overview on osteoporosis and its risk factors. *Ther Clin Risk Manag.* (2018) 14:2029–49. doi: 10.2147/TCRM.S138000

6. Levis S, Lagari VS. The role of diet in osteoporosis prevention and management. *Curr Osteoporos Rep.* (2012) 10:296–302. doi: 10.1007/s11914-012-0119-y

7. Chiavarini M, Naldini G, Fabiani R. The role of diet in osteoporotic fracture healing: a systematic review. *Curr Osteoporos Rep.* (2020) 18:138–47. doi: 10.1007/s11914-020-00573-8

8. Veronese N, Reginster JY. The effects of calorie restriction, intermittent fasting and vegetarian diets on bone health. *Aging Clin Exp Res.* (2019) 31:753–8. doi: 10.1007/s40520-019-01174-x

9. Vestergaard P. Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes—a meta-analysis. *Osteopor Int.* (2007) 18:427–44. doi: 10.1007/s00198-006-0253-4

10. Oei L, Rivadeneira F, Zillikens MC, Oei EH. Diabetes, diabetic complications, and fracture risk. *Curr Osteoporos Rep.* (2015) 13:106–15. doi: 10.1007/s11914-015-0260-5

11. Muka T, Trajanoska K, Kiefte-de Jong JC, Oei L, Uitterlinden AG, Hofman A, et al. The association between metabolic syndrome, bone mineral density, hip bone geometry and fracture risk: The Rotterdam Study. *PLoS ONE.* (2015) 10:e0129116. doi: 10.1371/journal.pone.0129116

12. Napoli N, Strollo R, Paladini A, Briganti SI, Pozzilli P, Epstein S. The alliance of mesenchymal stem cells, bone, and diabetes. *Int J Endocrinol.* (2014) 2014:690783. doi: 10.1155/2014/690783

13. Marino S, Bellido T. PTH receptor signalling, osteocytes and bone disease induced by diabetes mellitus. *Nat Rev Endocrinol.* (2024) 20:661–72. doi: 10.1038/s41574-024-01014-7

14. Hofbauer LC, Busse B, Eastell R, Ferrari S, Frost M, Müller R, et al. Bone fragility in diabetes: novel concepts and clinical implications. *Lancet Diabetes Endocrinol.* (2022) 10:207–20. doi: 10.1016/S2213-8587(21)00347-8

15. Khosla S, Samakkarnthai P, Monroe DG, Farr JN. Update on the pathogenesis and treatment of skeletal fragility in type 2 diabetes mellitus. *Nat Rev Endocrinol.* (2021) 17:685–97. doi: 10.1038/s41574-021-00555-5

16. Mirmiran P, Esfandiari S, Bahadoran Z, Tohidi M, Azizi F. Dietary insulin load and insulin index are associated with the risk of insulin resistance: a prospective approach in tehran lipid and glucose study. *J Diabetes Metab Disord.* (2015) 15:23. doi: 10.1186/s40200-016-0247-5

17. Murakami K, Sasaki S, Takahashi Y, Okubo H, Hosoi Y, Horiguchi H, et al. Dietary glycemic index and load in relation to metabolic risk factors in Japanese female farmers with traditional dietary habits. *Am J Clin Nutr.* (2006) 83:1161–9. doi: 10.1093/ajcn/83.5.1161

18. Neuhouser ML, Schwarz Y, Wang C, Breymeyer K, Coronado G, Wang CY, et al. A low-glycemic load diet reduces serum C-reactive protein and modestly increases adiponectin in overweight and obese adults. *J Nutr.* (2012) 142:369–74. doi: 10.3945/jn.111.149807

19. Kelly KR, Haus JM, Solomon TP, Patrick-Melin AJ, Cook M, Rocco M, et al. A low-glycemic index diet and exercise intervention reduces TNF(alpha) in isolated mononuclear cells of older, obese adults. *J Nutr.* (2011) 141:1089–94. doi: 10.3945/jn.111.139964

20. Juanola-Falgarona M, Salas-Salvado J, Ibarrola-Jurado N, Rabassa-Soler A, Diaz-Lopez A, Guasch-Ferre M, et al. Effect of the glycemic index of the diet on weight loss, modulation of satiety, inflammation, and other metabolic risk factors: a randomized controlled trial. *Am J Clin Nutr.* (2014) 100:27–35. doi: 10.3945/ajcn.113.081216

21. Feskens EJM, Du H. Dietary glycaemic index from an epidemiological point of view. *Int J Obes.* (2006) 30:S66–71. doi: 10.1038/sj.ijo.0803495

22. Bao J, Atkinson F, Petocz P, Willett WC, Brand-Miller JC. Prediction of postprandial glycemia and insulinemia in lean, young, healthy adults: glycemic load compared with carbohydrate content alone. *Am J Clin Nutr.* (2011) 93:984–96. doi: 10.3945/ajcn.110.005033

23. Garcia-Gavilan JF, Bullo M, Camacho-Barcia L, Rosique-Esteban N, Hernandez-Alonso P, Basora J, et al. Higher dietary glycemic index and glycemic load values increase the risk of osteoporotic fracture in the PREvencion con DIeta MEDiterranea (PREDIMED)-Reus trial. *Am J Clin Nutr.* (2018) 107:1035–42. doi: 10.1093/ajcn/nqy043

24. Nouri M, Mahmoodi M, Shateri Z, Ghadiri M, Rajabzadeh-Dehkordi M, Vali M, et al. How do carbohydrate quality indices influence on bone mass density in postmenopausal women? A case-control study. *BMC Womens Health.* (2023) 23:42. doi: 10.1186/s12905-023-02188-4

25. Abdollahi M, Kalantari N, Abdollahi Z, Goodarzy F, Esmaeili M, Houshiar-Rad A. Theoretical and practical aspects of the of Iranian teachers cohort study: a methodology report. *Iran J Nutr Sci Food Technol.* (2021) 15:123–30.

26. Ahmad S, Harris T, Limb E, Kerry S, Victor C, Ekelund U, et al. Evaluation of reliability and validity of the General Practice Physical Activity Questionnaire (GPPAQ) in 60–74 year old primary care patients. *BMC Fam Pract.* (2015) 16:1–9. doi: 10.1186/s12875-015-0324-8

27. Looker AC, Harris T, Wahmer H. Assessing hip fracture risk in a population-based health survey: the NHANES III osteoporosis component. *Aging Clin Exp Res.* (1992) 4:53–60. doi: 10.1007/BF03324066

28. Cohen A, Shane E. Evaluation and management of the premenopausal woman with low BMD. *Curr Osteoporos Rep.* (2013) 11:276–85. doi: 10.1007/s11914-013-0161-4

29. Ferrari S, Bianchi M, Eisman J, Foldes A, Adami S, Wahl D, et al. Osteoporosis in young adults: pathophysiology, diagnosis, and management. *Osteopor Int.* (2012) 23:2735–48. doi: 10.1007/s00198-012-2030-x

30. World Health Organization. *Assessment of Fracture Risk and Its Application to Screening for Postmenopausal Osteoporosis: Report of a WHO Study Group [Meeting Held in Rome From 22 to 25 June 1992].* World Health Organization (1994).

31. Mirmiran P, Esfahani FH, Mehrabi Y, Hedayati M, Azizi F. Reliability and relative validity of an FFQ for nutrients in the Tehran lipid and glucose study. *Public Health Nutr.* (2010) 13:654–62. doi: 10.1017/S1368980009991698

32. Ghaffarpour M, Houshiar-Rad A, Kianfar H. The manual for household measures, cooking yields factors and edible portion of foods. *Tehran.* (1999) 7:42–58.

33. Bao J, De Jong V, Atkinson F, Petocz P, Brand-Miller JC. Food insulin index: physiologic basis for predicting insulin demand evoked by composite meals. *Am J Clin Nutr.* (2009) 90:986–92. doi: 10.3945/ajcn.2009.27720

34. Wolever TM, Yang M, Zeng XY, Atkinson F, Brand-Miller JC. Food glycemic index, as given in glycemic index tables, is a significant determinant of glycemic responses elicited by composite breakfast meals. *Am J Clin Nutr.* (2006) 83:1306–12. doi: 10.1093/ajcn/83.6.1306

35. Foster-Powell K. International tables of glycemic index and glycemic load values. *Diabetes Care.* (2008) 31:2281–3. doi: 10.2337/dc08-1239

36. Taleban F, Esmaeili M. *Glycemic Index of Iranian Foods.* National Nutrition and Food Technology Research Institute publication (1999).

37. Huang R, Wang H, Shen Z, Cai T, Zhou Y, Wang Y, et al. Increased glycemic variability evaluated by continuous glucose monitoring is associated with osteoporosis in type 2 diabetic patients. *Front Endocrinol.* (2022) 13:861131. doi: 10.3389/fendo.2022.861131

38. Levitan EB, Cook NR, Stampfer MJ, Ridker PM, Rexrode KM, Buring JE, et al. Dietary glycemic index, dietary glycemic load, blood lipids, and C-reactive protein. *Metabolism.* (2008) 57:437–43. doi: 10.1016/j.metabol.2007.11.002

39. Ricker MA, Haas WC. Anti-inflammatory diet in clinical practice: a review. *Nutr Clin Pract.* (2017) 32:318–25. doi: 10.1177/0884533617700353

40. Kawahito S, Kitahata H, Oshita S. Problems associated with glucose toxicity: role of hyperglycemia-induced oxidative stress. *World J Gastroenterol.* (2009) 15:4137–42. doi: 10.3748/wjg.15.4137

41. Ginaldi L, Di Benedetto MC, De Martinis M. Osteoporosis, inflammation and ageing. *Immun Ageing.* (2005) 2:14. doi: 10.1186/1742-4933-2-14

42. Amarasekara DS, Kim S, Rho J. Regulation of osteoblast differentiation by cytokine networks. *Int J Mol Sci.* (2021) 22:62851. doi: 10.3390/ijms22062851

43. Ferrari SL, Ahn-Luong L, Garnero P, Humphries SE, Greenspan SL. Two promoter polymorphisms regulating interleukin-6 gene expression are associated with circulating levels of C-reactive protein and markers of bone resorption in postmenopausal women. *J Clin Endocrinol Metab.* (2003) 88:255–9. doi: 10.1210/jc.2002-020092

44. Zha L, He L, Liang Y, Qin H, Yu B, Chang L, et al. TNF-α contributes to postmenopausal osteoporosis by synergistically promoting RANKL-induced osteoclast formation. *Biomed Pharmacother.* (2018) 102:369–74. doi: 10.1016/j.biopha.2018.03.080

45. Xu J, Yu L, Liu F, Wan L, Deng Z. The effect of cytokines on osteoblasts and osteoclasts in bone remodeling in osteoporosis: a review. *Front Immunol.* (2023) 14:1222129. doi: 10.3389/fimmu.2023.1222129

46. O'Brien CA, Gubrij I, Lin SC, Saylors RL, Manolagas SC. STAT3 activation in stromal/osteoblastic cells is required for induction of the receptor activator of NF-kappaB ligand and stimulation of osteoclastogenesis by gp130-utilizing cytokines or interleukin-1 but not 1,25-dihydroxyvitamin D3 or parathyroid hormone. *J Biol Chem.* (1999) 274:19301–8. doi: 10.1074/jbc.274.27.19301

47. Yoshitake F, Itoh S, Narita H, Ishihara K, Ebisu S. Interleukin-6 directly inhibits osteoclast differentiation by suppressing receptor activator of NF-kappaB signaling pathways. *J Biol Chem.* (2008) 283:11535–40. doi: 10.1074/jbc.M607999200

48. Kudo O, Sabokbar A, Pocock A, Itonaga I, Fujikawa Y, Athanasou NA. Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. *Bone.* (2003) 32:1–7. doi: 10.1016/S8756-3282(02)00915-8

49. Axmann R, Bohm C, Kronke G, Zwerina J, Smolen J, Schett G. Inhibition of interleukin-6 receptor directly blocks osteoclast formation *in vitro* and *in vivo*. *Arthritis Rheum.* (2009) 60:2747–56. doi: 10.1002/art.24781

50. Yamashita T, Yao Z, Li F, Zhang Q, Badell IR, Schwarz EM, et al. NF-kappaB p50 and p52 regulate receptor activator of NF-kappaB ligand (RANKL) and tumor necrosis factor-induced osteoclast precursor differentiation by activating c-Fos and NFATc1. *J Biol Chem.* (2007) 282:18245–53. doi: 10.1074/jbc.M610701200

51. Gilbert L, He X, Farmer P, Rubin J, Drissi H, van Wijnen AJ, et al. Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/Pebp2alpha A) is inhibited by tumor necrosis factor-alpha. *J Biol Chem.* (2002) 277:2695–701. doi: 10.1074/jbc.M106339200

52. Gilbert L, He X, Farmer P, Boden S, Kozlowski M, Rubin J, et al. Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. *Endocrinology.* (2000) 141:3956–64. doi: 10.1210/endo.141.11.7739

53. Wei S, Kitaura H, Zhou P, Ross FP, Teitelbaum SL. IL-1 mediates TNF-induced osteoclastogenesis. *J Clin Invest.* (2005) 115:282–90. doi: 10.1172/JCI200523394

54. Kim JH, Jin HM, Kim K, Song I, Youn BU, Matsuo K, et al. The mechanism of osteoclast differentiation induced by IL-1. *J Immunol.* (2009) 183:1862–70. doi: 10.4049/jimmunol.0803007

55. Atkinson FS, Brand-Miller JC, Foster-Powell K, Buyken AE, Goletzke J. International tables of glycemic index and glycemic load values 2021: a systematic review. *Am J Clin Nutr.* (2021) 114:1625–32. doi: 10.1093/ajcn/nqab233

56. Price CT, Langford JR, Liporace FA. Essential nutrients for bone health and a review of their availability in the average North American Diet. *Open Orthop J.* (2012) 6:143–9. doi: 10.2174/1874325001206010143

57. Gonick HC, Goldberg G, Mulcare D. Reexamination of the acid-ash content of several diets12. *Am J Clin Nutr.* (1968) 21:898–903. doi: 10.1093/ajcn/21.9.898

58. Gannon RH, Millward DJ, Brown JE, Macdonald HM, Lovell DP, Frassetto LA, et al. Estimates of daily net endogenous acid production in the elderly UK population: analysis of the National Diet and Nutrition Survey (NDNS) of British adults aged 65 years and over. *Br J Nutr.* (2008) 100:615–23. doi: 10.1017/S0007114508901240

59. Remer T, Manz F. Potential renal acid load of foods and its influence on urine pH. *J Am Diet Assoc.* (1995) 95:791–7. doi: 10.1016/S0002-8223(95)00219-7

60. Carnauba RA, Baptistella AB, Paschoal V, Hübscher GH. Diet-induced low-grade metabolic acidosis and clinical outcomes: a review. *Nutrients.* (2017) 9:538. doi: 10.3390/nu9060538

61. Frick KK, Bushinsky DA. Effect of metabolic and respiratory acidosis on intracellular calcium in osteoblasts. *Am J Physiol Renal Physiol.* (2010) 299:F418–25. doi: 10.1152/ajprenal.00136.2010

62. Bushinsky DA, Smith SB, Gavrilov KL, Gavrilov LF Li J, Levi-Setti R. Chronic acidosis-induced alteration in bone bicarbonate and phosphate. *Am J Physiol Renal Physiol.* (2003) 285:F532–9. doi: 10.1152/ajprenal.00128.2003

63. Saito M, Marumo K. Collagen cross-links as a determinant of bone quality: a possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. *Osteopor Int.* (2010) 21:195–214. doi: 10.1007/s00198-009-1066-z

64. Yan W, Li X. Impact of diabetes and its treatments on skeletal diseases. *Front Med.* (2013) 7:81–90. doi: 10.1007/s11684-013-0243-9

65. Ma L, Oei L, Jiang L, Estrada K, Chen H, Wang Z, et al. Association between bone mineral density and type 2 diabetes mellitus: a meta-analysis of observational studies. *Eur J Epidemiol.* (2012) 27:319–32. doi: 10.1007/s10654-012-9674-x

66. Wallander M, Axelsson KF, Nilsson AG, Lundh D, Lorentzon M. Type 2 diabetes and risk of hip fractures and non-skeletal fall injuries in the elderly: a study from the fractures and fall injuries in the elderly cohort (FRAILCO). *J Bone Miner Res.* (2017) 32:449–60. doi: 10.1002/jbmr.3002

67. Srikanthan P, Crandall CJ, Miller-Martinez D, Seeman TE, Greendale GA, Binkley N, et al. Insulin resistance and bone strength: findings from the study of midlife in the United States. *J Bone Miner Res.* (2014) 29:796–803. doi: 10.1002/jbmr.2083

68. Fu YH, Liu WJ, Lee CL, Wang JS. Associations of insulin resistance and insulin secretion with bone mineral density and osteoporosis in a general population. *Front Endocrinol.* (2022) 13:971960. doi: 10.3389/fendo.2022.971960

69. Napoli N, Conte C, Pedone C, Strotmeyer ES, Barbour KE, Black DM, et al. Effect of insulin resistance on BMD and fracture risk in older adults. *J Clin Endocrinol Metab.* (2019) 104:3303–10. doi: 10.1210/jc.2018-02539

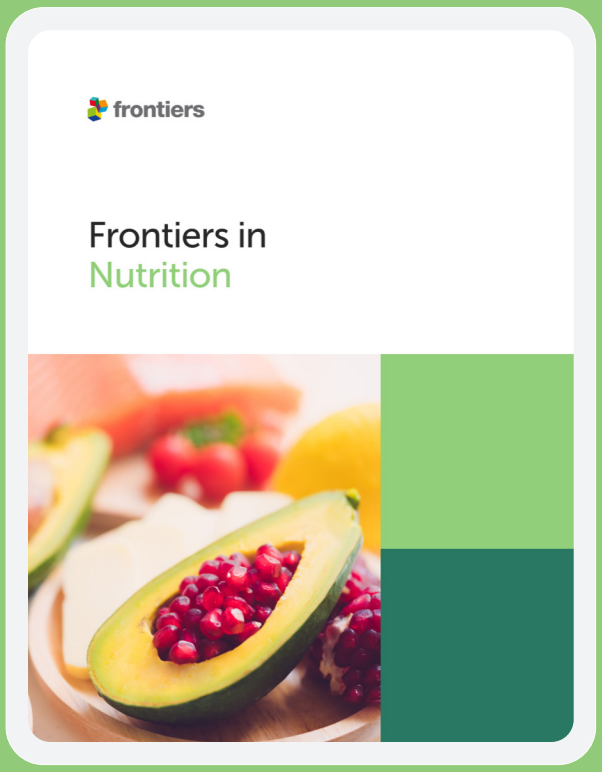
Frontiers in Nutrition

Explores what and how we eat in the context of health, sustainability and 21st century food science

A multidisciplinary journal that integrates research on dietary behavior, agronomy and 21st century food science with a focus on human health.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact