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ÉLIÁS ANNA JÚLIA

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című program

Programvezető: Dr. Földvári-Nagy Lászlóné Dr. Lenti Katalin, főiskolai tanár

Témavezetők: Dr. Földvári-Nagy Lászlóné Dr. Lenti Katalin, főiskolai tanár
Dr. Földvári-Nagy László, főiskolai tanár

PROBIOTIC SUPPLEMENTATION: A UNIVERSAL SOLUTION OR A CASE OF LIMITED EFFICACY?

PhD thesis

Anna Júlia Éliás

Semmelweis University Doctoral School

Health Sciences Division

Supervisors: Katalin Lenti Ph.D.

László Földvári-Nagy Ph.D.

Official reviewers: Mária Szekeres Ph.D.

Gábor Nagy-Grócz Ph.D. habil.

Head of the Complex Examination Committee: András Arató Ph.D.

Members of the Complex Examination Committee:

Gergely Agócs Ph.D.
Peter Banovcin Ph.D.
Krisztina Hagymási Ph.D. med.habil.
Vasile Drug Ph.D.

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List of abbreviations

16S rRNA: 16S ribosomal ribonucleic acid

ACE: Abundance-based coverage estimator

AGA: American Gastroenterological Association

ASV: Amplicon Sequence Variant

B:F ratio: *Bacteroidetes:Firmicutes* ratio

CFU: Colony forming unit

bid: twice a day

CI: Confidence interval

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbent assay

EU: European Union

GRADE: The Grading of Recommendations, Assessment, Development and Evaluation

IBD: Inflammatory bowel disease

ITT: Intention-To-Treat

MD: Mean difference

MedD: Median Differences

MD: Mean difference

mITT: Modified Intention-To-Treat

NA: Not Applicable

NI: No Information

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

PCR-DGGE: Polymerase chain reaction denaturing gradient gel electrophoresis

PP: Per-Protocol

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PROSPERO: International prospective register of systematic reviews

RCT: Randomized controlled trial

RoB: Risk of bias

SMD: Standardized mean difference

SD: Standard Deviation

tid: three times a day

TRFLP: Terminal restriction fragment length polymorphism

UK: United Kingdom

UniFrac: Unique fraction metric

USA: United States of America

Q1: First Quartile

Q3: Third Quartile

qid: four times a day

qPCR: Quantitative real-time polymerase chain reaction

WGO: World Gastroenterology Organisation

I. Introduction

1.1. Probiotics: definition, mechanisms, and applications

Probiotic products contain "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (1). Probiotics generally consist of microorganisms present in the native gut flora, encompassing bacteria, yeasts, or a combination thereof (2). Historically, species from the *Lactobacillus* and *Bifidobacterium* genera have dominated the probiotic landscape due to their safety profiles and believed health benefits (2). However, reclassifying the *Lactobacillus* genus in 2020 into 23 new genera has highlighted the genetic and functional diversity within these microbes and the need for strain-specific research (2,3). Additionally, promising probiotics from other genera, such as *Bacillus*, have gained attention due to their unique properties, such as spore formation, which improves survivability in the gastrointestinal tract (2).

Probiotic supplements are often associated with general health in society. The estimated value of the worldwide probiotics market was USD 77.12 billion in 2022, with an anticipated compound annual growth rate of 14.0% projected between 2023 and 2030 (4). While previously mostly functional fermented milk products such as yogurt were in focus, the market is now diversifying into probiotic food, drinks, and supplements (4). It is crucial to note that these products are also partly offered to healthy people as a general preventative method. The leading cause is increased public knowledge of the alleged health advantages of probiotics, including enhanced digestive system performance and gut health (4).

Despite their widespread popularity, probiotics remain a subject of scientific debate regarding their universal efficacy. While some strains have been extensively researched and have shown some clinical benefits, particularly in conditions such as antibiotic-associated diarrhea (5,6), the level of evidence and the generalizability of these effects to broader populations remain uncertain (2). The growing probiotics market reflects increasing consumer interest in these products as a means of improving gut health and overall well-being (4). However, many commercial probiotic products are marketed with broad health claims, often without robust clinical evidence supporting their efficacy in different conditions, especially in healthy individuals.

The mechanisms by which probiotics exert their effects are complex and vary between strains. These microorganisms contribute to gut homeostasis through several pathways,

including modulation of the immune system, competition with pathogenic bacteria, production of metabolic byproducts such as short-chain fatty acids, and interaction with host cells via chemical signaling (7). While these mechanisms suggest potential health benefits, their effectiveness highly depends on the specific strain, dosage, and host factors, including individual microbiome composition and disease condition (8).

Recent advances in microbiota research have led to investigating novel probiotic strains and microbial combinations, challenging the traditional view of probiotics as universally beneficial (9). The emerging concept of personalized probiotics, which considers individual microbiome profiles and host-specific factors, further highlights the limitations of a one-size-fits-all approach (10). Additionally, alternative strategies, such as next-generation probiotics, synbiotics, and microbiome-targeted interventions, are gaining traction as potential solutions to the limitations of conventional probiotic supplementation (11).

Addressing the questions around the applicability of probiotics requires a critical evaluation of the current evidence, including strain-specific effects, inter-individual variability, and the evolving landscape of microbiome research. While probiotics hold promise, their role in health promotion and disease prevention must be examined with a deeper understanding of their mechanisms, limitations, and real-world applicability.

1.2. The gut microbiota: composition, function, and stability

The human gut microbiota comprises a diverse and dynamic community of microorganisms, including bacteria, archaea, viruses, and fungi, that reside primarily in the gastrointestinal tract. These microbial populations are dominated by the phyla *Firmicutes* and *Bacteroidetes*, with bacteria from *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* also present in smaller proportions (12,13). The composition of the gut microbiota is influenced by various factors, including host genetics, diet, age, and environmental exposures (14–16).

The gut microbiota plays a fundamental role in human health by contributing to essential physiological processes (17). These include the fermentation of indigestible dietary components, production of short-chain fatty acids (SCFAs), synthesis of vitamins, and modulation of the immune system (18,19). Furthermore, the microbiota interacts with the gut epithelium to maintain intestinal homeostasis and protect against pathogenic

colonization (20,21). Dysbiosis, or an imbalance in microbial composition, has been associated with various metabolic, inflammatory, and neurological disorders (17,22–25). The stability of the gut microbiota is crucial for maintaining health, yet it exhibits both resilience and adaptability in response to internal and external perturbations. While short-term dietary changes can lead to transient shifts in microbial composition, long-term alterations in diet and lifestyle might induce more persistent modifications (26). Antibiotic exposure, infections, and stress can also disrupt microbiota stability, potentially leading to long-term health consequences (27,28). Despite these variations, the gut microbiota demonstrates a remarkable capacity for resilience, often returning to a stable state following disturbances, although the extent and speed of recovery may vary among individuals (17,29).

1.2.1. Methods for investigating gut microbiome

There are several existing approaches to investigating the composition of the gut microbiome. Traditionally, classical culture-based methods were regarded as the gold standard (30,31). While initially these methods dominated the field, it is now estimated that over 70% of human gut microbial species remain uncultured (32,33). Therefore, the limitations of culture-dependent techniques are now well recognized. These include the inability to cultivate many gut microbes on artificial media, the risk of misidentification, limited selectivity of culture media, and interspecies competition for nutrients, all of which may affect the growth and detection of certain strains (30,31).

The introduction of molecular techniques opened a new door for researchers to characterize intestinal microbiota in a culture-independent manner through rRNA gene detection (32,33). Methods such as fluorescent in situ hybridization, denaturing and temperature gradient gel electrophoresis, and 16S rRNA gene clone libraries were successfully applied to study microbial ecology (32,33). Among these, PCR-based techniques using specific primers have provided the most sensitive, rapid, and accessible approach. More recently, real-time quantitative PCR has enabled the specific detection and quantification of selected bacteria from fecal DNA, further enhancing analytical precision (32,33).

Another huge breakthrough was the development of next-generation, high-throughput sequencing techniques. The most used method nowadays is 16S rRNA gene sequencing, which targets hypervariable regions of the 16S gene (34). It offers a cost-effective and

computationally efficient way to characterize microbial communities (34). It allows reliable taxonomic profiling even with relatively low sequencing depth (as low as 18,000–20,000 reads per sample) (35). Outputs are represented as Operational Taxonomic Units (OTUs) or Amplicon Sequence Variants (ASVs), which support analyses of community diversity, richness, and evenness (35). However, this technique is not without limitations: the selection of variable regions (commonly V3–V4) may introduce taxonomic bias, and discrepancies in rRNA gene copy numbers and within-genome variation usually prevent species-level resolution and allow the detection at the genus level (34). Given that species within the same genus can exhibit distinct associations with either health or disease, accurate taxonomic classification at the species level is critical. Computational studies have demonstrated that longer read lengths in 16S rRNA gene sequencing enhance the accuracy of taxonomic annotation. The combination of full-length 16S rRNA gene sequencing with high-throughput output has become feasible with the advent of third-generation sequencing technologies (36). Although these platforms initially exhibited high error rates, recent improvements in sequencing chemistry and the application of deep learning-based error correction protocols have markedly increased data quality (36). Comparative analysis of full-length and short-read 16S rRNA gene amplicon sequencing in the context of human gut microbiota showed high concordance at the genus level between the two methods (37). However, full-length sequencing provided improved resolution at the species level. This allowed for more accurate discrimination between closely related taxa, thereby offering a more detailed and representative profile of microbial community composition (37).

Another technique used is shotgun metagenomic sequencing, which provides the most comprehensive view. Rather than targeting specific regions, this method sequences all genomic content in a sample, enabling species- and strain-level discrimination (34). Furthermore, it captures a broader range of microorganisms, including viruses, fungi, protozoa, archaea, and bacteria (34). By randomly fragmenting and sequencing long DNA molecules, this method delivers not only taxonomic composition but also gene-level insights assessing the functional contributions of individual community members (35). These functional datasets provide valuable information that is not obtainable through 16S rRNA gene sequencing alone (35). Despite these advantages, shotgun sequencing has traditionally been limited by higher costs, the presence of host DNA contamination, and

the need for more complex bioinformatic analyses (34). Both 16S and shotgun metagenomics are widely applied, but comparing outputs across these platforms remains difficult. Key obstacles include differing taxonomic resolutions, biases inherent to sequencing and amplification, and the use of distinct reference databases that might differ in size, update frequency, content, and curation protocols (34).

1.2.2. Diversity indices

Diversity refers to the variety of life forms present in a biological system (38). In the case of the gut microbiome, diversity includes richness (the number of unique taxonomic units) and evenness (the distribution of species to each other) (39–41). A healthy gut microbiome is characterized by high richness and evenness, with a relatively balanced proportion of various bacterial species (42,43). This diversity is essential for maintaining a stable and functional gut ecosystem (17,44). Microbial diversity is a descriptive feature and a fundamental determinant of ecosystem stability and functional resilience (45). Diverse communities can perform a wide array of essential tasks, including the breakdown of complex nutrients, the synthesis of bioactive metabolites, and regulating host immune responses (16). High diversity enhances the system's ability to resist external perturbations such as dietary shifts, infections, or antibiotic exposure, and facilitates faster recovery following disturbances (45). Moreover, greater diversity reduces the risk that any species or functional group might dominate and disrupt ecological equilibrium (45). Preserving and promoting microbial diversity is thus a central goal in maintaining a healthy and robust gut ecosystem. It is important to note that several methods exist to measure the diversity of the gut microbiome. Standard measures include alpha diversity, focusing on species richness and evenness within a sample. Each alpha diversity index is calculated differently, depending on factors like how the presence or absence of particular rare species is assessed and interpreted (46). Beta diversity, on the other hand, looks at compositional differences between microbial communities (39). The metric used may affect the interpretation of the study results. α- and β-diversity indices reported in this thesis are described in **Table 1.**

Table 1. Definitions of gut microbiome diversity indices reported in the thesis

α-diversity indices	Definition	Reference
Shannon diversity index	The Shannon diversity index shows how diverse the species in a given community are. It rises with the number of species and the	(46–49)

	evenness of their abundance. The higher the index is, the more diverse the species are in the habitat. If the index equals 0, only one species is present in the community. The index has no upper limit. According to the current data, there are no clearly defined reference values for the "ideal" Shannon diversity of the microbiome. For the definition of low diversity, the cut-off points in the literature range from 2.0 to 4.0.	
Observed OTUs	An OTU table contains the number of sequences that are observed for each operational taxonomic unit (OTUs) in each sample. An OTU can be defined as a collection of 16S rRNA sequences that have a certain percentage of sequence divergence. Columns usually represent samples, and rows represent genera or species-specific taxonomic units (OTUs).	(46,50,51)
Chao1 index	Chao1 is a nonparametric method for estimating the number of species in a community. The Chao richness estimator is based on the concept that rare species infer the most information about the number of missing species.	(46,52,53)
Simpson index	Simpson's index is a weighted arithmetic mean of proportional abundance, used to measure the probability that two individuals randomly chosen from a sample belong to the same species. The index reflects both the richness (number of species) and evenness (distribution of individuals among species) within a sample. The value of the index (D) ranges between 0 (infinite diversity) and 1 (no diversity). To emphasize diversity, the index is often transformed into Gini-Simpson index, the Simpson's index of Diversity (1-D) which ranges from 0 (maximum homogeneity) to 1 (maximum diversity).	(54–57)
PD whole tree / Faith Phylogenetic diversity	A quantitative measure of phylogenetic diversity, "PD", has been defined as the minimum total length of all the phylogenetic branches required to span a given set of taxa on the phylogenetic tree.	(46,54,58)
Strong's dominance index	Strong's dominance index measures the maximum departure between the observed proportions and a perfectly even community.	(46,59)
Pielou's evenness	Pielou's evenness is an index that measures diversity along with species richness. While species richness is the number of different species in a given area, evenness is the count of individuals of each species in an area. A calculated value of Pielou's evenness ranges from 0 (no evenness) to 1 (complete evenness).	(46,54,60)
ACE (Abundance-based coverage estimator) of species richness index	The ACE is a nonparametric method for estimating the number of species using sample coverage, which is defined as the sum of the	(46,54,61,62)

	probabilities of the observed species. By the ACE method the groups can be categorized as abundant and rare groups according to the observed frequencies.	
Inverse Simpson index	This is the inverse of Simpson dominance and is often used to measure species diversity. It gives an estimate of the effective number of equally abundant species that would result in the same level of dominance. A higher Simpson reciprocal dominance value signifies higher species diversity or richness, with a more even distribution of individuals among species.	(54,57,63)
Shannon effective count	The number of equally-common species required to give a particular value of an index is called the "effective number of species". It provides an intuitive interpretation of diversity as the equivalent number of equally abundant species in a community.	(56,63,64)
β-diversity indices	**Definition**	**Reference**
Bray-Curtis (dis)similarity index	Bray-Curtis dissimilarity measures the compositional dissimilarity between the microbial communities of two samples. Its value ranges between 0 and 1, where 0 means that the two sites have the same composition (i.e., they share all the species), and 1 means that the two sites do not share any species.	(46,65,66)
Euclidean distance	When two samples are compared, Euclidean distance puts more weight on differences in species abundances than on difference in species presences. As a result, two samples not sharing any species could appear more similar (with lower Euclidean distance) than two samples which share species, but the species largely differ in their abundances	(46,67)
(un)weighted UniFrac distance	Both weighted (quantitative) and unweighted (qualitative) variants of UniFrac are widely used in microbial ecology. Unweighted UniFrac measures the distance between communities based on the lineages they contain. This approach is more powerful than nonphylogenetic distance measures because it exploits the different degrees of similarity between sequences. Weighted UniFrac is a newer variant of the original unweighted UniFrac measure that weights the branches of a phylogenetic tree based on the abundance of information. It serves as a quantitative measure of β diversity that can detect changes in how many sequences from each lineage are present, as well as detect changes in which taxa are present. This ability is important because the relative abundance of different kinds of bacteria can be critical for describing community changes. In contrast, the original, unweighted UniFrac is a qualitative β diversity	(46,68,69)

	measure because duplicate sequences contribute no additional branch length to the tree.	
Jensen-Shannon divergence	The Jensen-Shannon divergence is an asymmetric measure that quantifies the relative entropy or informational difference between two distributions. It provides a way to evaluate the distance between two data distributions, highlighting how distinct they are from one another.	(70)
Horn-Morisita distance metrics	The Horn-Morisita index evaluates the probability that individuals drawn from two separate vectors belong to different species, relative to drawing from each vector independently. It is applicable to both transformed counts and proportions.	(71)
Spearman correlation distance	The Spearman distance is based on the Spearman rank correlation coefficient, which evaluates the monotonic relationship between two variables. The Spearman distance is calculated as one minus the absolute value of the Spearman correlation coefficient, offering a measure of dissimilarity between ranked data. It is particularly useful when the data exhibit a monotonic association rather than a linear one.	(72)
Canberra distance	Canberra distance calculates a sum of relative differences where the species-specific absolute values of difference are relativized by the sum of the numbers being compared, i.e., for each species-wise comparison the values are bounded [0, 1]. Because the absolute difference is relativized by the sum of the respective values, the value of a given arithmetic difference exhibits an extremely concave distribution declining from one and asymptotic to zero as values increase.	(73)
Jaccard similarity coefficient	The Jaccard similarity index (sometimes called the Jaccard similarity coefficient) compares members of two sets to see which members are shared and which are distinct. It is a measure of similarity for the two sets of data, with a range from 0% to 100%. The higher the percentage, the more similar the two populations are.	(74)

Source: Éliás et al. (46)

1.2.3. Disruption of the gut microbiota: antibiotic-induced dysbiosis

Antibiotic treatment affects the gut bacterial microbiota quantitatively and qualitatively, causing a decrease or even extinction of certain species, leading to a low-diversity microbiome, and allowing some potentially harmful bacteria to become dominant, e.g., *Clostridium perfringens*, *Staphylococcus aureus*, or *Clostridioides difficile* (46,75,76). This microbial imbalance is called dysbiosis. The deviation from the normal

microbiome has been linked to obesity, malnutrition, inflammatory bowel disease (IBD), neurological dysfunctions, and cancer (17,46). The gut microbiota can spontaneously recover, but it is influenced by various host and external factors like age, health status, the geographical area of origin of patients, dose, duration, and the spectrum of antibiotic treatment (29,46,77,78). Young, healthy adults have stable microbial community functions (46,79), but repeated perturbation of the ecosystem is particularly detrimental if there is insufficient time for recovery after the initial impairment. Previous research has shown that gut microbiota recovers within about 2 weeks after a single antibiotic exposure in adults, but repeated exposures can significantly prolong the recovery time (27,29,46,80,81). Probiotics are commonly used to prevent dysbiosis; however, the effects of concurrent supplementation on fecal microbiota diversity and taxonomical composition during antibiotic therapy are not fully understood. The effects of these products on clinical outcomes during antibiotic therapy have been intensely researched; however, most research did not focus on investigating the composition of the gut microbiome (46). This aspect is also missing from the current guidelines on the use of probiotics of the American Gastroenterological Association (AGA) (82) and World Gastroenterology Organization (WGO) (2,46) .

1.3. Zonulin and intestinal barrier integrity

In recent years, increasing attention has been given to intestinal barrier integrity and permeability, which play a crucial role in gastrointestinal function. Intestinal permeability is regulated by the intestinal barrier, particularly by tight junctions, specialized cell junctions that tightly connect the endothelial cells of the intestinal wall, preventing the passage of undesirable substances into the bloodstream (20,83). In addition to serving as a protective barrier, the intestinal epithelium is responsible for absorption, ensuring a controlled and large-volume transport of nutrients from the intestinal lumen into the body. Besides the transcellular transport occurring through epithelial cells, paracellular transport, regulated primarily by tight junctions, is also significant, allowing the passage of water-soluble molecules and ions (83,84).

A key protein involved in both functions is zonulin, which is produced by intestinal epithelial cells. Zonulin modulates tight junction permeability by loosening intercellular connections, thereby increasing the permeability of the intestinal wall. However,

excessive loosening of these junctions can lead to the uncontrolled passage of larger molecules, such as proteins or bacteria, into the bloodstream, contributing to a condition known as leaky gut syndrome. This phenomenon can negatively affect overall health and increase the risk of disease development (83,85).

Several studies and clinical trials have suggested the potential therapeutic role of probiotics in modulating intestinal permeability. However, current research findings remain inconclusive, as some studies report minimal or no beneficial effects of probiotic supplementation on intestinal permeability (83,86). Given these discrepancies, further investigation is required to elucidate the exact mechanisms by which probiotics influence intestinal permeability (83).

1.4. The role of systematic reviews and meta-analyses in health sciences

Systematic reviews employ rigorous scientific methods to minimize bias when evaluating existing literature. Their key components include formulating a well-defined research question, conducting a comprehensive literature search to identify all relevant studies, systematically compiling studies that address the question, critically assessing the methodological quality of the included research, extracting and analyzing data – both statistically and non-statistically – and evaluating the applicability of the synthesized evidence (87). Meta-analysis is the statistical approach that combines data from multiple independent studies to generate a comprehensive and quantitative assessment of a predefined research question (88). This methodology is widely used in various scientific fields, particularly medicine, nutrition, psychology, and epidemiology, where evidence from individual studies may be inconsistent or inconclusive (89). Meta-analysis offers several advantages, including increased statistical power, improved precision of effect estimates, and the ability to identify patterns across studies. By synthesizing evidence, it helps resolve discrepancies among individual studies and informs clinical and policy decision-making (88,89). However, it is not without limitations. The validity of a meta-analysis depends on the quality of the included studies, and its results can be influenced by methodological heterogeneity, selective reporting, and potential biases. Furthermore, while meta-analysis can establish strong associations, it cannot fully eliminate confounding factors or prove causality (87–89).

Despite its challenges, meta-analysis remains a cornerstone of evidence-based research,

providing a structured and quantitative approach to summarizing scientific knowledge. When conducted rigorously, it serves as a powerful tool for drawing meaningful conclusions and guiding future research directions.

II. Objectives

Study I. To evaluate the effect of probiotic supplementation on gut microbiome diversity and composition during antibiotic treatment by systematically reviewing and synthesizing available evidence based on randomized controlled human trials.

Study II. To assess the impact of probiotic supplementation in healthy populations by systematically reviewing and synthesizing available evidence based on randomized controlled human trials. In this thesis, I intend to report results on the following specific outcomes:

a) gut microbiome diversity
b) zonulin levels

2.1. Hypotheses

It is hypothesized that probiotic supplementation does not have a statistically significant or clinically relevant overall effect on gut microbiome diversity, or zonulin levels, both during antibiotic treatment and in healthy populations. This assumption is based on the variability in individual microbiome responses, strain-specific probiotic effects, and inconsistencies in current literature.

III. Methods

All investigations were designed following Cochrane recommendations (88). The findings follow the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 Statement (90). Study protocols were pre-registered with the International Prospective Register of Systematic Reviews (PROSPERO) under the following registration numbers:

1. The effect of probiotic supplementation on the gut microbiome during antibiotic treatment: CRD42021282983 (46)
2. The effect of probiotic supplementation in healthy populations: CRD42022286137 (83)

3.1. Search strategies and selection criteria

We formulated our clinical questions and defined the eligibility criteria using the PICO-S framework, encompassing Population, Intervention, Comparison, Outcome, and Study Design. The included studies met the following criteria:

1. *Population (P)* — people treated with antibiotics regardless of indication; *Intervention (I)* — probiotic supplementation along with antibiotic treatment; *Comparison (C)* — no probiotic supplementation (placebo or no intervention); *Outcome (O)* — gut microbial diversity (any reported diversity indices) and composition at the end of the intervention (and after a follow-up period) (46).
2. *Population (P)* — healthy individuals as specified in the articles;
   Intervention (I) — probiotic supplementation;
   Comparison (C) — no probiotic supplementation (placebo or no intervention);
   Outcome (O) — the primary outcome was gut microbial diversity (any reported diversity indices) at the end of the intervention (and after a follow-up period). Additionally, we aimed to assess any other outcomes as reported in the identified studies. In this thesis, I report results on zonulin levels (83).

We applied no restrictions on sex, age, or ethnicity. Only randomized controlled trials (RCTs) were included (46,83).

A systematic search was conducted across three medical databases – MEDLINE (via PubMed), Embase (via embase.com), and the Cochrane Central Register of Controlled Trials (CENTRAL) – without applying filters or restrictions (46,83).

Study I.

The search was completed on 15/10/2021 using the following search key: (probiotic OR probiotic* OR bifidobac* OR lactobac* OR escherichia OR streptococcus OR saccharomyces OR bacillus OR pediococc* OR leuconostoc OR enterococc* OR lactococc*) and ((microbio* or microbiome or flora or microflora) and (diversity or composition or abundance or alteration or restoration or reconstitution or recovery or correction or correct* OR disrupt*)) OR (OTU or OTUs OR "operational taxonomic unit*" OR dysbiosis OR dysbacteriosis OR "16S rRNA" OR "rRNA, 16S" OR "16S Ribosomal RNA" OR "RNA, 16S Ribosomal" OR "Ribosomal RNA, 16S" OR "16S rDNA" OR "rDNA, 16S" OR "16S Ribosomal DNA" OR "DNA, 16S Ribosomal" OR "Ribosomal DNA, 16S") and random* (46).

Study II.

The search was completed on 12/04/2024 using the following search key: (normal OR general OR healthy) and (population OR participant OR participants OR volunteer OR volunteers OR subject OR subjects OR adult OR adults OR adolescent OR adolescents OR child OR children OR infant OR infants OR newborn OR newborns OR birth cohort OR pediatric* OR elderly OR elders) AND (probiotic OR probiotic* OR bifidobac* OR lactobac* OR escherichia OR streptococcus OR saccharomyces OR bacillus OR pediococc* OR leuconostoc) AND random* (83).

In both cases, if published protocols for eligible studies were not identified, additional searches were conducted on the EU Clinical Trials Register (91) and ClinicalTrials.gov (92).

Study selection was facilitated using Rayyan, a web-based tool for systematic reviews, alongside EndNote X9 (Clarivate Analytics, Philadelphia, PA, USA) for reference management (93). Following automated and manual duplicate removal, a stepwise manual selection was performed by two independent researchers (46,83). The initial screening was based on titles and abstracts, followed by full-text assessments against the eligibility criteria. Cohen's kappa coefficient was calculated at each stage to measure inter-rater agreement, and any discrepancies were resolved through consensus. Eligible studies were analyzed by outcome (46,83).

3.2. Data collection

In the data extraction process, two independent authors manually gathered information from each article, crosschecking each other's data pool. Any discrepancies were resolved through consensus (46,83). The collected information was then summarized using a standardized data collection form. The extracted data included: study characteristics (first author, year of publication, country, number of centers, and setting), study sample description (sample size, sex distribution, age, and any specificity of the sample as stated in each study), details for probiotics (probiotic type, dose, and duration) and outcomes as reported in each article (46,83). In instances where data were presented solely in graphical format, we utilized GetData Graph Digitizer software (version 2.26.0.20) and using PlotDigitizer software (3.3.9, 2025) to extract the information (94,95).

3.3. Synthesis methods

3.3.1. The effect of probiotic supplementation on the gut microbiome during antibiotic treatment

The statistical analysis was performed by a biostatistician using the *R* software (v4.2.1.) (96) with meta (97) and *dmetar* (98) packages. A meta-analysis was performed if the evaluated outcome was reported in at least three articles (46). For the effect size measure, we calculated mean differences (MD, probiotic, and antibiotic minus only antibiotic treatment) with 95% confidence intervals (CIs) (46). If available, the mean and the corresponding standard deviations (SD) were extracted from each study. In other cases, to estimate the mean and standard deviation based on 0,1,2,3,4 quartiles (extracted from box plots), Luo (99) and Shi (100) methods were used as implemented in the *meta* package (46). Based on the included article by Oh et al. (101), where the raw data of Shannon, Chao1, and observed OTUs (operational taxonomic units) diversity indices were given, we could assume that the distribution of these indices did not differ from a normal distribution to a relevant extent. Therefore, the estimation of mean and SD from the quantiles could be acceptable (46). As the main result, we pooled the values of Shannon and Chao1, observed OTU diversity indices after treatment, and used the inverse variance weighting method for each separately (46). We included only RCTs; therefore, we could assume that the characteristics before the treatment were not different in the intervention and control groups. As an additional sensitivity analysis, we performed a separate analysis for data before the treatment and a meta-analysis for the “before-after”

change values (46). For the change calculations, we used the correlation coefficient determined from the data of Oh et al. (101). As we anticipated considerable between-study heterogeneity, a random-effects model was used to pool the effect sizes. We did not apply the Hartung-Knapp adjustment (102,103). The maximum-likelihood estimator was applied with the Q profile method for confidence interval to estimate the heterogeneity variance measure τ^2 (104). Additionally, between-study heterogeneity was described using the Cochran's Q test and Higgins&Thompson's I^2 statistics (105). As the study number was low (<10), we could not assess the publication bias or additional influence analysis (e.g., leave-one-out analyses) (46). Forest plots were used to summarize the results graphically. Individual study confidence intervals were presented on the plot using *t*-distribution estimation. We report the results as (MD, [95% CI lower limit – 95% CI upper limit]) (46).

3.3.2. The effect of probiotic supplementation on the gut microbiome diversity in healthy populations

As we assumed considerable between-study heterogeneity in all cases, a random-effects model was used to pool effect sizes in a frequentist framework. In most studies, the quartiles of diversity index values could be extracted from box plots. Therefore, the effect size was expressed as the difference in medians (MedD) between groups (probiotic-treated minus control) instead of the usual mean difference (although we assume that the two do not differ relevantly). From the study MedD, we estimated the mean of median differences. Since only RCT studies were included, we used post-treatment data to estimate the difference between the treated and control groups. Results were considered statistically significant if the pooled 95% CI did not contain the null value ($p < 5\%$). We used the inverse variance weighting method for pooling MedDs. To estimate the heterogeneity variance measure τ^2, the restricted maximum-likelihood estimator with the Q profile method for confidence interval (104) was applied. Potential outlier publications were explored using different leave-one-out influence measures and plots following the recommendation of Harrer et al. (106). We performed subset analyses for studies of different types of probiotics and additional analyses based on the intervention time and the risk of bias. Small study publication bias was assessed by visual inspection of funnel plots and calculating Egger's test p-values (107). We assumed possible small study bias if the p-value was less than 10%. All statistical

analyses were performed with *R* (v4.4.1) (108) using the *meta* (v7.0.0) (109) and *metamedian* (v1.1.1) (110) packages for basic meta-analysis calculations and plots, *metafor* (v4.6.0) (111) and *dmetar* (v0.1.0) (112) package for additional influential analysis calculations and plots. Results were presented using forest plots illustrating MedD with corresponding 95% CI lower and upper limits for individual studies. The findings are reported as (MedD [95% CI lower limit – 95% CI upper limit]).

3.3.3. The effect of probiotic supplementation on zonulin levels in healthy populations

As we assumed considerable between-study heterogeneity in all cases, a random-effects model was used to pool effect sizes in a frequentist framework (83). As the source of the zonulin level was different in the studies, we used a standardized mean difference (SMD, Hedges's g (113)) to express the difference in the zonulin level between the probiotics and control groups (83). As all the included study was RCT, we assumed that the baseline values were equal in the probiotics and control group; therefore, the effect could be expressed by using the "after-treatment" values – available in all eligible studies (83). To calculate the SMD, the mean and standard deviation were extracted or estimated from the studies. We reported the difference as "probiotics minus control". We summarized the findings related to the meta-analysis on forest plots (83). Between-study heterogeneity was described by the between-study variance (τ^2) (104) and the Higgins&Thompson's I^2 statistics (105). Results were considered statistically significant if the pooled 95% CI did not contain the null value ($p < 5\%$). All statistical analyses were performed with *R* (v4.4.1) (108) using the *meta* (v7.0.0) (109) package for basic meta-analysis calculations and plots (83).

3.4. Risk of bias assessment

Two authors independently assessed the risk of bias using the revised Cochrane risk-of-bias tool (RoB2) (114). Discrepancies were resolved by consensus. Risk of bias assessment is a critical step in evaluating the reliability and validity of studies included in a systematic review or meta-analysis (46,83). The evaluation covered biases related to the randomization process, deviations from intended interventions, missing data, outcome measurement, and the selection of reported results. Each domain was rated, and the tool automatically determined the overall risk level, categorized as low, some concerns, or high (46,83).

3.5. Certainty assessment

The certainty of the evidence was independently evaluated by two investigators using the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) framework (115). It is a systematic approach used to assess the strength of recommendations in healthcare research and guideline development. We evaluated the quality of evidence based on factors such as risk of bias, inconsistency, indirectness, imprecision, and publication bias. We classified evidence into four levels as recommended: high, moderate, low, and very low certainty. Any disagreements were resolved by consensus (46,83).

IV. Results

4.1. The effect of probiotic supplementation on gut microbiome during antibiotic treatment

4.1.1. Study selection

The results of the search and selection processes are summarized in **Figure 1**. Our search key identified 19,596 records (46). Cohen's kappa index for the title and abstract selection was 0.86, whereas it was 0.95 for the full-text selection (46). Of the 15 articles eligible for the qualitative synthesis (877 patients) (31,101,116–128), five were suitable for the quantitative synthesis of the Shannon diversity index (335 patients) (101,119–122), and three for the quantitative synthesis of Chao1 and observed OTUs indices (236 patients) (101,120,122). No additional articles were found by screening the reference lists of the papers included. We included only non-overlapping populations in our review. Study characteristics are summarized in **Appendix - Table S1** (46)**.** Most of the studies investigated adult populations. One article investigated neonates (120), and one study included an adolescent population aged 15 years (120). In eight of the studies, the indication of antibiotic therapy was *Heliobacter pylori* eradication (31,101,117,118,120,122,125,126). One study focused on *Clostridioides difficile* infection (119), and two investigated patients with various infections outside the gastrointestinal tract (116,123). Four studies investigated healthy populations without any medical indication for antibiotic therapy (121,124,127,128). For the investigation of the microbial composition, nine studies used the 16S rRNA sequencing technique (101,117–124), three used standard microbiological culturing techniques (31,125,126), one study combined DNA-based terminal restriction fragment length polymorphism (TRFLP) analysis and standard culturing methods (127), and two studies used other polymerase chain reaction (PCR)-based techniques (116,128). All included articles were available in full text and were published in peer-reviewed journals, except the study of Amarri et al., in which case only study protocol with results were published (46,116).

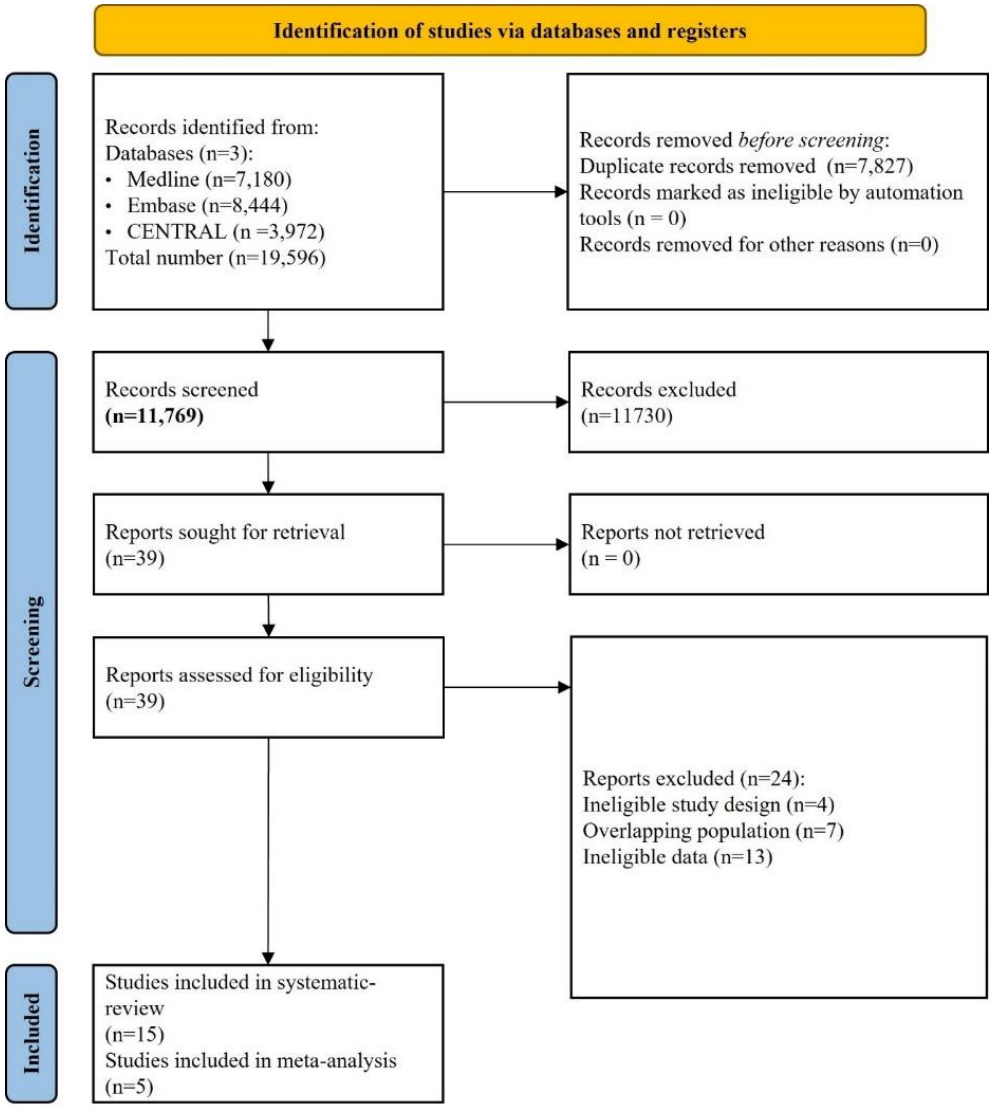

Figure 1. PRISMA flowchart of the selection process - The effect of probiotic supplementation on gut microbiome during antibiotic treatment

Source: Éliás et al. (46)

4.1.2. Quantitative synthesis

Six eligible articles reported results on the Shannon diversity index (101,119–123). One article that reported on the neonate population exclusively (123) was not included in the meta-analysis due to the impact on the indirectness of our results (105). Based on the synthesis of five articles (335 patients), the Shannon diversity index was not significantly different between the probiotic-supplemented and antibiotic-only treated groups when measured immediately at the end of antibiotic treatment (MD=0.23 [(-)0.06 – 0.51] **(Figure 2. A)** (46)**.** We identified three eligible articles with 236 patients for the meta-

analysis of the number of Observed OTUs and Chao1 index (101,120,122). The results are presented in **Figure 2. B-C**. The results of Kabbani et al. were previously excluded due to the time point of measurement, which was not reported precisely (124). For Observed OTUs, the neonate population was also excluded (123). In both cases, the diversity of the intestinal flora of the probiotic and control groups did not significantly differ from each other (Observed OTUs: MD=17.15 [(-)9.43 – 43.73]; Chao1: MD=11.59 [(-)18.42 – 41.60]) (46).

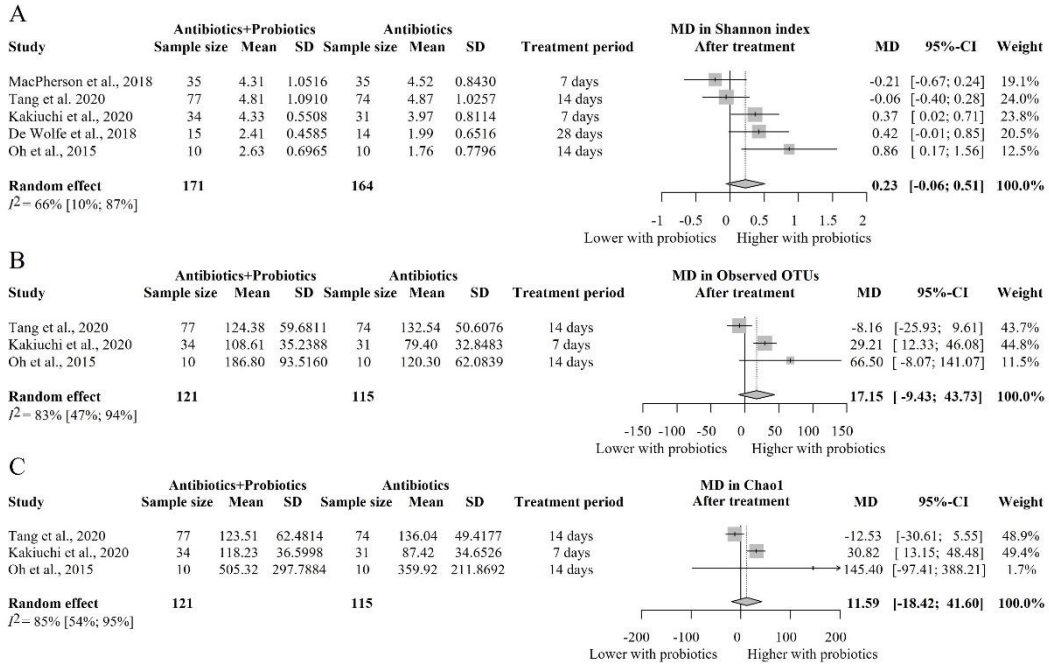

Figure 2. After antibiotic treatment, the Shannon diversity index (A), Observed OTUs (B) and Chao1 index (C) are not significantly higher in patients receiving concurrent probiotic supplementation than in those treated with antibiotics alone, as measured immediately after antibiotic treatment. CI: confidence interval; MD: mean difference

Source: Éliás et al. (46)

Although all the included studies were RCTs, the baseline values in the article by De Wolfe et al. (119) showed a marked difference in Shannon diversity index (MD=0.64 [(0.05 – 1.22] (46)), and the study of Kakiuchi et al.(120) in Chao1 index (MD=21.57 [3.47 – 39.68] – **Appendix – Figure S1** (46)) between the probiotic and control groups. Therefore, as a sensitivity analysis, we performed a separate calculation for data before the treatment (46) (**Appendix – Figure S1**), and for the magnitude of change, assessing the difference between "before-after" values in each study (**Figure 3. A-C**) (46). We did

not find any significant difference in the change values between the experimental and control groups regarding either of the diversity indices (Shannon index: MD=0.07 [(-)0.19 – 0.32]); Observed OTUs: MD=8.09 [(-)3.87 – 20.05]); Chao1 index: MD=3.77 [(-)10.17 – 17.71]) (46).

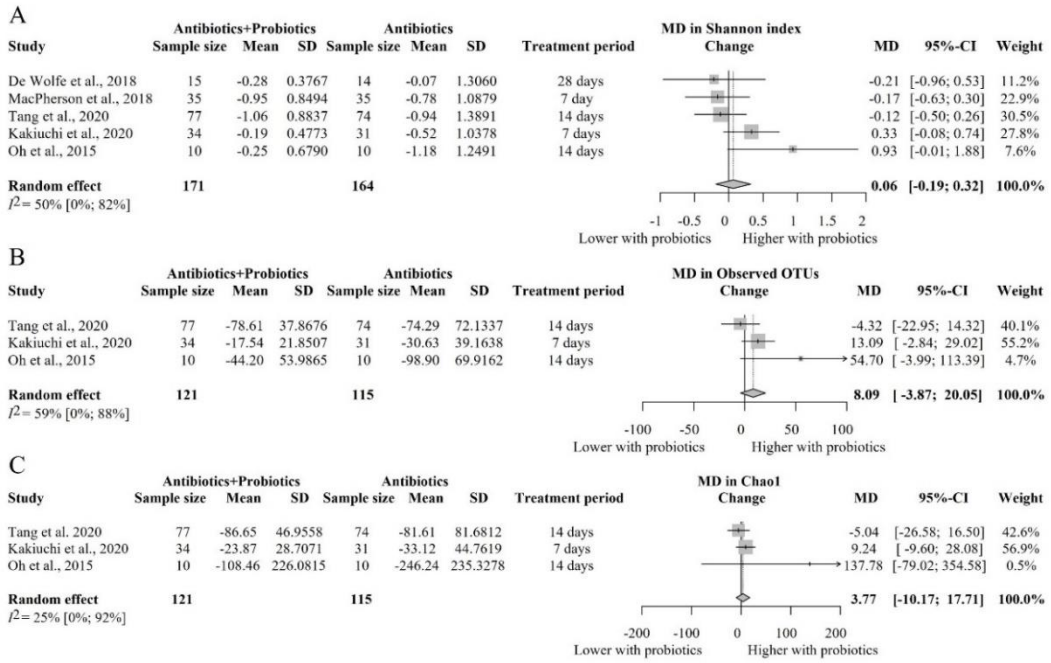

Figure 3. The magnitude of change compared to pre-treatment values of the Shannon diversity index, Observed OTUs, and Chao1 indices is not significantly different in the group receiving concurrent probiotic supplementation than in the group treated with antibiotics alone. CI: confidence interval; MD: mean difference

Source: Éliás et al. (46)

4.1.3. Qualitative synthesis

4.1.3.1. α- and β diversity indices

α-diversity indices were lower in both the intervention and the control groups after the antibiotic administration (46). The three articles – that were not suitable for the meta-analysis – reporting on the Shannon diversity index revealed no significant difference between the groups (117,118,123). As for the Observed OTUs, the three articles not included in the meta-analysis reported no significant difference between the two groups (117,123,124). Regarding the Chao1 index, Kabbani et al. reported significantly higher values in the control group (124). For most of the α-diversity indices unsuitable for meta-

analysis, the studies did not reveal a significant difference (5% significance level) between the probiotic and control groups (46,101,117–124). A detailed summary is in **Appendix – Table S2.**

The most used ß-diversity indices were Bray-Curtis dissimilarity index and both weighted and unweighted UniFrac (unique fraction metric) distances. Most studies found no significant difference (5% significance level) between the groups (46). Only Engelbrektson et al. reported a significantly improved ß-diversity by the Euclidean distance (127) by showing almost no change in the probiotic group, with a large shift toward diminished ß-diversity in the control group (46). None of the studies reported significant differences between the two groups regarding other indices (46,117,119–122,127). A detailed summary can be found in **Appendix – Table S3** (46).

4.1.3.2. Taxonomic composition

At the phylum level, a decreasing trend in the proportion of *Firmicutes* and *Bacteroidetes* with a higher relative abundance of *Proteobacteria* was observed after antibiotic therapy in both groups, regardless of probiotic supplementation (46). There was also a reduction in the *Bacteroidetes:Firmicutes* (B:F) ratio at the cessation of treatments. This was confirmed by several studies; however, in the study of Oh et al. the reduction was significantly greater in the control group (101,118,122). Importantly, these changes in phyla abundances disappeared at day 56 in the studies of Chen et al. and Tang et al. (118,122).

Changes in the level of the *Enterobacteriaceae* family were inconsistent across the studies. Several articles reported an increasing trend of *Enterobacteriaceae* in the probiotic-supplemented group only (117,124); however, according to other studies (125,126), this increase was observed only in the control group (46). Meanwhile, Forssten et al. and MacPherson et al. reported a higher relative abundance of *Enterobacteriaceae* in both groups after antibiotic treatment, which normalized after two weeks of follow-up (121,128). Changes in the other bacterial families were heterogeneously reported (see **Appendix -Table S4**).

At the genus level, *Bacteroides* showed a decreasing trend in the probiotic-supplemented group (117,119,126). However, some studies reported a reduction of *Bacteroides* in both groups after antibiotic treatment, which showed a re-growing tendency during 3-8 weeks of follow-up (122,125). Patients with probiotic supplementation had a higher proportion

of *Escherichia* spp. according to Cárdenas et al. (117), while two other studies reported that the addition of probiotics reduced the overgrowth of *Escherichia* compared to the control group (101,124). According to the study of Tang et al., where probiotic supplementation was continued for two more weeks after antibiotics cessation, the abundance of the genus *Enterococcus* increased at weeks 2 and 4 of follow-up in the intervention group only (122). Meanwhile, Wang et al. reported this increasing tendency in both groups at week 2. In their study, probiotics were suspended after the antibiotic cessation. However, by weeks 6, 8, and 9 of follow-up, the enrichment of *Enterococcus* had disappeared in both intervention and control groups, as reported in both studies (31,122). Probiotic supplementation seems to help maintain the level of the *Bifidobacterium* genus (120,123,127). According to Plummer et al. *Bifidobacterium* decreased in both groups during antibiotic therapy but tended to increase after therapy cessation to day 35 of follow-up (125). In the study of Kabbani et al., *Roseburia* prevalence was decreased by antibiotic treatment only; however, Tang et al. reported a significant reduction in both groups (122,124). Probiotic supplementation resulted either in an increase of *Blautia* in the intervention group or a decrease in the control group only, according to two studies (120,123). However, Tang et al. described a lower abundance of *Blautia* in both groups after antibiotic treatment, with a re-growing tendency with time regardless of probiotic supplementation (122).

The summarized results of the taxonomic analysis of microbiome composition, as measured immediately at the end of simultaneous antibiotic and probiotic treatment, are presented in **Appendix – Table S4** (46).

4.1.4. Risk of bias

The overall risk of bias was low to high for the indices included in the meta-analyses (46). The high risk of bias was caused mainly by the baseline differences between the interventional and control groups regarding some of the diversity indices (46,119,120). A detailed summary can be found in **Appendix – Figure S2-S7** (46).

4.1.5. GRADE assessment

Based on the GRADE assessment, the quality of evidence for the meta-analyses was low (**Appendix – Table S5)** (46).

4.2. The effect of probiotic supplementation on gut microbiome

diversity in healthy populations

4.2.1. Study selection

The results of the search and selection processes are illustrated in **Figure 4**. The initial search yielded 13,625 records. Cohen's kappa coefficient was calculated to assess inter-rater agreement, achieving values of 0.94 and 0.81 during the title and abstract screening phase and 0.94 and 0.98 during the full-text selection phase. Ultimately, 47 articles met the eligibility criteria for qualitative synthesis of gut microbiome diversity indices.

Study characteristics are summarized in **Appendix - Table S5**. To ensure robustness, the review includes only non-overlapping populations based on the available information. Most eligible studies focused on adult populations (129–163) three investigated elderlies (164–166). Six articles specifically examined infants (167–172), while three studies focused on children (173–175). All studies were randomized and placebo-controlled; six used a crossover design (138,140,144,156,158,162). Although we identified forty-seven eligible articles reporting the results of gut microbiome diversity, only twenty-two for the Shannon diversity index (130,131,134–137,140,142–147,149,151,152,154,160,161,163,165,166), seven for the OTUs index (131,133,139,142,144,147,161), nine for the Chao1 index (130,138,139,143,144,146,154,163,165), and ten for the Simpson's Index of Diversity (134,142–144,146,147,151,152,161,165) provided data, either in acceptable numerical format or via boxplots, for including into the meta-analysis. We handled articles reporting data on the infant or children population separately to prevent the indirectness of our findings. We excluded the above articles from the meta-analysis and included them only in the systematic review to preserve the reliability of our results.

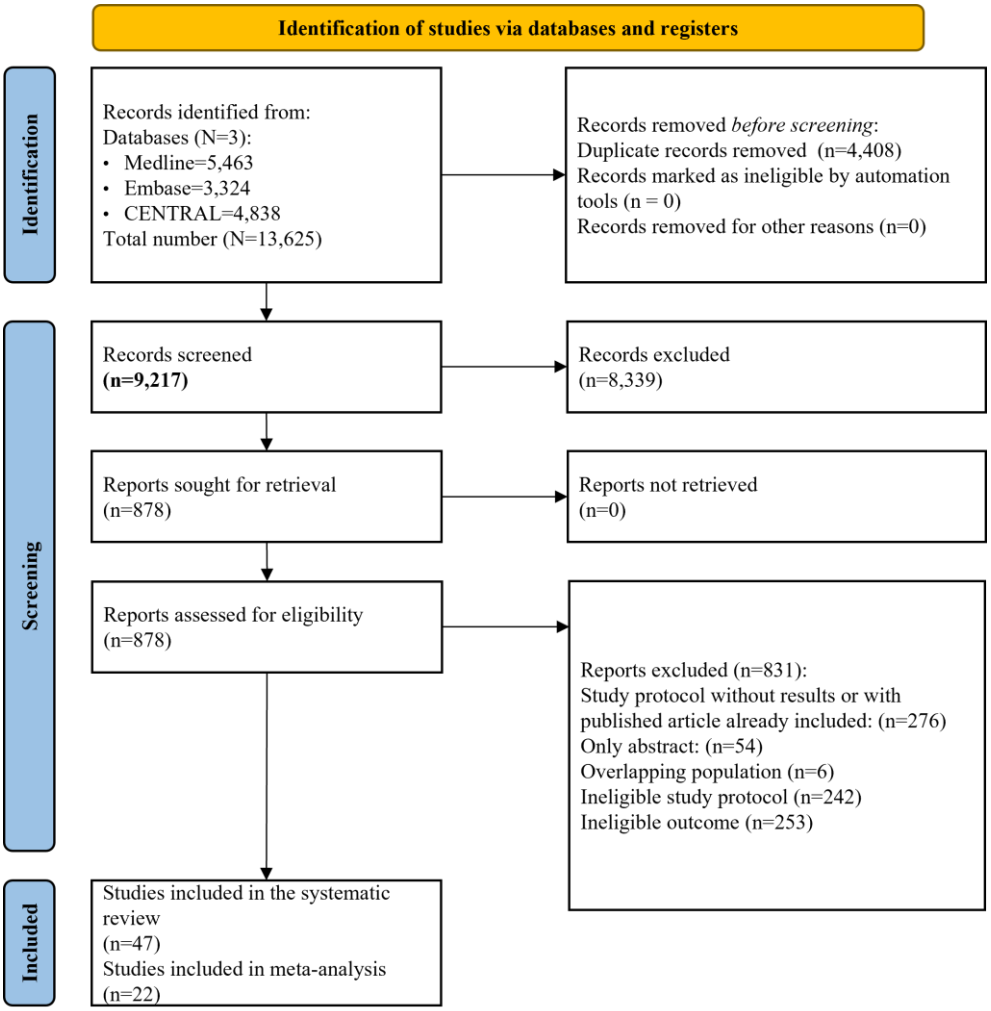

Figure 4. PRISMA flowchart of the selection - The effect of probiotic supplementation on gut microbiome diversity in healthy populations

Source: own work, unpublished

4.2.2. Quantitative synthesis

The results of the meta-analysis of the Shannon diversity index, including twenty-two articles with 1068 individual participants (130,131,134–137,140,142–147,149,151,152,154,160,161,163,165,166) are summarized in **Figure 5. A.** We did not find a significant difference between the intervention and control groups when measured immediately at the end of treatment (MedD=(-)0.08 [(-)0.16 – 0.01]). We performed subgroup analysis based on the probiotic composition used in each study. Three major groups of articles have been identified that investigated the effect of bacteria belonging to the family of *Lactobacillaceae*, *Bifidobacteriaceae*, and *Bacillaceae* or, additionally, a mixture of these. Neither of these groups showed any significant effect as shown in **Figure 5. B.**

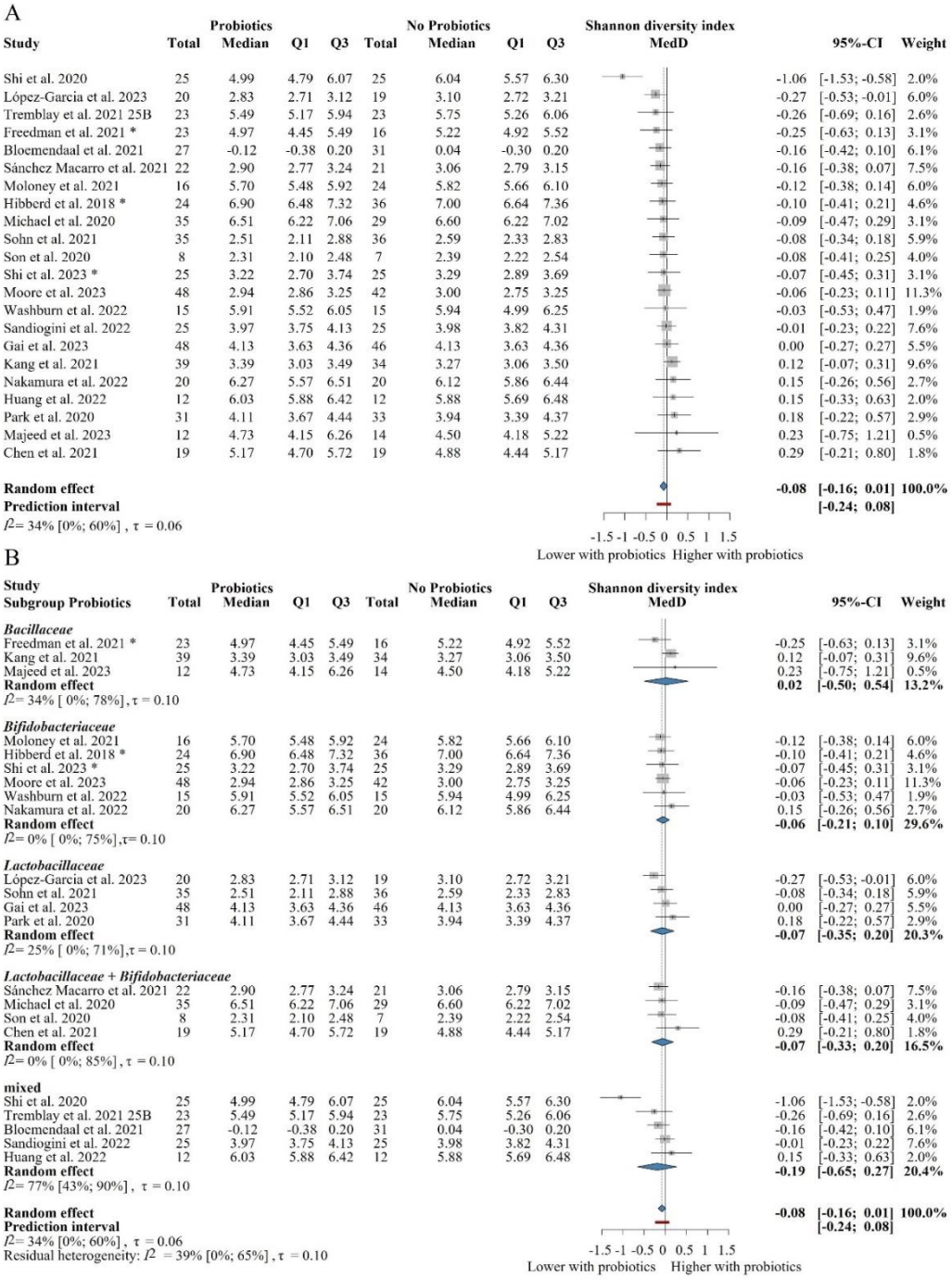

Figure 5. Shannon diversity index is not significantly different in healthy people receiving probiotic supplementation than in those in the control group, as measured immediately after the treatment period (A), sub-grouped based on probiotic strain family (B). CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

Source: own work, unpublished

We identified seven eligible articles with 447 individual participants for the meta-analysis of the number of Observed OTUs (131,133,139,142,144,147,161). The results are

presented in **Figure 6. A,** showing no significant difference between the two groups (MedD=2.19[(-)2.20 – 6.57]). Subgroups based on the probiotic composition resulted in no significant and clinically irrelevant differences either, as shown in **Figure 6. B.**

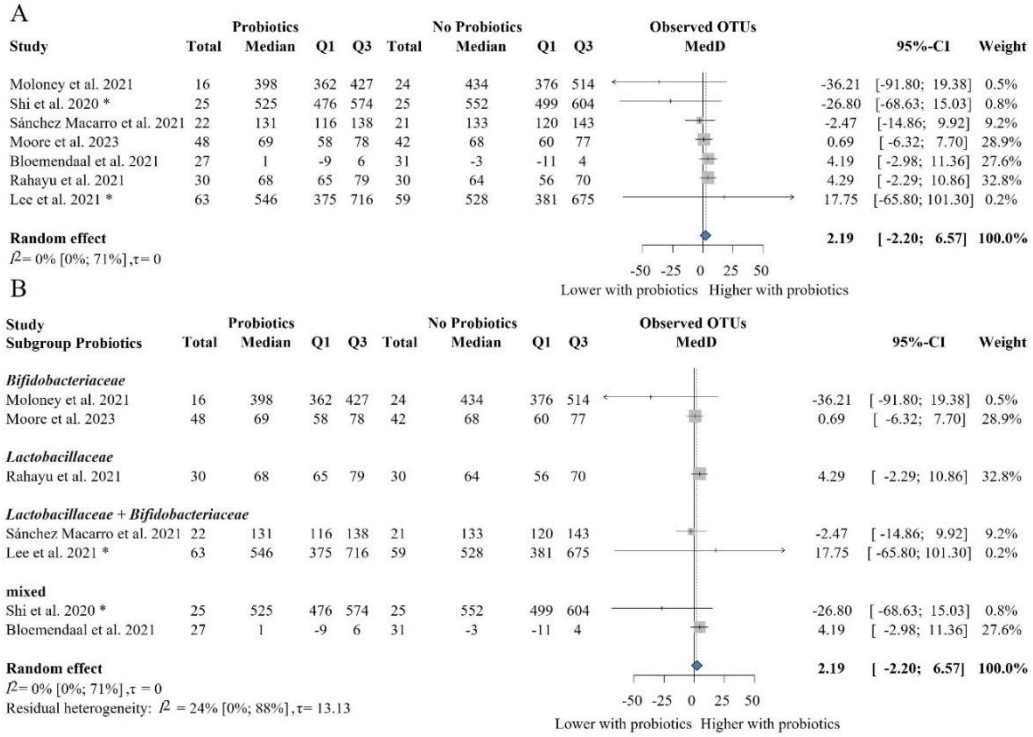

Figure 6. The number of Observed OTUs is not significantly different in healthy people receiving probiotic supplementation than in those in the control group, as measured immediately after the treatment period (A), sub-grouped based on probiotic strain family (B). OTU: operational taxonomic unit; CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.
Source: own work, unpublished

Nine eligible articles with 456 individual participants provided the data on the Chao1 index for quantitative analysis (130,138,139,143,144,146,154,163,165) as shown in **Figure 7. A.** Probiotic supplementation did not result in a significantly different Chao1 index compared to the control group (MedD=(-)3.19 [(-)27.28 – 20.89]), even when we performed the subgroup analysis based on the probiotic composition (**Figure 7. B**).

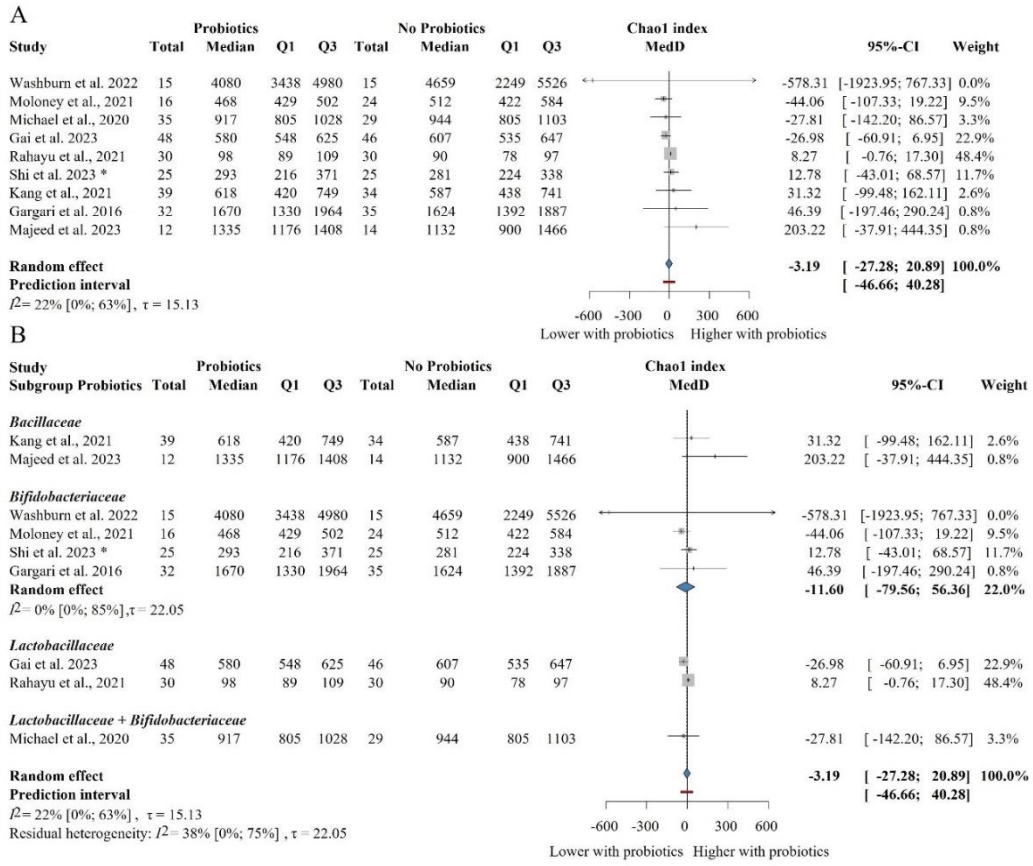

Figure 7. Chao1 index is not significantly different in healthy people receiving probiotic supplementation than in those in the control group, as measured immediately after the treatment period (A), sub-grouped based on probiotic strain family (B). CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

Source: own work, unpublished

We performed the meta-analysis using the Simpson's Index of Diversity, standardizing all data to reflect higher values indicating greater diversity. However, it was not always explicitly stated in each article whether Simpson's Index of Diversity (1-D) or Simpson's Index (D) was used. Based on the reported values (typically ranging from 0.8 to 0.9), it is likely that the Simpson's Index of Diversity was applied, rather than the traditional Simpson's Index, which measures the probability of two random samples belonging to the same species. This approach allowed consistency in interpreting higher values as greater diversity across studies.

Ten eligible articles with 455 individual participants were included in the meta-analysis on the Simpson's Index of Diversity (134,142–144,146,147,151,152,161,165) (**Figure 8. A)**, with no observed significant difference between probiotic and control groups

(MedD=(-)0.01 [(-)0.02 – 0.00]). Similarly, the subgroup analysis based on the composition of probiotic supplementation did not bring significant results in all cases (**Figure 8. B**).

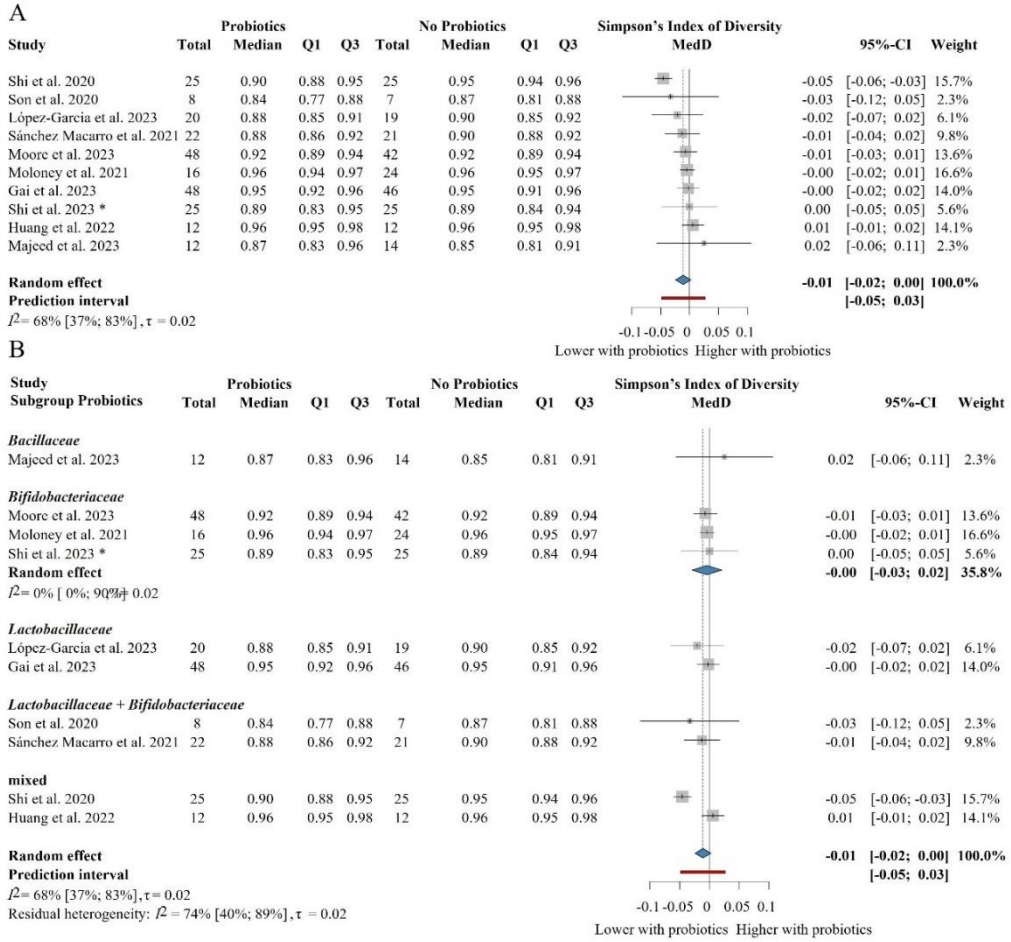

Figure 8. Simpson's Index of Diversity is not significantly different in healthy people receiving probiotic supplementation than in those in the control group, as measured immediately after the treatment period (A), sub-grouped based on probiotic strain family (B). CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

Source: own work, unpublished

Additional sensitivity analyses and subgroup analyses based on the risk of bias assessment (low, some concerns, or high) along with the result of the meta-regression analysis investigating the effect of the duration of intervention were not significant in any of the cases (**Appendix – Figure S6-S17**). Small study publication bias assessment and leave-out analyses did not raise serious concerns and influential articles regarding the

results of our meta-analyses (**Appendix – Figure S18-S25**).

4.2.3. Qualitative synthesis

Most of the included studies reported no significant and relevant effect or difference in α-diversity values following probiotic consumption compared to the control group.

Shi et al. (2020) found a decrease in Shannon and Simpson's Index of Diversity after the multi-strain probiotic intervention, while the control group microbiota remained stable. There was no difference in the number of Observed OTUs in either of the groups (161). Plaza-Diaz et al. investigated several bacterial strains, comparing them to a placebo group. In their study, treatments with *Lacticaseibacillus paracasei* CNCM I-4034 or *Lacticaseibacillus rhamnosus* CNCM I-4036 significantly increased the Shannon index at the end of the intervention. At the same time, *Bifidobacterium breve* CNCM I-4035 or the combined supplementation of *B. breve* CNCM I-4035 and *L. rhamnosus* CNCM I-4036 did not affect α-diversity. Notably, the results of the placebo group have not been mentioned in the article, and no comparison between the groups has been performed (141). Rahayu et al. reported significant increases in Chao1 and Observed OTUs in the *Lactiplantibacillus plantarum* Dad-13-supplemented group; however, a comparison with the placebo group was not performed (139). According to our comparative meta-analysis the mean of median differences was not significantly nor relevantly different in the two groups after the intervention period.

Paytuvi-Gallart investigated the children population. According to their results, *Bacillus subtilis* DE111 significantly increased both Shannon and Simpson's Index of Diversity α-diversity indices but not the richness of the gut microbiota. However, no comparison with the control group was performed (174). Interestingly, Gan et al. reported an increase in alpha diversity in the stools of the placebo group among children; however, they did not specify which of the four investigated indices was exactly affected. The probiotic group consuming a multi-strain product did not change over time (173). Investigating the infant population, Li et al. reported increased Simpson's Index of Diversity, Chao1, and ACE indices after *L. paracasei* N1115 intake. Notably, the Shannon index was significantly higher in both the probiotic and control groups after the intervention (172). All other studies with other probiotic strains reported no significant changes or differences in α-diversity indices (**Appendix – Table S7.**) (129–138,140,142–147,149–156,158–160,162–170). β-diversity analyses largely confirmed the absence of a

significant and relevant effect of probiotics on altering the overall structure of the gut microbiota, with some exceptions. Ferrario et al. revealed that treatment with *L. paracasei* DG significantly altered the overall faecal microbiota composition of participants, as demonstrated by repeated-measures ANOVA of paired distances between the probiotic and placebo treatments based on weighted UniFrac distance (156). Plaza-Diaz et al. found that probiotic treatment altered β-diversity over time in participants taking *L. rhamnosus,* based on weighted UniFrac distance. Participants in this group tended to have more similar overall structures after the intervention. This effect was still observed after 15 days of follow-up (141). Similarly, Wischmeyer et al. found a significant difference in β-diversity based on the Bray-Curtis distance between the probiotic-supplemented (*L. rhamnosus*) and control groups (129). Sohn et al. reported a significant difference between the control and the *L. plantarum* K50-supplemented group based on Bray-Curtis distance (145). According to Majeed et al., significant differences in β-diversity between placebo and *Heyndrickxia coagulans*-supplemented groups using both weighted and unweighted UniFrac distances were observed (146). In the study of Gan et al., the microbiota of the children in the multi-strain probiotic group remained stable when comparing weeks in contrast to the placebo group, which displayed significant variability across the same time points based on weighted UniFrac distance (173). On the other hand, Lau et al. reported a significant change in Bray-Curtis distance in the *Bifidobacterium longum* BB536-supplemented children after the treatment (175). All the other studies with other probiotic strains did not report any significant change or difference in β-diversity indices (**Appendix – Table S8**) (130–138,140,142,143,148–150,153–155,157–160,163,165–169,172).

4.2.4. Risk of bias

The overall risk of bias was low to high for the indices included in the meta-analyses (**Appendix – Figure S26-S29**).

4.2.5. GRADE assessment

Based on the GRADE assessment, the quality of evidence for the meta-analyses was moderate (**Appendix – Table S9**).

4.3. The effect of probiotic supplementation on zonulin levels in healthy populations

4.3.1. Study selection

The results of the search and selection processes are illustrated in **Figure 9**. We identified 13,625 records (83). Cohen's kappa coefficient was calculated to assess inter-rater agreement, achieving values of 0.94 and 0.90 during the title and abstract screening phase and 0.94 and 0.98 during the full-text selection phase (83). Ultimately, 5 articles met the eligibility criteria for the qualitative synthesis of zonulin levels (176–180). An additional study was included in the systematic review that measured stool zonulin concentration; therefore, it could not be compared with data from blood levels (181). Study characteristics are summarized in **Appendix - Table S10.** The review includes only non-overlapping populations based on the available information to ensure robustness (83). The included studies exclusively investigated adult populations, although no initial age criteria were specified. Two studies investigated male athletes (177,181), while one included only pregnant women (180). Other studies investigated both males and females, who were either normal weight to mildly obese (176,178) adults or had minimal complaints of abdominal bloating, burping, or flatulence (179).

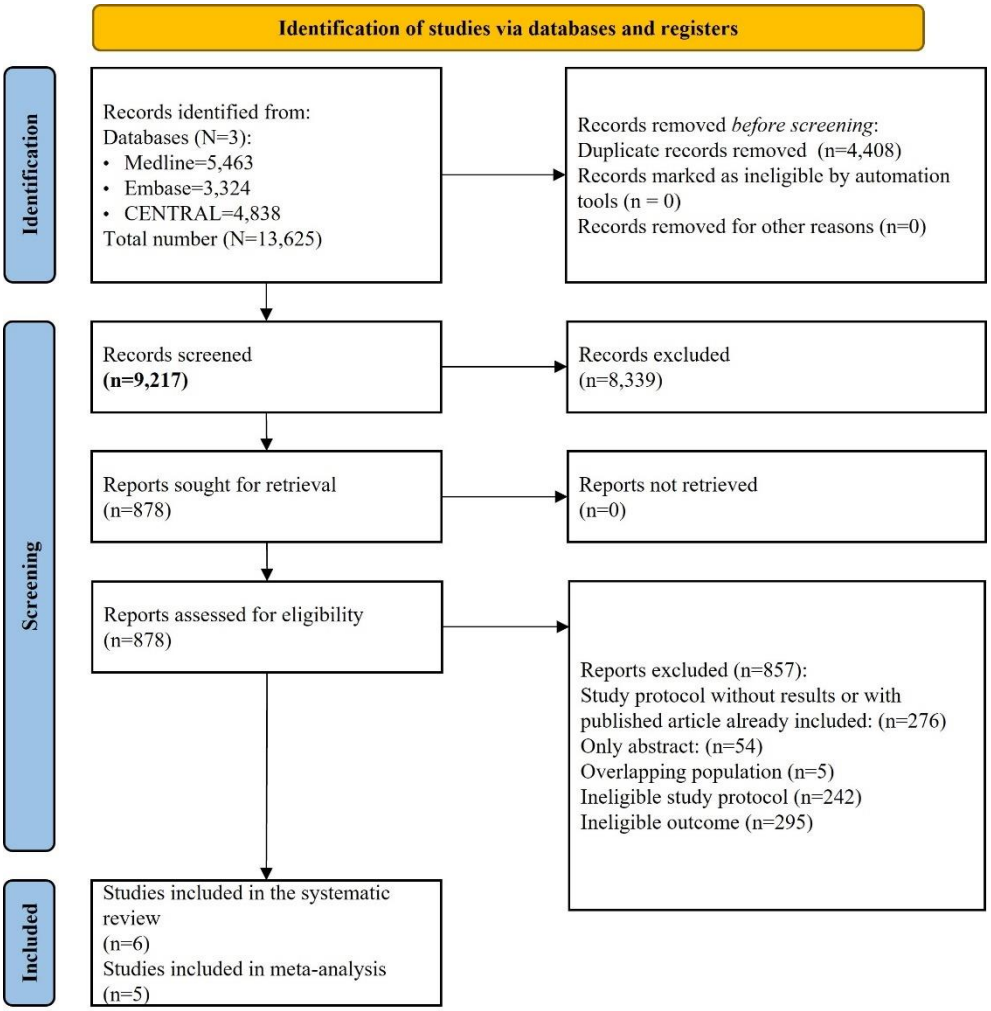

Figure 9. PRISMA flowchart of the selection - The effect of probiotic supplementation on zonulin levels in healthy populations

Source: Földvári-Nagy et al. (83)

4.3.2. Quantitative synthesis

The results of the meta-analysis of blood zonulin levels, including five articles with 307 individual participants (83), are summarized in **Figure 10.** We did not find a statistically significant or clinically relevant difference between the intervention and control groups when measured immediately at the end of treatment (SMD=(-)0.01 [(-)0.39 – 0.37]) (83).

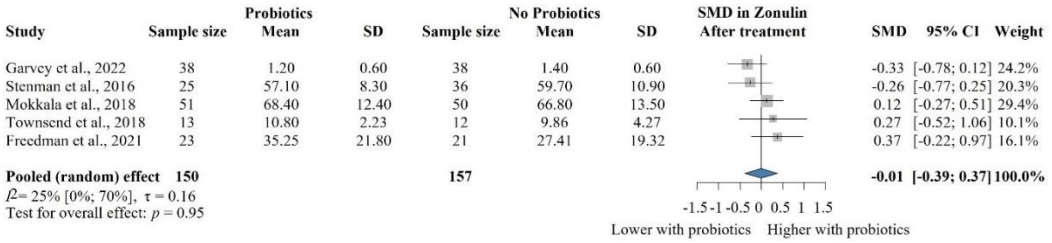

Figure 10. Zonulin levels are not significantly different in healthy people receiving probiotic supplementation than in those in the control group, as measured immediately after the treatment period. Concentrations are expressed as ng/ml. CI: confidence interval; SD: standard deviation; SMD: standardized mean difference

Source: Földvári-Nagy et al. (83)

4.3.3. Qualitative synthesis

As confirmed by our meta-analysis, individual studies did not report differences in zonulin in response to probiotic supplementation compared to placebo (83). According to Freedman et al., no significant differences in plasma zonulin were reported between or within groups (178). Plasma zonulin was not affected by the study products in the study of Garvey et al. (179) The consumption of probiotic supplements had no impact on the serum zonulin concentration, as no statistically significant differences were detected among the groups from early to late pregnancy or at late pregnancy according to Mokkala et al. (180) Levels of circulating zonulin seemed to remain consistently lower throughout the study in the probiotic groups compared to placebo, the difference was however not statistically significant in the factorial analysis (176). In their study, Townsend et al. found no differences in plasma zonulin concentrations following a 12-week intervention (177). The Mazur-Kurach et al. study was not included in the meta-analysis, because they measured zonulin levels from the feces, unlike others (181). In their study, the baseline stool zonulin levels did not differ significantly between probiotic and control groups, with both exceeding normal reference values. The 16-week probiotic supplementation

significantly reduced zonulin levels compared to baseline and placebo (83,181) .

4.3.4. Risk of bias

The overall risk of bias was mostly low for the studies included in the systematic review and meta-analysis (160,177,179,180). The high risk of bias for Stenman et al. was caused by the high dropout rate due to protocol deviations (83,176) (**Appendix – Figure S30-S31**).

4.3.5. GRADE assessment

Based on the GRADE assessment, the quality of evidence for the meta-analyses was very low (83) (**Appendix – Table S11**).

V. Discussion

The studies included in this thesis represent the first systematic reviews and meta-analyses to quantitatively summarize the findings of available randomized controlled trials investigating the impact of probiotic supplementation on gut microbiome diversity under antibiotic treatment and for healthy populations (46). Moreover, we systematically investigated the effect of probiotics on zonulin levels among healthy people (83). Our findings do not support probiotic supplementation for preserving or modifying microbiome diversity, whether in the context of antibiotic therapy or generally healthy individuals, and we did not confirm the effect of probiotics on zonulin in healthy populations (46,83).

5.1. Probiotic supplementation and microbiome

5.1.1. Diversity indices

The imbalance of the bacterial composition in the gut microbiome is called dysbiosis (46). One form of this can be low-diversity dysbiosis, which is often caused by broad-spectrum antibiotic therapy (75). Decreased gut microbiome diversity has been associated with obesity, inflammatory bowel disease (IBD), liver disease, and recurrent *Clostridioides difficile* infection, among other pathologies (46). Maintaining gut microbial diversity during periods of potential impairment seems important (17,44). Probiotics are widely used to support gut health and prevent dysbiosis, particularly during antibiotic therapy. However, robust scientific evidence for their effectiveness in maintaining microbiome diversity remains limited (2,82). Our systematic review and meta-analysis challenge the assumption that probiotics significantly impact microbiome composition, particularly in preventing low-diversity dysbiosis induced by antibiotic treatment (46). The meta-analysis of key alpha diversity indices (Shannon, Chao1, and Observed OTUs) demonstrated no significant effect of probiotics on preserving diversity (46). Moreover, most studies analyzing beta diversity found no meaningful differences between probiotic and control groups, reinforcing the conclusion that probiotics do not prevent antibiotic-induced dysbiosis (46). Similarly, probiotics were found to have no statistically significant impact on alpha or beta diversity in healthy individuals. Although some individual studies reported minor changes, our overall findings suggest that the gut microbiome of a healthy adult is highly resilient, making it unlikely that probiotics induce

meaningful alterations (46).

5.1.1.1. Strain-specific contradictions and similarities

Historically, species from the *Lactobacillus* and *Bifidobacterium* genera have dominated the probiotic landscape due to their safety profiles and believed health benefits (2). However, reclassifying the *Lactobacillus* genus in 2020 into 23 new genera has highlighted the genetic and functional diversity within these microbes and the need for strain-specific research (2,3). The current nomenclature, as provided by the NCBI Taxonomy Database, was used to update the bacterial strain names used in each article when interpreting our results (182). Additionally, promising probiotics from other genera, such as *Bacillus*, have gained attention due to their unique properties, such as spore formation, which improves survivability in the gastrointestinal tract (2).

Due to the lack of publications in the antibiotic study, we could only perform a sub-group meta-analysis, grouped by the taxonomic family of probiotics in the case of healthy populations. Overall, we did not reveal a statistically significant effect of supplementation on gut microbiome diversity in healthy populations. However, a closer examination of specific strains highlighted notable differences, emphasizing the importance of strain-level specificity in probiotic research.

Ferrario et al. reported that *Lacticaseibacillus paracasei* DG significantly altered β-, but not α-diversity (156), while Plaza-Diaz et al. and Li et al. observed changes in α-diversity with *L. paracasei* CNCM I-4034 and *L. paracasei N1115* but no corresponding changes in β-diversity (141,172).

While Rahayu et al. found significant increases in Chao1 and Observed OTUs with *Lactiplantibacillus plantarum* Dad-13, other studies using *L. plantarum* K50, LPQ180, or IMC 510® reported no changes in α-diversity (145,149,153). This aligns with our meta-analysis finding no significant difference in the Rahayu et al. study when performing a comparison to a placebo (139). Notably, however, there was a significant difference in the overall composition based on Bray-Curtis distance in the study of Sohn et al using *L. plantarum* K50 (145).

Supplementation with *Lacticaseibacillus rhamnosus* CNCM I-4036 (141) and *L. rhamnosus GG* (ATCC 53103) (129) resulted in changes in α- and/or β-diversity over time, while another study using *L. rhamnosus* LRa05 did not report significant changes

in either α- or β-diversity (143).

The conflicting results may be attributed to differences in variant-specific properties, doses, or study populations. On the other hand, other species investigated from the *Lactobacillaceae* family, such as *Lactiplantibacillus pentosus* (152), *Lactobacillus helveticus* (170), *Ligilactobacillus salivarius* (162,169), *Lactobacillus johnsoni*i (157), and *Limosilactobacillus reuteri* (150) showed concordant ineffectiveness in modulating microbiome diversity. These species are, however, less represented in our review.

We observed a contradictory effect in the *Bifidobacteriaceae* family, especially for *Bifidobacterium longum* BB536. Lau et al. found significant β-diversity changes (Bray-Curtis distance) in children (175), but no such effects in adults, according to Nakamura et al. were reported (140). Similarly, another variant, *B. longum* BB68S, showed no significant changes in β-diversity in elderly participants (165). Notably, α-diversity was not affected in the above studies, along with Moloney et al., who used *B. longum* AH1714 as probiotic supplementation (144).

This incongruency may be due to differences in specific bacterial formulations or host responses. However, other species from the taxonomic family, such as *Bifidobacterium animalis* (136,168,169), *Bifidobacterium bifidum* (138,170), *Bifidobacterium breve* (141,142,148), and *B. longum subsp. infantis* (130,170,171), remained concordantly ineffective in modifying diversity indices compared to placebo across several studies in this review.

The *Bacillaceae* family also showed variable effects. Paytuvi-Gallart reported increases in Shannon and Simpson's Index of Diversity indices with *Bacillus subtilis* DE111 in children (174); however, in adults, another study using the same variant reported no significant changes in diversity metrics (160). Similarly, *B. subtilis* R0179 was not effective, according to another study (155).

Majeed et al. observed significant changes in β-diversity with *Heyndrickxia coagulans* (146), while Kang et al. found no such effects with *H. coagulans* SNZ 1969 (154).

Interestingly, multiple studies that used multi-strain probiotic formulations tend to not affect α- and β-diversity indices (131–135,137,147,150,151,159,164,166,167,173). According to the study by Shi et al. (2020), Shannon and Simpson's Index of Diversity was even significantly lower than the control group after the intervention (161).

5.1.1.2. Age group differences

Probiotic effects on diversity also varied across age groups, with children showing more pronounced responses in some cases. Li et al. reported increases in multiple α-diversity indices (Simpson's Index of Diversity, Chao1, ACE) in infants supplemented with *L. paracasei* N1115,(172) while Paytuvi-Gallart found increased Shannon and Simpson's Index of Diversity indices in children with *B. subtilis DE111*.(174) Moreover, Lau et al. observed significant β-diversity changes in children supplemented with *B. longum* BB536 (175). These findings suggest that the developing microbiome in younger individuals may be more susceptible to probiotic-induced changes. On the other hand, most other studies did not reveal the modifying effect of probiotics in infants (167–171) or children (173). Interestingly, Zhong et al. reported no effect of probiotics on gut microbiome diversity in infants during antibiotic treatment either (123).

Most adult studies reported no significant changes in α- or β-diversity indices. Similarly, studies focusing primarily on elderlies found no significant changes in diversity metrics (164–166). This aligns with the hypothesis that a mature, stable microbiome is less responsive to probiotic interventions (183,184).

5.1.1.3. Clinical relevance of diversity indices

The clinical utility of diversity indices in diagnosing or predicting disease risk remains uncertain. Currently, there is no robust evidence supporting their diagnostic value, and further research is needed to better understand the relationship between microbiome diversity and systemic outcomes. An important challenge is the lack of standardized cut-off values or normal ranges for most indices, complicating their interpretation in clinical settings.

It is well-studied that microbiome diversity evolves with age, with lower diversity observed in childhood and greater stability achieved in adulthood (183).

In the context of healthy populations, the participants in most studies included in this review were adults, who generally exhibit high baseline diversity. Therefore, any statistically significant differences reported are not necessarily clinically important. This high baseline diversity may partly explain the limited effects of probiotics in these populations, as interventions are less likely to induce measurable changes in an already diverse and stable microbiome (184).

For instance, a study from the literature suggests that a "normal" Shannon diversity index often exceeds 3 (185), with others setting the threshold as high as 4 (47) in clinical settings. In this review, most healthy adult participants had Shannon index values within or above these values, except for children, whose lower diversity levels align with developmental norms. In studies where the median Shannon diversity index values of participants did not exceed 3.5 (therefore, could be considered as “low”), probiotics generally failed to impact the index statistically or clinically significantly (142,147,151,152,154). The only exception was the study by Sohn et al., which reported a significant difference in β-diversity compared to the control group, but not in α-diversity (145). This suggests that the effect of probiotics on diversity does not necessarily depend on baseline values in healthy populations, particularly when the microbiota represents an otherwise stable community. Similarly, the Simpson’s Index of Diversity with cut-offs for high diversity around 0.75 (47), was consistently achieved across most adult populations in the studies reviewed, except for paediatric participants. Other indices, such as Chao1 and Observed OTUs, lack well-defined cut-off values, making their interpretation challenging. Consistent with the existing literature, children in our studies exhibited lower values for these indices, reflecting the developmental stage of their gut microbiota, which typically has lower diversity than adults (183,186).

In the context of antibiotic treatment, an interesting observation was that the Shannon diversity index dropped below 3.5 at the end of treatment in only two out of five studies (101,119) in our meta-analysis. In these cases, both the probiotic and control groups exhibited this decline. Notably, Oh et al. reported a higher Shannon index in the probiotic group compared to the control (101), whereas DeWolfe et al. did not observe this difference (119). The remaining studies maintained Shannon diversity within “normal” levels (120–122).

5.1.2. Taxonomic composition

Antibiotic-induced changes in the gut microbial communities, such as decreased *Bacteroidetes:Firmicutes* (B:F) ratio – or a higher *Firmicutes:Bacteroidetes* (F:B) ratio, as often used in the literature – have been associated with obesity and metabolic syndrome (187,188). This tendency of reduction in the B:F ratio was observed regardless of probiotic supplementation during antibiotic therapy according to several included studies,

but it also normalizes in both groups during follow-up (46,118,122). An increased proportion of *Proteobacteria* was reported by studies in both groups, which is a possible microbial signature of several diseases, such as metabolic disorders and IBD (189). These changes, however, showed a restoration tendency after 8 weeks of follow-up (46,101,118,122).

The enrichment of the *Enterobacteriaceae* family is commonly associated with specific antibiotic resistance genes for aminoglycosides, beta-lactams, and carbapenems, thus being a potentially dangerous source of antibiotic resistance gene transfer (121,190). Although the members of this family are considered normal intestinal residents, some may become opportunistic pathogens. They have a higher abundance in IBD patients, but their underlying pathological mechanisms are still under investigation (191). Changes in the level of the *Enterobacteriaceae* family were inconsistently reported in the included articles; therefore, we cannot draw strong conclusions about the consequences of probiotic supplementation (46). The tendency of abundance normalization after the cessation of the antibiotic treatment suggests that the changes in *Enterobacteriaceae* induced by treatment are transient (46,121,128).

Reduced abundance of several species in *Bacteroides* might be associated with the risk of *Clostridioides difficile* infection (192). The reduction of this genus was prevalent in the probiotic-supplemented group in several cases, the background of which is unclear (117,119,126). The re-growth of these bacteria during follow-up suggests that the changes are not permanent (46,122,125).

Escherichia coli and *Enterococcus* family species are commensal inhabitants of the gastrointestinal tract that may become pathogens in a dysbiotic environment for several diseases, such as antibiotic-associated diarrhea, vomiting, or permanent intestinal inflammation. Moreover, they are characterized by antibiotic resistance (193,194). Probiotic supplementation seems to reduce Escherichia overgrowth during antibiotic therapy according to Kabbani et al. and Oh et al. (46,101,124). Nevertheless, the level of both *Escherichia* and *Enterococcus* tends to normalize after antibiotics cessation regardless of probiotics supplementation (46). This brings the efficacy of probiotics in preventing this type of antibiotic-induced dysbiosis into question (31,122,124).

Probiotic supplementation seems to maintain the level of *Bifidobacteria* during antibiotic therapy (120,123,127). Several species and/or strains of this genus may be useful for

health, including modulating gut microbial homeostasis, inhibiting pathogens, and modulating immune responses. They can suppress oncogenic activity within the microbiome, and they can produce vitamins and transform food compounds into bioactive molecules (195). *Bifidobacteria* also play a crucial role during early life. They are among the first colonizers of the human gut. According to previous studies, children with allergic diseases have a reduced gut microbial diversity with a lower abundance of *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* compared to healthy controls (196). Therefore, the results of Zhong et al. are especially important as they showed that probiotic supplementation maintained the level of *Bifidobacteria* in newborns during antibiotic therapy (46,123).

Some of the included articles suggested that probiotic supplementation during antibiotic therapy has a protective effect on *Blautia* and *Roseburia* spp. levels (120,123,124). Recently, *Blautia* has been associated with alleviating inflammatory and metabolic diseases by regulating host health, and it has also been characterized by antibacterial activity (197). Gut *Roseburia* spp. produce short-chain fatty acids, modulate colonic motility, support immunity, and have anti-inflammatory effects (198). These findings suggest that probiotic supplementation may have some benefits but the tendency for *Blautia* levels to normalize spontaneously after antibiotic discontinuation casts doubt on them (46,122).

5.2. Probiotic supplementation and zonulin

Based on the five included studies, probiotic supplementation only showed a (-)0.01 SMD in blood zonulin levels compared to placebo after the intervention, which is clinically negligible (83). Furthermore, the 95% confidence interval ((-)0.39 to +0.37) strengthens the absence of a significant difference between probiotic users and non-users (83).

5.2.1. Clinical relevance

A 2023 meta-analysis focused on broader populations provides a distinct perspective on the effect of probiotic supplementation on intestinal barrier function, as measured by zonulin levels (199). Their pooled data from five randomized controlled trials (RCTs), including 385 subjects, demonstrated a significant improvement in gut barrier function following probiotic intervention compared to placebo (83,199). However, when stratified

by population characteristics, a notable difference emerged, since only one study included healthy athletes (177), while the remaining four (200–203) were conducted in diseased populations. The subgroup analysis revealed a remarkable decrease in serum zonulin levels among patients, suggesting a beneficial effect of probiotics in individuals with pre-existing gut barrier dysfunction (199). Conversely, in athletes, probiotic supplementation did not result in significant changes in serum zonulin levels, indicating a limited effect in populations without underlying permeability issues, which aligns with our findings (83,199). On the other hand, the pooled effect of five RCTs (201,203–206) that measured fecal zonulin levels showed no significant difference between probiotics and placebo groups (199).

Our aim was, however, to identify those apparently healthy people who can benefit from probiotic supplementation, being at risk of increased permeability (83). It might be challenging since normal reference values for zonulin levels vary depending on the measurement method (83). According to CE-marked competitive enzyme-linked immunosorbent assay (ELISA) kits from Immunodiagnostik AG (Bensheim, Germany), studies on apparently healthy individuals have estimated a mean reference value of 34 ng/mL (±14 ng/mL) (207). The analysis of individuals with minimal gastrointestinal symptoms showed that initial concentrations were low, with mean plasma zonulin below 1.5 ng/mL (83). No clinically significant changes were observed in clinical chemistry, hematology, plasma lipids, intestinal permeability, or inflammatory markers after probiotic treatment (179). On the other hand, the study by Mokkala et al., which investigated pregnant women, found that serum zonulin levels were consistently elevated in both the probiotic and control groups (180). Their findings demonstrated that serum zonulin concentration – an indicator of intestinal permeability – increased from early to late pregnancy in overweight and obese pregnant women, and probiotic supplementation had no effect on preventing this increase (180). Elevated zonulin levels may be associated not only with pregnancy but also with obesity (83). In the study by Stenman et al., changes in zonulin levels were found to be significantly correlated with variations in trunk fat mass among overweight and obese individuals (176). Notably, in their study, probiotic supplementation failed to produce a statistically significant reduction in elevated zonulin levels (mean serum concentration >55 ng/mL) (176). This aligns with the ineffectiveness reported by Freedman et al. investigating normal to mildly obese people (178). As an

additional risk factor, some research has reported compromised gut permeability in response to acute exercise stress following endurance and interval training (208,209). Thus, it is important to characterize intestinal permeability following acute resistance exercise or a competitive event in athletes (83). Among our included studies, Townsend et al. investigated division I male baseball athletes but reported mean serum zonulin levels remaining below 11 ng/ml, which could be considered normal (177). On the other hand, Mazur-Kurach et al. reported that their group of cyclists exceeded the normal reference range of fecal zonulin (>60 ng/mL - as defined in their article). After four months, zonulin levels declined by 43.87% in the probiotic group but rose by 25.07% in the placebo group (181). This aligns with a previous work that found that 14 weeks of probiotic supplementation resulted in significantly decreased levels of fecal zonulin, indicating an improvement in intestinal barrier integrity in trained men (83,205).

5.2.2. Sample sources

The question arises of which biomarker – fecal or blood zonulin – is more suitable for assessing zonulin levels (83). The relationship between circulating zonulin and intestinal zonulin remains unclear, as circulating zonulin may originate from sources beyond the gut (85). Nevertheless, a decrease in circulating zonulin has been associated with a lower lactulose/mannitol ratio, a well-established marker of intestinal permeability (200,210) . Therefore, it can be inferred that changes in circulating zonulin may serve as an indicator for monitoring alterations in gut barrier function (83). However, this assumption was challenged by a cross-sectional study, which found that plasma zonulin showed no or inconsistent associations with the lactulose/mannitol ratio. This discrepancy may be attributed to the limited specificity of human ELISA kits (211,212). According to other studies, fecal zonulin, unlike serum zonulin, showed no significant correlation with markers of microecological dysregulation or inflammation. This suggests that serum zonulin may be a more reliable indicator of intestinal permeability than fecal zonulin (83,199). This question should be, however, further investigated.

5.2.3. Strain-specificity

In vitro and *in vivo* studies suggest that probiotic supplementation may play a role in the upregulation and redistribution of interepithelial tight junction proteins, but these effects

appear to be strain-specific (213). Certain probiotic strains may exert their effects through distinct pathways, highlighting the importance of strain selection in research and clinical applications (83).

For example, Peng et al. demonstrated that *Bacillus subtilis* CW14 could protect intestinal epithelial cell microvilli and tight junction proteins (ZO-1 and claudin-1) from damage induced by ochratoxin A, while also helping to maintain genome stability in Caco-2 cells (213,214). In our review, three articles investigated the effect of *Bacillus subtilis* DE111 (177,178) and BS50 (179) supplementations, but showed no effect of zonulin levels in healthy populations (83) .

Bifidobacteria has also been shown potential in preclinical settings. *Bifidobacterium infantis* helped to prevent barrier damage caused by interleukin-1 stimulation by normalizing the expression of tight junction proteins occludin and claudin-1 in an *in vitro* Caco-2 intestinal epithelial cell model (213,215). Furthermore, in a neonatal mouse model of necrotizing enterocolitis (NEC), *Bifidobacterium infantis* contributed to maintaining intestinal barrier integrity by modulating the proper localization of claudin-4 and occludin within tight junctions, which in turn reduces intestinal permeability and lowers the incidence of NEC (213,216) *Bifidobacterium animalis subsp. lactis* alone (180) or in combination with *Lacticaseibacillus rhamnosus* (176) was not able to decrease elevated zonulin levels in healthy humans (83). The only study that showed a modulating effect of probiotics used a mixture of 13 strains belonging to the *Lactobacillaceae*, *Bifidobacteriaceae,* and *Streptococcaceae* families (181). However, comparing different probiotic mixtures is challenging due to variations in strain composition, dosage, and potential synergistic effects, highlighting the need for standardized research approaches to better understand their specific impacts (83).

5.3. Hypothesis testing

The hypothesis that probiotic supplementation does not have a statistically significant or clinically relevant effect on gut microbiome diversity under antibiotic treatment, in healthy populations, or zonulin levels in healthy people was systematically examined through a systematic review and meta-analysis of existing literature (44,79). The results supported the null hypothesis, indicating that probiotic supplementation did not produce a statistically significant or clinically meaningful change in gut microbiome diversity or

zonulin levels (44,79). Subgroup analyses, where feasible, did not reveal substantial variations in response based on probiotic strain, treatment duration, or the risk of bias in included studies. These findings align with the premise that the gut microbiome exhibits a high degree of interindividual variability, potentially limiting the effectiveness of generalized probiotic interventions. Additionally, the lack of a consistent effect on zonulin levels suggests that probiotics may not significantly modulate intestinal permeability in healthy individuals (44,79).

In conclusion, the hypothesis was confirmed, with evidence supporting the null hypothesis across all assessed outcomes (44,79).

5.4. Implication for practice and research

The summary of the available literature facilitates utilizing scientific results in daily practice, which is crucially important (46,217,218).

Probiotic supplementation has a limited effect on the diversity of the gut microbiome in healthy individuals and during antibiotic treatment (46). Personalized recommendations, considering individual factors and the benefits of each strain, would be essential (46,83). In clinical practice, probiotics should be used selectively, focusing on functional benefits and targeted use in vulnerable populations, e.g., to prevent *Clostridioides difficile* infection or antibiotic-associated diarrhea, as advised by the current guideline of the AGA and WGO on the use of probiotics (2,82). On the other hand, patients with low risk would reasonably select no probiotics, thus avoiding potential harm and additional costs (82,219).

Probiotic use in healthy populations should emphasize evidence-based functional outcomes rather than microbiome diversity changes (46). Educating patients about the limitations and potential benefits of probiotics is essential (46,83).

Future research should standardize diversity assessment methods, consider functional and clinical outcomes alongside diversity metrics, explore strain-specific mechanisms, and evaluate long-term effects (46). Aligning diversity data with broader health outcomes will provide a clearer understanding of the role of probiotics in promoting gut and overall health (46). Combining microbiome data with metagenomics, metabolomics, and transcriptomics could provide a more comprehensive understanding of probiotic effects (83). Increasing the number of studies would be beneficial, incorporating variations in

probiotic strains, dosages, and intervention durations. This would enable a more comprehensive and reliable assessment of probiotic effects and allow differentiation between specific strains and dosing regimens (46,83). A unified methodological approach would facilitate more reliable and comprehensive conclusions (46,83).

5.5. Strengths and limitations

Our systematic reviews and meta-analyses provide the highest level of evidence, including only randomized controlled trials (46,83). To our knowledge, these are the first studies conducting both qualitative and quantitative synthesis on these specific topics. We followed Cochrane's recommendations and the PRISMA Statement with strict methodology (46,83).

Despite including all relevant studies without restrictions, limited data was available. Given the low number of studies, subgroup analysis based on age, gender, probiotics, duration of intervention, or risk of bias assessment was impossible in some cases (46,83). The population's heterogeneity further makes interpretation challenging. These factors should be considered when interpreting the results and highlight the need for further research to better understand the impact of these variables on the outcomes (46,83).

VI. Conclusions

The summarized results from the currently available randomized controlled trials do not support probiotic supplementation as an effective strategy to modify gut microbiome diversity during antibiotic treatment or in healthy populations. In these contexts, the meta-analyses of the most common diversity indices, including Shannon, Chao1, Observed OTUs, and Simpson's Index of Diversity, revealed no significant effect of probiotics on modulating or increasing microbial diversity. While not all reported outcomes could be analyzed quantitatively, the strong overall trend across studies suggests a lack of influencing effect on both α- and β-diversity metrics. Furthermore, our meta-analysis of five studies with 307 healthy individuals revealed no significant effect of probiotics on circulating zonulin levels.

There is a strong need for standardized normal ranges and consistent reporting of diversity metrics to support more robust and comparable analyses. A consensus for appropriate methods and clinically important outcomes is critical for further research. Studies should focus on the potential clinical relevance of probiotics in specific populations and on understanding the functional impacts of microbiota modulation.

VII. Summary

Probiotics are widely used to support gut health, particularly in maintaining microbiome diversity and intestinal barrier integrity. However, their effectiveness remains uncertain. Three recent systematic reviews and meta-analyses examined the impact of probiotics on gut microbiome composition during antibiotic therapy, in healthy individuals, and on intestinal permeability measured by blood zonulin levels.

The systematic review and meta-analysis of randomized controlled trials assessed whether concurrent probiotic supplementation mitigates antibiotic-induced microbiome alterations. Among 11,769 screened articles, 15 were eligible for qualitative synthesis, and five were included in the meta-analysis. The pooled results for Shannon, Chao1, and Observed OTUs indices showed no statistically significant differences between probiotic-supplemented and control groups. Additionally, most studies reported no meaningful differences in other α- and β-diversity indices. Although taxonomic shifts varied, microbiome composition tended to return to baseline levels in both groups after 3–8 weeks. The limited number of studies and variations in antibiotic and probiotic regimens were key limitations.

In the second meta-analysis, we evaluated the effect of probiotics on microbiome diversity in healthy individuals. A systematic search identified 47 eligible studies, with 22 (1,068 individuals) included in the meta-analysis. No statistically significant effects were observed for Shannon, Observed OTUs, Chao1, or Simpson's Diversity Index. These findings suggest that probiotic supplementation does not alter microbiome diversity in healthy populations.

The third meta-analysis explored the potential of probiotics to improve intestinal barrier integrity by assessing blood zonulin levels. A systematic search identified 9,217 articles, with data from 307 individuals across five studies included in the meta-analysis. The results revealed no significant difference in blood zonulin concentrations between probiotic and control groups, indicating that probiotic supplementation does not affect intestinal permeability.

Collectively, these findings challenge the widespread assumption that probiotics significantly modulate microbiome diversity or enhance intestinal barrier function. Further studies are needed to determine the contexts in which probiotics may provide meaningful benefits, particularly regarding strain-specific and condition-specific effects.

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IX. Bibliography of the candidate's publications

9.1. Publications related to the topic of the dissertation

The cumulated impact factor of the indicated journals (based on the latest data currently known): **7.1**

1. **Éliás AJ,** Barna V, Patoni C, Demeter D, Veres DS, Bunduc S, Erőss B, Hegyi P, Földvári-Nagy L, Lenti K. Probiotic supplementation during antibiotic treatment is unjustified in maintaining the gut microbiome diversity: a systematic review and meta-analysis. BMC Med. 2023;21(1):262. doi: 10.1186/s12916-023-02961-0

2. Földvári-Nagy KCs, Simon V, Csősz Cs, Schnabel T, Veres DS, Lenti K, Földvári-Nagy L, **Éliás AJ**. The effect of probiotic supplementation on zonulin levels in healthy individuals – A systematic review and meta-analysis. Developments in Health Sciences. in press Paper: in press (2025) doi: 10.1556/2066.2025.00081

9.2. Publications unrelated to the topic of the dissertation

The cumulated impact factor of the indicated journals (based on the latest data currently known): **8.6**

1. Gul L, **Elias AJ**, Tambaku T, Olbei M, Watters E, Bohar B, Modos D, Madgwick M, Korcsmaros T. Protocol for predicting host-microbe interactions and their downstream effect on host cells using MicrobioLink. STAR Protoc. 2025;6(1):103570. doi: 10.1016/j.xpro.2024.103570

2. Pokhilenko I, Afentou N, Fu L, Hiligsmann M, Witthoft C, Hefni M, Nzefa LD, Randelli F, **Elias AJ**, Bartos K, Csajbókné Csobod É, Ouguerram K, Parnet P, Ruiz-de-Maya S, Ferrer-Bernal E, Frew E. What's for lunch? Eliciting preferences for food on university campus: discrete choice experiment protocol [Preprint, not peer-reviewed]. *Research Square*. 2024 Jun 7. Available from: https://doi.org/10.21203/rs.3.rs-4436883/v1.

3. **Éliás AJ**, Bodor Z, Benedek C. Xanthohumol (*Humulus lupulus* L.): Potential of bioactive polyphenols in beer. In: Goyal MR, Nath A, Kovács Z, editors. Sustainable and Functional Foods from Plants: Health Impact, Bioactive

Compounds, and Production Technologies. 1st ed. Boca Raton: CRC Press; 2024. p. 253–90.

4. **Éliás AJ,** Balogh L, Brányik T, Mák E, Csajbókné Csobod É, Veresné Bálint M, Benedek C. Xanthohumol-Enriched Beer Does Not Exert Antitumorigenic Effects on *HeLa* Cell Line *In Vivo*. Molecules. 2023;28(3):1070. doi: 10.3390/molecules28031070.

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6. **Éliás AJ**, Juhász R, Hajas L, Benedek C. *In vitro* study of the protein digestibility of various lentil-based gluten-free biscuits. Új Diéta: A Magyar Dietetikusok Lapja (2001-). 2022;31(1):22-25. Hungarian.

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Table S1. Study characteristics - The effect of probiotic supplementation on the gut microbiome during antibiotic treatment

Study	Country	Study design*	Population		Antibiotic (and additional) treatment			Probiotic supplementation		Microbiome analysis method
			Number of randomized patients (female %)	Age (years - mean ± SD) in the intervention (and control) groups	Indication	Type and dose	Duration (days)	Type and dose	Duration (days)	
Amarri (2008)	Italy	open-label, national, parallel RCT	58 (50)	40 ± 18.9 months (42.1 ± 18.9 months)	Bacterial upper respiratory tract infections	amoxicillin 50 mg/kg/day divided in 3 daily doses	5-10	antibiotic-resistant *Bacillus clausii* 2 x $2x10^9$ CFU/day	12-17	PCR-DGGE
Cárdenas 2020)	Ecuador	single-blinded RCT	38 (60.5)	37.9 ± 7.2 (39.5 ± 10.7)	*Helicobacter pylori* infection	amoxicillin 1 g tid, tinidazole 1 g qid, omeprazole 40 mg bid	14	*Saccharomyces boulardii CNCM I-745* $22.5x10^9$ CFU/day	14	16S rRNA sequencing
Chen et al. (2018)	China	open-label RCT	70 (78.5)	43.89 ± 12.50 (43.20 ± 12.45)	*Helicobacter pylori* infection	pantoprazole 40 mg, amoxicillin 1000 mg, furazolidone 100 mg, colloidal bismuth pectin 0.4 g, bid	14	*Clostridium butyricum* 3 x 40 mg/day	14	16S rRNA sequencing
De Wolfe (2018)	USA	double-blinded, placebo-controlled RCT	31 (N.D.)	N.D. (N.D.)	*Clostridium difficile* infection	standard of care antibiotics (*vancomycin, metronidazole, or fidaxomicin*)	28	*Lactobacillus acidophilus NCFM® (ATCC 700396), Lactobacillus paracasei Lpc-37 (ATCC SD5275), Bifidobacterium lactis Bi-07 (ATCC SC5220), and Bifidobacterium lactis Bl-04 (ATCC SD5219).* $1.7x10^{10}$ CFU/day	28	16S rRNA sequencing
Kabbani (2017)	USA	open-label RCT	24 (59)	N.D. (N.D.)	Healthy volunteers no indication	amoxicillin-clavulanate 875/125 mg, bid	7	*Saccharomyces boulardii* (SB) CNCM I-745 (syn. CBS 5926) 2 x 500 mg/day	14	16S rRNA gene pyrosequencing (bTEFAP).
Kakiuchi	Japan	open-label	65 (44.6)	15.31 ± 0.32	*Helicobacter*	vonoprazan 20 mg,	7	*Enterococcus faecium 129 BIO 3B-R.*	7	16S rDNA sequencing

(2020)		RCT		(15.08 ± 0.28)	*pylori* infection	amoxicillin 750 mg, clarithromycin 400 mg bid		3 tablets/day		
MacPherson. (2018)	Canada	double-blinded, placebo-controlled RCT	70 (N.D.)	N.D. (N.D.)	Healthy volunteers no indication	amoxicillin trihydrate 875 mg, potassium clavulanate 125 mg	7	*Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 $3.8x10^9$ CFU and $0.2x10^9$ CFU/day	14	16S rRNA gene amplicon, shotgun metagenomics sequencing
Oh (2016)	Korea	RCT	20 (30)	51.7 ± 0.79 (49.3 ± 3.56)	*Helicobacter pylori* infection	clarithromycin 500 mg, amoxicillin 1000 mg, lansoprazole 30 mg bid	14	*Streptococcus faecium* and *Bacillus subtilis* 2 x ($9x10^8$ and $1x10^8$)/day	14	16S rRNA gene-pyrosequencing
Tang (2021)	China	placebo-controlled, multi-center RCT	151 (34.4)	43.29 ± 11.30 (45.32 ± 10.98)	*Helicobacter pylori* infection	esomeprazole 20 mg, amoxicillin 1000 mg furazolidone 100 mg, bismuth potassium citrate 220 mg bid	14	*Enterococcus faecium* and *Bacillus subtilis* 3 x ($4.5x10^8$ and $5.0x10^7$) CFU/day	28	16S rRNA high-throughput sequencing
Zhong (2021)	China	open-label parallel RCT	42 (52.4)	all neonates (all neonates)	15 neonates with neonatal pneumonia 5 neonates with urinary tract infection 35 neonates with non-specific infection	piperacillin–tazobactam 100 mg/kg bid	7	*Bifidobacterium longum, Lactobacillus acidophilus,* and *Enterococcus faecalis* 3 x $1.0×10^7$ CFU/day	7	high-throughput sequencing of 16S rRNA amplicons
Engelbrektson (2009)	USA	placebo-controlled RCT	40 (77.5)	36.5 ± N.D. (39.5 ± N.D.)	Healthy volunteers – no indication	augmentin (amoxicillin and clavulanic acid) 875 mg bid	7	*Bifidobacterium lactis* Bl-04 ($5x10^9$ CFU), *Bifidobacterium lactis* Bi-07 ($5x10^9$ CFU), *Lactobacillus acidophilus* NCFM ($5x10^9$ CFU) *Lactobacillus paracasei* Lpc-37 ($5x10^9$ CFU) and *Bifidobacterium bifidum* Bb-02 ($5x10^8$ CFU) 2 x $2.05x10^{10}$ CFU/day	21	DNA-based TRFLP analysis and culture-based microbiological techniques

Forssten (2014)	Finland	double-blinded, parallel RCT	80 (50)	33.7 ± 9.4 (30.9 ± 10.3)	Healthy volunteers – no indication	amoxicillin 875 mg, clavulanate 125 mg	7	*Lactobacillus acidophilus (L. acidophilus) ATCC 700396 and Bifidobacterium animalis (B. animalis) ssp. Lactis ATCC SD5220 (Danisco)* $12.5x10^9$ and $12.5x10^9$ CFU/day	14	qPCR and flow cytometry
Madden. (2005)	UK	pilot-scale, double-blinded RCT	13 (53.8)	60 ± N.D. (49 ± N.D.)	*Helicobacter pylori* infection	amoxycillin 500 mg qid, metronidazole 400 mg tid, lansoprazole 30 mg bid	8	*Lactobacillus acidophilus (CLT60 and CUL21) and two strains of Bifidobacterium bifidum (CUL17 and B. bifidum Rhodia* $2.5x10^{10}$ CFU/day	14	culture-based microbiological techniques
Plummer (2005)	UK	double-blinded RCT	155 (N.D.)	N.D. N.D.	*Helicobacter pylori* infection	amoxicillin 1 g bid, clarithromycin 500 mg bid, lansoprazole 30 mg bid in case of penicillin allergy metronidazole 400 mg tid was substituted	7	*Lactobacillus acidophilus* (CUL60 and CUL21) and two strains of *Bifidobacterium spp* $2.5x10^{10}$ CFU/day	21	culture-based microbiological techniques
Wang (2017)	China	double-blinded RCT	20 (45)	37.1 ± 12.3 (42.8 ± 13.8)	*Helicobacter pylori* infection	esomeprazole 20 mg bid, amoxicillin 1000 mg bid, clarithromycin 500 mg bid tinidazole 500 mg bid	14	*Saccharomyces boulardii* CNCM I-745® 2 x 500 mg	14	culture-based microbiological techniques

Abbreviations: RCT: randomized controlled trial; USA: United States of America; UK: United Kingdom; N.D.: no data; bid: twice a day; tid*:* three times a day; qid: four times a day; bTEFAP: bacterial tag–encoded FLX amplicon pyrosequencing; DNA: deoxyribonucleic acid; TRFLP: terminal restriction fragment length polymorphism; qPCR: quantitative real-time polymerase chain reaction PCR-DGGE: polymerase chain reaction denaturing gradient gel electrophoresis; rRNA – ribosomal ribonucleic acid; CFU – Colony Forming Unit

*If not otherwise mentioned, the studies were single centers.

Source: Éliás et al. (46)

Table S2. Changes in the microbiome α-diversity indices as measured after the antibiotic treatment – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment

Study	Shannon diversity index	Chao1 index	Observed OTUs	Pielou's evenness	Strong's dominance index	ACE index	Faiths Phylogenetic Diversity
Cárdenas et al. (2020)							
Chen et al. (2018)							
de Wolfe et al. (2018)							
Kabbani et al. (2017)							
Kakiuchi et al. (2020)							
MacPherson et al. (2018)							
Oh et al. (2016)							
Tang et al. (2021)							
Zhong et al. (2021)							

	no significant difference in α-diversity between groups
	there is a higher α-diversity in the intervention group (antibiotics + probiotics)
	there is a higher α-diversity in the control group (antibiotics only)
	not applicable*

*If a study did not investigate a specific outcome, "not applicable" is indicated.

Abbreviations: OTU: operational taxonomic unit; ACE: Abundance-based coverage estimator

Source: Éliás et al. (46)

Table S3. Changes in the microbiome β-diversity indices as measured after the antibiotic treatment – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment

Study	Bray-Curtis (dis)similarity index	Euclidean distance	Jaccard similarity coefficient	Canberra distance	Weighted UniFrac distance	Unweighted UniFrac distance
Cárdenas et al. (2020)						
Engelbrektson et al. (2009)						
de Wolfe et al. (2018)						
Kakiuci et al. (2020)						
MacPherson et al. (2018)						
Tang et al. (2021)						

	no significant difference in ß-diversity between groups
	there is an improved ß-diversity in the intervention group (antibiotics + probiotics)
	there is an improved ß-diversity in the control group (antibiotics only)
	not applicable*

*If a study did not investigate a specific outcome, “not applicable” is indicated.

Abbreviations: UniFrac: unique fraction metric

Source: Éliás et al. (46)

Table S4. The summarized results of taxonomic analysis of microbiome composition as measured immediately at the end of simultaneous antibiotic and probiotic treatment – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment

Study	Changes in the intervention group	Changes in the control group	Comparison of the two groups immediately after cessation of the simultaneous antibiotic and probiotic treatment
Studies with sequencing methods outcomes			
Cárdenas et al. (2020)	↑ Family: *Clostridiales, Lachnospiracea* Genus: *Bacteroides, Prevotella Lactobacillus* ↓ Family: *Ruminococcaceae* Genus: *Bacteroides* and other undefined genera OTUs	No difference observed	Family: A higher abundance of *Enterobacteriaceae* was found in the intervention group, compared to control group.

Chen et al. (2018)	↑ Phylum: *Proteobacteria, Cyanobacteria, Actinobacteria* Species: *Clostridium butyricum* (not statistically significant) ↓ Phylum: *Firmicutes, Bacteroidetes, Verrucomicrobia, Tenericutes* *Bacteroidetes:Firmicutes* ratio Class: *Fusobacteria*	↑ Phylum**:** *Proteobacteria, Cyanobacteria* Family: *Enterobacteriaceae, Leuconostocaceae* Species: *Lactococcus raffinolactis, Lactobacillus sakei, Acinetobacter baumannii* NIPH60 ↓ Phylum: *Firmicutes, Bacteroidetes, Verrucomicrobia, Lentisphaerae* *Bacteroidetes:Firmicutes* ratio Family: *Lachnospiraceae, Ruminococcaceae, Rikkenellaceae, Christensenellaceae, Peptococcaceae, Clostridiales* Family XI., *Victivallaceae*	Species: *Clostridium butyricum* in probiotic group was significantly higher compared to control group.
De Wolfe et al. (2018)	↓ Genus*: Bacteroides*	N.D.	N.D.
Kabbani et al. (2017)	↑ Genus: *Escherichia, Parabacteroides, Enterobacter, Odoribacter, Stenotrophomonas*	↑ Genus: *Escherichia, Parabacteroides, Enterobacter* ↓ Genus: *Roseburia, Ruminococcus*	Genus*: Ralstonia* and *Proprionibacterium* levels were higher in probiotic group, whereas *Parabacteroides* levels were higher in the antibiotic group. The increase in *Escherichia* prevalence was less (but not statistically significant) in the probiotic group.

Kakiuchi et al. (2020)	↑ Genus: *Blautia*	↓ *Genus*: *Collinsella* and *Bifidobacterium*	N.D.
MacPherson et al. (2018)	↑ Family: *Bacteroidaceae Enterobacteriaceae, Porphyromonadaceae* ↓ Family: *Coriobacteriaceae, Peptostreptococcaceae, Lachnospiraceae, Ruminococcaceae*, unidentifed *Clostridiales*	↑ Family: *Bacteroidaceae Enterobacteriaceae, Porphyromonadaceae* ↓ Family: *Coriobacteriaceae, Peptostreptococcaceae, Lachnospiraceae, Ruminococcaceae*, unidentifed *Clostridiales*	Family: In the family of *Porphyromonadaceae* (specifically the genus *Parabacteroides*) there was an increase in the probiotic group compared to the control.
Oh et al. (2015)	↑ Phylum: *Proteobacteria* Genus: *Escherichia* ↓ Phylum: *Firmicutes*	↑ Phylum: *Proteobacteria* Genus: *Escherichia* ↓ Phylum: *Firmicutes*	Phylum: The changed proportions of the phyla were larger in the antibiotics group than in the probiotics group. Genus: Changed genera were larger in the antibiotics group.
Tang et al. (2020)	↑ Phylum: *Proteobacteria* Genus: *Shigella, Klebsiella, Streptococcus, Veillonella, Enterococcus, Citrobacter, Oscillospira* Phylum: *Firmicutes, Baceriodetes* ↓ *Bacteroidetes:Firmicutes* ratio	↑ Phylum: *Proteobacteria* Genus: *Shigella, Klebsiella, Streptococcus, Veillonella, Dialister, Anaerotruncus, and Megasphaera* Phylum: *Firmicutes, Baceriodetes* ↓ Genus: *Bacteroides, Faecalibacterium, Roseburia, Phascolarctobacterium, and Blautia*	N.D.

	Genus: *Bacteroides, Faecalibacterium, Roseburia, Phascolarctobacterium, Blautia*		
Zhong et al. (2021)	N.D.	 Phylum: *Actinobacteria* Genus: *Bifidobacterium, Erysipelatoclostridium, Blautia, Lactobacillus, Clostridium_sensu_stricto_1, Peptoclostridium, Propionibacterium, Staphylococcus, Parabacteroides, Bacillus, Bifdobacterium, Lactobacillus*	Phylum: Increased relative abundance of *Actinobacteria* and *Proteobacteria* were found in the probiotic group Genus: Higher relative abundance of *Bifidobacterium* was found in the probiotic group.
Engelbrektson et al. (2009)	N.D.	 Relative increase Family: *Enterobacteraceae* Genus: *Clostridium, Eubacterium, Bacteroides*  Relative decrease Genus: *Bifidobacterium* No statistical difference between the mean bacterial counts before antibiotic treatment compared to the days after antibiotic treatment was	Family: Counts on MacConkey agar (*Enterobactereaceae*) were significantly higher in the probiotic group. Genus: The counts on *Bifidobacterium* iodoacetate medium agar (*Bifidobacterium*) were significantly higher in the probiotic group).

		observed due to large subject-to-subject variability.	
Studies with bacterial count outcomes (standard microbiological methods)			
Madden et al. (2005)	↓ Total anaerobes	↑ Total facultative anaerobes Family*: Enterobactereaceae*	Genus: Numbers of *Bacteroides* in probiotic group were significantly lower compared to the control group.
Plummer et al. (2005)	↑ Yeast Total bacterial count ↓ Family: *Enterobacteriaceae* Genus: *Bacteroides, Bifidobacteria*	↑ Yeast, *Candida albicans* Total bacterial count, Total facultative anaerobes ↓ Family: *Enterobacteriaceae* Genus: *Bacteroides, Bifidobacteria, Lactobacilli*	Yeast: The number of *Candida albicans* in the placebo group was significantly higher than in the probiotic group.
Forssten et al. (2014)	↑ Family: *Enterobacteriaceae* Total bacterial counts, *Clostridium cluster XIV* ↓	↑ Family: *Enterobacteriaceae* Total bacterial counts, *Clostridium cluster XIV* ↓	Species: The probiotic group had significantly higher levels of *Bifidabecterium lactis* and *Lactobacillus acidophilus* ATCC 700396 compared to the placebo group.
Wang et al. (2017)	↑ Number of total aerobs Genus*: Enterococcus*	↑ Yeast Number of total aerobs Genus*: Enterococcus*	N.D.

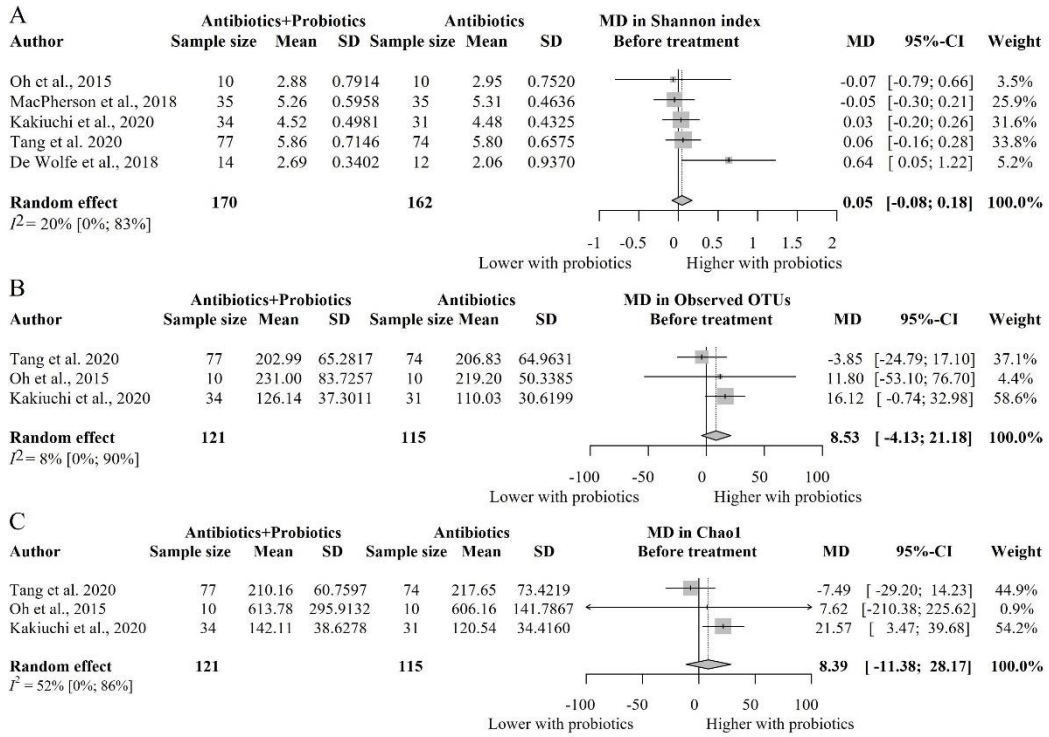
Amarri et al. (2008)	No significcant difference	No significant difference	N.D.

Legends: ↑: increase in the number/proportion of given taxonomic group; ↓: reduction in the number/proportion of given taxonomic group

Abbreviations: *N.D.*: no data

Source: Éliás et al. (46)

Figure S1. Additional sensitivity analyses for the baseline values of Shannon, Observed OTUs and Chao1 diversity indices - The effect of probiotic supplementation on the gut microbiome during antibiotic treatment

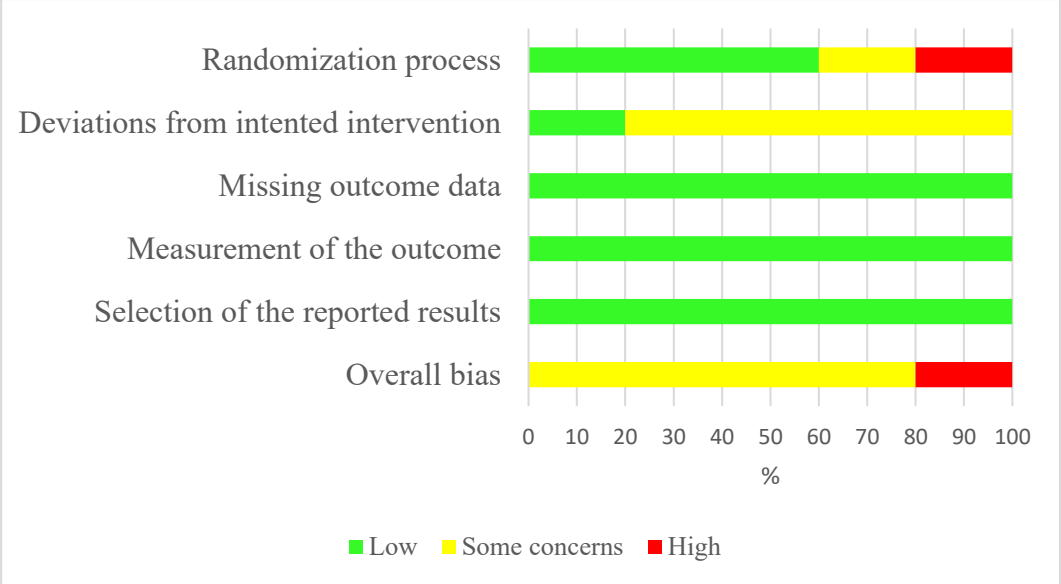


A: Shannon diversity index, B: Observed OTUs, C: Chao1 index

Abbreviations: SD: standard deviation CI: confidence interval OTU: Operational Taxonomic Unit

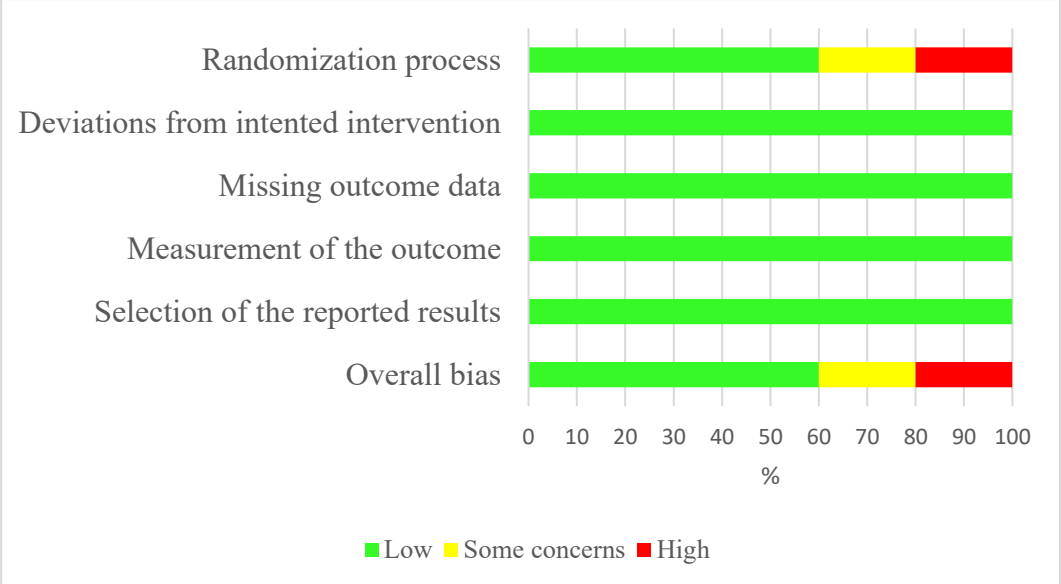
Source: Éliás et al. (46)

Figure S2. Risk of bias assessment for the main meta-analysis of Shannon diversity index - Assignment to intervention (the "intention-to-treat" effect) (n=5) – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment



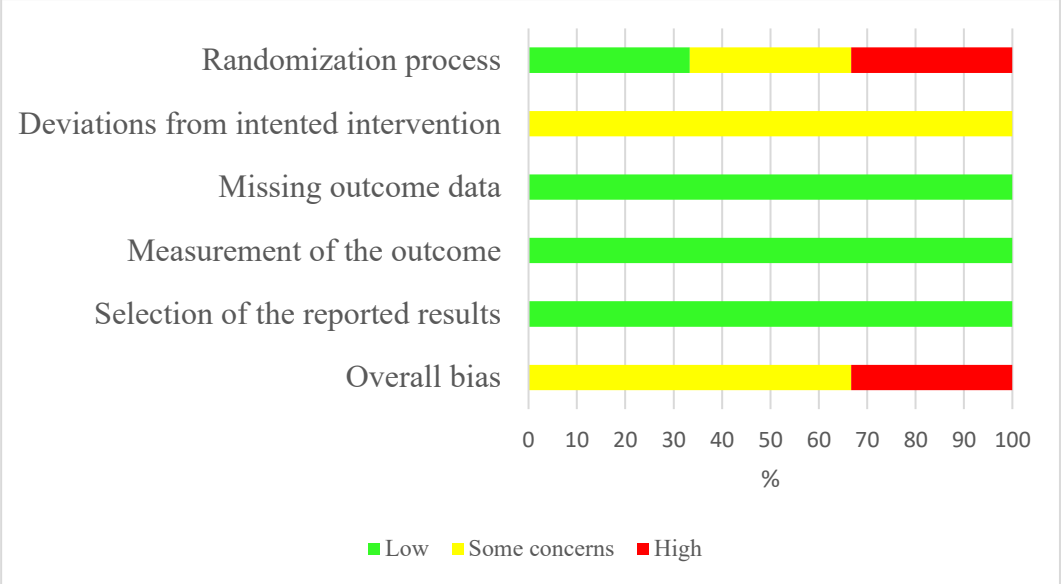
Source: Éliás et al. (46)

Figure S3. Risk of bias assessment for the main meta-analysis of Shannon diversity index - Adhering to intervention (the "per-protocol" effect) (n=5) – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment



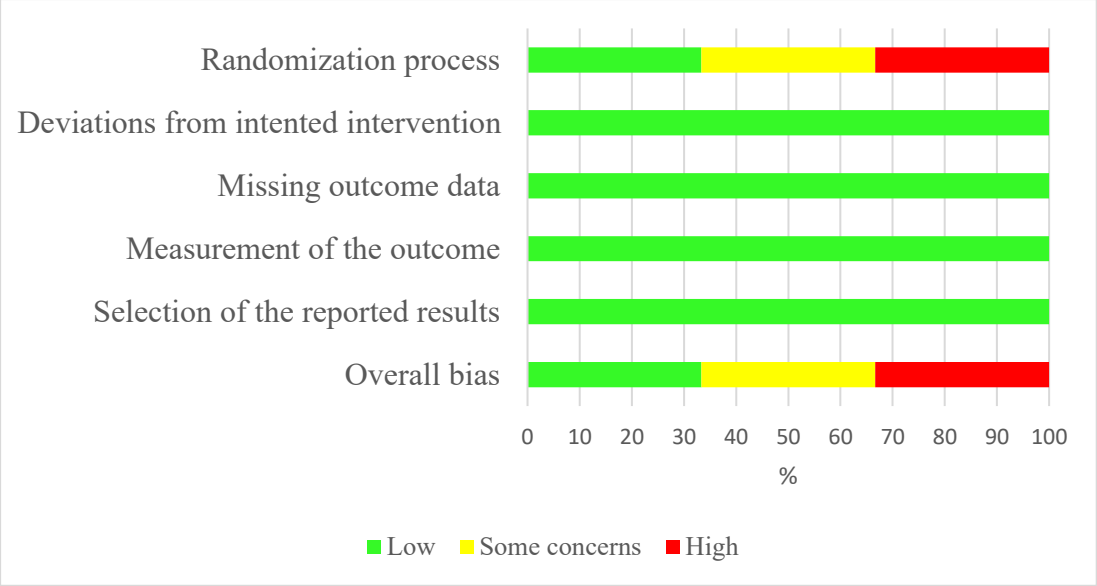
Source: Éliás et al. (46)

Figure S4. Risk of bias assessment for the meta-analysis of Chao1 index - Assignment to intervention (the "intention-to-treat" effect) (n=3) – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment



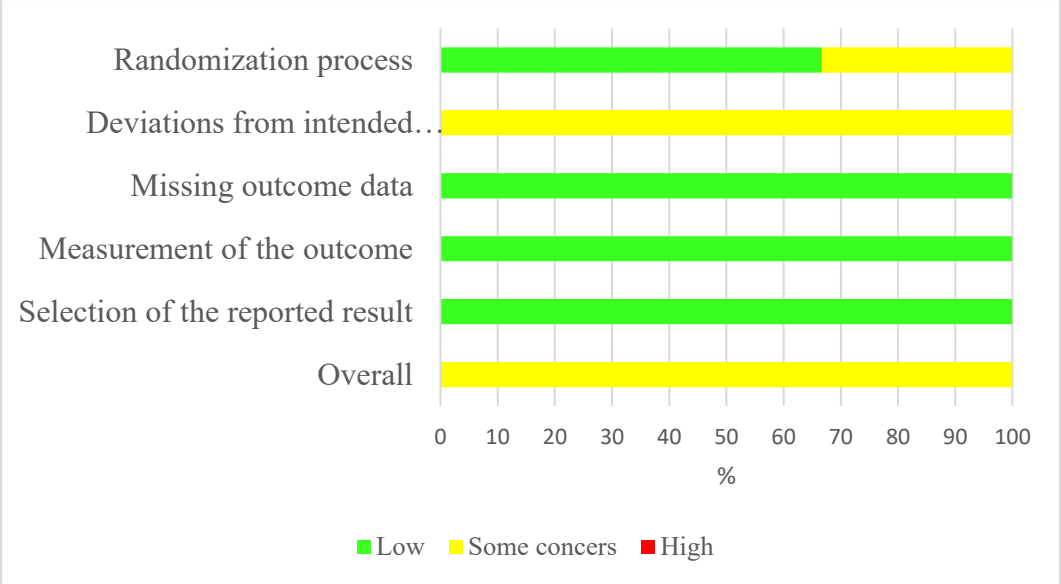
Source: Éliás et al. (46)

Figure S5. Risk of bias assessment for the meta-analysis of Chao1 index - Assignment to intervention (the "intention-to-treat" effect) (n=3) – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment



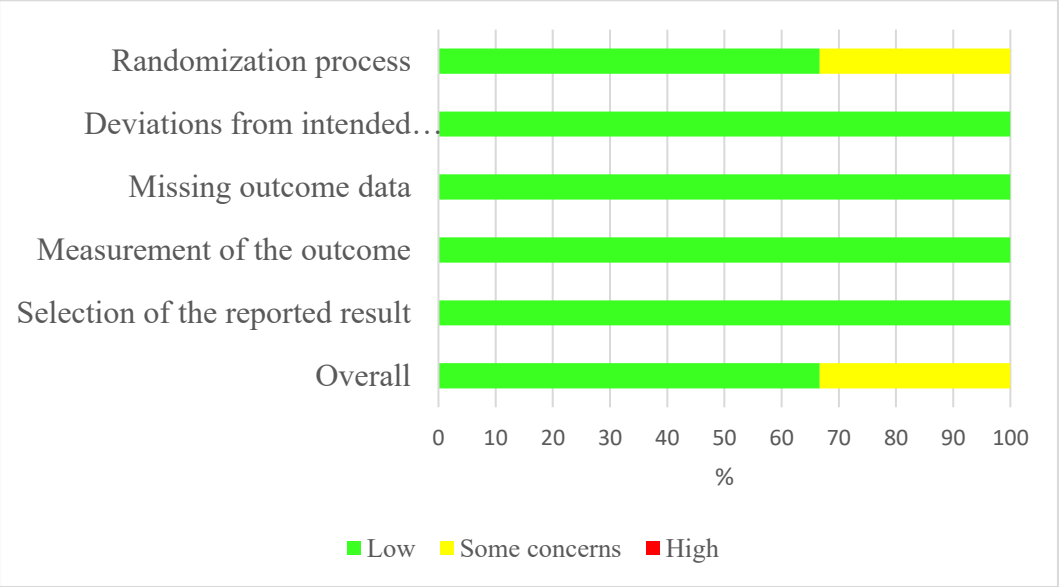
Source: Éliás et al. (46)

Figure S6. Risk of bias assessment for the meta-analysis of Observed OTUs - Assignment to intervention (the "intention-to-treat" effect) (n=3) – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment



Source: Éliás et al. (46)

Figure S7. Risk of bias assessment for the meta-analysis of Observed OTUs - Adhering to intervention (the "per-protocol"' effect) (n=3) – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment



Source: Éliás et al. (46)

Table S5. GRADE assessment for the meta-analyses of Shannon, Chao1 and Observed OTUs diversity indices – The effect of probiotic supplementation on the gut microbiome during antibiotic treatment

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	probiotics	no probiotics	Relative (95% CI)	Absolute (95% CI)		
Shannon diversity index (follow-up: range 7 days to 28 days; assessed with: sequencing; Scale from: 0 to 10)												
5	randomised trials	serious	serious	not serious	serious	none	171	164	-	MD **0.23 Shannon diversity index higher** (0.06 lower to 0.51 higher)	⊕⊕◯◯ Low	IMPORTANT
Observed OTUs (follow-up: range 7 days to 14 days; assessed with: sequencing; Scale from: 0 to 1000)												
3	randomised trials	not serious	serious	not serious	serious	none	121	115	-	MD **17.15 Observed OTUs higher** (9.43 lower to 43.73 higher)	⊕⊕◯◯ Low	IMPORTANT
Chao1 index (follow-up: range 7 days to 14 days; assessed with: sequencing; Scale from: 0 to 1000)												
3	randomised trials	serious	serious	not serious	serious	none	121	115	-	MD **11.59 Chao1 index higher** (18.42 lower to 41.6 higher)	⊕⊕◯◯ Low	IMPORTANT

CI: confidence interval; **MD:** mean difference (46)

Table S6. Study characteristics - The effect of probiotic supplementation on the gut microbiome in healthy populations

Study	Country	Study design (No. of centers*)	Population (Randomized)					Probiotic type (as reported in each study**) and dose	Reclassified probiotic nomenclature (if applicable)	Placebo type and dose	Duration (days)	Wash-out period (days) if applicable
			Number of randomized subjects (female %)	Number of subjects in probiotic group	Number of subjects in control group	Age (years - mean ± SD) in the intervention (and control) groups	Specification of the population					
Axelrod (2019)	USA	randomized, double-blind, placebo-controlled crossover study	9 (N.D.)	5	4	Whole population: 31 ± 2.3	healthy, trained endurance athletes	*Lactobacillus salivarius* UCC118 (2 x 10^8 CFU/day)	*Ligilactobacillus salivarius* UCC118	200 mg corn starch with magnesium stearate	28 days	28 days
Bagga (2018)	Austria	randomized, double-blind, placebo-controlled clinical trial	30 (47)	15	15	28.27 ± 4.2 (27.25 ± 5.78 and 26.87 ± 4.97) *	healthy volunteers	*Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Bifidobacterium lactis* W51, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, *Bifidobacterium lactis* W52, *Lactobacillus plantarum* W62 and *Bifidobacterium bifidum* W23 (7.5x10^9 CFU/g x 3 / day)	*Lacticaseibacillus casei* W56 *Lacticaseibacillus paracasei*W20 *Ligilactobacillus salivarius* W24 *Bifidobacterium animalis subsp. lactis* W19 *Lactiplantibacillus plantarum* W62	3 g maize starch and maltodextrins	28 days	NA
Bazanella (2017)	Germany	double-blind, randomized, and placebo controlled clinical trial	97 (64)	48	49	newborns (newborns)	healthy infants	control formula plus a total concentration of 10^8 CFU/g with equal amounts of *Bifidobacterium bifidum* BF3, *Bifidobacterium breve* BR3, *Bifidobacterium longum subspecies infantis* BT1, and *Bifidobacterium longum* BG7	NA	whey based infant formula	1 year	NA

Bloemendaal (2021)	The Netherlands	exploratory analysis of a double-blind, randomized, placebo-controlled study	67 (100)	31 (MITT)	33 (MITT)	NA *criteria: 18-40	healthy female subjects	*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, and *Lactococcus lactis* W58 (2.5×10^9 CFU/g x 2 / day)	*Bifidobacterium animalis subsp. lactis* W51 and W52*Levilactobacillus brevis* W63 *Lacticaseibacillus casei* W56 *Ligilactobacillus salivarius* W24	2 g maize starch, maltodextrin, vegetable protein and a mineral mix	28 days	NA
Boesmans (2018)	Belgium	randomized, double-blind, placebo-controlled crossover trial	30 (53)	15	15	32 Range: 26–45 (28 Range: 25–33)	healthy volunteers	*Butyricicoccus pullicaecorum* 25-3T (10^8 CFU/day)	NA	maltodextrin	28 days	21 days
Castanet (2020)	France, Greece, Austria	multicenter, randomized, double-blind, controlled trial (six sites from three countries)	127 (ITT N=202 with not randomized reference group)	44	40	newborns (newborns)	healthy full-term vaginal born infants	Control formula + native bovine lactoferrin (1 g/l) and *Bifidobacterium animalis subsp lactis* CNCM I-3446 ($3.7 \pm 2.1 \times 10^4$ CFU/g powder formula)	NA	The control formula was designed to match as closely as possible early maternal milk energy and proteins levels	28 days	NA
Chen (2020)	China	double-blinded randomized controlled trial	40 (100)	20	20	22.7 ± 1.5 (23.0 ± 1.4)	healthy males	*Lactobacillus. rhamnosus* GG, *Lactobacillus acidophilus, Bifidobacterium animalis* and *Bifidobacterium longum* (1.32×10^{11} CFU/day)	*Lacticaseibacillus rhamnosus* GG	starch, maltodextrin and sugar	28 days	NA
Chen (2023)	China	three-arm, randomized, double-blind, placebo-controlled study	88 (mITT 86 → 57)	29	31	1.2 (0.5) (month) 1.3 (0.5)	healthy infants	*Lactobacillus salivarius* AP-32 (2.5×10^9 CFU x 2 / day)	*Ligilactobacillus salivarius* AP-32	0.5 g maltodextrin	4 months	NA
				28		1.3 (0.4) (months) 1.3 (0.5)		*Bifidobacterium animalis subspecies lactis* CP-9 (2.5×10^9 CFU.x 2 / day)	NA			
De Andrés (2018)	Spain	seconday analysis of a	219 (ITT) 202 (mITT)	23	23	NA - only for the original population	Infants from 3 to 12 months of age,	*Bifidobacterium infantis* (3×10^9 CFU/day)	*Bifidobacterium longum subsp.*	potato starch	56 days	NA

		randomized, double-blind, placebo controlled, multicenter intervention study (NI)	198 (PP) - subgroup of 92 infants				breastfed and/or formula fed		*infantis*			
	Spain			23				*Lactobacillus helveticus* R0052 (3×10^9CFU/day)	NA			
	Spain			23				*Bifidobacterium bifidum* R0071 (3×10^9CFU/day)	NA			
Ferrario (2014)	Italy	randomized, double-blind, crossover placebo-controlled study	30 (60)	30	30	35 ± 10,7	healthy volunteers	*Lactobacillus paracasei* DG (24 x 10^9 CFU/day)	*Lacticaseibacillus paracasei DG*	placebo (NI)	28 days	28 days
Freedman (2021)	USA	parallel arm, double-blind, randomized, placebo-controlled intervention study	46 (61)	25	21	36.9 ± 12.9 (34.4 ± 13.0)	normal weight to mildly obese healthy adults	*Bacillus subtilis* strain DE111 (1 x 10^9 CFU/day)	NA	maltodextrin	28 days	NA
Gai (2023)	China	single-blind placebo-controlled trial	100 (analyzed 94 → 66)	50	50	only available for the analyzed population 22.6 ± 1.6 (23.0 ± 2.4)	healthy volunteers	*Lacticaseibacillus rhamnosus* strain LRa05 (1×10^{10} CFU/day)	NA	2.0 g maltodextrin	28 days	NA
Gan (2022)	China	randomized, single-blind, placebo-controlled, multicenter clinical trial (2)	100 (analyzed 92 (50)	50	50	only available for the analyzed population 8.4 (8.1)	children with functional constipation according to Rome III criteria.	*Lactobacillus acidophilus* DDS-1 R and *Bifidobacterium animalis subsp. lactis* UABla-12TM (5×10^9 CFU x 2 / day)	NA	hydroxymethyl cellulose magnesium stearate,	28 days	NA
Gargari (2016)	Italy	randomized, double-blind, crossover, and placebo-controlled intervention study	35	35	35	NI for the randomized population	healthy volunteers	*Bifidobacterium bifidum* Bb (3.8 x 10^9 CFU/day)	NA	maltodextrin, cellulose powder, dextrose, a separating agent (magnesium salts of edible fatty acid), and silica	28	28
Hanifi (2015)	USA	double-blinded, placebo-	83 → 41 (59)	21	20	Median and range 23 (20-49) (23 (20-46))	healthy volunteers	*Bacillus subtilis* R0179 (0.1×10^9 CFU/day)	NA	placebo (NI)	28 days	NA

		controlled, randomized trial	83 → 40 (48)	20		Median and range 22 (20-31) (23 (20-46))		*Bacillus subtilis* R0179 (1×10^9 CFU/day)	NA			
			83 → 42 (NI)	22		Median and range NI (23 (20-46))		*Bacillus subtilis* R0179 (10×10^9 CFU/day)	NA			
Hibberd (2018)	Finland	PP subset of a double-blind, randomized, parallel, placebo-controlled clinical trial (4)	225 → PP 134 → 61 (72)	25	36	49.1±11.9 (48.3±8.6)	overweight or obese (body mass index (BMI) 28.0-34.9) but otherwise healthy volunteers	*Bifidobacterium animalis subsp lactis* 420™ (B420) (10^{10} CFU/day)	NA	12 g/day of microcrystalline cellulose	6 months	NA
Hiraku (2023)	Japan	placebo-controlled, double-blinded, randomized trial	111 (only available for compliant participants: 52)	57	54	newborns (newborns)	healthy full-term infants	*Bifidobacterium infantis* M-63 (1×10^9 CFU / 1.0 g of sachet)	*Bifidobacterium longum subsp. infantis* M-63	sterilized dextrin only / 1.0 g of sachet	3 months	NA
Huang (2022)	China	randomized-controlled trial	31 (100)	15	16	analyzed population 27.42 ± 3.09 (27.33 ± 2.90)	Pregnant women before 32 weeks of gestation.	*Bifidobacterium longum* (0.5×10^7 CFU x 4), *Lactobacillus delbrueckii supsp. bulgaricus* (0.5×10^6 CFU x 4), and *Streptococcus thermophilus* (0.5×10^6 CFU x 4) / day	NA	nothing	52 ± 7.08 days.	NA
Kang (2021)	Korea	randomized, double-blind, placebo controlled, parallel-group trial	80 (88)	40	40	(mean ± SE) 44.4 ± 2.2 (45.3 ± 1.8)	modified Rome III functional constipation criteria fulfilling adults, otherwise healthy	Spore-forming *Bacillus coagulans* SNZ 1969 (1.0×10^9 CFU/day)	*Heyndrickxia coagulans* SNZ 1969	maltodextrin	56 days	NA
Kim (2021)	Korea	randomized, double-blind, placebo-controlled, multicenter clinical trial (2)	63	32	31	NI for the randomized population only for analyzed population n=27 71.11 ± 5.02 (n=26 72.00 ± 3.36)	community-dwelling older adults (65+)	*Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI in soybean oil (1×10^9 CFU / day)	NA	500 mg of soybean oil	84 days	NA
Lau (2018)	Malaysia	randomised, double-blind, parallel and placebo-controlled	520 (52)	259	261	4.2±1.3 (4.1±1.3)	healthy pre-school children aged 2-6 years	*Bifidobacterium longum* BB536 (5×10^9 CFU)	NA	maltodextrin (1g)	10 months	NA

		study										
Lee (2021)	Korea	randomized, double-blind, placebo-controlled trial PP analysis	156 (NI)	78	78	NI for the randomized population, only for the analyzed population n=63 38.86 ± 10.89 (n=59 37.63 ± 11.04)	healthy adults aged 19 to 65 years with psychological stress and subclinical symptoms of depression or anxiety	*Lactobacillus reuteri* NK33 (2×10^9 CFU x 2 / day) and *Bifidobacterium adolescentis* NK98 (0.5×10^9 CFU x 2 / day)	*Limosilactobacillus reuteri* NK33	500 mg maltodextrin	56 days	NA
Li (2023)	China	single-center, randomized, triple-blind placebo-controlled trial	109 (101 finished → 52%)	51	50	NI (6-24 months)	healthy infants delivered by C-section	*Lactobacillus paracasei* N1115 (2×10^{10} CFU/g)	*Lacticaseibacillus paracasei* N1115	maltodextrin	3 months	NA
López-Garcia (2023)	Spain	randomized, placebo-controlled, single-blind study	39 (51)	20	19	31.45 ± 8.28 (33.63 ± 6.96)	healthy volunteers	*Lactiplantibacillus pentosus* LPG1 (1×10^{10} CFU/day)	*Lactiplantibacillus pentosus* LPG1	dextrose	30 days	NA
Majeed (2023)	India	randomized, double-blind, placebo-controlled trial	30 (63)	15	15	37.67 ± 10.65 (39.50 ± 9.15)	healthy adults	*Bacillus coagulans (Weizmannia coagulans)* microbial type culture collection 5856 (LactoSpore®) (2×10^9 CFU/day)	*Heyndrickxia coagulans*	maltodextrin	28 days	NA
Marcial (2017)	USA	randomized double-blind placebo-controlled parallel study	42 (72)	21	21	Mean and range 23 (18–36) ((21 (18–48))	healthy adults	*Lactobacillus johnsonii* N6.2 (10^5 CFU/day)	NA	NI	56 days	
Michael (2020)	United Kingdom	exploratory, block-randomised, parallel, double-blind, single-centre, placebo-controlled superiority study	220 (60)	110	110	45.30 ± 10.20 46.52 ± 9.93	volunteers with a waist circumference >89 cm (women) or>100 cm (men); a body mass index (BMI, kg/m2) between 25 and 34.92	*Lactobacillus acidophilus* CUL60 (NCIMB 30157*), Lactobacillus acidophilus* CUL21 (NCIMB 30156), *Lactobacillus plantarum* CUL66 (NCIMB 30280) *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis subsp. lactis* CUL34 (NCIMB	*Lactiplantibacillus plantarum* CUL66 (NCIMB 30280)	microcrystalline cellulose	180 days	NA

								30172) on a base of microcrystalline cellulose (5×10^{10} CFU/day)				
Moloney (2021)	Ireland	double-blind, randomized, placebo-controlled, repeated measures, cross-over design.	30 (0)	15 (first)	15 (second)	20.7 (SEM 0.28)	male university students	*Bifidobacterium longum* AH1714 (1×10^9 CFU/day)	NA	corn starch, magnesium stearate, hypromellose, titanium dioxide	56 days	unknown
Moore (2023)	Ireland	single-center, double-blind, placebo-con-trolled, randomized controlled trial	160 (100)	80	80	all 33.6 +/- (3.9)	pregnant women	*Bifidobacterium breve* 702258 (minimum 1×10^9 CFU/day)	NA	standard excipients	from 16 weeks gestation until 3 months postpartum	NA
Mutoh (2024)	Japan	randomized, double-blind, placebo-controlled, parallel-group clinical trial	30 (47)	15	15	46.3 ± 11.6 (47.9 ± 11.6)	volunteers	*Bifidobacterium breve* M-16 V (2×10^9 CFU/day)	NA	maltodextrin	42	NA
Nakamura (2022)	Japan	randomized, double-blind, controlled crossover trial	24 (60)	12	12	47.7 ± 5.8 (all participants)	healthy volunteers with constipation	*Bifidobacterium longum* BB536 (5.0×10^9 CFU/day)	NA	potato starch	14 days	28 days
Pagliai (2023)	Italy	randomized, double-blinded parallel controlled trial	40 (50)	20	20	51 ± 12.7 (51 ± 14.7)	overweight or obese (body mass index (BMI) 28.0-34.9) but otherwise healthy population (BMI ≥ 25 kg/m2)	*Lactiplantibacillus plantarum* IMC 510® (1.5×10^{10} CFU / capsule)	NA	placebo (NI)	3 months	NA
Park (2020)	Korea	double-blind, randomized, placebo-controlled	70 (66)	35	35	48.3 ± 11.6 (8.3 ± 13.2)	healthy volunteers	*Lactobacillus plantarum* LPQ180 (2×400mg / $2 \times 4 \times 10^9$ CFU/day)	*Lactiplantibacillus plantarum* LPQ180	2 x 400 mg maltodextrin	84 days	NA
Paytuvi-Gallart (2020)	Spain	randomised, parallel, double-blind, placebo-controlled study	102 (only available for the at-the-end investigated population)	51	51	only available for the at-the-end investigated population 2-6 years	healthy children attending day-care	*Bacillus subtilis* DE111 (1×10^9 CFU /dose)	NA	dextrose, tapioca maltodextrin, natural flavor and l-leucine	56 days	NA
Plaza-Diaz	Spain	randomized,	25 (36)	4	5	25,5 ± 6,9	healthy volunteers	*Bifidobacterium breve*	NA	NI	30 days	NA

(2015)		placebo-controlled trial				(26,6 ± 3,9)		CNCM I-4035 (9×10^9 CFU/day)				
				5		23,6 ± 4,5 (26,6 ± 3,9)		*Lactobacillus rhamnosus* CNCM I-4036 (9×10^9 CFU/day)	*Lacticaseibacillus rhamnosus* CNCM I-4036			
				4		25,5 ± 4,2 (26,6 ± 3,9)		*Bifidobacterium breve* CNCM I-4035 and *Lactobacillus rhamnosus* CNCM I-4036 (9×10^9 CFU/day)	*Lacticaseibacillus rhamnosus* CNCM I-4036			
				5		27,2 ± 2,1 (26,6 ± 3,9)		*Lactobacillus paracasei* CNCM I-4034 (9×10^9 CFU/day)	*Lacticaseibacillus paracasei* CNCM I-4034_			
Qian (2020)	China	controlled trial	total of 36, in the groups of our interest n=18 (50)	9	9	M ± SEM 55.8±8.8 (51.7±5.0)	healthy volunteers typicalli consuming high-fat diet	*Bifidobacterium longum* ($\geq 1.0 \times 10^7$ CFU/g), *Lactobacillus acidophilus* ($\geq 1.0 \times 10^7$ CFU/g) and *Enterococcus faecalis* ($\geq 1.0 \times 10^7$ CFU/g)	NA	no intervention	4 months	NA
Rahayu (2021)	Indonesia	randomized, double-blind, placebo controlled study	60 (60)	30	30	44.07 ± 6.23 (44.67 ± 5.66)	healthy overweight adults (body mass index (BMI) equal to or greater than 25)	*Lactobacillus plantarum* Dad-13 (2×10^9 CFU/gram/sachet)	*Lactiplantibacillus plantarum* Dad-13	1 g skimmed milk powder	90 days	NA
Sánchez Macarro (2021)	Spain	randomized double-blind and controlled single-center clinical trial	44 (0)	22	22	25.3 ± 7.2 years ((27.1 ± 8.4 years)	caucasian healthy male volunteers who performed aerobic physical exercise between 2 and 4 times a week	*Bifidobacterium longum* CECT 7347, *Lactobacillus casei* CECT 9104, and *Lactobacillus rhamnosus* CECT 8361 (in a ratio 1:4.5:4.5, 1 x 10^9 total CFU /day)	*Lacticaseibacillus casei* CECT 9104 *Lacticaseibacillus rhamnosus* CECT 8361	300 mg capsules with maltodextrin and sucrose	42 days	NA
Sandiogini (2022)	Italy	placebo-controlled, randomized, double-blind, clinical trial	50 (72)	25	25	probiotics: 63:71 ± 5:28 (female) 60:00 ± 3:32 (male) // placebo 60:00 ± 3:32 (female) 60:00 ± 3:32 (male)	flu-vaccinated healthy elderly subjects	*Lactiplantibacillus plantarum subsp. plantarum* (formerly *Lactobacillus plantarum*) PBS067 (1×10^9 CFU), *Bifidobacterium animalis subsp. lactis* BL050 (1×10^9 CFU) *Bifidobacterium longum subsp. infantis* BI221 (1×10^9 CFU), *Bifidobacterium longum subsp. longum* BLG240 (1×10^9	NA	placebo (NI)	28 days	NA

								CFU/day)				
Shi (2020)	China	prospectively randomized controlled	50 (70)	25	25	40:6 ± 11:0 (43:2 ± 12:2)	adults with the gastrointestinal symptoms of abdominal pain, abdominal bloating, abdominal distension, or bowel habit abnormalities (constipation, diarrhea, or mixed constipation and diarrhea)	Medilac-S (live combied *Bacillus subtilis* and *Enterococcus faecium* (500 mg per time, x 3 / day)	NA	no intervention	28 days	NA
Shi (2023)	China	a randomized, double-blind, placebo-controlled trial	60 (58)	30	30	64.10 3.40 (64.50 ± 3.79)	healthy elderly people aged 60–75 years,	*Bifidobacterium longum* BB68S (BB68S, CGMCC No. 14168) (5×10^{10} CFU/sachet)	NA	maltodextrin	56 days	NA
Simon (2015)	Germany	double-blind, 1:1 randomized, prospective, longitudinal pilot trial	21 (52)	11	10	50 +/- 6.7 (all population)	glucose-tolerant volunteers,	*Limosilactobacillus reuteri* SD5865 (2×10^{10} CFU/day)	NA	NI	28 days	NA
Sohn (2021)	South Korea	randomized, double-blind controlled clinical trial	81 (60)	41	40	47.8 ± 11.7 (45.5 ± 10.0)	healthy men and women aged 20 to 65 years with a BMI of 25–30 kg/m2	*Lactobacillus plantarum* K50 (4×10^9 CFU/day)	*Lactiplantibacillus plantarum* K50	microcrystalline cellulose powder,	84 days	NA
Son (2020)	Korea	randomized-controlled trial	20 (0)	10	10	without dropouts 26.50 ± 5.01 (27.14 ± 5.93)	Bodybuilders who consumed an extremely high-protein/low-carbohydrate diet	*Lactobacillus acidophilus*, *Lacticaseibacillus casei, Lactobacillus helveticus*, and *Bifidobacterium bifidum* (10^{12} CFU of each / day)	NA	corn starch	60 days	NA
Tremblay (2021)	United States of America	double-blind, randomized, parallel design study	69 (68 ended -- 63%)	23	23	median and range 22 (18–30) (27 (18–31)(	volunteers	*Lactobacillus helveticus* R0052, *Lactobacillus rhamnosus* R0011, *Lactobacillus casei* R0215, *Pediococcus acidilactici* R1001, *Bifidobacterium breve* R0070, *Bifidobacterium longum subsp. longum* BB536 *Lactobacillus plantarum* R1012,	*Lacticaseibacillus rhamnosus* R0011 *Lacticaseibacillus casei* R0215 *Lactiplantibacillus plantarum* R1012	potato starch, magnesium stearate, and vitamin C	28 days	NA

								Lactococcus lactis subsp. lactis R1058 (5×10^9 CFU/day)				
Washburn (2022)	United States of America	randomized placebo-controlled trial	32 → 30 (50)	16	16	analyzed population 29 (25)	self-reported healthy adults	*Bifidobacterium infantis* (1×10^9 CFU/day)	*Bifidobacterium longum subsp. infantis*	emply gelatine capsules	30 days	NA
Wischmeyer (2024)	United States of America	a randomized, double-blind, placebo-controlled trial	182 (63)	91	91	NI	exposed household contacts (individuals living with someone recently diagnosed with COVID-19)	*Lacticaseibacillus rhamnosus GG. (ATCC 53103)* 10^{10} CFU/capsule (age < five, one capsule daily, age > five, two capsules daily)	NA	325 mg of microcrystalline cellulose	28 days	NA

Abbreviations: NI: no information; NA: not applicable; CFU: Colony Forming Unit; ITT: intention-to-treat; mITT: modified intention-to-treat; PP: per-protocol; subsp.: subspecies

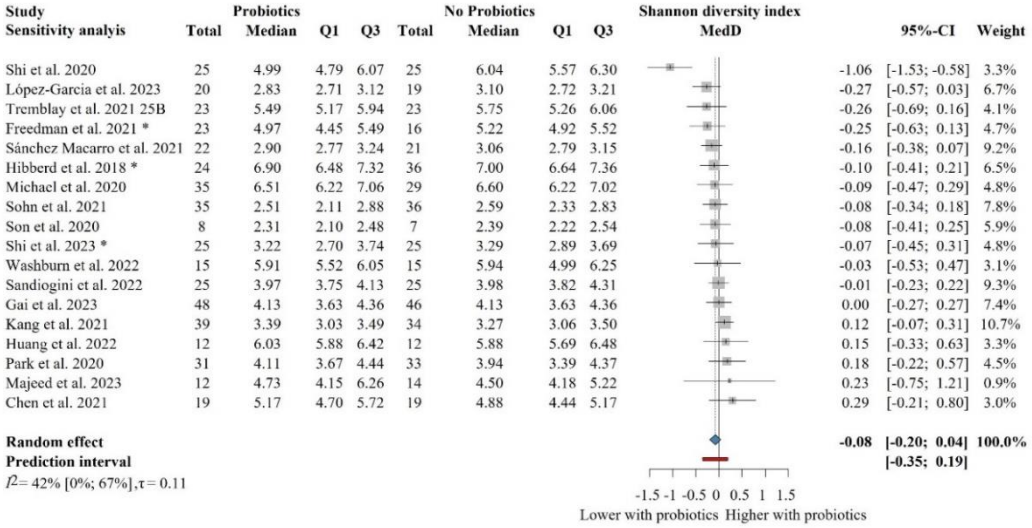
*If not otherwise mentioned, the studies were single centres.

**There has been a major reclassification of bacterial genera, resulting in some articles using the "old" nomenclature while others adopt the updated names. We have included the corresponding information in the baseline table, aligning with the terminology used in each publication. Bacterial strains that have undergone reclassification are underlined, with their updated names, accurate as of January 2025, provided in the following column.

Source: own compilation, unpublished

Figure S6. Additional sensitivity analysis for the more restricted analysis of Shannon diversity index – The effect of probiotic supplementation on the gut microbiome in healthy populations

As a sensitivity analysis, we performed a separate calculation with more restricted inclusion criteria, without studies performed with a cross-over design (140,144) providing change data only (131) or with no clear number of participants (142) (MedD=(-)0.08 [(-)0.20 – 0.04]).

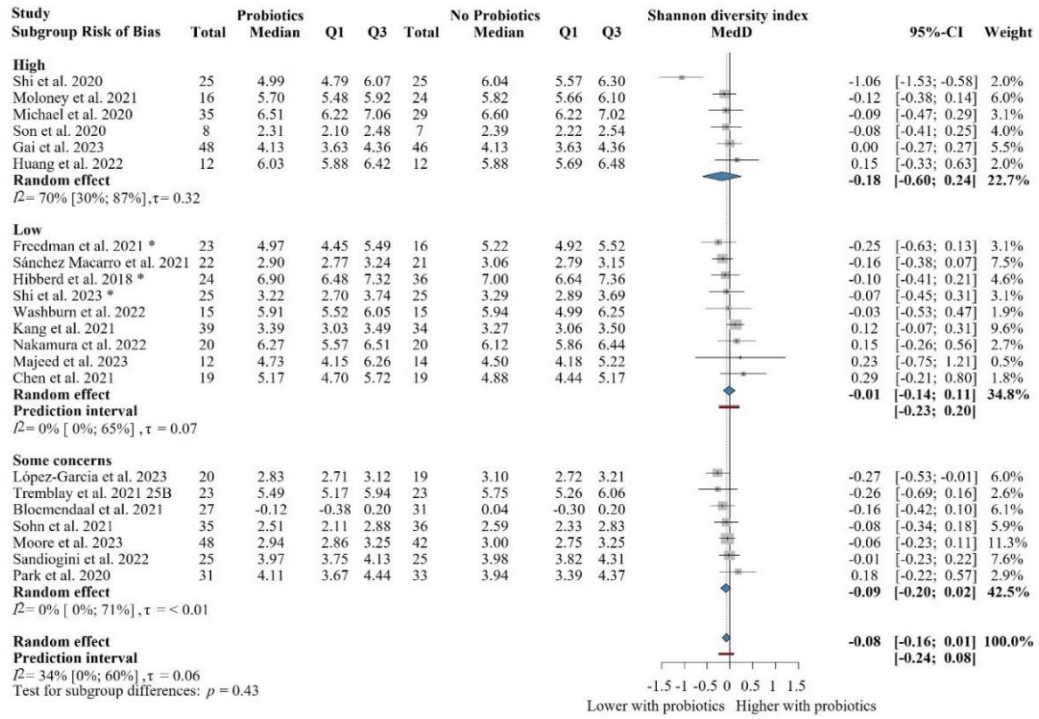


Abbreviations: CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

The "*" indicates that the median and q1, q3 are estimated from mean and standard deviation in that study.

Source: own work, unpublished

Figure S7. Additional sensitivity analysis for the subgroups based on risk of bias assessment for Shannon diversity index – The effect of probiotic supplementation on the gut microbiome in healthy populations

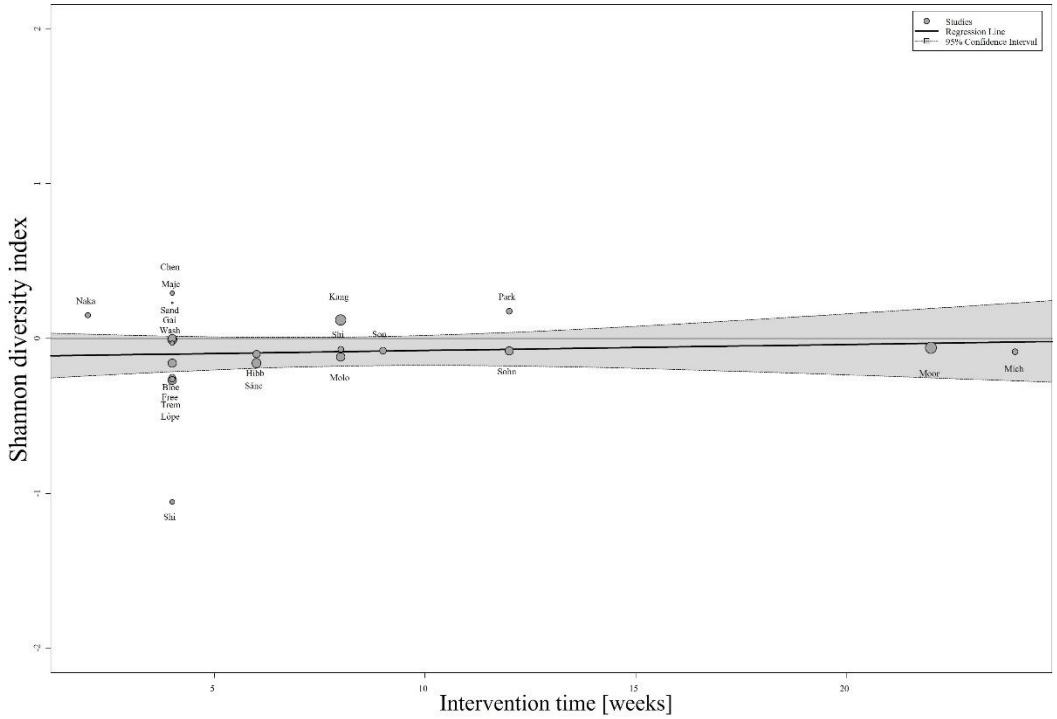


Abbreviations: CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

The "*" indicates that the median and q1, q3 are estimated from mean and standard deviation in that study.

Source: own work, unpublished

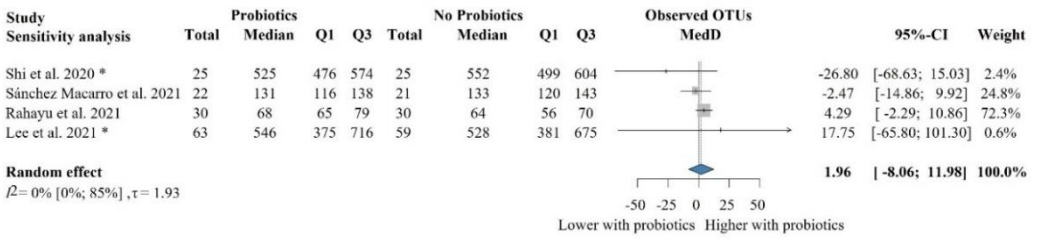
Figure S8. Meta-regression analysis investigating the relationship between intervention time and the Shannon diversity index - The effect of probiotic supplementation on the gut microbiome in healthy populations



Source: own work, unpublished

Figure S9. Additional sensitivity analysis for the more restricted analysis of Observed OTUs - The effect of probiotic supplementation on the gut microbiome in healthy populations

We did not identify significant differences when removing studies with not clear data on the number of participants (142), cross-over design (144), and change results (131).

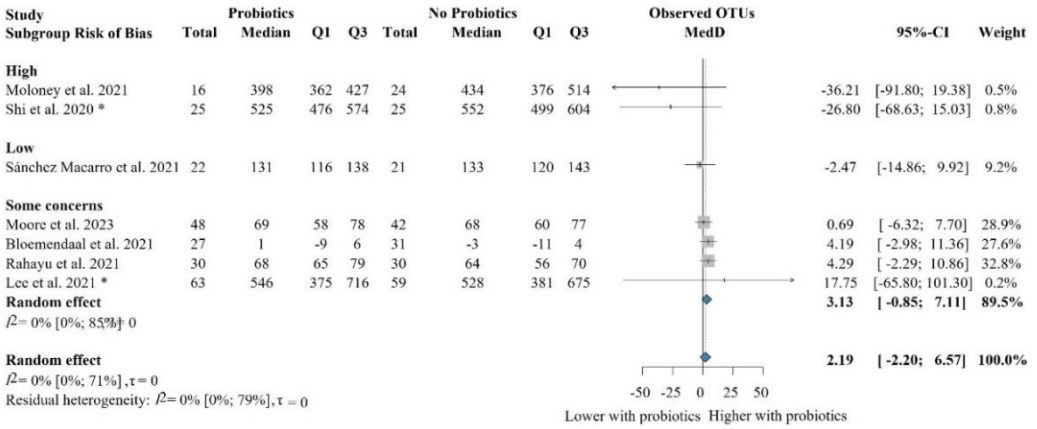


Abbreviations: OTU: Operational Taxonomic Unit; CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

The "*" indicates that the median and q1, q3 are estimated from mean and standard deviation in that study.

Source: own work, unpublished

Figure S10. Additional sensitivity analysis for the subgroups based on risk of bias assessment for Observed OTUs- The effect of probiotic supplementation on the gut microbiome in healthy populations

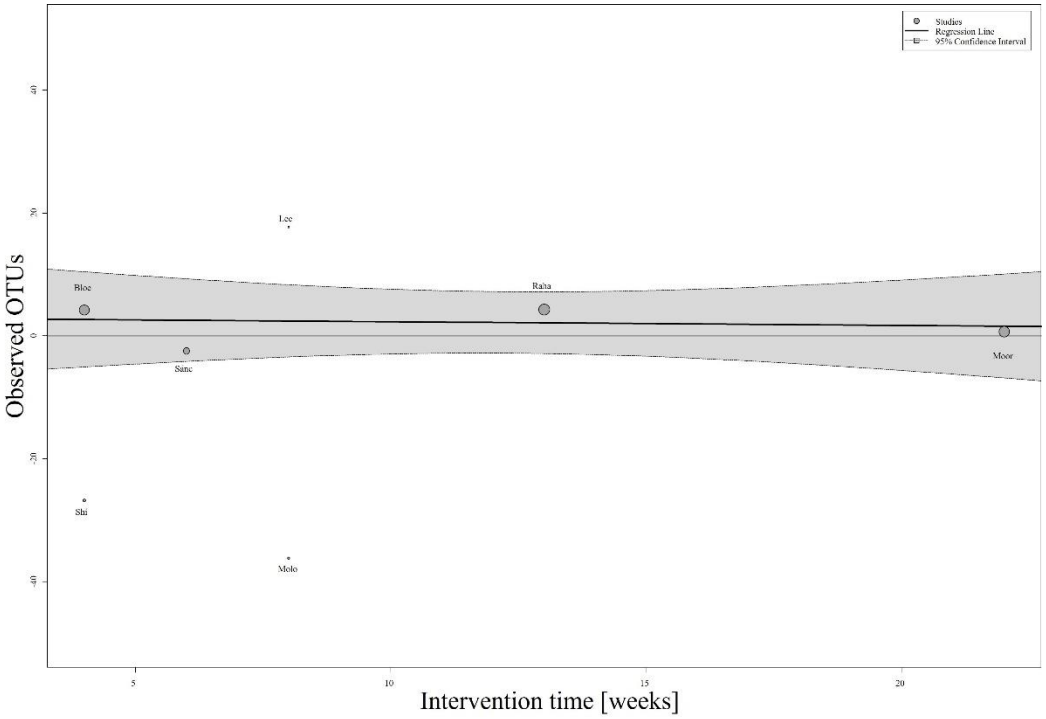


Abbreviations: CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

The "*" indicates that the median and q1, q3 are estimated from mean and standard deviation in that study.

Source: own work, unpublished

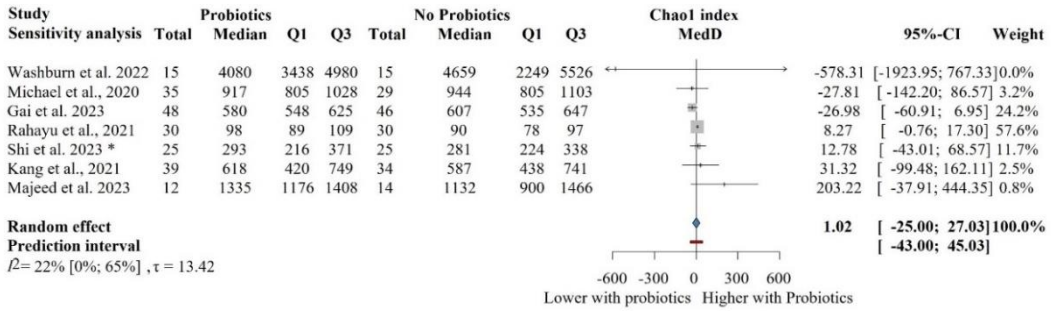
Figure S11. Meta-regression analysis investigating the relationship between intervention time and the number of Observed OTUs - The effect of probiotic supplementation on the gut microbiome in healthy populations



Source: own work, unpublished

Figure S12. Additional sensitivity analysis for the more restricted analysis Chao1 index - The effect of probiotic supplementation on the gut microbiome in healthy populations

The more restricted sensitivity analysis removing studies with cross-over design (138,144) revealed no significant or relevant difference between groups either (Supplementary Figure 7) (MedD=1.02 [(-)25.00 – 27.03]).

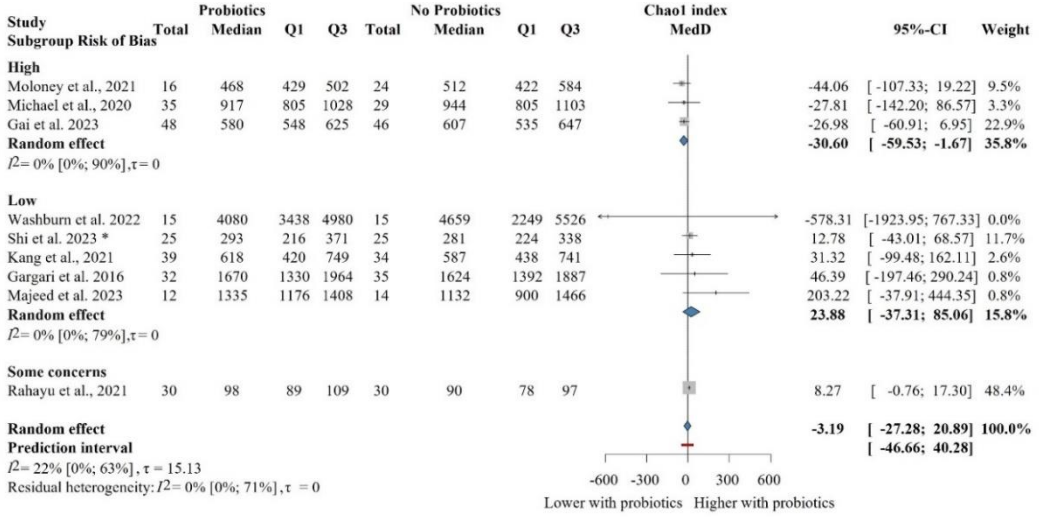


Abbreviations: CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

The "*" indicates that the median and q1, q3 are estimated from mean and standard deviation in that study.

Source: own work, unpublished

Figure S13. Additional sensitivity analysis for the subgroups based on the risk of bias assessment Chao1 index - The effect of probiotic supplementation on the gut microbiome in healthy populations

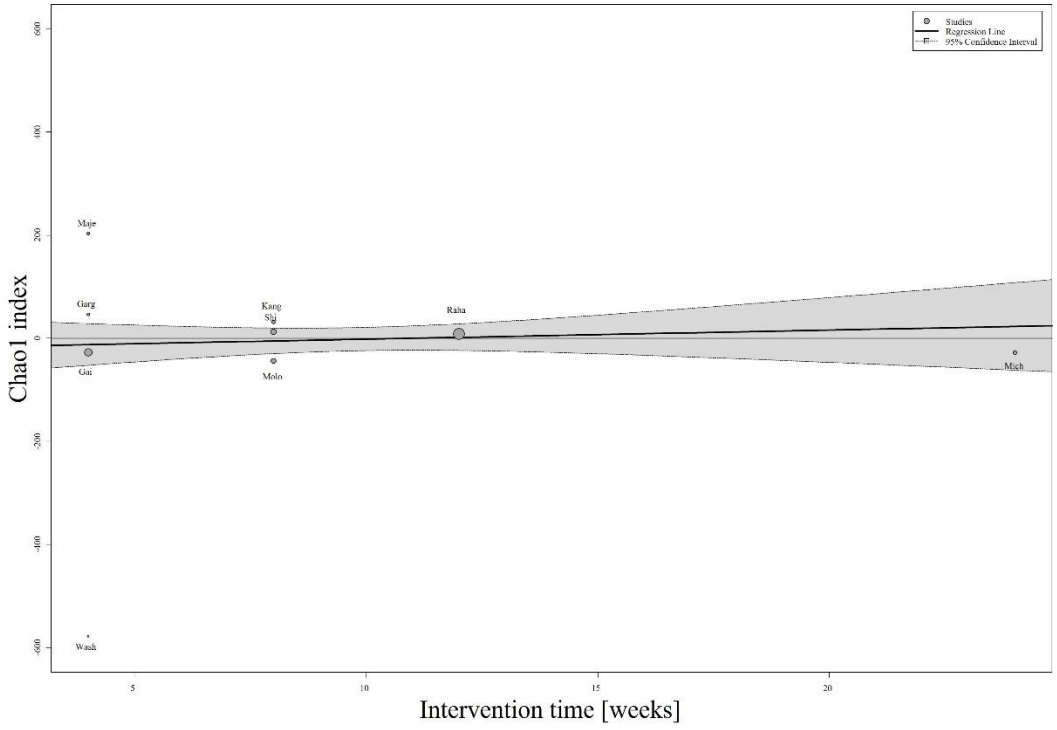


Abbreviations: CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

The "*" indicates that the median and q1, q3 are estimated from mean and standard deviation in that study.

Source: own work, unpublished

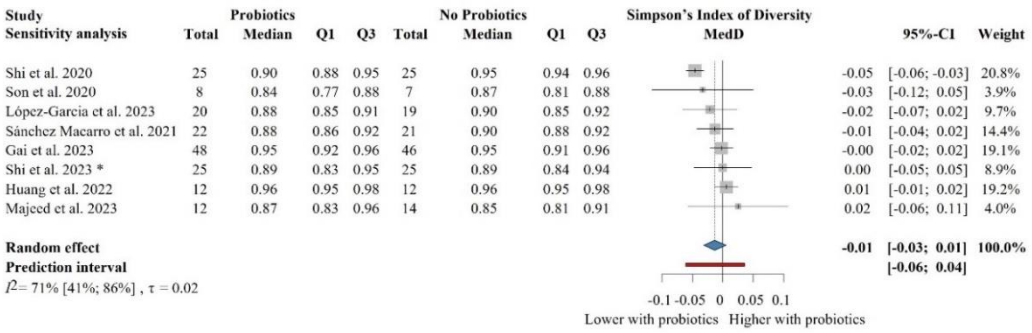
Figure S14. Meta-regression analysis investigating the relationship between intervention time and the Chao1 index - The effect of probiotic supplementation on the gut microbiome in healthy populations



Source: own work, unpublished

Figure S15. Additional sensitivity analysis for the more restricted analysis of Simpson's Index of Diversity - The effect of probiotic supplementation on the gut microbiome in healthy populations

In the sensitivity analysis, we removed the study with cross-over design (144) and the one with no clear number of participants (142), but we did not reveal any effect of probiotics.

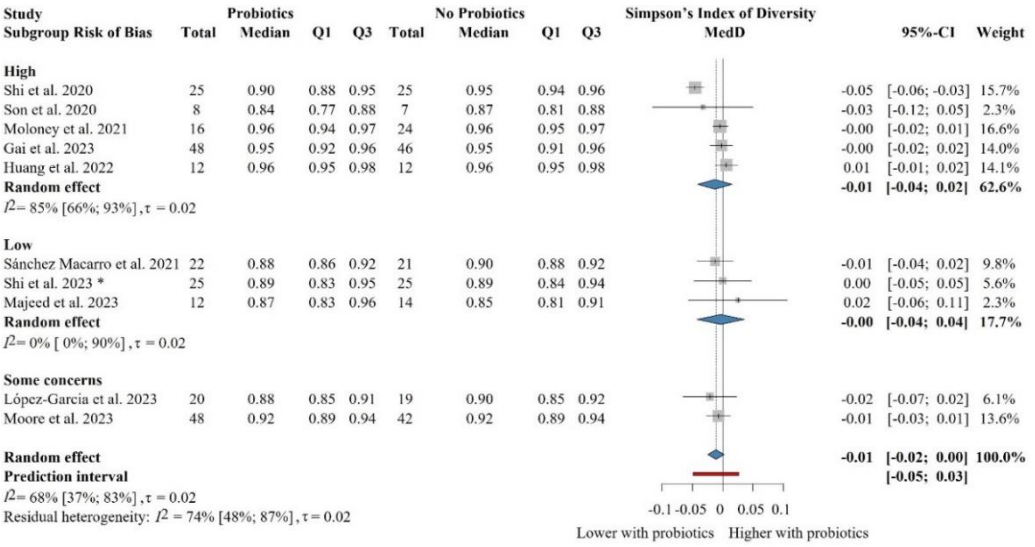


Abbreviations: CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

The "*" indicates that the median and q1, q3 are estimated from mean and standard deviation in that study.

Source: own work, unpublished

Figure S16. Additional sensitivity analysis for the subgroups based on risk of bias assessment of Simpson's Index of Diversity - The effect of probiotic supplementation on the gut microbiome in healthy populations

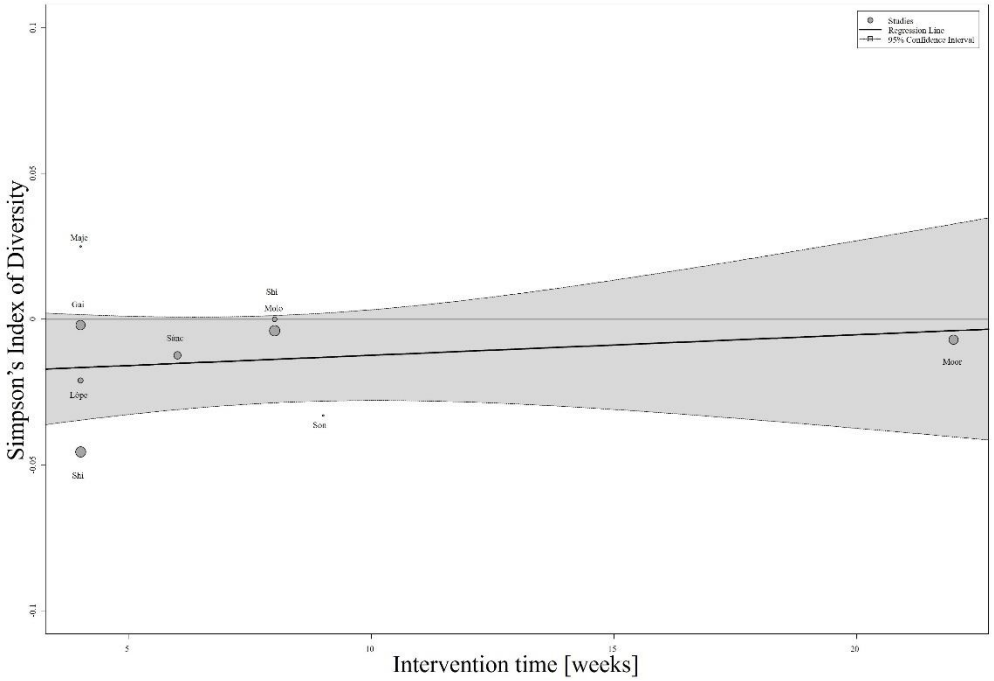


Abbreviations: CI: confidence interval; MedD: mean of median differences. Q1: first quartile; Q3: third quartile.

The "*" indicates that the median and q1, q3 are estimated from mean and standard deviation in that study.

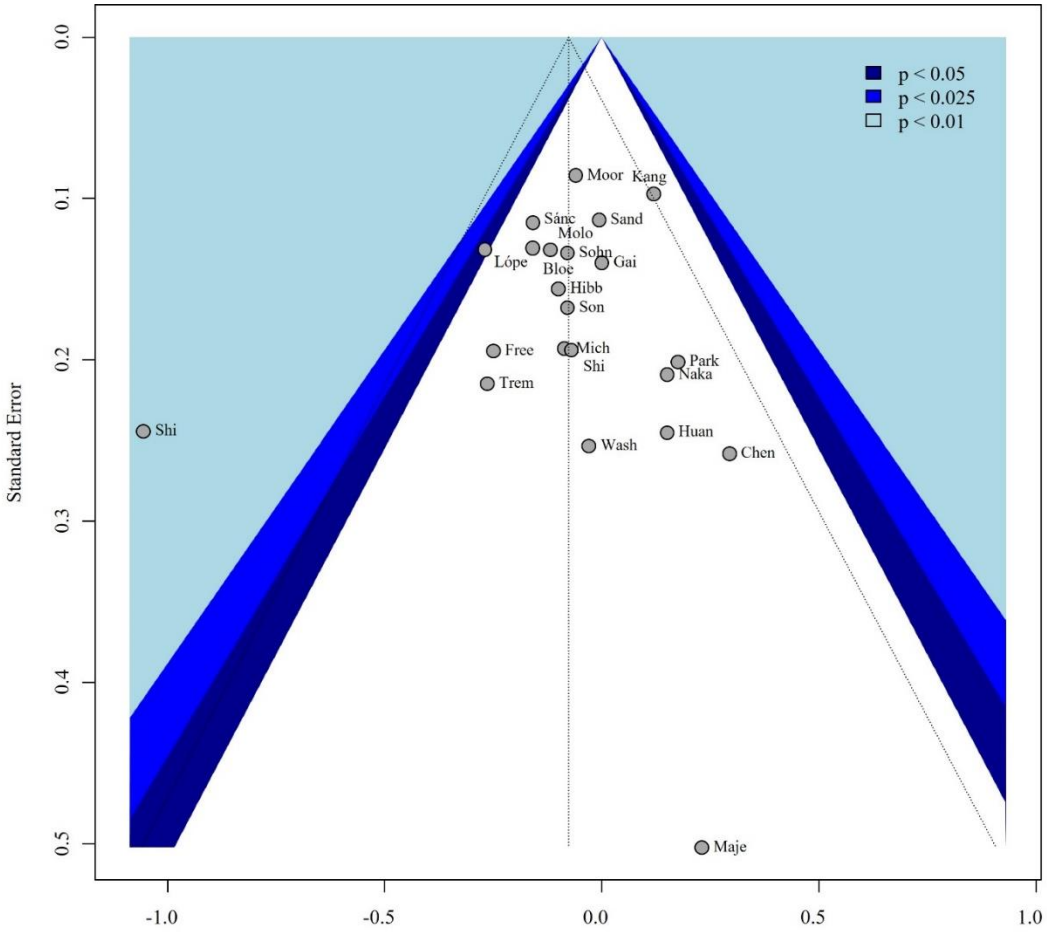
Source: own work, unpublished

Figure S17. Meta-regression analysis investigating the relationship between intervention time and the Simpson's Index of Diversity - The effect of probiotic supplementation on the gut microbiome in healthy populations



Source: own work, unpublished

Figure S18. Funnel plot to assess publication bias for Shannon diversity index - The effect of probiotic supplementation on the gut microbiome in healthy populations

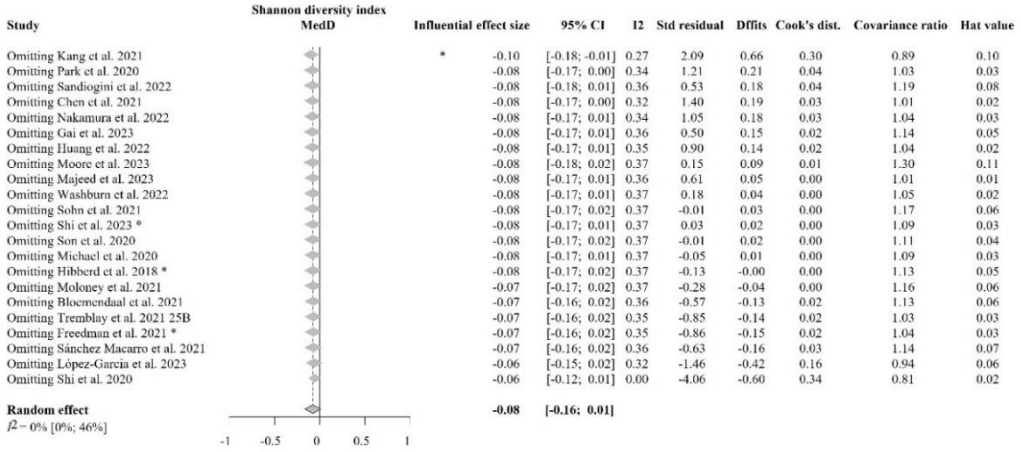


The points on the funnel plots for assessing publication bias represent the different studies. It shows the residuals on the x-axis against their corresponding standard errors. Here we could assess the small study bias: at the bottom of the funnel, if the studies are distributed not symmetrically, and out of the funnel, it indicates potential publication bias.

Source: own work, unpublished

Figure S19. Additional leave-one-out analysis for Shannon diversity index- The effect of probiotic supplementation on the gut microbiome in healthy populations

In the leave-one-out analysis, the Kang et al. 2021 (154) study can be considered statistically influential. However, the analysis indicates that it does not clinically relevantly affect the point estimate of the effect size or its confidence interval.



Study	Influential	effect size	95% CI	I2	Std residual	Dffits	Cook's dist.	Covariance ratio	Hat value
Omitting Kang et al. 2021	*	-0.10	[-0.18; -0.01]	0.27	2.09	0.66	0.30	0.89	0.10
Omitting Park et al. 2020		-0.08	[-0.17; 0.00]	0.34	1.21	0.21	0.04	1.03	0.03
Omitting Sandiogini et al. 2022		-0.08	[-0.18; 0.01]	0.36	0.53	0.18	0.04	1.19	0.08
Omitting Chen et al. 2021		-0.08	[-0.17; 0.00]	0.32	1.40	0.19	0.03	1.01	0.02
Omitting Nakamura et al. 2022		-0.08	[-0.17; 0.01]	0.34	1.05	0.18	0.03	1.04	0.03
Omitting Gai et al. 2023		-0.08	[-0.17; 0.01]	0.36	0.50	0.15	0.02	1.14	0.05
Omitting Huang et al. 2022		-0.08	[-0.17; 0.01]	0.35	0.90	0.14	0.02	1.04	0.02
Omitting Moore et al. 2023		-0.08	[-0.18; 0.02]	0.37	0.15	0.09	0.01	1.30	0.11
Omitting Majeed et al. 2023		-0.08	[-0.17; 0.01]	0.36	0.61	0.05	0.00	1.01	0.01
Omitting Washburn et al. 2022		-0.08	[-0.17; 0.01]	0.37	0.18	0.04	0.00	1.05	0.02
Omitting Sohn et al. 2021		-0.08	[-0.17; 0.02]	0.37	-0.01	0.03	0.00	1.17	0.06
Omitting Shi et al. 2023 *		-0.08	[-0.17; 0.01]	0.37	0.03	0.02	0.00	1.09	0.03
Omitting Son et al. 2020		-0.08	[-0.17; 0.02]	0.37	-0.01	0.02	0.00	1.11	0.04
Omitting Michael et al. 2020		-0.08	[-0.17; 0.01]	0.37	-0.05	0.01	0.00	1.09	0.03
Omitting Hibberd et al. 2018 *		-0.08	[-0.17; 0.02]	0.37	-0.13	-0.00	0.00	1.13	0.05
Omitting Moloney et al. 2021		-0.07	[-0.17; 0.02]	0.37	-0.28	-0.04	0.00	1.16	0.06
Omitting Bloemendaal et al. 2021		-0.07	[-0.16; 0.02]	0.36	-0.57	-0.13	0.02	1.13	0.06
Omitting Tremblay et al. 2021 25B		-0.07	[-0.16; 0.02]	0.35	-0.85	-0.14	0.02	1.03	0.03
Omitting Freedman et al. 2021 *		-0.07	[-0.16; 0.02]	0.35	-0.86	-0.15	0.02	1.04	0.03
Omitting Sánchez Macarro et al. 2021		-0.07	[-0.16; 0.02]	0.36	-0.63	-0.16	0.03	1.14	0.07
Omitting López-García et al. 2023		-0.06	[-0.15; 0.02]	0.32	-1.46	-0.42	0.16	0.94	0.06
Omitting Shi et al. 2020		-0.06	[-0.12; 0.01]	0.00	-4.06	-0.60	0.34	0.81	0.02
Random effect		**-0.08**	**[-0.16; 0.01]**						
I^2 = 0% [0%; 46%]									

Cases which are considered as possible influential with respect to any of the shown measures are marked with "*" at column "*Influential*". Note that the chosen cut-offs are (somewhat) arbitrary based on *dmetar* package. "*Effect size*": the pooled effect size without the given study.

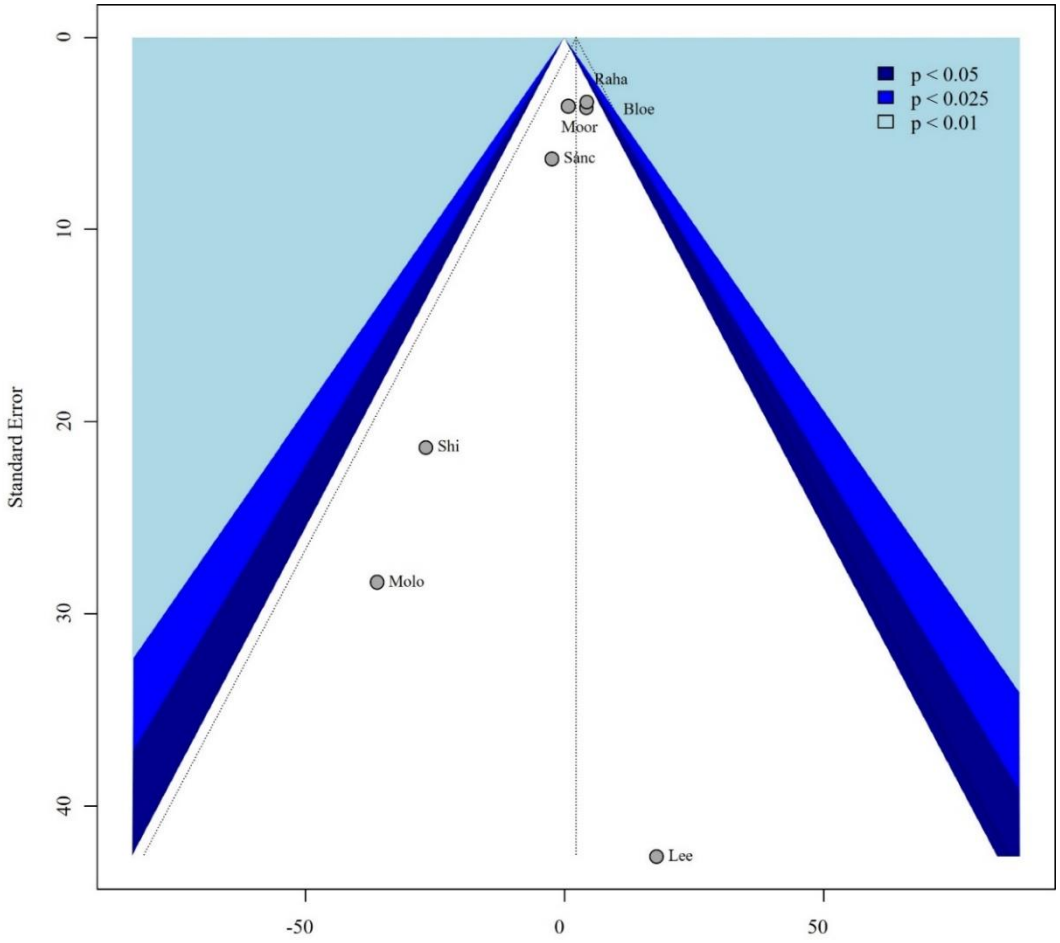
"*95% CI*": the 95% confidence interval of the pooled effect size without the given study. "I^2": the Higgins&Thomson I^2 heterogeneity value without the given study. "*Std residual*": the studentized residuals. It shows the deleted residual divided by its estimated standard deviation.

"*Dffits*": the difference in fits. It quantifies the number of standard deviations that the fitted value changes without the given study. (Typical threshold is $3 * \sqrt{(p/(k-p))}$, where p is the number of model coefficients and k is the number of cases). "*Cook's dist.*": Cook's distance. It depends on both the residual and leverage of the omitted study. (Typical threshold value is 2). "*Covariance ratio*": the covariance ratio. It shows the change in the determinant of the covariance matrix of the effect size. (Typical threshold value is 1). "*Hat value*": the value of the hat matrix without the given study. (Typical threshold is $3 * p/k$)

Abbreviations: MedD: mean of median differences; CI: confidence interval.

Source: own work, unpublished

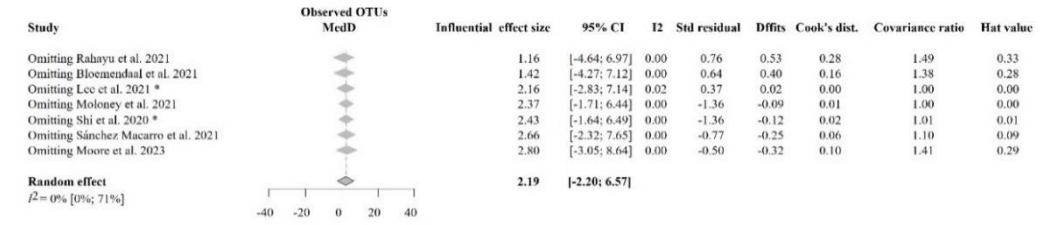
Figure S20. Funnel plot to assess publication bias for the Observed Operational Taxonomic Units (OTUs) - The effect of probiotic supplementation on the gut microbiome in healthy populations



The points on the funnel plots for assessing publication bias represent the different studies. It shows the residuals on the x-axis against their corresponding standard errors. Here we could assess the small study bias: at the bottom of the funnel if the studies are distributed not symmetrically and out of the funnel, it indicates potential publication bias.

Source: own work, unpublished

Figure S21. Additional leave-one-out analysis for the Observed Operational Taxonomic Units (OTUs) - The effect of probiotic supplementation on the gut microbiome in healthy populations



Cases which are considered as possible influential with respect to any of the shown measures are marked with "*" at column "*Influential*". Note that the chosen cut-offs are (somewhat) arbitrary based on *dmetar* package. "*Effect size*": the pooled effect size without the given study.

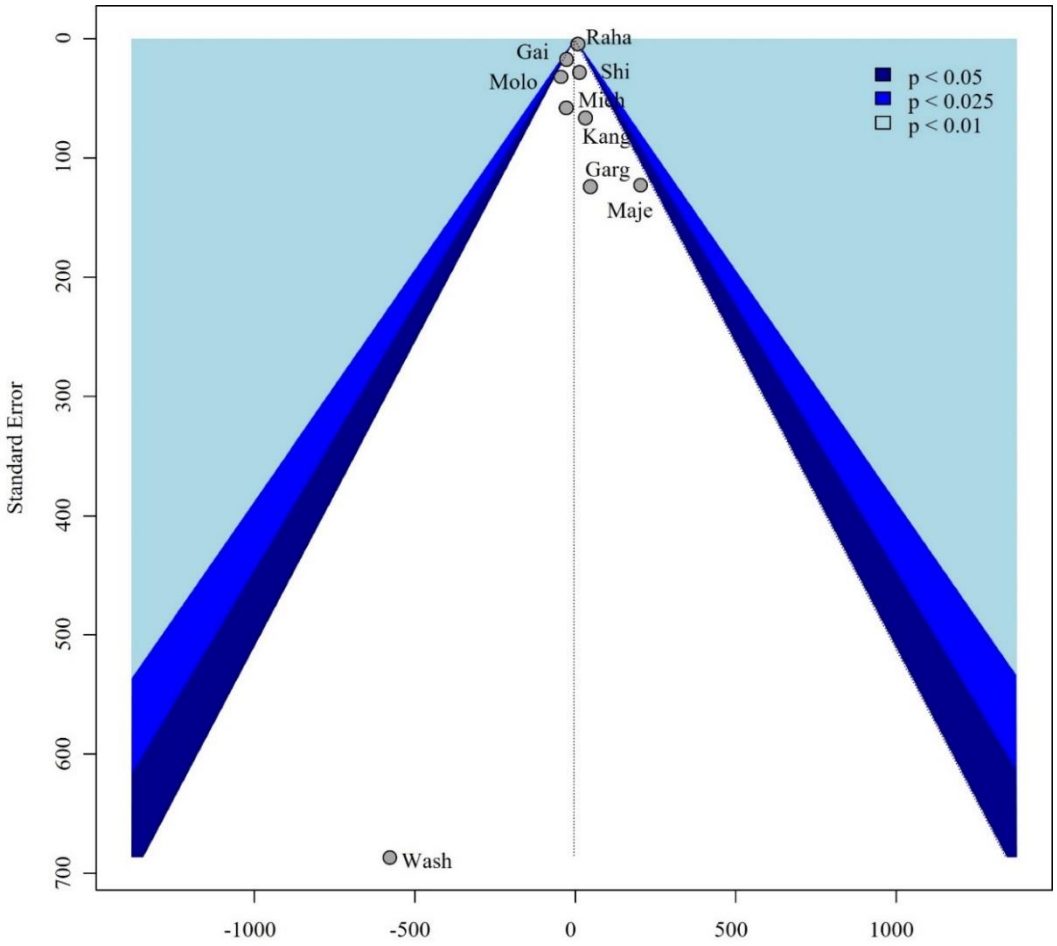
"*95% CI*": the 95% confidence interval of the pooled effect size without the given study. "I^2": the Higgins&Thomson I^2 heterogeneity value without the given study. "*Std residual*": the studentized residuals. It shows the deleted residual divided by its estimated standard deviation.

"*Dffits*": the difference in fits. It quantifies the number of standard deviations that the fitted value changes without the given study. (Typical threshold is $3 * \sqrt{(p/(k-p))}$, where p is the number of model coefficients and k is the number of cases). "*Cook's dist.*": Cook's distance. It depends on both the residual and leverage of the omitted study. (Typical threshold value is 2). "*Covariance ratio*": the covariance ratio. It shows the change in the determinant of the covariance matrix of the effect size. (Typical threshold value is 1). "*Hat value*": the value of the hat matrix without the given study. (Typical threshold is $3 * p/k$)

Abbreviations: MedD: mean of median differences; CI: confidence interval.

Source: own work, unpublished

Figure S22. Funnel plot to assess publication bias for Chao1 index - The effect of probiotic supplementation on the gut microbiome in healthy populations

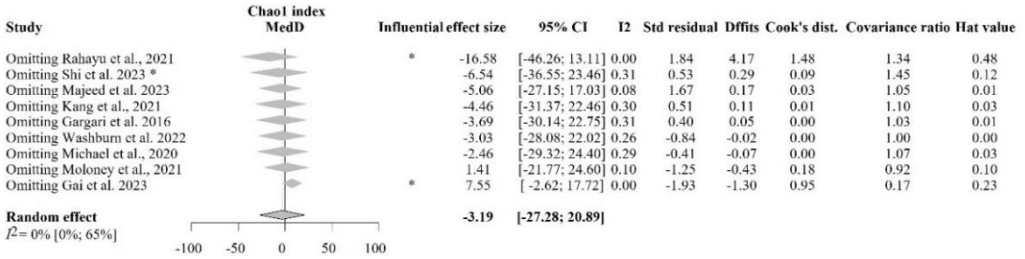


The points on the funnel plots for assessing publication bias represent the different studies. It shows the residuals on the x-axis against their corresponding standard errors. Here we could assess the small study bias: at the bottom of the funnel if the studies are distributed not symmetrically and out of the funnel, it indicates potential publication bias.

Source: own work, unpublished

Figure S23. Additional leave-one-out analysis for Chao1 index - The effect of probiotic supplementation on the gut microbiome in healthy populations

In the leave-one-out analysis, the Shi et al. 2023 (165), Moloney et al. 2021 (144), and Gai et al. 2023 (143) studies can be considered statistically influential studies. However, the analysis indicates that they do not clinically relevantly affect the point estimate of the effect size or its confidence interval.



Cases which are considered as possible influential with respect to any of the shown measures are marked with "*" at column "*Influential*". Note that the chosen cut-offs are (somewhat) arbitrary based on *dmetar* package. "*Effect size*": the pooled effect size without the given study.

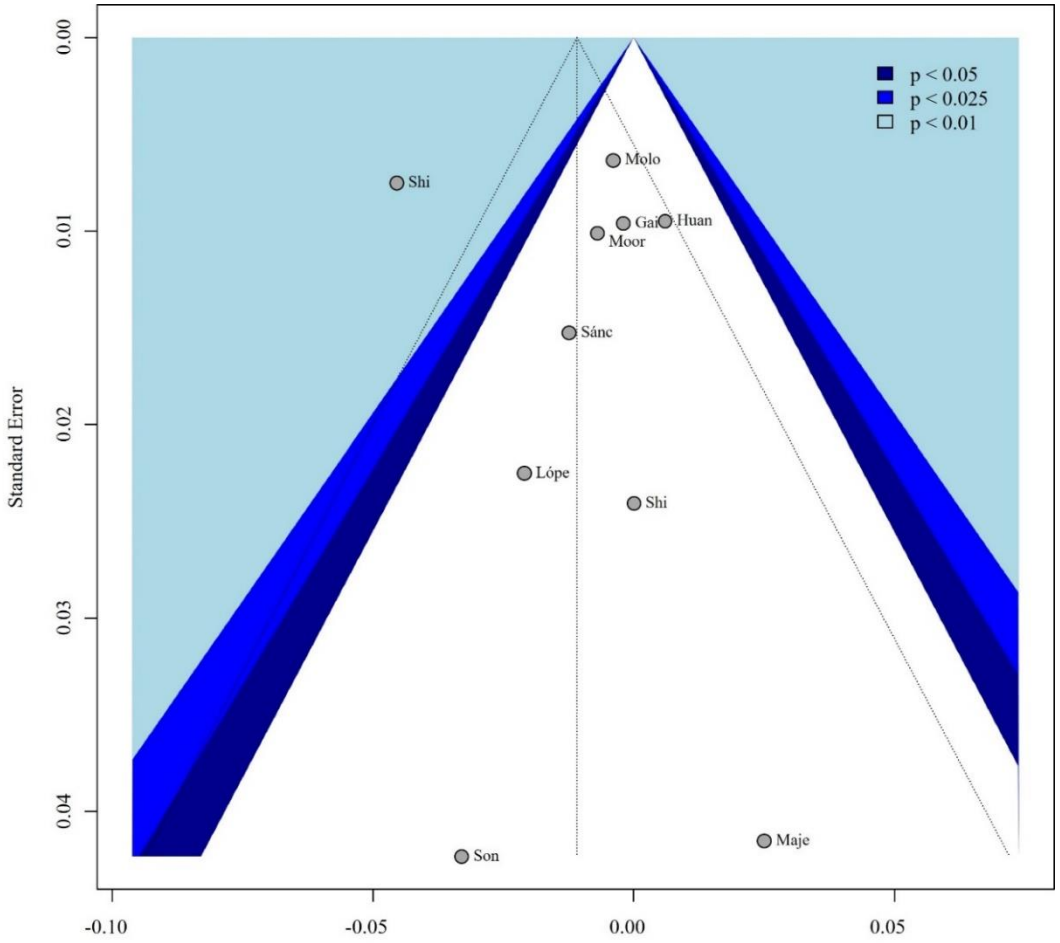
"*95% CI*": the 95% confidence interval of the pooled effect size without the given study. "I^2": the Higgins&Thomson I^2 heterogeneity value without the given study. "*Std residual*": the studentized residuals. It shows the deleted residual divided by its estimated standard deviation.

"*Dffits*": the difference in fits. It quantifies the number of standard deviations that the fitted value changes without the given study. (Typical threshold is $3 * \sqrt{(p/(k-p))}$, where p is the number of model coefficients and k is the number of cases). "*Cook's dist.*": Cook's distance. It depends on both the residual and leverage of the omitted study. (Typical threshold value is 2). "*Covariance ratio*": the covariance ratio. It shows the change in the determinant of the covariance matrix of the effect size. (Typical threshold value is 1). "*Hat value*": the value of the hat matrix without the given study. (Typical threshold is $3 * p/k$)

Abbreviations: MedD: mean of median differences; CI: confidence interval.

Source: own work, unpublished

Figure S24. Funnel plot to assess publication bias for Simpson's Index of Diversity - The effect of probiotic supplementation on the gut microbiome in healthy populations

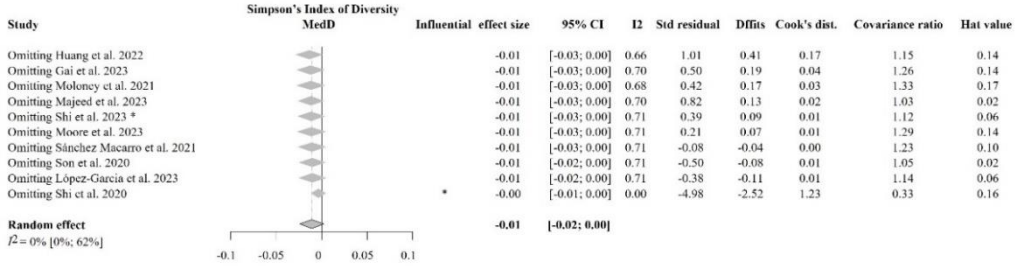


The points on the funnel plots for assessing publication bias represent the different studies. It shows the residuals on the x-axis against their corresponding standard errors. Here we could assess the small study bias: at the bottom of the funnel if the studies are distributed not symmetrically and out of the funnel, it indicates potential publication bias.

Source: own work, unpublished

Figure S25. Additional leave-one-out analysis Simpson's Index of Diversity - The effect of probiotic supplementation on the gut microbiome in healthy populations

In the leave-one-out analysis, the Shi et al. 2023 (161) study can be considered a statistically influential study based on the I^2 value and the covariance ratio. However, the analysis indicates that it does not clinically relevantly affect the point estimate of the effect size or its confidence interval.



Study	Influential	effect size	95% CI	I2	Std residual	Dffits	Cook's dist.	Covariance ratio	Hat value
Omitting Huang et al. 2022		-0.01	[-0.03; 0.00]	0.66	1.01	0.41	0.17	1.15	0.14
Omitting Gai et al. 2023		-0.01	[-0.03; 0.00]	0.70	0.50	0.19	0.04	1.26	0.14
Omitting Moloney et al. 2021		-0.01	[-0.03; 0.00]	0.68	0.42	0.17	0.03	1.33	0.17
Omitting Majeed et al. 2023		-0.01	[-0.03; 0.00]	0.70	0.82	0.13	0.02	1.03	0.02
Omitting Shi et al. 2023 *		-0.01	[-0.03; 0.00]	0.71	0.39	0.09	0.01	1.12	0.06
Omitting Moore et al. 2023		-0.01	[-0.03; 0.00]	0.71	0.21	0.07	0.01	1.29	0.14
Omitting Sánchez Macarro et al. 2021		-0.01	[-0.03; 0.00]	0.71	-0.08	-0.04	0.00	1.23	0.10
Omitting Son et al. 2020		-0.01	[-0.02; 0.00]	0.71	-0.50	-0.08	0.01	1.05	0.02
Omitting López-García et al. 2023		-0.01	[-0.02; 0.00]	0.71	-0.38	-0.11	0.01	1.14	0.06
Omitting Shi et al. 2020	*	-0.00	[-0.01; 0.00]	0.00	-4.98	-2.52	1.23	0.33	0.16
Random effect		**-0.01**	**[-0.02; 0.00]**						
I^2 = 0% [0%; 62%]									

Cases which are considered as possible influential with respect to any of the shown measures are marked with "*" at column "*Influential*". Note that the chosen cut-offs are (somewhat) arbitrary based on *dmetar* package. "*Effect size*": the pooled effect size without the given study.

"*95% CI*": the 95% confidence interval of the pooled effect size without the given study. "I^2": the Higgins&Thomson I^2 heterogeneity value without the given study. "*Std residual*": the studentized residuals. It shows the deleted residual divided by its estimated standard deviation.

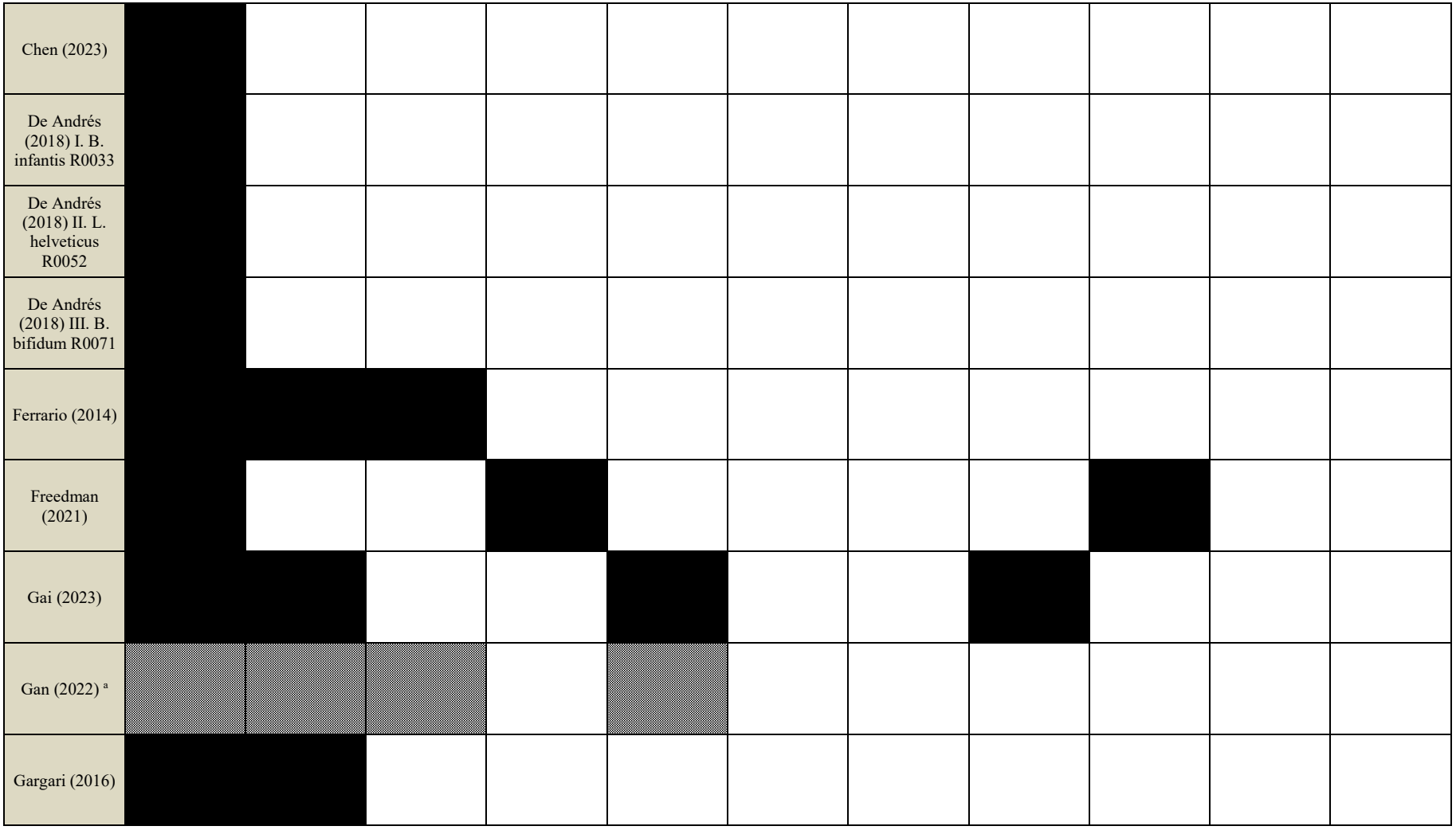
"*Dffits*": the difference in fits. It quantifies the number of standard deviations that the fitted value changes without the given study. (Typical threshold is $3*\sqrt{(p/(k-p))}$, where *p* is the number of model coefficients and *k* is the number of cases). "*Cook's dist.*": Cook's distance. It depends on both the residual and leverage of the omitted study. (Typical threshold value is 2). "*Covariance ratio*": the covariance ratio. It shows the change in the determinant of the covariance matrix of the effect size. (Typical threshold value is 1). "*Hat value*": the value of the hat matrix without the given study. (Typical threshold is $3*p/k$)

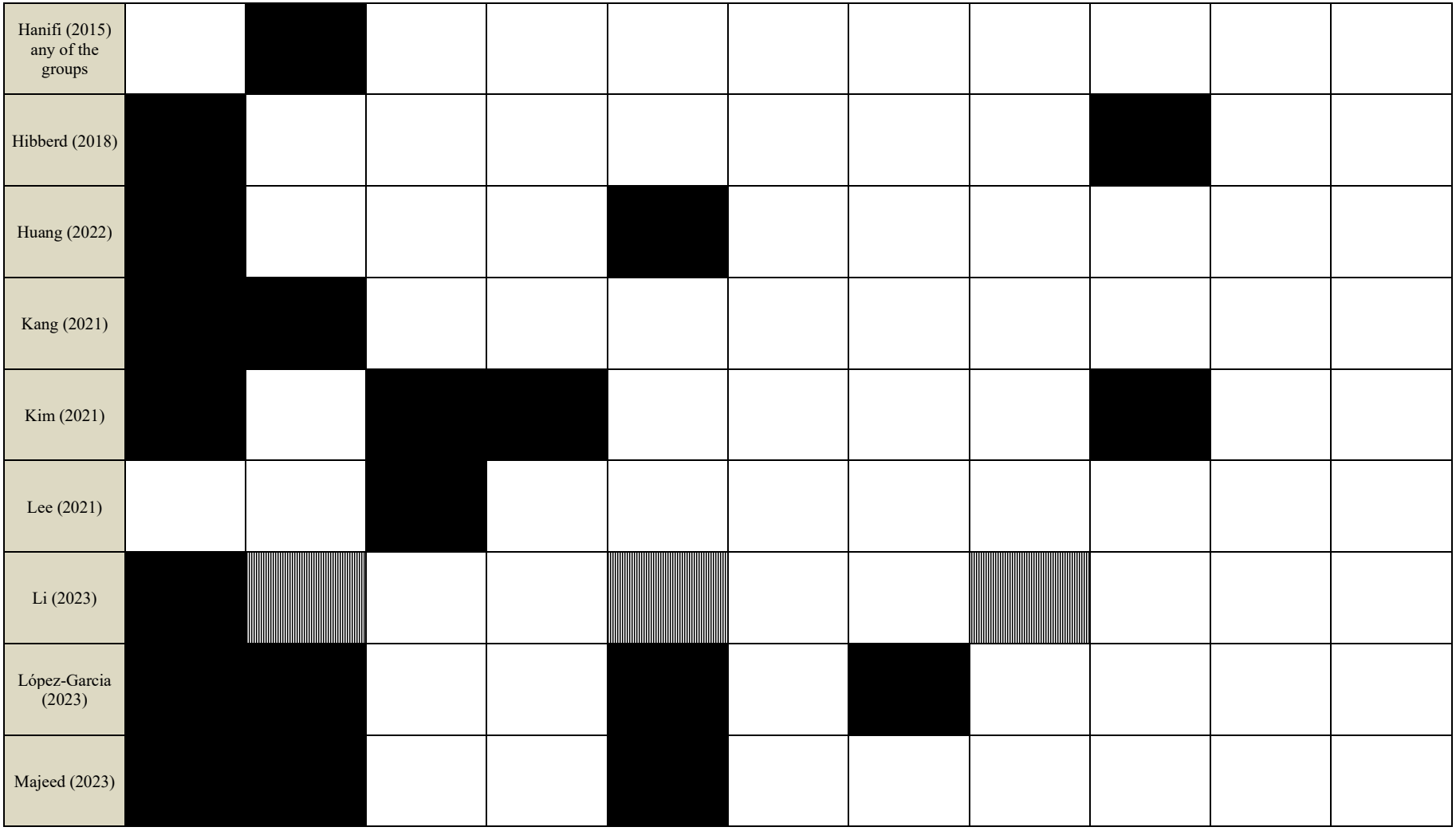
Abbreviations: MedD: mean of median differences; CI: confidence interval.

Source: own work, unpublished

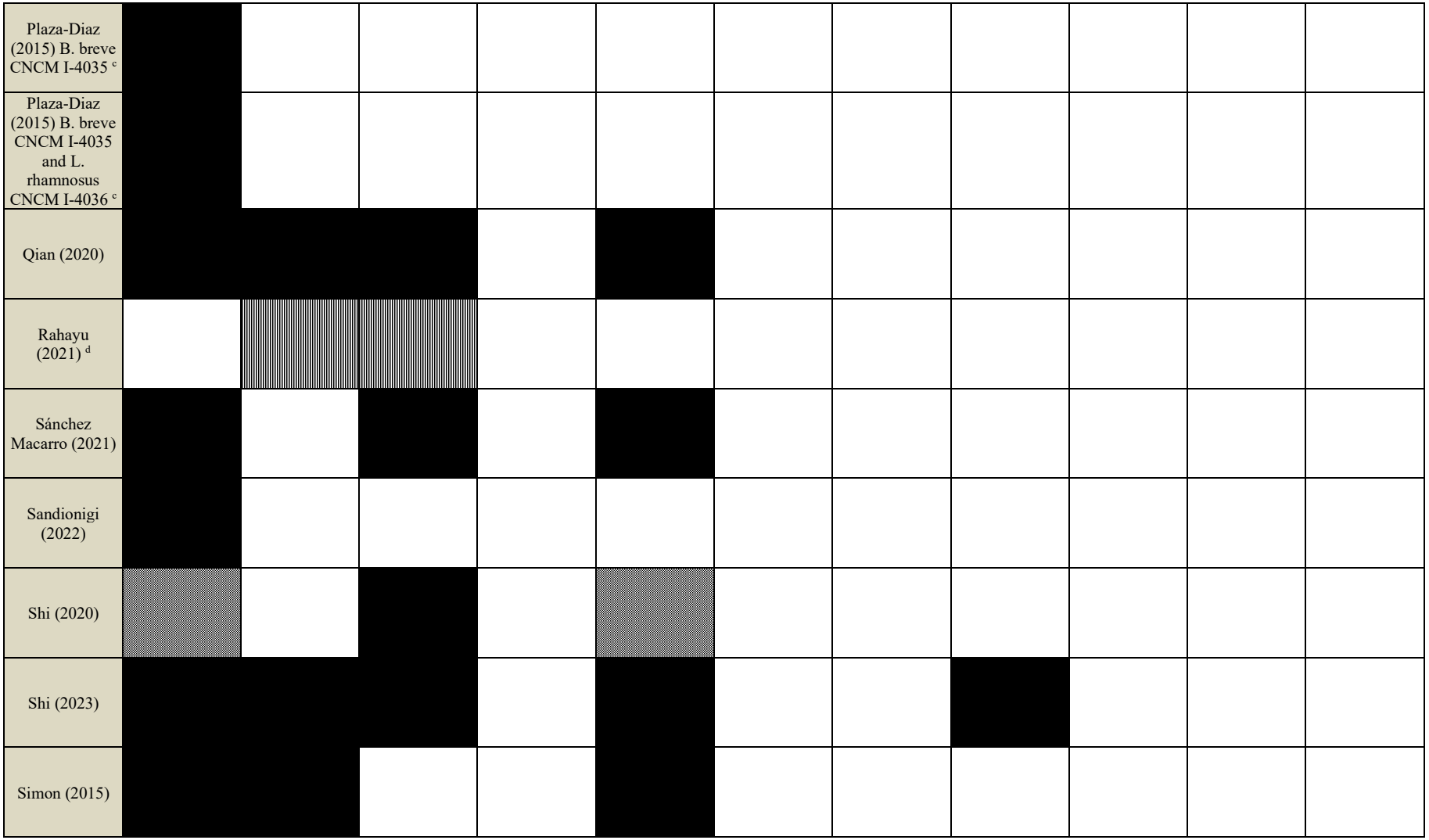
Table S7. Changes in the microbiome α-diversity indices as measured after the intervention period – The effect of probiotic supplementation on the gut microbiome in healthy populations

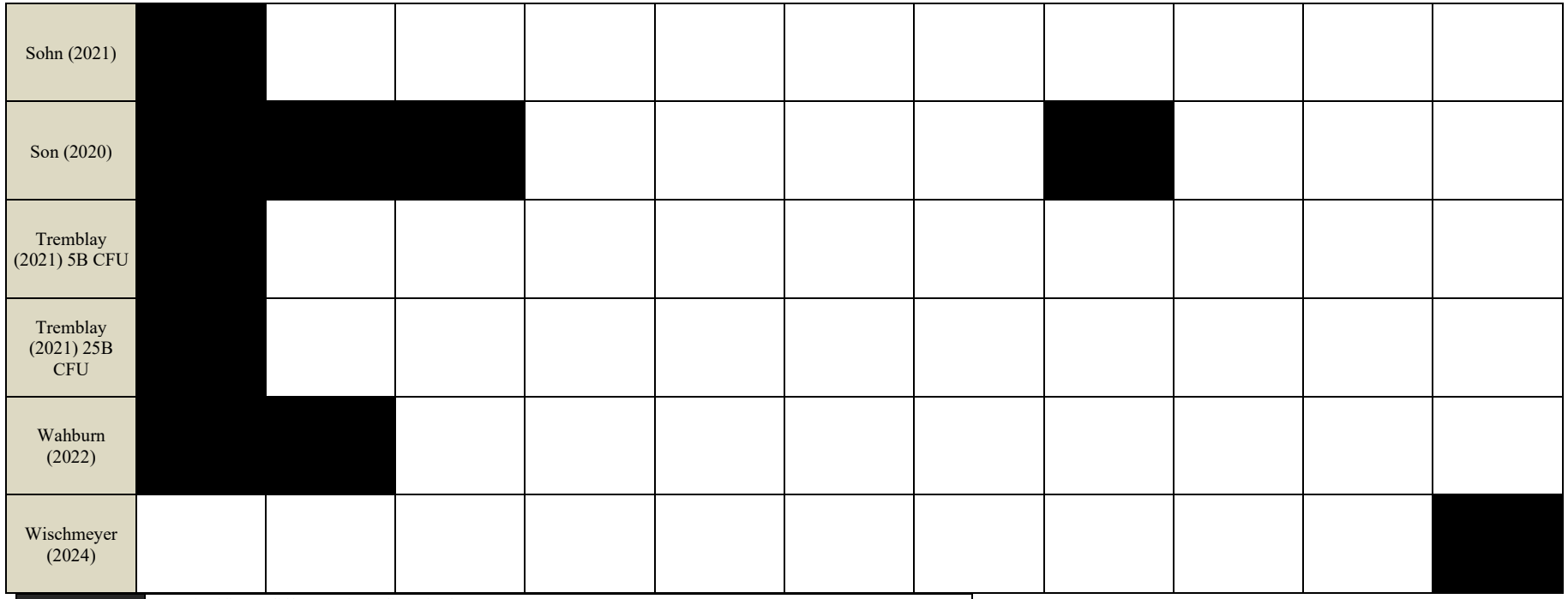
Study	Shannon diversity index	Chao1 index	Observed OTUs / Richness	Pielou's evenness	Simpson's Index of Diversity	Inverse Simpson index	Strong's dominance index	ACE index	Faiths Phylogenetic Diversity	Shannon effective count	Not specified
Axelrod (2019)	■	■			■			■			
Bagga (2018)						■					
Bazanella (2017)			■							■	
Bloemendaal (2021)	■		■						■		
Boesmans (2018)		■		■							
Castanet (2020)											■
Chen (2020)	■										


Chen (2023)
De Andrés (2018) I. B. infantis R0033
De Andrés (2018) II. L. helveticus R0052
De Andrés (2018) III. B. bifidum R0071
Ferrario (2014)
Freedman (2021)
Gai (2023)
Gan (2022) a
Gargari (2016)


Hanifi (2015) any of the groups
Hibberd (2018)
Huang (2022)
Kang (2021)
Kim (2021)
Lee (2021)
Li (2023)
López-Garcia (2023)
Majeed (2023)

Michael (2020)											
Moloney (2021)											
Moore (2023)											
Nakamura (2022)											
Pagliai (2023) [b]											
Park (2020)											
Paytuvi-Gallart (2020)											
Plaza-Diaz (2015) L. rhamnosus CNCM I-4036 [c]											
Plaza-Diaz (2015) L. paracasei CNCM I-4034 [c]											


Plaza-Diaz (2015) B. breve CNCM I-4035 c
Plaza-Diaz (2015) B. breve CNCM I-4035 and L. rhamnosus CNCM I-4036 c
Qian (2020)
Rahayu (2021) d
Sánchez Macarro (2021)
Sandionigi (2022)
Shi (2020)
Shi (2023)
Simon (2015)



	no significant difference in α-diversity between groups
	favors probiotics (higher α-diversity in the intervention group compared to baseline and/or to placebo)
	favors placebo (higher α-diversity in the control group compared to baseline and/or to probiotics)
	not applicable*

[a] Gan et al. reported an increase in alpha diversity in the stools of the placebo group; however, did not specify which index was exactly affected.

[b] Instead of Observed OTUs, Observed Amplicon Sequence Variants (ASV) were reported.

[c] No information about the placebo group.

[d] Rahayu et al. reported significant increases in the probiotic group; however, a comparison with the placebo group was not performed. According to our comparative meta-analysis, the mean of median differences was not significantly different in the two groups after the intervention period.

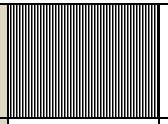
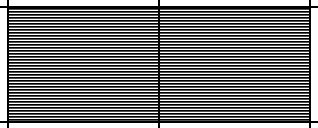
*If a study did not investigate a specific outcome, “not applicable” is indicated.

Abbreviations: OTU: operational taxonomic unit; ACE: Abundance-based coverage estimator. Source: own compilation, unpublished

Table S8. Changes in the microbiome β-diversity indices as measured after the intervention period – The effect of probiotic supplementation on the gut microbiome in healthy populations

Study	Bray-Curtis (dis)similarity index	Euclidean distance	Weighted UniFrac distance	Unweighted UniFrac distance	Generalized UniFrac distance	Jensen-Shannon divergence	Morisita-Horn distance metrics.	Spearman correlation distance	Not clearly specified
Bagga (2018)	■								
Bazanella (2017)					■				
Bloemendaal (2021)			■						
Boesmans (2018)	■								
Castanet (2020)									■
Chen (2020)	■								
Chen (2023)	■								

Ferrario (2014)									
Freedman 2021									
Gai (2023)									
Gan (2022)									
Gargari (2016)									
Hanifi (2015) *any of the groups									
Hibberd (2018)									
Huang (2022)									
Kang (2021)									

Lau (2018)									
Lee (2021)									
Li (2023)									
Majeed (2023)									
Marcial (2017)									
Michael (2020)									
Moore (2023)									
Mutoh (2024)									
Nakamura (2022)									

Pagliai (2023)									
Park (2020)									
Paytuvi-Gallart (2020)									
Plaza Diaz (2015) L.rhamnosus group [a]									
Plaza-Diaz (2015) L. paracasei CNCM I-4034 [a]									
Plaza-Diaz (2015) B. breve CNCM I-4035 [a]									
Plaza-Diaz (2015) B. breve CNCM I-4035 and L. rhamnosus CNCM I-4036 [a]									
Qian (2020)									
Sandionigi(2022)									

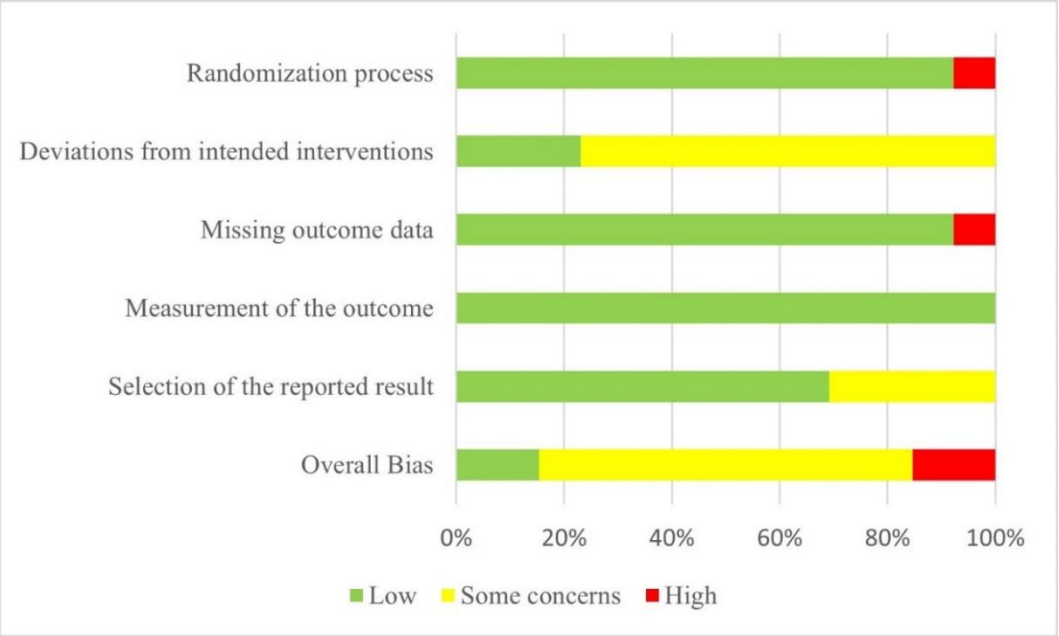
Shi (2020)									
Shi (2023)									
Simon (2015)									
Sohn (2021)									
Tremblay (2021) 5B CFU									
Tremblay (2021) 25B CFU									
Washburn (2022)									
Wischmeyer (2024)									

	no significant difference in β-diversity between groups
	there is a significant change in the probiotic group compared to baseline
	there is a significant change in the placebo group compared to baseline
	there is a significant difference between the groups
	not applicable*

[a] No information about placebo.

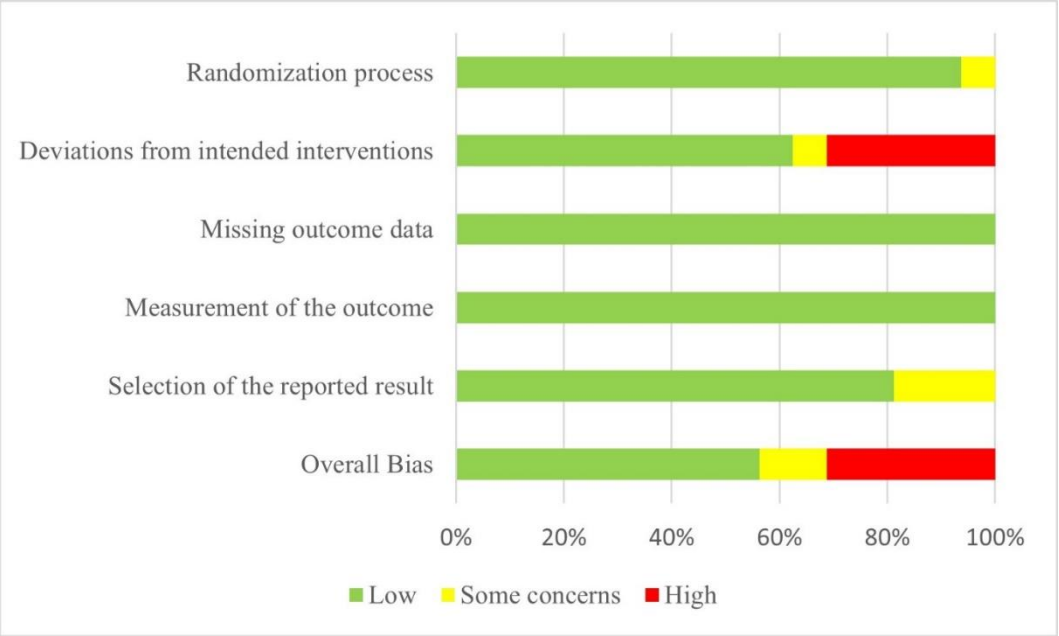
*If a study did not investigate a specific outcome, “not applicable” is indicated. Abbreviations: UniFrac: unique fraction metric. Source: own compilation, unpublished

Figure S26. Risk of bias assessment for parallel design studies - Assignment to intervention (the 'intention-to-treat' effect) (n=13) - The effect of probiotic supplementation on the gut microbiome in healthy populations



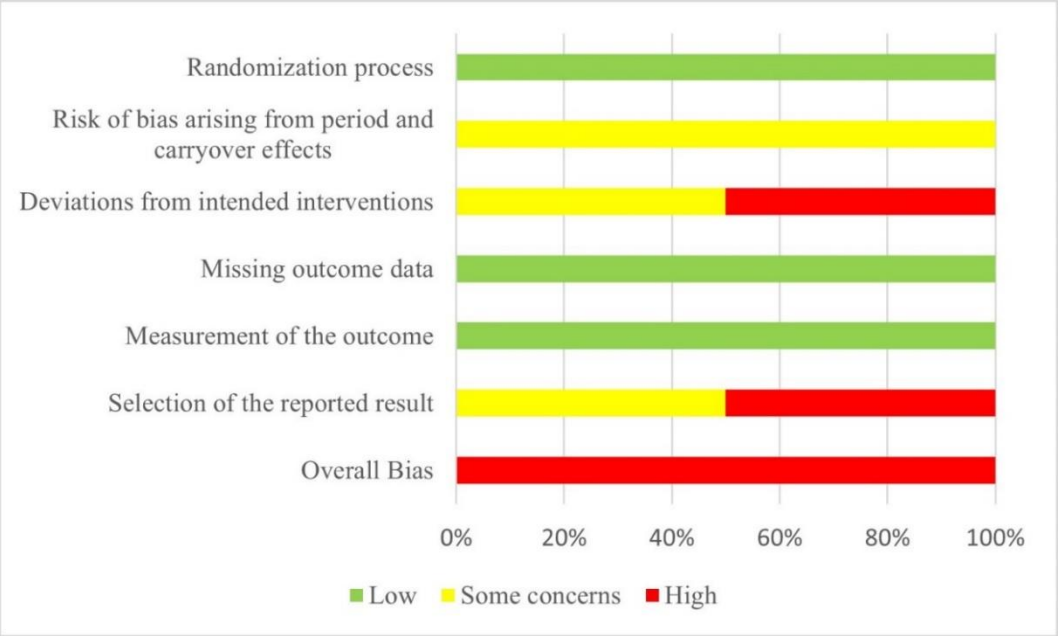
Source: own work, unpublished

Figure S27. Risk of bias assessment for parallel design studies - Adhering to intervention (the 'per-protocol' effect) (n=16) - The effect of probiotic supplementation on the gut microbiome in healthy populations



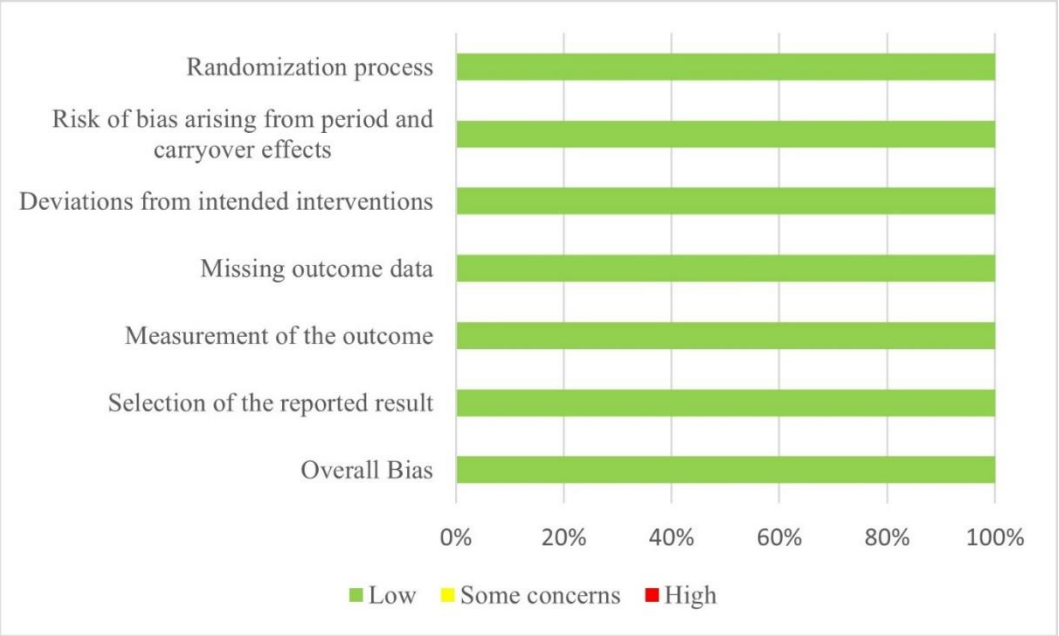
Source: own work, unpublished

Figure S28. Risk of bias assessment for cross-over design studies - Assignment to intervention (the 'intention-to-treat' effect) (n=2) - The effect of probiotic supplementation on the gut microbiome in healthy populations



Source: own work, unpublished

Figure S29. Risk of bias assessment for crossover design studies - Adhering to intervention (the 'per-protocol' effect) (n=4) - The effect of probiotic supplementation on the gut microbiome in healthy populations



Source: own work, unpublished

Table S9. GRADE assessment for the meta-analyses of Shannon, Observed OTUs, Chao1 and Simpson's Index of Diversity indices - The effect of probiotic supplementation on the gut microbiome in healthy populations

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	probiotics	control	Relative (95% CI)	Absolute (95% CI)		
Shannon diversity index (follow-up: range 2 weeks to 6 months; assessed with: 16S rRNA sequencing)												
22	randomised trials	serious	not serious	not serious	not serious	none	552	552	-	MedD **0.08 lower** (0.16 lower to 0.01 higher)	⊕⊕⊕◯ Moderate	IMPORTANT
Observed OTUs (follow-up: range 4 weeks to 22 weeks; assessed with: 16S rRNA sequencing)												
7	randomised trials	serious	not serious	not serious	not serious	none	231	232	-	MedD **2.19 higher** (2.2 lower to 6.57 higher)	⊕⊕⊕◯ Moderate	IMPORTANT
Chao1 index (follow-up: range 4 weeks to 24 weeks; assessed with: 16S rRNA sequencing)												
9	randomised trials	serious	not serious	not serious	not serious	none	252	252	-	MedD **3.19 lower** (27.28 lower to 20.89 higher)	⊕⊕⊕◯ Moderate	IMPORTANT
Simpson's Index of Diversity (follow-up: range 4 weeks to 22 weeks; assessed with: 16S rRNA sequencing)												

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	probiotics	control	Relative (95% CI)	Absolute (95% CI)		
10	randomised trials	serious	not serious	not serious	not serious	none	236	235	-	MedD **0.01 lower** (0.02 lower to 0.00 higher)	⊕⊕⊕◯ Moderate	IMPORTANT

CI: confidence interval; MedD: mean of median differences.

Source: own compilation, unpublished

Table S10. Study characteristics - The effect of probiotic supplementation on zonulin levels in healthy populations

Study	Country	Study design (No. of centers*)	Population (Randomized)					Probiotic type (as reported in each study**) and dose	Reclassified probiotic nomenclature (if applicable)	Placebo type and dose	Duration	Sample source
			Number of randomized subjects (female %)	Number of subjects in the probiotic group	Number of subjects in the control group	Age (years - mean ± SD) in the intervention (and control) groups	Specification of the population					
Freedman (2021)	USA	parallel arm, double-blind, randomized, placebo-controlled intervention study	46 (61)	25	21	36.9 ± 12.9 (34.4 ± 13.0)	normal weight to mildly obese healthy adults	*Bacillus subtilis* strain DE111 (1×10^9 CFU/day)	N.A.	maltodextrin	28 days (4 weeks)	plasma
Garvey (2022)	USA	randomized, double-blind, placebo-controlled, parallel clinical trial	76 (55)	38	38	50.4 ± 10.0 (50.5 ± 8.8)	healthy people with at least minimal complaints of abdominal bloating, burping, or flatulence	*Bacillus subtilis* BS50 (2×10^9 CFU/day)	N.A.	maltodextrin	42 days (6 weeks)	plasma
Mazur-Kurach (2022)	Poland	double-blind, randomized, and placebo-controlled trial	26 (0)	13	13	23.25 ± N.D. (21.28 ± N.D.)	healthy, male competitive road cyclists	*Lactobacillus plantarum, Lactobacillus casei, Lactobacillus rhamnosus, Bifidobacterium breve, Lactobacillus acidophilus, Bifidobacterium longum, Bifidobacterium bifidum, Bifidobacterium infantis, Lactobacillus helveticus,*	*Lactiplantibacillus plantarum, Lacticaseibacillus casei, Lacticaseibacillus rhamnosus, Bifidobacterium longum subsp. infantis, Limosilactobacillus*	potato starch	112 days (16 weeks)	stool

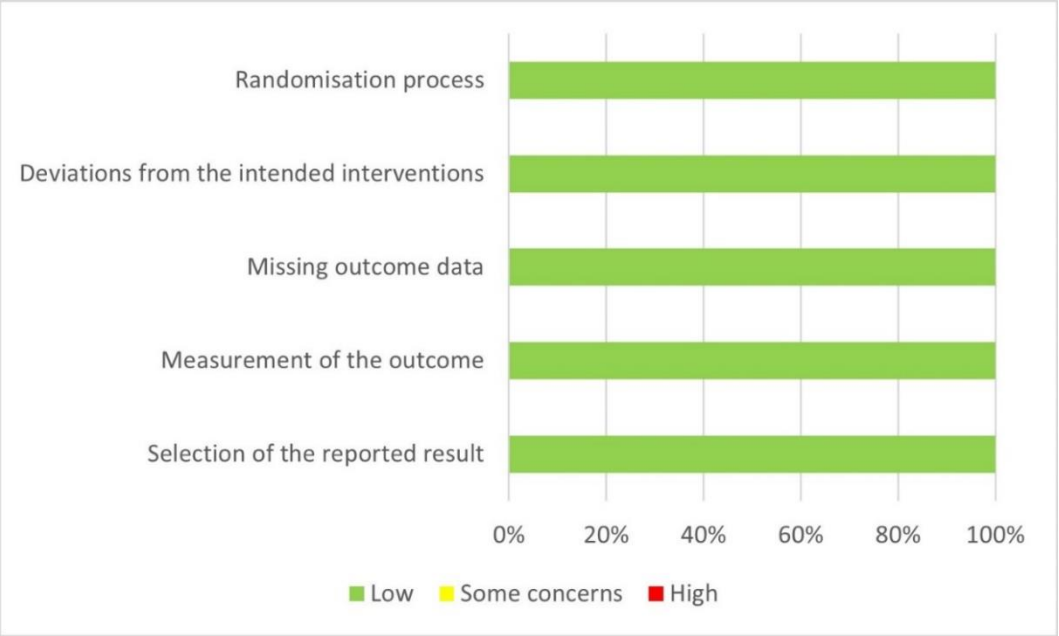
								Lactobacillus fermentum, Lactobacillus bulgaricus, Lactococcus lactis, and *Streptococcus thermophilus* (1×10^{11} CFU/day)	*fermentum, Lactobacillus delbrueckii subsp. bulgaricus*			
Mokkala (2018)	Finland	randomized double-blind placebo-controlled clinical trial	200 in 4 groups → 102 in the eligible 2 groups (100)	51	51	30.5 ± 4.9 (30.2 ± 3.9)	healthy overweight pregnant women	*Bifidobacterium animalis ssp. lactis* 420 (DSM 22089, *Lactobacillus rhamnosus* HN001 (ATCC SD5675) (2×10^{10} CFU/day)	*Lacticaseibacillus rhamnosus*	microcrystalline cellulose + medium chain fatty acids (capric acid C8 54.6% and caprylic acid C10 40.3%)	21.8 ± 2.6 weeks in the probiotic and 21.3 ± 2.3 weeks in the placebo group	serum
Stenman (2016)	Finland	double-blind, randomized, placebo-controlled, multicenter clinical trial (4)	225 in 4 groups → 112 in the eligible two groups (available for the ITT population) (80)	55	57	available for the ITT population (48 and 56 subjects) 50.6 ± 10.6 (49.9 ± 8.5)	overweight or obese (body mass index (BMI) 28.0-34.9) but otherwise healthy volunteers	*Bifidobacterium animalis subsp. lactis 420*™ (B420) (1×10^{10} CFU/day)	N.A.	12 g/day of microcrystalline cellulose	6 months	serum
Townsend (2018)	USA	double-blind, placebo-controlled, randomized study	25 (0)	13	12	20.1 ± 1.5 years for all participants	healthy division I male baseball athletes	*Bacillus subtilis* DE111 (1×10^{9} CFU/day)	N.A.	maltodextrin	84 days (12 weeks)	serum

Abbreviations: USA: United States of America; N.D.: no data; N.A.: not applicable; CFU: Colony Forming Unit; ITT: intention-to-treat

*If not otherwise mentioned, the studies were single centers.

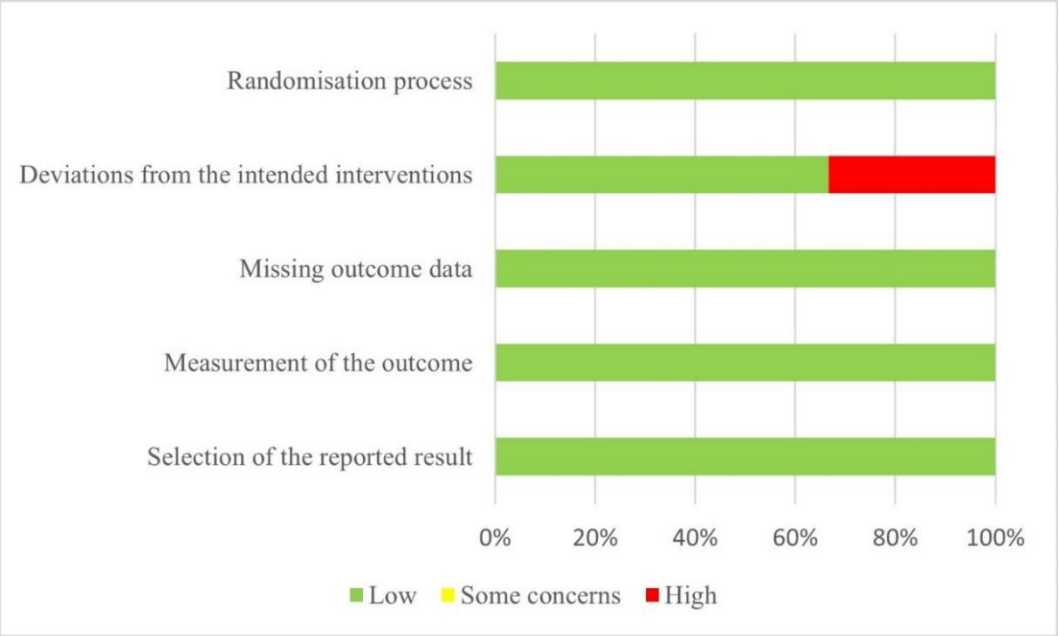
**There has been a major reclassification of bacterial genera, resulting in some articles using the "old" nomenclature while others adopt the updated names. We have included the corresponding information in the baseline table, aligning with the terminology used in each publication. Bacterial strains that have undergone reclassification are underlined, with their updated names, accurate as of February 2025, provided in the following column (182). Source: Földvári-Nagy et al. (83)

Figure S30. Risk of bias assessment for studies included in the meta-analysis – Assignment to intervention (the 'intention-to-treat' effect) (n=2) - The effect of probiotic supplementation on zonulin levels in healthy populations



Source: Földvári-Nagy et al. (83)

Figure S31. Risk of bias assessment for studies included in the meta-analysis – Adhering to intervention ('per-protocol' effect) (n=3) - The effect of probiotic supplementation on zonulin levels in healthy populations



Source: Földvári-Nagy et al. (83)

Table S11. GRADE assessment for the meta-analyses of zonulin levels - The effect of probiotic supplementation on zonulin levels in healthy populations

Certainty assessment							№ of patients		Effect		Certainty	Importance
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	probiotics	placebo	Relative (95% CI)	Absolute (95% CI)		
Zonulin (assessed with: ELISA)												
5	randomized trials	not serious	not serious	serious	not serious	none	150	157	-	SMD **0.01 SD lower** (0.39 lower to 0.37 higher)	⨁◯◯◯ Very low	IMPORTANT

CI: confidence interval; SD: standard deviation; SMD: standardized mean difference

Source: Földvári-Nagy et al. (83)