The impact of mixed carotenoid supplementation on skin carotenoid status as affected by probiotics and gut microbiota: A randomized, double-blind, controlled trial

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Abstract

Carotenoids are yellow-red plant pigments with antioxidant potential that humans primarily obtain from their diet. Carotenoid intake and blood and skin carotenoid status are consistently associated with a reduced risk of skin, age-related, and non-communicable diseases, but intervention studies have demonstrated a broad range of responses. Among various host-related factors that may account for this interindividual variation, the gut microbiome has sparsely been explored. Probiotics have also been proposed to enhance phytochemical status, but no human intervention studies have been conducted.

In the present work, a 10-week, double-blind, randomized controlled trial involving 37 premenopausal women (average age of 40) was conducted. All participants took two supplements daily: a mixed carotenoid supplement (17 mg of total carotenoids) and either a probiotic or a placebo. The primary outcome was skin carotenoid status (SCS) measured using a spectrophotometer. Subgroup analyses were conducted according to baseline SCS, carotenoid intervention responsiveness, and body mass index (BMI). Secondary outcomes included the analysis of stool microbiota composition, plasma carotenoids, and markers of antioxidant capacity, inflammation, and skin health (e.g., hydration, wrinkling).

Baseline gut microbial composition was associated with baseline SCS and carotenoid intervention Responder status. Probiotic supplementation enhanced the effects of carotenoid supplementation on SCS, but not plasma carotenoid concentrations. Subgroup analyses showed that the effect of probiotic supplementation was strongest in participants with one or more of the following characteristics: (a) normal BMI; (b) high baseline SCS; and (c) those who did not respond to carotenoid supplementation. Probiotic + carotenoid supplementation was associated

with changes in gut microbial composition, while carotenoid supplementation was not. There were no notable changes in plasma antioxidant capacity, immune status or skin health measures. Overall, these findings indicate the potential of probiotics to enhance SCS and highlight the potential role of the gut microbiota in the SCS response to carotenoid intervention.

Résumé

Les caroténoïdes sont des pigments végétaux jaune-rouge avec un potentiel antioxydant que les humains obtiennent principalement de leur alimentation. L'apport en caroténoïdes et le statut en caroténoïdes dans le sang et la peau sont systématiquement associés à une réduction du risque de maladies de la peau, de maladies liées à l'âge et de maladies non transmissibles, mais les études d'intervention ont démontré une large gamme de réponses. Parmi les divers facteurs liés à l'hôte qui peuvent expliquer cette variation interindividuelle, le microbiome intestinal a été peu exploré. Les probiotiques ont également été proposés pour améliorer le statut des composés phytochimiques, mais aucune étude d'intervention humaine n'a été menée.

Dans le présent travail, un essai contrôlé randomisé en double aveugle de 10 semaines impliquant 37 femmes préménopausées (âge moyen de 40 ans) a été réalisé. Toutes les participantes ont pris deux suppléments quotidiens : un supplément de caroténoïdes mixtes (17 mg de caroténoïdes totaux) et soit un probiotique, soit un placebo. Le principal résultat était le statut des caroténoïdes de la peau (SCS) mesuré à l'aide d'un spectrophotomètre. Des analyses de sous-groupes ont été menées en fonction du SCS de départ, de la réponse à l'intervention en caroténoïdes et de l'indice de masse corporelle (IMC). Les résultats secondaires comprenaient l'analyse de la composition du microbiote fécal, des caroténoïdes plasmatiques, et des marqueurs de la capacité antioxydante, de l'inflammation et de la santé de la peau (par exemple, hydratation, rides).

La composition microbienne intestinale de base était associée au SCS de départ et au statut de répondeur à l'intervention en caroténoïdes. La supplémentation en probiotiques a renforcé les effets de la supplémentation en caroténoïdes sur le SCS, mais pas les concentrations

plasmatiques de caroténoïdes. Les analyses de sous-groupes ont montré que l'effet de la supplémentation en probiotiques était le plus fort chez les participantes ayant une ou plusieurs des caractéristiques suivantes: (a) un IMC normal; (b) un SCS de départ élevé; et (c) celles qui n'ont pas répondu à la supplémentation en caroténoïdes. La supplémentation en probiotiques + caroténoïdes a été associée à des changements dans la composition du microbiote intestinal, tandis que la supplémentation en caroténoïdes seule ne l'était pas. Il n'y a eu aucun changement notable dans la capacité antioxydante plasmatique, le statut immunitaire ou les mesures de la santé de la peau. Dans l'ensemble, ces résultats indiquent le potentiel des probiotiques à améliorer le SCS et le rôle du microbiote intestinal dans la réponse du SCS à l'intervention en caroténoïdes.

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Contribution of authors

Lucas Gabriel Roldos Saibene (Candidate) was responsible for the design, coordination, and completion of the ethics and clinical trial applications, the overall study and recruitment, staff management, data collection and standard operating procedures, and experimental protocol development and analysis of plasma and supplement carotenoids and antioxidant capacity. Lucas was also responsible for the interpretation of data, the performance of statistical analyses and the production of the visualizations. Finally, Lucas wrote the original draft of the entirety of this dissertation.

Dr. Stan Kubow (Candidate's supervisor) was responsible for the conceptualization of the research project, with critical input into study design, methodology and any ongoing guidance and feedback where required. Dr. Kubow supervised all aspects of the dissertation and provided extensive feedback and input.

Dr. Michele Iskandar was involved in methodology development, statistical analysis, and data interpretation. Dr. Iskandar also provided extensive support in reviewing and editing the dissertation, along with ongoing guidance and feedback where required.

Dr. Kebba Sabally was involved in the development of the methodology and data processing for the analysis of plasma and supplement carotenoids and antioxidant capacity.

Ms. Marie-Laure Oula provided support for the ethics and clinical trial application and overall clinical trial coordination. Ms. Oula was also the primary point of contact between Lallemand Health Solutions and McGill University.

Mr. Jeremie Auger was primarily responsible for the bioinformatics analysis for 16S rRNA sequencing, including the methodology, most of the statistics, and most of the visualizations from the analysis.

Ms. Amanda Piano was involved in the analysis of cytokines, hormones, and stool sample qPCR and 16s rRNA sequencing.

Dr. Thomas A Tompkins was responsible for critical input into study design and methodology.

Dr. Chiranjeev Dash was responsible for the ultra-performance liquid chromatography-mass spectrometry analysis of plasma short-chain fatty acids.

Contribution to original knowledge

This is the first human randomized controlled trial (RCT) to evaluate the potential of probiotic supplementation to enhance mixed carotenoid supplement-induced changes in tissue carotenoid status and various health markers. This is also the first study to comprehensively evaluate associations between baseline gut microbiota composition and SCS, and baseline gut microbiota composition and response to carotenoid supplementation. The comprehensive analysis of response to carotenoid intervention using a distribution-based minimum clinically important difference (MCID) and analysis of response according to High or Low baseline SCS has not previously been conducted. This is also the first study to demonstrate a steady state in SCS after carotenoid supplementation and explicitly measure changes in gut microbiota composition after mixed-carotenoid supplementation. Finally, this is also the first supplementation RCTs to use sophisticated machine-learning algorithms, in addition to more traditional statistical methods, to analyze gut microbiota composition.

This is the first demonstration of the potential for probiotics to enhance phytochemical status in a human RCT. This study contributes to the understanding of the interindividual variation observed regarding phytochemical bioactives, specifically concerning carotenoids and the gut microbiota composition. It also forms a foundation for future work to investigate determinants of interindividual variation and interventions to overcome differences in response to phytochemical intervention.

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List of abbreviations

AA: Ascorbic acid

ABTS: 2,2′-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid

AES: Australian Eating Survey

ARE: Antioxidant response element

ARFS: Australian recommended food score

ASV: Amplicon sequence variant

BCO1: Beta-carotene monooxygenase-1

BCO2: Beta-carotene dioxygenase-2

BEV: Bacterial extracellular vesicle

BMI: Body mass index

CD36: Cluster determinant 36

CDCA: Chenodeoxycholic acid

CFU: Colony forming unit

CI: Confidence interval

CIE: International Commission on Illumination

CHD: Coronary heart disease

CKD: Chronic kidney disease

CRP: C-reactive protein

CVD: Cardiovascular disease

DC: Dark chocolate

DCA: Deoxycholic acid

DNA: Deoxyribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EV: Extracellular vesicle

FAVVA: Fruit and vegetable variety index

FDR: False discovery rate

FFQ: Food frequency questionnaire

FPP: Farnesyl pyrophosphate

FRAP: Ferric reducing antioxidant power

FVs: Fruits and vegetables

GIT: Gastrointestinal tract

GRAS: Generally recognized as safe

HDL: High-density lipoprotein

HPLC: High-performance liquid chromatography

IBD: Irritable bowel syndrome

IL: Interleukin

IPAQ: International physical activity questionnaire

KEGG: Kyoto encyclopedia of genes and genomes

LC-MS: Liquid chromatography-mass spectrometry

LDL: Low-density lipoprotein

LEfSe: Linear discriminant analysis effect size

LMM: Linear mixed model

MBQC: Microbiome quality control

MCID: Minimum clinically important difference

MCS: Mental component score

MDA: Malondialdehyde

MET: Metabolic equivalent of task

ML: Machine learning

MRM: Multiple reaction monitoring

MS: Mass spectrometry

NAFLD: Non-alcoholic fatty liver disease

NPC1L1: Niemann Pick C1-like 1

Nrf-2: Nuclear factor erythroid 2-related factor 2

NF-κB: Nuclear factor kappa B

ORAC: Oxygen radical absorbance capacity

PBS: Phosphate buffer solution

PCoA: Principal coordinates analysis

PCS: Physical component score

PSQI: Pittsburgh sleep quality index

PUFA: Polyunsaturated fatty acids

QoL: Quality of life

qPCR: Quantitative polymerase chain reaction

RCT: Randomized controlled trial

RFU: Relative fluorescence units

RNA: ribonucleic acid

ROS: Reactive oxygen species

rRNA: Ribosomal ribonucleic acid

RRS: Resonance Raman spectroscopy

RS: Reflection spectroscopy

SCFA: Short-chain fatty acid

SCS: Skin carotenoid status

SEM: Standard error of the mean

SFA: Saturated fatty acid

SR-B1: Scavenger receptor class B type 1

TAC: Total antioxidant capacity

TEWL: Trans-epidermal water loss

TNF-α: Tumor necrosis factor-α

TPTZ: 2,4,6-Tri (2-pyridyl)-1,3,5-triazine

UPLC-MS: Ultra-performance liquid chromatography-mass spectrometry

US: United States

UVR: Ultraviolet radiation

Chapter 1: Introduction

1.1 General introduction

A plant-based dietary pattern high in fruit and vegetables (FVs) is perhaps one of the most well-established aspects of a health-promoting diet [1]. A 2017 systematic review of 95 prospective observational studies demonstrated a dose-response relationship between FV intake and decreased relative risk for cardiovascular diseases (CHD, CVD, and stroke), cancer, and all-cause mortality [2]. Beyond the high fiber and micronutrient content and low-calorie density, increasing evidence suggests that dietary phytochemicals are partially responsible for the inverse association between plant-based dietary patterns and various diseases [3,4].

Carotenoids are a class of yellow-red phytochemicals that humans cannot synthesize and are primarily obtained from FV intake [5]. When ingested and following immediate usage for their provitamin A or antioxidant potential, a portion of absorbed carotenoids are readily stored in the skin allowing for non-invasive, optical assessment of skin carotenoid status (SCS) [6]. SCS has been suggested as a marker of overall health, is associated with markers of skin health, and correlates moderately with FV intake and strongly with blood carotenoid concentrations [7–12]. Results from carotenoid intervention trials generally align with observational associations between tissue carotenoid status and health (e.g., skin, noncommunicable and age-related diseases and outcomes), attributed to their antioxidant and anti-inflammatory potential, but can be limited by relatively high interindividual response variability [13]. Beyond food-related factors that affect carotenoid bioaccessibility (e.g., matrix, processing, other food components) and many factors (e.g., lifestyle, socioeconomic) that have been associated with varied responsiveness to carotenoid interventions, the gut microbiome is a host-related factor that has sparsely been investigated [14].

The gut microbiome is a complex ecosystem of microbes that interact with the host and are linked with metabolism and health [15]. Although gut microbiome research is generally in a descriptive phase, diet has emerged as a key modulator of gut microbial composition and function [16–18]. Dietary phytochemicals may play a considerable role in the relationship between diet and the gut microbiome [19,20]. Some preclinical trials have suggested improved carotenoid bioaccessibility, bioavailability, and metabolite production from gut microbial fermentation and biotransformation, but underlying mechanisms are unclear [21–25]. A few human observational studies and only a couple of intervention studies have reported associations between carotenoids and gut microbiome composition, but further well-designed human trials are needed [26–29].

Probiotics are live microorganisms that benefit the host in various ways, such as enhancing the gut-skin axis and gut microbial composition and metabolic capacity [30–32]. Probiotic intervention has been associated with improved skin health and the status of some micronutrients (e.g., B vitamins, calcium, iron) [33–35]. However, beyond suggestions for enhanced phytochemical bioaccessibility and status and the production of carotenoids, the influence of probiotics on carotenoid status is uncertain [19,36–38].

1.2 Research objectives

Conduct a double-blind randomized, controlled trial to:

1. Determine whether baseline gut microbiota composition is associated with (a) higher baseline SCS or (b) responsiveness to supplementation with mixed carotenoids.

2. Determine whether probiotic supplementation will enhance mixed carotenoid supplement-induced changes in tissue carotenoid status.

3. Determine whether supplementation with a mixed carotenoid supplement paired with and without probiotics is associated with changes in gut microbiota composition.

4. Determine whether supplementation with a mixed carotenoid supplement affects markers of a) antioxidant capacity, b) inflammation, and c) skin health when taken alone or in combination with a probiotic supplement.

1.3 Guiding hypotheses

Hypothesis for Objective 1:

Baseline gut microbiota composition will differ between (a) individuals with High and Low baseline SCS and (b) Responders and Nonresponders to carotenoid intervention.

Hypothesis for Objective 2:

Probiotic supplementation will potentiate the increasing effect of mixed-carotenoid supplementation on SCS.

Hypotheses for Objective 3:

i) Mixed carotenoid supplementation will lead to measurable changes in gut microbiota composition.

ii) The addition of probiotics to mixed carotenoid supplementation will produce more pronounced shifts in gut microbiota composition compared to the mixed carotenoid supplementation alone.

Hypotheses for Objective 4:

i) Mixed carotenoid supplementation will improve markers of (a) antioxidant capacity, (b) inflammation, and (c) skin health.

ii) The addition of probiotics to mixed carotenoid supplementation will produce more pronounced improvements in markers of (a) antioxidant capacity, (b) inflammation, and (c) skin health compared to mixed carotenoid supplementation alone.

Chapter 2: Literature Review

2.1 Carotenoids: An overview

Carotenoids are a class of naturally occurring yellow, orange, and red pigments commonly found in plants, algae, bacteria, and some fungi [39]. They were first discovered in the 19th century when chemists isolated yellow, fat-soluble pigments from food materials such as carrots, egg yolk, and tomatoes [5,40]. It was not until the 20th century that the structures of carotenoids, including β-carotene, lutein, and lycopene, were elucidated by the groups of Karrer and Kuhn (who received the 1937 and 1938 Nobel prizes in chemistry, respectively, for their work) and, subsequently, the presence of β-carotene in human blood and tissue was first reported [39,41]. The study of the roles of carotenoids in human health began in the 1930s when the conversion of β-carotene to retinol was demonstrated, but their function was relatively limited to vision until the 1970s when the link between carotenoids and protection against noncommunicable diseases was first suggested [5]. Since then, much has been learned about carotenoids and their absorption, distribution, metabolism, excretion, human health benefits, and purported underlying mechanisms [13,42,43].

2.1.1 Structure and classification

Carotenoids are a class of hydrocarbons generally made of 40 carbons in an eight-isoprenoid unit structure [44,45]. Their central conjugated double-bond structure confers the characteristic chromophore responsible for their color, makes them susceptible to non-enzymatic cleavage and isomerization, and results in a delocalization of electrons that allows for resonance-stabilization with antioxidant potential [46,47]. The hydrocarbon structure of carotenoids makes them lipophilic, contributing to their low bioavailability, complex absorption and metabolism,

and localization within cell membranes. At the same time, the variety of end groups and isomers can introduce polarity that alters solubility, absorption, and localization in cells and tissues [48,49].

Carotenoids can be classified according to different criteria [50] (see Fig. 2.1 A). Depending on the presence or absence of oxygen, often in their end groups, carotenoids are classified as xanthophylls (e.g., β-cryptoxanthin, lutein, and zeaxanthin) or carotenes (e.g., α-carotene, β-carotene, and lycopene), respectively. The relatively more polar xanthophylls are more soluble, dispersed (i.e., less prone to aggregation), evenly distributed in lipoproteins (both low-density lipoprotein (LDL) and high-density lipoprotein (HDL)) for transport, vertically oriented in the lipid membrane, and can be highly concentrated in certain tissues (e.g., lutein and zeaxanthin in the macula), compared to carotenes [51–55].

Carotenoids are also classified according to their ability to produce vitamin A. Provitamin A carotenoids have at least one substituted beta-ring (e.g., β-carotene, α-carotene and β-cryptoxanthin) and are preferentially and centrally cleaved by β-carotene 15,15'-monooxygenase (BCO1) to form retinal that is further transformed into other retinoids [56]. In contrast, non-provitamin A carotenoids can be eccentrically, enzymatically (by β-carotene 15,15'-dioxygenase (BCO2)) or nonenzymatically cleaved (e.g., oxidized) to form other carotenoid cleavage products called apocarotenoids (these also include the cleavage products without substituted beta-rings from α-carotene and β-cryptoxanthin) [57] (see Fig. 2.1 B).

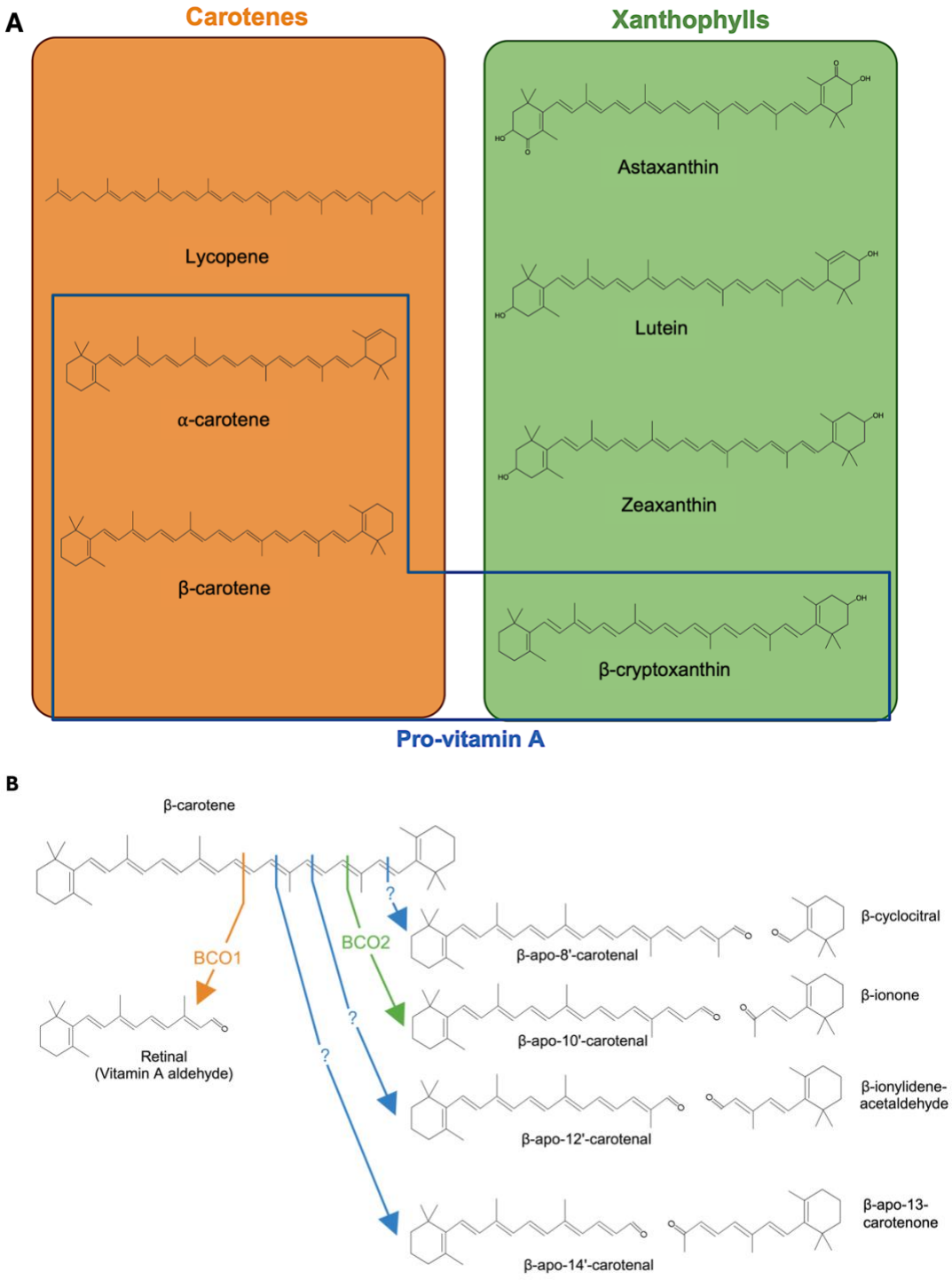


Figure 2.1. Common carotenoids and cleavage enzymes. (A) Structure and classification of common carotenoids (adapted from Lucas et al. (2022)). (B) Products of central and eccentric cleavages of β-carotene (adapted from Harrison & Quadro (2018)). BCO1 = β-carotene 15,15'-monooxygenase; BCO2 = β-carotene 15,15'-dioxygenase. Illustrations created with BioRender.com

2.1.2 Sources and intake

Carotenoids are synthesized by plants and many microorganisms, but not by humans who must obtain them from the diet, primarily through FV intake [39]. Among the over 1100 carotenoids that have been described, about 50 are present in the human food chain and six carotenoids account for a majority of all carotenoids identified in human tissue [58–60]. The major carotenoids commonly noted in the human diet and tissue include: (a) α-carotene and β-carotene from yellow-orange FVs such as carrots and peppers; (b) β-cryptoxanthin predominantly located in citrus fruits; (c) lutein and zeaxanthin found in deep green colored vegetables (where chlorophyll masks the carotenoid color); and (d) lycopene primarily found in red FVs such as tomato and grapefruit [61]. Levels of apocarotenoids in food and plasma are small (<1% of carotenoid content) and limited studies suggest that apocarotenoids are either not absorbed or are rapidly metabolized into derivatives (e.g., retinoids, aldehydes, alcohols) [57,62]. Carotenoid content of FVs can be influenced by various factors including, growing season, geographical and cultivation variation, maturity, and storage [63,64]. Tissue carotenoid levels have been suggested as a biomarker for FV intake as they are often moderately correlated, although the relationship can depend on the type of FVs and carotenoids consumed, as well as the measured tissue (e.g., blood vs skin) and confounding factors (e.g., lifestyle, demographics) [10,61,65,66].

Globally, carotenoid intake varies across countries according to many factors such as dietary pattern and socioeconomic status [67,68]. Data from the Latin American Health and Nutrition Study involving 9,218 individuals from urban areas of eight Latin American countries showed that total carotenoid intake ranges from 2.5 to 7 mg/day with major sources being carrots, beets, and tomatoes [67]. The average dietary carotenoid intake among US adults is about

10 mg/day, with tomatoes, carrots, and spinach observed as major food sources, according to data from 22,339 adults who participated in the National Health and Nutrition Examination Survey 2009 to 2018 cycles [69]. When adjusted for energy intake, carotenoid intake was greater in older individuals, women, non-smokers, supplement users, those with normal body mass index (BMI), those above the poverty threshold, and those who practice vigorous physical activity. In a 2020 review that evaluated carotenoid intake data primarily from European and North American countries, carotenoid intake ranged from 1 to 22 mg/day with an average of 11.8 mg/day (4.6 mg lycopene, 4.1 mg β-carotene, 2.2 mg lutein/zeaxanthin, 0.7 mg α-carotene, and 0.3 mg β-cryptoxanthin) [70].

2.1.3 Functional roles, safety and recommendations

The main biological role of carotenoids in humans is their provitamin A activity. The intake of provitamin A carotenoids supports vitamin A status, which plays roles in cell proliferation and differentiation, growth and development, and visual and immune function [64,71–73]. Some limited evidence suggests that apocarotenoids (besides retinoids) may act similarly to retinoic acid (an active metabolite of retinol) by interacting with nuclear hormone receptors, such as retinoic acid and retinoid-X receptors, but extensive analyses involving systems biology and multi-omics approaches are needed to consolidate and substantiate these preliminary findings [74–77]. Vitamin A toxicity from provitamin A carotenoid intake is unlikely as a negative feedback loop exists between the body's vitamin A status and the activity of BCO1 [78,79].

Beyond supporting vitamin A status and preventing vitamin A deficiency and associated complications (e.g., xerophthalmia, reduced immune function), carotenoids (both intake and tissue status) are consistently associated with improved skin health and a lower risk of mortality

and various non-communicable and age-related diseases [42,72,80–87]. Tissue carotenoid status (both blood and skin) has even been suggested as an indicator of overall health, with higher levels not only being associated with health status but also healthier lifestyle behaviors (e.g., no smoking or alcohol, regular exercise) [9,82,88–91]. Furthermore, intervention studies using FV extracts or synthetic carotenoids have shown improvements in cortisol and self-reported quality of life (physical and cognitive) and sleep [92–94], as well as outcomes for conditions characterized by chronic low-grade inflammation and oxidative stress, like obesity [95], neurodegenerative diseases [96–98], and age-related macular degeneration [99,100].

The health benefits of carotenoids are primarily attributed to their antioxidant and anti-inflammatory potential [81,101]. Based on in vitro and human and animal supplementation trials, carotenoids are indicated to directly contribute to the overall antioxidant network by acting as singlet oxygen quenchers and scavengers of reactive oxygen species (ROS), as well as indirectly through the induction of cellular redox signalling [101,102]. The conjugated double-bond structure of carotenoids allows them to absorb and safely dissipate excitation energy from high-energy compounds (e.g., singlet oxygen) and delocalize (i.e., stabilize) an unpaired electron after neutralizing a free radical [102,103]. Preclinical evidence also suggests that carotenoids may exhibit indirect antioxidant and anti-inflammatory effects by modulating transcription factors and their respective downstream targets relating to oxidative stress and inflammation [74,76]. Carotenoids have been shown to inhibit nuclear factor kappa B (NF-κB) signalling that generally involves the initiation of pro-inflammatory genes (e.g., tumor necrosis factor-α, interleukin (IL)-6) and is activated in response to stress signals (e.g., free radicals, cytokines) [104,105]. Carotenoids have also been shown to enhance the activation of nuclear factor erythroid 2-related factor 2 (Nrf-2) signalling that generally upregulates antioxidant response element (ARE)-driven genes (e.g.,

glutathione-S-transferases) [106,107]. ARE-driven genes are integral to the endogenous antioxidant system and their activation can also inhibit NF-κB and further decrease pro-inflammatory cytokines [108]. However, whether these observations result from direct antioxidant effects from carotenoids or indirect interactions with transcription factors, as well as whether physiological levels in humans or certain body sites (e.g., digestive tract) exert the same influence, is unclear [76,109].

Improvements in markers of oxidative distress (e.g., DNA damage, protein and lipid oxidation) and antioxidant capacity (e.g., ferric reducing antioxidant power (FRAP), total antioxidant capacity (TAC)) have been associated with increased serum carotenoid levels in some human carotenoid intervention studies [101,110–113]. However, results are not consistent and may depend on the health status of the population, attributed to differences in redox homeostasis; individuals with disease generally have excess ROS that overcome the antioxidant response and lead to oxidative stress [101,114]. Human observational studies generally support an inverse association between markers of oxidative stress and carotenoid status, especially when considering individuals with chronic disease [101]. However, results from human intervention studies generally demonstrated beneficial effects in individuals with chronic disease, but small or no change in healthy individuals [101]. A meta-analysis of human carotenoid intervention trials involving astaxanthin showed improvements in malondialdehyde (MDA, an indicator of lipid peroxidation), but not TAC, C-reactive protein (CRP, an indicator of systemic inflammation), IL-6 or TNF-α [115]. When stratified by health status, improvements in MDA and IL-6 were observed in individuals with type 2 diabetes, but no other markers and not in healthy individuals. A meta-analysis of mixed carotenoid supplements in healthy individuals reported improvements in FRAP and oxygen radical absorbance capacity (ORAC) but not antioxidant enzymes (e.g.,

superoxide dismutase) [116]. However, the individual meta-analysis for each outcome in these studies only included 2-5 studies, which limits the overall confidence in the findings. A 2021 meta-analysis of 26 RCTs reported reductions in inflammatory markers, such as significant decreases in CRP and IL-6), after carotenoid supplementation [117]. However, there was a high heterogeneity among included studies, attributed to the type and dosage of carotenoids, intervention duration, and participant age and health status.

On the other hand, some in vitro studies have demonstrated prooxidant effects from β-carotene and lycopene that may manifest as a function of the redox potential of the biological environment (e.g., oxygen levels, interactions with/presence of other antioxidants, carotenoid concentrations) [49,118–120]. A meta-analysis of 8 RCTs involving men and women aged 40-84 reported that β-carotene supplementation (6 mg/d to 50 mg/every other day for 4-13 years) was positively associated with lung cancer in smokers and asbestos workers but not associated with overall or other site-specific cancers [121]. A similar meta-analysis focused only on lung cancer risk observed a similar result and reported a general increasing trend with a greater dose [122]. Notably, some studies did not show adverse effects on lung cancer in smokers, but they differed from those that did in their dose and frequency of β-carotene supplementation and number of smokers. In the Physicians Health Study, 22,071 men (11% smokers, 39% former smokers; aged 40-84) took 50 mg β-carotene every other day for 12 years [123]. In the Linxian trial, 29,450 healthy men and women (30% smokers; aged 40-69) took 15 mg of β-carotene + 30 mg α-tocopherol + 50 µg selenium daily for 5 years [124]. It is also worth noting that a systematic review of 17 observational studies reported an inverse association between plasma carotenoids (including β-carotene alone) and lung cancer risk or mortality, although there was not enough data to include a stratified analysis by smoking status [125].

The primary role of photooxidative protection conferred by carotenoids in plants also manifests in humans via skin protection and maintenance [39,83]. In vitro studies using cultured human skin fibroblasts have demonstrated improvements in skin antioxidant defenses, such as radical scavenging and inhibition of lipid peroxidation from ultraviolet radiation (UVR), with carotenoid treatment [83]. Mixed and single carotenoid supplement human intervention trials have demonstrated improvements in skin health outcomes including UVR-damage resistance (e.g., prevention or decrease in UVR-induced erythema), wrinkling, hydration, and elasticity [8,126–129]. A 2008 meta-analysis of seven RCTs showed that supplementation with β-carotene (15 to 180 mg/day) lasting at least 10 weeks can protect against sunburn (i.e., increases the minimal erythema dose) [130]. A review of 25 human clinical trials showed improvements in various markers of oxidative stress and inflammation (e.g., matrix metalloproteinase 1 gene expression, IL-6, TNF-α), as well as photoprotection against UV radiation, after supplementation with carotenoid (e.g., β-carotene, lutein, lycopene) and carotenoid-rich supplements [131]. Generally identified by yellow-orange skin coloration (most noticeable on the palms and soles) and defined as carotenaemia, an excess intake of carotenoids in humans appears to be benign, however high tissue carotenoid levels leading to carotenemia can result from diseases and disorders (e.g., hypothyroidism, genetic defects in carotenoid cleavage enzymes) linked to changes in carotenoid metabolism in some rare cases [132,133].

Although carotenoids have demonstrated various health benefits and no risks (assuming physiological doses of about 12 mg total carotenoids/day and apart from the increased risk of lung cancer in smokers), there are no clear recommended values for the daily intake of carotenoids [70,134,135]. This is primarily because they are not classified as essential nutrients and many factors complicate the quantification of clear intake needs (e.g., dietary and host-related

factors, interindividual variability, different population needs) [13,70]. The latest guidelines from the National Academy of Medicine report that there is insufficient data to estimate requirements (e.g., dietary reference intakes, tolerable upper intake limits) for β-carotene and other carotenoids; they support existing recommendations for increased carotenoid-rich FVs (≥ 5 servings/d providing 3-6 mg β-carotene) and do not advise β-carotene supplementation [136]. The European Food Safety Authority also concluded that, although there is no indication that β-carotene intake from the background diet (even as a food additive) is associated with adverse health effects, there is insufficient data to establish an upper limit for β-carotene supplementation [137]. They also recommend that β-carotene supplementation should be limited to meeting vitamin A requirements by the general population and avoided by smokers. The closest to an official guideline may be meeting vitamin A recommendations with provitamin A conversion rates: 900 µg of retinol equivalents for a healthy adult man would require about 11 or 22 mg/day of β-carotene or α-carotene and β-cryptoxanthin, respectively, assuming carotenoids would be the only source [70].

2.1.4 Liberation, absorption, distribution, metabolism and excretion

A fraction of carotenoids is released from the food matrix via food processing or mastication in the oral phase, and stomach enzymes in the gastric phase, before travelling through the duodenum where they are incorporated into the lipid phase of the meal and are transferred into mixed micelles [138]. An in vitro simulated digestion model indicated that about 10% of carotenoids are bioaccessible (i.e., released from the food matrix during digestion and potentially available for further uptake and absorption) in the small intestine [21]. Carotenoid-containing micelles are taken up by enterocytes, primarily in the proximal jejunum, and

combined with apolipoproteins before being incorporated into chylomicrons for transport throughout the body [73,139–141]. Micelle uptake by enterocytes occurs through both passive diffusion, via a concentration gradient, and facilitated diffusion, which is mediated by some of the same proteins used in cholesterol and fat-soluble vitamin transport (e.g., scavenger receptor class B type 1 (SR-B1), cluster determinant 36 (CD36), Niemann Pick C1-like 1 (NPC1L1)) [142,143]. Inside the enterocyte, carotenoids can be processed into vitamin A or apocarotenoids through enzymatic cleavage (BCO1 or BCO2) and are packaged into chylomicrons for transport in the serum along with the other carotenoids [57,73,139,141].

The absorption, distribution and storage of carotenoids have been suggested to follow the triage concept: transport via serum to cover immediate needs, such as ROS neutralization and vitamin A replenishment, while excess carotenoids are stored in the liver, adipose tissue, retina, and skin to maintain these body systems [73,82,144]. Across human clinical trials, the absorption efficiency of isotopically labelled β-carotene (food and supplement form) varied greatly (~3-80%), generally ranging between 10-30% [138,145]. In vitro intestinal cell culture models involving Caco-2 cell (and clones with BCO1 activity, TC7) monolayers report up to 11% absorption of pure carotenoids [146,147]. After chylomicron metabolism, carotenoids are predominantly transported in LDL and HDL in the bloodstream for longer-term distribution throughout the body. The more non-polar carotenes are predominantly found in LDL and the more polar xanthophylls are more evenly distributed among LDL and HDL [53,55]. Although the transport mechanism from serum to tissues is uncertain, it likely involves the transporters mentioned (e.g., SR-B1) and LDL receptors (LDLR) that recognize HDL and LDL [52]. Lastly, given the relatively poor absorption of carotenoids, it is not surprising to note that large amounts of

ingested carotenoids were excreted in human stool (10-85% excretion) when examined in 101 healthy adults (45-65 years) [148].

Various food-related factors, spanning from the food matrix, processing, and storage of carotenoids to interactions with other food components, can influence the bioaccessibility and absorption of carotenoids [25,149,150]. Given their hydrophobic, hydrocarbon structure, carotenoid absorption is enhanced with dietary fat consumption. Salad consumption with full-fat dressing results in greater chylomicron carotenoid content than low-fat or fat-free dressed salad consumption [151]. Longer-chain fatty acids appear to facilitate micellization of carotenoids, while the influence of the degree of fatty acid saturation still needs to be clarified [152–154]. The enhanced absorption of lutein from eggs compared to either spinach or lutein supplements (matched doses of 6 mg/day over 9 days in a crossover RCT in 10 healthy men) was attributed to the food matrix of the egg and its accompanying lipid and cholesterol content [155]. Fiber, protein, and plant cell wall resistance to digestion and degradation or complex formation with other components (e.g., minerals), as well as the form (e.g., solid vs liquid or crystalline vs globular) of carotenoids in the food matrix, can limit the bioavailability of carotenoids [25,64,156,157]. An RCT involving 11 participants who consumed 10 mg lycopene from different tomato juices showed that *cis*-isomers of lycopene, stored in a lipid-dissolved globular state, were more than eight times more bioavailable than the *trans*-isomers, stored in an aggregated crystalline state [158]. Bioavailability was evaluated by sampling and analyzing plasma triglyceride-rich lipoprotein fractions (predominantly chylomicrons) from participants for 12 h after intake (i.e., measured at 2, 3, 4, 5, 6, 8, 10 and 12 h). Moreover, carotenoids can compete with each other or other fat-soluble compounds for incorporation into micelles and absorption [64,152,159–161].

Emerging innovations, including nanotechnology and supramolecular transporters (i.e., carriers), demonstrate the potential for improved carotenoid bioaccessibility and bioavailability [162]. This can involve emulsions and size and surface modifications that can enhance solubility, dispersion, and cellular uptake or even nanocarriers and other nutrients or compounds that improve mucus penetration or regulate the expression of carotenoid transport proteins [162–164]. Some studies have demonstrated the potential of biological vesicles, such as extracellular or outer membrane vesicles, to enhance lipophilic phytochemical uptake and intestinal permeability [165–167]. Extracellular vesicles (EVs) are lipid bilayered nanoparticles, produced by eukaryotes and prokaryotes, that can package lipids, protein, DNA, RNA, and organic compounds for intercellular communication and waste control [168]. Jang et al. (2023) demonstrated high stability and good antioxidant and anti-inflammatory activity from fetal bovine serum extracellular vesicles loaded with saponin and astaxanthin [166]. Once absorbed, EVs can directly fuse with plasma membranes of target cells or are internalized by mammalian cells through (clathrin or lipid raft-mediated) endocytic pathways [169,170].

Bacterial EVs (BEVs) have been observed in human plasma, cerebrospinal fluid and stools [171] and hold great therapeutic and diagnostic potential for understanding complex interactions between commensal microbes and the host [172,173]. BEVs have previously been used for vaccination against pathogenic bacteria as well as loaded with bioactive molecules when isolated from, or within, probiotic bacteria [174,175]. While the validity of BEVs is debated, the presence of these membranous entities, whether formed from membrane fractions (an ‘artificial vesicle’) or controlled release, might serve as a delivery system for improved carotenoid bioaccessibility and bioavailability [167,176,177].

2.1.5 Assessment and skin carotenoid status

Various methods for assessing carotenoid intake and status, essential for understanding the relationships between carotenoids, diet, and disease, have been developed with their own strengths and weaknesses [6]. Carotenoid intake is primarily estimated by matching subjective recall or tracking of dietary intake, from burdensome questionnaires or diaries such as 24-h recalls or food records, with often incomplete and inaccurate nutrient databases [178,179]. Furthermore, dietary assessment does not account for differences in absorption and metabolism of carotenoids [180]. Carotenoid status is frequently assessed with complex analytical chemistry techniques, generally including high-performance lipid chromatography (HPLC) paired with UV-visible light or mass spectroscopy detection, involving invasive tissue sampling (often blood), preparation that may introduce oxidation and expensive equipment [181]. Moreover, blood is a transport medium for carotenoids meaning it represents shorter-term status and may have higher intra-individual variability than storage tissues (e.g., skin) [182–184].

The recent developments in non-invasive, optical methods for skin carotenoid status (SCS) assessment balance the convenience of dietary assessment and the objectivity of blood analysis [6]. Carotenoids accumulate in the epidermis and dermis, with the highest concentrations in the stratum corneum (outermost layer), allowing for the non-invasive assessment of SCS [83,185]. Carotenoids are thought to be transported to the stratum corneum via surface penetration after sweat or sebaceous gland secretion and during the proliferation of keratinocytes [186]. Since greater carotenoid levels are observed in skin regions with a higher density of sweat glands, such as the palms and forehead, these regions serve as primary measurement areas for SCS

assessment [129,187,188]. Moreover, melanin interference is less likely in the palm, regardless of ethnicity [10,189].

There are two main optical methods for SCS assessment. Resonance Raman spectroscopy (RRS) measures unique vibrational energy transitions after laser excitation that are highly specific and sensitive but exhibit weak signals and require expensive and complex equipment [190,191]. In contrast, reflection spectroscopy (RS) measures relatively strong wavelengths reflected off the skin that fit within the absorption maxima (450-480 nm) of key dietary carotenoids to estimate SCS [185,189]. RS slightly sacrifices specificity and sensitivity for cost, convenience, and accessibility when compared to RRS, but both optical methods generally share moderate ($0.3 < r \leq 0.5$) correlations with dietary intake and strong to very strong correlations ($0.5 < r \leq 1.0$) with blood carotenoid concentrations [10,187,192–195].

Colorimetry (often measured using a spectrophotometer) extends spectral reflectance measurements of the skin by calculating the yellow color saturation, which is characteristic of the presence of carotenoids in the skin, as an estimate of SCS [7,10,189,196]. The carotenoid-associated yellow coloration is quantified by calculating the b* value, a component of the device independent and perceptually uniform International Commission on Illumination (CIE) L*a*b* color space, that is a measure of blue (-120) to yellow (120) coloration [7,197,198]. Colorimetry maintains the ability to objectively track changes in SCS after FV and carotenoid supplementation (Table 2.1) [11,199–202]. With properly controlled capture and processing conditions, colorimetry may also be used to track SCS via digital photo analysis [201,203]. Colorimetry-measured SCS has been moderately correlated with dietary intake in various studies [10,204] and weakly with blood carotenoid concentrations in one study [200] (Table 2.2). However, this latter study included nine different skin regions in the colorimetry measurement for SCS,

and the melanin concentrations of varying skin regions that can interfere with the SCS estimation were not controlled for, which may account for the weak correlation [198,205,206].

Table 2.1. Summary of carotenoid intervention studies using colorimetry-based SCS assessment.

Reference	Study design	Intervention (dose)	Population (characteristics)	SCS Outcomes	Areas of skin for SCS assessment	Tool used
Tan et al., 2015	6-wk RCT (3-wk FU)	Fruit smoothie (~25 mg/d total carotenoids) vs. mineral water	68 Malaysian university students (mean age: 20; 58% women)	↑Δb* value at wk 5 (3.4; $p < 0.001$), wk 7 (3.8; $p < 0.001$), and wk 9 (3.3; $p < 0.001$) compared to wk 0 (18.33 b* value); no changes in control group	Three areas: forehead and both cheeks	CM2600D spectrophotometer (Konica Minolta)
Pezdirc et al., 2016	4-wk crossover RCT (2-wk washout between alternate inteventions)	High vs. low carotenoid-containing FV intake (↑5 vs. ↓ 2mg/day total carotenoids)	30 women (mean age: 22; mean BMI: 23; 83% Caucasian)	↑Δb* value (0.6; $p < 0.001$) in high carotenoid-containing FV intake group compared to low	Nine areas: both cheeks, forehead, outer and inner arm, shoulder, hip, palm, and sole of foot	CM700D spectrophotometer (Konica Minolta)
Stephen et al., 2011	8-wk trial	β-carotene supplement (15 mg/d)	10 Caucasian adults (aged 19-22; 80% women)	↑ Δb* value (≥ 1.08) of the palm ($p < 0.001$), forehead ($p = 0.033$), shoulder ($p = 0.028$) and both cheeks($p < 0.01$), but not inner arm ($p = 0.186$)	Six areas: palm, forehead, shoulder, both cheeks and inner arm	CM2600D spectrophotometer (Konica Minolta)
Foo et al., 2017	12-wk RCT	β-carotene supplement (18 mg/d) vs. lactose capsule (400 mg)	42 Caucasian men (mean age: 22)	↑Δb* value (2.70; $p < 0.001$) after β-carotene, but not placebo, treatment (0.38; $p = 0.22$)	Three areas: forehead and both cheeks	Analysis of color-calibrated images captured in standardized conditions
Coetzee & Perrett, 2014	8-wk trial	β-carotene supplement (15 mg/d)	10 black African women (mean age: 28)	↑ Δb* value of the palm (1.67; $p = 0.001$) and inner arm (1.14; $p = 0.008$), but not forehead, outer arm, and both cheeks ($p \geq 0.11$)	Six areas: palm, forehead, both cheeks, and inner and outer arm	CM2600D spectrophotometer (Konica Minolta)
Roldos et al., 2018	2-week trial (4-wk FU)	β-carotene-rich carrot juice (25 mg/d in 250 mL)	25 university students (mean age: 22; 64% women)	↑ Δb* value of the palm at wk 2, 4, and 6 (0.82-1.18; $p < 0.05$), compared to baseline; No changes in the forehead or top of hand	Three areas: palm, forehead, top of hand	CM600D spectrophotometer (Konica Minolta)

BMI measures in kg/m^2; FU = follow up; FV = Fruit and Vegetable; RCT = randomized controlled trial; SCS = skin carotenoid status characterized by skin yellow color saturation (b* value).

Table 2.2. Summary of studies evaluating the relationship between colorimetry-based SCS and FV intake and plasma carotenoid concentrations.

Reference	Design (intervention or assessment)	Population (characteristics)	Outcomes	Areas of skin for SCS assessment	Tool used
Pezdirc et al. (2016)	4-wk crossover RCT (High vs. low carotenoid-containing FV intake; ↑5 vs ↓ 2mg/day total carotenoids)	30 women (mean age:22; mean BMI: 23; 83% Caucasian)	Weak correlation between SCS and total plasma carotenoids (r = 0.27; p <0.05)	Nine areas: left and right cheek, forehead, outer and inner arm, shoulder, hip, palm, and sole of foot.	CM700D spectrophotometer (Konica Minolta)
Ashton et al. (2018)	Cross-sectional (ARFS and FAVVA)	148 adults (mean age: 22; 55% women; mean BMI: 24)	Moderate correlation between SCS and FV intake (ρ = 0.30 and 0.39; p <0.001)	Three areas: inner and outer arm and palm.	CM700D spectrophotometer (Konica Minolta)
Bixley et al. (2018)	Cross-sectional (FFQ)	30 Caucasian men (mean age: 22; mean BMI: 24)	SCS strongly correlated with carotenoid intake (r = 0.6; p <0.001) and moderately with FV intake (r = 0.42; p = 0.02)	Four areas: forehead, palm, inner arm, and sole of foot.	Spectro-Guide 450 Gloss 6801 spectrophotometer (BYK Gardner)
Coyle et al. (2018)	Cross-sectional (AES 2010)	118 Caucasian women (median age: 25; median BMI: 23)	Weak to moderate correlation between SCS and FV intake (β = 0.29; p < 0.001)	Nine areas: left and right cheek, forehead, outer and inner arm, shoulder, hip, palm, and sole of foot.	CM700D spectrophotometer (Konica Minolta)
Pezdirc et al. (2015)	Cross-sectional (AES 2010)	91 Caucasian women (median age: 22; median BMI: 23)	Strong correlation between SCS and FV intake (β = 0.80; p = 0.017 **|** ρ = 0.65; p <0.01))	Nine areas: left and right cheek, forehead, outer and inner arm, shoulder, hip, palm, and sole of foot.	CM700D spectrophotometer (Konica Minolta)
Whitehead et al. (2012)	Longitudinal (FFQ at 0, 3 and 6 wks)	35 adults (mean age: 21; 60% women; 97% Caucasian)	Moderate correlation between SCS and FV intake (ρ = 0.25; p = 0.038)	Seven body locations: Left and right cheek, forehead, inner and outer arm, shoulder, and palm.	CM2600D spectrophotometer (Konica Minolta)
Stephen et al. (2011)	Cross-sectional (FFQ)	82 Caucasian adults (aged 18-26; 59% women)	Moderate correlation between SCS and FV intake (ρ = 0.25; p = 0.026)	Four body locations: inner and outer arm, shoulder, and palm.	CM2600D spectrophotometer (Konica Minolta)

Spearman's correlation coefficients used for non-normal distributed data were described as poor < 0.20, moderate 0.2-0.6, or strong > 0.6, as previously suggested within dietary validation studies [207,208]. BMI measures in kg/m^2; AES = Australian Eating Survey; ARFS = Australian Recommended Food Score; FAVVA = Fruit and Vegetable Variety Index; FFQ = Food Frequency Questionnaire; FV = Fruit and Vegetable; SCS = skin carotenoid status characterized by skin yellow color saturation (b* value).

2.1.6 Interindividual variability in carotenoid status

A prominent obstacle in carotenoid research is the rather large interindividual variation in carotenoid status [13,180,209]. Interindividual differences in tissue carotenoid status have been observed in observational and intervention studies using various sources (e.g., FVs, FV extracts, synthetic carotenoids), supplement types (e.g., carotenes, xanthophylls), and doses (1-120 mg/d), ranging from 40-240% relative standard deviation of tissue carotenoid levels [13,210–212]. Differences in response to 2 weeks of daily carrot juice intake (250 mL; 25 mg β-carotene) were observed in an open-label trial involving 25 participants [201]. Half of the participants significantly increased SCS after supplementation which was sustained for the full four-week follow-up (i.e., responders). On the other hand, seven participants demonstrated significant, sustained increases at weeks 4 and 6 compared to baseline (i.e., slow responders), while six participants did not show any change in SCS (i.e., nonresponders). Interestingly, the baseline SCS of each responder group was different, with the nonresponders having the lowest value and responders the highest, suggesting that carotenoid intervention responsiveness may have to do with the triage concept mentioned earlier (i.e., use vs storage/threshold for storage).

Beyond the differences in food- and diet-related factors that influence carotenoid bioaccessibility and bioavailability, the interindividual variation in carotenoid status has been associated with various host-related factors, ranging from genetic differences in cleavage enzymes and transport proteins to differences in sociodemographic and lifestyle factors [13,213]. The host-related factors have primarily been explored in preclinical and observational studies. Lifestyle habits and health status may modulate the use or storage of carotenoids, such as smoking and greater BMI that are associated with lower carotenoid status, presumably because

of increased oxidative stress, inflammation, or adipose tissue sequestration of carotenoids [214–217]. Genes (i.e., different single nucleotide polymorphisms) and diseases related to digestive enzymes, bile production (e.g., Crohn's disease), or transporters can influence carotenoid absorption and metabolism [13,48,217]. Women have demonstrated higher carotenoid levels than men [218] even when consuming equal amounts of fruits and vegetables [219], but there is no plausible explanation beyond conjecture, such as the role of estrogen in lipid metabolism [220,221]. Although it is unclear whether the gut microbiome contributes to carotenoid metabolism or whether carotenoids can be absorbed in the colon, differences in gut microbial composition have been suggested as another host-related factor that may contribute to interindividual variations in carotenoid status [27,209].

2.2 The gut microbiome

The gut microbiome is a complex ecosystem of microbes present in the gastrointestinal tract (GIT) that interact with the host and are linked with metabolism and health [15,222]. The human gut provides a habitable environment and a steady supply of nutrients, while the gut commensal microbiota competes with pathogenic bacteria and supports host metabolism and immune function [223]. Dietary fibers that the host cannot digest are fermented by anaerobic microbes to produce short-chain fatty acids (SCFAs) that serve as energy for colonocyte growth and maintenance and confer various benefits to the host (e.g., anti-inflammatory, metabolic and immune regulation) [224,225]. Gut microbiota also synthesize essential vitamins and amino acids and modify compounds, such as bile acids and phytochemicals [226].

Despite strides in human gut microbiome research driven by next-generation sequencing technologies, the field is still mostly in a descriptive phase [16,227]. Certain gut microbial

differences in composition and function have been observed between healthy individuals and those with disease, but a clear definition of a healthy microbiome does not seem to exist [228–231]. For example, an increased ratio of Firmicutes to Bacteroides, the two most abundant phyla in the gut microbiome, was initially suggested as a biomarker for obesity, complete with a proposed underlying mechanism of more efficient energy extraction from fiber fermentation by Firmicutes [232–234]. However, further data and analyses showed positive, null, and negative associations between this proposed biomarker and obesity [235]. Another example of contradictory conclusions is exemplified by a 2019 systematic review of 16 studies comparing gut microbiota between Parkinson's disease patients and healthy individuals [236]. Although several taxa, including *Lactobacillaceae* and *Bacteroidetes*, were significantly increased in Parkinson's disease patients in four studies, they significantly decreased in two other studies. Furthermore, over 100 differentially abundant taxa across all taxonomic levels (from phylum to species) were identified overall. As gut microbiome research progresses, the variation in results across studies is being attributed to key aspects of technical variability in microbiome research as well as biological and environmental factors, such as age, genetics, geography and lifestyle, that characterize the complexity and variability of the gut microbiome [235–238].

2.2.1 Technical variation in human gut microbiome studies and best practices

Beyond the variability in gut microbiome research that arises from biological and environmental factors such as diet (covered in Section 2.2.2), several key technical factors involving sample collection, processing, and analysis introduce variability [239,240]. This is exemplified by the Microbiome Quality Control (MBQC) project baseline study where blinded specimen sets from human stool and two positive controls (chemostats, a large quantity of fecal-

derived material grown in a chemostat bioreactor; and ‘artificial colonies’ made up of common human stool and oral cavity microbiota) were sequenced by 15 different laboratories (using 16S rRNA sequencing) and analyzed using nine bioinformatics protocols [241]. Variability depended mostly on the specimen type and origin, followed by DNA extraction, sequencing, and bioinformatics data processing. Although each step had the potential to introduce variation of comparable effect size to that of biological differences, many were typically smaller. While other studies have echoed issues related to a lack of standardization in microbiome research to tackle technical variation, initiatives like the MBQC project have been criticized for not providing clear unified protocols and a gold standard of microbiome research methods is yet to be established [239,242]. Thus, it is worth characterizing key steps and methods (e.g., strengths, limitations, frequency of use) to provide some direction for current best practices in gut microbiome research.

Certain sample handling procedures and sequencing methods may improve the accuracy, reproducibility, and efficiency of microbiome studies. Technical variation in sample handling can be minimized and characterized by using the same reagent kits for all samples in a study and collecting multiple samples among time points (to characterize and internalize intrinsic variability) [239,243]. Stool sample preprocessing can also improve reproducibility (e.g., reduce a ratio bias that can occur due to the inefficient lysis of Gram-positive bacteria) and accuracy [244]. Mechanical lysis by bead-beating has been positively associated with bacterial diversity and is considered necessary for efficient DNA extraction from Gram-positive bacteria (because of the thick peptidoglycan layer in their cell wall) [242,245–247]. Although metagenomic sequencing (e.g., whole-genome shotgun sequencing) is more comprehensive, providing taxonomic and functional information and allowing for strain-level resolution, it is more expensive and computationally

demanding than marker gene sequencing (e.g., 16S rRNA gene amplicon sequencing) that is most often used for its cost-effectiveness [248]. Furthermore, when comparing shotgun and 16S rRNA sequencing, many aspects of bacterial community characterization were found to be consistent between methods [249]. However, V3-V4 region primers have been recommended for greater accuracy if using 16S rRNA sequencing as they show the lowest degree of deviation between observed and expected taxa abundance and are frequently used [249,250]. Still, when enough reads are available, shotgun sequencing has more power to detect less abundant taxa that may be biologically meaningful compared to 16S rRNA sequencing [251].

The high dimensionality of gut microbiome data, presenting hundreds to thousands of different taxa, requires careful processing for meaningful results [239]. A 2021 systematic review involving 419 microbiome studies on human participants showed a considerable level of heterogeneity in data analysis strategies, with alpha and beta diversity being the most investigated, followed by dimension reduction, differential abundance, clustering, and predictive models [252]. The authors noted a shift away from general descriptions (diversity metrics) to more focused questions and sophisticated methods (differential abundance). Overall patterns in microbiome variation are often assessed using alpha and beta diversity that correspond to within-sample and between-sample diversity, respectively. Quantitative metrics that incorporate abundance and evenness or presence vs. absence of features, such as the Shannon index and weighted UniFrac metrics (for alpha and beta-diversity, respectively), are generally more sensitive and integrative measures for species diversity (compared to qualitative metrics) and most often used [253]. Ordination techniques, such as principal coordinates analysis (PCoA), are often used to visualize beta-diversity as they reduce large complex distance matrices into manageable three-dimensional representations of sample distances [252].

The identification of differentially abundant taxa between groups (i.e., treatment vs. control) is particularly challenging, not only because of the high dimensionality of gut microbiome data but also because of its sparsity (i.e., many zeros present) and compositionality (i.e., based on relative abundance specific to the sample, not actual abundance) [254]. This prohibits the use of classic statistical techniques that make specific assumptions about the data (e.g., normality, independence of observations, linearity) and often lead to high false discovery rates (FDR) [252,254–256]. Nonparametric tests that target differential abundance (e.g., linear discriminant analysis effect size or LEfSe) can be useful to identify specific taxa differences, but they present high variability in the number of significant taxa identified, can still have high false positive rates (especially in small datasets), and do not integrate the compositional nature of the microbiome data [252,257,258]. Furthermore, commonly used methods do not explicitly adjust for repeated measures or multiple covariates that can substantially influence results [259].

Table 2.3. Comparative table summarizing the strengths and limitations of various statistical and machine learning (ML) methods used for analyzing gut microbiome data [252,260–263]

Method	Description	Strengths	Limitations
Alpha Diversity (e.g., Shannon)	Measures the diversity within a single sample based on species richness and evenness.	- Simple to compute - Quantifies diversity within a sample - Easily interpretable	- Ignores differences between samples (inter-sample diversity) - Sensitive to rare species - Limited in distinguishing subtle community changes
Beta Diversity (e.g., UniFrac)	Assesses differences in microbial community composition between multiple samples.	- Captures differences between samples - Used for clustering - Works well for exploratory analysis	- Requires large sample sizes - Sensitive to sampling depth - Does not handle compositional data challenges well
Ordination (e.g., PCoA)	Techniques to visualize complex, multi-dimensional data by reducing dimensions while preserving relationships.	- Helps visualize relationships between samples - Reduces high-dimensional data - Interpretability	- Can be difficult to interpret biologically - Sensitive to distance metrics used - May oversimplify relationships in high-dimensional spaces
Differential Abundance (e.g., LEfSe)	Statistical methods for identifying taxa that differ in abundance across different conditions or groups.	- Tailored for count data - Identifies specific taxa differences - Commonly used in biomarker discovery	- Compositional nature of microbiome data complicates interpretation - False positives in small datasets - Assumptions vary between methods
Random Forests	An ensemble learning method that builds multiple decision trees for classification or regression tasks.	- Robust to noise and overfitting - Can handle complex, non-linear relationships - Feature importance	- Difficult to interpret - Requires tuning - Computationally expensive for large datasets
Support Vector Machines	A supervised learning algorithm that finds the optimal hyperplane to separate classes in high-dimensional space.	- Effective in high-dimensional spaces - Performs well with small to medium datasets	- Sensitive to parameter tuning - Limited interpretability - Not as effective for large, sparse datasets
Gradient Boosting Machines (e.g., XGBoost)	Boosting algorithms that build trees sequentially to minimize prediction error and improve performance.	- High prediction accuracy - Handles non-linear data well - Feature importance ranking	- Computationally intensive - Requires careful tuning - Prone to overfitting if not regularized
Logistic Regression	A statistical method used for binary classification that estimates probabilities based on independent variables.	- Simple and interpretable - Effective for binary classification - Works well as a baseline	- Assumes linear relationships - Sensitive to multicollinearity - Limited with high-dimensional microbiome data

Machine learning (ML) has emerged as a novel method of gut microbiome data analysis that can evaluate the complex structure of the whole microbial community and identify associations between community structure and classification (e.g., treatment vs. control) [263]. Considering that even core taxa that are consistently present across individuals exhibit variability in abundance and presence, attributed to biological and environmental variation, it is often not possible to differentiate disease versus healthy states by focusing on individual taxa [264,265]. This highlights a great advantage of ML models over traditional statistical approaches that consider each taxa in isolation since ML models simultaneously consider the relative abundance of multiple taxa and their context dependency [266]. ML models implicitly capture relationships and interactions between microbial features as they look for combinations of microbial abundance (i.e., multivariate interactions) that optimize the prediction accuracy of a label [267]. On the other hand, this also complicates the interpretation of ML analysis results, especially since ML methods are often criticized for being 'black box systems' that generate predictions without much context on the classification process [268].

Although the application of ML models to gut microbiome analysis is relatively recent, some have demonstrated better performance and interpretability than others. When the disease prediction ability of various supervised ML classification models (based on gut microbiome sequences) was compared, random forest models were among the top performers [266,269]. Random forest methods involve creating multiple decision trees using different randomly selected subsets of training data to vote for classification (e.g., treatment vs. control), whereby the classification with the most votes is chosen as the final prediction and taxa are ranked according to the level of impact they have on the classification decision (i.e., important taxa) [261]. The identification of important taxa is a key feature of decision tree-based algorithms as it

shows which factors or taxa are associated with the predicted task, meaning they are not completely 'black box systems' [261]. Random forest models have also demonstrated a high degree of collinearity (i.e., permutations or the removal of data points had a minimal impact on model performance) [266]. This can be attributed to the ability of random forest models to not only evaluate the impact of each individual predictor variable, like traditional univariate statistical methods, but also evaluate the impacts of multivariate interactions with other predictor variables [267]. Thus, despite their difficult interpretation (i.e., partial 'black box system') when compared to more traditional statistical methods, ML models provide a more comprehensive analysis of gut microbiota composition by characterizing key taxa and implicitly capturing their interactions and relationships [270].

2.2.2 Diet as a key modulator of the gut microbiome

The high interindividual variability in gut microbiome composition is often attributed to lifestyle-associated factors (e.g., diet, exercise, antibiotics); a greater understanding of these factors could aid in the determination of what constitutes a "normal" and/or "healthy" microbiome [15,271]. The definition of a healthy microbiome may depend on the individual as the same diet consumed by different people can elicit different metabolic health effects and microbial responses [272,273]. Moreover, how the diverse metabolic potential of the gut microbiome may be manipulated through relatively low-cost, highly accessible lifestyle-associated interventions, such as dietary intervention or supplementation, make them an attractive avenue for possible disease management/prevention and health maintenance [17,32,274].

Among the key modulating factors of the gut microbiome (e.g., disease, environment, genetics), diet is a critical modulating factor of gut microbial composition and function [15,17,18].

A traditional "Western diet" that is high in processed foods (i.e., fat and sugar) and low in FVs (i.e., fiber and phytochemicals) is associated with increased opportunistic bacteria, their harmful metabolites, and inflammatory cytokines. Conversely, a plant-based diet that is high in FVs and whole grains generally has health-promoting effects on gut microbiome composition and function (e.g., enhanced bacterial diversity and abundance and increased SCFAs) [275]. Beyond the established capacity of the gut microbiome to metabolize most macronutrients, often benefitting the host and gut microbiome, emerging evidence reveals a relationship between phytochemicals and the gut microbiome that may also be mutually beneficial [18,20,276].

2.2.2.1 Interactions between the gut microbiome and phytochemicals

Microbial metabolites of phytochemicals have demonstrated enhanced bioavailability and bioactivity compared to their parent phytochemicals [20]. Gut microbial metabolism of glucosinolates, sulfur-containing phytochemicals abundant in cruciferous vegetables that are not protective in native form, is partially responsible for the generation of metabolites called isothiocyanates (e.g., sulforaphane) that have demonstrated health-promoting properties (e.g., antioxidant, anti-inflammatory) in preclinical and human studies [277–279]. Equol is a gut microbial metabolite of soy isoflavones (e.g., daidzein) that is more bioavailable and bioactive, having demonstrated cardioprotective and osteoprotective effects in preclinical studies and human trials [280–282].

Although it has become clear that gut microbial metabolism of phytochemicals can depend on the composition and metabolic capacity of the gut microbiome, even individuals with metabolite-producing bacteria do not always produce the metabolite [20,279,280]. In a cross-sectional study involving 58 women (mean age: 56), although 97% had equol-producing

bacteria, only 17% were equol-producers [283]. Interestingly, equol-producers showed significantly higher alpha diversity and different dietary patterns (e.g., higher meat and vegetable intake), than equol nonproducers. This suggests that differences in the overall gut microbial composition, dietary patterns, and their interaction can influence the capacity for microbial metabolism of phytochemicals. This aligns with other studies that show high interindividual variation in gut microbiota composition and/or metabolite production after phytochemical intervention [284–286]. Jamieson et al. (2024) demonstrated a high interindividual variation in metabolite production from the polyphenol xanthohumol (found in the hop plant) after supplementation in an 8-week RCT involving 30 healthy adults (mean age: 31; 53% women) despite no differences in diversity metrics or microbial community structure [285]. It has been suggested that this interindividual variation in response to phytochemical intervention should be explored by separating individuals accordingly (e.g., responders vs. nonresponders) [287].

Phytochemicals have also been shown to influence gut microbiome composition. Several preclinical and some human studies involving phytochemicals (e.g., polyphenols, carotenoids, alkaloids) have demonstrated beneficial changes in gut microbiome composition (e.g., increases in *Lactobacillus*, *Bifidobacterium*, *Akkermansia*, *Roseburia*; decreases in pathogenic bacteria [19,20,29,280,288,289]. However, the few existing phytochemical supplementation clinical trials report conflicting results, with some reporting no change in gut microbiota composition and others reporting enrichment of members of certain families (e.g., *Lachnospiraceae*, *Bacteroides*) (Table 2.3); many also report high interindividual variation in the gut microbiota composition. Furthermore, other clinical trials that have reported changes in gut microbiome composition used phytochemical-enriched extracts that often contain other components (e.g., macronutrients, minerals) that confound the results [29,290–293]. Finally, these studies primarily report descriptive

analyses or use traditional statistical analyses whose assumptions are often violated by the compositionality (e.g., normality, independence) of the gut microbiome data (discussed in Section 2.2.1 on technical variation) leading to a high risk of false positives [252,255]. Consequently, there is a need for more omics and systems biology science that emphasizes functional analysis and microbial metabolites, and leverages improved computational methods (e.g., machine learning), to evaluate the complex structures of whole microbial communities (not just specific taxa) [20,74,261,263].

Table 2.4. Summary of current human trials evaluating the effect of phytochemical supplementation on gut microbiome composition.

Reference	Study design	Intervention (dose)	Population (characteristics)	Results
Jamieson et al., 2024	8-wk RCT	Polyphenol xanthohumol (24 mg/d) vs. placebo	30 healthy adults (mean age: 31; 53% women)	No differences in alpha and beta diversity or microbial community structure; High variation in xanthohumol metabolite production observed between individuals & by enterotype; individual taxa associated with metabolites in enterotype dependent manner.
He et al., 2024	6-mo RCT	Curcumin (500 mg/d) vs. placebo	80 patients with NAFLD (mean age: 41; 61% men; mean BMI: 28)	Curcmin increased Firmicutes to Bacteroidetes ratio and ↑ *Bacteroides* abundance.
Mathrani et al., 2023	12-wk RCT	Polyphenol rutin (500 mg/d) vs. rutin (500 mg/d) supplemented yogurt vs. placebo	87 adults with overweight (mean age: 44; 55% women; mean BMI: 28)	No differences in alpha and beta diversity or microbial community structure; High interindividual variation in gut microbiota observed.
Wiese et al., 2019	4-wk-RCT	Lycopene (7 mg) in dark chocolate (10 mg) vs. lycopene (7 mg) with SFA vs. lycopene (30 mg) with SFA vs. lycopene (30 mg) with PUFA vs. dark chocolate (10 mg; control), daily	30 adults with obesity (mean age: 58; 50% men; mean BMI: 32)	Dose dependent changes in gut microbiome composition, with notable ↑ in *Bifidobacterium adolescentis* & *longum* , for all three lycopene + fatty acid supplement groups.
Pivari et al., 2022	6-mo trial	Curcumin (100 mg/d curcuminoids)	11 adults with CKD (mean age: 72; 55% women; mean BMI: 28) with age- and sex-matched control (20 healthy adults; mean age: 74; 40% women; mean BMI: 25)	Curcumin promoted gut microbial shift towards health control (↑ alpha and different beta diversity in CKD compared to control at baseline that decreased to similar values after intervention); notably, *Lactobacillaceae spp.* ↑ in the last 3 mos of supplementation.
Guadamuro et al., 2021	4-wk-trial	Soy isoflavones (80 mg/d)	8 menopausal women (3 equol-producers; 5 equol non-producers)	Isoflavones ↑ *Slackia* genus abundance; equol producers had ↑ in members of *Lachnospiraceae* family and *Pseudoflavonifractor* genus. Similar beta diversity between equol producers & non-producers became different after intervention.
Guadamuro et al., 2015	6-mo trial	Soy isoflavones (80 mg/d)	16 menopausal women (mean age: 53)	Wide interindividual and inter-sample count variationa & no general patterns of change due to isoflavone intervention or associated with equol production.

BMI measures in kg/m^2; CKD = chronic kidney disease; NAFLD = Non-alcoholic fatty liver disease; PUFA = polyunsaturated fatty acid; RCT = randomized controlled trial; SFA = saturated fatty acid.

2.2.3 The gut microbiome and carotenoids: A bidirectional relationship?

Interactions between the gut microbiome and carotenoids are highly plausible as most ingested carotenoids (up to 95%) are not absorbed in the upper GIT and reach the colon [138,142,148]. Emerging preclinical evidence suggests that carotenoids may interact with the gut microbiome by modulating bacterial abundance (i.e., prebiotic effects), reducing oxidative stress in the gut, fostering SCFA production, maintaining healthy gut barrier function, and influencing gut metabolism and immunity [19,27]. Furthermore, bacteria have demonstrated the ability to biotransform and/or produce carotenoids or apocarotenoids that may be more bioaccessible [24]. Gut microbiota can also produce biosurfactants and secondary bile acids that can emulsify carotenoids and may facilitate their absorption [294–296]. However, very few human studies have been conducted in this context, as will be discussed later.

Despite suboptimal conditions for carotenoid absorption in the colon (without the presence of lipids, bile salts, and enzymes), the presence of lipid transporters (e.g., SR-B1, CD36) throughout the GIT and the known passive diffusion of carotenoids suggests that carotenoids may still be absorbed in the colon [138,142,148]. Although this has sparsely been investigated, one animal study supports the concept of colonic absorption of carotenoids [297]. Both intragastric and intracolonic administration of lycopene (12 mg) in corn oil in rats showed comparable lycopene levels in the liver 24 h after administration, while lycopene was not detected in control rats. Furthermore, although not significant, intragastric administration to colostomized rats resulted in lower liver lycopene levels when compared to non-colostomized rats (1.26 vs. 1.71 µg/mL). The extent to which colonic carotenoids may be absorbed in humans remains to be investigated.

2.2.3.1 Prebiotic potential of carotenoids

Several studies have suggested a prebiotic potential of carotenoids. Preclinical studies have demonstrated improvements in gut microbiome diversity and composition (e.g., increased *Lactobacillus, Bifidobacterium, Akkermansia*) after carotenoid intervention. [21,298,299]. Potential mechanisms include bactericidal and immune effects, improvement of tight junction integrity, and reductions in oxidative stress [27]. Higher carotenoid status [26,28] and intake [300,301] have been positively associated with gut microbial diversity in human observational studies involving various populations (e.g., healthy, pregnant, diseased), with varying differences in specific genera and species. Nonetheless, it has been suggested that associations may reflect high fiber intake or diet quality, as carotenoids are a biomarker of FV intake [28].

The few existing human intervention studies have reported mixed findings regarding the prebiotic effect of carotenoids. In a dietary intervention trial, 88 healthy adults (74% women) with an increased risk of colon cancer were randomized to receive dietary counselling for either a Healthy Eating or a Mediterranean diet. Participants with higher serum carotenoids had greater colonic microbiome beta-diversity and lower abundance of *Lachnospiraceae* (*Blautia* and *Roseburia*) at baseline [26]. Interestingly, colonic bacteria did not change after doubling FV intake (increase of 3-4 servings/d) for 6 months, suggesting that greater carotenoid intake (11 mg/d at baseline to 22 mg/d at 6 months) may not substantially alter gut microbiome composition [26,302].

Only one human intervention study involving synthetic carotenoid supplementation that examined its effects on the gut microbiome has been conducted. In a 4-week RCT, 30 adults with obesity (mean age: 58; 50% men; mean BMI: 32) were randomized into five groups with

different daily supplementation: I) 10 g dark chocolate (DC), II) 10 g DC with 7 mg lycopene, III) 7 mg lycopene with saturated fatty acids (SFAs), IV) 30 mg lycopene with SFAs, and V) 30 mg lycopene with polyunsaturated fatty acids [29]. Participants who took supplemental lycopene (7 or 30 mg) without DC (groups III, IV, and V) demonstrated dose-related changes in gut microbiome composition, with notable increases in *Bifidobacterium adolescentis* and *Bifidobacterium longum*, but the only statistically significant difference observed was an increase in Actinobacteria (which includes *Bifidobacterium*) from baseline to endpoint in group IV. This study has several limitations that limit the overall confidence in the reported findings. The use of traditional statistical methods, small sample size, lack of control for lycopene alone, and inclusion of various different supplement formulations increase the risk of false positives, even with an adjustment for FDR [252,255]. Furthermore, there was no monitoring or adjustment of dietary intake, a known critical modulator of gut microbiome and composition [15,17,18], and intake of DC and different fatty acids have been shown to alter gut microbial composition [303,304].

2.2.3.2 Gut microbial metabolism and carotenoids

Preclinical evidence suggests that gut microbiota may improve the bioaccessibility of carotenoids via fermentation or biotransformation, although underlying mechanisms are unclear. A bioaccessibility model involving in vitro enzymatic digestion and colonic fermentation of FVs (e.g., tomato, lettuce, mango) using rat cecal content reported an increase in lycopene and β-carotene release following colonic fermentation [22]. *Lactobacillus gasseri* strains have been shown to ferment carrot juice and enhance its carotenoid content in vitro but, despite the author's suggestions of microbial carotenoid production, the underlying mechanism was unclear and not

investigated [305]. Beneficial microbial biotransformation may also involve the isomerization of carotenoids from their *trans*-configuration to *cis*-configuration, which is more bioaccessible as it does not aggregate in a crystalline structure [158]. An in vitro fermentation study involving lactic acid bacteria (*Lactobacillus sakei* and *Pediococcus pentosaceus*) and tomato pulp reported increased total lycopene content with a greater *cis/trans* lycopene ratio when compared to control [306]. However, it is important to note that improvements in the bioaccessibility of carotenoids from gut microbial action also depend on factors including the overall diet (e.g., food matrix, types of macromolecules), type of carotenoid, fermentation conditions, and gut microbiome composition [24].

Gut microbial action may disrupt complexes of macromolecules (e.g. fibers, proteins) with lipids and lipid-soluble compounds like carotenoids, that inhibit lipid digestion and carotenoid bioaccessibility [25,307]. The human gut microbiome is known to metabolize carbohydrates (especially fiber), proteins, and (to a lesser extent) fats that reach the lower GIT [276]. During the process of upper intestinal digestion, some plant fibers can sequester lipid soluble components by creating a gel with bivalent cations. For example, the plant fiber pectin, that is present in most plant cell walls, can sequester carotenoids by creating a gel with calcium (a bivalent cation present in many leafy greens). The gel can inhibit carotenoid solubilization and micellization, but it could be disrupted by microbial action when reaching the colon [307,308]. Furthermore, some carotenoids may complex with proteins (often within the food matrix) that constitutes another structural barrier that restricts carotenoid bioaccessibility [25]. Thus, if carotenoids sequestered by fibers or proteins reach the colon, it is conceivable that gut microbial fermentation of these macromolecules may liberate carotenoids to render them available for biotransformation and/or absorption.

Other in vitro fermentation studies involving fecal slurries of healthy participants have demonstrated reduced concentrations of parent carotenoid compounds in conjunction with increases in vitamin A (i.e., retinal and retinoic acid) and SCFA production, as well as changes in microbiota composition, suggesting gut microbial metabolism of carotenoids [21,23]. Dai et al. (2022) performed 24-h in vitro fermentation of various common carotenoids, such as β-carotene and lutein, with fecal slurries of healthy participants. The degradation rate of carotenoids increased (up to more than 5 times), SCFA production doubled with carotenoid treatments compared to the control, and alpha and beta-diversity measures of the microbiota increased with carotenoid treatment [21]. In another study, after 24 h of in vitro β-carotene anaerobic fermentation, the degradation of β-carotene was 1.46 times greater than control (i.e., no gut microflora), while the production of vitamin A and SCFAs were two times that of control [23]. These studies suggest that gut microbiota can metabolize carotenoids into beneficial metabolites (e.g., vitamin A, SCFAs). The presence of commensal bacteria have previously been associated with high concentrations of retinoids (e.g., retinol, retinoic acid), attributed to bacterial metabolic capacity (e.g., aldehyde dehydrogenase activity), in vitro and in vivo [309,310].

2.3 Probiotics

Probiotics, or live microorganisms that benefit the host, have demonstrated the capacity to alter gut microbial composition and metabolism in seemingly favorable ways [30,32]. Probiotics are primarily known to compete with and inhibit the growth of pathogens, modulate gut microbiome composition, improve the host's immune response and gut barrier function (e.g., mucins, tight junction proteins, SCFA production), and enhance digestion and absorption of nutrients [30]. Meta-analyses of RCTs report improvements in various markers of health,

including markers of gut barrier integrity (e.g., zonulin, lactulose/mannitol ratio, endotoxins), cardiometabolic health (e.g., BMI, fasting glucose, lipid profile) and inflammation (e.g., CRP, TNF-α) [311–313]. However, it should be noted that conclusions are often limited by high study heterogeneity (e.g., dosages, duration, strains, baseline status) and a limited understanding of underlying mechanisms (e.g., alterations in gut microbial composition and activity, synergistic effects) [314,315]. Moreover, probiotic effects are often strain-specific, may depend on the baseline gut microbiome, and may alter gut microbial function and not composition (or vice versa) [316,317].

2.3.1 Enhanced absorption and metabolism of nutrients

Probiotics appear to enhance the absorption and metabolism of various nutrients. Probiotics (e.g., species belonging to *Lactobacillus*, *Bifidobacterium*, *Bacillus* genera) have been shown to improve protein absorption and metabolism in preclinical and clinical studies, attributed to their ability to induce host digestive proteolytic activity, enhance gut barrier integrity, and ferment proteins [318–320]. Probiotics have also been shown to reduce abdominal pain and other symptoms of lactose intolerance in systematic reviews and meta-analyses of human trials, primarily attributed to the presence of β-galactosidase that can hydrolyze lactose [321,322]. A systematic review from 2021 indicated that the intake of certain probiotic strains of *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* in healthy participants was associated with positive impacts on the status of certain micronutrients (e.g., vitamin B12, calcium, folate, iron and zinc) [33]. There are various possible mechanisms underlying potential probiotic-induced improvements in micronutrient status, such as vitamin production (e.g., B vitamins and vitamin K by *Lactobacillus* and *Bifidobacterium* species), decreased pH from organic acid (e.g., lactic

acid) production, and inhibition of pathogenic bacteria that may reduce competition with the host for available nutrients, but they require further investigation [34,323].

2.3.2 Potential synergy between probiotics and phytochemicals

Probiotics and phytochemicals can have similar beneficial effects on human health outcomes and may have complementary activity. A systematic review from 2022 involving 32 human trials showed that both probiotic and phytochemical interventions can improve symptoms of gastrointestinal disorders (e.g., irritable bowel syndrome (IBD), functional constipation) and, although no study combined the two, the authors suggested a potential synergy that should be investigated [324]. Probiotics have been proposed to enhance host metabolic capacity (e.g., introduce enzymatic activity) to ferment or biotransform phytochemicals into more bioavailable and bioactive forms. In vitro fermentation studies have demonstrated the capacity of probiotic lactobacilli and bifidobacteria to cleave off the bulky sugar groups from glycoconjugated phytochemicals (e.g., isoflavones like genistin), attributed to the presence of several glycosyl-hydrolases (e.g., β-glucosidases), that have reduced bioavailability compared to their more bioactive aglycone forms (e.g., genistein and equol) [325–328]. Additionally, some cell culture studies suggest that phytochemicals may promote the survivability and growth of probiotics, potentially by enhancing probiotic adhesion (to Caco-2 and HT29-MTX human epithelial cell cocultures and mucin) [329,330] and inhibiting pathogenic bacteria [36,331,332]. Only three human RCTs have evaluated the effects of combined probiotic and phytochemical supplementation, showing improved health outcomes (Table 2.5) [333–335]. To date, no human study has evaluated phytochemical status enhancement from probiotic supplementation.

Table 2.5. Summary of current human trials evaluating the effect of probiotic + phytochemical supplementation on health outcomes.

Reference	Study design	Intervention (dose)	Population (characteristics)	Results
Lambert et al., 2017	12-mo RCT	Isoflavone-rich extract (60 mg/d) and probiotics (proprietary lactic acid bacteria) vs. control	78 postmenopausal women with established osteopenia (mean age: 62; mean BMI: 26)	Isoflavone + probiotics ↓ bone loss (↑ bone mineral density vs. control).
Wang et al, 2022	3-mo RCT	Berberine (1.2 g/d) + multi-strain probiotic(*Lactobacillus* & *Bifidobacterium*) vs berberine vs probiotic vs placebo	365 adults with type II diabetes (mean age: 52; 55% men; mean BMI: 26)	Berberine + probiotic was superior to either supplement alone in improving postprandial lipidemia (total and LDL cholesterol).
Bouilly-Gauthier et al., 2010	6-wk RCT	Carotenoids (β-carotene & lycopene, 7.2 mg/d) and probiotic (*Lactobacillus johnsonii,* 5 × 10^8 CFU/d) vs. placebo	43 healthy women (mean age: 35; skin type III & IV)	Carotenoids + probiotic ↑ resistance to sunburn (minimal erythema dose) vs. no change in placebo.

BMI measures in kg/m^2; CFU = colony forming units; RCT = randomized controlled trial.

2.3.2.1 Probiotic-produced carotenoids

Various studies have demonstrated the capacity of microorganisms to produce carotenoids, whether naturally or with genetic modification (e.g., enhancing enzyme expression or precursor biosynthesis via plasmid transformation into bacteria), but only a couple of studies have evaluated their use as probiotics to enhance carotenoid status [27,336]. A few Bacillus species have been isolated from human feces and shown to produce carotenoids that are readily absorbed [37,38]. For example, significantly higher blood concentrations of apocarotenoids (methyl-glycosyl-apo-8'-lycopenoate and glycosyl-apo-8'-lycopene) produced by *Bacillus indicus PD01* were observed following three and six weeks of supplementation (5×10^9 CFUs), compared to placebo, in 62 healthy adults with overweight or obesity (mean age: 53; 57% women) [37]. In another study involving in vitro digestion, bacteria-produced apocarotenoids were reported to be more bioaccessible than β-carotene, whether in pure extracts (5x more) or when embedded in

their natural matrix (2x more; lyophilized cells and carrot puree) [38]. The absorption efficiency of the bacterial apocarotenoids and β-carotene (incorporated in mixed micelles) by Caco-2 cell monolayers was similar [38]. The recovery of the bacterial apocarotenoids in rat tissue (mostly in the liver) after a 3-day multiple-dose gavage intervention was considerably higher compared to β-carotene (2-9 nmol vs <1 nmol recovered) and there was no evidence of conversion to vitamin A (i.e., no increases in plasma retinol) [38]. The differences in bioaccessibility and tissue recovery were attributed to the polar glycosyl end-groups, and potentially because of inefficient degradation, of the bacterial apocarotenoids (i.e., potential resistance to BCO1 and BCO2 due to uncommon structures) [38].

2.3.2.2 Probiotics, carotenoids, and the gut-skin axis

As the largest human organ and initial barrier to extrinsic influence, the skin serves as an indicator of overall health status and biological age [337,338] and may be partly mediated through the delicate balance between the host and the gut microbiome (i.e., gut-skin axis) [31]. Gut microbial imbalances (e.g., increased pathogens, reduced diversity) are often observed in various inflammatory skin pathologies, including atopic dermatitis, psoriasis, rosacea, and acne vulgaris [339,340]. Commensal bacteria maintain gut immune system homeostasis by facilitating pathogen recognition, modulating immune responses and inflammatory signaling pathways, and maintaining intestinal epithelial barrier function [341–343]. Although mechanisms are not completely clear, modulation of systemic immunity, along with the production of metabolites such as SCFAs, appear to play a vital role in skin homeostasis [31]. Production of the SCFA butyrate by commensal gut microbiota has been shown to suppress immune responses and improve intestinal inflammation by inducing regulatory T cells in a mouse model [344].

Supplementation of probiotics, carotenoids and their combination have improved various markers of skin health and systemic inflammation that is often associated with skin health. Successful probiotic treatment has been shown to improve measures of skin hydration, trans-epidermal water loss (TEWL, a measure of skin barrier function), elasticity, and wrinkling [345,346], as well as systemic inflammation that is often associated with skin disorders [35,347–349]. Carotenoid intervention has also demonstrated improvements in similar skin health markers (e.g., skin hydration, elasticity, wrinkling, resistance to sunburn), as well as systemic inflammation (covered in Section 2.1.3) [8,126–131]. To date, only one study has evaluated the skin health effects of a combined probiotic and carotenoid supplement. In a series of three clinical trials involving 16 to 80 healthy women (mean age: 31 to 42), Bouilly-Gauthier et al. (2010) reported that daily supplementation with a mix of carotenoids (7.2 mg β-carotene and lycopene) together with *L. Johnsonii* (5×10^8 CFU) showed decreases in UV-induced immune cell activation (e.g., Langerhans cell density), accelerated post-UV skin recovery, and improved minimal erythema dose (shortest exposure of UV radiation to induce skin reddening) [333].

It is unclear whether the improvements in skin health from carotenoid and probiotic supplementation are related to changes in gut microbiome composition because of the lack of studies that have investigated this relationship. Although there are no carotenoid intervention studies available, one pilot study of 25 healthy adults (7 with noncystic acne; mean age: 31; sex not mentioned) who took a probiotic supplement (four billion spores from *Bacillus indicus, B. subtilis, B. coagulans, B. licheniformis,* and *B. clauisii*) for 4 weeks (after taking a placebo for 4 weeks) was performed [350]. Probiotic supplementation showed trending increases in hydration and decreases in sebum excretion rate in the entire cohort, and decreases in acne lesions in participants with acne, compared to placebo. Despite no changes in the diversity of fecal

microbiota, probiotic supplementation increased the relative abundance of certain bacterial genera (e.g., *Lachnospiraceae, Prevotella*; known for SCFA production) in association with an increased blood acetate/propionate ratio. In the absence of direct investigations, it is still conceivable that the benefits of probiotics and carotenoids on skin health may be mediated via alterations to the gut microbiome as the gut microbiome has been proposed to be a major regulator of the gut-skin-axis [31,351] and carotenoids and probiotics have demonstrated improvements in systemic inflammation, and gut microbial metabolites (e.g., SCFAs) [19,21,23,27,131,348].

Chapter 3: Methods

3.1 Ethical compliance

The study was approved by the Institutional Review Board of the Faculty of Medicine and Health Sciences at McGill University on April 13, 2020 (IRB/Info-Ed study number: A12-M57-19B/19-11-057) and approved by Health Canada's Natural and Non-Prescription Health Products Directorate on April 8, 2020 (File number: 246862). All participants provided informed consent. The study is registered at clinicaltrials.gov (NCT04511052).

3.2 Study design

The probiotics for enhanced tissue carotenoid status (ProCar) study was a double-blind, randomized controlled trial that evaluated the effects of a 10-week mixed carotenoid supplement intervention, with or without probiotic supplementation, on tissue carotenoid status, gut microbial composition, and various markers of health (Fig. 3.1). Interested participants were instructed to complete a pre-screening survey that evaluated their conditional eligibility. If potential participants passed the pre-screening survey, they were sent the informed consent form, contacted to discuss the study in more detail, and offered to schedule a screening visit. The screening visit was conducted two weeks before the start of the intervention to confirm participant eligibility, willingness to participate, and understanding of the study. The participants were also familiarized with the study and visit protocol (e.g., intervention, assessments, questionnaires) and provided with a stool collection kit to be filled and delivered by the participant one week before the baseline visit. Demographic information and medical history were collected at the baseline visit (week 0). All assessments were conducted at weeks 0, 5 and 10 and included: the primary outcome of SCS, stool and blood sample collection, skin health

measures (including standardized photo capture for wrinkling assessment), and questionnaires for dietary intake, physical activity, sleep, and quality of life.

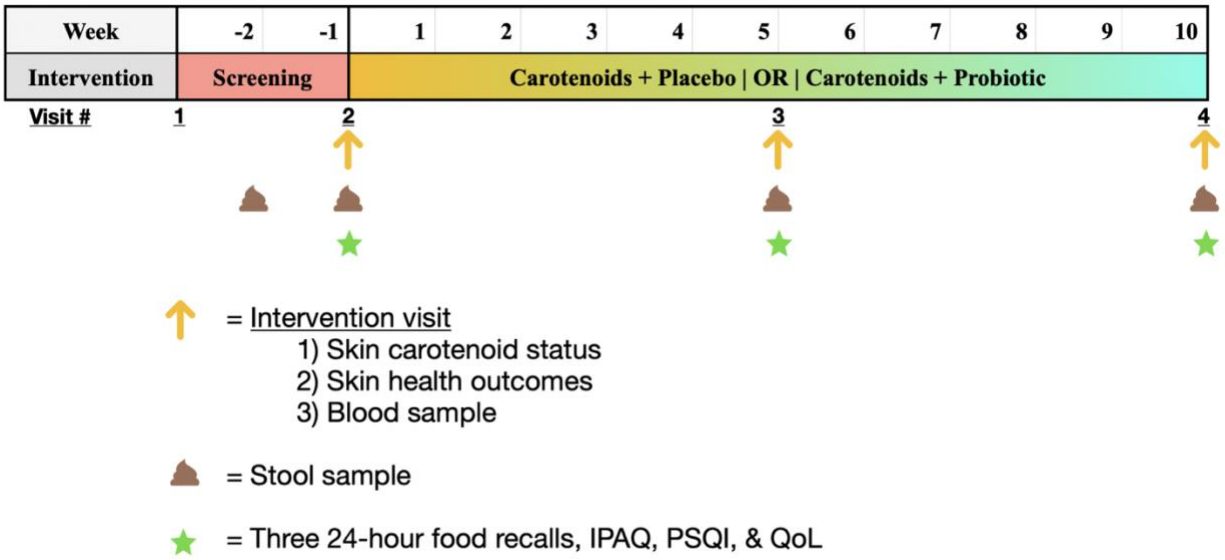


Figure 3.1. The Probiotics for enhanced tissue Carotenoid status (ProCar) study design. All outcomes were assessed at baseline (week 0), midpoint (week 5) and endpoint (week 10). A stool sample was also collected one week before baseline (week –1) to characterize baseline variation in gut microbiota composition. IPAQ = International Physical Activity Questionnaire; PSQI = Pittsburgh Sleep Quality Index; QoL = Quality of Life.

3.3 Participants

Healthy premenopausal women between the ages of 30 and 50 were recruited from Montreal (Quebec, Canada) primarily through advertisements (e.g., Facebook, fliers). A prescreening questionnaire was used to determine conditional eligibility whereby participants were invited for a screening visit to ensure they met the following eligibility criteria: BMI of

18.5 – 29.9 kg/m^2 (Normal to Overweight); not pregnant or breastfeeding; no known immunodeficiency, chronic or acute illness, skin condition, or use of any type of drug or supplement that may interfere with the intervention; no use of antibiotics within 1 month of study start; Fitzpatrick scale of skin types I, II, & III; no recent facial surgery or chemical treatment. A total of 37 individuals were enrolled and 4 dropped out for personal reasons, leaving 33 participants who completed the study (Fig. 3.2).

The participant visits were conducted at two study sites by the same staff and in the same manner: the Mary Emily Clinical Nutrition Research Unit (McGill University Macdonald Campus; November 6, 2021 to May 14, 2022); and the Health and Fitness Promotion Laboratory (McGill University Downtown Campus; May 28, 2022 to September 13, 2022).

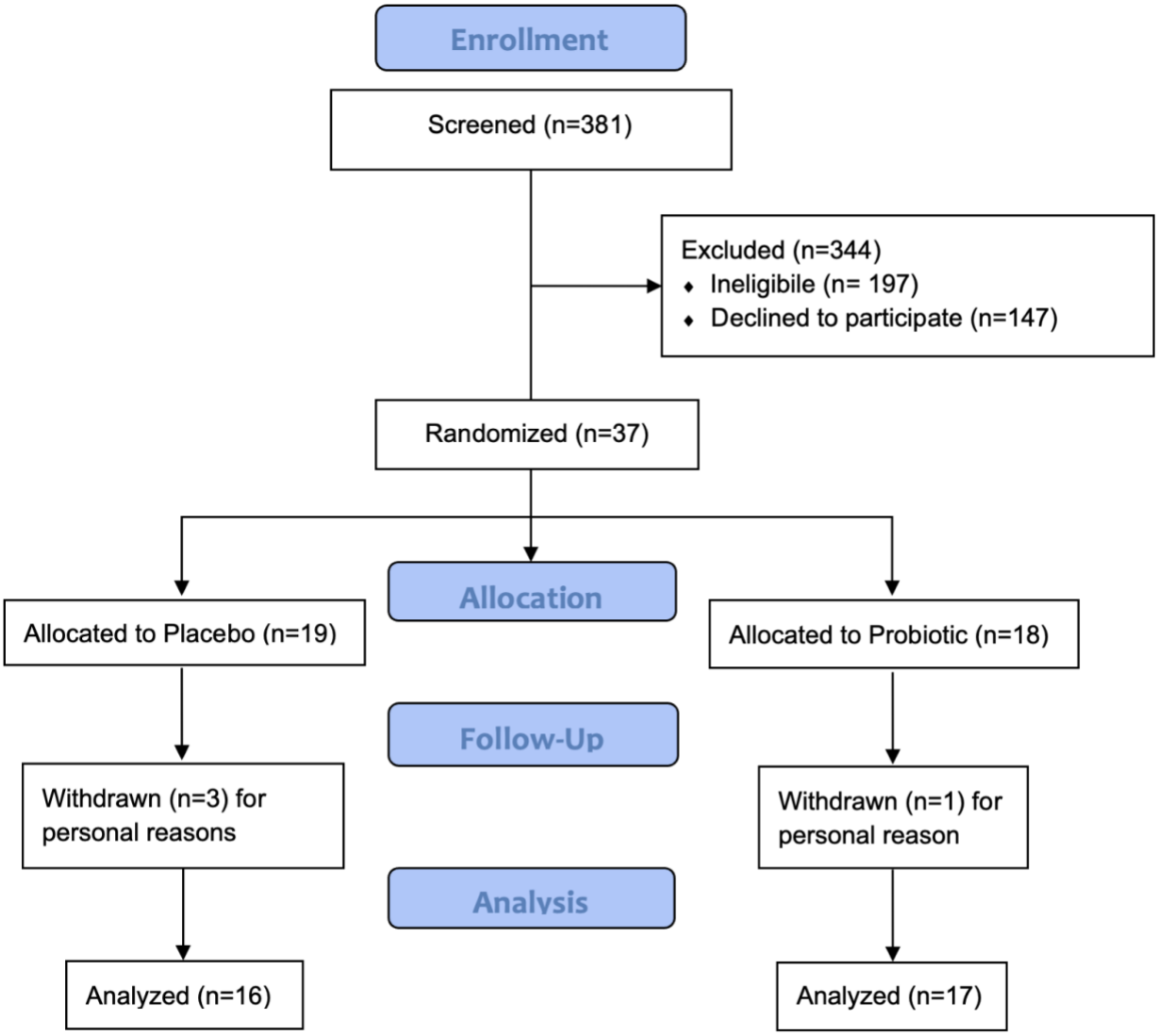


Figure 3.2. Participant flowchart including the numbers of enrolled and randomized participants.

3.4 Intervention

All participants were provided a mixed carotenoid supplement (17 mg total carotenoids including β-carotene, lutein, lycopene, astaxanthin, and zeaxanthin) to take daily. The carotenoid concentrations from the manufacturer's (Country Life, LLC; Long Island, NY) supplement facts

label for the Country Life Carotenoid Complex supplement can be found in the appendix (Appendix Table 1).

The participants were randomized (block randomization scheme; block size of 4) to take either a placebo or an identical-looking and tasting probiotic supplement daily [352]. Block randomization was chosen to ensure an equal distribution of participants across treatment groups. A block size of four means that within the first four participants, two are randomized to treatment and two to placebo. This pattern continues for each group of 4 participants (1-4, 5-8, 8-12 etc.). The randomized lot # for the treatment (placebo or probiotic) was assigned to the participant ID # before the participant was enrolled to maintain blinding. The probiotic contained 10 billion colony-forming units (CFUs) of *Bacillus* (*B.*) *subtilis* R0179 and was manufactured, labelled and blinded by Rosell® Institute for Microbiome and Probiotics (Montreal, QC, Canada). Compliance was measured through self-report and returned capsule count.

Participants were instructed to maintain current lifestyle habits (e.g., physical activity, diet), which were monitored using self-reported questionnaires at each visit. Participants were asked to abstain from using anti-aging creams containing retinol or other anti-aging ingredients and routine antioxidant, anti-inflammatory, herbal, prebiotic and probiotic supplements. Antibiotic use was monitored throughout the study.

3.5 Probiotic strain selection

The *B. subtilis* species can be found in soil and fermented soy-based foods and is considered a normal human gut commensal [353]. *B. subtilis* R0179 was initially chosen for its known safety profile (a safety and efficacy report has been filed for Fermalac SB, a combination

probiotic including *B. subtilis* previously used in various studies) [354] and current production and use. This strain survives passage through the human gastrointestinal tract and has not shown any adverse effects on general wellness or gastrointestinal function in healthy adults [355]. *B. subtilis* R0179 is generally recognized as safe (GRAS) and is known to promote intestinal microflora balance and reduce functional gastrointestinal disorders as a part of probiotic formulations [356–359]. Furthermore, preliminary work has shown that *B. subtilis* can enhance carotenoid status in a *Caenorhabditis* (*C.*) *elegans* model (Appendix Fig. 1).

3.6 Dietary intake

The Keenoa (Les Solutions Keenoa Inc., Montreal, QC) smartphone image-based dietary assessment application was used to assess dietary intake. Participants were instructed on how to download, register for, and use the application at the baseline visit. The application uses artificial intelligence to recognize and identify food items in photos captured by participants. If the application recognizes a food item, it displays options for the user to choose from including aspects that may have been missed (e.g., dressing if a salad is identified). Otherwise, participants can manually search and record foods that are linked to the Canadian Nutrient File (2015) food composition database. The tool is validated and comparable to the common 3-day food diary [360]. Participants were asked to document their food intake (e.g., food items and portion sizes) using Keenoa for two weekdays and one weekend day within the week before each visit. The study staff (trained to identify food items that were missed or misidentified by the user) checked the self-reported data with the participant. Food records were exported and reviewed by study staff for any missing items or misrecorded portion sizes according to the images provided. Randomized codes were used for file labels to maintain confidentiality.

3.7 Blood samples

Phlebotomists collected blood samples in ethylenediaminetetraacetic (EDTA)-coated tubes from overnight fasted participants after an overnight fast, via venipuncture, at weeks 0, 5 and 10 of the intervention. Immediately following collection, tubes were inverted 10 times before being centrifuged (2,000 x *g* for 10 m), aliquoted and stored at −80°C until analysis.

3.8 Stool samples

The participants collected stool samples using the Easy Sampler (ALPCO, Salem, NH), stored them in their household freezer (−20°C), and brought them to the study site within 24 h of collection. Participants provided a sample one week before the study started (week -1), as well as at each visit (weeks 0, 5 and 10), and samples were stored at −80°C until analysis.

3.9 Primary outcome: Skin carotenoid status

Skin carotenoid status (SCS) was estimated using the average b* value (yellow color saturation) of triplicate measures of skin of the forehead and palm (closer to the base of the thumb). This was measured using a CM-600d spectrophotometer (Konica Minolta, Tokyo, JPN), a device designed to evaluate the color of any object (in CIELab color space) that has previously been used to measure skin coloration associated with carotenoid and FV intake [11,200–202,361]. The b* value is a measure of blue (-120) to yellow (120) coloration [197]. Participants were asked to clean their hands with soap and their foreheads with an alcohol swab to ensure the absence of dirt or debris that may interfere with the spectrophotometric measurements. The device was calibrated between participants following the manufacturer's recommended protocol.

3.10 High-performance liquid chromatography (HPLC) analysis of carotenoids in plasma and supplement

Carotenoids and retinol were extracted from plasma (200 µL) and supplement samples twice with hexane/ethanol (2:1; 500 µL) with 0.1% BHT solution (vortexed 1 m, centrifuged at 2,100 *g* for 5 m), similar to Hrvolová et al. (2016) [362]. Since HPLC is considered the gold standard technique for carotenoid analysis [61], separation was done on an HPLC system (1260 Infinity II LC, Agilent, Santa Clara, CA), equipped with a C18 reverse phase column YMC Carotenoid (4.6 × 250 mm, 3 µm, (p/n CT99S032546WT) (Waters, Milford, MN), and coupled with a diode array detector (1260 Infinity II DAD WR, Agilent, Santa Clara, CA) set to 450 and 330 nm for carotenoid and retinol detection, respectively. Mobile phase A was methanol and mobile phase B was methyl tert-butyl ether: methanol (4:1), each with ammonium acetate at 0.7 g/L and with 0.1% acetic acid. The following linear gradient of mobile phase A was used (*t* (min), %A): (0.0, 90); (10.0, 75); (20.0, 50); (25.0, 30); (35.0, 10); (37.0, 6); (39.0, 90); (50.0, 90). Pooled hexane extracts were evaporated under a stream of nitrogen and subsequently dissolved in 100 µL of mobile phase B before separation and detection. Total run time of the analysis was 50 m. The mobile phase flow rate was 600 µL/m, and 20 µL of the extracted sample or standard (α-carotene, β-carotene, lutein, zeaxanthin, lycopene, astaxanthin, and retinol; Sigma-Aldrich, Canada Co.) was injected into the HPLC system. The standard curve concentrations included 0.05, 0.1, 0.5, 1.0, and 10 µg/mL. The samples were injected and analyzed in triplicate. Photodiode array and spectral analysis (450 and 330 nm) paired with carotenoid and retinol standards and sample spiking (i.e., internal standards) were used to identify the molecules. The measured carotenoid content of the carotenoid supplement is presented in Appendix Table 1.

3.11 DNA extraction

DNA from 150 mg of stool was extracted using the ZymoBIOMICS™96 MagBead DNA kit (Zymo Research, Irvine, CA, USA) automated on the KingFisher Flex Purification System (ThermoFisher Scientific Waltham, MA, USA). The following modifications were made to the manufacturer's protocol: a bead beating step was included before centrifugation consisting of 5 rounds, each lasting 1 m, at 6.5 m/s with 1 m dwell intervals on the Bead Ruptor Elite Bead Mill (Omni International, Kennesaw, GA, USA), and β-mercaptoethanol was added to the MagBinding Buffer to a final dilution of 0.5% (v/v). DNA purity and yield were assessed using a NanoDrop One UV/Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

3.12 Detection of probiotic strain by real-time quantitative polymerase chain reaction (qPCR)

The absolute quantification of *B. subtilis* R0179 was performed using Real-Time qPCR. DNA was diluted 5-fold in molecular-grade water before qPCR analysis. Each 10 µL qPCR reaction consisted of 300 nM of the appropriate forward and reverse primers (R179_VN_HP2_F/R) 1X SYBR Select Master Mix (ThermoFisher Scientific Waltham, MA, USA), and 1 µL of diluted DNA. The 384-well plate preparation for qPCR was performed at room temperature and automated using the epMotion 5075tc liquid handling robot (Eppendorf). The template DNA for the standard curve was generated from feces spiked with 10^9 lyophilized *B. subtilis* R0179, with total cell counts obtained using the fluorescent nucleic acid stain SYTO™ 24 (ThermoFisher Scientific, Waltham, MA, USA) on the CytoFLEX flow cytometer (Beckman Coulter Life Sciences, Indianapolis, IN, USA). DNA was recovered from spiked feces using the ZymoBIOMICS 96 MagBead protocol mentioned above, and serially diluted (10-fold)

to generate the template for the standard curve. The CFX384 ™ Touch Real-Time PCR Detection System (Bio-Rad, Montreal, QC, Canada) was used to perform qPCR analysis and results were viewed, and graphs were generated, using the CFX Maestro Software 1.1 version 4.1 (Bio-Rad, Montreal, QC, Canada) and GraphPad Prism 8 (GraphPad software, Boston, MA), respectively. The cycling conditions included a 2-m hold at 50°C, a 2-m hold at 95°C and 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, with a dissociation curve from 60°C to 95°C to confirm primer specificity.

3.13 16S rRNA gene amplicon sequencing and bioinformatics

DNA extracted from stool samples was analyzed using 16S rRNA gene amplicon sequencing. The data in FASTQ format were imported into one QIIME artefact (demux.qza). All reads were quality-filtered with the same parameters and trimmed at 240 bp on the forward read. QIIME 2 and the Deblur denoiser were used to process raw 16s rRNA sequences into amplicon sequence variant (ASV) abundances and representative samples. Taxonomic profiles were generated by merging representative sequences trained on the GreenGenes database. QIIME's visualization tools were used to generate and examine the principal coordinates analysis (PCoA), weighted UniFrac, and individual taxonomic profiles. Group differences were highlighted using the QIIME2 sample classifier, a supervised machine learning (ML) classification algorithm that can predict labels ('from which dataset does the taxonomic profile come from'), similar to Dahl et al. (2021) [363]. A total of 9 or 10 separate iterations were run and iterations with the median or best overall accuracy were chosen as the representative result. Pirate plots (generated using the R library PiratePlot; R version 3.5.3) of the most important features used by the algorithm (i.e., those that have the greatest influence on distinguishing between different classes or labels) were

generated. Labels were based on time (baseline = week -1 and 0 vs. treatment = week 5 and 10) and treatment group (probiotic vs. placebo). The ML algorithm was also used to investigate differences in gut microbial composition at baseline for participants with different BMI, baseline SCS and carotenoid intervention responsiveness. The relative abundance of bacterial genera was explored using MaAsLin2 (Microbiome Multivariable Association with Linear Models) [259]. All samples and time points were included, with time (baseline and treatment) and treatment (placebo and probiotic) as fixed effects and the sample ID as a random effect to control for repeated measures. The default MaAsLin2 parameters were used (e.g., variance-stabilizing log transformation and normalization by total sum scaling) with reporting based on $p < 0.05$ and $q < 0.05$ for false discovery rate adjustment by the Benjamini-Hochberg procedure.

3.14 Ferric reducing antioxidant power (FRAP) assay

The FRAP reagents were prepared as previously described [364]. The reaction was carried out in a 96-well plate with 30 µL dH_2O, 10 µL standards or samples, and 200 µL FRAP solution (pre-incubated at 37°C, 10:1:1 v/v/v of 300 mM sodium acetate at pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$). Samples were left to react at room temperature for 4 m at 37°C and absorbance was read at 593 nm in a microplate reader (BioTek Instruments, Winooski, VT, USA). All measurements were performed in three repetitions. A 6-point standard curve was produced using ascorbic acid (AA; 0-1000 µM) and the results were expressed as µM AA equivalents.

3.15 Total antioxidant capacity (TAC) assay

ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) and potassium persulfate were separately dissolved in water and combined following complete solubilization for a final 7 mM ABTS and 2.45 mM potassium persulfate solution similar to Re et al. (1999) [365]. The mixture was left to react for 12–16 h in the dark at room temperature to produce the ABTS radical cation stock solution. The dark blue solution was diluted with phosphate buffer saline (PBS) pH 7.4 to an absorbance of 0.70 at 734 nm and equilibrated at room temperature. After the addition 10 µL of 10x diluted sample or Trolox (an analog of α-tocopherol) standards in methanol to a 96-well plate, 200 µL of diluted ABTS radical cation solution was added and absorbance was measured at 734 nm following 4 m of incubation. All measurements were performed in three repetitions. A 6-point standard curve was produced using Trolox (final concentration 0-1000 µM) and the results were expressed as µM Trolox equivalents.

3.16 Multiplex analysis of cytokines

The MILLIPLEX MAP Human High Sensitivity T Cell Magnetic Bead Panel kit (Millipore, HSTCMAG-28K) was used to quantify IL-1β, IL-6, IL-8 and TNF-α in plasma samples collected during weeks 0, 5 and 10. Samples were diluted two-fold with the provided serum matrix before analysis according to the manufacturer's protocol. The analysis occurred on the Bio-Plex 200 Luminex System (Bio-Rad Laboratories, Montreal, QC, Canada). Values that were below the limit of detection were replaced with a value that was half of the limit of detection [366–368].

3.17 Plasma hormones

Testosterone was quantified using the Testosterone ELISA Kit 96T (Biomatik, EKU07605) according to the manufacturer's protocol. Plasma samples were diluted two-fold in PBS buffer before analysis. Undiluted plasma was analyzed for cortisol, estradiol, and progesterone levels using ELISA kits (Eagle Biosciences; COR31-K01, ESD31-K01, PRG31-K01, respectively) according to the manufacturer's protocol. In an attempt to capture and quantify samples with low estradiol and progesterone concentrations, calibrator "G", a two-fold dilution of the lowest standard (calibrator B) at a concentration of 10 pg/mL for estradiol and 0.15 ng/mL of progesterone, was added to the standard curve according to Eagle Biosciences' recommendation. Assay preparation was automated using the MicroPro300 Electronic 96-channel pipette (Mettler Toledo), followed by analysis on the Varioskan Lux Multimode Microplate Reader (Thermo Fisher Scientific).

3.18 Skin health

Skin health measures were evaluated using the Courage and Khazaka Cutometer dual multi-probe adapter 580 system (Courage and Khazaka, Cologne, Germany). Skin hydration was measured capacitively (in arbitrary conductance units) with a Corneometer CM825 (Courage and Khazaka, Cologne, Germany). Skin elasticity was measured using suction with the Cutometer MPA 580 (Courage and Khazaka, Cologne, Germany) and three separate mechanical properties were evaluated: 1) pliability (firmness, or how easily the skin lengthens or stretches under load; in mm); 2) viscoelasticity (simultaneous stiffness and damping behaviors, from both solid and liquid components of the skin, under load; unitless ratio); and 3) net elasticity (excludes viscous changes focusing on the solid components of the skin; unitless ratio) [369]. Skin trans-epidermal

water loss (TEWL; an indicator of skin barrier function; in g/m^2/h) was measured using the Tewameter TM300 (temperature and humidity sensors). Measurements were conducted in triplicate on the forehead and cheek and the values were averaged. All participants had rested ≥20 m before measurements, but we were unable to control for optimal and stable environmental conditions (room temperature 20°C and humidity 40-60%). The average room temperature was 22.9 ± 1.9°C (range: 15-26°C) and relative humidity was 38.5% ± 15.8% (range: 13-65%) across all study visits.

3.19 Skin wrinkling and coloration

Facial photos of participants used for skin wrinkling analysis were captured with a Canon EOS REBEL T1i digital camera (Canon, Tokyo, Japan) under specific conditions adapted from Foo et al. (2017) and Hamer et al. (2015) [203,370]. All photos were taken at a standard distance of 60 cm where participants sat upright in front of a white fabric background with a neutral expression. Participants also wore a headband to keep their hair from covering any skin on the face (e.g., bangs). A total of three photos were captured with participants facing straight at the camera and at 45° to the left and right of the camera. A Neewer photo studio kit was used to standardize illumination conditions (e.g., background, lighting) for image capture (Neewer, Shenzhen, China). Photos were color-calibrated using the ColorChecker Passport (X-Rite, Grand Rapids, MI, USA).

Skin wrinkling and coloration were assessed with photonumeric ratings of participant images from trained and blinded specialists (e.g., dermatologists) based on the Skin Aging Atlas Volume 5 – photo-aging face and body (Editions MED'COM, 2017) [371] in four areas: forehead, periocular, and perioral skin wrinkling and skin coloration. The Skin Aging Atlas is based on

several published scales and has been used to develop image analysis techniques to measure skin aging features [370]. The Skin Aging Atlas defines the type and area of wrinkling, evaluation factors (e.g., depth and number of folds, pigmentation and skin tone), and photo examples for each rating (from 0 up to 7), to guide specialists in the assessment of skin wrinkling and coloration. The facial photos of participants were assigned randomly generated codes to reduce bias and maintain blinding, integrity, and objectivity.

3.20 Quality of life

The self-administered, 36-item, short-form health survey (SF-36v.2) was used to evaluate health-related quality of life. It covers eight domains including physical functioning, pain, vitality, and emotional well-being. Physical component summary (PCS) and mental component summary (MCS) scores were calculated according to Ware (1994) [372]. The use of Canadian normative data was attempted, but factor score coefficients were not readily available. Since the magnitude of differences between Canadian and U.S. norms has been reported to be small and likely not clinically and socially meaningful, the U.S. normative dataset from Ware (1994) was used [372]. Still, it should be noted that Canadian norms were slightly higher in every domain, with the largest differences in the vitality and general health perceptions domains [373].

3.21 Sleep quality

Sleep quality was evaluated with the 19-item, self-administered Pittsburgh Sleep Quality Index Questionnaire (PSQI) [374]. The PSQI covers seven dimensions of sleep including time to sleep, sleep duration, sleep efficiency, and daytime dysfunction, to calculate an overall score of sleep quality. A higher score corresponds to a worse sleep quality.

3.22 Physical activity

Habitual physical activity was assessed using the short form of the International Physical Activity Questionnaire (IPAQ), a self-reported questionnaire that demonstrates adequate reliability and validity against accelerometry [375] and is used in several countries [376]. The questionnaire consists of 7 questions that evaluate the frequency and duration of "vigorous", "moderate", and "walking" activity during the previous week. The number of minutes reported for each physical activity type was truncated at 180 for weekly minutes according to the IPAQ truncation protocol [377]. Total metabolic equivalents of tasks (METs) per week were calculated (METs factors of 3.3, 4.0, 8.0 for walking, moderate, and vigorous activity, respectively) and categorized into High (≥ 3000 METs), Moderate (> 600 and < 3000 METs), and Low (≤ 600 METs) levels of physical activity.

3.23 Plasma short-chain fatty acids (SCFAs)

Plasma samples were sent to the Metabolomics Shared Resource at Georgetown University's Lombardi Comprehensive Cancer Center and analyzed by ultra-performance liquid chromatography – mass spectrometry (UPLC-MS) using previously described methods [378]. Briefly, 400 µL of water was added to plasma samples (250 µL), homogenized for 2 m on ice, and centrifuged at 13,000 rpm for 20 m at 4°C and the supernatant was collected. The supernatant was further diluted 10 times in methanol containing lactic acid-^{13}C3 as internal standard prior to quantitation. For derivatization of the SCFAs, 20 µL of each 4-acetamido-7-mercapto-2,1,3-benzoxadiazole, triphenyl phosphine, and 2,2'-dipyridyl disulfide (20 mM solution prepared in dichloromethane) were added to 400 µL supernatant and vortexed for 5 m.

at room temperature. The reaction mixture was dried under nitrogen and reconstituted with 200 µL methanol.

The mobile phase was composed of water with 0.2% formic acid (solvent A) and acetonitrile with 0.2% formic acid (solvent B). The samples were resolved on an Acquity BEH C18 1.7 µm, 2.1 x 50 mm column online with a triple quadrupole mass spectrometer (Xevo-TQ-S, Waters Corporation, USA) operating in the multiple reaction monitoring (MRM) mode for targeted quantification. Analyses were performed with a calibration curve (0.01 to 31,250 nM) for acetic acid (C2), propionic acid (C3), butyric acid (C4) and valeric acid (C5). The quality and reproducibility of LC-MS data was ensured using several measures. The sample queue was randomized in prior, and solvent blanks were injected between sets of samples to monitor and ensure there was no sample-to-sample carry-over. QC standard was injected periodically (after every 10 sample injections) to monitor shifts in signal intensities and retention time as measures of reproducibility and to ensure high quality LC-MS data. The coefficient of variation for QC standard for all SCFAs with the exception of acetic acid were well within permissible limits (<5%). MRM data were processed using MultiQuant 3.0.3 software. Metabolite quantitation was estimated by calculating the area under the curve for each metabolite normalized to the respective internal standard used for a particular SCFA.

3.24 Statistical analyses

The primary outcome was the change in SCS (i.e., average skin yellow color saturation or b* value), measured using a spectrophotometer. A sample size of 25 participants per group was estimated for differences in response change from baseline (over time) based on previous studies [201,202] to detect a minimum difference of 1.0 in SCS (b* value) following carotenoid

supplementation. Formulations from Lu et al. (2008) [379] for repeated measures analysis experiments with two levels of treatment were used. Recruitment was disrupted and encumbered due to the COVID-19 pandemic and 37 total participants were finally enrolled.

Baseline characteristics and nutrient intake estimations were presented as mean ± standard error of the mean (SEM). Quantitative baseline characteristics were compared between groups using an independent t-test or Mann-Whitney test and categorical data were compared using Chi-square testing. All repeated measures outcome data were presented as mean change from baseline (Δ) ± SEM, checked for normality using tests (e.g., Shapiro-Wilks) and visual inspection (e.g., histogram, QQ plot) and transformed if necessary (e.g., log, square root). A linear mixed model (LMM) was used to assess differences between treatment groups (probiotic vs placebo) and across time (week 0, week 5, week 10) for all repeated measures outcomes. The participant ID was included as a random effect to account for repeated measures between participants. Post-hoc comparisons with the Holm correction for multiple comparisons were used to identify significant differences. Subgroup analyses for SCS were conducted according to BMI (Normal vs Overweight; 18.5 – 24.9 vs 25 – 29.9 kg/m^2), baseline SCS (High vs Low; above vs below median, respectively) and week 5 intervention responsiveness (Responder vs Nonresponder; above vs below half of the standard deviation (SD) of the mean change in SCS from week 0 to week 5, respectively) [380]. The strength of positive correlations between different methods of carotenoid assessment (SCS, plasma carotenoids, and daily FV intake) was evaluated using Spearman correlations as some data were not normally distributed. Statistical significance was set at $p < 0.05$. The Benjamini-Hochberg adjusted pairwise Kruskal-Wallis test was used to identify differences between and within groups for alpha-diversity metrics. Results were considered statistically significant if $q < 0.05$.

Chapter 4: Results

4.1 Baseline characteristics

Overall, 89% of all randomized participants (n = 33) completed the study (Fig. 3.2). The mean compliance rate was 96%. There were no significant differences in baseline characteristics, SCS, plasma carotenoids (Table 4.1), nutrient intake (Table 4.2) or physical activity (Table 4.3) between the placebo and probiotic groups.

Table 4.1. Baseline study characteristics and tissue carotenoid status.

Characteristic	Placebo	Probiotic	*p*-Value
Age (years)	40.88 (1.21)	39.24 (1.52)	0.405
BMI (kg/m^2)	25.25 (0.79)	24.02 (0.85)	0.295
BMI = Normal (%)	8 (50.0)	10 (58.8)	0.611
SCS (b* value)	16.9 (0.41)	17.5 (0.30)	0.239
SCS = High (%)	7 (43.8)	9 (52.9)	0.598
Smoker = Yes (%)	3 (18.8)	4 (23.5)	1
Cannabis = Yes (%)	5 (31.2)	1 (5.9)	0.151
Alcohol = Yes (%)	12 (75.0)	11 (64.7)	0.792
Excessive alcohol intake (>7 drinks/week) = Yes (%)	5 (31.2)	3 (17.6)	0.614
Race/Ethnicity (%)			0.453
Caucasian	13 (81.2)	9 (52.9)	
Caucasian/East Asian	0 (0.0)	1 (5.9)	
East Asian	0 (0.0)	1 (5.9)	
Latino/Hispanic	1 (6.2)	3 (17.6)	
Middle Eastern	2 (12.5)	2 (11.8)	
South Asian	0 (0.0)	1 (5.9)	
Plasma carotenoid concentrations			
Total carotenoids (µg/mL)	1.94 (0.15)	1.77 (0.13)	0.355
β-carotene (µg/mL)	0.90 (0.10)	0.79 (0.09)	0.428
α-carotene (µg/mL)	0.30 (0.02)	0.25 (0.03)	0.090
Lycopene (µg/mL)	0.50 (0.06)	0.51 (0.04)	0.86
Lutein (µg/mL)	0.21 (0.02)	0.19 (0.02)	0.219
Zeaxanthin (µg/mL)	0.026 (0.003)	0.027 (0.004)	0.914
Retinol (µg/mL)	0.74 (0.04)	0.69 (0.04)	0.171

Data are presented as mean ± SEM unless otherwise specified. *p*-values were obtained by Mann-Whitney and independent t-test for not normally distributed and normally distributed quantitative data, respectively, and by Chi-square test for categorical data.

4.2 Nutrient intake and physical activity levels throughout the study

Nutrient intake (Table 4.2) and physical activity levels (Table 4.3) throughout the study did not change and were not different between groups at any time point.

Table 4.2. Nutrient intake estimations throughout the study

	Week 0			Week 5			Week 10		
	Placebo	**Probiotic**	p_{holm}	**Placebo**	**Probiotic**	p_{holm}	**Placebo**	**Probiotic**	p_{holm}
Number of days logged	2.88 (0.09)	2.94 (0.06)	1.000	3.00 (0.00)	2.53 (0.26)	0.881	2.63 (0.22)	2.35 (0.30)	1.000
Energy (kcal)	1559 (82.0)	1435 (90.6)	1.000	1411 (76.1)	1434 (99.0)	1.000	1423 (111)	1509 (129)	1.000
Carbohydrate (g)	188 (12.8)	160 (12.1)	1.000	163 (9.01)	169 (15.5)	1.000	168 (13.9)	173 (18.9)	1.000
Fiber (g)	22.0 (2.03)	15.5 (1.66)	0.148	17.5 (1.53)	16.8 (1.59)	1.000	17.5 (1.30)	15.8 (2.50)	1.000
Protein (g)	66.3 (3.55)	67.2 (4.72)	1.000	59.8 (3.56)	67.4 (5.79)	1.000	60.2 (4.17)	67.1 (5.09)	1.000
Fat (g)	64.4 (3.86)	58.1 (4.31)	1.000	56.3 (3.40)	53.9 (3.77)	1.000	61.6 (4.56)	61.8 (5.47)	1.000
Monounsaturated (g)	21.9 (1.86)	20.6 (1.37)	1.000	19.1 (1.45)	19.9 (1.49)	1.000	21.8 (1.97)	24.1 (2.39)	1.000
Polyunsaturated (g)	14.4 (0.97)	11.4 (0.84)	0.744	11.4 (1.27)	11.1 (1.09)	1.000	13.4 (1.17)	11.8 (1.47)	1.000
Saturated (g)	20.2 (1.48)	18.8 (1.98)	1.000	18.4 (1.28)	17.4 (1.61)	1.000	20.1 (1.75)	18.7 (2.02)	1.000
Cholesterol (g)	186 (23.0)	300 (23.7)	0.173	204 (22.3)	210 (29.7)	1.000	236 (48.8)	280 (42.0)	1.000
Daily FV servings	3.60 (0.33)	3.29 (0.36)	1.000	3.10 (0.31)	3.31 (0.42)	1.000	3.17 (0.36)	2.39 (0.29)	1.000
β-carotene (µg/day)	5621 (820)	4037 (746)	1.000	3713 (653)	3990 (601)	1.000	5239 (1267)	6232 (1598)	1.000
α-carotene (µg/day)	513 (127)	570 (131)	1.000	711 (194)	826 (169)	1.000	707 (245)	2046 (775)	0.496
Lycopene (µg/day)	2782 (869)	3153 (924)	1.000	3265 (835)	3501 (1237)	1.000	3270 (860)	4902 (1391)	1.000
Lutein & Zeaxanthin (µg/day)	5500 (1088)	2574 (702)	0.413	2384 (459)	2736 (638)	1.000	3210 (822)	2362 (477)	1.000
Vitamin A (µg/day)	752 (65.8)	610 (71.8)	1.000	571 (65.0)	576 (85.1)	1.000	727 (116)	805 (171)	1.000
Vitamin C (mg)	124 (22.3)	88.4 (13.3)	1.000	111 (13.8)	100 (13.8)	1.000	101 (12.8)	69.1 (10.8)	0.674

Data are presented as mean ± SEM with post hoc, Holm-corrected *p*-values for between-group comparisons at each timepoint. Nutrient intake estimations for each timepoint were based on three 24-h recalls (two weekdays, one weekend day).

Table 4.3. Physical activity level by treatment group and time point

	Physical activity level	Probiotic	Placebo	*p*-value
Week 0	Low	5	1	0.225
	Moderate	7	9	
	High	5	6	
Week 5	Low	3	2	0.755
	Moderate	7	6	
	High	6	8	
Week 10	Low	3	3	0.805
	Moderate	8	7	
	High	4	6	

Count data for each activity level (Low, medium, high) are presented per treatment group and time point. *p*-values were obtained by Chi-square test of association.

4.3 *B. subtilis* and carotenoid supplementation were safe and tolerable

No serious or severe adverse events were observed. All reported adverse events are summarized in Appendix Table 2.

4.4 Probiotic *B. subtilis* detected in fecal samples after supplementation

The absolute quantification of *B. subtilis* R0179 is shown in Appendix Figure 2. All but one (16/17) probiotic group samples collected at week 5 had quantifiable levels of *B. subtilis* R0179 with an average quantity of 7.72 LOG bacteria/g of feces. At week 10, the average quantity of *B. subtilis* R0179 across all probiotic group samples was 7.99 LOG bacteria/g of feces. *B. subtilis* R0179 was not detected in any of the placebo group samples. One participant in the probiotic group (CAR031) had strain detection in their baseline samples (weeks -1 and 0).

4.5 *B. subtilis* supplementation enhanced the effect of carotenoid supplementation on SCS

The mean SCS values (± SEM) over 10 weeks are shown in Figure 4.1 A. Overall, in the fixed effect omnibus test, there was an effect of time ($p < 0.001$) and treatment ($p = 0.044$), but no interaction effect ($p = 0.127$). Both groups significantly increased SCS at weeks 5 and 10 compared to week 0 ($p_{holm} \leq 0.004$). The probiotic group also significantly increased SCS from week 5 to week 10 (19.8 ± 0.38 vs. 18.9 ± 0.35; $p_{holm} = 0.032$). The probiotic trended strongly toward significantly higher SCS at week 10 when compared to placebo (19.8 vs. 18.3 b* value; $p_{holm} = 0.056$) and remained when comparing the change in SCS from baseline (ΔSCS; Fig. 4.1 D) despite becoming weaker (2.31 ± 0.34 vs. 1.46 ± 0.33; $p_{holm} = 0.093$). No difference was observed between groups at week 0 or week 5. The differences in ΔSCS over time were similar to those in SCS. The achieved power was 100% for within-group differences (α = 0.05; effect size f = 1.16; n = 33), 69% for between-group differences (effect size f = 0.378), and 95% for within-between interaction (effect size f = 0.262; G*Power v 3.1, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) [381].

The mean SCS values (± SEM) for the different skin regions assessed are shown in Figures 4.1 B (palm) and 4.1 C (forehead). Carotenoid supplementation alone (i.e., placebo) significantly increased SCS in the palm (week 0 vs. week 5 and week 10, $p_{holm} \leq 0.001$), but not the forehead. Probiotic + carotenoid supplementation significantly increased palm and forehead SCS at weeks 5 and 10 compared to week 0 ($p_{holm} \leq 0.003$) and palm SCS at week 10 compared to week 5 ($p_{holm} = 0.024$). The differences in ΔSCS for palm and forehead regions (Fig. 4.1 E and

4.1 F) over time were similar to those in SCS. Beyond a weak trend for greater forehead ΔSCS compared to placebo at week 10 (p_{holm} = 0.092), there were no differences between groups.

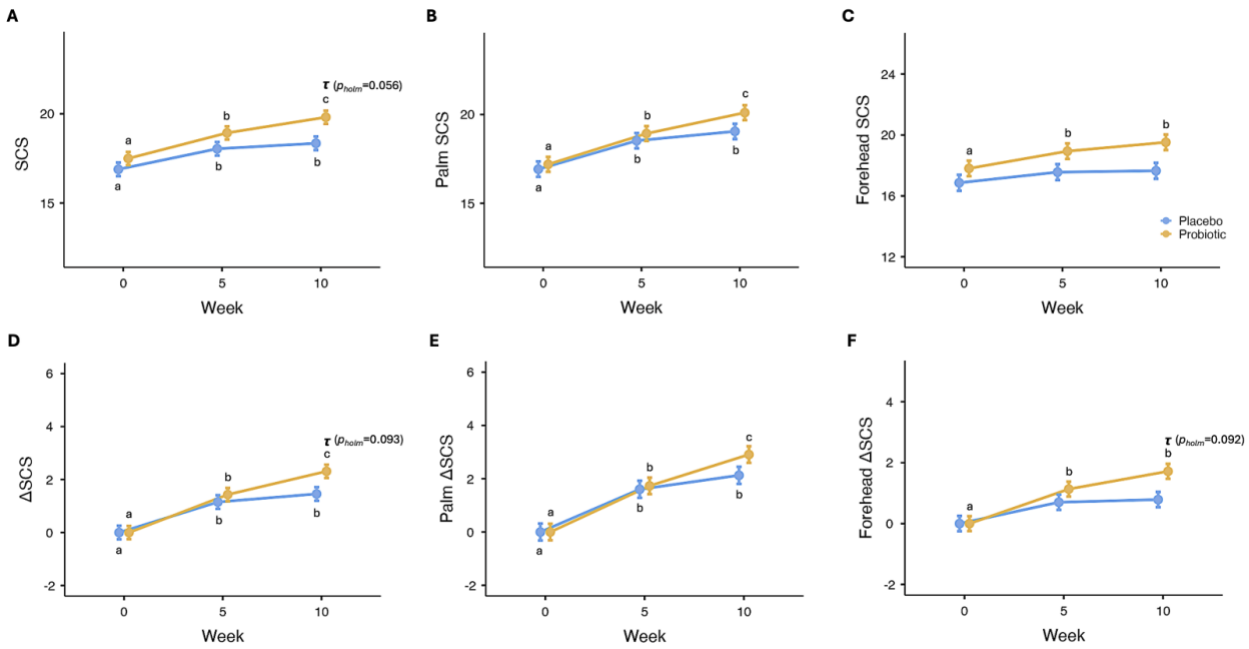


Figure 4.1. The primary outcome of SCS. Mean skin yellow color saturation (b*) values ± SEM are shown at weeks 0, 5 and 10 for both skin regions combined (A), the palm only (B), and the forehead only (C). Changes in SCS from baseline (ΔSCS) are shown for both skin regions combined (D), the palm only (E), and the forehead only (F). The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. All post-hoc analyses were conducted using the Holm method to adjust for multiple comparisons. Significant differences reported reflect the interaction effects between time and treatment (time*treatment) observed in the model. Significant differences ($p < 0.05$) within groups are represented by different letters (a, b, c), and trends for between-group differences are represented by tau (**τ**).

The ΔSCS values (± SEM) over 10 weeks for subgroups are shown in Figure 4.2. Subgroup analyses for SCS were conducted according to BMI (Normal vs. Overweight; 18.5 – 24.9 vs. 25 – 29.9 kg/m^2; n = 18 vs. 15; Fig. 4.2 A and 4.2 B, respectively), baseline SCS (High vs Low; above vs below median; n = 16 vs. 17; Fig. 4.2 C and 4.2 D, respectively) and week 5 intervention responsiveness (Responder vs Nonresponder; above vs below half of the standard deviation (SD) of the mean change in SCS from week 0 to week 5; n = 22 vs. 11; Fig. 4.2 E and

4.2 F, respectively) [380]. The probiotic improved SCS over time (week 0 vs. week 10, $p_{holm} \leq$ 0.020) in all subgroups except participants with Overweight BMI. Notably, the ΔSCS at week 10 was significantly higher than in week 5 in participants with Normal BMI who took the probiotic (p_{holm} = 0.030). Carotenoid supplementation without probiotic (i.e., placebo) increased ΔSCS over time in Responders (p_{holm} < 0.001) and participants with Low baseline SCS (p_{holm} < 0.001) and Overweight BMI (p_{holm} = 0.045). However, it is worth noting that the ΔSCS at week 10 for participants with overweight was slightly larger in the probiotic group compared to placebo (1.4 vs. 1.5). Given the subgroup analysis and slightly smaller sample size in the probiotic group (n = 7 vs. n = 8), the lack of a statistically significant difference may be attributed to limited power. Notably, in Nonresponders and participants with Normal BMI and high baseline SCS, the probiotic significantly increased ΔSCS over time while the placebo did not. This suggests that the SCS-enhancing effect of the probiotic may be mostly beneficial for carotenoid intervention Nonresponders and individuals with Normal BMI and High baseline SCS. There were no differences between treatment groups in the subgroup analysis.

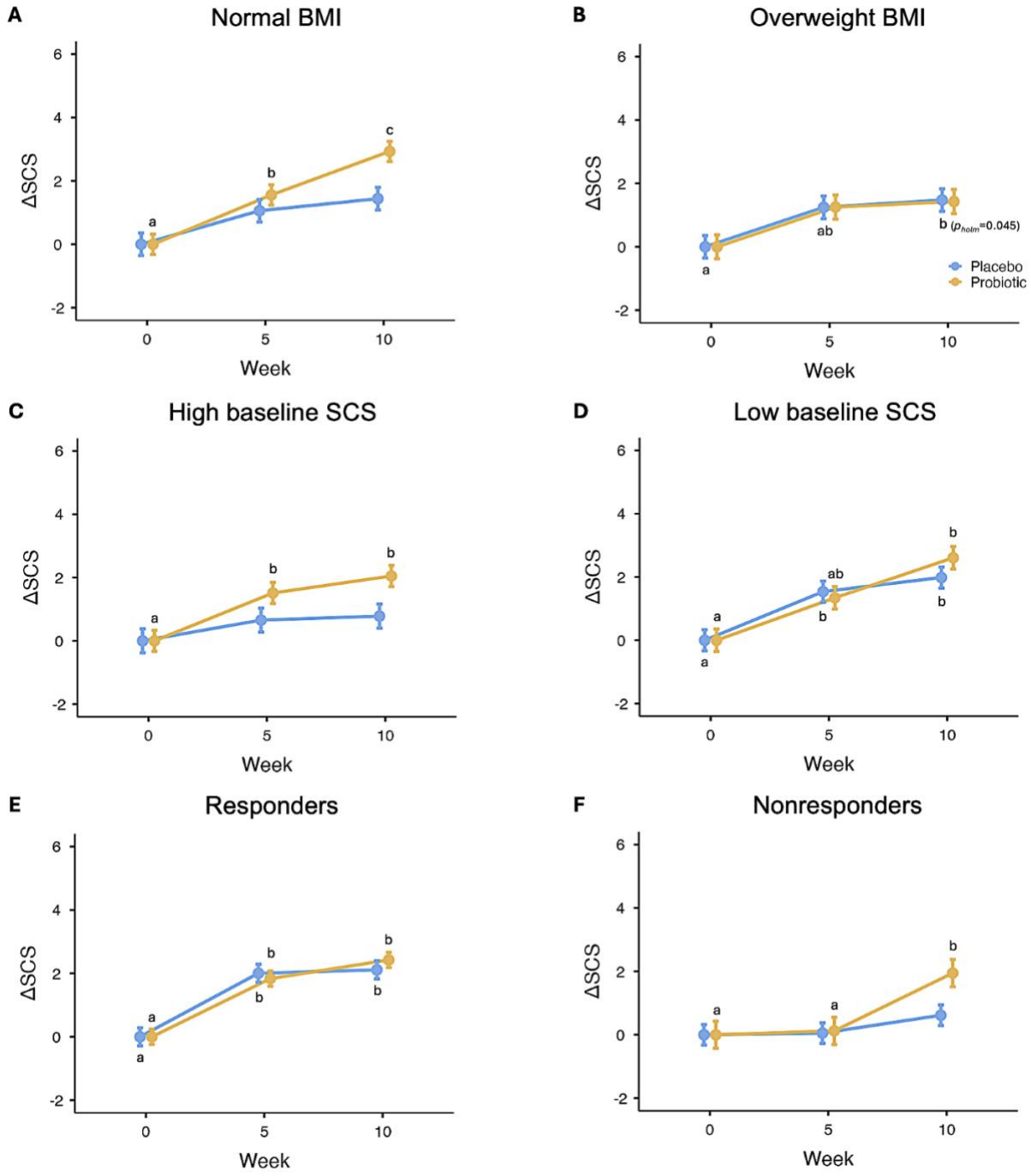


Figure 4.2. Subgroup analyses of SCS. Mean changes in SCS from baseline (ΔSCS) ± SEM are shown at weeks 0, 5 and 10. The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. (A) Normal BMI, (B) Overweight BMI, (C) High baseline SCS, (D) Low baseline SCS, and week 5 intervention (E) Responders and (F) Nonresponders. Significant differences ($p < 0.05$) within groups are represented by different letters (a, b, c). All post-hoc analyses were conducted using the Holm method to adjust for multiple comparisons. Significant differences reported reflect the interaction effects between time and treatment (time*treatment) observed in the model. Normal vs. Overweight BMI = 18.5 – 24.9 vs 25 – 29.9 kg/m^2 (n = 18 vs 15); High vs Low baseline SCS = above vs below the sample mean (n = 16 vs 17); carotenoid intervention Responders vs Nonresponders = above vs below 1/2 SD of the mean ΔSCS from week 0 to 5 (n = 22 vs 11).

4.6 *B. subtilis* supplementation does not enhance the effect of carotenoid supplementation on plasma carotenoid concentrations

The mean plasma carotenoid and retinol concentrations are shown in Figure 4.3. Total and individual plasma carotenoids generally increased over time ($p < 0.001$), with levels at week 10 being significantly greater than week 0 ($p_{holm} \leq 0.006$) in both groups for all carotenoids except lycopene. Lycopene increased at week 5 compared to week 0 ($p_{holm} = 0.013$) only in the probiotic group (Fig. 4.3 C). In both groups, all participants showed adequate retinol levels at baseline that increased at week 5 compared to week 0 ($p_{holm} \leq 0.005$) and returned to baseline levels at week 10 (Fig 4.3 G). No differences between groups in plasma carotenoid and retinol concentrations were observed.

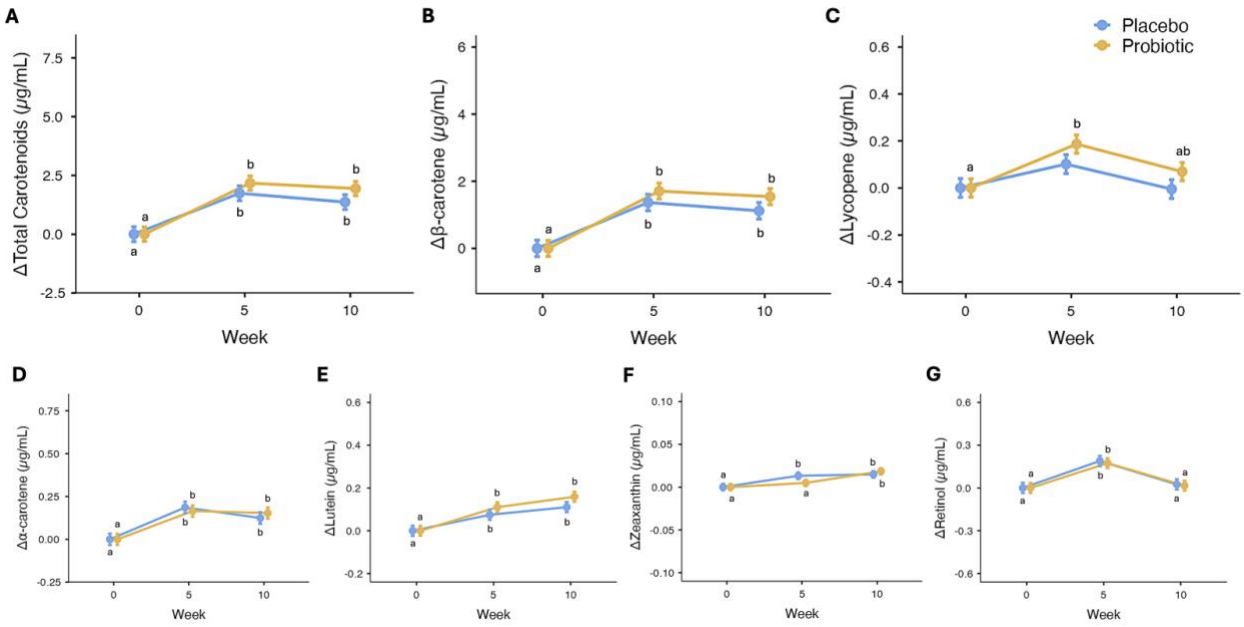


Figure 4.3. Plasma carotenoid and retinol concentrations. Mean changes from baseline (Δ) in plasma concentrations (μg/mL) ± SEM are shown at weeks 0, 5 and 10 for (A) total carotenoids, (B) β-carotene, (C) lycopene, (D) α-carotene, (E) lutein, (F) zeaxanthin, and (G) retinol. The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. Significant differences ($p < 0.05$) within groups are represented by different letters (a, b). All post-hoc analyses were conducted using the Holm method to adjust for multiple comparisons. Significant differences reported reflect the interaction effects between time and treatment (time*treatment) observed in the model.

4.7 *B. subtilis*, but not carotenoid, supplementation alters gut microbiota composition

Overall, alpha diversity was not different between treatment groups (Fig. 4.4). Only one of four metrics showed lower alpha diversity in the Probiotic group compared to the Placebo group at baseline: faith phylogenetic distance (an indicator of phylogenetic diversity; $q \leq 0.01$; Fig. 4.4 B). The other metrics did not show any differences between or within groups.

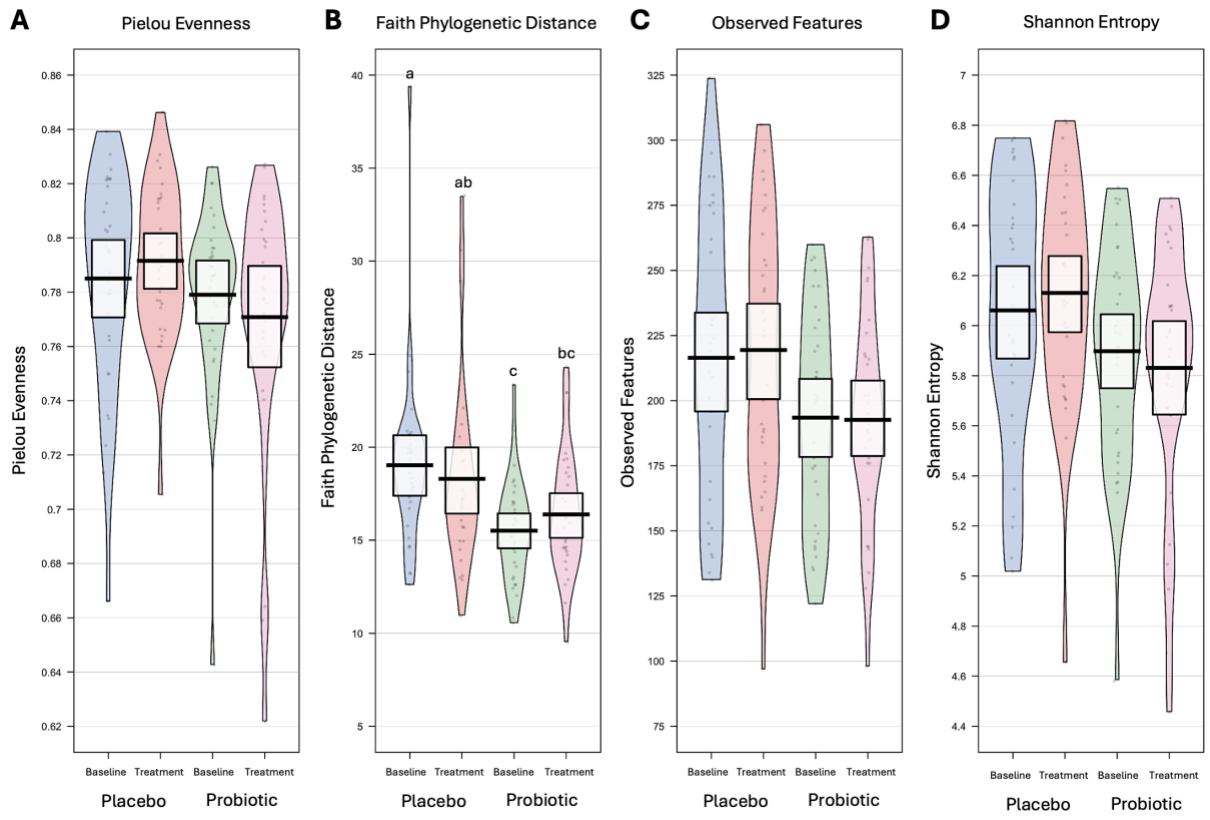


Figure 4.4. Alpha diversity metrics over time and after treatment. Mean alpha diversity ± 95% CI of the fecal microbiota datasets of participants in the placebo group (left) at baseline (blue) and after treatment (red) and probiotic (right) at baseline (green) and after treatment (pink) for four metrics: (A) Pielou Evenness (lower value indicates dominance by few species), (B) Faith Phylogenetic Distance (lower value indicates less phylogenetic diversity), (C) Observed Features (amplicon sequence variant counts; lower value indicates less species richness), and (D) Shannon Entropy (lower values indicate less species richness and evenness). Significant differences ($q < 0.05$) between treatment groups from Benjamini-Hochberg adjusted pairwise Kruskal-Wallis test are represented by different letters (a, b, c).

Beta-diversity by Weighted UniFrac PCoA from QIIME 2 was visualized using R and color-coded according to the sample's treatment allocation at baseline and after treatment (Fig. 4.5). Each data point represents a fecal sample, and the distance matrix between them is based on ASV counts and phylogeny. The larger circles represent the centroid or mean position of all the data points within a particular group. There were small differences in gut microbial composition between groups at baseline (red vs. blue centroid) that became slightly more different after

treatment (yellow vs. green centroid). PiratePlots of the mean relative abundance (%) ± 95% CI of the 36 most abundant taxa (by rank) in the placebo and probiotic groups at baseline and after treatment are shown in Appendix Figures 3-5. No obvious differences were observed between groups at baseline or after treatment.

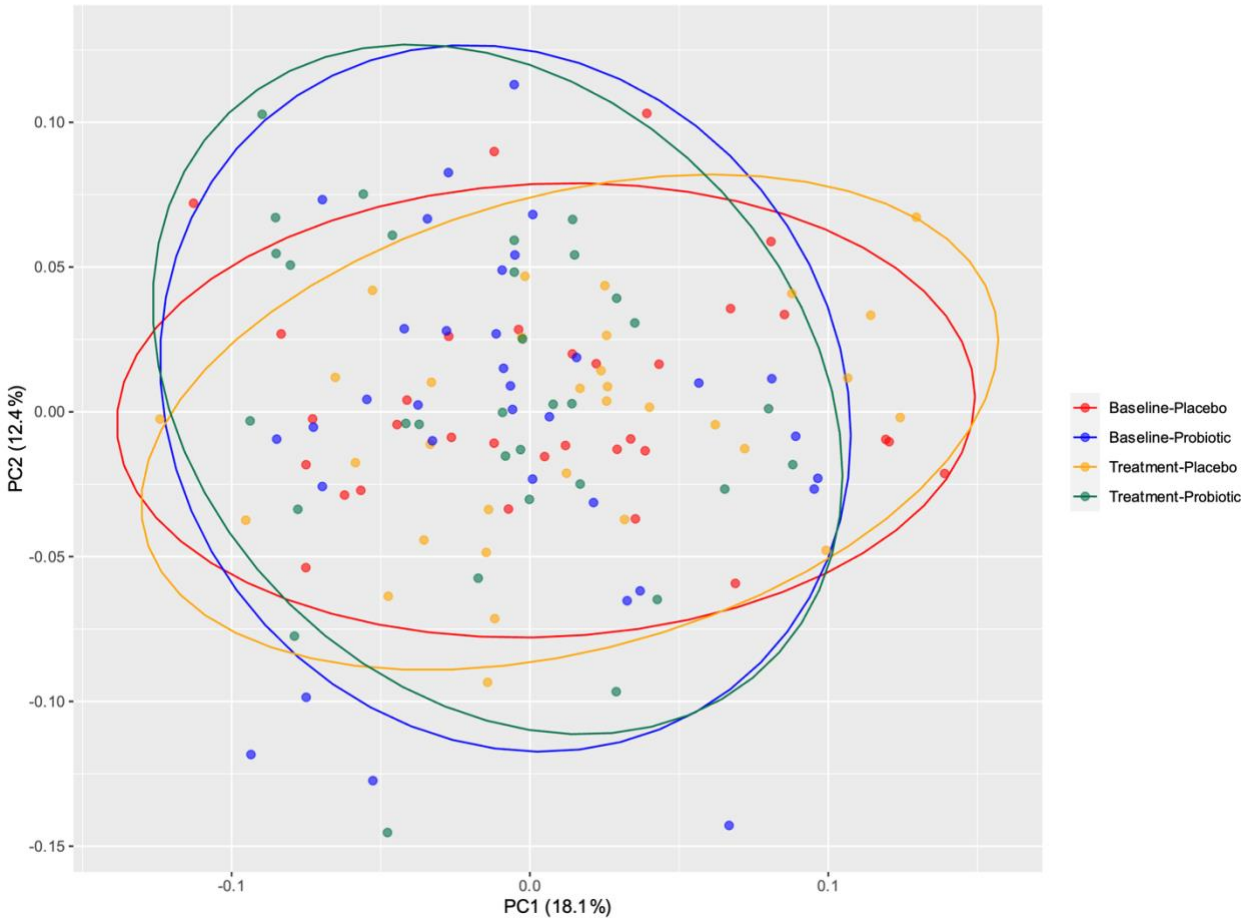


Figure 4.5. Beta diversity over time and after treatment. Beta diversity by Weighted UniFrac (phylogenetic distance between species weighted according to their relative abundance) Principal Coordinates Analysis (PCoA) of the fecal microbiota datasets of participants in the probiotic group at baseline (blue) and after treatment (green) and of participants in the placebo group at baseline (red) and after treatment (yellow).

The QIIME2 sample classifier, a supervised classification machine learning (ML) algorithm, was implemented to classify the gut microbiota profiles on sample origin (i.e., treatment group and timepoint) and to highlight the taxa that have the greatest influence on distinguishing between different classes (e.g., probiotic vs. placebo). Given that the ML algorithm randomly generates training and test datasets, 10 iterations were conducted for each comparison and the accuracy results were compiled (Appendix Table 3). One of the iterations that met the median overall accuracy value of all 10 conducted iterations was randomly selected as the representative result. Figure 4.8 shows the ML model-accuracy results for the Extra-Tree classification trained on the merged dataset samples for between-group comparisons at baseline (Fig. 4.6 A) and after treatment (Fig. 4.6 B) and for within-group comparisons over time (Fig. 4.6 C and D). The confusion matrices show strong accuracy scores on the diagonal and good overall accuracy results (i.e., correctly classified instances) for between-group comparison at baseline (80% final accuracy; Fig. 4.6 A) and after treatment (100% final accuracy; Fig. 4.6 B). This suggests that the groups already showed some differences at baseline, but clear differences between groups after treatment.

It is worth noting that the accuracy ratio was 1.5, meaning the model's accuracy was 1.5 times higher (i.e., 50% better) than random chance (which is represented by a baseline accuracy of 0.53 for binary classification). This ratio indicates that the machine learning algorithm was able to learn meaningful patterns from the baseline classification labels, leading to predictions better than random guessing. However, the model still faced challenges in perfectly distinguishing between the groups, as its accuracy did not reach the maximum possible value (2.0 for perfect accuracy compared to random chance). Optimally, randomization would have generated groups with similar gut microbiota composition at enrollment, such that the ML

algorithm would not be able to distinguish between groups beyond random chance (i.e., demonstrate an accuracy ratio closer to the baseline accuracy value of 0.5), but this was not the case. Thus, one could then interpret the accuracy ratio of 1.5 as the "enrollment bias" (i.e., new baseline accuracy according to participant randomization). Finally, we can then interpret the accuracy ratio of 2.0 for the between-group comparison after treatment as the ML algorithm having learned the most it can (100% better than random chance) to clearly distinguish both groups within the existing differences at baseline that showed an accuracy ratio of 1.5.

For within-group comparisons, a final accuracy of 100% was observed for changes over time within the probiotic group (Fig. 4.6 D) and a poor final accuracy of 35% was observed for changes over time within the placebo group (Fig. 4.6 C). This suggests that carotenoid supplementation did not substantially alter overall gut microbiota composition over time, but probiotic supplementation did.

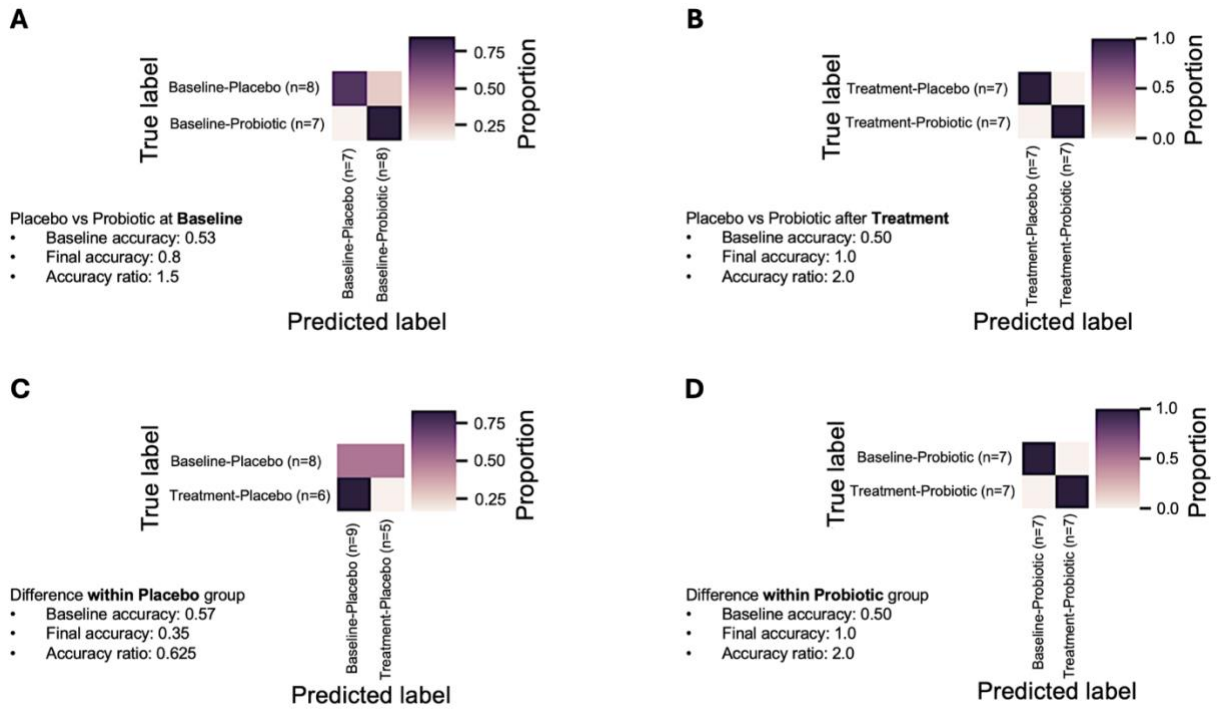


Figure 4.6. Confusion matrices evaluating the performance of the ML classification model over time and after treatment. The ML model classified group labels (A) at baseline and (B) after treatment, and treatment labels within (C) the placebo group and (D) the probiotic group, according to gut microbiota composition.

To explore the taxa that the ML model used to distinguish between labels (e.g., probiotic baseline and probiotic treatment), the lists of important taxa and associated importance scores were obtained from the 'feature_importance.qza' file generated (QIIME 2). The important taxa are ranked according to which taxa has the greatest predictive accuracy to distinguish between different classes or labels (e.g., between groups at baseline and after treatment, and within groups over time). The important taxa that were included in at least 2 of 3 iterations that met the median overall accuracy value of all 10 conducted iterations were manually compared. The manual selection consisted of identifying taxa from the top 36 important taxa that showed obvious differences in mean relative abundance with no overlap of CIs for each classification comparison

[382]. Expectedly, the *Bacillus* genera were ranked first for their ability to classify participants according to *B. subtilis* probiotic supplementation, with the probiotic group showing a higher relative abundance after treatment compared to baseline (Fig. 4.7 A). No other taxa showed obvious differences (i.e., mean differences without overlapping confidence intervals (CI); Appendix Fig. 6-7). *Bacillus* genera were also the most important taxa (rank 1) for the classification of groups after treatment (i.e., probiotic higher than placebo) and were accompanied by two other taxa that were different between groups: *Oscillospiraceae, UCG*-003 and *Coriobacteriales, incertae sedis (uncultured)* that showed lower and higher relative abundance, respectively, in the probiotic group compared to placebo (Fig. 4.7 B). There were three other taxa with some group differences that served as important taxa at both baseline and after treatment (Fig. 4.7 C): *Ruminococcaceae, Siraeum group* was lower in the probiotic group at baseline, but similar after treatment; *RF39, RF39* was lower in the probiotic group at baseline and after treatment (suggesting an innate difference between groups); and *Anaerovoracaceae, Family XIII AD3011 group* was lower in the probiotic group after treatment, but similar at baseline. No obvious differences were observed in the placebo group over time which aligns with the low classification model accuracy scores.

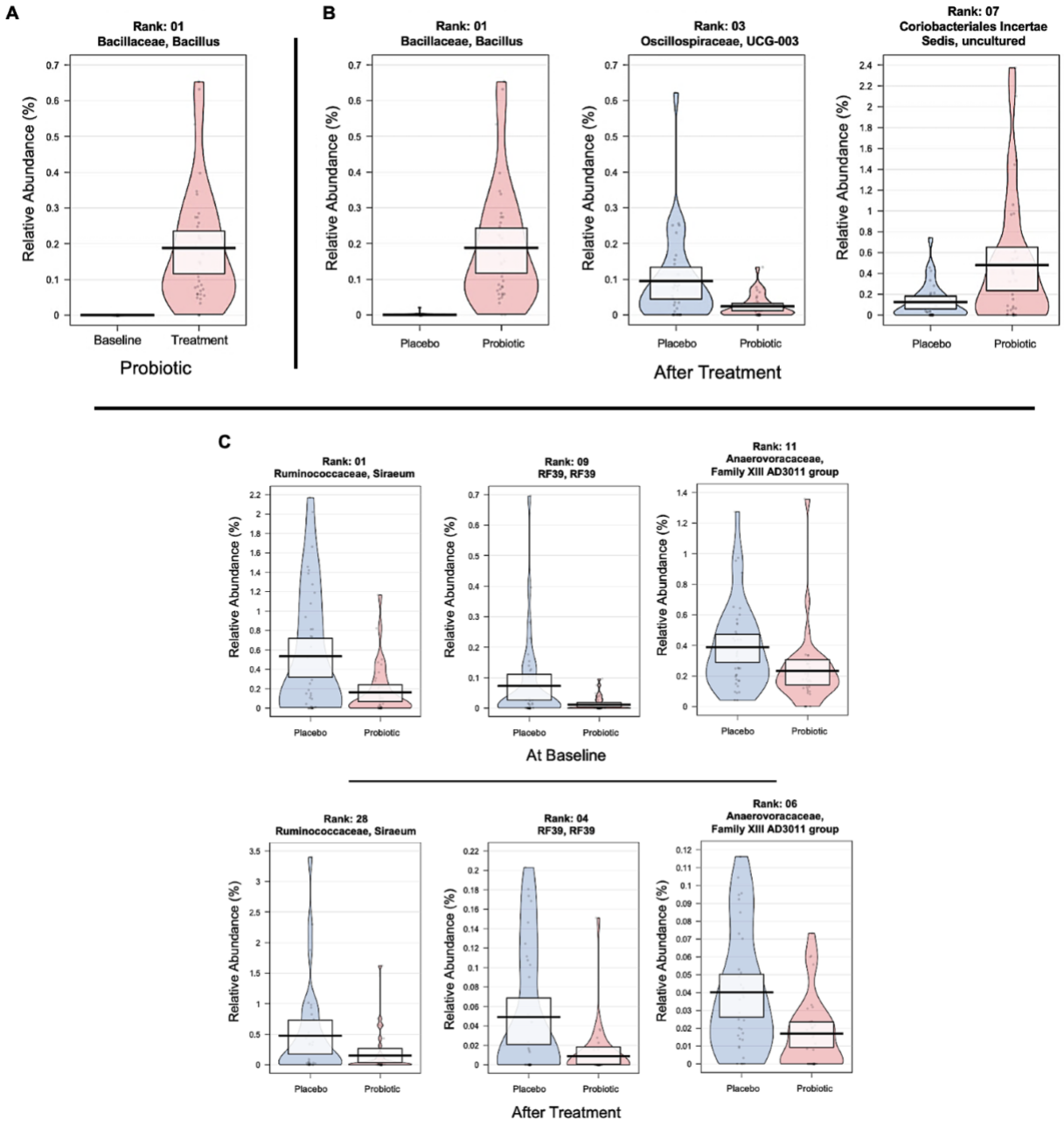


Figure 4.7. Pirate plots of important taxa for classifier distinction over time and after treatment. Pirate plots of the mean relative abundance (%) ± 95% CI of important taxa that showed obvious differences between classifiers (i.e., no overlapping CI) from the ML classification models for (A) probiotic at baseline vs. after treatment and (B) probiotic vs. placebo after treatment. (C) Taxa with obvious between-group differences that served as important taxa for ML classification models distinguishing groups at both baseline and after treatment.

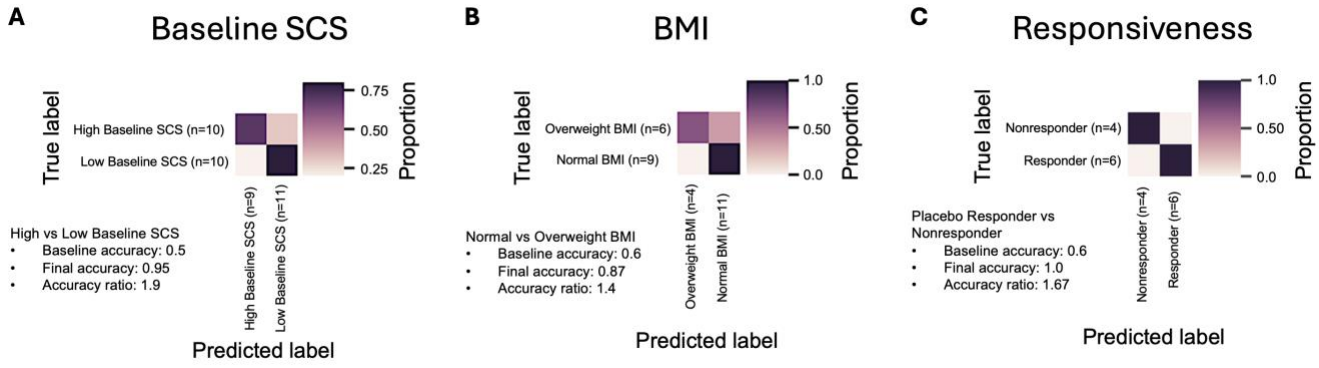
The relative abundance of bacterial genera was explored using MaAsLin2 (Microbiome Multivariable Association with Linear Models). All samples and time points were included, with time (baseline and treatment) and treatment (placebo and probiotic) as fixed effects and the sample ID as a random effect to control for repeated measures. Overall, the results aligned with those from the ML model. No differences were found in the placebo group over time. The only significant difference identified in the probiotic group was an increase in relative abundance in *Bacillus* genera after treatment compared to baseline (coefficient estimate = 3.44; $p < 0.001$; Appendix Table 4). After treatment, the probiotic group showed higher relative abundance of *Bacillus* (coefficient estimate = 3.41; $p < 0.001$) and *Acidaminococcus* (coefficient estimate = 0.91; $p < 0.001$), and mostly showed lower relative abundance of taxa from the Clostridia class (e.g., *Lachnospiraceae, Moryella* and *Oscillospiraceae, UCG-003*; coefficient estimates = -1.00 to -2.13; $p = 0.01$ to 0.003) compared to placebo. Several other taxa from the Clostridia class were lower in the probiotic group compared to placebo at baseline (e.g., *Oscillospiraceae, UCG-002 and Peptostreptococcaceae, Terrisporobacter;* coefficient estimates = -0.97 to -1.75; $p = 0.01$) and were not significant after treatment. The only differences that remained after the adjustment for false discovery rate (FDR) were those related to *Bacillus* ($q < 0.001$). This suggests that the other differences in specific taxa may be spurious and represent false positives (i.e., random variation rather than true biological effects) that may not hold after the adjustment for multiple comparisons.

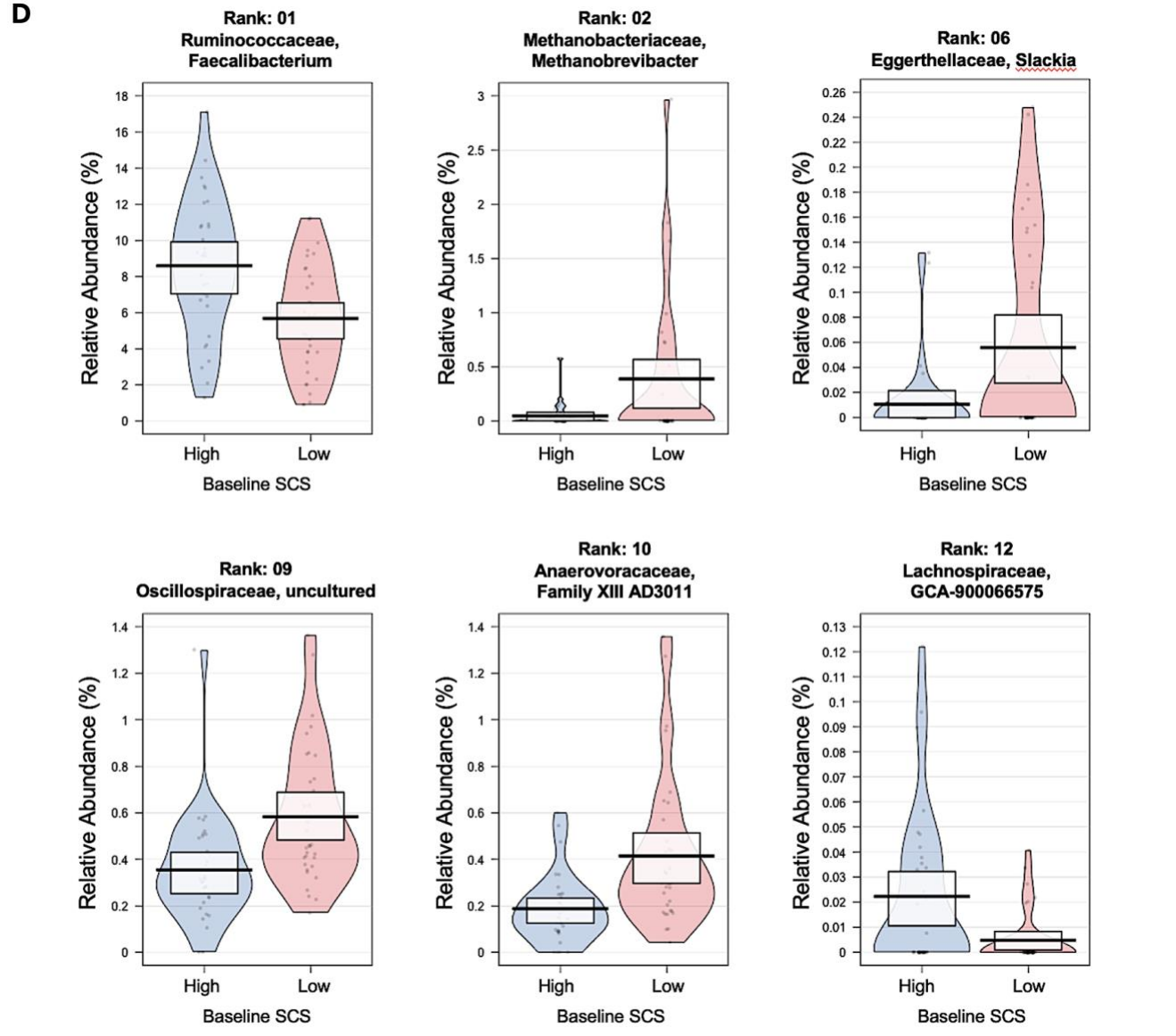
4.8 Baseline gut microbiota composition is associated with baseline SCS and response to carotenoid intervention, but not associated with BMI

The ML model-accuracy results for the Extra-Tree classification trained on the merged baseline gut microbiota dataset samples (i.e., weeks –1 and 0) of classifiers for baseline SCS, BMI, and carotenoid intervention responsiveness are shown in Figure 4.8. The confusion matrices show strong accuracy scores on the diagonal and good overall accuracy results for baseline SCS (95% final accuracy; Fig. 4.8 A) and carotenoid intervention responsiveness (100% overall accuracy; Fig. 4.8 C, placebo group only: Responders, n = 9; Nonresponders, n= 7). Although the best iteration of comparisons between classifiers for BMI suggested predictive potential with a final accuracy of 87% (Fig. 4.8 B), the median final accuracy and accuracy ratio across all iterations were relatively low (70% and 1.17, respectively; Appendix Table 5). The latter finding suggests that baseline gut microbiota composition is not associated with overweight or normal BMI. The top 6 ranked important taxa with obvious differences between labels that were shared among the 2 or 3 iterations with the highest overall accuracy for classifying between baseline SCS and responsiveness are shown in Figure 4.8 D and E. Interestingly, none of the important taxa with obvious differences between labels were shared among the two classification models (i.e., Baseline SCS and Responders). However, two important taxa from the Baseline SCS classification (Fig. 4.8 D) that also showed significant differences in the MaAsLin2 analysis (Appendix Table 6) were *Oscillospiraceae uncultured* and *Family XIII AD3011 group*.

The MaAsLin2 analysis showed a lower relative abundance of taxa in the Clostridia class for both High Baseline SCS (*Negativibacillus*, *Family XIII AD3011 group*, *Oscillospiraceae uncultured*) and Responders (*Negativibacillus*, *Fournierella*), as well as *Enterorhabdus* in

Responders (coefficient estimates = -0.81 to -1.47; $q \leq 0.025$), and higher *Bifidobacterium* for High Baseline SCS (coefficient estimate = 2.75; $q = 0.034$). Although there were other taxa with significant differences identified in the MaAsLin2 analysis (including some according to BMI), with most differences observed in the Clostridia class, some notable others (e.g., increased *Akkermansia* for Responders and *Moryella* for Overweight BMI), and some that overlapped with the ML models (e.g., decreased *Holdemanella* for Responders and *Slackia* for High Baseline SCS), they did not maintain significance when adjusted for FDR (Appendix Table 6). Furthermore, when age and daily FV servings were added as covariates in the MaAsLin 2 analysis (Appendix Table 7), *Bifidobacterium* was no longer associated with Baseline SCS, *Family XIII AD3011 group* was no longer negatively associated with Baseline SCS after FDR adjustment, and genera *CAG 352* and *Holdemanella* – two taxa used by the ML model to distinguish Responders (Fig. 4.8 E) – were significantly associated with daily FV servings after FDR adjustment. Despite not being significant after FDR adjustment, the associations between Responders and *CAG 352* and *Holdemanella* showed almost double the coefficient estimates, suggesting an association beyond only FV intake. Nonetheless, one taxon used by the ML model to distinguish Baseline SCS, *Slackia*, that was negatively associated with High Baseline SCS before adjustment for daily FV intake and FDR, ended up being associated with daily FV intake instead (without FDR adjustment).


A
Baseline SCS
True label
High Baseline SCS (n=10)
Low Baseline SCS (n=10)
0.75
0.50
0.25
Proportion
High Baseline SCS (n=9)
Low Baseline SCS (n=11)
Predicted label
High vs Low Baseline SCS
• Baseline accuracy: 0.5
• Final accuracy: 0.95
• Accuracy ratio: 1.9
B
BMI
True label
Overweight BMI (n=6)
Normal BMI (n=9)
1.0
0.50
0.0
Proportion
Overweight BMI (n=4)
Normal BMI (n=11)
Predicted label
Normal vs Overweight BMI
• Baseline accuracy: 0.6
• Final accuracy: 0.87
• Accuracy ratio: 1.4
C
Responsiveness
True label
Nonresponder (n=4)
Responder (n=6)
1.0
0.50
0.0
Proportion
Nonresponder (n=4)
Responder (n=6)
Predicted label
Placebo Responder vs Nonresponder
• Baseline accuracy: 0.6
• Final accuracy: 1.0
• Accuracy ratio: 1.67


D
Rank: 01
Ruminococcaceae, Faecalibacterium
Rank: 02
Methanobacteriaceae, Methanobrevibacter
Rank: 06
Eggerthellaceae, Slackia
Rank: 09
Oscillospiraceae, uncultured
Rank: 10
Anaerovoracaceae, Family XIII AD3011
Rank: 12
Lachnospiraceae, GCA-900066575
Relative Abundance (%)
High
Low
Baseline SCS

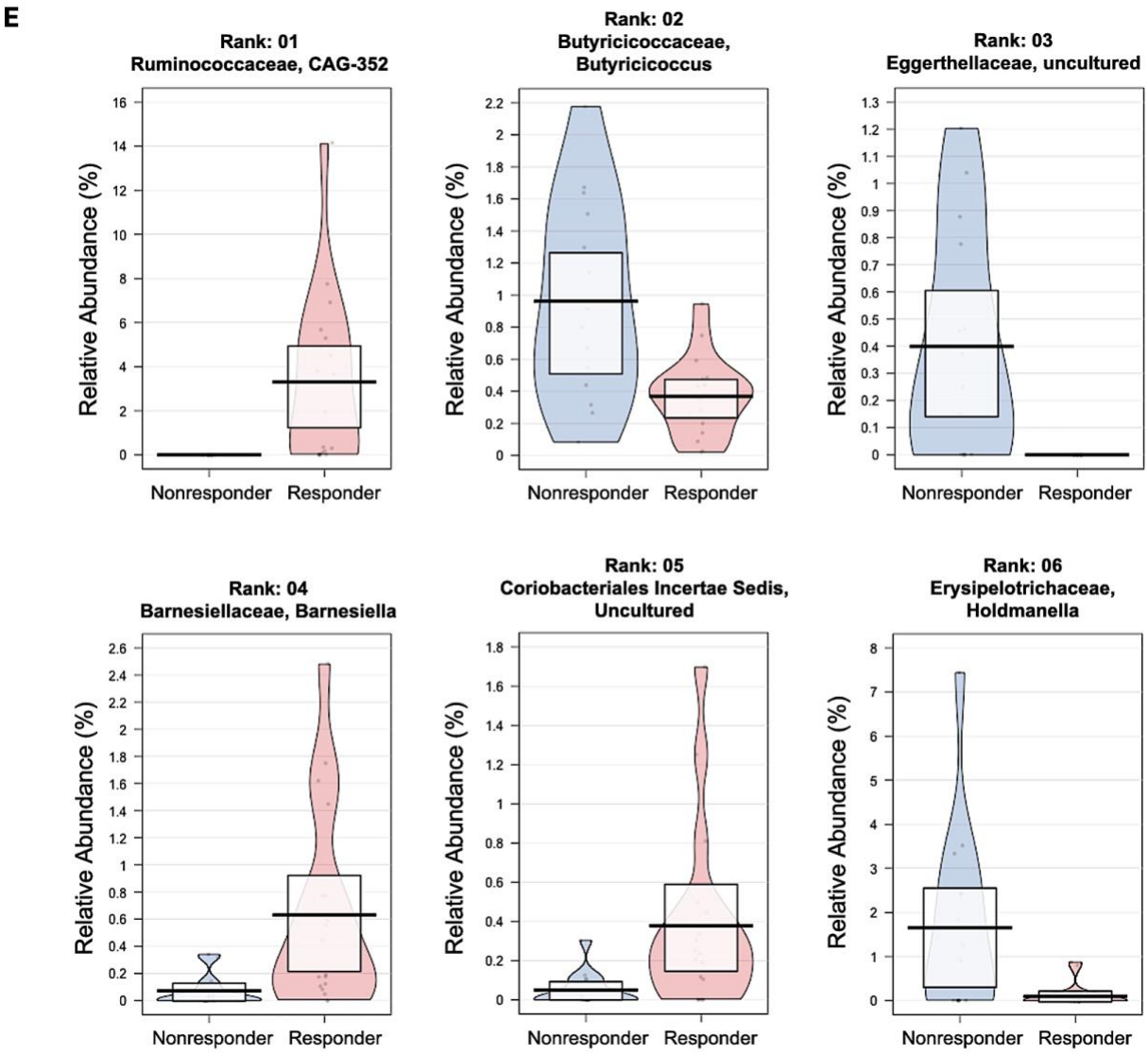


Figure 4.8. Confusion matrices evaluating the performance of the ML classification model at baseline. The ML model classified individuals into different groups according to gut microbiota composition at baseline: (A) baseline SCS (High vs Low; above vs below the sample mean; n = 16 vs 17), (B) BMI (Normal vs Overweight; n = 18 vs 15) and (C) carotenoid intervention responsiveness (Responder vs Nonresponders in placebo group only; above vs below 1/2 SD of the mean ΔSCS from week 0 to 5; n = 9 vs 7). Mean relative abundance ± 95% CI for the top 6 ranked important taxa identified by the ML classifier for the distinction of (D) baseline SCS and (E) responsiveness status that show obvious differences between labels (i.e., different means without overlapping CI).

4.9 Carotenoids, with or without *B. subtilis*, did not change antioxidant or immune status

Overall, there was no treatment or interaction effect on ABTS, but a time effect was observed (p = 0.002). Post-hoc analysis revealed a significant decrease from week 5 to week 10 (p_{holm} = 0.001) that was only observed in the placebo group (p_{holm} = 0.020; Fig. 4.9 B) in the interaction analysis. Subgroup analysis revealed that the decrease was only in carotenoid intervention Nonresponders who took the placebo (p_{holm} = 0.002). No other differences within groups and no differences between groups were observed. No between or within-group differences in plasma FRAP or cytokine concentrations were observed (Fig. 4.9).

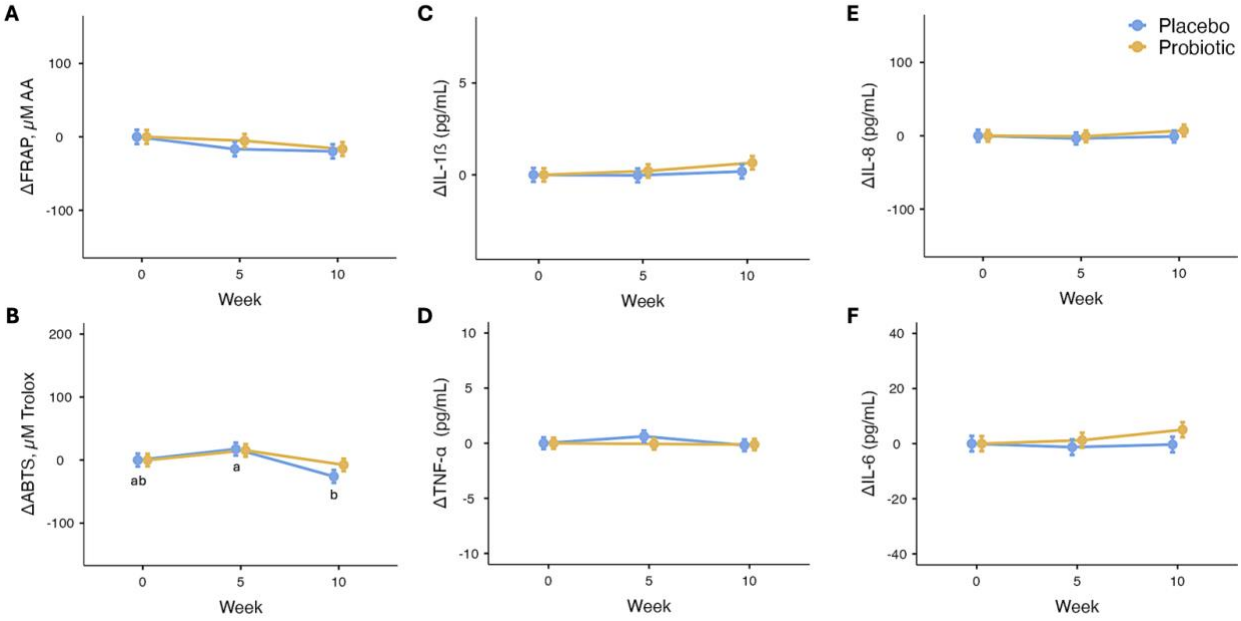


Figure 4.9. Mean changes from baseline (Δ) ± SEM for antioxidant capacity and immune status measures. The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. (A) FRAP (μM AA), (B) ABTS, (C) IL-1β, (D) TNF-α, (E) IL-8, and (F) IL-6. No difference between groups were observed. Significant differences (p < 0.05) within groups are represented by different letters (a, b). All post-hoc analyses were conducted using the Holm method to adjust for multiple comparisons. Significant differences reported reflect the interaction effects between time and treatment (time*treatment) observed in the model. FRAP = ferric reducing antioxidant power; AA = ascorbic acid; ABTS = 2,2′- azino-bis 3-ethylbenzothiazoline-6-sulfonic acid; IL = interleukin; TNF-α = tumor necrosis factor alpha.

4.10 *B. subtilis* may slightly increase plasma cortisol levels

Although no time or treatment effects were observed for cortisol, a time*treatment interaction effect was observed ($p = 0.021$). Post-hoc analysis showed a statistically significant minor increase in cortisol levels in the probiotic group from week 0 to week 10 (8.74 to 11.0 µg/dL; $p_{holm} = 0.017$; Fig. 4.10 A). At week 10, the probiotic group also showed slightly higher cortisol levels compared to placebo (mean difference of 2.73 µg/dL; $p_{holm} = 0.020$). In subgroup analysis, only carotenoid intervention Responders that took the probiotic increased cortisol levels from week 0 to week 10 ($p_{holm} = 0.030$) and showed increased cortisol level at week 10 compared to placebo ($p_{holm} = 0.025$). However, this difference is relatively small when considering natural fluctuations in cortisol according to the time of day (2-25 µg/dL) and variation according to menstrual cycle phases (up to 0.5 µg/dL) [383,384]. No between or within-group differences were observed for estradiol, testosterone, or progesterone.

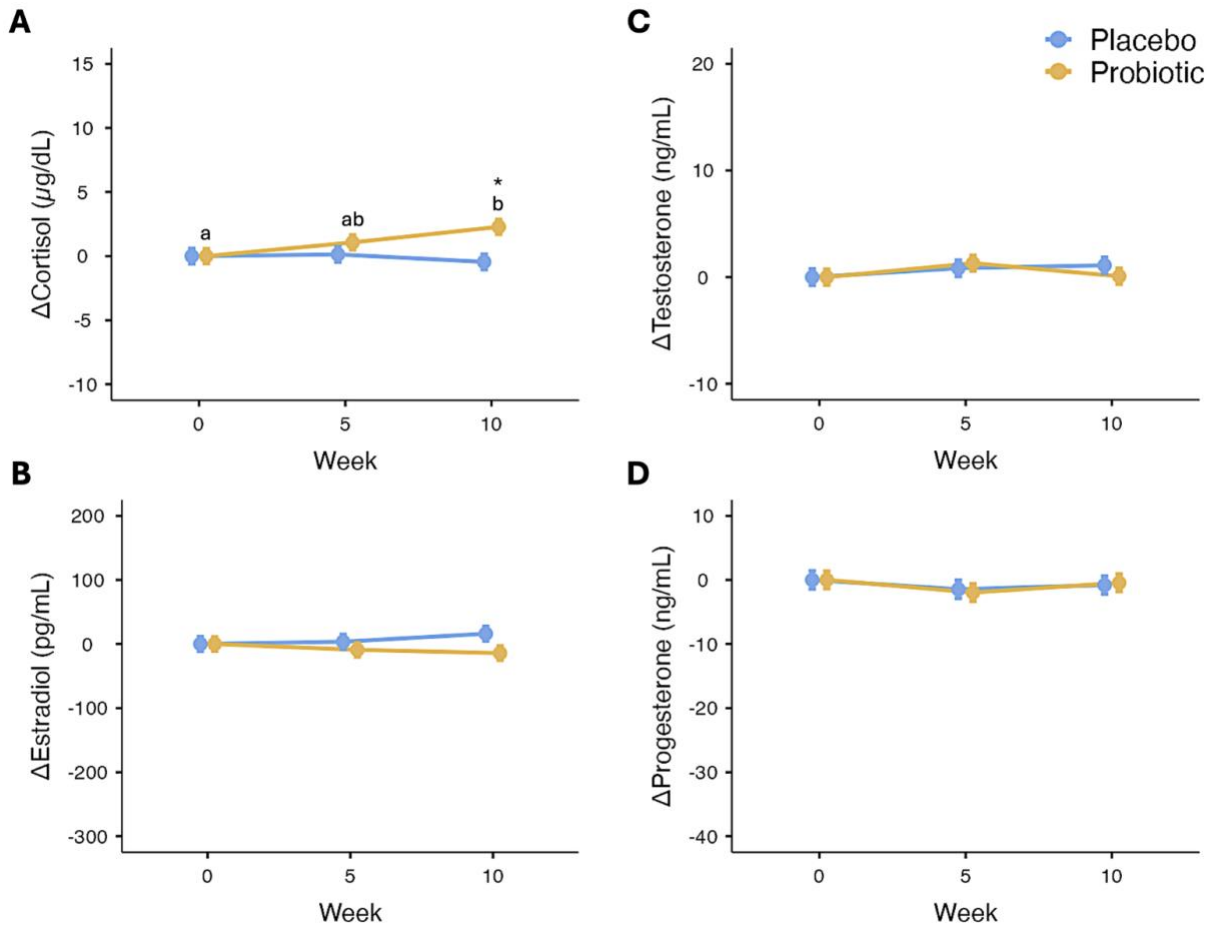


Figure 4.10. Mean changes from baseline (Δ) ± SEM for plasma hormone concentrations. The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. (A) Cortisol (µg/mL), (B) Estradiol (pg/mL), (C) Testosterone (ng/mL), and (D) Progesterone (ng/mL). Significant differences ($p < 0.05$) within groups are represented by different letters (a, b) and between-group differences are represented with an asterisk (*). All post-hoc analyses were conducted using the Holm method to adjust for multiple comparisons. Significant differences reported reflect the interaction effects between time and treatment (time*treatment) observed in the model.

4.11 Carotenoids with or without *B. subtilis* do not substantially affect skin health

No between-group differences were observed for any skin health measures (Fig. 4.11). Pliability showed a slight significant decrease from week 0 to week 10 in the placebo group only (-0.05 mm skin stretch; p_{holm}= 0.005; Fig. 4.11 C). No other within-group differences were observed. At week 10, there was a trend for a significant decrease from baseline in the placebo group compared to the probiotic group (-0.05 vs -0.01 mm; p_{holm}= 0.091). Subgroup analyses showed a stronger trend (p_{holm}= 0.055) for the decrease in the placebo group compared to the probiotic group in participants with high baseline SCS. The average room temperature was 22.9 ± 1.9 °C (range: 15-26 °C) and relative humidity was 38.5% ± 15.8% (range: 13-65%; Fig. 4.11 F), but no changes were observed after the inclusion of these variables as covariates in the LMM.

No between-group differences were observed for skin wrinkling or coloration (Appendix Fig. 5). Skin coloration (Appendix Fig. 8 D) increased from week 0 to week 5 (p_{holm} = 0.003) and to week 10 (p_{holm} < 0.001) in the placebo group, and from week 0 to week 10 in the probiotic group (p_{holm} = 0.029). An increasing trend was observed in the probiotic group from week 0 to week 5 (p_{holm} = 0.064). No other differences within groups (i.e., over time) were observed.

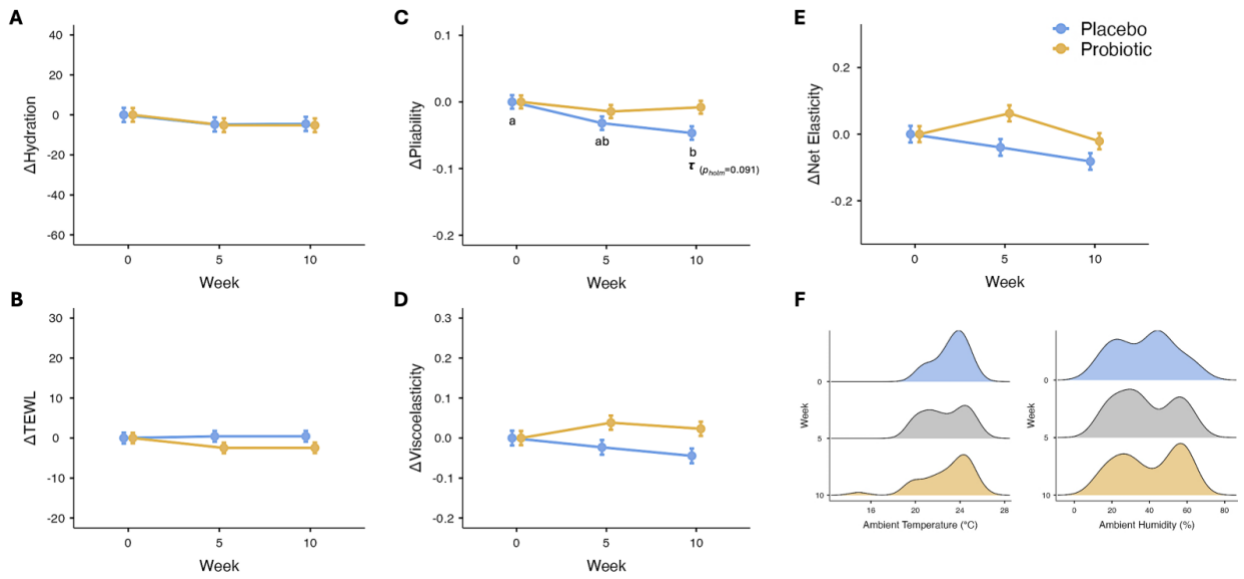


Figure 4.11. Mean changes from baseline (Δ) ± SEM for skin health measures. The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. (A) Hydration, (B) TEWL, (C) Pliability, (D) Viscoelasticity, (E) Net Elasticity. Significant differences ($p < 0.05$) within groups over time are represented by different letters (a, b) and trends for between-group differences are represented by tau (**τ**). All post-hoc analyses were conducted using the Holm method to adjust for multiple comparisons. Significant differences reported reflect the interaction effects between time and treatment (time*treatment) observed in the model. (F) Density distribution plots for ambient temperature (°C) and humidity (%). TEWL = Transepidermal Water Loss.

4.12 Carotenoids with or without *B. subtilis* do not change plasma SCFAs

No between or within-group differences were observed for plasma SCFAs (nM) (Appendix Fig. 9). A decreasing trend from week 0 to week 5 (p_{holm} = 0.080) in the probiotic group was observed. Subgroup analysis revealed significantly higher propionic acid levels at week 5 in the placebo group compared to the probiotic group (p_{holm} = 0.005) in participants with low baseline SCS.

4.13 Carotenoids with or without *B. subtilis* do not change the quality of life or sleep

No between or within-group differences were observed for physical or mental component scores of quality of life or sleep quality (Appendix Fig. 10).

4.14 Baseline SCS, total plasma carotenoids, and daily FV servings are moderately correlated

Spearman correlations were conducted to compare different carotenoid-related measurements at baseline. Daily FV servings showed a moderate positive correlation with SCS (ρ = 0.411; p = 0.009) and baseline total plasma carotenoids (ρ = 0.368; p = 0.018), a strong correlation with palm SCS (ρ = 0.600; $p < 0.001$), and no correlation with forehead SCS (ρ = 0.190; p = 0.145). Baseline total plasma carotenoids showed a strong trend for a moderate positive correlation with baseline SCS (ρ = 0.281; p = 0.057), a significant moderate positive correlation with baseline palm SCS (ρ = 0.323; p = 0.033), and no correlation with baseline forehead SCS (ρ = 0.135; p = 0.227).

When comparing the overall dataset (all time points and treatment), daily FV servings were not correlated with SCS or total plasma carotenoids, but SCS was strongly correlated with total plasma carotenoids (ρ = 0.618; $p < 0.001$). Furthermore, total plasma carotenoids were strongly correlated with palm SCS (ρ = 0.657; $p < 0.001$) and moderately with forehead SCS (ρ = 0.355; $p < 0.001$). Spearman's correlation coefficients used for non-normally distributed data were described as poor < 0.20, moderate 0.2-0.6, or strong > 0.6, as previously suggested within dietary validation studies [207,208].

Chapter 5: Discussion

This is the first study to explore the interplay between probiotic and carotenoid supplementation on human tissue carotenoid status. The key findings from this 10-week double-blind, randomized, controlled trial, were:

(a) Baseline gut microbiota composition differed between individuals with High vs. Low baseline SCS and between the carotenoid intervention Responders vs. Nonresponders.

(b) Mixed carotenoid supplementation increased SCS overall, but the impact varied according to baseline SCS. In addition, some individuals did not respond to the carotenoid intervention.

(c) Probiotic supplementation enhanced the effects of carotenoid supplementation on SCS, but not plasma carotenoid concentrations. Subgroup analyses showed that probiotic supplementation may be most beneficial for SCS enhancement in participants with normal BMI or high baseline SCS, or participants who do not respond to carotenoid supplementation.

(d) Probiotic + carotenoid supplementation was associated with changes in gut microbiota composition, while carotenoid supplementation alone was not.

(e) Neither carotenoid supplementation alone nor in combination with probiotic supplementation improved markers of antioxidant capacity, inflammation, or skin health.

5.1 The association between baseline gut microbiota composition and SCS

This is the first study to directly investigate associations between gut microbiota composition and SCS, evidenced by both the random forest ML model and the MaAsLin2 results. This suggests that gut microbiota composition may affect carotenoid status and is an important consideration for carotenoid status assessment. The methods identified two common defining taxa. Both an *uncultured* genus from the *Oscillospiraceae* family and the *Family XIII AD3011 group* from the *Anaerovoracaceae* family were negatively associated with baseline SCS. However, these taxa are not well-characterized, and little is known about their metabolic capacity beyond inferences from genomic analysis; the genome is often dominated by genes with either unknown functions or general function prediction only [385,386]. Despite often appearing in high-throughput sequencing data and some genera (e.g., *Oscillospira*) having been positively associated with SCFAs, leanness (i.e., low BMI), and plant fiber degradation (with relatively few genes with inferred involvement in carbohydrate transport and metabolism), many *Oscillospiraceae* remain uncultured (likely due to challenges with their anaerobic nature) [387–390]. The few studies that have shown changes in *Anaerovoracaceae Family XIII AD3011 group* have reported conflicting results in animals and humans (e.g., greater abundance associated with a high-fat diet in one study [391] and with a low-fat diet in another [392]), but some consistency regarding an association with increased inflammation (IL-5) in animal and human studies [386,393,394].

Our findings overlap with previous observational studies that have reported associations of dietary and plasma carotenoids with gut microbiota composition, but the specific taxa differ [26,28]. Djuric et al. (2018) reported a decreased Firmicutes abundance, mostly from the

Lachnospiraceae family and *Blautia* and *Roseburia* genera, associated with greater serum total carotenoids in 76 adults (mean age: 53; 75% women; mean BMI: 27) with a high colon cancer risk [26]. Although some taxa from the *Lachnospiraceae* family decreased in our study, they did not include the *Blautia* and *Roseburia* genera, were associated with Overweight BMI (conversely, those associated with baseline SCS increased), and did not remain significant after adjusting for FDR (Appendix Table 5). Two key factors, among many, that could account for the differences observed in comparison to our study are: 1) the use of LEfSe differential abundance analysis, which produces high false positives compared to MaAsLin2 [258], and 2) colonic biopsies (i.e., mucosa), whose microbiota community composition is known to differ from stool samples [395].

Schmidt et al. (2021) used a generalized linear mixed model to compare individual plasma carotenoids to specific taxa from stool samples of 23 pregnant women (mean age: 30; mean BMI: 27) and reported differing associations with different individual plasma carotenoids and certain taxa (*Akkermansia*, *Prevotella*, *Phascolarctobacterium*, and *Ruminococcaceae UCG-002*) [28]. The only overlap in results with our study is the positive association between *Ruminococcaceae UCG-002* and plasma β-carotene concentrations, as *Ruminococcaceae Faecalibacterium* was ranked first as an important taxon for the distinction between High and Low baseline SCS by the ML model in our study. The *Ruminococcaceae* family are known butyrate (an anti-inflammatory SCFA) producers that are often inversely associated with inflammatory conditions (e.g., IBD, dermatitis) and positively associated with gut barrier and overall health [396]. This aligns with the previously established positive associations between higher SCS and overall health [9,88,90]. Although the analysis conducted by Schmidt et al. (2021) was more similar to MaAsLin2 than LEfSe, and the same adjustment for FDR was used, they did

not adjust for confounders (e.g., BMI). Conversely, our study involved two baseline samples to better characterize intra-individual variation in gut microbiota composition and MaAsLin2 allows for the incorporation of confounders and random effects and accounts for the non-independence of samples [259]. Furthermore, our study used an ML model that implicitly characterizes the interactions between gut microbiota, serving as an evaluation of the complex community structure of a whole microbial community, not only the impact of each individual taxa [263,266,267].

5.2 Adjustment for FV intake in the association between carotenoid status and gut microbiota composition

Our study showed a moderate positive correlation between FV intake and SCS at baseline, consistent with previous studies [10,204]. One key limitation of the available human studies evaluating gut microbiota composition is that they have not properly controlled for other dietary components that have demonstrated their own prebiotic effects. Dietary and plasma carotenoids reflect FV intake and FVs are rich in fiber and other prebiotic components (e.g., polyphenols) [61,66]. Previously reported associations of dietary and plasma carotenoids with gut microbial composition may reflect high fiber or diet quality rather than a specific effect of carotenoids [28]. Fiber intake is associated with increased gut microbial diversity and favorable changes of genera in the *Ruminococcaceae* and *Lachnospiraceae* families, both which are involved in macronutrient fermentation [300,397–399].

In our study, when FV intake was included in the MaAsLin 2 analysis, we observed a shift in associations between certain taxa and SCS. The previously noted association between *Anaerovoracaceae Family XIII AD3011 group* and SCS was no longer significant after the

adjustment for FDR. In addition, statistically significant associations (after FDR adjustment) were observed between daily FV servings and two bacterial genera (*CAG 352* and *Holdemanella*; Appendix Table 7). Although not very well characterized, *CAG 352* has been associated with healthy individuals [400] and belongs to the *Ruminococaceae* family that is generally associated with overall health and degradation of dietary fibers [300,396]. *Holdemanella* has been associated with meat and dairy intake (not FV intake) [401,402] and the production of long-chain fatty acids (from fructooligosaccharide fermentation) that exhibited anti-inflammatory properties in a mouse model of colitis [403]. These genera were also identified by the ML model as key markers distinguishing Responders (Fig. 4.8 E). However, their associations with Responders did not maintain statistical significance after FDR adjustment in the MaAsLin 2 model. This suggests that FV intake may be a more consistent factor associated with these genera than Responder status. The nearly double coefficient estimates between these genera and Responders imply that additional factors, beyond FV intake, may account for some of the differences in these genera between Responders and Nonresponders. Although this may be a false positive as the association was not significant after FDR adjustment in the MaAsLin2 analysis, it is (once again) important to highlight the advantage of the ML model to implicitly characterize interactions between gut microbiota and evaluate the whole microbial community [263,266,267].

Although insignificant after FDR adjustment, another example of changes in association after the inclusion of FV intake can be seen for *Slackia.* A negative association between *Slackia* and High Baseline SCS that was identified in the initial MaAsLin2 and ML analyses (Figure 4.8 D and Appendix Table 6) was not observed following the inclusion of daily FV servings as a factor in the MaAsLin 2 analysis. Instead, daily FV servings were negatively associated with

Slackia (Appendix Table 7), suggesting that this association is more dependent on daily FV intake than Baseline SCS. Although not well characterized, one human intervention trial reported increased *Slackia* after 6-weeks on a modified Mediterranean-ketogenic diet [404]. Our study highlights the need for future studies that evaluate the association between carotenoid status and gut microbiota composition to adjust for FV intake or other dietary components that may have their own impact on gut microbiota composition, and use ML algorithms that provide insights into the complex community structure of the gut microbiome.

5.3 SCS increases to a plateau with mixed carotenoid intervention

Studies consistently show that carotenoid supplementation, whether through dietary sources, extracts, or supplements, significantly increases SCS measured via RRS, RS, and colorimetry (i.e., skin yellow color saturation) [10,188,405–407]. When using skin yellow color saturation (b* value) as the measure for SCS, significant increases in SCS from carotenoid supplementation have been detected in as little as two weeks (Table 2.1), with greater changes in SCS observed with greater doses [6,194,201]. However, previous carotenoid intervention studies that measured skin b* value primarily included β-carotene supplements (15-18 mg/d) or carotenoid-rich dietary supplementation (5-25 mg/d). In our study, SCS significantly increased after mixed carotenoid supplementation (17 mg/d) to a similar degree (↑ Δ SCS of 1.5) when compared to other studies with similar supplement doses (15-18 mg/d) of β-carotene [11,199]. The mixed carotenoid supplement was chosen to reduce any bias from the presence of other dietary components (e.g., fiber). In addition, the dose of mixed carotenoids aligns more with a relevant intake of multiple carotenoids within a diet when compared to a high-dose single carotenoid supplement (i.e., carotenoids are generally not consumed individually, especially at high doses ≥

15 mg/d) [70]. However, it should be noted that the increase in total carotenoids was primarily driven by β-carotene (Fig. 4.3), which was the most abundant carotenoid in our mixture (Appendix Table 1) as well as often in the dietary context [70].

Previous studies evaluating SCS had not characterized steady-state conditions with continued supplementation. The few previous studies that monitored repeated measures of a change in b* value for SCS did so after supplementation had ended (Table 2.1) [201,202]. Without continued supplementation, the plateau in SCS observed after supplementation ended could be interpreted as a delay in the storage of plasma carotenoids from the initial increase in intake. Jilcott Pitts et al. (2023) measured plasma carotenoids and SCS via RS after 3 and 6 weeks of 4-8 mg/d total carotenoids in an RCT involving 162 adults (mean age: 32; 49% men; mean BMI: 25) [183]. Plasma carotenoids increased by 30 and 60% and SCS by 20 and 40% over time for the 4 and 8 mg/d total carotenoid intervention groups, respectively. The plasma carotenoids demonstrated a plateau after week 3 of intervention, which aligns with previous work on plasma carotenoids (> 90% fraction of plasma steady-state concentrations at 2-3 weeks) [408], while no plateau effect was observed in SCS [183]. Our study is the first to show that continued supplementation with carotenoids leads to a relative steady state (i.e., no difference between weeks 5 and 10) in SCS after 5 weeks of supplementation. However, we do not know if SCS would continue to increase, albeit more slowly, to eventually reach a significant increase after a longer intervention period (e.g., 3-6 months). Future studies with more frequent measurement time points and different doses are needed to confirm the robustness of the achieved steady state. Moreover, it is unclear whether there is a mechanism for the degradation or increased excretion of carotenoids when a steady state is reached at a specific dose.

5.4 Baseline SCS and response to carotenoid intervention

To expand on the concept of reaching a steady state in SCS after carotenoid supplementation and explore whether SCS saturation may influence response to carotenoid intervention, we conducted a subgroup analysis according to baseline SCS (High vs Low; above vs below median baseline SCS). We showed that participants with High baseline SCS did not increase SCS after carotenoid supplementation, while those with low baseline SCS did. This study is the first to show that not only can a steady state in SCS be achieved, but there may also be a carrying capacity for carotenoid storage in the skin that goes on to influence an individual's response to carotenoid intervention. This aligns with the concept of micronutrient absorption that can depend on baseline status, with significant improvements shown for individuals with an existing deficiency and diminished effects (and increased excretion) observed with higher baseline levels (i.e., when needs are met) [409–411]. However, this is speculation, and the effect could be related to other factors such as increased degradation, excretion or other adaptations, similar to how provitamin A carotenoid cleavage enzymes are regulated via a feedback mechanism whereby high vitamin A status inhibits their activity to prevent vitamin A toxicity [78,79].

Previous studies involving carotenoid supplementation have demonstrated differences in carotenoid intervention response according to baseline SCS status. In an RCT involving 56 breast cancer survivors who consumed a high FV diet for three years, changes in blood carotenoids were inversely correlated with baseline blood carotenoid concentrations (lycopene, α-carotene, β-carotene, lutein, and β-cryptoxanthin) [412]. Baseline SCS was also found to be a significant covariate of the change in SCS after a 6-week intervention with carotenoid-rich juice

(8 mg carotenoids/day) [183]. This suggests that baseline carotenoid status is relevant when considering supplementation. Our study adds to the literature supporting the need to properly monitor and characterize baseline status and excretion in the context of carotenoid supplementation studies.

With the known interindividual variation in carotenoid status, responsiveness to carotenoid intervention has been entertained by researchers but rarely explicitly evaluated in the context of a study [13,180]. Only two previous studies have attempted to characterize the responder relationship with carotenoid intervention. Borel et al. (1998) reported high variability in plasma β-carotene response (17-fold difference between highest and lowest concentrations) to a supraphysiological dose of β-carotene supplementation (120 mg) in 79 young men (mean age: 24; mean BMI: 22) [210]. The authors claimed that since β-carotene was detected in all participants, there is likely a very small proportion of true Nonresponders to pharmacological doses, but no other criteria for the classification of Responders was discussed. In previous work by our group, Responders were classified as individuals having demonstrated an increase ≥ 1.0 in SCS (b* value) compared to baseline in their 2-week, open-label trial involving 25 participants who drank one cup of carrot juice daily (25 mg β-carotene) [201]. Half of the sample were classified as Responders at week 2, a fourth were classified as slow responders (reaching the Responder classification 2 or 4 weeks after supplementation ended), and the remaining participants did not show any change in SCS and were classified as Nonresponders. However, the classification criteria were rather arbitrary (using an anchor-based minimum clinically important difference (MCID)[380]), and individual variation was not considered; the data reported was not the change from baseline (Δ) and the baseline SCS of each responder group was different (e.g., the Nonresponders had the lowest baseline value and the Responders the highest).

The present work explored the response to carotenoid intervention by classifying Responders according to a distribution-based MCID [380]. Responders were classified according to whether they were above half of the SD of the mean change in SCS from week 0 to week 5 (Nonresponders were below this cutoff). Moreover, the data was presented as the mean change from baseline (Δ) to reduce any bias from the baseline SCS level. It is also worth noting that, Responder and Baseline SCS classifications were not associated with each other ($\chi^2 = 0.728$, $p = 0.4$) and Responders and Nonresponders shared a similar mean baseline SCS (17.1 ± 0.32 vs. 17.4 ± 0.41, respectively). The results demonstrate a clear relationship where Nonresponders did not change SCS from baseline throughout the study duration, whereas Responders demonstrated a significant increase at week 5 compared to week 0 which was maintained at week 10. This supports the concept that, while some individuals can improve their SCS, others may not be able to or have a limited capacity (as the slope began to increase, although not significantly different, from week 5 to week 10) to improve their SCS. Future studies need to be designed specifically (i.e., with appropriate power) to explore what drives these differences in Responders and Nonresponders, such as genetic polymorphisms in digestive or metabolic enzymes, lifestyle factors, or gut microbiota composition [13,27,213,217].

5.5 SCS and BMI

Previous carotenoid supplementation studies have reported an influence of BMI on carotenoid status, with observational studies showing inverse correlations between BMI and plasma or skin carotenoids [91,216,217,413–415]. In an RCT involving 56 breast cancer survivors who consumed a high FV diet for three years, changes in blood carotenoids were inversely correlated with BMI [412]. BMI was also identified as a significant covariate for the change in plasma

carotenoid concentrations in a 6-week carotenoid supplementation RCT [183]. While BMI was not a significant covariate in our study, we found a borderline significant increase in SCS for individuals with Overweight but not with Normal BMI. The difference between our study and the overall literature may be attributed to the small sample size for the subgroup analysis (n = 8 per BMI group) and the small difference in change in SCS (ΔSCS for Normal BMI = 1.4, ΔSCS for Overweight BMI = 1.5). Moreover, differences in FV intake were not always accounted for in previous studies, which is a considerable confounder as FV intake is also inversely associated with BMI [416,417]. Also, BMI does not exactly account for adiposity [418], whereby adipose tissue sequestration of carotenoids and increased oxidative stress and inflammation are the principal proposed mechanisms underlying the association between BMI and carotenoids [217].

5.6 Carotenoids and antioxidant capacity, immune status, and skin health

The present work showed no clear changes in antioxidant capacity or immune status after carotenoid intervention beyond a small decrease in TAC (ABTS) in Nonresponders. Previous carotenoid intervention trials in healthy humans have generally shown no to small positive changes in measures of oxidative stress, including antioxidant capacity and immune status [101]. Elmadfa et al. (2004) showed a small decrease in TAC after 5-weeks of supplementation with different doses of β-carotene (5, 10, 20, and 40 mg/d) in 42 healthy men (mean age: 23; mean BMI: 22) [111]. Conversely, Kim et al. (2011) showed increases in TAC after 3-weeks of supplementation with different doses of astaxanthin (5, 20, or 40 mg/d) in 39 smokers (mean age: 24; 97% men; mean BMI: 24) [419]. Meta-analyses involving 12 and 7 carotenoid supplementation RCTs demonstrated reductions in IL-6 and TNF-α, respectively, but both

exhibited high levels of heterogeneity ($I^2 \geq 82\%$) and the relationship was no longer detected when stratified by health status in healthy individuals [117].

Although the protection of macromolecules (e.g., protein, lipids, DNA) related to antioxidants is generally considered a beneficial physiological effect, the EFSA does not acknowledge many frequently measured markers of oxidative stress on their own, such as antioxidant capacity (e.g., FRAP, TAC), because changes in the overall antioxidant capacity of plasma have not been robustly established as a beneficial physiological effect in humans [420]. This is attributed to the high interindividual variability observed with antioxidant capacity measurements likely resulting from various factors (e.g., environmental, behavioral) that are integrated within the body for redox balance [421]. It has been suggested that the effects of dietary antioxidant supplementation (e.g., 600 g of FVs or multivitamins) may only be observed among poorly nourished individuals (e.g., individuals with low baseline nutrient status) that have existing oxidative distress (e.g., high levels of oxidized DNA damage and low repair activity) [422]. Thus, the participants in our study may have maintained an adequate redox balance such that added carotenoids did not significantly improve antioxidant capacity or immune status. This corroborates our findings showing a decrease in TAC only in individuals that did not respond to carotenoid intervention, suggesting that these participants had insufficient antioxidants (e.g., carotenoids) to maintain redox balance compared to those that responded to carotenoid supplementation. Moreover, given the concentration of the lipophilic carotenoids in the lipid membrane, markers of lipid peroxidation, such as F2 isoprostanes or oxidized LDL, are likely more sensitive and suitable to detect potential beneficial effects on oxidative stress related to carotenoid intervention [101,420,423]. Many methods of antioxidant capacity that are widely used, primarily for their ease of use, typically rely on aqueous or alcoholic extracts of plasma that fail

to completely account for lipophilic antioxidants and are not considered to be physiologically relevant [101].

Although existing studies have reported improvements in skin health measures (e.g., hydration, elasticity, UV protection), after carotenoid supplementation [8,126–129], no significant changes in skin health measures were found in this work beyond a small decrease in pliability in the placebo group only. The decrease in pliability aligns with the decrease in TAC, also only observed in the placebo group (specifically the carotenoid intervention Nonresponders), but the difference is also very small (-0.05 mm skin stretch) and fits within natural variations that may occur according to seasonal or temperature changes (0.03 to 0.08 mm) [424–426]. Differences in study design are not likely to account for the differences in results as intervention duration (8-12 weeks,) dose (6-24 mg), and supplement composition (lutein/zeaxanthin; β-carotene; β-carotene, lutein, lycopene; astaxanthin) from previous studies were not largely different from our design [126–128].

One considerable limitation of our study was the lack of control over the ambient temperature and humidity. Although the skin probes used can work in a range of 5-40℃ and 30-70% relative humidity (RH), the skin probe manufacturer recommends optimal room conditions of 20℃ and 40-60% RH for consistency across repeated measurements (Courage + Khazaka electronic GmbH, Germany). The mean ambient temperature and RH over all the measurements conducted in our study were 22.9°C (range: 15-26°C) and 38.5% (range: 13-65%), respectively, suggesting that the skin health measurements were not consistent. Thus, even if there were changes that may be attributed to the intervention, the high variability in ambient temperature and humidity conditions in our study likely reduced our ability to detect them.

5.7 The potential prebiotic effect of carotenoids

Carotenoids have been suggested to exhibit prebiotic effects [14,27,299,427]. A few in vitro fermentation studies of various common carotenoids (e.g., β-carotene, lycopene, lutein) involving fecal slurries of healthy participants have reported increased relative abundance of *Lachnospiraceae*, *Roseburia*, and *Parasutterella* and decreased *Dialister*, *Collinsella*, and *Enterobacter* [21,23]. Although the in vitro studies reported relatively consistent results, the few available observational studies identified different key taxa or changes that contradict the in vitro work. Schmidt et al. (2021) reported associations of dietary and plasma carotenoids with higher α-diversity and varying changes in *Akkermansia*, *Prevotella*, *Phascolarctobacterium*, and *Ruminococaceae UCG-002* associated with specific carotenoids [28]. Djuric et al. (2018) reported associations between total plasma carotenoids and decreased Firmicutes abundance, mostly from the *Lachnospiraceae* family and *Blautia* and *Roseburia* genera [26].

Two existing carotenoid intervention studies have evaluated changes in gut microbiota composition. Djuric et al. (2018) reported no changes in colonic bacteria (measured from colonic mucosal biopsies) after doubling carotenoid intake (11 mg/d at baseline to 22 mg/d at 6 months) from increased FV intake over 6 months [26]. Wiese et al. (2019) reported dose-related changes in gut microbiota profile after 4 weeks of co-supplementation with dark chocolate/fatty acids, with notable increases in *Bifidobacterium adolescentis* and *longum* [29]. However, this study used various formulations of a single-carotenoid lycopene supplement involving different doses of lycopene in combination with different fatty acids or dark chocolate, had a small sample size and no control for lycopene alone, and the lower dose lycopene (7 mg) + dark chocolate combination showed the greatest increase in blood lycopene concentrations. This makes it difficult to

determine which component (or combination) elicited the subtle changes in gut microbiome composition reported, especially when changes in gut microbiota composition have been shown after the intake of dark chocolate and associated with different fatty acids [303,304]. Moreover, they used operational taxonomic units (OTUs) to identify microbial sequences, evaluated differences over a rather short period (4 weeks) using traditional statistical methods (FDR corrected ANOVA), and did not control for or monitor dietary intake. ASVs provide higher resolution and accuracy for the identification of microbial sequences by detecting single-nucleotide differences, compared to OTUs which cluster sequences based on a similarity threshold (generally 97-99%) that can bias results and reduce reproducibility across studies [428–431]. Finally, it is important to note that none of the previously reported changes in specific taxa from the literature, whether associations between carotenoid status or human and in vitro carotenoid intervention, overlapped between studies (some were even conflicting: decrease [26] and increase [21] in *Roseburia*).

In our study, the comprehensive ML algorithm could not distinguish a difference in overall gut microbiota composition, diversity measures did not substantially change, and the MaAslin 2 analysis did not identify differences in any individual taxa after the mixed carotenoid intervention. As mentioned previously in the discussion on the association between baseline gut microbiota composition and SCS (Section 5.1), previous studies have not properly controlled for other dietary components that can have their own prebiotic effects and used methods of microbiome analysis that are prone to false positives (e.g., differential abundance analysis such as LEfSe) or do not account for the complexity of the interactions between gut microbiota [28,252,257–259,263,266,267,270,432]. These factors, along with variability from other technical and biological factors (e.g., sample and data processing, lifestyle) may account for the differences observed. Future studies need to use more sophisticated data analysis (e.g., ML models) in

concert with a functional evaluation of the gut microbiome (e.g., proteomics, metabolomics) for a more robust evaluation of the potential prebiotic effect of carotenoids [433–436].

5.8 The *Bacillus* genus as a select probiotic

The spore-forming *Bacillus* genus has emerged as a potent probiotic as it can survive extreme conditions, germinate and reside within the host, provide various benefits (e.g., gut barrier integrity, biofilm production), and is present in various fermented foods [437,438]. The *B. subtilis* species can be found in soil and fermented soy-based foods and is considered a normal human gut commensal [353]. Natto is a traditional Japanese food made from soybeans fermented with *B. subtilis* that has demonstrated various probiotic properties, such as regulation of gut microbiota and improved gut health [439,440]. Although natto intake was not reported by participants in our study, the potential intake of fermented soybeans could potentially explain why one participant showed low levels of the probiotic strain before the intervention began. Natto consumption has also been significantly correlated with blood concentrations of total carotenoids, lutein, and β-carotene in a cross-sectional study of 805 healthy Japanese individuals [91]. Although gut microbiome composition and *B. subtilis* were not measured in the above study, it suggestively supports the role of *B. subtilis* in carotenoid status enhancement, although mechanisms remain to be investigated.

The gastrointestinal viability and dose-response tolerance of *B. subtilis* R0179 has been shown over 4 weeks of supplementation at doses of 0.1, 1.0, and 10 $\times 10^9$ CFUs/capsule/day [355]. There were no clear changes in gut microbial composition despite recovery of the probiotic after treatment, suggesting longer durations of intervention may be needed; a low recovery of the probiotic following a one-week washout indicated that the bacteria do not persist in the GIT.

Probiotic supplementation generally does not demonstrate large changes in gut microbiome composition but rather small changes in specific genera, and changes are often transient, returning to pre-treatment levels within 1 to 3 weeks once supplementation stops [441,442]. We demonstrated that 10 weeks of supplementation with 10 billion CFUs of *B. subtilis* R0179 was sufficient to increase *B. subtilis* R0179 levels in all participants who took the probiotic (none were detected in the placebo group) without any serious adverse effects. These increases were also associated with changes in gut microbiome composition and the enhancement of SCS (covered in the following sections), suggesting effective dose and duration of intervention.

5.9 Probiotics enhance SCS increases from carotenoid supplementation

This is the first study to explore and demonstrate the potential of probiotic supplementation to enhance phytochemical status. We showed an effect of treatment (Placebo vs. Probiotic; $p = 0.044$) in the fixed effect omnibus test that demonstrated a strong trend for significantly higher SCS in the Probiotic group compared to placebo at week 10 in post hoc analysis. Although this trend became weaker after adjusting for baseline SCS levels, the relatively limited power (69%) to detect between-group differences (likely due to the reduced participant enrollment compared to our sample size calculation; 37 vs. 50, respectively) suggests that the difference may be significant with an appropriate sample size. Moreover, when within-group (i.e., over time) differences are compared (Fig. 4.1), the Probiotic group continued to increase SCS (week 5 was significantly higher than week 10) while the Placebo group reached a plateau (week 5 was not significantly different from week 10). This suggests that the probiotic supplementation enhanced SCS increases from the mixed carotenoid supplementation.

In subgroup analyses, we showed that the probiotic enhancement of SCS was only observed in participants with Normal BMI, High baseline SCS, and Nonresponders. Within these subgroups, SCS showed statistically significant increases in the Probiotic group over time, while the Placebo group did not show significant changes. This suggests that the SCS-enhancing effect of probiotic supplementation may only be effective in individuals with Normal BMI and may enhance responsiveness to carotenoid intervention in individuals with High baseline SCS and Nonresponders. In the other subgroups (Overweight BMI, Low baseline SCS, and Responders), changes in SCS over time in the Probiotic group were similar to those in the Placebo group. Previously, a higher BMI was associated with decreased carotenoid status, presumably because of increased oxidative stress, inflammation, or adipose tissue sequestration of carotenoids [216,217,443], as well as an altered gut microbiome [444] and a decreased response to probiotic intervention [445].

The difference in SCS enhancement response to *B. subtilis* intervention according to BMI may also be related to secondary bile acid production. Although we did not analyze secondary bile acids in our study, plasma secondary bile acids increased in participants with obesity (BMI ≥ 30) and nonsignificantly decreased in participants with normal or overweight BMI in a 6-week crossover RCT involving *B. subtilis* intervention [446]. The authors speculated that participants with obesity may have begun the study with more microbes that could metabolize bile acids when combined with *B. subtilis* but emphasized the reduced power for the subgroup analysis and preliminary nature of the conclusion. Moreover, bile acids (both primary and secondary) have been shown to alter gut barrier integrity in different ways making it difficult to interpret these subgroup differences [294]. In preclinical studies, primary bile acid chenodeoxycholic acid (CDCA) and secondary bile acid deoxycholic acid (DCA) increased intestinal permeability and

inflammation [294]. In contrast, secondary bile acids lithocholic acid and ursodeoxycholic acid did not have direct effects on intestinal permeability or inflammation and reduced CDCA and DCA effects [294]. Furthermore, since certain secondary bile acids like DCA increase intestinal permeability [447,448], it may be speculated that this could facilitate the passive diffusion of carotenoids for enhanced absorption. However, this speculation contradicts the association between secondary bile acids and BMI reported previously, indicating further research is necessary to understand the potential role of secondary bile acids, and their association with BMI, on carotenoid absorption.

According to previous work, one probable explanation for the enhancement of carotenoid status from probiotic supplementation is probiotic production of carotenoids. A few *Bacillus* species have been isolated from human feces and shown to produce carotenoids that are readily absorbed [37,38]. Carotenoids produced by *Bacillus* species were reported to be more bioaccessible, after in vitro digestion, and bioavailable, in rat tissue after a 3-day multiple-dose gavage intervention, compared to β-carotene [38]. Carotenoids produced by a *Bacillus indicus* PD01 accumulated in blood following 3 and 6 weeks of supplementation (5×10^9 CFU's), compared to placebo, in 67 humans with overweight and obesity [37]. These results contrast with our subgroup analysis showing that individuals with overweight BMI did not increase SCS with probiotic supplementation, suggesting that the *B. subtilis* probiotic from our study may not produce carotenoids.

Genome analysis of *B. subtilis* R0179 shows that it has the metabolic machinery (enzymes in the methylerythritol pathway) to produce isoprenoids that are precursors to carotenoid synthesis (Kyoto encyclopedia of genes and genomes (KEGG) pathway map).

Although the capacity of *B. subtilis* R0179 stops at the production of farnesyl pyrophosphate (FPP; C15), genome analysis of some bacteria commonly found in the human gut microbiome shows the genetic capacity to produce carotenoids from FPP. *Lactobacillus plantarum* strains have dehydrosqualene synthase (*crtM*) and dehydrosqualane desaturase (*crtN*) genes, which are major genes required for the biosynthesis of apocarotenoids (e.g., 4,4'-diaponeurosporene) from FPP [449]. Although this carotenoid biosynthesis pathway may not be their primary function in the gut, and this strain was not identified in our analyses, it is feasible that the elevated presence of carotenoid precursors could result in carotenoid biosynthesis by other gut microbiota with the appropriate metabolic capacity. However, this is far from clear and requires functional analysis with the integration of multi-omics (e.g., full gene analysis, proteomics, and metabolomics) to explore this potential mechanism by which probiotics may enhance carotenoid status [435,436,450].

Another potential explanation for the SCS-enhancing effect of the probiotic supplementation is enhanced metabolic capacity. The human gut microbiome is known to metabolize macromolecules (e.g., fiber, proteins) that can sequester lipid-soluble compounds, such as carotenoids [25,276,307]. *B. subtilis* is known to produce several enzymes (e.g., proteases, amylases) that facilitate the breakdown of macromolecules [451–453]. This enzymatic activity increases the accessibility of nutrients for the growth of the bacterial community [454]. It has been suggested to serve a "public good", similar to the antimicrobial metabolites that inhibit pathogens [455] and structural components of the biofilm extracellular matrix [456] that *B. subtilis* also produces [454]. *Bacillus* probiotics have also demonstrated the potential to improve macronutrient metabolism and absorption in preclinical and human trials [319,438]. Thus, it is reasonable to speculate that the *B. subtilis* probiotic supplementation may enhance the bioaccessibility of carotenoids by improving the metabolic capacity of the host to breakdown

macromolecules that may sequester carotenoids. Furthermore, although we did not see large changes in gut microbiota composition or plasma SCFAs, changes in function have been observed without changes in composition (even when considering diet and probiotics) [17,265,316,317,457], and there is low concordance between plasma and stool SCFAs suggesting they may reflect different physiological processes (e.g., gut-localized vs systemic) [458,459]. Again, future studies using multi-omics are necessary to understand potential underlying mechanisms for the SCS-enhancing effect of *B. subtilis* [435,436,450].

Certain microbes can also produce biosurfactants that may serve to emulsify lipophilic substances and enhance their bioaccessibility and bioavailability[296]. *Bacillus subtilis* produces biosurfactants (e.g., surfactin) that can form and stabilize oil in water emulsions in various conditions [295], similar to how bile acids facilitate micelle formation during upper intestinal digestion and absorption. Furthermore, surfactin from *B. subtilis* has been shown to enhance gut-intestinal barrier function and immunity in vivo, demonstrated by elevated expression of intestinal mucosal (e.g., IgA, Mucin 1 and 2) and tight junction proteins (e.g., claudin-1, occludin) and increased mucin secretions and intestinal villi height in mice, which may support enhanced nutrient absorption [460]. Given that the gut microbiome is known to metabolize macromolecules and may produce carotenoids, carotenoid metabolites, biosurfactants and secondary bile acids, and carotenoid transporters are known to be expressed in the colon, it is conceivable that initially unabsorbed carotenoids may be freed and carotenoids or their metabolites may be produced, then emulsified, and finally absorbed within the colon.

The SCS enhancement seen with the *B. subtilis* R0179 treatment may be related to the generation of BEVs that facilitate carotenoid absorption. *Bacillus subtilis* 168 was shown to release BEVs that pass through Caco-2 cells in a transwell human intestinal cell model [461].

Lipid-based nanoparticles (e.g. liposomes or micelles – similar to BEVs) have previously been used as cancer drug delivery systems to improve the bioavailability of hydrophobic molecules [462]. Administration of a nano-emulsion loaded lycopene-enriched tomato extract improved lycopene bioaccessibility in vitro [463]. Following a short incubation period with curcumin, isolated EVs have been successfully loaded with the hydrophobic phytochemical and have demonstrated improvements in solubility, stability, and bioactivity *in vivo*, as well as bioavailability *in vitro* [464]. Hence, a potential avenue for improved carotenoid bioavailability could be conferred via BEVs generated by specific gut microbiota that can package dietary or produced carotenoids or their metabolites and facilitate carotenoid emulsion and uptake.

Without a placebo group for the probiotic supplement, the only differences between groups in changes over time for the health markers observed (decrease in TAC and skin pliability in the placebo group over time) cannot be attributed to the probiotic supplement. The maintenance of TAC and skin pliability within the probiotic group is more likely associated with the SCS-enhancing effect of the probiotic. This is supported by the subgroup analyses that show the decrease in TAC was only observed in Nonresponders and a strong trend for decreased skin pliability in the placebo group compared to the probiotic group in participants with High baseline SCS. This suggests that the probiotic may contribute to better maintenance of antioxidant capacity and skin pliability by enhancing SCS. The minimal effects observed on health markers in our study may be primarily attributed to the healthy status of the individuals. The few previous studies that have demonstrated improvements in health markers with combined probiotic and phytochemical supplementation either involved individuals with impaired health (e.g., osteopenia, type II diabetes) over longer durations (3 and 12 months) and with higher sample

sizes (n = 78 and 365) [334,335] or measured the response of the skin to a stressor (UV radiation) [333] (Table 2.5).

5.10 Probiotic supplementation is associated with changes in gut microbiota composition

In this work, qPCR, ML, and MaAsLin 2 analysis all confirmed the presence of the supplemented probiotic, *B. subtilis*, in stool samples collected from participants who took the probiotic. However, the relative abundance of few other taxa changed in association with probiotic supplementation when compared to placebo. In the ML analysis, the probiotic was associated with a lower relative abundance of *Oscillospiraceae UCG-003* and *Anaerovoracaceae Family XIII AD3011 group* and higher relative abundance of Coriobacteriales *incertae sedis, uncultured*. *Anaerovoracaceae Family XIII AD3011 group* and other uncultured taxa from the *Oscillospiraceae* family were also inversely associated with high baseline SCS, suggesting internal consistency within the analyses regarding associations with SCS, but as mentioned previously, these taxa and their potential role in this context are not well characterized. *Coriobacteriales incertae sedis* was also associated with Responders in the ML analysis, but beyond an association with blueberry intake in a mouse study and having been suggested to metabolize phytochemicals [465,466], nothing is known about these taxa regarding their role in carotenoid metabolism. Future research that explores the functional characteristics of these taxa is necessary to further understand the interplay between probiotic supplementation, gut microbiota, and SCS.

5.11 Hormones, probiotics, and SCS

Hormone (estradiol, progesterone, testosterone, and cortisol) levels can differ according to various factors including menstrual cycle, body composition, hormonal contraceptives, and age [467–469]. Hormone fluctuations related to the menstrual cycle have also been associated with differences in skin health markers (e.g., hydration, TEWL, elasticity), including hydration being positively associated with estrogen levels and negatively with menstrual cycle irregularity [470–472]. A few studies have also demonstrated associations between fluctuations in menstrual cycle hormones and circulating carotenoids, but results were not adjusted for carotenoid intake and some observed changes were not significant after adjustment for cholesterol [180,473,474]. We did not observe any differences in most plasma hormone levels over time and did not observe any differences between groups.

The small increase in cortisol levels over time observed in the probiotic group may be a product of natural variation according to the circadian rhythm or menstrual cycle. Cortisol levels are known to follow the circadian rhythm, with secretion peaking about 30 minutes after awakening (up to 25 µg/dL) and progressively decreasing by night (down to 2 µg/dL) [384]. A meta-analysis of 35 human studies showed that women in the follicular phase have slightly higher cortisol levels (up to 0.5 µg/dL more) than those in the luteal phase [383]. Although all study visits were conducted in the morning and early afternoon, visits were scheduled according to availability which could have biased the result. The magnitude of change observed in our study (2.73 µg/dL) is quite small compared to the natural variation of cortisol levels throughout the day (> 20 µg/dL). Furthermore, probiotic interventions have not been shown to affect cortisol levels [475,476]. Future studies should adequately monitor and adjust for hormones to reduce any

influence of menstrual cycle variation on other outcomes such as carotenoid status and skin health.

5.12 Skin vs. blood carotenoid status

In this study, we observed an initial increase in total plasma carotenoids (week 0 vs. week 5) that reached a plateau (week 5 not different from week 10) in both treatment groups (Fig. 4.3). It is possible that steady-state conditions were reached earlier, as a previous trial that monitored the plasma kinetics of carotenoid supplementation reported a steady-state of plasma concentrations at 2-3 weeks [408]. Although SCS and blood carotenoid concentrations are strongly correlated and both are associated with a reduced risk of noncommunicable disease, SCS has been suggested as a better long-term biomarker for carotenoid status [61,477]. Skin is a storage medium that is less sensitive to acute changes in carotenoid intake, whereas blood is a transport medium that is more sensitive [182] and reaches a steady state more quickly [183]. This may explain why we only observed an enhancement in SCS (i.e., an increase beyond what was observed in the placebo group) and not blood carotenoids after probiotic supplementation.

Case studies and interventions involving excess intake of carotene-rich foods (e.g., carrots, sweet potato) and large doses of supplemental β-carotene (15-300 mg/d, in patients with erythropoietic protoporphyria characterized by acute photosensitivity of the skin), respectively, have been shown to induce carotenaemia (noticeable yellow-orange skin discoloration) [133,478–480]. Carotenaemia is presumably caused by the excess storage of carotenoids in the skin, but SCS has not been quantitatively evaluated in these studies, blood concentrations were only sometimes reported, and doses were often different for each participant, making it difficult to conclude differences between skin and blood carotenoid kinetics. Von Laar et al. (1995) showed that β-

carotene serum levels increased from day 0 to 30, but did not increase further up to day 150, of daily β-carotene supplementation (50-150 mg/d depending on the participant) in 14 patients with erythropoietic protoporphyria; carotenaemia or skin color were not mentioned [481]. Future studies that compare the different kinetics of blood and skin carotenoids after interventions with different doses are needed to further understand differences and potential limits in the transport and storage of carotenoids.

5.13 Skin regions included in SCS assessment

When using SCS assessment methods, the region of skin measured is an important consideration [10]. The reporting of the Δ in b* value as an average of 3 to 9 areas (forehead, cheeks, palm, etc.) in previous studies has been shown to influence results (Table 2.1). The skin of the palm, forehead, inner arm (bicep), and sole were more strongly correlated with FV intake, compared to the skin of the cheek, shoulder, outer arm, and back of the hand, in 30 Caucasian men [482]. This is presumably because the latter skin regions are more exposed to UVR (i.e., more carotenoid oxidation) and have a lower density of sweat glands (i.e., less carotenoid deposition) [129,187,188]. In previous studies that measured several skin regions and reported individual b* values, the palm consistently showed improvements with carotenoid supplementation, while the others did not [11,199,201]. Coetzee & Perrett (2014) showed that the palm was the only region that increased in SCS after eight weeks of β-carotene supplementation (15 mg/d) in 10 African women (mean age: 28) and after adjustments for multiple comparisons [199]. This is likely because of the higher density of the interfering chromophore of melanin in the other skin regions assessed (inner arm, forehead, outer arm, and cheeks), and lower melanin interference in the palm regardless of ethnicity [10,189,198].

The importance of the selection of skin regions included in the SCS assessment is clear when evaluating the correlation with blood carotenoid concentrations and supports the need for context when describing SCS methods. The only previous study to compare colorimetry-based SCS with blood carotenoid concentrations found a weak correlation between the two measures (Table 2.2) [200]. It included nine different skin regions in the colorimetry measurement for SCS and there was no adjustment for melanin concentrations of different skin regions that can interfere with the SCS estimation [198,205,206]. In our study, baseline total plasma carotenoids showed a strong trend for a moderate positive correlation with baseline SCS, a significant moderate positive correlation with baseline palm SCS, and no correlation with baseline forehead SCS. Furthermore, when the full dataset was included, total plasma carotenoids were strongly correlated with SCS and palm SCS, and moderately with forehead SCS. Our results highlight the importance of selecting skin regions as an indicator of carotenoid status, demonstrating the greater sensitivity of the palm region compared to the forehead region.

We chose to include measurements of the palm and forehead for total SCS as these regions have a high density of sweat glands and have demonstrated higher carotenoid levels [129,187,188]. Although the forehead also has a higher melanin density than the palm, we controlled for this by limiting eligible participants to lighter skin types (Fitzpatrick I, II, and III) characterized by lower melanin density [483,484]. We also wanted to consider some of the external stressors, such as UVR (e.g., sun exposure) that is associated with the skin health outcomes we also measured, in the assessment of SCS [83,485]. The skin of the forehead is relatively sensitive (e.g., thin epidermis, frequent sun exposure) and may provide some context of skin health within the SCS measurement, without being overly sensitive like the skin of the cheek [486]. Conversely,

the palm receives limited sun exposure and the skin of the palm is a relatively poor indicator of skin health (e.g., thick epidermis, low sensitivity) [487,488].

Our results align with the literature regarding the reduced sensitivity of forehead SCS as an indicator of carotenoid status [10,11,199,201]. We only observed changes in the palm SCS measures after mixed carotenoid supplementation and forehead SCS was not correlated with total plasma carotenoids while palm SCS was. This suggests that forehead SCS may not be a viable companion to palm SCS for overall SCS assessment. One previous carotenoid supplementation study using colorimetry-based SCS reported increased palm SCS along with a borderline significant increase in forehead SCS [11], but most studies using RS-based SCS assessment have shifted to the palm or thumb as the primary measurement region to avoid the interference of melanin [10]. Future research requires a more rigorous assessment of the various skin regions that may be combined for an optimal overall assessment of SCS that may integrate aspects of skin health.

5.14 Strengths and limitations

The strengths of our study include the use of a double-blind, randomized controlled trial design, which minimizes bias and enhances the reliability of our findings. We also employed sophisticated statistical [259,489] and comprehensive ML methods [261,270] to control for interindividual differences, identify between and within-group differences and determine associations with gut microbiota composition and identify key taxa. The monitoring and control of various lifestyle and host-related factors (e.g., dietary intake, physical activity, quality of life and sleep, sex, health status) and the use of cosmetics and other supplements also contribute to the strength of the study. However, our study also has notable limitations. The absence of a

placebo group for the carotenoid and combination interventions and monitoring of all host-related factors (e.g., genetic variants of carotenoid cleavage enzymes), along with the baseline differences in gut microbial composition between groups, limit the interpretation and generalizability of our results. Our study was adequately powered to detect differences in the primary outcome (SCS) over time, but there was limited statistical power for between-group comparisons and the secondary and subgroup analyses. Although spectrophotometry is considered less sensitive and robust in comparison to other optical methods for SCS assessment [6], we were still able to demonstrate expected changes as well as parallels with other objective measures (HPLC plasma carotenoid concentrations) for tissue carotenoid status. Future studies should include larger and more diverse populations, a placebo control group for all intervention arms, and comprehensive measurement and analysis of as many known food and host-related factors as possible. The European Cooperation in Science and Technology (COST) is driving initiatives, such as the COST POSITIVe (Interindividual variation in response to consumption of plant food bioactives and determinants involved) Action to systematically analyze and identify key determinants of the interindividual variation regarding both bioavailability and bioactivity of the main families of phytochemicals [209]. This work will contribute to this initiative, as the COST POSITIVe group has identified the gut microbiota composition and specific strains and functionalities as essential for a better understanding of the interindividual variation observed regarding phytochemical bioactives, despite its lack of investigation. Lastly, the incorporation of more sensitive and advanced methods for SCS (e.g., RS) and gut microbial composition and metabolite assessment, such as the comprehensive ML algorithms and MaAsLin 2 analyses we used, may provide more detailed insights into the effects and underlying mechanisms of

probiotics on carotenoid intervention, as well as the relationship between the gut microbiome and carotenoids.

Chapter 6: Conclusions

The present work provides compelling evidence that probiotic supplementation can enhance the effects of carotenoid supplementation on SCS, but not plasma carotenoid concentrations. In this context, probiotic supplementation appears to be particularly beneficial for individuals with normal BMI, high baseline SCS, or those who typically do not respond to carotenoid supplementation alone. This is the first demonstration of the potential for probiotics to enhance phytochemical status in a human RCT. This is also the first study to suggest that probiotic supplementation may enhance the response of an individual to phytochemical supplementation, paving the way for potentially novel, relatively low-cost, highly accessible strategies for possible disease management/prevention and health maintenance. The associations between gut microbiota composition and SCS, carotenoid intervention responder status, and probiotic supplementation support the role of the gut microbiome as a host-related factor that can influence carotenoid status. This is the first human RCT to explicitly evaluate these relationships while also adjusting for confounders (e.g., FV intake). However, future studies are needed to fully understand the underlying mechanisms and interplay between the gut microbiome, carotenoid status and health markers. More high-quality human studies that adjust for other host-related factors and confounders and incorporate the use of multi-omics and ML analyses are needed to understand the functional and metabolic capacity of the gut microbiome that may be associated with carotenoid status and associated health effects.

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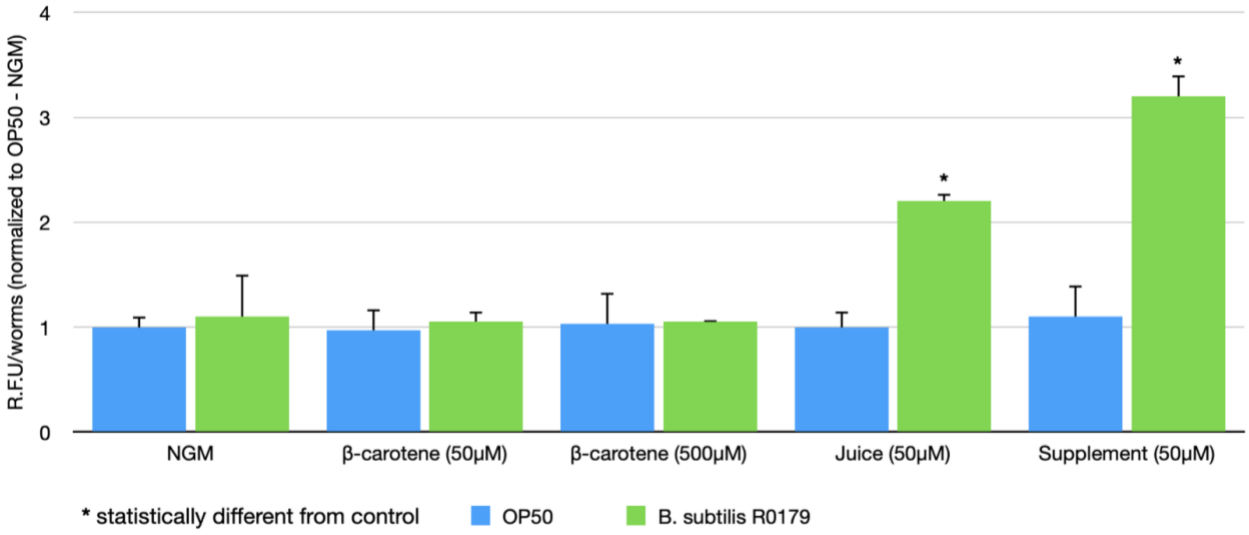
Appendices

Appendix Table 1. Carotenoid concentrations in the mixed-carotenoid supplement.

	Lutein	Zeaxanthin	α-carotene	β-carotene	Lycopene	Astaxanthin	Total carotenoids
Our analysis	3.40 (0.74)	0.20 (0.05)	0.65 (0.32)	10.49 (3.75)	2.62 (1.33)	n.d.	17.4 (5.39)
Supplement label	5.0	0.3	0.29	7.5	3.0	1.0	16.8

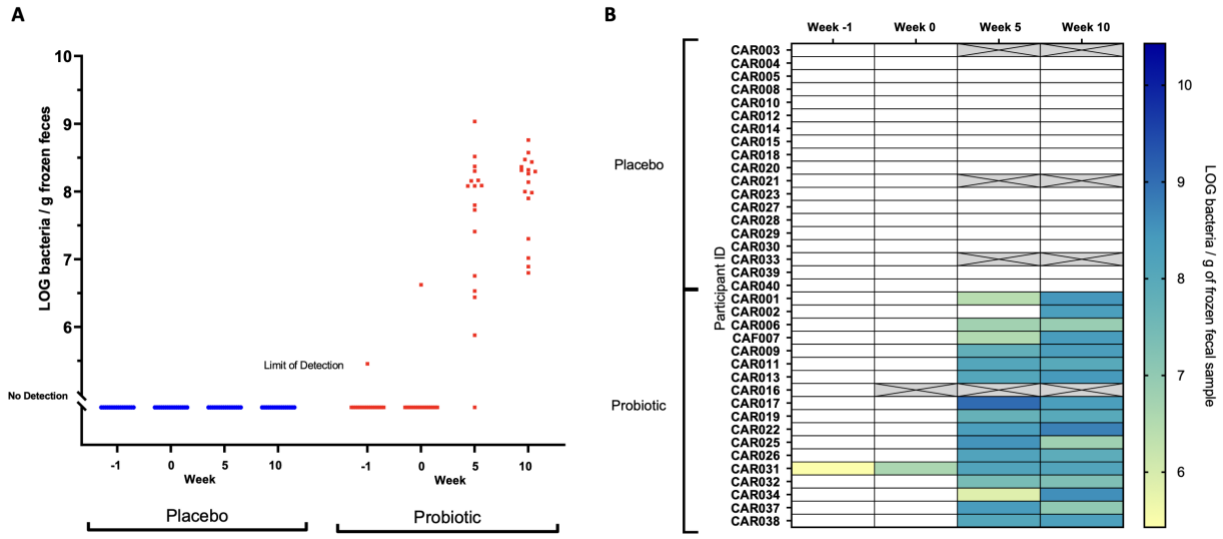
Data are presented as mean ± standard deviation in µg. n.d. = not detected.



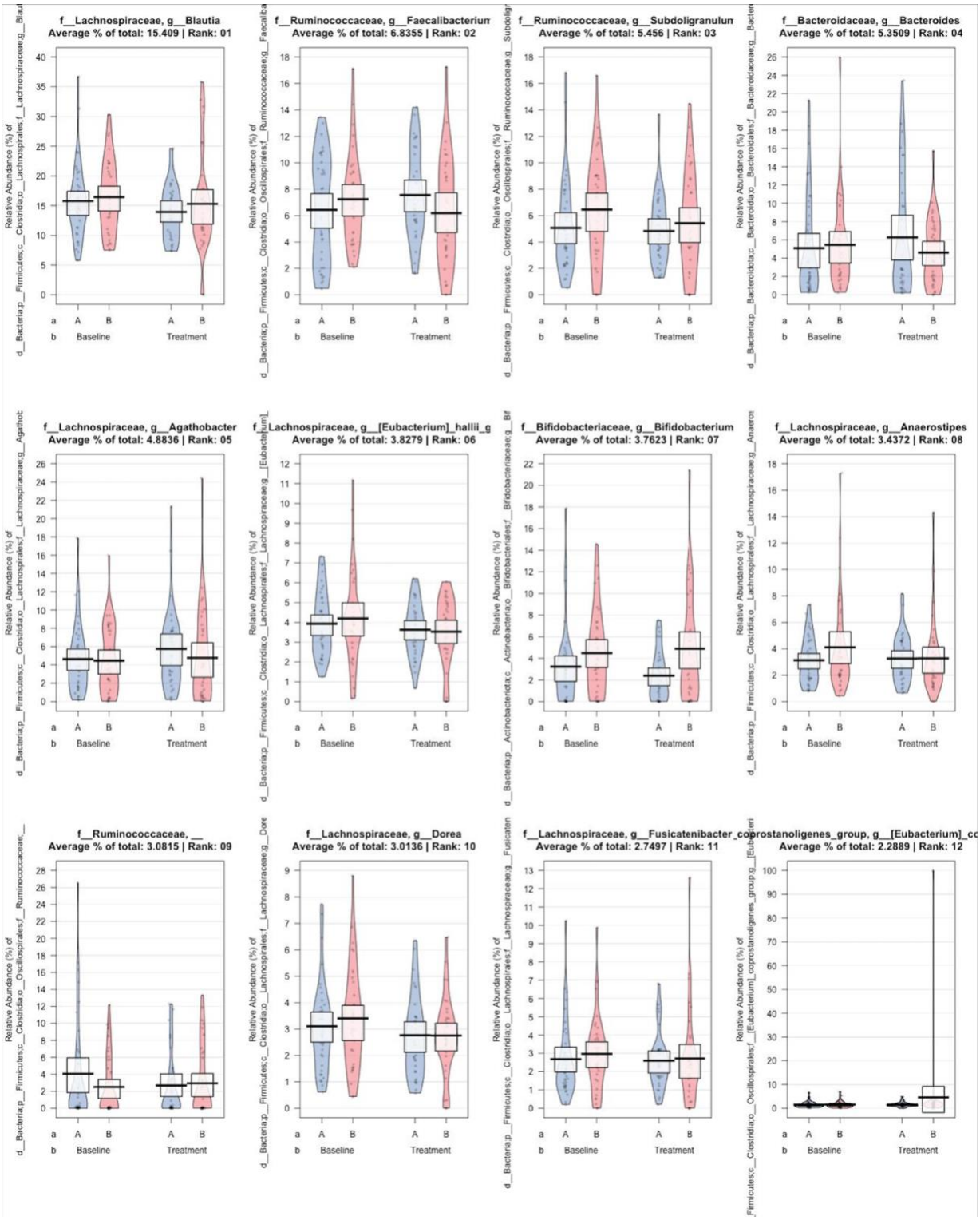
Appendix Figure 1. Probiotic selection screening study results. Carotenoid concentrations were measured in *C. elegans* following 24 h incubation with *B. subtilis* and various combinations of carotenoid supplementation. Relative fluorescence units (RFU) at the absorbance maxima of carotenoids (488 nm) indicate carotenoid concentration. Significantly different ($p < 0.01$) R.F.U./ # of worm compared to control (OP50; bacterial strain conventionally used as food for maintenance of *C. elegans*) with Dunnett's test is represented by an asterisk (*). The nematode *C. elegans* is used as a model system for high-throughput drug screening and molecular pathway elucidation [490]. Carotenoid fluorescence has previously been used to identify carotenoid content in vivo [491] and was adapted to a *C. elegans* model to evaluate the uptake kinetics of carotenoids in the presence of different probiotic strains (*B. subtilis* and other confidential strains) with known safety profiles. *C. elegans* grown with *B. subtilis* R0179 demonstrated the highest relative fluorescence (a three-fold increase after 24-h incubation) at carotenoid absorbance maxima (488 nm) when supplemented with the mixed carotenoid supplement (Country Life Carotenoid Complex) used in this study (Appendix Table 1). The increase in relative fluorescence indicates a higher concentration of carotenoids present in the nematodes. A two-fold increase was also observed with *B. subtilis* and carotenoid-rich carrot juice while the other strains tested only showed slight increases with the mixed carotenoid supplement and the carrot juice.

Appendix Table 2. Summary of reported adverse events.

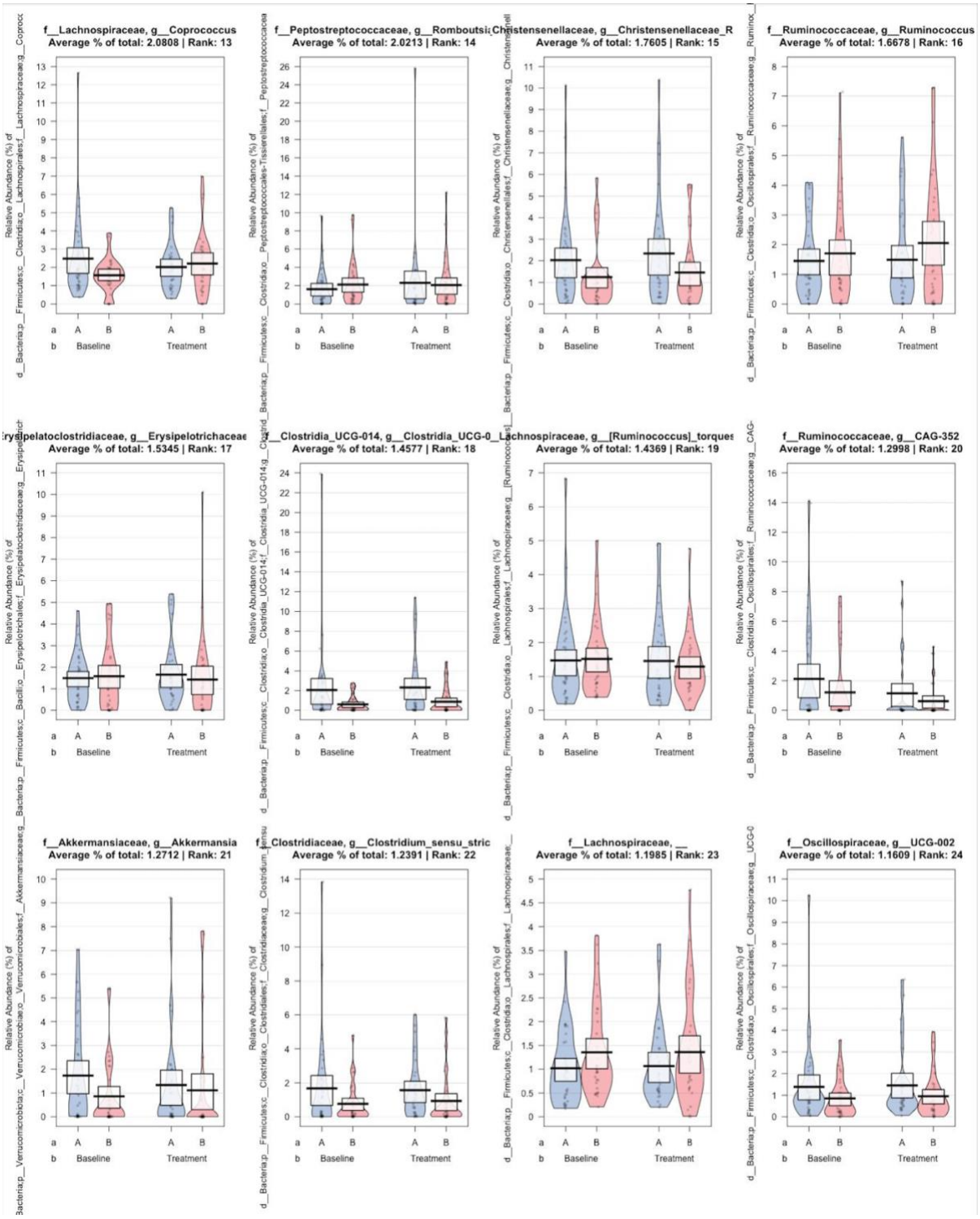
Adverse events	Placebo	Probiotic	Total
Gastrointestinal discomfort	3	0	3
COVID-19	6	3	9
Urinary tract infection	1	1	2
Bacterial infection	1	0	1
Acute respiratory tract infection	0	1	1



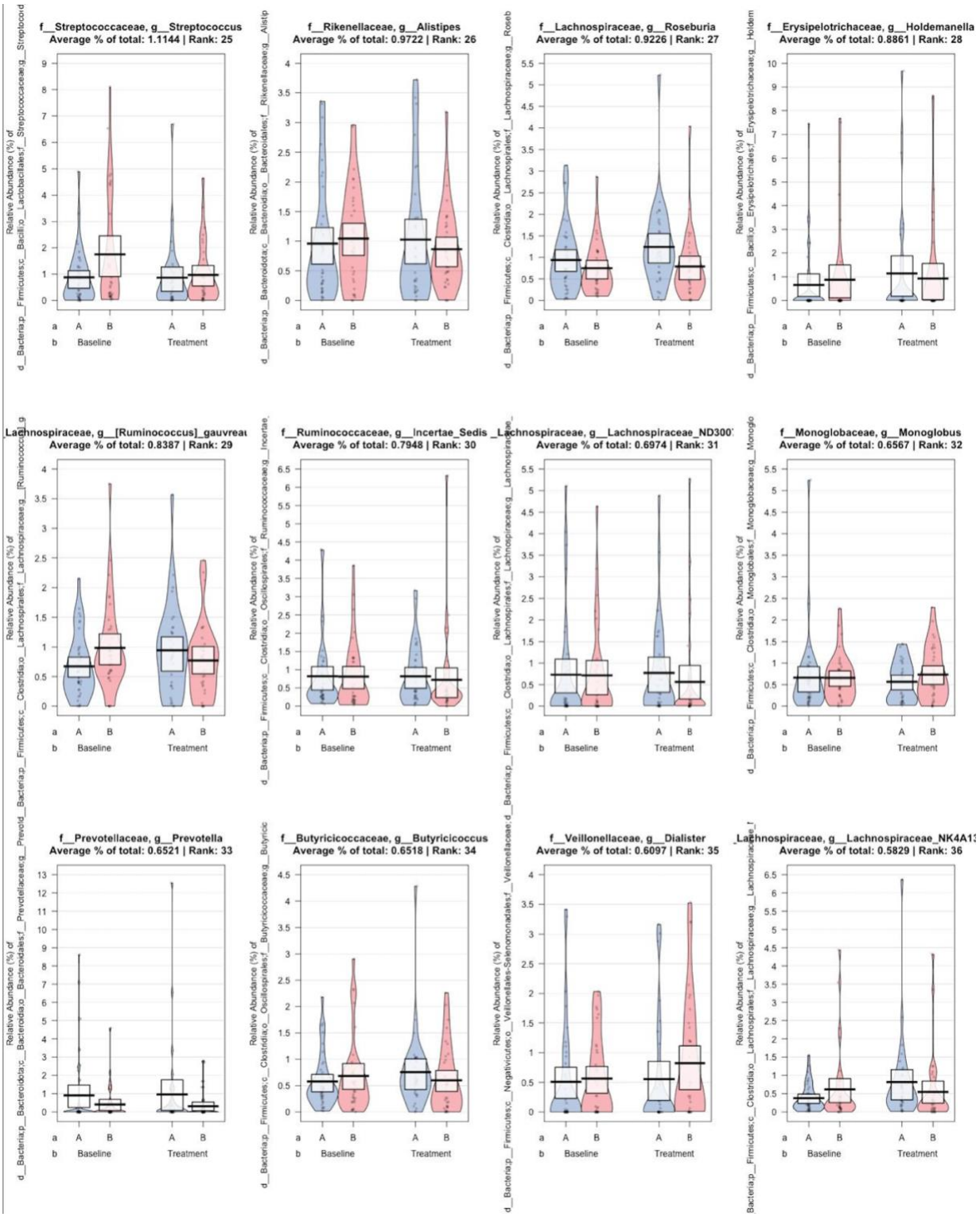
Appendix Figure 2. Absolute quantification of *B. subtilis* R0179 in fecal samples (LOG bacteria/g frozen feces) collected during baseline (weeks -1 and 0) and treatment (weeks 5 and 10). (A) Absolute quantification of *B. subtilis* R0179 in fecal samples for the placebo (n= 16; blue) and probiotic group (n= 17; red) participants. (B) Heat map of the absolute quantification results of *B. subtilis* R0179 in fecal samples for the placebo (n= 19) and probiotic group (n= 18) participants. All participants are included.



Appendix Figure 3. Pirate plots of the 1-12/36 most abundant taxa over time and after treatment. Pirate plots of the mean relative abundance (%) ± 95% CI of 1-12 of the 36 most abundant taxa (by rank) in the placebo (blue; "A") and probiotic (red, "B") groups at baseline (left) and after treatment (right).



Appendix Figure 4. Pirate plots of the 13-24/36 most abundant taxa over time and after treatment. Pirate plots of the mean relative abundance (%) ± 95% CI of 13-24 of the 36 most abundant taxa (by rank) in the placebo (blue; "A") and probiotic (red, "B") groups at baseline (left) and after treatment (right).

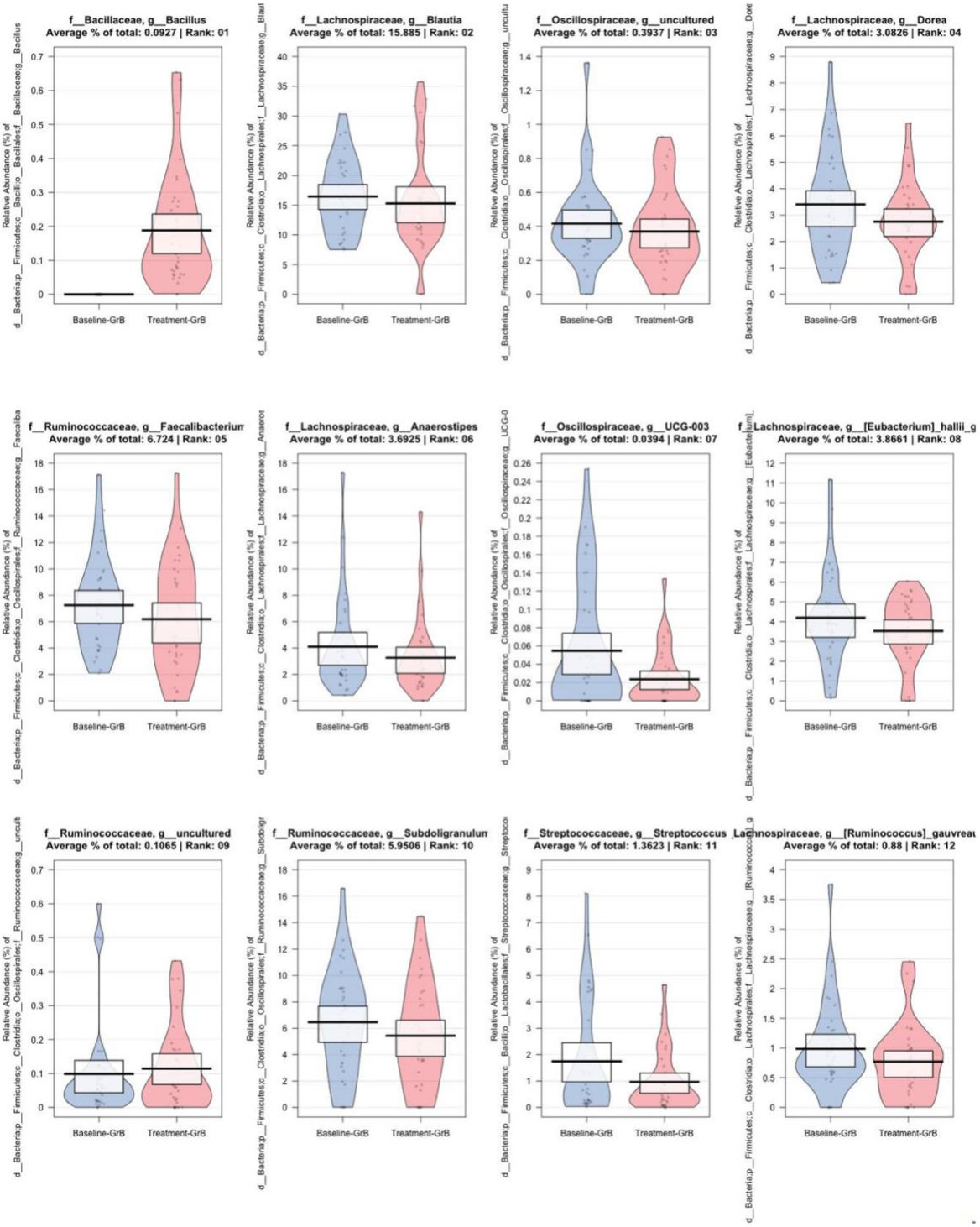


Appendix Figure 5. Pirate plots of the 25-36/36 most abundant taxa over time and after treatment. Pirate plots of the mean relative abundance (%) ± 95% CI of 13-24 of the 36 most abundant taxa (by rank) in the placebo (blue; "A") and probiotic (red, "B") groups at baseline (left) and after treatment (right).

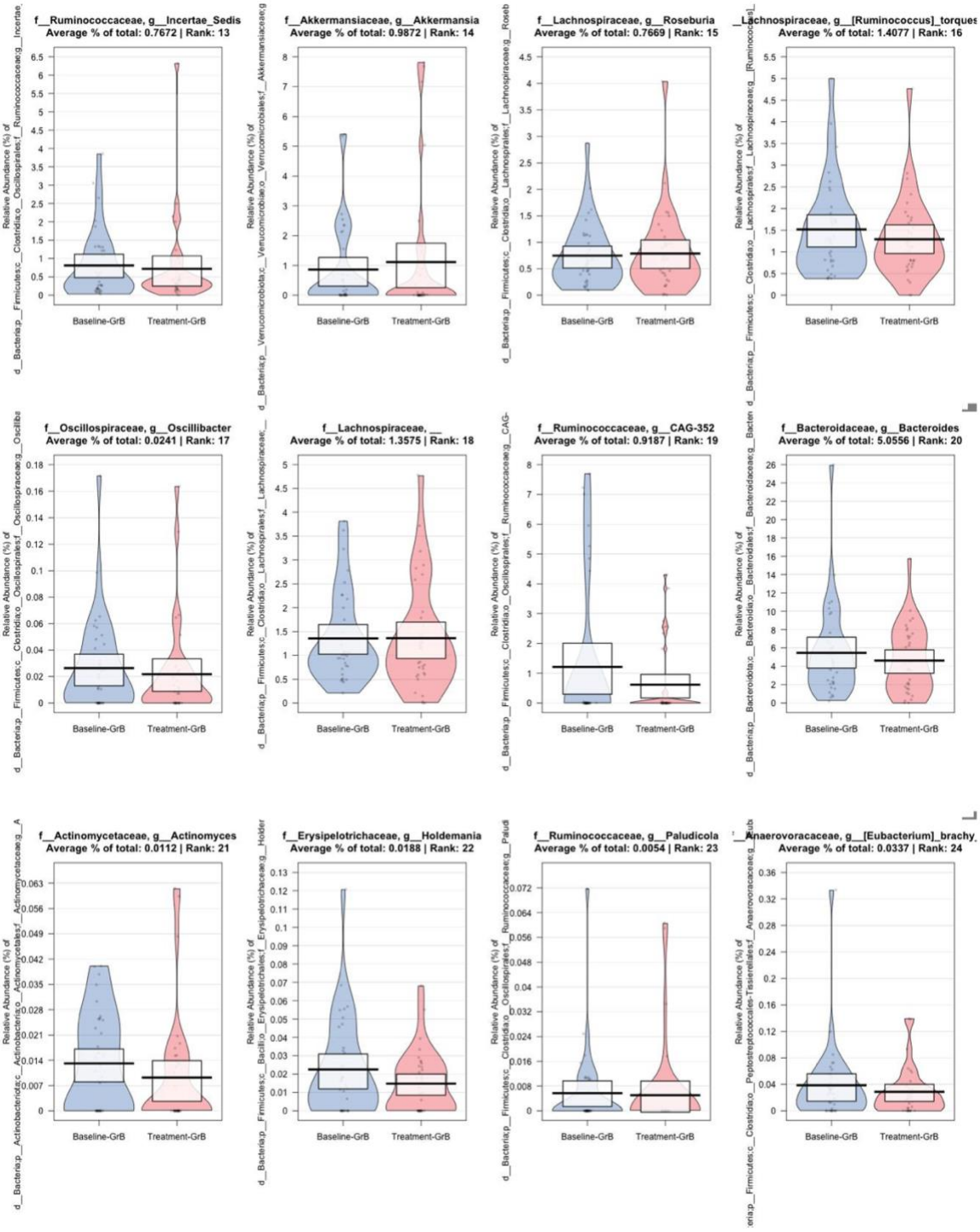
Appendix Table 3. Confusion matrix accuracy scores for the main group analysis.

ClassifierIteration_ID	Accuracy Ratio	Final Accuracy	Baseline Accuracy
Baseline-ProbioticVsPlacebo--0	1.25	0.67	0.53
Baseline-ProbioticVsPlacebo--1	1.50	0.80	0.53
Baseline-ProbioticVsPlacebo--2	1.63	0.87	0.53
Baseline-ProbioticVsPlacebo--3	1.75	0.93	0.53
Baseline-ProbioticVsPlacebo--4	1.75	0.93	0.53
Baseline-ProbioticVsPlacebo--5	1.50	0.80	0.53
Baseline-ProbioticVsPlacebo--6	1.63	0.87	0.53
Baseline-ProbioticVsPlacebo--7	1.25	0.67	0.53
Baseline-ProbioticVsPlacebo--8	1.63	0.87	0.53
Baseline-ProbioticVsPlacebo--9	1.50	0.80	0.53
Median	1.56	0.83	0.53
Treatment-ProbioticVsPlacebo--0	1.86	0.93	0.50
Treatment-ProbioticVsPlacebo--1	2.00	1.00	0.50
Treatment-ProbioticVsPlacebo--2	2.00	1.00	0.50
Treatment-ProbioticVsPlacebo--3	2.00	1.00	0.50
Treatment-ProbioticVsPlacebo--4	2.00	1.00	0.50
Treatment-ProbioticVsPlacebo--5	2.00	1.00	0.50
Treatment-ProbioticVsPlacebo--6	1.86	0.93	0.50
Treatment-ProbioticVsPlacebo--7	1.86	0.93	0.50
Treatment-ProbioticVsPlacebo--8	2.00	1.00	0.50
Treatment-ProbioticVsPlacebo--9	2.00	1.00	0.50
Median	2.00	1.00	0.50
Placebo-BaselineVsTreatment--0	0.63	0.36	0.57
Placebo-BaselineVsTreatment--1	0.50	0.29	0.57
Placebo-BaselineVsTreatment--2	0.88	0.50	0.57
Placebo-BaselineVsTreatment--3	0.25	0.14	0.57
Placebo-BaselineVsTreatment--4	0.50	0.29	0.57
Placebo-BaselineVsTreatment--5	0.50	0.29	0.57
Placebo-BaselineVsTreatment--6	0.63	0.36	0.57
Placebo-BaselineVsTreatment--7	0.63	0.36	0.57
Placebo-BaselineVsTreatment--8	0.63	0.36	0.57
Placebo-BaselineVsTreatment--9	0.63	0.36	0.57
Median	0.63	0.36	0.57
Probiotic-BaselineVsTreatment--0	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--1	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--2	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--3	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--4	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--5	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--6	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--7	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--8	2.00	1.00	0.50
Probiotic-BaselineVsTreatment--9	2.00	1.00	0.50
Median	2.00	1.00	0.50

Confusion matrix accuracy scores for all iterations of comparisons between groups (Probiotic vs. Placebo at baseline and after treatment) and within groups over time (baseline vs. treatment for Placebo and Probiotic groups). Color coding indicates poor (red) to moderate (yellow) to good (green) accuracy.



Appendix Figure 6. Pirate plots of the 1-12/24 important taxa from the ML model classification for before and after probiotic treatment. Pirate plots of the mean relative abundance (%) ± 95% CI of 13-24 of the 24 important taxa (by rank) from the ML model classifying the probiotic group at baseline (blue, "Baseline-GrB") and after treatment (red, "Treatment-GrB").



Appendix Figure 7. Pirate plots of the 13-24/24 important taxa from the ML model classification for before and after probiotic treatment. Pirate plots of the mean relative abundance (%) ± 95% CI of 13-24 of the 24 important taxa (by rank) from the ML model classifying the probiotic group at baseline (blue, "Baseline-GrB") and after treatment (red, "Treatment-GrB").

Appendix Table 4. MaAsLin2 results for all significant taxa in the main group analysis.

Comparison	Feature	coef	stderr	pval	qval
Probiotic Baseline vs. Treatment	d__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Bacillaceae.g__Bacillus	3.44389666	0.18066535	3.33E-39	1.64E-36
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Bacilli.o__Bacillales.f__Bacillaceae.g__Bacillus	3.41264666	0.18346653	3.36E-38	5.51E-36
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptostreptococcales.Tissierellales.f__Anaerovoracaceae.g__Family_XIII_UCG.001	-1.5302674	0.49983246	0.0026839	0.11287387
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Moryella	-1.0832489	0.3575988	0.00296662	0.11287387
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG.003	-1.5249079	0.51640518	0.00374621	0.12287567
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__uncultured	-1.1030812	0.39621533	0.00618327	0.14536549
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Enterococcaceae.g__Enterococcus	-1.0046033	0.36099703	0.00620462	0.14536549
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Negativicutes.o__Acidaminococcales.f__Acidaminococcaceae.g__Acidaminococcus	0.91404738	0.33474522	0.00721419	0.14789091
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Sellimonas	0.83263488	0.32090987	0.0105745	0.19269096
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Eubacterium._xylanophilum_group	-1.5751414	0.62362052	0.01276345	0.22022509
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_NK4A136_group	-1.3954428	0.55787363	0.01363368	0.22359234
Treatment Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__.Eubacterium._siraeum_group	-2.1311196	0.86299519	0.01484967	0.23567856
Baseline Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Bacilli.o__Izemoplasmatales.f__Izemoplasmatales.g__Izemoplasmatales	-0.7418235	0.25150849	0.00378536	0.17259981
Baseline Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Ruminococcus._gnavus_group	0.99854494	0.38136678	0.00990196	0.24563568
Baseline Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptostreptococcales.Tissierellales.f__Peptostreptococcaceae.g__Terrisporobacter	-1.7466441	0.66820393	0.01002352	0.24563568
Baseline Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Clostridia_vadinBB60_group.f__Clostridia_vadinBB60_group.g__Clostridia_vadinBB60_group	-0.9655205	0.37616545	0.0114166	0.24563568
Baseline Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__UCG.002	-1.1401107	0.44930745	0.0123653	0.24563568
Baseline Probiotic vs. Placebo	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG.008	-1.0086738	0.39829114	0.01253365	0.24563568

Mixed-effects linear models using a variance-stabilizing log transformation on relative abundance genera data were used to determine the significance. Results were controlled for repeated measures. Significant differences that remained after the adjustment for false discovery rate are highlighted in light green.

Appendix Table 5. Confusion matrix accuracy scores for subgroup analysis of baseline gut microbiota.

Classifier Iteration ID	Accuracy Ratio	Overall Accuracy	Baseline Accuracy
Baseline-Placebo-ResponderVsNonResponder--1	1.333	0.80	0.6
Baseline-Placebo-ResponderVsNonResponder--2	1.333	0.80	0.6
Baseline-Placebo-ResponderVsNonResponder--3	1.500	0.90	0.6
Baseline-Placebo-ResponderVsNonResponder--4	1.500	0.90	0.6
Baseline-Placebo-ResponderVsNonResponder--5	1.500	0.90	0.6
Baseline-Placebo-ResponderVsNonResponder--6	1.500	0.90	0.6
Baseline-Placebo-ResponderVsNonResponder--7	1.667	1.00	0.6
Baseline-Placebo-ResponderVsNonResponder--8	1.667	1.00	0.6
Baseline-Placebo-ResponderVsNonResponder--9	1.667	1.00	0.6
Median	1.500	0.90	0.6
Baseline-HighVsLowSkinCarotenoidStatus--1	1.900	0.95	0.5
Baseline-HighVsLowSkinCarotenoidStatus--2	1.600	0.80	0.5
Baseline-HighVsLowSkinCarotenoidStatus--3	1.500	0.75	0.5
Baseline-HighVsLowSkinCarotenoidStatus--4	1.500	0.75	0.5
Baseline-HighVsLowSkinCarotenoidStatus--5	1.300	0.65	0.5
Baseline-HighVsLowSkinCarotenoidStatus--6	1.500	0.75	0.5
Baseline-HighVsLowSkinCarotenoidStatus--7	1.400	0.70	0.5
Baseline-HighVsLowSkinCarotenoidStatus--8	1.800	0.90	0.5
Baseline-HighVsLowSkinCarotenoidStatus--9	1.700	0.85	0.5
Median	1.500	0.75	0.5
Baseline-BMICategory-0	1	0.6	0.6
Baseline-BMICategory-1	1.444444444	0.866666667	0.6
Baseline-BMICategory-2	1.333333333	0.8	0.6
Baseline-BMICategory-3	1.222222222	0.733333333	0.6
Baseline-BMICategory-4	1.111111111	0.666666667	0.6
Baseline-BMICategory-5	1.111111111	0.666666667	0.6
Baseline-BMICategory-6	1	0.6	0.6
Baseline-BMICategory-7	1.222222222	0.733333333	0.6
Baseline-BMICategory-8	1	0.6	0.6
Baseline-BMICategory-9	1.3125	0.75	0.57
Median	1.167	0.70	0.6

Confusion matrix accuracy scores for all iterations of comparisons of baseline gut microbiota composition between classifiers for carotenoid intervention responsiveness (Responder vs Nonresponder), baseline SCS (High vs Low), and BMI (Normal vs Overweight). Color coding indicates poor (red) to moderate (yellow) to good (green) accuracy.

Appendix Table 6. MaAsLin2 results for all significant taxa in the subgroup analysis of baseline gut microbiota.

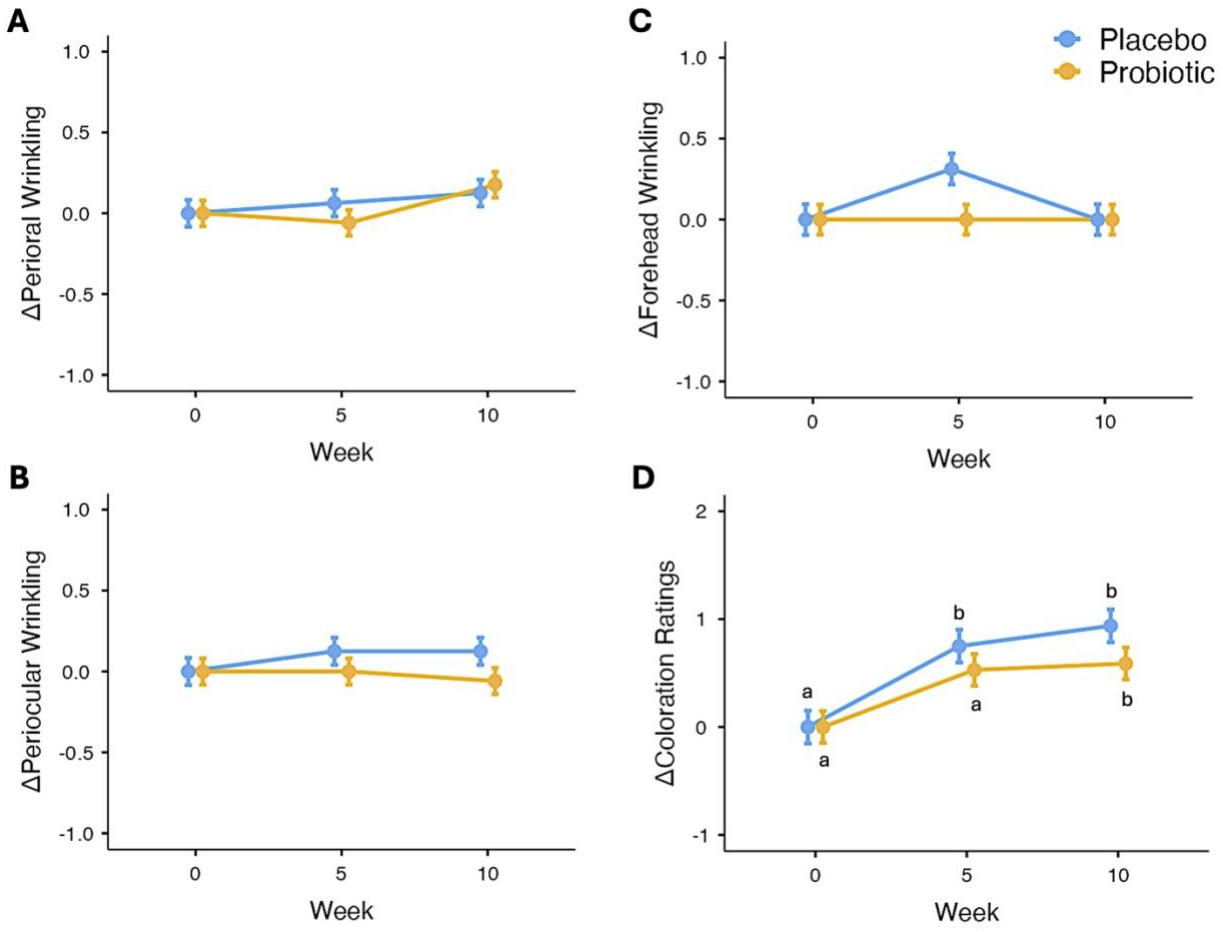
Subgroup Category	Feature	value	coef	stderr	pval	qval
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptostreptococcales.Tissierellales.f__Anaerovoracaceae.g__Family_XIII_AD3011_group	High	-1.1715219	0.2900204	0.00015009	0.02445751
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Negativibacillus	High	-1.2852636	0.32433381	0.00019411	0.02445751
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Negativibacillus	Responder	-1.4698658	0.34656043	7.54E-05	0.02445751
Responders	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Enterorhabdus	Responder	-1.4262561	0.35964486	0.0001922	0.02445751
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Fournierella	Responder	-1.2873907	0.33063549	0.00024422	0.02461784
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__uncultured	High	-0.8134779	0.21231599	0.00029983	0.0251856
BaselineSCS	d__Bacteria.p__Actinobacteriota.c__Actinobacteria.o__Bifidobacteriales.f__Bifidobacteriaceae.g__Bifidobacterium	High	2.74819607	0.74412162	0.00046997	0.03383772
BMI	d__Bacteria.p__Actinobacteriota.c__Actinobacteria.o__Bifidobacteriales.f__Bifidobacteriaceae.g__Bifidobacterium	Normal	2.62804416	0.75040124	0.00086186	0.05429694
Responders	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Prevotellaceae.g__Alloprevotella	Responder	-1.6450763	0.47665758	0.00101001	0.05656075
BMI	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Moryella	Normal	-1.1540494	0.35608013	0.00191789	0.09666146
BMI	d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Faecalitalea	Normal	1.67966237	0.53847618	0.00275007	0.12600303
BMI	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Agathobacter	Normal	-1.375572	0.44866563	0.00321272	0.13447502
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__CAG.56	High	1.92470444	0.63324897	0.0034686	0.13447502
BMI	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Coprococcus	Normal	-0.8135138	0.27354865	0.00418443	0.15063959
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Faecalibacterium	High	0.6234423	0.2156066	0.00527946	0.15652033
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Incertae_Sedis	High	-1.1258674	0.38786356	0.00511682	0.15652033
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__GCA.900066575	High	1.07430693	0.36736405	0.00481499	0.15652033
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.__	Responder	-3.1175873	1.10535893	0.00643295	0.15923021
BaselineSCS	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Slackia	High	-1.3267156	0.46954093	0.00634259	0.15923021
Responders	d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__uncultured	Responder	-0.7675541	0.26958423	0.00597444	0.15923021
BMI	d__Bacteria.p__Firmicutes.c__Clostridia.o__Eubacteriales.f__Eubacteriaceae.g__Eubacterium	Normal	0.63106649	0.22464124	0.00663459	0.15923021
Responders	d__Bacteria.p__Verrucomicrobiota.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Akkermansiaceae.g__Akkermansia	Responder	2.85305635	1.02626414	0.00718702	0.16464814
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG.001	Responder	-1.3211266	0.48198524	0.00799215	0.16560752
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptostreptococcales.Tissierellales.f__Anaerovoracaceae.g__Eubacterium._brachy_group	High	-1.485102	0.54567814	0.00842328	0.16560752
Responders	d__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Burkholderiales.f__Sutterellaceae.g__Parasutterella	Responder	1.39929102	0.50691051	0.00758175	0.16560752
BMI	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Eubacterium._fissicatena_group	Normal	0.84515698	0.31113995	0.00854324	0.16560752
BaselineSCS	d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Carnobacteriaceae.g__Granulicatella	High	0.95129233	0.35564076	0.0095475	0.17821999
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG.003	High	0.4916959	0.18587552	0.01032854	0.18591376
Responders	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__uncultured	Responder	-0.8061056	0.30718581	0.01092194	0.18981578
BaselineSCS	d__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Burkholderiales.f__Sutterellaceae.g__Parasutterella	High	1.22922662	0.47439985	0.01191212	0.19561968
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Sellimonas	High	0.81711812	0.31582072	0.01203216	0.19561968
BMI	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Enterorhabdus	Normal	-0.859098	0.33941944	0.01392211	0.21927328
Responders	d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Holdemanella	Responder	-0.8289622	0.33238934	0.01531392	0.232858
Responders	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Marinifilaceae.g__Odoribacter	Responder	1.26161067	0.51254256	0.01663271	0.232858
Responders	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Prevotellaceae.g__Paraprevotella	Responder	0.87510337	0.35385607	0.01615141	0.232858
BMI	d__Bacteria.p__Firmicutes.c__Clostridia.o__Christensenellales.f__Christensenellaceae.g__Christensenella	Normal	0.65597601	0.26586808	0.01638894	0.232858

Mixed-effects linear models using a variance-stabilizing log transformation on relative abundance genera data were used to determine the significance. Results were controlled for repeated measures. Significant differences that remained after the adjustment for false discovery rate are highlighted in light green.

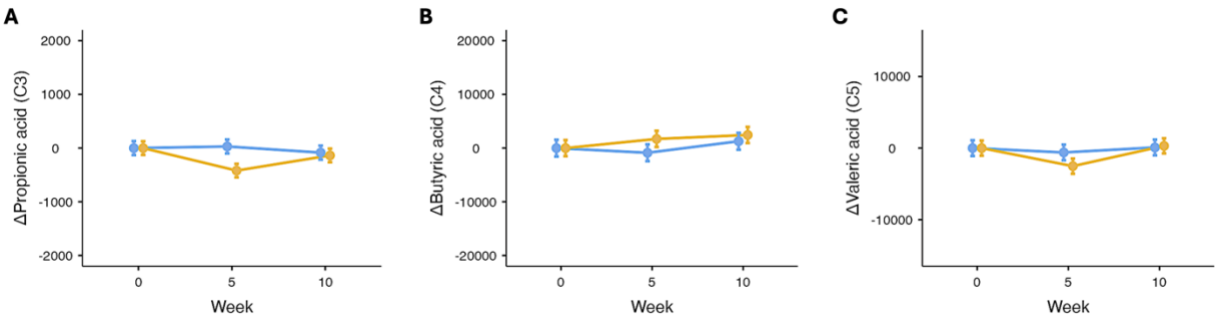
Appendix Table 7. MaAsLin2 results for all significant taxa in the baseline gut microbiota subgroup analysis with age and daily FV servings as confounders.

metadata	feature	value	coef	stderr	pval	qval
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__uncultured	High	-1.212175	0.23653991	3.35E-06	0.00281066
Age	d__Archaea.p__Euryarchaeota.c__Methanobacteria.o__Methanobacteriales.f__Methanobacteriaceae.g__Methanosphaera	Age	0.06516965	0.01392984	1.69E-05	0.00710095
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Negativibacillus	High	-1.4608918	0.35407568	0.00011554	0.01941143
Responders	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Enterorhabdus	Responder	-1.5070082	0.36282033	0.00010521	0.01941143
DailyFVservings	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Defluviitaleaceae.g__Defluviitaleaceae_UCG.011	DailyFVservi	0.7180846	0.17276727	0.00010423	0.01941143
DailyFVservings	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__CAG.352	DailyFVservi	1.31242531	0.32401212	0.00014892	0.0208491
DailyFVservings	d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Holdemanella	DailyFVservi	-0.4817523	0.12180424	0.00020466	0.02148962
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Negativibacillus	Responder	-1.2797557	0.3225308	0.00019621	0.02148962
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Fournierella	Responder	-1.2529404	0.32810239	0.00032033	0.02989771
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.__	Responder	-3.7043539	1.03273301	0.00067327	0.05655472
DailyFVservings	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__.Clostridium._methylpentosum_group.g__.Clostridium._methylpentosum_group	DailyFVservi	-0.5379471	0.15288214	0.00083389	0.06367872
Age	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Muribaculaceae.g__Muribaculaceae	Age	0.10944686	0.03146541	0.00094545	0.06618158
Responders	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Prevotellaceae.g__Alloprevotella	Responder	-1.6420912	0.49047942	0.00141042	0.09113469
BMI_Cat	d__Bacteria.p__Actinobacteriota.c__Actinobacteria.o__Bifidobacteriales.f__Bifidobacteriaceae.g__Bifidobacterium	Normal	2.49556171	0.76752638	0.00188582	0.11314923
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__CAG.352	Responder	2.51455787	0.81038931	0.00292103	0.12881589
Responders	d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Holdemanella	Responder	-0.9637891	0.30464557	0.00244593	0.12881589
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptostreptococcales.Tissierellales.f__Anaerovoracaceae.g__Family_XIII_AD3011_group	High	-1.0402328	0.34026518	0.00333473	0.12881589
BMI_Cat	d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__Faecalitalea	Normal	1.74502807	0.5535226	0.00252661	0.12881589
Age	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG.008	Age	0.10282617	0.03371622	0.00340624	0.12881589
BMI_Cat	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Moryella	Normal	-1.1057429	0.36401631	0.0035271	0.12881589
BaselineSCS	d__Archaea.p__Euryarchaeota.c__Methanobacteria.o__Methanobacteriales.f__Methanobacteriaceae.g__Methanosphaera	High	0.53863998	0.17593068	0.00329141	0.12881589
Age	d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptostreptococcales.Tissierellales.f__Anaerovoracaceae.g__.Eubacterium._brachy_group	Age	-0.1500589	0.04807256	0.00276701	0.12881589
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Paludicola	High	-0.8635315	0.28166416	0.00325215	0.12881589
Age	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.__	Age	-0.2623512	0.08976714	0.00488883	0.14338119
Responders	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__uncultured	Responder	-0.8920206	0.30771323	0.00522413	0.14338119
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Lachnospiraceae_UCG.001	Responder	-1.4084776	0.47735048	0.00451799	0.14338119
Age	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Adlercreutzia	Age	-0.1210507	0.04136518	0.00483674	0.14338119
DailyFVservings	d__Archaea.p__Euryarchaeota.c__Methanobacteria.o__Methanobacteriales.f__Methanobacteriaceae.g__Methanosphaera	DailyFVservi	-0.1859764	0.06407435	0.00517131	0.14338119
Responders	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Prevotellaceae.g__Paraprevotella	Responder	1.00746344	0.34808762	0.00529145	0.14338119
BaselineSCS	d__Bacteria.p__Firmicutes.c__Bacilli.o__Lactobacillales.f__Carnobacteriaceae.g__Granulicatella	High	1.23562567	0.41997353	0.00462708	0.14338119
BMI_Cat	d__Bacteria.p__Firmicutes.c__Clostridia.o__Eubacteriales.f__Eubacteriaceae.g__Eubacterium	Normal	0.66794347	0.23016353	0.00517811	0.14338119
BMI_Cat	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Agathobacter	Normal	-1.2626885	0.44831748	0.00656169	0.17224431
Responders	d__Bacteria.p__Firmicutes.c__Bacilli.o__Erysipelotrichales.f__Erysipelotrichaceae.g__uncultured	Responder	-0.7734598	0.27761351	0.00713054	0.17616626
Responders	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Barnesiellaceae.g__uncultured	Responder	0.99764743	0.35790803	0.00710458	0.17616626
DailyFVservings	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Paludicola	DailyFVservi	0.28430863	0.10258273	0.00741949	0.17806766
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__CAG.352	High	-2.3948186	0.88964882	0.00919427	0.17960897
BMI_Cat	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Coprococcus	Normal	-0.7527755	0.27841831	0.00890674	0.17960897
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__CAG.56	High	1.98531416	0.73680752	0.00913038	0.17960897
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__uncultured	High	-1.0910074	0.40041367	0.00842012	0.17960897
Age	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Tyzzerella	Age	-0.1440213	0.05341328	0.00908472	0.17960897
Responders	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Barnesiellaceae.g__Barnesiella	Responder	1.64005736	0.60118452	0.00834475	0.17960897
BaselineSCS	d__Bacteria.p__Firmicutes.c__Clostridia.o__Peptostreptococcales.Tissierellales.f__Anaerovoracaceae.g__.Eubacterium._brachy_group	High	-1.6723502	0.60714552	0.00777079	0.17960897
Age	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Ruminococcaceae.g__Negativibacillus	Age	0.07617995	0.028035	0.00858885	0.17960897
Responders	d__Bacteria.p__Verrucomicrobiota.c__Verrucomicrobiae.o__Verrucomicrobiales.f__Akkermansiaceae.g__Akkermansia	Responder	2.76435384	1.04823461	0.01063038	0.18603172
BaselineSCS	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Adlercreutzia	High	-1.3887481	0.52243289	0.01005447	0.18603172
DailyFVservings	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Slackia	DailyFVservi	-0.5144496	0.19434966	0.01035684	0.18603172
BaselineSCS	d__Bacteria.p__Firmicutes.c__Negativicutes.o__Veillonellales.Selenomonadales.f__Veillonellaceae.g__Veillonella	High	1.46418462	0.55209181	0.01021988	0.18603172
Age	d__Bacteria.p__Firmicutes.c__Bacilli.o__Staphylococcales.f__Gemellaceae.g__Gemella	Age	0.07114076	0.02694915	0.01055602	0.18603172
DailyFVservings	d__Bacteria.p__Firmicutes.c__Clostridia.o__Oscillospirales.f__Oscillospiraceae.g__uncultured	DailyFVservi	0.22494636	0.08614837	0.01138208	0.19512142
Responders	d__Bacteria.p__Proteobacteria.c__Gammaproteobacteria.o__Burkholderiales.f__Sutterellaceae.g__Parasutterella	Responder	1.33985304	0.51636122	0.01187928	0.19957198
Responders	d__Bacteria.p__Firmicutes.c__Clostridia.o__Monoglobales.f__Monoglobaceae.g__Monoglobus	Responder	-1.1480171	0.4446701	0.0122908	0.20243669
BaselineSCS	d__Bacteria.p__Actinobacteriota.c__Actinobacteria.o__Bifidobacteriales.f__Bifidobacteriaceae.g__Bifidobacterium	High	2.26561395	0.89081983	0.01357706	0.21483481
DailyFVservings	d__Bacteria.p__Bacteroidota.c__Bacteroidia.o__Bacteroidales.f__Rikenellaceae.g__Alistipes	DailyFVservi	0.49184072	0.19389284	0.01381081	0.21483481
Age	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Roseburia	Age	0.08285548	0.03261793	0.01368623	0.21483481
BMI_Cat	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__.Eubacterium._fissicatena_group	Normal	0.79890328	0.31786167	0.0146615	0.22392104
DailyFVservings	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Gordonibacter	DailyFVservi	0.54802742	0.21982988	0.0154453	0.23167954
BaselineSCS	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Eggerthellaceae.g__Gordonibacter	High	-1.4974093	0.60359283	0.01592913	0.23474501
Age	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__uncultured	Age	-0.0783244	0.03170395	0.01635184	0.23681978
Age	d__Bacteria.p__Firmicutes.c__Clostridia.o__Lachnospirales.f__Lachnospiraceae.g__Blautia	Age	-0.027984	0.01139005	0.01692408	0.23830742
Age	d__Bacteria.p__Actinobacteriota.c__Coriobacteriia.o__Coriobacteriales.f__Coriobacteriaceae.g__Collinsella	Age	0.11746227	0.04785402	0.01702196	0.23830742

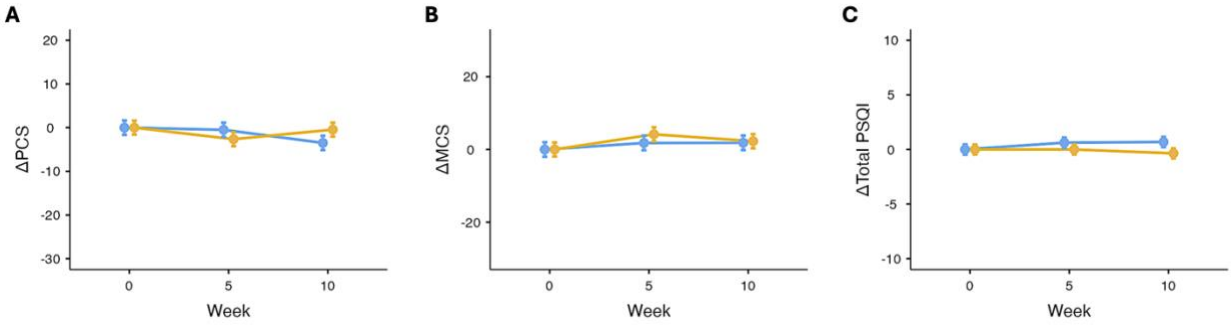
Mixed-effects linear models using a variance-stabilizing log transformation on relative abundance genera data were used to determine the significance. Results were controlled for repeated measures. Significant differences that remained after the adjustment for false discovery rate are highlighted in light green.



Appendix Figure 8. Mean changes from baseline (Δ) ± SEM for skin wrinkling and coloration ratings. The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. (A) Perioral wrinkling, (B) Periocular wrinkling, (C) Forehead wrinkling, and (D) Coloration rating. Significant differences ($p < 0.05$) within groups over time are represented by different letters (a, b). All post-hoc analyses were conducted using the Holm method to adjust for multiple comparisons. Significant differences reported reflect the interaction effects between time and treatment (time*treatment) observed in the model.



Appendix Figure 9. Mean changes from baseline (Δ) ± SEM for plasma SCFAs (nM). The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. (A) Propionic acid (C3), (B) Butyric acid (C4), and (C) Valeric acid (C5). SCFAs = short-chain fatty acids.



Appendix Figure 10. Mean changes from baseline (Δ) ± SEM for quality of life and sleep. The probiotic group is represented with yellow lines and the placebo group is represented with blue lines. (A) PCS, (B) MCS, and (C) Total PSQI. PCS = Physical component score; MCS = Mental component score; PSQI = Pittsburgh Sleep Quality Index.