CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

INTERACTION BETWEEN CAROTENOIDS AND THE INTESTINAL MICROBIOTA AND ITS IMPACT ON FUNCTIONAL PROPERTIES

by

Maria Helena de Almeida Ribeiro Rocha

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

By
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To the ones that always supported me:

"The art and science of asking questions is the source of all knowledge."

Thomas Berger

Resumo

As doenças crónicas não transmissíveis (DNTs) são responsáveis por 41 milhões de mortes anualmente e podem estar associadas a hábitos alimentares não saudáveis, razão pela qual levou a que várias organizações de nutrição e saúde recomendassem o consumo regular de frutas e vegetais. Os carotenoides e os seus metabolitos estão associados a benefícios para a saúde e são os fitoquímicos lipossolúveis mais prevalentes na dieta humana, encontrados em quantidades da ordem do micromolar no sangue e suscetíveis a múltiplas reações de oxidação e isomerização.

Na natureza, os carotenoides têm uma biodisponibilidade reduzida, o que leva à sua acumulação no colon, que é colonizado por diversos microrganismos importantes para a digestão e saúde intestinal. A biodisponibilidade destes compostos é influenciada por vários fatores, com destaque para a importância da microbiota intestinal na sua absorção e metabolismo. A dieta desempenha um papel fundamental na regulação da microbiota, podendo afetar a eficácia de ação dos carotenoides. No entanto, a interação específica entre carotenoides e microbiota intestinal ainda não está bem documentada e carece de evidências claras. Para entender melhor a possível interação entre os carotenoides e a microbiota intestinal, três carotenoides (beta (β)-caroteno, luteína e licopeno), uma mistura desses pigmentos e a alga *Osmundea pinnatifida* (como fonte de carotenoides) foram submetidos a uma simulação *in vitro* da digestão gastrointestinal. Após a caracterização do impacto desse processo em cada grupo testado, os carotenoides digeridos foram avaliados em amostras frescas de fezes humanas de dadores voluntários por fermentação para aferir o efeito destes sobre a dinâmica metabólica e populacional da microbiota intestinal.

A digestão dos grupos testados originou diferentes tipos de carotenoides ao longo do trato gastrointestinal (TGI), sendo o β-caroteno a única condição em que foi identificada a absorção de um caroteno (2,49%). Nenhum carotenoide foi detetado na *O. pinnatifida*, o que sugere que numa matriz complexa como algas, os carotenoides podem estar comprometidos na sua bioacessibilidade sem pré-tratamento de lise da alga. A composição da microbiota intestinal foi analisada, e foi demonstrado que *Bacteroidota*, *Bacillota*, *Pseudomonadota* e *Actinomycetota* são os principais filos presentes, e que os carotenoides estimularam o aumento da abundância relativa (AR) da família *Lachnospiraceae* e a diminuição da AR das bactérias pertencentes aos géneros *Lactobacillus*, *Enterococcus*, *Streptococcus* e *Bifidobacterium*, o que é consistente com estudos anteriores. Em geral, também a produção de ácidos gordos de cadeia curta (AGCC) foi estimulada na presença dos carotenoides, o que mais uma vez corrobora resultados obtidos em estudos anteriores e apoia o papel positivo destes pigmentos na saúde intestinal. Em termos de propriedades funcionais, as soluções testadas apresentaram atividades antioxidante e antidiabética consideráveis, sendo os valores mais altos obtidos para a luteína na fração absorvida e para a mistura de carotenoides na fração retida no intestino. Por fim, foi demonstrado que as amostras de carotenoides apresentaram efeitos anti-mutagénicos, independentemente da concentração, mas não apresentaram citotoxicidade mesmo quando aplicadas nas concentrações mais altas.

Palavras-chave: pigmentos naturais; trato gastrointestinal; metabolitos; microbiota intestinal; saúde.

Abstract

Noncommunicable diseases (NCDs) are responsible for 41 million deaths annually and can be linked to unhealthy dietary habits, prompting various nutrition and health organizations to advocate for regular fruit and vegetable consumption. Carotenoids and their metabolites are associated with positive health benefits and are the most prevalent lipid-soluble phytochemicals in the human diet, found in small amounts in human blood, and susceptible to oxidation and isomerization reactions.

In nature, carotenoids have reduced bioavailability, leading to their accumulation in the colon, which is colonized by a diverse population of microorganisms playing crucial roles in digestion, and maintaining intestinal health. The bioavailability of these compounds can be influenced by various factors, highlighting the significance of the intestinal microbiota in their absorption and metabolism. The diet plays a fundamental role in the regulation of the microbiota, which can affect the effectiveness of carotenoids' action. However, the specific interaction between carotenoids and intestinal microbiota is not well-documented yet and lacks clear evidence.

To better understand the possible interaction between carotenoids and the intestinal microbiota, three carotenoids (beta(β)-carotene, lutein, and lycopene), a mixture of these pigments, and the algae *Osmundea pinnatifida* (as a source of carotenoids) were subjected to an *in vitro* simulation of the gastrointestinal digestion. After the characterization of the impact of this process on each tested condition, the digested carotenoids were evaluated on fresh human faecal samples from volunteer donors via fermentation to assess the effect on the intestinal microbiota's metabolic and population dynamics.

The digestion of the carotenoids in the tested conditions, in a general way, originated different types of carotenoids along the gastrointestinal tract (GIT), being β-carotene the only condition in which was identified the absorption of a carotene (2.49%). No carotenoids were detected in *O. pinnatifida*, which suggests that in a complex matrix like algae, carotenoids may be compromised in their bioaccessibility without prior lysis treatment of the algae. The intestinal microbiota composition was analysed, being demonstrated that the Bacteroidota, Bacillota, Pseudomonadota, and Actinomycetota are the main phyla present and that carotenoids stimulated the increase of the relative abundance (RA) of *Lachnospiraceae* family and the decrease of *Lactobacillus, Enterococcus*, *Streptococcus* and *Bifidobacterium* genera which was consistent with the previous studies. In general, the production of short-chain fatty acids (SCFAs) was also stimulated in the presence of carotenoids, which once again corroborates results obtained in previous studies and supports the positive role of these pigments in intestinal health.

In terms of functional properties, the tested carotenoid solutions presented considerable antioxidant and antidiabetic activities, being the highest values obtained for lutein in the fraction absorbed and for the mixture of carotenoids in the fraction retained in the intestine. Finally, it was demonstrated the antimutagenic effect of these carotenoid samples regardless of the concentration and also the absence of cytotoxicity even when applied at the highest concentrations.

Keywords: natural pigments; gastrointestinal tract; metabolites; intestinal microbiota; health.

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Abbreviations

AA – Antioxidant activity

AAPH – 2,2'-azo-bis-(2-methylpropionamidine)-dihydrochloride

ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AMD – Age-related macular degeneration

ATCC – Trace mineral solution

BCO – β,β-carotene-15,15'-oxygenase

CCD – Carotenoid cleavage dioxygenase

CD36 – Cluster determinant 36

C^+ – Positive control

C^- – Negative control

CVDs – Cardiovascular diseases

DNA - deoxyribonucleic acid

DPPH – 2,2-diphenyl-1-picrylhydrazyl

FOS – Fructooligosaccharides

GIT – Gastrointestinal tract

HHP – High-hydrostatic pressure

HPLC – High-performance liquid chromatography

IL-1β – Interleukine-1-beta

IL-6 – Interleukine 6

IM – Inside the membrane

IN – Inside

LPS – Lipopolysaccharide

LDL – Low-density lipoproteins

LPS – Lipopolysaccharide

NCDs – Non-communicable diseases

NMDS – Non-metric multidimensional scaling

NI – No identified

NPC1L1 – NPC1-like transporter 1

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

OM – Outside the membrane

OUT – Outside

OH – Ohmic heating

OTU – Operational taxonomic unit

PCA – Principal component analysis

PCR – Polymerase chain reaction

PBS – Phosphate buffer solution

PCoA – Principal coordinates analysis

RA – Relative abundance

ROS – Reactive oxygen species

RGE – Resuspended rabbit gastric extract

SCFAs – Short-chain fatty acids

SD – Standard deviation

SGF – Simulated gastric fluid

SGP – Simulated gastric phase

SIF – Simulated intestinal fluid

SIP – Simulated intestinal phase

SFE – Super-critical fluid extraction

SPE – Solid phase extraction

SSF – Simulated salivary fluid

SR-BI – Scavenger receptor class B type 1

TE – Trolox equivalent

TNF-α – Tumour necrosis factor-alpha

TSB – Tryptone Soya Broth

T2DM – Type 2 diabetes mellitus

Tween 20 – Polysorbate 20M

UV – Ultraviolet

XTT – (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)

CHAPTER I

Introduction

1.1 Carotenoids

1.1.1 General characteristics

Carotenoids are colourful lipid-soluble pigments (1,2), responsible for a wide spread of colours present in various autotrophs such as microalgae, bacteria, fungi, and plants (2,3). These natural pigments are part of the tetraterpenes family, characterized by a central chain with 40 atoms of carbon and alternating single and double bonds and various cyclic or acyclic end groups, depending on the carotenoid (2).

Carotenoids exhibit diverse molecular structures and are naturally occurring in various sources, existing in over 750 different carotenoids. However, unlike autotrophs, Humans and animals lack the ability to synthesize carotenoids on their own. Instead, only 40 carotenoids are commonly consumed through diet (1,4,5) and, a smaller subset is absorbed, stored, and consequently detected in our bloodstream and body tissues (2,6–9).

According to their chemical constituents, carotenoids can be classified as carotenes or xanthophylls (10), existing as pure hydrocarbons in the first case (e.g., α-carotene, β-carotene, and lycopene) and, having oxygenated derivatives on their terminal rings in the case of xanthophylls (e.g., lutein, β-cryptoxanthin, and zeaxanthin) (9).

Xanthophylls are synthesized within the plastids and have an important function, as accessory pigments, to capture certain wavelengths of sunlight that are not absorbed by chlorophyll (5). The presence of a polar group in the chemical structure of xanthophylls can affect the polarity of the carotenoids as well as their biological functions (1,2,9,11).

Carotenes are orange pigments responsible for transmitting the light energy they absorb from chlorophyll and absorbing the energy from singlet oxygen formed in photosynthesis to protect the plant tissues (5).

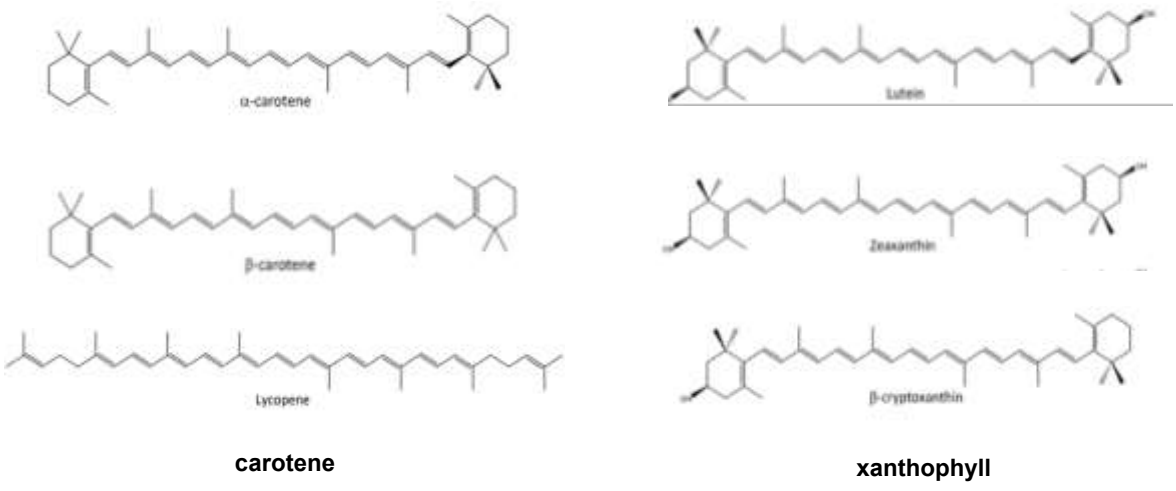


Figure 1.1 - Chemical structures of the major carotenoids.

In terms of physicochemical properties, carotenoids are associated with cytosolic lipid droplets and membrane lipid bilayers and, can modify some properties related to the permeability and fluidity of the membrane (12,13). Carotenoids can also be classified according to their functional properties into primary and secondary carotenoids, with the photosynthetic carotenoids included in the primary group and having an important role in photosynthesis (1,14).

So far, only 50 carotenoids are known to have provitamin A activity (14). The most important precursors of vitamin A in humans identified were α-carotene, β-carotene, gamma (δ)-carotene, and β-cryptoxanthin (14–17). When exposed to light, heat, oxygen, or acids, carotenoids are very susceptible to multiple reactions such as oxidation and isomerization (5).

Lutein, β-carotene, and lycopene are 3 of the 40 carotenoids present in a usual human diet (2). The natural pigment β-carotene has the highest vitamin A activity, once is the only molecule that presents symmetry, and a unique molecule of β-carotene is sufficient to be converted into two molecules of vitamin A (15). Since humans aren't able to synthesize vitamin A *de novo*, it is necessary to obtain proper amounts of this nutrient through diet, in orange and yellow vegetables and fruits such as carrots and mangoes and, in vegetables with dark green leaves (15).

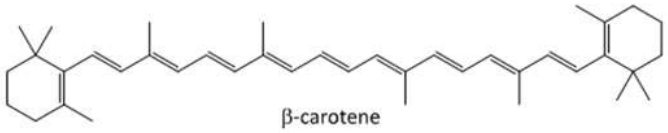


Figure 1.2 - Chemical structure of β-carotene (3).

Lutein is a xanthophyll abundant in dark green vegetables (18) and in egg yolk (19) and is present in the eye's lens, dispersed all over the retina (20,21). The nine double bonds that are characteristic of the structure of this xanthophyll are responsible to scavenge oxygen intermediates (22) and to absorb and emit certain light wavelengths that lead to the characteristic colour properties of lutein (23).

Lutein as a xanthophyll is less hydrophobic than carotenes which makes this first pigment better absorbable into micelles and, with a higher bioaccessibility (13).

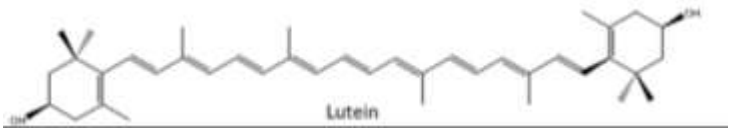


Figure 1.3 - Chemical structure of lutein (3).

Lycopene is a predominant unsaturated carotenoid present in fruits and vegetables and, is the pigment responsible for the red colour of tomatoes, apricots, papaya, watermelons, and red grapefruits (24,25). With the molecular formula $C_{40}H_{56}$, this hydrocarbon carotenoid has an acyclic open-chain structure with 13 double-bounds and is usually longer than the other carotenoids (11,24), contributing to its superior free radical-quenching abilities among carotenoids (1).

This carotenoid doesn't have symmetrical planarity, which justifies the absence of vitamin A-like activity (24). It is a lipophilic molecule that is transported in lipoproteins through the blood and, consequently, accumulated in the vasculature and the tissues (25).

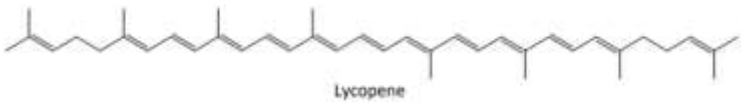


Figure 1.4 - Chemical structure of lycopene (3).

While carotenoids are known for their vibrant colours and potential health benefits (1,2), their bioavailability plays a crucial role in determining their actual impact on human health (2,15). Since these natural pigments are fat-soluble compounds, they require the presence of dietary fats for optimal absorption (26,27), which means that consuming carotenoid-rich foods along with healthy fats can enhance their bioavailability. On the other hand, as the demand for natural and functional ingredients continues to rise (17), the development of efficient and sustainable extraction techniques for carotenoids (28) remains a focus of ongoing research and technological innovation.

In addition, these natural pigments have several applications such as in feed, food, nutraceutical, and pharmaceutical industries (5), being applied as colourants in food, beverages and cosmetics, as nutrient supplements, feed additives, and animal feed supplements (17).

1.1.2 Biological functions

Carotenoids have several important biological functions such as antioxidant, antibacterial, immunological, and anti-inflammatory activities, and beneficial effects in the treatment of some cancers, and diabetes, as well as in eye infections, neurological disorders, and CVDs (1,24,29,30).

Due to these bioactive properties that promote health, the use of carotenoids as food ingredients has increased exponentially (1).

a) Antioxidant activity

These natural pigments have antioxidant activity since they regulate the production of antioxidant enzymes, scavenge radicals produced in various pathologies and during aerobic metabolism (2,31,32) and, as they have conjugated double bounds, can accept electrons from the reactive species, neutralizing the free radicals (1,2,7,12,33).

Carotenoids are able to absorb short wavelengths and high-energy blue light, protecting, for example, the retina from photochemical damage (9). In addition, as lipid-soluble molecules, they are present in lipid/water interfaces and in lipophilic environments, playing the important role of protecting cellular membranes and lipoproteins against the damage caused by radicals (34). At the lipid/aqueous interface of cellular membranes are found xanthophylls since they are less hydrophobic than carotenes. These pigments can scavenge, therefore, radicals of both lipid and aqueous phases. On the other hand, carotenes are present in the apolar core of lipidic membranes such as that of the outer cell membrane, mitochondrial membrane, and nucleus membrane, scavenging radicals of the lipid phase (34).

b) Anti-cancer properties

Cancer is the second most frequent cause of death in the world, being oxidative stress responsible for deoxyribonucleic acid (DNA) mutations, unstable genome, and cell proliferation, which contributes to cancer development (35).

Carotenoids have shown potential in treating certain cancers, such as lung, prostate, and colon cancer, acting as chemopreventive or chemotherapeutic agents (36,37). The protective effect of carotenoids against cancer may be associated with their antioxidant property (38).

The intake of food that contains retinol and high blood levels of retinol or β-carotene (precursor of vitamin A) are associated inversely with the risk of developing cancer in humans (37). Although high levels of vitamin A and retinoids can be prophylactic agents for the prevention of cancer, β-carotene is not toxic, even if administered at high levels for a long period (37).

Lutein, zeaxanthin, and lycopene are known to reduce the development of inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), interleukine-1-beta (IL-1β), and interleukin 6 (IL-6) by blocking the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (9,39). These natural pigments can inhibit cell proliferation and dysregulation, enhance immune system functioning, signal the growth factor, and reduce the expression of metastasis regulators, being lycopene more effective than β-carotene in long-period treatments (36–38,40).

c) Immunological role

Carotenoids have several anti-inflammatory functions such as stimulating the expression of antioxidant genes and controlling signal pathways to induce inflammatory mediators, which have beneficial effects on inflammation and redox imbalances (28).

These natural pigments can also activate the adaptive immune response, maintaining an adequate defence to protect against bacterial infection and radiation (28,41).

Besides that, carotenoids can stimulate the proliferation of B and T-lymphocytes, the macrophages and cytotoxic T-cells activity, the function of effector T-cell, the production of cytokines, the induction of apoptosis and affect cell growth (2,15,25).

d) Prevention of diseases

i. CVDs

CVDs are the leading causes of incapacity and death worldwide (42) and, are usually associated with an unhealthy diet, physical inactivity and tobacco and alcohol consumption, which lead to elevated blood pressure, blood glucose and blood lipids, overweight and obesity (42). CVDs are associated with atherosclerotic lesions and the formation of foam cells, provoked by low-density lipoproteins (LDL) altered by oxidation (35).

Carotenoids, particularly lycopene, have been inversely associated with CVDs, protecting against atherosclerosis through the inhibition of the development of reactive oxygen species (ROS) and LDL oxidation (43–45).

Besides that, carotenoids can decrease the risk of these diseases through mechanisms such as lowering blood pressure, decreasing pro-inflammatory cytokines and markers of inflammation and enhancing liver, muscle, and adipose tissue's sensitivity to insulin (2).

ii. Eye disorders

The most prevalent eye disorders in the elderly are cataracts, age-related macular degeneration (AMD), glaucoma and diabetic retinopathy (46), which are mainly caused due to oxidative stress (oxygen and light sensitivity), adverse environmental factors, high polyunsaturated fatty acids, exposure to blue and ultraviolet (UV) light, aging, and smoking (47).

A deficit in Vitamin A is also a concern since it influences immunity, destroying receptors that are sensitive to light and can cause xerophthalmia that progresses to irreversible blindness (48,49).

There are two xanthophylls present in the eye's lens: in the central macula is concentrated zeaxanthin and all over the retina is dispersed lutein (20,21). Many clinical trials show that a diet containing a high proportion of lutein and zeaxanthin lowers the concentration of lutein and zeaxanthin in the retina, plasma, and tissue (50). These can be explained once the two natural pigments have antioxidant activity, filtering blue light to protect photoreceptor cells from light-generated oxygen radicals (51,52). Therefore, carotenoids can be used to prevent and treat a wide range of eye disorders such as age-related macular degradation, cataracts, and retinitis pigmentosa (9).

iii. Neurological problems

Neurodegenerative diseases such as Alzheimer, Parkinson, and lateral amyotrophic sclerosis (53) are associated with mutations in the mitochondrial DNA and oxidative stress, contributing to DNA damage accumulation. Carotenoids are antioxidant agents capable of scavenging free radicals (9), suppressing proinflammatory cytokines (54) and reducing oxidative stress (55). Carotenoids such as lycopene, zeaxanthin, and lutein were shown to limit neuronal damage, reducing cognitive decline (9).

Below, in Table 1.1, are presented the principal biological functions of β-carotene, lutein, and lycopene.

Table 1.1 - Main biological properties of β-carotene, lutein, and lycopene.

Carotenoid	Biological functions	References
β-carotene	Stimulates the proliferation of lymphocytes Reduces the LDL susceptibility to oxidation Activates cell communication Reduces inflammation	(1,15,25)
Lutein	Scavenges oxygen intermediates Blue light filter Decreases the proliferation of breast cancer cells Reduces oxidative stress and apoptosis	(2,23,25,56–59)
Lycopene	Inhibits lipid peroxidation Eliminates ROS Reinforces the immune system Free radical quencher	(1,25)

1.2 Bioaccessibility and bioavailability of carotenoids

The maximum quantity of a carotenoid released from the food matrix that is available to be absorbed in the epithelial cells of the intestine is defined as bioaccessibility (60). Bioavailability is the fraction of an ingested compound that enters the bloodstream and plays its physiological functions (5,61).

In nature, the bioavailability of carotenoids is reduced since there is a resistance to digestion and degradation from the protein complexes of carotenoids and the cell walls of plants to achieve adequate release (2,15). The bioavailability can be reduced depending on the localization of carotenoids within the chloroplasts and chromoplasts of plants (9,62). For example, although β-carotene has a high activity and conversion to vitamin A, its absorption from plant sources is approximately 65%, being the recommended intake of 2-4 mg per day not achieved (15,63,64).

The carotenoids' bioavailability and consequently absorption can be affected by several factors such as the dietary sources, season, food composition, the structure and breakup of the food matrix, the presence of lipids, the dosage, transport and rate of absorption, the presence of other soluble compounds/carotenoids, the cooking temperature and mode (11,24,65,66). These conditioning factors, when well designed, can help provide the release of the carotenoid from the food matrix, enhancing its bioavailability or transforming it into an isomer that is better absorbed by the human organism (11).

The carotenoids' release from the food matrix depends on their state, as the carotenoids immersed entirely in lipid droplets are more easily released than the ones in the microcrystalline form like lycopene in tomato and β-carotene in carrot, respectively (67). The bioavailability of carotenoids is also significantly influenced by dietary composition (68,69). Since carotenoids are lipophilic compounds, their bioavailability increases when it is consumed in food containing a fat source (26,27). On the other hand, the absorption of carotenoids can decrease with the simultaneous consumption of dietary fibre such as pectin at the same meal (70).

Cooking, boiling, blanching and steaming disrupts the cellular membrane, facilitating the release of carotenoids from the matrix (2,15,25,66,71). Although this lowers the amount of carotenoid content, when compared to uncooked food, it increases the carotenoids' bioavailability and absorption (2,9,15,71). For example, in cooked tomatoes, the lycopene availability is higher than in raw tomatoes, and the more prolonged the heat treatment, the lower the carotenoid content becomes(25,66).

In Figure, 1.5 are presented the main variables influencing the bioavailability of the carotenoids, either increasing (left) or lowering (right) it.

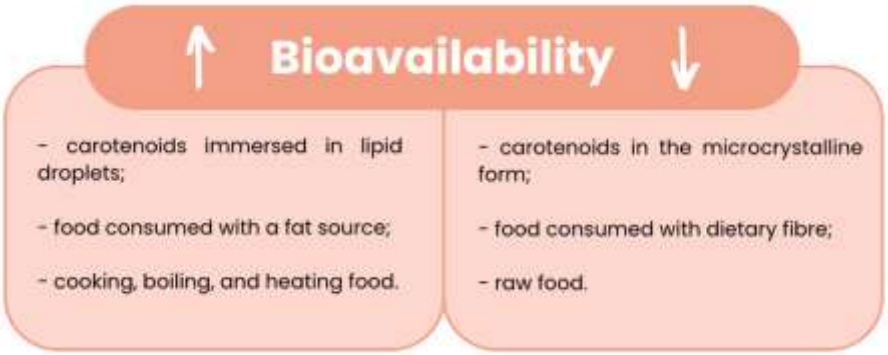


Figure 1.5 - Factors that condition the increase (↑) or decrease (↓) of the carotenoids' bioavailability. Image from (72).

Therefore, different technologies are required to increase carotenoids' solubility and bioavailability (28). The traditional methodology has been extracting carotenoids from food matrices using organic solvents such as hexane, acetone, and petroleum ether, once carotenoids are highly hydrophobic, meaning that they are insoluble in water and methanol (28). However, most of these organic solvents are toxic to human health and the extracts must be purified if they are to be used in the food industry (28).

Alternative methods to recover carotenoids have been proposed, such as Super-critical fluid extraction (SFE), High-hydrostatic pressure (HHP), and, more recently, Ohmic Heating (OH) (73). The OH method uses an electric current that is passed through a conductor matrix such as food and generates heat from the electrical resistance of the matrix (74). The authors used this methodology to extract bioactive compounds from tomato by-products and only used a mixture of 70% water:ethanol absolute (v/v) as a solvent to obtain both carotenoids and polyphenols (73,74). The authors showed that OH is a selective method and that it is possible to extract these bioactive compounds without organic solvents, substituting the traditional methods (73,74). However, OH can't extract those bioactive compounds that remain bound to dietary fibres, and it is still unknown how their potential antioxidant properties and main bioactive compounds are affected by the gastrointestinal tract during digestion (75).

1.3 Carotenoids absorption mechanism

The carotenoids' pathway throughout the gastrointestinal tract (GIT) begins in the mouth, where they are released from the food matrices and, as they progress through the stomach and intestine, become susceptible to modifications such as solubilization by the intestinal fluids (75). Subsequently, the bioactive compounds within the intestine undergo a process of selection through permeation, making them accessible for absorption into the bloodstream (75). Meanwhile, the non-bioaccessible compounds are used directly by the intestinal microbiota (75,76).

The mechanism of carotenoids' absorption can be divided into release from the food matrix, transfer to the oil phase, formation of mixed micelles and absorption itself, as illustrated below in Figure 1.6.

1.3.1 Release

The carotenoids' absorption pathway begins with mastication, which consists of the physical disruption of the food matrix that leads to their release (15). The physical form of these natural pigments is a limiting factor which conditions their release during digestion (15,77). For example, β-carotene within the food can be present in liquid crystalline form, such as in mango and papaya, or in solid crystalline form like in carrot and tomato (15,77), being the β-carotene's bioavailability higher in foods where this carotenoid is in liquid crystalline form (15,77).

1.3.2 Transfer to the oil phase

The next step consists of the carotenoids' dissolution in the gastric emulsion (digest). If carotenoids aren't completely digested from the food matrix, they cannot be transferred to the oily phase since there is no direct contact between them and the oil (15). The incorporation of carotenoids into the gastric

emulsion is also affected by several factors: soluble proteins, the surface charge of the gastric emulsion, the oil, and the amount of the carotenoid present (15).

In the case of β-carotene, soluble proteins (e.g., casein) can be highly surface-active molecules, and the particles that are formed tend to be highly negatively charged, which prevents lipid droplet aggregation and, consequently, inhibits the incorporation of these natural pigments into the gastric emulsion. However, if the pH is decreased, the concentrations of soluble proteins decrease, which means that the transfer of β-carotene to oil increases (15). The adherence of oil to the carotene-containing matrix and the β-carotene solubilization increases when the surface charge of the gastric emulsion is decreased since it allows for higher adherence of oil to the carotene-containing matrix (15). In addition, the oil and the amount of the carotenoid present will affect the carotenoid's solubility in the oily phase, determining the extension of the carotenoid transfer to the digesta (15,78).

1.3.3 Micelle formation

Bile salts are released during the passage through the small intestine, promoting the formation of mixed micelles (15). These micelles are composed of free fatty acids, monoglycerides, phospholipids, and the carotenoid, (79) and result from the action of bile salts as surfactants that reduce the size of the gastric emulsion to micelles with a diameter of approximately 80 Å (79). The amphiphilic structure of the micelles allows the lipophilic nutrients to remain soluble in the aqueous digesta (11).

This step is crucial for the absorption of carotenoids since this only occurs if they are in the form of mixed micelles, meaning that the factors that affect the micelle formation also affect the bioavailability of carotenoids in the digestion process (15). Lipids are necessary for the incorporation into micelles and to stimulate the release of bile to form mixed micelles, being the dietary fat a factor that influences the formation of micelles (15,69). However, Roodenburg et al. (69) showed that an increase in dietary fat is only beneficial to the formation of micelles until an optimal threshold. Besides the amount, also the type of fat affects the micelle formation: the longer the fatty acyl chains, the more extensive the micelle formation, and the bioavailability of the carotenoids (15).

In addition, plant-based foods contain fibres such as alginate, guar, and pectin that in the presence of β-carotene act as limiting factors since they inhibit micelle formation and decrease the bioavailability of this carotenoid (80–82).

1.3.4 Absorption

Finally, the micelles containing the carotenoid come into contact with the apical side of the intestinal epithelial cells (15), enter the enterocytes, are incorporated along with other lipids of the diet into chylomicrons, are transported across the basolateral membrane (40) and, then go into the lymphatic system and are released into circulation and distributed throughout the body (11,40).

It was initially believed that the absorption of carotenoids occurred through passive diffusions, such as dietary lipids, due to the concentration difference between the micelle and the plasma membrane of the intestinal cell (13,40). Although this mechanism is still believed to be partially valid, it has been discovered that the absorption of the carotenoids can be facilitated by membrane transporters (83), such as the scavenger receptor class B type 1 (SR-BI), the cluster determinant 36 (CD36), and NPC1- like

transporter 1 (NPC1L1) (13,40). While certain studies have demonstrated that these proteins enhance the absorption of carotenoids (84), the specific mechanisms responsible for these effects remain unclear (85).

In addition, absorption can be affected by viscosity, since it inhibits the formation of micelles and consequently decreases the amount of carotenoid in a form that can be absorbed (15), by the individual's genetic susceptibility and dose ingested (2).

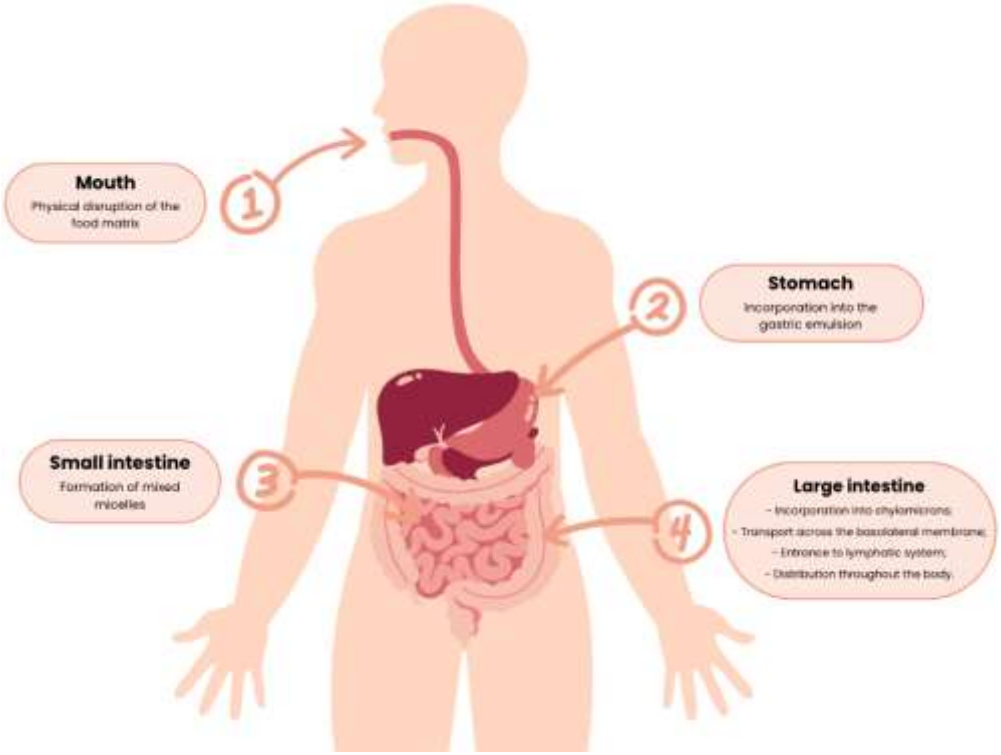


Figure 1.6 - Major steps of carotenoids absorption mechanism. Image from (72).

1.4 Intestinal Microbiota

The intestinal microbiota is composed of a complex community of microorganisms (86), which include bacteria, viruses, and some eukaryotes that colonize the digestive tract just after birth (87), and live in the digestive tracts of humans and animals (88).

Even though more than 50 bacterial phyla have been identified so far (89), the human intestinal microbiota is primarily dominated by just two of them: *Bacteroidota* and *Bacillota*, while other phyla like *Pseudomonadota*, *Verrucomicrobiota*, *Actinomycetota*, *Fusobacteriota*, and *Cyanobacteria* are found in smaller amounts (90). The *Bacillota* phylum comprises over 200 different genera, including *Lactobacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, and *Ruminicoccus* (90). On the other hand, the *Bacteroidota* phylum is primarily composed of dominant genera such as *Bacteroides* and *Prevotella* (90).

The microbial composition of the intestinal microbiota is different along the GIT (86). Although in the stomach and small digestive tract are only present few species of bacteria (91), in the colon, the microbial population is very dense with approximately 10^{12} bacterial cells per gram of intestinal content

(86). Almost 99% of the bacteria present in the intestine are anaerobes, but the cecum which connects the small intestine to the colon is composed of a high density of aerobic microorganisms (86).

Below, in Figure 1.7, are presented the principal microbial differences in abundance and composition along the entire GIT.

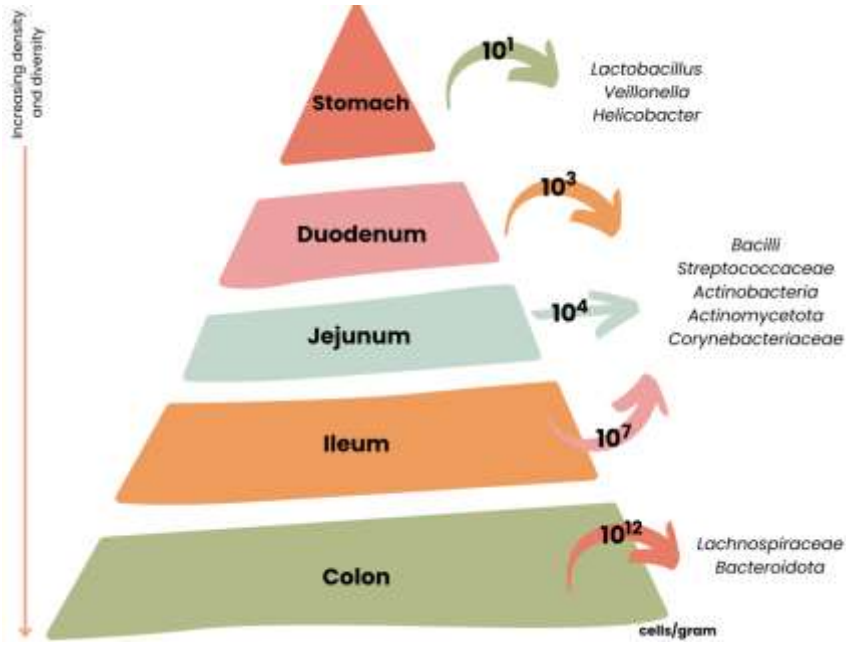


Figure 1.7 - Main differences across the GIT according to microbial abundance and composition. Adapted from (92). Image from (72).

Some of the biological functions of the intestinal microbiota are protection against pathogens by colonizing mucosal surfaces (86), enhancement of the immune system (93), production and release of different antimicrobial substances (86), and control of epithelial cell proliferation and differentiation (94), among others. Therefore, the intestinal microbiota plays a significant role in digestion and metabolism (95) as well as in maintaining normal intestinal physiology and health (86). This consists of a mutualistic relationship since the host intestine provides the necessary conditions for the bacteria to survive and reproduce and the microbiota modulates the various physical functions like nutrient processing and digestion and immune cell growth and response (86).

However, several factors can affect the composition and function of the intestinal microbiota (86). These can be individual intrinsic factors such as age, ethnicity and genetic markers or environmental factors like geographic area, lifestyle, diet, and drug intake (96,97). These factors can lead to an increment or decrease of some species, as well as modifications of their metabolites produced (98). In the case of diet, it plays a crucial role in modulating the intestinal microbiota, either in a useful or harmful way (86).

1.5 Intestinal Microbiota Metabolites

The intestinal microbiota is confined within the gastrointestinal lumen, leading to the potential for intestinal bacteria to translocate and trigger local or systemic inflammation (99,100). To overcome this spatial limitation, the intestinal microbiota releases numerous diverse metabolites (100), including bile

acids, vitamins, tryptophan, and SCFAs (101). These metabolites have far-reaching effects on various organs within the host's body, regulating local and systemic immune responses, nutrient absorption, host metabolism, and intestinal microbiota composition, all of which play a crucial role in maintaining health or contributing to the development of diseases (100).

A diverse range of metabolites can be produced and released by the intestinal microbiota, which can either be originated directly from dietary compounds, be generated by the host and chemically transformed, or be newly synthesized by the intestinal microbiota itself (102). In addition, within the intestinal microbiota, millions of microbial genes facilitate the synthesis of numerous enzymes. These enzymes can ferment dietary compounds that remain undigested by human enzymes, such as fibre or primary bile acids (103).

Carotenoids need to go through a series of processes to become biologically active and play their effects on the host: absorption, transfer to the circulating system, delivery to the target sites in the body, and metabolic conversion to the active vitamin form (100,104). Carotenoid cleavage products, like apocarotenoids, are formed through the action of specific enzymes, such as carotenoid cleavage dioxygenase (CCD) in plants (105) and β, β-carotene-15,15′-oxygenase (BCO) in vertebrates (104).

On the other hand, depending on their chemical nature, these metabolites can also play significant roles in the development and progression of diseases such as cancer, hypertension, Parkinson's, and non-alcoholic fatty liver diseases (106–109).

1.6 Interaction between carotenoids and the Intestinal Microbiota

Although the relationship between carotenoids and the intestinal microbiota remains a subject with limited available information and conclusive evidence, some studies suggested that the intestinal microbiota could play a crucial role in determining the effectiveness of carotenoids' actions (110).

Jalal et al. (111) found that excessive growth of harmful bacteria from the *Pseudomonadota* phylum led to damage to mucosal epithelial cells and increased intestinal permeability, resulting in decreased carotenoid absorption. Another study using colonic faecal samples demonstrated that carotenoids were metabolized by the intestinal microbiota, producing new compounds (112). Therefore, these studies have shown that the composition of the intestinal microbiota significantly influences the absorption and metabolism of carotenoids (110).

In addition, dietary supplementation with carotenoids such as lycopene can regulate the intestinal microbiota composition, inhibiting harmful bacteria like *Pseudomonadota* while promoting the growth of beneficial bacteria like those belonging to the *Bifidobacterium* and *Lactobacillus* genera, thereby supporting intestinal immunity and alleviating symptoms related to anxiety, colitis, and depression (113). Supplementation with β-carotene has also shown positive effects on intestinal health by increasing *Bacteroidota* and *Pseudomonadota* while decreasing harmful bacteria like *Dialister* and *Enterobacter* (110). Moreover, the administered dose of β-carotene influenced the composition of the intestinal microbiota, with different doses impacting the abundance of specific bacterial strains (110). The impact of xanthophylls, such as lutein and zeaxanthin, on the intestinal microbiota composition appears to be more significant than that of carotenes, highlighting the structural differences among carotenoids and their varied effects on the intestinal microbiota (114).

These findings suggest a structure-activity relationship between carotenoids and the intestinal microbiota, making the latter a potential target for carotenoid utilization (114). However, a comprehensive understanding of the direct interaction between carotenoids and the intestinal microbiota and their relationship is still lacking (115).

1.7 Aim of the thesis

Carotenoids, in their natural state, tend to have reduced bioavailability without having undergone any processing or treatment. This phenomenon leads to their accumulation in the colon, which is colonized by a diverse community of microorganisms that play important functions such as digestion, metabolism, and overall intestinal health. The impact of diet on the intestinal microbiota is well-established, with diet acting as one of the most influential regulators. Surprisingly, despite the significance of carotenoids in human nutrition, there is limited information available about their relationship with intestinal microbiota.

One of the challenges of carotenoids lies in their hydrophobic nature, and with their high susceptibility to light, heat, oxygen, or acids. Therefore, the formation of by-products with harmful or unknown effects may occur, impacting the overall bioaccessibility, bioavailability, and absorption of carotenoids.

In this sense, the main objective of this thesis was to study the interaction between carotenoids and the intestinal microbiota and how such may impact on important functional properties. To do so, a set of specific objectives were established and included (i) the study and evaluation of the gastrointestinal delivery system for carotenoids, (ii) the evaluation of the processes occurring during digestion, from mastication to absorption, and (iii) the assessment of the impact of the intestinal microbiota and its metabolites on the stability and functionality of carotenoids.

CHAPTER II

Materials and Methods

2.1 Material and Methods

2.1.1 Samples preparation

For the preparation of the carotenoids' samples, it was taken into account for the fact that pure β-carotene is rapidly degraded in the gastric phase during digestion, and for this reason, it is necessary to protect it from degradation without inhibiting the intestinal release (15,116). Therefore, since carotenoids are lipophilic compounds and it is known that their bioavailability increases when they are consumed with a fat source (26,27), the carotenoids samples were prepared with a solution containing polysorbate 20M (Tween 20) that is a lipidic additive frequently used in the food industry.

Hence, the solution of β-carotene was prepared with 11.9 mg of β-carotene (Extrasynthese - Lyon, France), 19 mL of a phosphate buffer solution (PBS) at pH 7.4 and 1 mL of Tween 20 (Sigma-Aldrich, Missouri, USA). This solution was then submitted to solid-phase extraction to obtain better results in further analysis since it permits compounds to be extracted, cleaned up after, and concentrated before being quantified. The lutein and lycopene solutions (Extrasynthese - Lyon, France) were prepared similarly with 500 µL of the respective carotenoid (concentration: lutein = 1 mg/mL; lycopene = 2.5 mg/mL), 19 mL of PBS pH 7.4 and 1 mL of Tween 20.

In the case of the mixture of the three carotenoids (β-carotene, lutein and lycopene) it was prepared with 500 µL of each carotenoid (using the solutions previously prepared) and 1.5 mL of the solution of PBS pH 7.4 and Tween 20 (prepared before).

For the algae *Osmundea pinnatifida* (Praia da Agudela, Portugal) solution, the first step was the algae grinding to homogenize the sample, which was done in a kitchen robot (Bimby TM6 Vorkwerk). Then, a suspension with 3 g of the algae and 3 mL of a solution with PBS pH 7.4 and Tween 20 was prepared.

2.1.2 *In vitro* gastrointestinal digestion (INFOGEST)

Simulated complete digestion of the carotenoid solutions was performed according to the standardized static digestion model INFOGEST 2.0 protocol described by Brodkorb et al. (117). The procedure consists of the simulation of the sequential phases that happen along the GIT. For each sample, one replica was performed and a negative control without carotenoid sample (PBS) was used. First, to simulate the oral phase, 3 mL of a carotenoid solution (previously prepared) was mixed with 2.4 mL of Simulated Salivary Fluid (SSF), 0.015 mL of $CaCl_2$ and 0.540 mL of α-amylase from human saliva (84 U/mg of powder; A1031-5KU; Sigma-Aldrich, Missouri, USA) and the mixture was incubated in an orbital (Orbital Shaker MaxQ 6000) for 2 min at 37°C at 200 rpm. Then, 4 mL of Simulated Gastric Fluid (SGF), 0.00250 mL of $CaCl_2$ and 0.40 mL of RGE (resuspended rabbit gastric extract - lipase and pepsin at 15 U/mg of powder; Lipolytech, Marseille, France) were added and the resulting mixture incubated in the above mentioned orbital for 2 h at 37 °C at 130 rpm to simulate the gastric phase. The intestinal phase was simulated by adding 4 mL of Simulated Intestinal Fluid (SIF), 0.021 mL of $CaCl_2$, 1.57 mL of bile salts (0.2 g/mL; bile extract porcine – B8631; Sigma-Aldrich, Missouri, USA) and 2.625 mL of pancreatin from porcine pancreas (6 U/mg of powder; P7545; Sigma-Aldrich, Missouri, USA) and then incubating in the orbital for 2 h and 30 min at 37 °C at 45 rpm. To understand the possible absorption of the carotenoids in the samples, upon digestion, a dialysis process was performed with 3.5 kDa

membranes (Pre-wetted RC Tubbing, Spectra/Por®6 Dialysis Membrane; 734–0652; VWR Chemicals, Pennsylvania, USA). The final content of the simulated digestion process was placed inside the membrane and a significant volume of water was used in the outside (enough to cover all sample that is inside the membrane). The volumes inside and outside the membrane were measured. The dialyses samples were placed in the orbital to mimic the peristaltic movements, overnight at 37 °C at 50 rpm. At the end of the process, the proportion that was found outside (OUT) of the dialysis membrane represented the sample fraction that was available for absorption and the proportion that was left inside (IN) the dialysis membrane represented the non-absorbable fraction (75,118,119). During the gastrointestinal digestion simulation, aliquots of 1 mL in each step were collected: mouth, stomach, small intestine, colon, and basolateral fraction for further analysis.

2.1.3 Bioaccessibility and recovery indexes of carotenoids compounds throughout the GIT

Bioaccessibility was defined as the proportion of the bioactive compound that could become available for absorption into the blood system:

Bioaccessibility index (%) = (BC/BCDSI) × 100 (1)

where BC is the bioactive compound content (mg) in the digested sample after the dialysis step and BCDSI is the bioactive compound content (mg) in the digested sample after the small intestinal step.

The recovery index determines the main compound concentration during each step of gastrointestinal digestion following the equation:

Recovery index (%) = (BCDF/BCTM) × 100 (2)

where BCDF represents the bioactive compound content (mg) in each digested fraction and BCTM is the initial bioactive compound content (mg).

2.1.4 High-Performance Liquid Chromatography (HPLC) Analysis

The identification and quantification of each component in the sample were obtained on HPLC-DAD Agilent 1260 InfinityII, according to the method proposed by Wright et al. (120). The samples were analysed in a reverse-column C18 (2.1 x 150 mm, 2.7 µm) and the solvents used with a flow rate of 1 mL/min were: Solvent A – acetonitrile:water (90:10 v/v); Solvent B – ethyl acetate (100%); Solvent C – Methanol:ammonium acetate (80:20 v/v). The acetonitrile and ethyl acetate were from Merck (Algés, Portugal), methanol from Fischer Scientific (Portugal) and ammonium acetate from Sigma-Aldrich, Sintra, Portugal. The pigment detection was at 436 nm for carotenoids and the standard curves of β-carotene, lutein, lycopene, β-cryptoxanthin, and zeaxanthin were performed, and the results expressed in mg/100 g of sample. Samples were analyzed in duplicate.

All samples were first submitted to solid-phase extraction (SPE) according to the method described by Shen et al. (121). C_{18} cartridges (SEP-PAK Vac 3cc, 500 mg, Waters,) were conditioned with 3 mL of acetone and 3 mL of a mixed solvent (isopropanol + ethyl acetate + water, 1 + 1 + 1, v/v/v) in sequence, respectively. The sample solution was then loaded onto the cartridge at a 1 mL/min flow rate.

The cartridge was washed with 5 mL of a mixed solvent (ethanol + water, 1 + 1, v/v), and eluted with 1 mL of acetone in sequence. The acetone, isopropanol and ethanol were from Merck (Algés, Portugal). The eluate was collected and passed through a 0.22 μm mm filter from Millipore (Billerica, MA, U.S.). The final solution was ready for the HPLC analysis. Later, the same aliquots were analysed by UPLC-qTOF MS to evaluate the metabolites that may have been generated during gastrointestinal digestion.

2.1.5 UPLC-qTOF MS analysis

The UPLC-qTOF MS analysis was used since HPLC doesn't allow a complete elucidation of the structure and identification of fragmentation patterns of the compounds. The carotenoids' analysis was performed according to the method described by Monforte et al. (122), with slight modifications in an Ultimate 3000 Dionex UHPLC coupled to an ultra-high resolution Qq-time of flight mass spectrometer (Impact II, Bruker Daltonics, Germany) with the separation of metabolites performed using a reversed-phase column ACQUITY UPLC® BEH 130Å C18, 1.7 μm, 2.1×100 mm (Waters, Milford, MA). The mobile phases used were: A (acetonitrile:water (90:10 v/v)); B (ethyl acetate); Flow: 0.250 mL/min; 0-2 min: 15 % A; 2-11.6 min: 15 to 0% A; 11.6-14 min: 0 to 15% A; 14-15 min: 15% A (123). For carotenoids, parameters for MS analysis were set using electrospray ionization (ESI) in positive ionization mode with spectra acquired over a range from m/z 50 to 2000 in an Auto MS scan mode (123). The selected parameters were as follows: capillary voltage, 4.5 kV (positive mode); drying gas temperature, 200 °C; drying gas flow, 8.0 L/min; nebulizing gas pressure, 2 bar; collision radio frequency (RF), 300 Vpp; transfer time, 120 μs; and pre-pulse storage, 8 μs. The post-acquisition internal mass calibration used sodium formate clusters, with sodium formate delivered by a syringe pump at the start of each chromatographic analysis. The data was then analysed with Metaboscape® software.

2.1.6 *In vitro* faecal fermentations

a) Collection and preparation of faecal inocula

A pool of fresh faecal samples was provided by 5 healthy unrelated anonymous volunteer donors (3 men and 2 women with ages between 23 to 39 years old meeting the criterion of ≥18 and <50 years old), selected by established criteria according to health status and dietary habits, namely absence of chronic diseases and allergies, no consumption of probiotics or prebiotics (food or supplements) in the preceding 6 months or had not received antibiotics in the preceding six months. Furthermore, an informed consent form was distributed among donors to provide information about the study and the consent certificate was signed individually by each participant. The faecal samples were maintained under anaerobic conditions for a maximum of 2 h before being used. The faecal inoculum was prepared by diluting the faecal matter in PBS pH 7,4 in an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK; 10% CO2, 5% H2 and 85% N2), and autoclaved in a stomacher bag at 230 rpm for 1 min. The inocula were then preserved in DMSO at -80 ºC until use.

b) Fermentation Medium preparation

The fermentation medium comprised 5.0 g/L of tryptone Soya Broth (TSB) without dextrose (Fluka Analytical, St. Louis, Missouri, EUA), 5.0 g/L bactopeptone (Becton Dickinson Biosciences, New Jersey,

USA), 5.0 g/L yeast nitrogen base, 0.5 g/L cysteine hydrochloride (Merck, Darmstadt, Germany), 1.0% (v/v) of salt solution A [100.0 g/L NH_4Cl (Merck, Darmstadt, Germany), 10.0 g/L $MgCl_2 \cdot 6H_2O$ (Merck, Darmstadt, Germany), 10.0 g/L $CaCl_2 \cdot 2H_2O$ (Carlo Erba, Chaussée du Vexin, France)], 0.2% (v/v) of salt solution B [200.0 g/L $K_2HPO_4 \cdot 3H_2O$ (Merck, Darmstadt, Germany)], 0.2% (v/v) of a 0.5 g/L resazurin solution (Sigma-Aldrich Chemistry, St. Louis, USA) and 1% (v/v) of a 10 mL/L of trace mineral solution (ATCC, Virginia, USA). The medium final pH value was adjusted to 6.8 and was then bubbled with N_2. Fructooligosaccharides (FOS; Nutripar, Matosinhos, Portugal) were used as a positive control and the digested carotenoids' fluids retained inside the dialysis membrane were added to the respective flasks at a final concentration of 2%. The flasks were capped and autoclaved.

c) Faecal fermentations

The flasks prepared before (2.6.2) were inoculated at 2% (v/v) with faecal inocula and incubated for 48 h at 37 ºC under an anaerobic atmosphere (10% CO_2, 5% H_2 and 85% N_2). Samples were collected after 0, 6, 12, 24 and 48 h of incubation and the pH values were measured using a MicropH 2002 pH meter (Crison, Barcelona, Spain), equipped with a 52-07 pH electrode (Crison, Barcelona, Spain). The positive and negative controls were, respectively, designated as C^+ (FOS) and C^- (plain medium), while the digested carotenoids' fluids were named β-carotene, Lutein, Lycopene, Mix and Algae. Afterwards, the samples were stored at -20 ºC until analysis. All the steps considered in this section were carried out inside an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK).

d) Faecal fermentation samples' processing

Aliquots of each sample were centrifuged for 10 min at 4000 g at 4 ºC. The resulting supernatants were used to evaluate sugars organic acids, according to section 2.7, and the pellet was used to extract the genomic DNA, according to section 2.8.

2.1.7 Sugars and organic acids analysis

Sugar consumption and organic acid production during faecal fermentation were analyzed using an HPLC system comprised of a Knauer K-1001 pump (Berlin, Germany), an ion exchange Aminex HPX87H (300 x 7.8 mm) (Bio-Rad, Hercules, USA) column and two detectors assembled in series, namely a UV-vis detector (220 nm) and a refractive index detector, both from Knauer (Berlin, Germany) at a temperature of 65 ºC. An isocratic gradient was used (13 mM H_2SO_4 Merck, Darmstadt, Germany) at a 0.6 mL/min flow rate. The injection volume was 40 µL and the running time was 30 min. Fermentation supernatants were filtered through a 0.22 µm syringe filter and each sample was injected in duplicate.

2.1.8 Bacterial population analysis

Fresh human faeces samples were examined to determine the influence of various carotenoids on the metabolic and population dynamics of the intestinal microbiota after characterising the impact of the digesting process on each sample. In order to do that, a method based on DNA extraction and 16S

ribosomal RNA gene (rRNA) amplification was employed, which may be utilised with polymerase chain reaction (PCR) and metagenomics sequencing to characterise the dynamics of the microbes.

a) DNA extraction

An NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal) was used to extract DNA with some modifications. Briefly, pellets were washed with TE buffer (pH 8.0; Tris EDTA buffer), vortexed and centrifuged at 4000 x g for 10 min at 4 ºC. Then, supernatants were discarded and 180 µL of a freshly prepared lysozyme solution (10 mg/mL lysozyme in TE buffer 1x) was added to the samples and incubated for a period of 2 h, at 37 ºC. Afterwards, samples were centrifuged at 4000 x g for 10 min at 4 ºC and supernatants were discarded. The remaining steps were performed according to the manufacturer's instructions. After extraction, DNA's purity and concentration were assessed using a Thermo Scientific™ µDropTM Plate coupled with a Thermo Scientific™ Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Waltham, USA).

2.1.9 Total antioxidant capacity

The total antioxidant capacity (AA) was measured through 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. The ABTS scavenging method was performed in a black 96-well microplate, following the method described by (124) with some modifications. 20 µL of the sample, Trolox, or solvent were added to 180 µL of $ABTS^{\bullet+}$ working solution. The mixture was incubated for 5 min at 25 °C, and the absorbance at 734 nm was measured with a Multidetection plate reader (Synergy H1, Vermont, USA).

The DPPH scavenging method was performed according to the method described by (125) with some modifications. The assay was performed in a 96-well microplate. To 25 µL of the sample, Trolox or solvent was added 175 µL of $DPPH^{\bullet}$ working solution. The mixture was incubated for 30 min at 25 °C, and the absorbance at 515 nm was measured with a Multidetection plate reader (Synergy H1, Vermont, USA).

For the antioxidant capacity assessment, the DPPH reagent (free radical, 44150) was obtained from Alfa Aesar (Kandel, Germany). Methyl-β-cyclodextrin (332615), fluorescein (F6377), ABTS reagent (A1888), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 238813) and 2,2′-azobis-(2-methyl-propionamidine)-dihydrochloride (AAPH, 440914) were purchased from Sigma-Aldrich.

2.1.10 Antidiabetic activity

The antidiabetic activity was quantified through the α-glucosidase inhibition assay that consists of a colorimetric-based quantitative method as described by Oliveira et al. (2022). 50 µL of the solutions obtained after the dialysis phase (fraction available for absorption - outside the membrane - and the non-absorbable fraction - inside the membrane - of each carotenoid condition tested and 100 µL of 0.1 M PBS (pH 6.9) containing α-glucosidase solution (1.0 U/mL) were incubated in 96-well plates at 25 °C for 10 min. After preincubation, 50 µL of a 5 mM ρ-nitrophenyl-α-D-glucopyranoside solution prepared in 0.1 M phosphate buffer (pH 6.9) was added to each well. The plate was incubated at 25 °C for 5 min, and absorbance readings were recorded at 405 nm with a microplate reader (Synergy H1, Vermont,

USA). The results were compared with the control, which contained 50 µL of buffer solution instead of an active extract. Acarbose was used as the positive standard in a concentration of 2,5 mg/mL. All assays were performed in triplicate.

2.1.11 Mutagenic and Anti-mutagenic activity

The Ames test was used to assess the mutagenicity and anti-mutagenicity of the carotenoids' samples after the colon simulated digestive process, using *Salmonella typhimurium* (His-) strain TA-98 following the method described by Coelho et al. (127). The positive control used was quercetin (Sigma, St. Louis, MO). without metabolic activation (without rat liver extract). The number of revertants was analyzed and compared with the negative control. All the assays were performed in triplicate.

2.1.12 Cytotoxicity

Human Caucasian colon carcinoma epithelial cells (Caco-2, ECACC 86010202) were acquired from the European Collection of Authenticated Cell Cultures. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 as monolayers. Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS),1% (v/v) of Penicillin-Streptomycin-Fungizone and 1% (v/v) Non-Essential Amino Acids.

Cytotoxicity evaluation was performed according to the ISO 10993-5:2009 standard. Cells were grown to 80–90% confluence, detached using TrypLE Express (ThermoScientific, Waltham, MA, USA) and seeded at 1×10^4 cells/well in a 96-well microplate (Nunclon Delta, ThermoScientific, Waltham, MA, USA). After 24 h, the culture medium was carefully removed and replaced with culture medium supplemented with test samples (at the desired concentration). DMSO (Sigma, St. Louis, MI, USA) at 30% (v/v) in culture medium was used as a death control, and plain culture medium was used as growth control. After 24 h of incubation, Presto Blue (ThermoFisher, Waltham, MA, USA) was added to each well and incubated. After this period, fluorescence (Ex: 560 nm; Em: 590 nm) was measured using a microplate reader (Synergy H1, Biotek Instruments, Winooski, VT, USA). All assays were performed in quadruplicate.

2.1.13 Statistical analysis

Data's statistical analysis was done using IBM SPSS Statistics v21.0 (IBM, Chicago, USA). The normality of the data's distribution was evaluated through Shapiro-Wilk's test. As the data proved to follow a normal distribution, One-way ANOVA, coupled with Tukey's post hoc test, was used to determine the significance of carotenoids' effect on bacterial populations at each time point. Repeated Measures ANOVA was used to evaluate the effect of the carotenoids solutions on the bacterial population over time. Differences were considered significant for p-values ≤ 0.05.

CHAPTER III

Results and Discussion

3.1 Carotenoids throughout the GIT

3.1.1 HPLC analysis

An *in vitro* simulation of gastrointestinal digestion was performed on the five samples studied: β-carotene, Lutein, and Lycopene (in 0.1% of Tween solution), a combination of these carotenoids (Mix), and the Alga *Osmundea pinnatifida*. To evaluate the impact of this process on the stability and bioavailability of the carotenoid components, the samples obtained at each stage of the simulated digestion (simulated salivary phase - SSP; simulated gastric phase – SGP; simulated intestinal phase – SIP; dialysis phase inside the membrane – IM; dialysis phase outside the membrane – OM) were analysed through HPLC and the results are presented in Table 3.1.

In Table 3.1, it is observed that β-carotene is just detected in the final stages of the process - the dialysis phases (inside and outside the membrane) - which means that a fraction of this carotene remains in the dialysis membrane to be directly used by the intestinal microbiota, and another fraction is absorbed (2.49%), being distributed throughout the body. In the salivary, gastric and intestinal phases carotenoids such as β-cryptoxanthin, lycopene and zeaxanthin are surprisingly detected, and their concentrations decrease throughout the GIT. This can be explained since carotenoids are very susceptible compounds that, in the presence of light, heat, oxygen, or acids exposure, suffer multiple reactions, particularly, conversion between carotenoids (128). These results obtained may indicate that during digestion, the carotenoids may be degraded by, for example hydrolytic enzymes, possibly originating different compounds from the original carotenoid, but also different carotenoids exposed to different conditions may lead to the synthesis of other carotenoids and/or compounds.

In the case of lutein (Table 3.1), this xanthophyll is detected from the primary step (salivary phase) until the intestinal phase along with other carotenoids such as zeaxanthin, β-cryptoxanthin, lycopene, and β-carotene. This can be explained by the fact that a fraction of lutein remained intact (although the concentration decreased throughout the tract) and the other fraction was converted into different carotenoids. However, in the dialysis phases, lutein was no longer detected: inside the membrane only zeaxanthin and β-cryptoxanthin were detected and outside the membrane, no type of carotenoid was detected, which can mean that lutein is lost for intestinal microbiota use inside the membrane but is also not absorbed. Nevertheless, other compounds generated from lutein can be released in circulation, acting in a beneficial way.

Lycopene by itself is never detected throughout the GIT (Table 3.1). However, inside the membrane is detected the carotenoid zeaxanthin that can be generated from the possible reactions (cyclization into carotene rings and hydroxylation thereof) that lycopene suffered. In addition, lycopene can originate other types of compounds that remain in the membrane and/or are absorbed, playing its beneficial functions for the body.

In the case of the Mix, none of the three carotenoids studied (β-carotene, lutein, and lycopene) were detected along the GIT (Table 3.1). From the salivary phase until the dialysis phase inside the membrane carotenoids such as zeaxanthin and β-cryptoxanthin were detected whose final concentrations were higher than in the primary phase (salivary phase), evidencing the possible occurrence of biosynthetic reactions across the GIT. In terms of the dialysis phase, it was observed that no carotene was absorbed (outside the membrane). Nevertheless, other metabolites that possibly were

generated throughout the digestion process, could have been absorbed and acted in a way that can provide benefits for human health.

Table 3.1 – The carotenoids profile found in each tested condition at each simulated gastrointestinal (GIT) phase, with their respective concentrations (Mean ± standard deviation (SD)) in mg/L. SSP - simulated salivary phase; SGP - simulated gastric phase; SIP - simulated intestinal phase; IM - dialysis phase inside the membrane; OM - dialysis phase outside the membrane; NI - not identified.

	β-carotene		**Lutein**		**Lycopene**		**Mix**	
GIT phase	**Carotenoids identified**	**Mean ± SD (mg/L)**	**Carotenoids identified**	**Mean ± SD (mg/L)**	**Carotenoids identified**	**Mean ± SD (mg/L)**	**Carotenoids identified**	**Mean ± SD (mg/L)**
SSP	β-cryptoxanthin	$3x10^{-3} \pm 9x10^{-5}$	Lutein	$9x10^{-6} \pm 3x10^{-7}$	NI		Zeaxanthin	$2x10^{-6} \pm 3x10^{-8}$
	Lycopene	$3x10^{-2} \pm 9x10^{-4}$	Zeaxanthin	$1x10^{-6} \pm 1x10^{-8}$			β-cryptoxanthin	$6x10^{-6} \pm 1x10^{-7}$
			β-cryptoxanthin	$9x10^{-6} \pm 1x10^{-8}$				
			Lycopene	$2x10^{-7} \pm 4x10^{-9}$				
			β-carotene	$2x10^{-7} \pm 2x10^{-9}$				
SGP	β-cryptoxanthin	$9x10^{-4} \pm 6x10^{-5}$	Lutein	$4x10^{-4} \pm 6x10^{-5}$	NI		β-cryptoxanthin	$2x10^{-5} \pm 2x10^{-7}$
	Lycopene	$5x10^{-3} \pm 6x10^{-5}$	Zeaxanthin	$6x10^{-5} \pm 6x10^{-7}$				
			β-cryptoxanthin	$5x10^{-4} \pm 4x10^{-7}$				
			Lycopene	$4x10^{-6} \pm 8x10^{-7}$				
			β-carotene	$2x10^{-5} \pm 3x10^{-8}$				
SIP	β-cryptoxanthin	$9x10^{-4} \pm 3x10^{-5}$	Lutein	$2x10^{-3} \pm 1x10^{-4}$	NI		β-cryptoxanthin	$1x10^{-4} \pm 2x10^{-6}$
	Zeaxanthin	$8x10^{-4} \pm 3x10^{-4}$	Zeaxanthin	$9x10^{-5} \pm 4x10^{-6}$				
			β-cryptoxanthin	$8x10^{-4} \pm 5x10^{-6}$				
IM	β-cryptoxanthin	$5x10^{-4} \pm 2x10^{-4}$	Zeaxanthin	$1x10^{-4} \pm 5x10^{-6}$	Zeaxanthin	$2x10^{-2} \pm 4x10^{-5}$	Zeaxanthin	$4x10^{-4} \pm 2x10^{-6}$
	β-carotene	$6x10^{-2} \pm 3x10^{-4}$	β-cryptoxanthin	$1x10^{-3} \pm 1x10^{-5}$			β-cryptoxanthin	$2x10^{-3} \pm 4x10^{-6}$
OM	β-carotene	$1x10^{-2} \pm 5x10^{-4}$	NI		NI		NI	

For the *O. pinnatifida* algae no type of carotenoid whatsoever was found and identified by HPLC. This can be an indicator that in a complex matrix such as algae, the bioaccessibility of carotenes can be strongly affected, requiring the application of methods of extraction to release these pigments and deliver them separately to be effectively bioaccessible (129). The process of extracting carotenoids frequently includes the extraction of additional substances like chlorophylls, lipids, and esters, being essential to proceed to eliminate these potentially disruptive compounds that might affect the analysis (129), possibly the reason why no carotenoids were detected by HPLC.

Therefore, all the samples from the different conditions assayed were then analysed through UPLC-qTOF MS since HPLC didn't allow a complete elucidation of the structure and identification of fragmentation patterns to identify the present compounds, and the results are presented below in Figures 3.1 and 3.9.

3.1.2 Bioaccessibility and recovery indexes of carotenoids throughout the GIT

It was not possible to calculate the bioaccessibility indexes for the different carotenoids tested in the different condition (plain, mixture and complex alga matrix) since after the dialysis step (OM) there were no carotenoids being identified for the majority of the conditions tested, which means that these natural pigments did not become available for absorption (or were degraded beforehand). In the case of β-carotene, although it was identified after the dialysis step, this carotenoid was not identified in the sample after the small intestinal step (SIP). Therefore, it is possible to conclude that these carotenoids are not bioaccessible for absorption under these tested conditions.

In the case of the recovery indexes, it was just possible to calculate them for the β-carotene and lutein groups (and for lutein just from the first three stages SSP, SGP and SIP), since in the other conditions, the initial carotenoids were converted into other natural pigments during the simulated digestion. Below, in Table 3.2, are presented the recovery indexes (%) from the samples mentioned before:

Table 3.2 - Recovery indexes (%) for the carotenoids β-carotene and lutein at the various sampling points.

Carotenoid	GIT phase	Recovery index (%)
β-carotene	IM	0.2
	OM	0.5
Lutein	SSP	0.000005
	SGP	0.0005
	SIP	0.004

In Table 3.2 it is possible to observe that the recovery indexes are very low for all samples, being the highest recovery obtained for β-carotene outside the membrane. However, in a general way, it is possible to conclude that during the digestion of these natural pigments it is almost impossible to recover them since, as observed, they are converted into other compounds during this process.

3.1.3 Metabolomics analysis throughout the GIT

The different samples from the different conditions assayed were analysed by UPLC-qTOF MS to understand the major differences in terms of compounds' fragments in the distinct phases of the simulated digestion: SSP, SGP, SIP, IM and OM.

Therefore, Figure 3.1 presents the results for plain β-carotene (Figure 3.1a), plain lutein (Figure 3.1b), plain lycopene (Figure 3.1c), the Mix (Figure 3.1d) and the *O. pinnatifida* algae (Figure 3.1e) conditions along the GIT through principal component analysis (PCA).

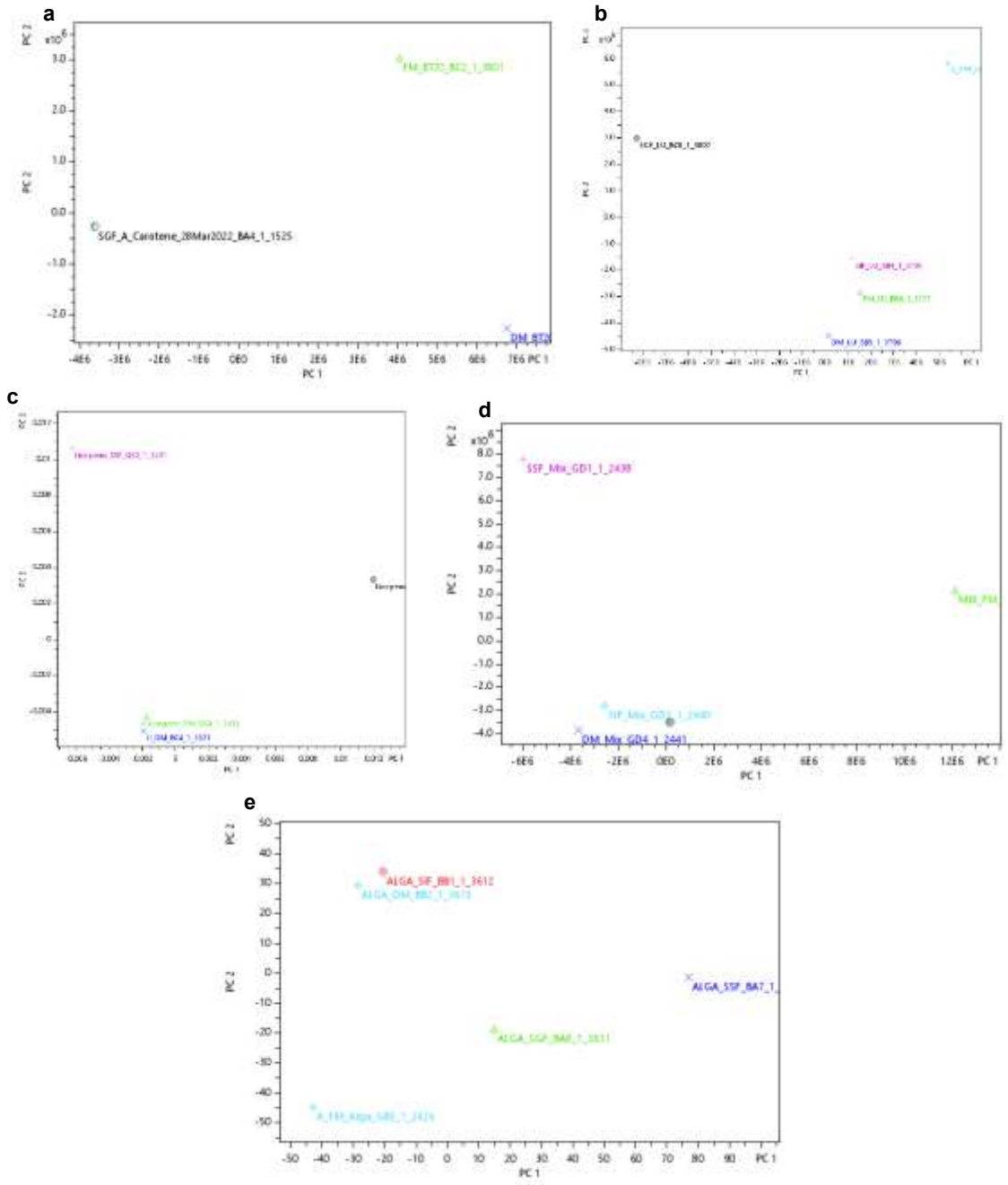


Figure 3.1 - PCA results of the carotenoids' profiles found in the different samples along the GIT from the different conditions assayed. Results were obtained through Metaboscape® software. + Mouth – SSP; ● Stomach – SGP; ◆ Intestine – SIP; × Colon – IM; △ Basolateral – OM.

PCA was the method chosen to analyse the results from the UPLC analysis to try and simplify the complex data obtained. PCA has a primary objective of decreasing the complexity of a dataset while preserving its underlying information. When dealing with datasets comprising multiple groups of variables, it is common to find different variables exhibiting similar behaviour, thus containing redundant information. In the context of mass spectra, these variables refer to the intensities at specific masses (130).

For β-carotene (Figure 3.1a), it is possible to observe that in the first 3 phases (SSP, SGP and SIP) the variance of the fragments' peaks obtained was very similar between them since they are clustered together, which can indicate underlying patterns or relationships between the fragments obtained in these phases. In the case of this carotene inside and outside the membrane, the variance of the peaks obtained are very distinct from each other and from the fragments obtained in the first 3 phases, since they are significantly deviated in the graph, which can indicate that the fragments originated are very distinct.

In the case of lutein (Figure 3.1b), the variance of the fragments' peaks in the first 2 phases (SSP and SGP) are considerably deviating in the graph suggesting that the fragments obtained in these phases have distinct origins. On the other hand, the fragments obtained in SIP, IM and OM may have some relationship since the variance of the fragments' peaks is closer in the graph, indicating an increasing on bioaccessibility by these fragments.

The PCA analysis for lycopene (Figure 3.1c) demonstrated that the fragments obtained in the SSP could be distinct from the ones obtained in the SGP and SIP since the variance of the fragments' peaks obtained in the first phase is different from the variance obtained in the gastric and intestinal phases. Inside and outside the membrane, the variance of the fragments' peaks is almost clustered, which can indicate some relationship between the fragments obtained in these 2 phases. However, the possible fragments obtained inside and outside the membrane are different from the SSP, SGP and SIP.

For the Mix group (Figure 3.1d), analysing the position of the variance of the fragments' peaks obtained along the GIT, it is possible to conclude that the fragments originated in each phase are very distinct from each other, except for SGP, SIP and IM which positions in the graph are similar possibly indicating a relationship between the fragments obtained.

In Figure 3.1e, it is possible to observe the PCA analysis for the *O. pinnatifida* algae condition. The variance of the fragments' peaks obtained in the SIP and IM are very similar, which can indicate any type of relationship between the fragments obtained in these 2 phases. On the other hand, the variance of the peaks obtained in these phases is significantly deviated in the graph from the variance of the peaks of SSP, SGP and OM and significantly deviated from each other. This can indicate that the fragments obtained in these phases may not have a relationship between them.

Therefore, it is possible to conclude that the relationship that the fragments obtained in each GIT phase may have, does not follow a tight correlation. The possible similarity that could exist between the fragments of these different phases is distinct between the carotenoids' groups.

3.1.4 Microbiota population analysis

After characterizing the impact of the digestion process on each sample the study proceeded to the evaluation of fresh human faecal samples to assess the effect of different carotenoids on the intestinal microbiota's metabolic and population dynamics. For that, a technique based on the extraction of DNA and the amplification of the 16S ribosomal RNA gene (rRNA) was used, which can be exploited with polymerase chain reaction (PCR) and metagenomics sequencing to characterize the microbial dynamics.

a) Relative abundance

The main taxa present in each tested condition (control, β-carotene, lutein, lycopene, Mix, and Alga) at phylum and genus levels were chosen based on the findings of the taxonomic annotation to create the distribution histogram of the relative abundance (RA) of taxa. The relative taxonomic abundances for phyla and genera found in each tested condition are illustrated in Figure 3.2a and 3.2b, respectively.

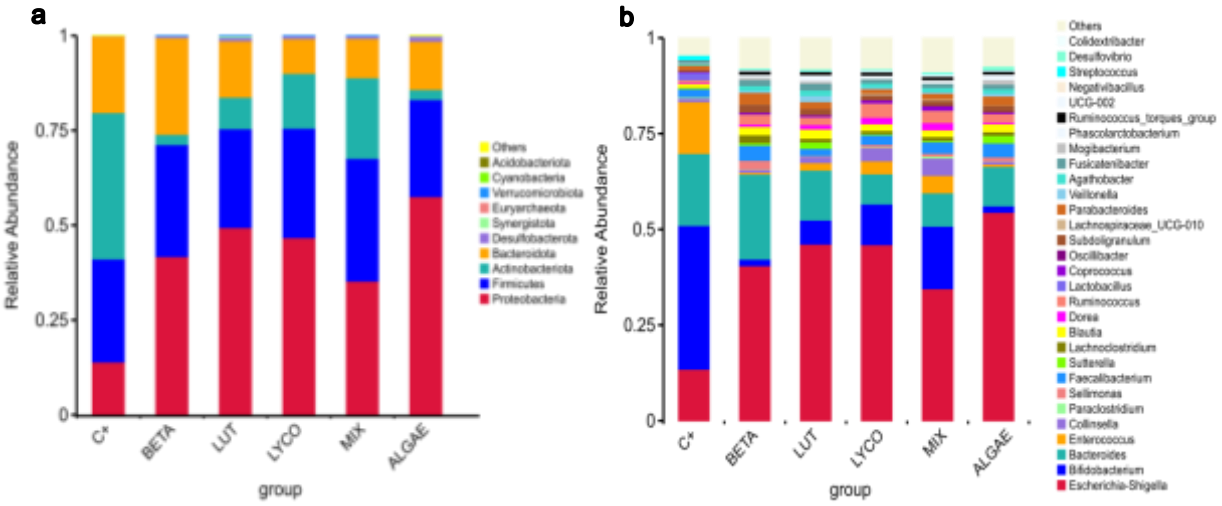


Figure 3.2 - Phyla and genera relative taxonomic abundance from each sample condition. (C^+: control; BETA: β-carotene; LUT: lutein; LYCO: lycopene; MIX: mix; ALGAE: *O. pinnatifida*).

In Figure 3.2, it is possible to observe the RA of some bacterial groups in the intestinal microbiota for each sample condition after 48 h of the simulated fermentation process.

At the phylum level (Figure 3.2a), the intestinal microbiota was mainly composed of *Bacteroidota*, *Bacillota*, *Pseudomonadota*, and *Actinomycetota*, which is consistent with the results obtained in other studies (110,114).

From the *Bacteroidota* phylum, it was detected the presence of two different genera (Figure 3.2b): *Bacteroides* and *Parabacteroides*. For the *Bacteroides* genus, it was observed that, when compared with the control, its RA decreased in all sample conditions, except for the plain β-carotene sample in which a slight increase in the respective RA was observed. In the case of the *Parabacteroides* genus, its RA increased in all sample conditions when compared with the control, meaning that carotenoids could provide good conditions for the growth of the bacteria from this genus.

In the case of the *Bacillota phylum*, 23 different genera were detected (Figure 3.2b) belonging to families such as: *Lachnospiraceae* (*Sellimonas*, *Lachnoclostridium*, *Blautia*, *Dorea*, *Coprococcus*, UCG-010, *Agathobacter*, *Fusicatenibacter*, *Ruminococcus torques* group), *Enterococcaceae* (*Enterococcus*), *Peptostreptococcaceae* (*Paraclostridium*), *Ruminococcaceae* (*Faecalibacterium*, *Ruminococcus*, *Subdoligranulum*, *Negativibacillus*), *Lactobacillaceae* (*Lactobacillus*), *Oscillospiraceae* (*Oscilibacter*, UCG-002, *Colidextribacter*), *Veillonellaceae* (*Veillonella*), *Anaerovoracaceae* (*Mogibacterium*), *Acidaminococcaceae* (*Phascolarctobacterium*), and *Streptococcaceae* (*Streptococcus*).

For the *Lachnospiraceae, Ruminococcaceae* and *Oscillospiraceae* families, the tendency observed was an increase of the RA (Figure 3.2b) for all the tested conditions (in comparison with the control), except for the UCG-010, *Faecalibacterium*, *Negativibacillus* and *Colidextribacter* genera which RA decreased when plain lutein was tested. The RA of the *Veillonellaceae*, *Anaerovoracaceae* and *Acidaminococcaceae* families was higher than the control for all conditions, which means that the samples favoured the growth of these bacteria. On the other hand, the RA of the genera *Enterococcus*, *Lactobacillus*, *Streptococcus* and *Paraclostridium* decreased in all carotenoid groups except for the Mix sample, in which RA increased compared to the control for the *Paraclostridium* genus.

In the case of the *Pseudomonadota* phylum, the *Escherichia, Shigella* and *Sutterella* genera were detected (Figure 3.2b), and their RA was higher than that of the control for all samples.

Regarding the *Actinobacteriota* phylum 2 different genera were detected (Figure 3.2b): *Bifidobacterium* and *Collinsella*. In the case of *Bifidobacterium,* the RA was lower than that reported for the control in all conditions tested; in *Collinsella*, the RA was higher for lutein, lycopene, and the Mix and lower for β-carotene and the *O. pinnatifida* algae.

In a general way, the carotenoid samples demonstrated a positive role in modulating the intestinal microbiota since they promoted the growth of beneficial phyla such as *Lachnospiraceae*, *Proteobacteria* and *Bacteroidetes*. However, there was also observed an increase in the RA of *Escherichia-Shigella* which are bacteria usually associated with the loss of beneficial functions, such as the production of SCFAs (131). Since below in point 3.1.7, the results obtained related to the organic acid production demonstrated that carotenoid sample groups induced the production of these types of acids (when compared with the control), we may affirm that the increase in the RA of *Escherichia-Shigella* bacteria was not significant.

b) Compositional analysis

According to the microbial abundance information on principal genera present in all sample conditions the heatmap (Figure 3.3a) was drawn to check whether the samples with similar processing are clustered or not, as well as the possible similarities and differences between groups. In addition, Figure 3.3b presents a cladogram that provides valuable insights into the microbial evolutionary relationships among the different tested groups, based on their genetic similarities and evolutionary history.

In Figure 3.3a it is possible to observe the most abundant genus and phylum in each sample. For plain β-carotene, the most abundant phyla were *Bacillota* and *Bacteroidota*. When compared with the control, the main differences were that the genus of the *Bacillota* family changed, increasing the ones

from the *Lachnospiraceae* family and that bacteria such as *Lactobacillus, Enterococcus, Streptococcus* and *Bifidobacterium* decreased its abundance, which was consistent with the results of other studies (110) and supports that the β-carotene can play positive roles in intestinal health.

In the case of plain lutein, the most abundant phylum continues to be *Bacillota* as in the control and in β-carotene, but more diverse at the family level, in contrast with the predominance of *Lachnospiraceae* family in β-carotene. Nevertheless, there is an increase in the *Lachnospiraceae* family and a decrease in *Lactobacillus, Enterococcus, Streptococcus* and *Bifidobacterium* bacteria, which corroborates previous results that sustain the beneficial roles of lutein in intestinal well-being (114).

In the plain lycopene and Mix samples, the *Bacillota* phylum continues to lead in terms of abundance. Regarding lycopene, the most abundant genus was *Ruminococcus* (from *Bacillota* phylum) and in the Mix was *Collinsella* (from *Actinobacteriota* phylum). In the alga sample, it was evident more differences compared with the control, since the genus of bacteria more abundant were from different phyla: *Desulfobacteriota, Bacillota* and *Pseudomonadota*.

Therefore, it can be said in a general way that an increase in *Bacteroidota* and *Bacillota* phyla, in particular, an increase in the *Lachnospiraceae* family and a decrease in *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium* bacteria, supports that carotenoids can play positive roles in intestinal health.

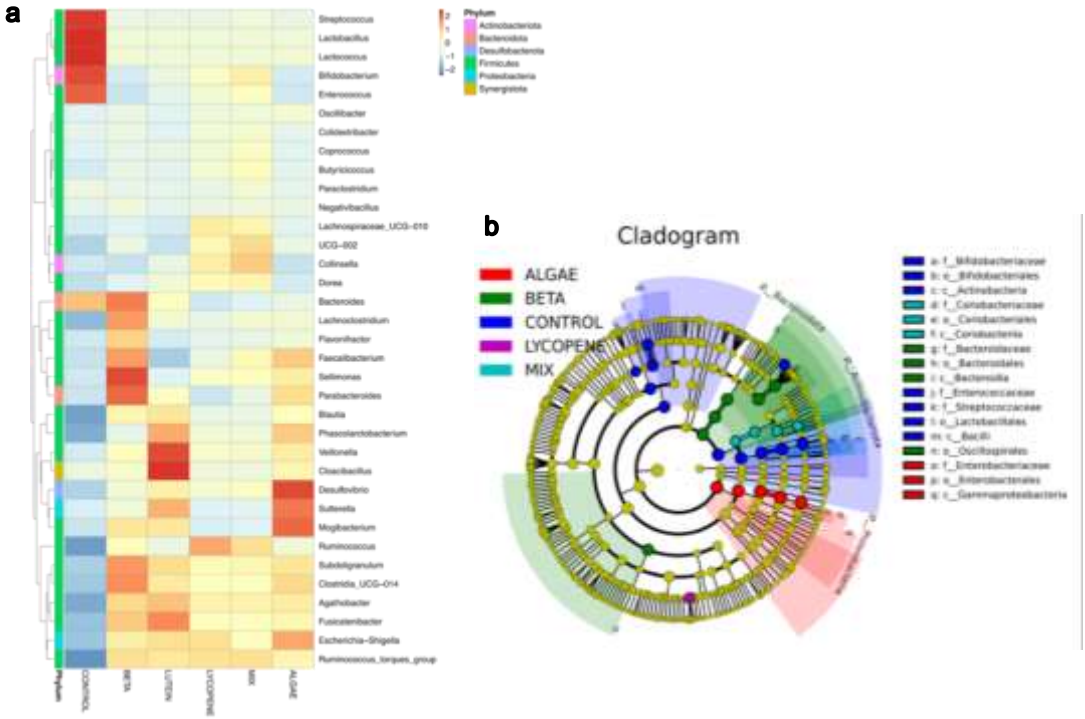


Figure 3.3 - Composition analysis of intestinal microbiota in each tested condition of carotenoids a: Taxonomic abundance cluster heatmap. b: Graphical representation of phylogenetic relationships – Cladogram.

In Figure 3.3b, it is possible to observe the phylogenetic relationships between each group. Each circle stands for a different taxon at the relevant taxonomic level, with the circles radiating from the inner side to the outer side representing the taxonomic rank from phylum to genus. Each circle's diameter

corresponds proportionately to the relative abundance of each taxon. Taxa with significant differences are highlighted in the colour of the respective group, whereas taxa with non-significant differences are highlighted in yellow.

Therefore, analysing Figure 3.3b, it is possible to corroborate the fact that the most abundant phyla present are *Bacteroidota, Pseudomonadota*, and *Actinomycetota*.

For plain β-carotene, in comparison with the other conditions, the most abundant phylum is the *Bacteroidota*, but the *Oscillopirales* order was significant in this condition. In the case of plain lutein, it is absent from the Figure, which indicates that there are no species with significant differences in this condition.

Lycopene has a phylogenetic relationship more distant from the other tested groups since its representation is not contained in the circles corresponding to the phyla with the highest relative abundance. However, by analysing the heatmap (Figure 3.3a) it is possible to observe that the phylum represented in the Cladogram (Figure 3.3b) may correspond to *Bacillota*, since it is the phylum with the highest relative abundance in this group.

In Figure 3.3b is also possible to observe that *Actinomycetota* is the most significant phylum in the Mix condition and the *Pseudomonadota* phylum in the Algae, in comparison with the other tested conditions.

Therefore, it is possible to conclude, that these results corroborate the ones previously analysed in Figure 3.2 and Figure 3.3a.

c) Flower diagram

Below, Figure 3.4 presents the flower diagram of each sample group. In the flower, each petal represents one group sample, different colours represent different groups, the middle core number represents the number of feature sequences shared by all groups, and the number on the petal represents the number of feature sequences unique to this group.

Therefore, it is possible to observe that the sample groups share 222 feature sequences with each other, and that the plain β-carotene and the Algae conditions have the highest number of unique feature sequences. This indicates that these two groups have more unique species of bacteria present, which means a higher bacterial diversity when compared with the other conditions.

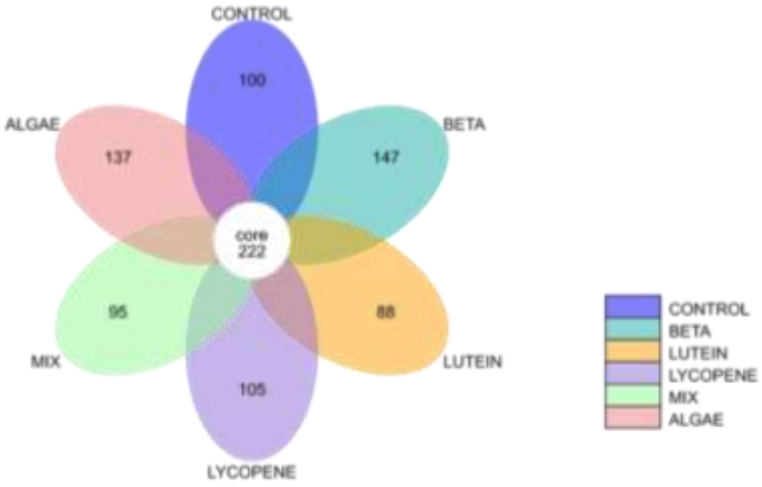


Figure 3.4 - Flower diagram of each sample group.

d) Ternary plot

To find the differences in dominant taxa among the three groups of samples at phylum rank, the top 10 taxa with the average abundance of the three groups of samples at phylum rank were selected to generate a ternary plot (131). The ternary plot in phylum is represented, below, in Figure 3.5:

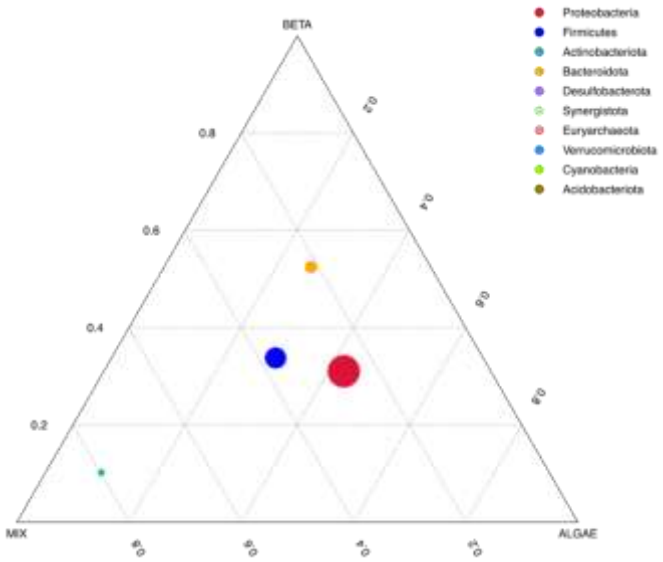


Figure 3.5 - Ternary plot of the plain β-carotene, Mix and Alga assay conditions.

The three vertexes represent the three groups: β-carotene, Mix and alga. The circles represent the dominant taxa, and the size of the circles is proportional to the relative abundance. The sample to which the circle is close to has a higher abundance of this phylum.

In Figure 3.5, it is possible to observe that the dominant taxa are the *Pseudomonadota, Bacillota, Bacteroidota* and *Actinobacteriota* phyla, which are in decreasing order of their relative abundance. The circle representative of the *Actinobacteriota* phylum is significantly closer to the Mix condition, indicating that this phylum is more abundant in this group when compared with the other two groups. Although the other three circles occupy a very central position on the ternary plot, it is possible to observe that the *Bacteroidota* phylum is slightly more abundant in the plain β-carotene sample, the *Pseudomonadota* phylum is to some degree more abundant in the alga sample and, finally, the abundance of the *Bacillota* phylum is to some extent higher in the Mix sample.

e) α-diversity analysis

The α-diversity is applied to the analysis of the microbial community diversity within a sample (132), in which the Shannon and Simpson indexes are calculated based on Operational Taxonomic Unit (OTU) species and abundance, allowing the analysis of the species richness and evenness (133).

The Shannon diversity index is calculated by considering the total number of taxa in the sample (richness), and the proportion of each taxon (abundance). On the other hand, the Simpson index characterizes the diversity and uniformity of species distribution within a community by calculating the probability that two randomly sampled individuals belong to different species. Therefore, the higher the Shannon index and lower the Simpson index, the greater the variety of species in the sample.

The boxplots were formed to analyse the differences in α-diversity indices between groups and are presented in Figure 3.6, which can reflect the maximum, minimum, median and abnormal value of the index from each assay condition:

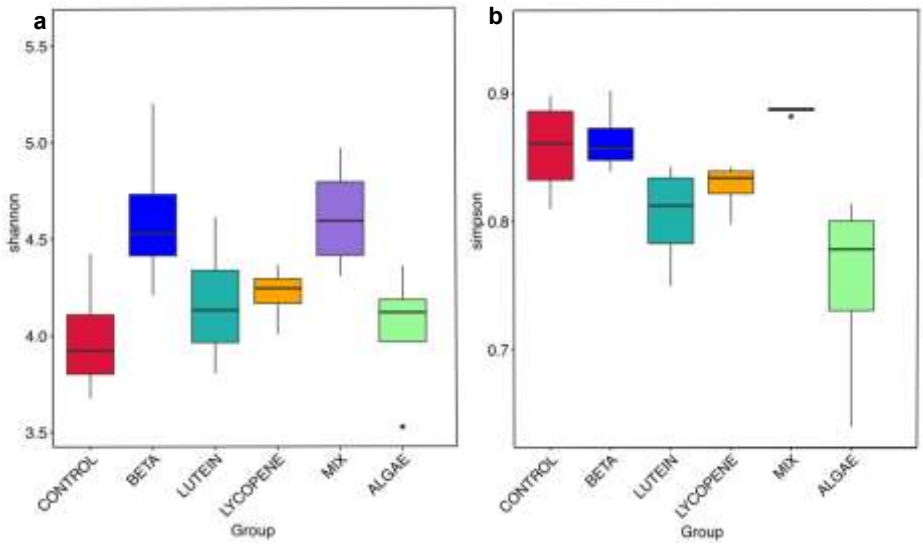


Figure 3.6 - Boxplots for the α-diversity indices through Shannon (a) and Simpson's (b) tests. Significant differences were obtained ($p < 0.05$).

In Figure 3.6a, it is possible to observe throughout the median value that the Mix and plain β-carotene samples have the highest Shannon index value and the Algae and Lutein samples have the lowest Shannon index values. When comparing the median value of the Simpson index (Figure 3.6b), it can be observed that the Simpson index value of the Algae samples is lower than that registered by the lutein samples, meaning that the samples with the greater variety of species belong to the Algae samples. In addition, Figure 3.6b shows that the Simpson index value of the plain β-carotene samples is lower than that reported for the Mix samples, which indicates a greater variety of species in the β-carotene samples, which is corroborated by the flower diagram presented in Figure 3.4.

f) β-diversity analysis

The β-diversity is a comparative analysis of the microbial community composition of different samples. First, according to the taxonomy annotation results of all samples and the abundance information of feature sequences, the feature sequence information of the same classification is combined to obtain the species abundance information table (Profiling Table). At the same time, the phylogenetic relationship between feature sequences is used to further calculate the unweighted Unifrac distance (134,135). Unifrac distance is to calculate the distance between samples by using the evolutionary information between microbial sequences in each sample. If there are more than two samples, a distance matrix can be obtained. Then, the weighted Unifrac distance (unweighted Unifrac) is further constructed using the abundance information of the feature sequence (Lozupone CA et al., 2007). Finally, the differences between different samples (groups) were found through β-diversity index inter-group difference analysis, multivariate statistical methods such as PCA, Principal coordinates Analysis (PCoA), and Non-Metric Multidimensional Scaling (NMDS). Weighted Unifrac distance and

Unweighted Unifrac distance were selected to measure the dissimilarity coefficient between pairwise samples, which is a widely used phylogenetic measurement method in microbial community sequencing projects. The heatmap based on the Weighted Unifrac and Unweighted Unifrac distances is plotted in Figure 3.7 as follows:

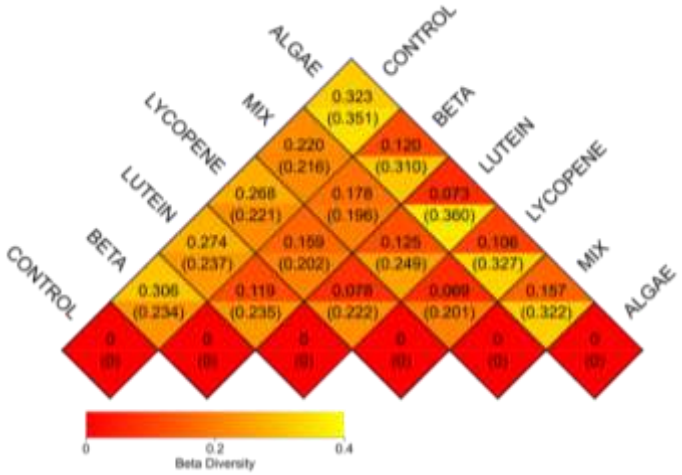


Figure 3.7 - β-diversity heatmap. Note: each grid represents a pairwise dissimilarity coefficient between pairwise samples, in which the Weighted Unifrac distance is displayed above and the Unweighted Unifrac distance conversely.

The analysis of Figure 3.7 corroborates, in a general way, the information previously discussed in section 3.4.2. The lower coefficients were obtained for the plain lycopene and the Mix samples (0.069), indicating that these two samples are not very distinct from each other, at a microbial community composition level. The higher coefficients are observed in the comparison between the control and each group, corroborating the fact that all samples are very distinct from the control.

In addition, it can be said that the plain β-carotene sample has a microbial community composition different from that of the plain lycopene and the Mix samples (coefficient of 0.159 and 0.178, respectively), as demonstrated before. Also, it can be said that the Mix sample is quite different from that of the Algae sample, presenting a coefficient of 0.157.

3.1.5 Impact of the digested carotenoids on pH evolution in faecal fermentation

The pH value is one of the main factors in the colon's environment that imposes selection pressure on the population of intestinal microbes, controlling the availability of nutrients, enzyme activity, and microbial development (136). Besides that, the pH value of the faecal fermentation medium is a very important indicator of whether the fermentation is occurring or not, and its decrease is associated with the presence of organic and short chain fatty acids (137).

When considering the variation of the pH values, it can be seen in Figure 3.8 that the tendency of its variation is common for both controls and samples: it decreases between the 0h-6h and 12h-24h of incubation and increases between 6h-12h and 24h-48h of the process, except for the positive control which pH value also decreases between the 24h and 48h.

Therefore, it can be said that during the decrease of the pH value between 0h-6h and 12h-24h of incubation, the production of organic acids could have occurred.

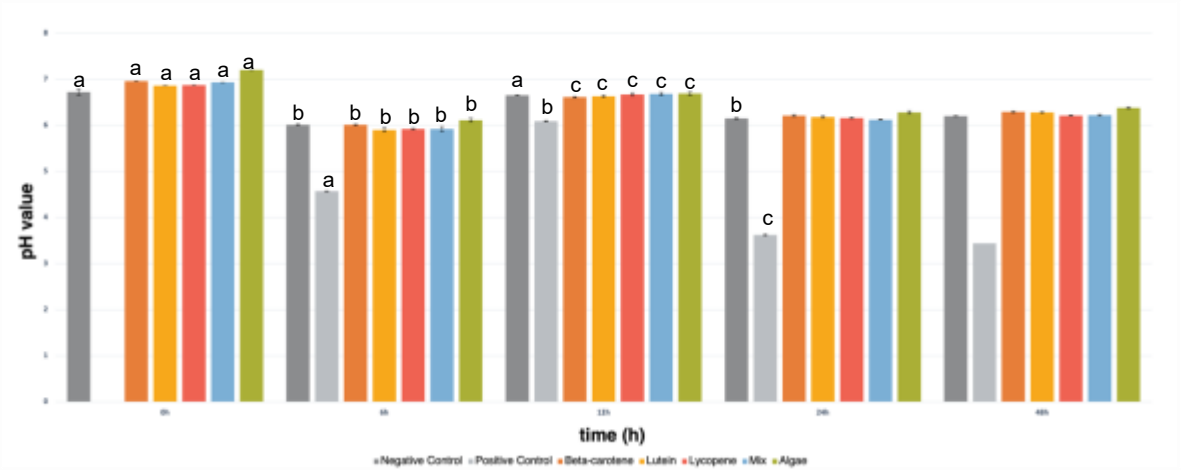
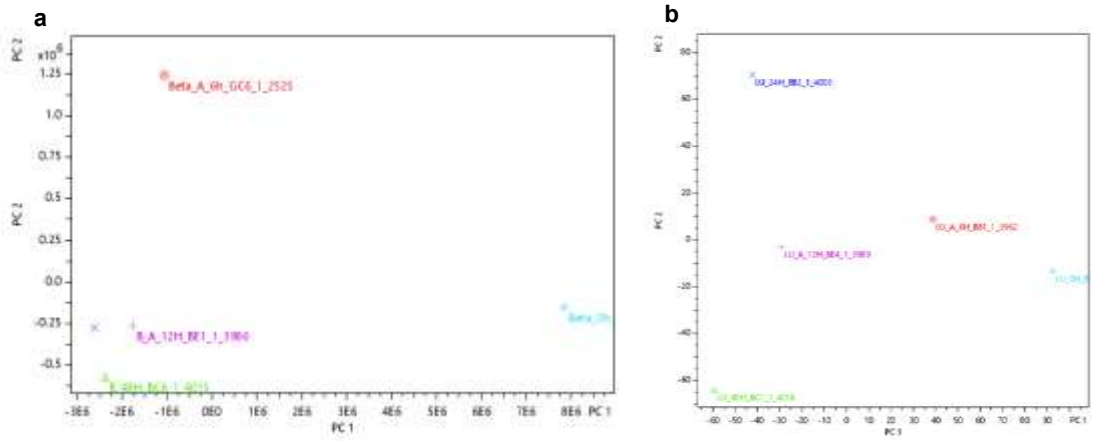


Figure 3.8 - pH values variation (Mean ± SD) over 48 h incubation of fermentation media without faecal inoculum (Negative control), with faecal inoculum (Positive control) and with inoculum in the presence of the different carotenoids' digesta sample conditions, obtained after simulated GIT digestion. Different letters mark statistically significant ($p < 0.05$) differences.

3.1.6 Metabolomics analysis of the non-absorbed residue (IM)

Each carotenoid group was also analysed by UPLC-qTOF MS to understand the influence that the intestinal microbiota during the 48 h of the simulated fermentation process may have in terms of compounds' fragments. Therefore, in Figure 3.9, it is presented the results of the plain β-carotene (Figure 3.9a), plain lutein (Figure 3.9b), plain lycopene (Figure 3.9c), Mix (Figure 3.9d) and Algae (Figure 3.9e) samples along the 48 h of fermentation through the principal component analysis (PCA).



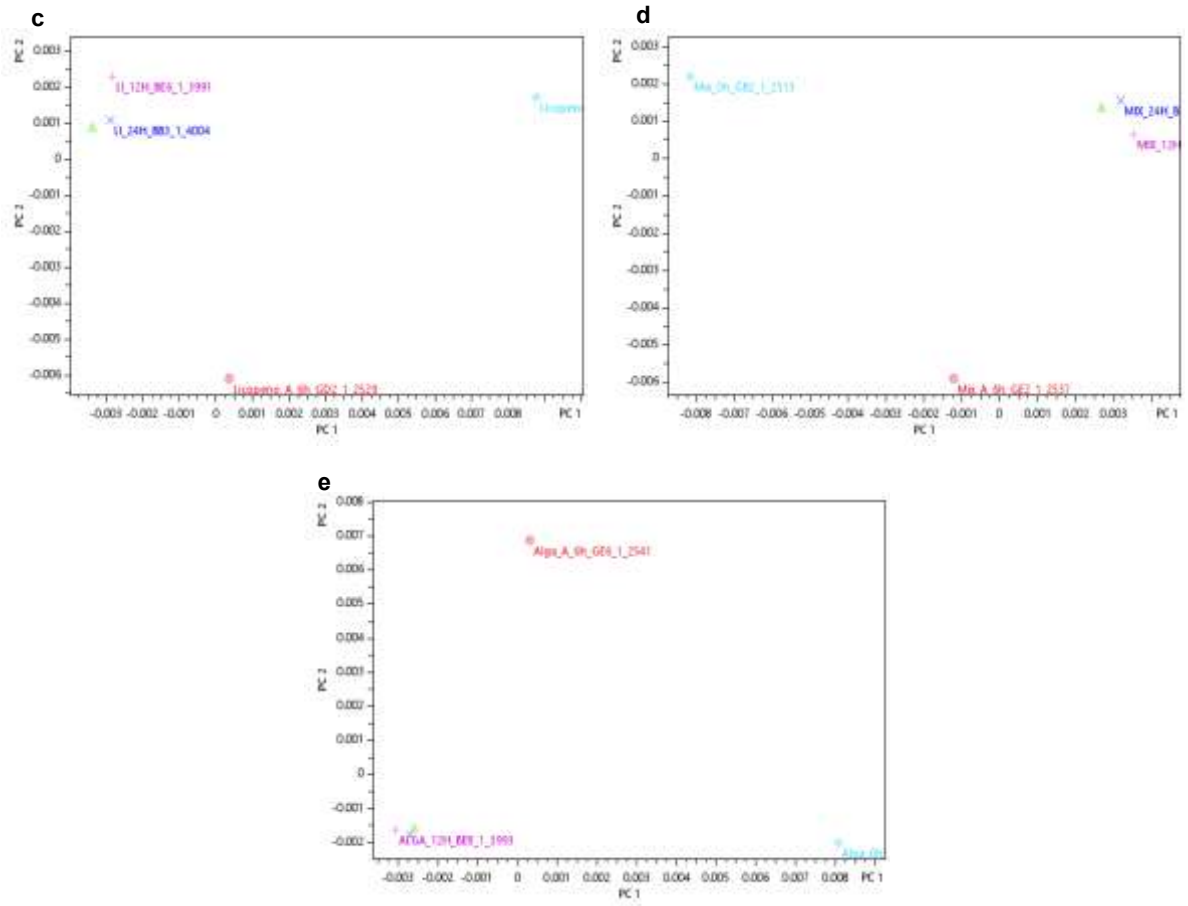


Figure 3.9 - PCA results of the carotenoids' sample groups along the 48h of fermentation obtained through Metaboscape® software. ◇ 0h; ● 6h; + 12h; × 24h; △ 48h.

For the β-carotene (Figure 3.9a), lycopene (Figure 3.9c), Mix (Figure 3.9d), and Algae (Figure 3.9e) samples it is possible to conclude that the behaviour of the fragments' peak variances was very similar throughout the 48 hours of fermentation. At the beginning of the fermentation process (first 2 time points: 0 h and 6 h), the variance of the peaks obtained are very distinct from each other and from the fragments obtained in the last 3 time points (12h, 24h and 48h), since they are significantly deviated in the graph, which can indicate that the fragments originated are very distinct. At 12h, 24h and 48h of fermentation the variance of the fragments' peaks obtained was very similar between them since they are close to each other, which can indicate some relationships between the fragments obtained in these time points.

However, in the case of lutein (Figure 3.9b), it is possible to observe that the fragments' peak variances are more dispersed in the graph, which can indicate that the fragments that originated at each time point are very distinct between them.

Therefore, it is possible to conclude that the relationship that the fragments obtained in each time point follows a tight correlation with the exception of lutein. The similarity that could exist between the fragments obtained happens for 12h, 24h and 48h of fermentation and the fragments obtained at 0h and 6h of this process are distinct from each other and from the fragments obtained in the last 3 time points.

3.1.7 Impact of the digested carotenoids on sugar consumption and organic acid production

The intestinal microbiota can break down and metabolize a variety of substrates. In the presence of the carotenoids' samples, the most significant metabolized sugar was glucose, as shown in Figure 3.10a.

The glucose concentration was initially high due to large amounts of this monosaccharide in the fermentation medium. After 6 h, there was a marked decrease in its concentration since the bacteria strains consume this substrate. After 12 h, the glucose concentration was even lower, for the Lutein, Lycopene and Mix samples, and in the Algae sample, the monosaccharide was completely consumed.

Peculiarly, in the case of the β-carotene sample, it was detected an increment of the glucose concentration after 12 h, which was also observed for maltose in a conventional extraction (SFCONV) of phytochemicals from tomato bagasse in a previous study (138). Recent research in humans demonstrated that compositional and functional differences in the intestinal microbiota contribute to metabolic dysfunctions, but there is still a lack of information regarding which compositional and/or functional characteristics of the intestinal microbiota are relevant for metabolic health, and through which mechanisms they affect host glucose homeostasis. Changes in bile acid metabolism and alterations in SCFAs production are examples of possible mechanisms, but the increase of systemic lipopolysaccharide (LPS) is the more feasible mechanism in this situation (139). In the β-carotene sample, in contrast with the other samples, there were detected more bacteria from the Bacteroidota phylum which includes Gram-negative opportunistic pathogens that contain LPS increasing this bacterial cell wall component that usually is associated with glucose intolerance pathology (139). After 24 h, the glucose in the β-carotene and lutein samples was completely consumed. The metabolic capacity to consume glucose did not differ significantly between the lycopene and the Mix samples, which stabilized after 12 h.

In Figure 3.10, it is also possible to observe that the intestinal microbiota can produce a variety of organic acids such as succinic, acetic, butyric, propionic and malic acids.

In the case of the succinic acid (Figure 3.10b), contrary to the positive control, the carotenoids' samples induced the production of this acid during the 48 h of incubation, after the simulated GIT digestion. For β-carotene and Lutein samples the concentration of acetic acid decreased until 24 h of incubation and increased from the 24 to 48 h of the process. The Lycopene sample had the same behaviour along the 48 h of incubation as the negative control, decreasing this acid concentration until the 12 h of process and between the 24 and 48 h and increasing from the 12 and 24h of incubation. For the Mix sample, the concentration of acetic acid increased in the first 6 h of incubation and decreased between 12 and 48 h. The concentration of this acid decreased during the 48 h of incubation in the Algae sample.

For the acetic acid (Figure 3.10c), the concentration variation along the 48 h of incubation was equal for Lutein and Lycopene samples, decreasing until 24 h of the process and increasing in the last 24 h of incubation. For β-carotene, the concentration of this acid decreased in the first 12 h of the process and increased after this time point until the end of the incubation time. The acid acetic concentration in the Mix sample increased between 0-6 h and 24-48 h of incubation and decreased between 6 h and 24

h of the fermentation process. In the Algae sample, the concentration variation during incubation time was the same as in the succinic acid. These results are not following those obtained in a previous study, in which for β-carotene, Lutein and Lycopene, the concentration of the acetic acid increased during all process times (114).

The concentration of the butyric acid (Figure 3.10d) varied in the same way for β-carotene and Lutein, decreasing during the incubation process, but in contrast with the positive control, the carotenoids' samples induced the production of this acid. For Lycopene and the Mix samples, this acid concentration increased after 12 h of the process, followed by a decrease and an increase, respectively, in the last 36 h of incubation. In the case of the Algae sample, the concentration of butyric acid decreased in the first 6 h of incubation and increased from this time point until the end of the process. Once again, these results are not in agreement with the ones obtained previously, which increased the butyric acid concentration during the 48 h of the process for β-carotene and Lycopene and for Lutein increased from 6 to 12 h and decreased from 12 to 24h (114).

For the propionic acid (Figure 3.10e), its concentration in the first 12 h of incubation in the presence of the carotenoids' samples, did not differ from the positive control except for the Algae sample that induced the decrease of this acid during this period. In the last 36 h of the process, the concentration variation of the propionic acid was different from the positive control, decreasing from the 12 h to the end of the process, except for Lycopene, Mix and Algae samples whose concentration increased in the last 24 h of incubation. In a previous study, the concentration of propionic acid increased until the end of the process for β-carotene and Lycopene and for Lutein increased from 6 to 12 h and decreased in the last 12 h of incubation (114).The production of malic acid (Figure 3.10f) was just induced for the β-carotene sample in the last 24 h of the process and for the Algae sample in the first 6 h of incubation.

In conclusion, although between the 0h-6 h and the 12h-24 h of incubation, the microbiota pH value decreased (Figure 3.8), it is not possible to establish a correlation with the production of organic acids (Figures 3.10b-e) since it occurred during all the 48 h of the process only variating the concentration of the acids along the incubation time. Moreover, the induction of these acids production by the tested groups corroborates the fact that *Lachnospiraceae*, *Lactobacillus*, and *Bifidobacterium* bacteria, which are known for their ability to produce these types of acids (140), showed an increase in their RA.

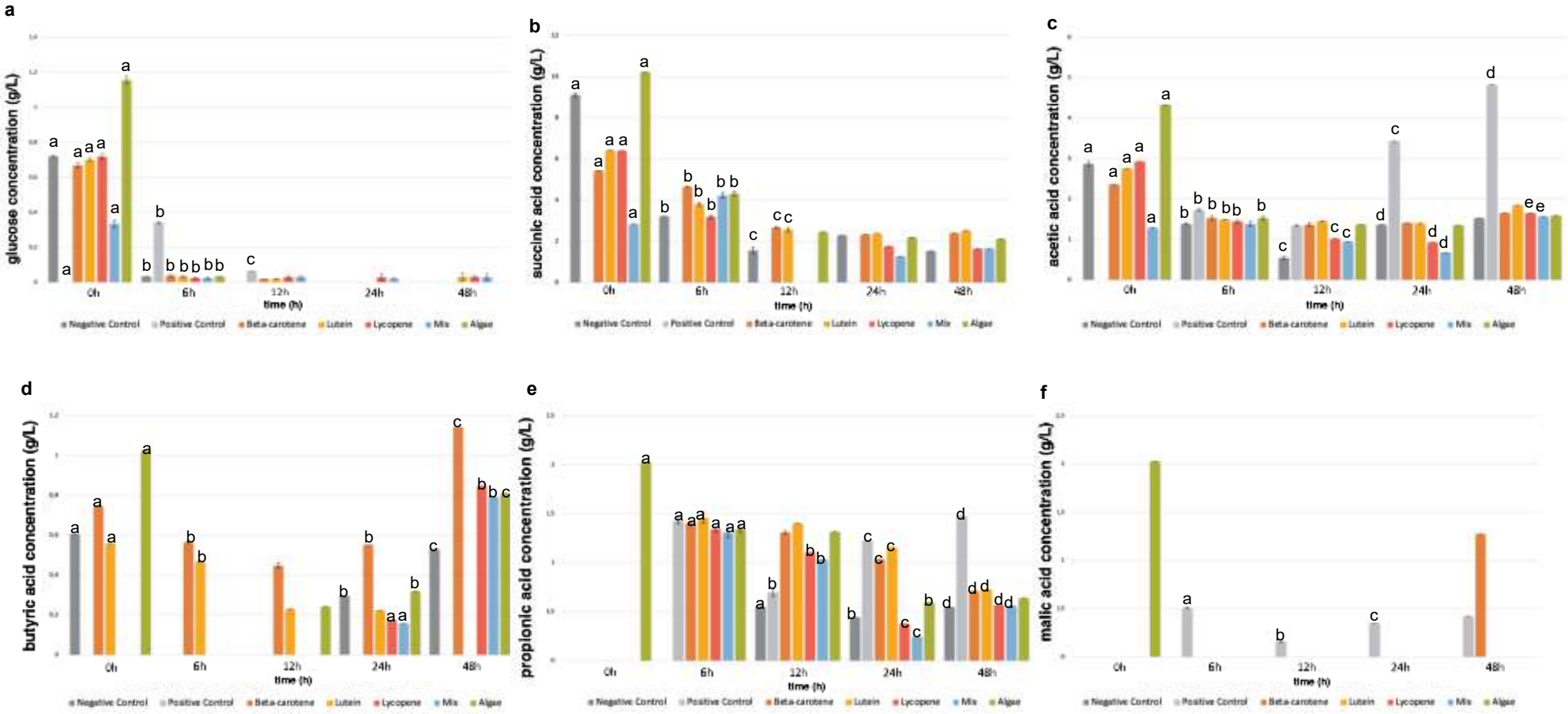


Figure 3.10 - Concentrations (Mean ± SD) of glucose consumed (a) and of organic acids (b-f) released, in g/L, during the 48 h of incubation in the presence of the carotenoids' sample groups, after simulated GIT digestion. Different letters mark statistically significant ($p < 0.05$) differences.

3.1.8 Antioxidant activity of carotenoids

The antioxidant activity of the solutions obtained after the dialysis phase (fraction available for absorption - outside the membrane - and the fraction non-absorbable - inside the membrane - of each carotenoid group was evaluated using ABTS and DPPH methods, and the samples showed similar behaviours. For all groups, it is observed in Figure 3.11 that the antioxidant activity is higher outside the membrane, meaning that the fraction of compounds that are absorbed and distributed throughout the body is more effective in its action than the fraction that remains inside the membrane.

The results obtained by the two methods also showed that the group with the higher antioxidant activity was Lutein, followed by Lycopene, the Mix, β-carotene and, finally the Algae group. This supports the fact of Lutein is a xanthophyll, being less hydrophobic than carotenes (such as β-carotene and Lycopene) and for this reason, is capable of scavenging radicals both from lipid and aqueous phases, increasing its action effectiveness.

In the case of Algae, its lower antioxidant activity can be justified since the breakup of the food matrix consists of one of several factors that affect the carotenoids' bioavailability and consequently absorption. Thereby, since the Algae was only triturated with no release of carotenoids, the bioavailability of these natural pigments was reduced and, consequently, their effectiveness as antioxidants were compromised.

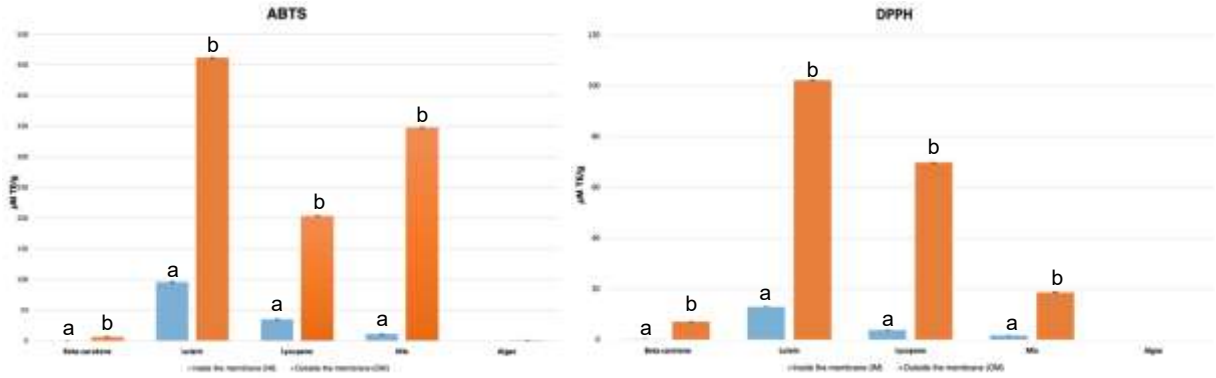


Figure 3.11 - Antioxidant activity (Mean ± SD) by DPPH (a), and ABTS (b) methods of the carotenoids' digested samples inside the membrane and outside the membrane. Different letters mark statistically significant ($p < 0.05$) differences.

3.1.9 Antidiabetic activity of carotenoids

The small intestine epithelial cell brush border contains an oligosaccharide hydrolase known as α-glucosidase, which converts oligosaccharides and disaccharides into monosaccharides (126). Inhibiting α-glucosidase activity may slow down the absorption of carbohydrates and reduce postprandial hyperglycemia, which will aid in the treatment of type 2 diabetes mellitus (T2DM) (141). Numerous polyphenols produced from plants have demonstrated potent inhibitory effects on α-glucosidase, which lowers the rate at which complex starches and oligo, tri, and disaccharides are broken down into absorbable glucose (142).

The carotenoids' digested samples were tested for α-glucosidase inhibition and compared with acarbose as a positive control. The most popular and extensively researched medicine in this family, acarbose, has been found to lengthen the lives of those with type 2 diabetes mellitus and lower the risk of cardiovascular events in those with impaired glucose tolerance (143).

The results of Figure 3.12 showed that for all sample groups, the percentage of inhibition was lower outside the membrane (fraction available for absorption) than for the non-absorbable fraction (inside it), which means that carotenoids have an anti-diabetic activity more effective for the fraction that becomes available in the intestine after absorption (IM). Acarbose presented an α-glucosidase inhibition of 92%.

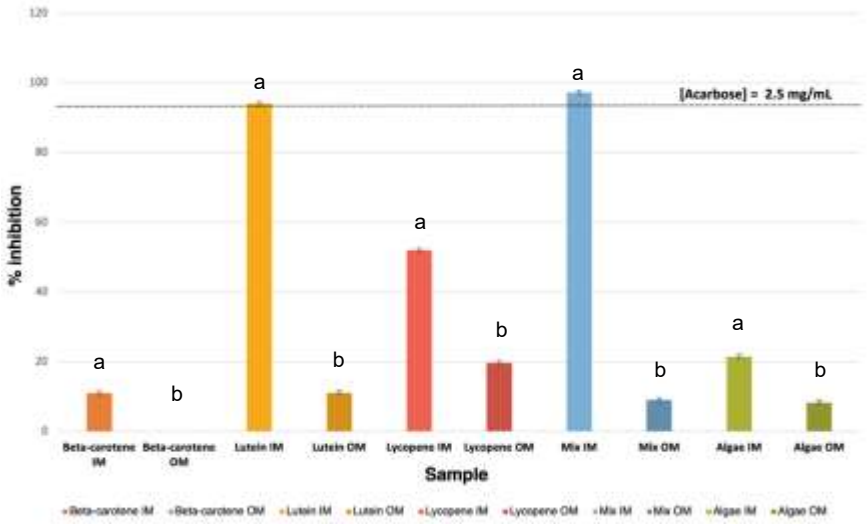


Figure 3.12 - Percentage of α-glucosidase activity inhibited (Mean ± SD) tested with carotenoids' digested samples. Acarbose (2.5 mg/mL) was used as a positive control. IM – inside the membrane; OM – outside the membrane. Different letters mark statistically significant ($p < 0.05$) differences.

For the β-carotene sample, the fraction inside the membrane presented an α-glucosidase inhibition of 11%, which indicates that this carotenoid has low anti-diabetic activity and inclusive, the lowest among all sample groups. In the case of Lutein, inside the membrane, the percentage of inhibition was higher than the positive control (94%) and outside the membrane Lutein presented 9.7% of inhibition, meaning that this xanthophyll has a higher anti-diabetic activity (inside the membrane) than acarbose.

The same happened for the Mix, in which the α-glucosidase inhibition inside the membrane of 97.2% was higher than the 92% of acarbose and higher than the 9.7% of inhibition that the Mix has outside the membrane. In comparison with all sample groups, the Mix has the highest anti-diabetic activity. Lycopene presented 52% of α-glucosidase inhibition inside the membrane and 17.6% of inhibition outside the membrane, which means that this carotenoid already presents considerable anti-diabetic activity inside the membrane.

Finally, the algae presented 21.4% of α-glucosidase inhibition inside the membrane and 7.6% of inhibition outside the membrane, which indicates that it has a promising anti-diabetic activity.

3.1.10 Mutagenicity and anti-mutagenicity of carotenoids

The term "mutagenicity" describes the production of long-lasting mutations in an organism's DNA sequence, which may cause a heritable change in the characteristics of living systems. Mutagens can be neutralised by antimutagenic substances (144). Therefore, after the simulated digestion, the carotenoids' samples were analysed through the AMES test to assess their mutagenicity or anti-mutagenicity effect (Figure 3.13).

The results showed that the samples were not mutagenic in none of the concentrations tested since there wasn't an observed increase in the number of revertants (Figure 3.13). Although it was observed an increase in the number of revertants for the β-carotene sample at a concentration of 100 μg, it doesn't represent a mutagenic effect. To confirm the mutagenic effect of this compound, further experiments with higher concentrations needed to be done.

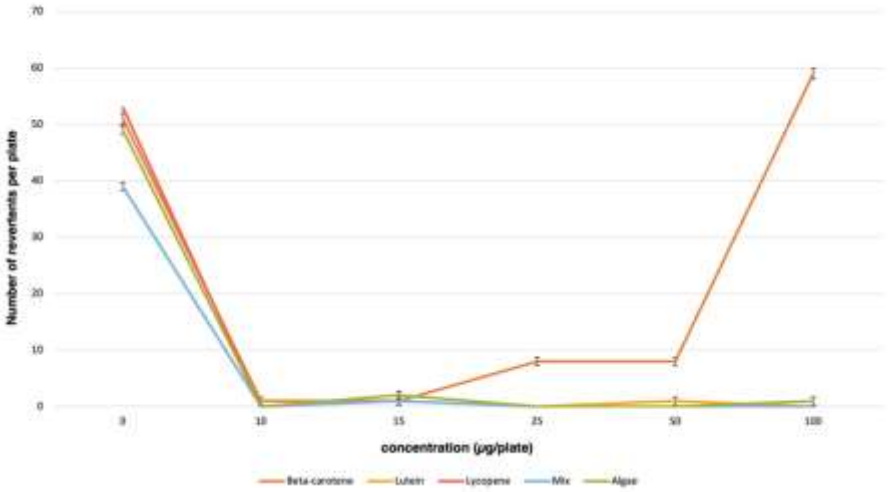


Figure 3.13 - Mutagenicity of carotenoids' samples. Results are the means of two determinations ± SD.

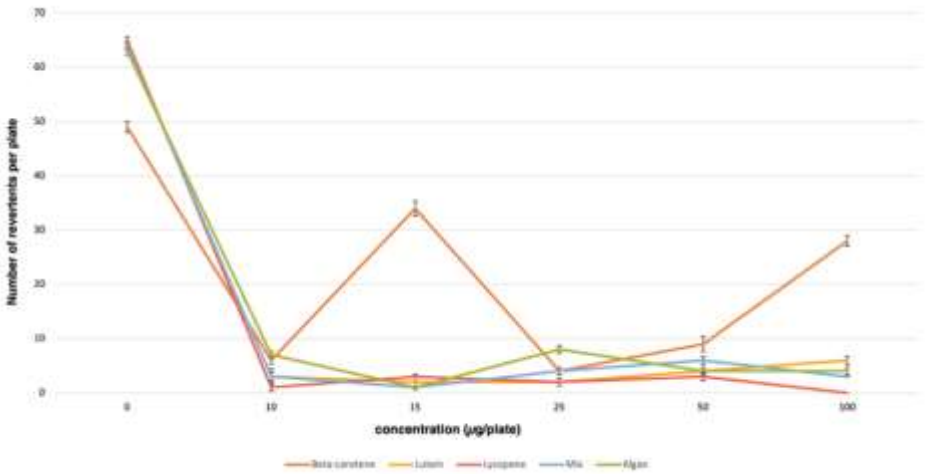


Figure 3.14 - Anti-mutagenicity of carotenoids' samples. Results are the means of two determinations ± SD.

In Figure 3.14 is possible to verify that in all samples were observed a decrease in the number of revertants, regardless of the applied concentration, which indicates that the extracts had an anti-mutagenic effect on the compound quercetin since they reversed its mutagenic effect.

3.1.11 Cytotoxicity of the digested carotenoids

The XTT method is an efficient technique for evaluating the potential cytotoxicity effect of the carotenoids digested samples.

The results, in Figure 3.15, show that the highest concentration of all carotenoids' samples (β-carotene: 0.57 mg/L; Lutein: 1mg/L; Lycopene: 2.5 mg/L; Mix: 6 mg/L; Algae: 103 mg/L) presented an inhibition above 80%, which indicates some inhibition of cell viability.

However, the samples of these carotenoids with lower concentrations (1:2, 1:4 and 1:8), did not inhibit the cellular metabolism since they present negative values of metabolism inhibition. Therefore, the carotenoids digested samples didn't show cytotoxicity for these cells, suggesting the promotion of cell growth. Although, some carotenoid samples presented positive values in dilution 1:2 and Lycopene also in dilution 1:4, the inhibition values were lower than 30%, being considered not relevant.

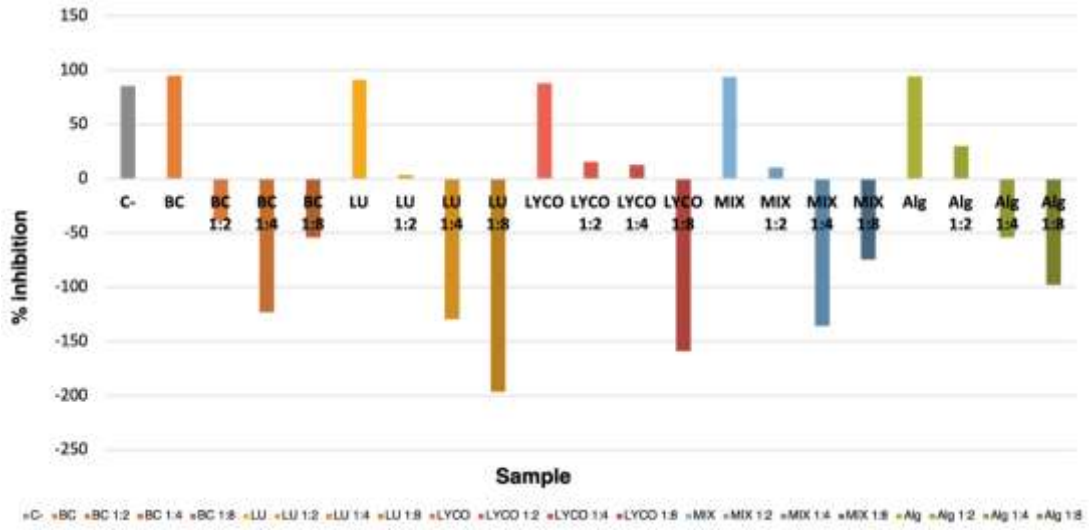


Figure 3.15 - Cytotoxicity analysed by the XTT method (Mean ± SD). C-: negative control; BC: β-carotene; LU: lutein; LYCO: lycopene; Alg: algae; 1:2 – half of the initial concentration; 1:4 – ¼ of the initial concentration; 1:8 – 1/8 of the initial concentration. The results below the line (negative values) represent the non-toxic extracts.

CHAPTER IV

Conclusions

NCDs have emerged as a global health challenge, responsible for a staggering 41 million deaths each year, which are usually associated with unhealthy dietary behaviours. In response to this alarming trend, various nutrition and health organizations advocate for the regular consumption of fruits, vegetables due to their potential to promote positive health outcomes. Among the numerous bioactive compounds found in these plant-based foods, carotenoids and their metabolites have gained particular attention for their association with these health benefits. Therefore, this Dissertation consists of an effort for shaping effective dietary recommendations and strategies through carotenoids to combat the rising burden of NCDs, by analysing the interaction of these natural pigments with the intestinal microbiota.

Initially, the *in vitro* simulation of gastrointestinal digestion in the tested groups allowed us to sustain the information present in the literature related to the low bioaccessibility of carotenoids. Also, although it was added a lipophilic compound to protect these natural pigments, it wasn´t enough to prevent chemical reactions, giving rise to other carotenoids and compounds as shown in the HPLC analysis. Then, through UPLC-qTOF MS TOF, it was possible to conclude that the fragments obtained along the GIT have different behaviours according to the GIT phase depending on the condition tested.

After characterizing the impact of the digestion process on each type of sample, it was analysed the intestinal microbiota composition through several methodologies. At the phylum level, the intestinal microbiota was mainly composed of *Bacteroidota, Bacillota, Pseudomonadota*, and *Actinomycetota*, which were consistent with the results obtained in previous studies and sustained by all types of analysis done. In a general way in terms of RA, carotenoids stimulated the growth of bacteria from the *Lachnospiraceae* family and the decreased of *Lactobacillus, Enterococcus, Streptococcus* and *Bifidobacterium*, which was consistent with the previous studies and supports that carotenoids play positive roles in intestinal health. In addition, it was possible to conclude that Lycopene and the Mix groups are not very distinct from each other, at a microbial community composition level, and that β-carotene and Algae groups allowed more diversified microbiota allowing unique species of bacteria present when compared with the other groups.

Furthermore, the carotenoid groups subjected to 48 h of the simulated fermentation process were analysed using UPLC-qTOF MS and it was possible to observe a tight correlation: the fragments obtained at 12 h, 24 h and 48 h of fermentation are similar between them and the fragments obtained at 0 h and 6 h of this process are distinct from each other and from the fragments obtained in the last 3 time points showing a faster activity in the beginning and then a stabilization, with the exception of the Lutein group which fragments originated at each time point are very distinct between them demonstrating a longer period of transformation for these sample.

The intestinal microbiota used glucose as a carbon source since it is possible to see a decrease in its concentration along the 48 h of fermentation. Moreover, these bacteria induced the production of organic acids such as succinic, acetic, butyric, propionic, and malic acids. Although different groups produce these key organic acids, this also supports the observation that *Lachnospiraceae* bacteria increased its RA, which are known for their ability to produce these types of acids.

In terms of the functional properties of the tested carotenoids solutions, Lutein was the sample with the highest antioxidant activity, followed by Lycopene, the Mix, β-carotene and, finally the Algae samples relative to the fraction absorbed. On the other hand, in the case of antidiabetic activity, and as desired,

more effective results were obtained for the fraction that remained in the colon (IM), being the highest activity achieved by the Mix group and the lowest by β-carotene.

The carotenoid samples were also evaluated for their mutagenicity/anti-mutagenicity and cytotoxicity. Not only was the absence of mutagenicity demonstrated in all the concentrations tested for all samples but also their anti-mutagenicity effect (regardless of the concentration applied) since carotenoids reversed the mutagenic effect of quercetin. In terms of cytotoxicity, although the highest concentration of all carotenoid samples presented an inhibition above 80% (indicates some inhibition of cell viability), the samples of these carotenoids with lower concentrations did not inhibit cellular metabolism, suggesting the promotion of cell growth.

Finally, it is worth noting that in the Algae sample, it was possible to observe that the bioavailability and stability of carotenoids were lower compared to the other groups where carotenoids were evaluated individually, as well as the antioxidant and antidiabetic activities. On the other hand, although the Mix group was created to assess the possible synergy/antagonism between the 3 carotenoids used, it was not possible to draw a conclusion regarding this effect since the results obtained were not significantly different compared to the carotenoids in their isolated form, showing a closer similarity in behaviour to Lycopene. Therefore, with the improvements suggested before, it will be possible to achieve clear evidence of the Mix effect as a synergistic, antagonistic, or neutral effect.

CHAPTER V

Future perspectives

This Master's Dissertation was dedicated to the study of a topic that still lacks associated information and clear evidence - the interaction between carotenoids and the intestinal microbiota. It contributed to understanding the impact of digestion on the bioavailability and stability of carotenoids, and explored the interaction that these carotenoids would have with the intestinal microbiota and, consequently, the functional properties of these natural pigments. However, some developments can be explored based on the findings and limitations of the current study.

Regarding the preparation of the solutions, future research could expand the concentrations used for each carotenoid, providing deeper insights to identify the most effective carotenoid concentrations to achieve better bioavailability and stability of these natural pigments. Nevertheless, the amount and type of the lipophilic compound can also play an important effect in the impact that digestion has on carotenoids, which may help to identify the most effective approach. In addition, it would be also interesting to evaluate if a technique to extract carotenoids in algae could have a different result in all points assessed in this Dissertation.

Concerning the UPLC-qTOF MS TOF analysis, exploring the results in terms of the identification of the fragments obtained in each phase of the simulated digestion and at each time-point of the fermentation would fulfill a major and crucial gap that exists in the literature about the theme of this Dissertation.

Finally, the mutagenicity and anti-mutagenicity effects of the carotenoids could also be improved, by analysing and comparing the results obtained previously with metabolic activation (e.g., liver homogenate S9 mix) that will allow us to understand the effect of these pigments during liver absorption.

CHAPTER VI

Appendixes

nutrients

MDPI

Review

Carotenoids Diet: Digestion, Gut Microbiota Modulation, and Inflammatory Diseases

Helena R. Rocha †, Marta C. Coelho *,†, Ana M. Gomes and Manuela E. Pintado *

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† These authors contributed equally to this work.

Abstract: Several epidemiologic studies have found that consuming fruits and vegetables lowers the risk of getting a variety of chronic illnesses, including several types of cancers, cardiovascular diseases (CVDs), and bowel diseases. Although there is still debate over the bioactive components, various secondary plant metabolites have been linked to these positive health benefits. Many of these features have recently been connected to carotenoids and their metabolites' effects on intracellular signalling cascades, which influence gene expression and protein translation. Carotenoids are the most prevalent lipid-soluble phytochemicals in the human diet, are found in micromolar amounts in human serum, and are very susceptible to multiple oxidation and isomerisation reactions. The gastrointestinal delivery system, digestion processes, stability, and functionality of carotenoids, as well as their impact on the gut microbiota and how carotenoids may be effective modulators of oxidative stress and inflammatory pathways, are still lacking research advances. Although several pathways involved in carotenoids' bioactivity have been identified, future studies should focus on the carotenoids' relationships, related metabolites, and their effects on transcription factors and metabolism.

Keywords: carotenoids; gastrointestinal tract; intestinal microbiota; metabolites; absorption

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1. Introduction

Carotenoids are natural pigments from the tetraterpenes family, characterized by a central chain with 40 atoms of carbon and alternating single and double bonds and various cyclic or acyclic end groups, depending on the carotenoid [1]. In terms of physicochemical properties, carotenoids are colourful lipophilic compounds [1,2], responsible for the variety of colours present in several autotrophs such as microalgae, bacteria, fungi, and plants [1,3].

Humans and animals cannot synthesize carotenoids by themselves, they can be found in their tissues due to the absorption and deposition of the carotenoids ingested in dietary food [2,4,5].

Carotenoids are natural organic pigmented compounds with structural variations, with more than 750 carotenoids being known, but only 40 of them are present in the human diet and 20 in human blood and tissues [1,6–8]. The 40 carotenoids present in a usual human diet [1] can be found in coloured fruits and vegetables, such as tomatoes, carrots, and spinach [9].

In terms of chemical constituents, these natural pigments can be divided into two categories: carotenes and xanthophylls [10]. If they are pure hydrocarbons, they can be classified as carotenes such as alpha(α)-carotene, beta(β)-carotene, and lycopene [8]. Xanthophylls are carotenoids with oxygenated derivatives on their terminal rings [8], with complex xanthophylls containing oxygen substituents, aldehyde groups, epoxide groups and oxo/keto groups [11]. Zeaxanthin, lutein, canthaxanthin, violaxanthin and neoxanthin are examples of complex xanthophylls.

Carotenes absorb light energy from chlorophyll and energy from singlet oxygen formed in photosynthesis, being responsible for transmitting this light and protecting the plant tissues [5]. Xanthophylls, synthesized within the plastids, work as accessory pigments, capturing the wavelengths of sunlight that chlorophyll cannot absorb [5].

Regarding functional properties, carotenoids can be classified as primary and secondary carotenoids, with the photosynthetic ones included in the primary group and playing an important role in photosynthesis [2,12].

These natural organic pigmented compounds, in terms of physicochemical properties, are associated with membrane lipid bilayers and cytosolic lipid droplets, which can affect some properties associated with the permeability and fluidity of the membrane [9].

The principal properties of carotenoids mentioned before are illustrated below, in Figure 1.

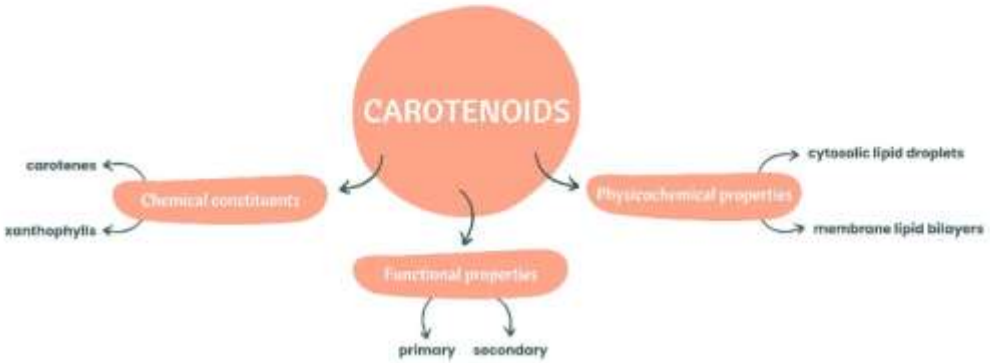

Figure 1. Chemical constituents and functional and physicochemical properties of carotenoids.

The regular consumption of fruits and vegetables is widely recommended due to their multiple health benefits such as the lower incidence of chronic diseases [13] such as cardiovascular diseases (CVDs), several types of cancers [14], and bowel diseases. Chron's disease and ulcerative colitis are two chronic inflammatory bowel diseases (IBDs) characterized by recurring episodes of inflammation in the gastrointestinal tract (GIT) [15] that cause damage to its tissues [16].

Several studies have attributed to bioactive compounds present in the diet [14], in particular carotenoids [13,17], the responsibility for beneficial health effects in various pathologies, namely IBDs. This can be explained by their several important biological functions such as antioxidant activity [2,18], meaning that these pigments can inhibit or downregulate the unstable compounds produced by the body [2,18] in various pathologies and during oxidative stress caused by reactive oxygen species (ROS) [1,19]. In addition to this, carotenoids have other important functions such as antibacterial, immunological, and anti-inflammatory activity, and beneficial effects on the treatment of diabetes, and in infectious, eye, and neurological diseases [2,18].

Some of the more important biological functions related to human health of the three most known carotenoids are presented in Table 1.

Table 1. Principal biological functions of β-carotene, lutein, and lycopene.

Carotenoid	Biological Functions	References
β-carotene	Stimulates the proliferation of lymphocytes; reduces the low-density lipoprotein (LDL) susceptibility to oxidation; activates cell communication; reduces inflammation; improves cardiovascular health.	[2,20,21]

Table 1. *Cont.*

Carotenoid	Biological Functions	References
Lutein	Scavenges oxygen intermediates; blue light filter; maintenance of eye health; decreases the proliferation of breast cancer cells; reduces oxidative stress and apoptosis.	[1,21–24]
Lycopene	Inhibits lipid peroxidation; eliminates reactive oxygen species (ROS); reinforces the immune system; free radical quencher; prevents skin damage.	[2,21]

These antioxidant phytochemicals are also important dietary sources of vitamin A and protect cells and tissues from oxidative damage, interacting with other antioxidants [11,25]. So far, only 50 carotenoids are known to have provitamin A activity [12], with α-carotene, β-carotene, gamma(γ)-carotene, and β-cryptoxanthin being the most important precursors of vitamin A in humans [12,20].

Vitamin A is important for proper visual, immune, and gastrointestinal functions, growth, and embryonal development [20]. Humans cannot synthesize vitamin A de novo, obtaining adequate amounts through dietary food, such as from orange and yellow vegetables and in vegetables with dark green leaves [20].

The recent discoveries about the health promotion properties of carotenoids have aroused interest in applying these natural pigments in diversified areas [2]. These natural pigments have several applications such as in feed, and in the food, nutraceutical, and pharmacology industries [5]. Carotenoids can be applied as colourants in food, beverages, and cosmetics, as food supplements, as feed additives, and as supplements [26].

In nature, the bioavailability of carotenoids is reduced [1,20] without processing or any type of treatment, leading to an accumulation in the colon [27], which is colonized by a large number of microorganisms [28] that play important roles in digestion and metabolism [29], as well as in maintaining normal gut physiology and health [30].

Diet is one of the most important regulators of the intestinal microbiota [31], but there is a lack of information about the relationship between carotenoids and the intestinal microbiota [32]. In addition to that, these natural pigments are hydrophobic molecules, which makes their solubility in water low and, when exposed to light, heat, oxygen, or acids, are very susceptible to multiple oxidation and isomerisation reactions [5]. Therefore, the polarity of carotenoids can change due to the polar functional groups attached to the main chain and some products with harmful or unknown effects can also be formed [1], which can affect the carotenoids' bioaccessibility, bioavailability, and absorption.

In this sense, this review aims to present an overview of the gastrointestinal delivery system for carotenoids, the processes occurring during digestion, from mastication to absorption, and the impact of the gut microbiota and its metabolites on the stability and functionality of carotenoids, and also their ability to modulate inflammatory and oxidative stress pathways.

2. Overview of the Publications

2.1. Methodology of Research

The research articles about carotenoids and intestinal microbiota were searched on Science Direct and on PubMed, using the keywords "carotenoids", "gut microbiota", and "interaction". Therefore, all publications available in these two databases which contained the words mentioned before as author-specified keywords in the title or abstract were considered.

Later, the abstracts of all of the articles were analysed and divided into two categories: carotenoids in human health and the interaction of carotenoids with the gut microbiota.

There are several articles that mention that phytochemicals/dietary lipids may influence the composition of the intestinal microbiota, the digestion process, and the occurrence or prevention of some diseases, among others. Although carotenoids belong to those categories, the detailed process is focused on other phytochemicals/dietary lipids. For carotenoids, the process is only a supposition, according to the chemical and structural similarities with other dietary lipids, highlighting the lack of information about these natural pigments. For this reason, articles where the role or the process that carotenoids perform is not the main focus were excluded.

2.2. Results

According to our search through the Science Direct database, only four relevant research articles were published (2018: 1; 2022: 3). The oldest article was published in 2018, which reveals that the interaction between carotenoids and the intestinal microbiota is a recent research theme.

The search performed on PubMed showed that 18 articles containing the keywords selected by us were published (2017: 2; 2018: 3; 2019: 2; 2020: 6; 2021: 0; 2022: 6). Although more articles were found through *PubMed* than through Science Direct, it corroborates the fact that this topic is a very recent research target and that more research needs to be conducted concerning the interaction between carotenoids and the intestinal microbiota. The articles published were categorized into carotenoids in human health (5 articles) and the interaction of carotenoids with the gut microbiome (7 articles).

The number of published articles related to carotenoids and their interaction with the intestinal microbiota, according to its year of publication, is represented in Figure 2.

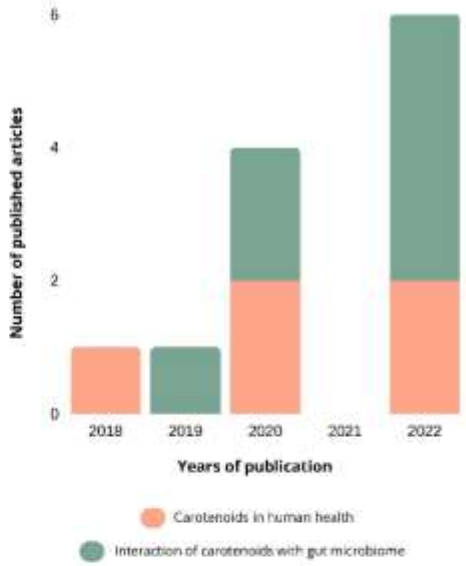

Figure 2. The number of published articles related to carotenoids and their interaction with the intestinal microbiota. The databases used were Science Direct and PubMed, using the keywords "carotenoids", "gut microbiota", and "interaction". The articles were categorized, based on their abstracts, into carotenoids in human health and the interaction of carotenoids with the gut microbiota.

Initially, the studies were more related to the role that carotenoids play in human health such as the antioxidant and anti-inflammatory activity and the beneficial effects that they have on the treatment of some cancers and CVDs. It is believed that due to these bioactive properties, the interest in carotenoids has increased exponentially, which is corroborated by the increase in articles about the interaction of carotenoids with the gut microbiome in more recent years.

However, it still lacks information related to the impact of digestion and interaction between the carotenoids and the gut microbiome on the stability and functionality of carotenoids.

3. Bioaccessibility and Bioavailability of Carotenoids

The bioaccessibility of a carotenoid is defined as the maximum quantity of a carotenoid released from the food matrix that is available to be absorbed in the epithelial cells of the intestine [33]. The fraction of an ingested compound that enters the bloodstream and performs its physiological functions is the definition of the bioavailability of a carotenoid [5,34].

In nature, the bioavailability of these natural pigments is reduced, since there is a resistance to digestion and degradation from the protein complexes of carotenoids and the cell walls of plants to achieve adequate release from the matrix [1,20]. In the case of β-carotene, the activity and conversion to vitamin A are high. However, the absorption from plant sources is approximately 65%, with the recommended daily intake of 2–4 mg per day not being achieved [20,35].

The carotenoids' bioavailability and consequently absorption have some limitations due to factors such as the dietary sources, food composition, cooking temperature, season, the breakup of the food matrix, presence of lipids, dosage, and presence of other soluble compounds/carotenoids [18,36]. These factors can lead to the release of carotenoids from the food matrix, improving its bioavailability or transforming the carotenoids into isomers that are better absorbed by the organism [36].

The release from the food matrix depends on the state of the carotenoid, as natural pigments immersed entirely in lipid droplets are more easily released than ones in the microcrystalline form [37]. This explains the low availability of lycopene in tomatoes and β-carotene in carrots [37].

The dietary composition also has a significant effect on the bioavailability of carotenoids [36]. Carotenoids are lipophilic compounds and for this reason their bioavailability increases when they are consumed allied with a fat source [38], but decreases when they are consumed with dietary fibre such as pectin [39].

Food thermal processes such as cooking, boiling, and heating disrupt the cellular membrane, allowing the release of carotenoids from the matrix [1,20,21], and although this leads to a decrease in the carotenoid content, it raises their bioavailability and absorption when compared with uncooked food [1,8,20]. For example, in cooked tomatoes, the lycopene availability is higher than in raw tomatoes, and the more prolonged the heat treatment, the lower the carotenoid content is [21].

The principal factors affecting the carotenoids' bioavailability, enhancing (left) or decreasing (right) it, are represented below, in Figure 3.

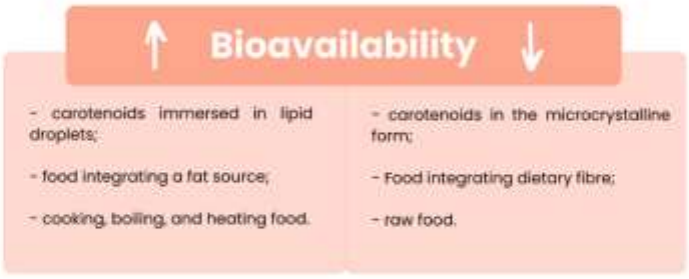

Figure 3. Conditioning factors that enhance (↑) or decrease (↓) the carotenoids' bioavailability [1,8,20,37].

Therefore, different extraction technologies are required to increase carotenoids' solubility and bioavailability [40]. The traditional methodology uses organic solvents such as hexane and acetone to extract carotenoids from food matrices, because of their hydrophobicity [40]. However, the toxicity of these organic solvents to human health, imposes the use of food-grade solvents to purify these carotenoids and use them in the food industry [40].

In the last few years, some alternative methods to recover carotenoids have been presented, such as super-critical fluid extraction (SFE), high hydrostatic pressure (HHP), and Ohmic heating (OH).

SFE is an extraction technique that reduces the toxic solvents used during the process and can generate a solvent-free extract at moderately high selectivity and yield temperatures [11]. Although it is a non-inflammable and non-toxic method, its non-polar nature demands the use of a stabiliser and a cosolvent, and carotenoid degradation and/or isomerization can occur [40,41]. This technique is advantageous insofar as the process is both environmentally benign and energy efficient and the sustainable solvent is simple to obtain. However, it presents some limitations, since it is an expensive method and the polar extracts are insoluble in the CO_2 mobile phase [42–44].

HHP is a simpler and more efficient technique than conventional extraction methods, that contributes to improve the bioaccessibility of bioactive compounds [45]. HHP is advantageous since it is a completely solvent-free procedure that uses tomato leaf waste at a high CO_2 pressure (180 bar), and at room temperature to obtain phylloquinone [41]. However, once again it is limited by its high cost, and by the necessary improvements in the associated recovery process [46].

More recently, Coelho et al. [47] proposed OH, which consists of the use of an electric current that passes across a conductor matrix (e.g., food) to generate heat from the electrical resistance of the matrix. This methodology is more advantageous than the ones mentioned before since it allows the extraction of bioactive compounds such as carotenoids and polyphenols from their matrices only using ethanol:water as a solvent [45,47], and the application of a low temperature prevents thermal losses [11].

The authors [47] showed that this method can replace traditional methods since it is selective, enabling bioactive compounds to be extracted without organic solvents. OH has some limitations given the impossibility of extracting some bioactive compounds that remain bound to dietary fibres and the lack of information about the potential antioxidant properties of these bioactive compounds, as well as how they are affected by the GIT during digestion [48].

The main advantages and limitations of OH as an alternative method to recover carotenoids without organic solvents are represented in Figure 4.

Figure 4. Advantages and limitations of using Ohmic heating (OH) in the extraction of bioactive compounds. Adapted from [11].

4. Carotenoid Absorption Mechanism

The carotenoids' pathway along the GIT starts in the mouth, where they are liberated from the food matrices and, passing through the stomach and intestine, become susceptible to modifications such as solubilization by the intestinal fluids [48,49]. Then, the bioactive compounds in the intestine suffer selection through permeation, becoming available for bloodstream absorption [48], and the non-bioaccessible ones are directly used by the gut microbiota [48,49].

The carotenoid absorption mechanism can be divided into release from the food matrix, transfer to the oil phase, formation of mixed micelles, and absorption, as represented below, in Figure 5.

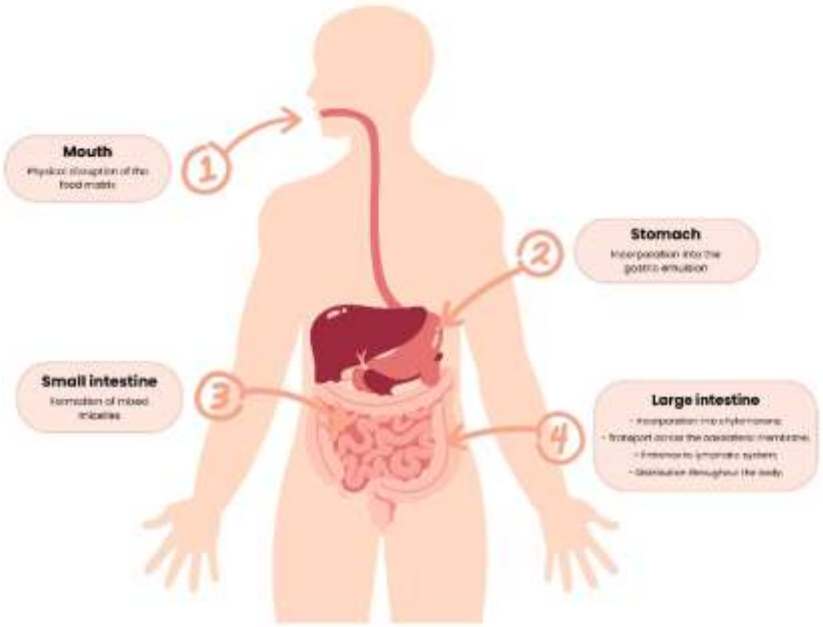

Figure 5. Major steps of the carotenoid absorption mechanism.

4.1. Release from the Food Matrix

The carotenoid absorption mechanism starts with mastication, the physical disruption that leads to the release of carotenoids from the food matrix [20]. This step is the first limiting factor affecting bioavailability since the physical form of carotenoids conditions their release during digestion [20,50]. In the case of β-carotene, for example, it can be within the food in liquid crystalline form, such as in mango and papaya, or in solid crystalline form, as in carrot and tomato [20,50], with the bioavailability of this carotenoid in food being higher in the liquid crystalline form [20,50].

4.2. Transfer to the Oil Phase

The second step consists of the dissolution of carotenoids into the gastric emulsion. The first limiting factor is the digestion from the food matrix: if it is not complete, carotenoids will not have direct contact with the oil and, consequently, will not be transferred to the oily phase [20].

The incorporation of carotenoids into the gastric emulsion also faces several limiting factors, such as soluble proteins, the surface charge of the gastric emulsion, the oil, and the amount of the carotenoid present [20].

In the case of β-carotene, the incorporation of this carotenoid into the gastric emulsion is inhibited by soluble proteins that affect the interfacial characteristics of the digesta. Proteins (e.g., caseins) have been suggested to help in the bioaccessibility of liposoluble food elements in a variety of ways. Proteins may stabilize oil-in-water (o/w) emulsions in the GI tract after adsorption to lipid droplet surfaces. This is due to the fact that proteins can be highly surface-active molecules, and the formed particles tend to be highly negatively charged, preventing lipid droplet aggregation. However, Qiu et al. [51] found that gliadin reduced enzymatic lipid degradation, most likely by preventing digestive enzymes from adsorbing to droplet surfaces or directly binding to enzymes, implying that proteins may have a negative influence on the micellization process. Whey protein isolate (WPI) inhibits

lipid oxidation and facilitates the formation of smaller lipid droplets, increasing β-carotene bioaccessibility. WPI has both hydrophobic and hydrophilic groups, and its conformation affects its properties at the oil/water interface. This study investigated the effect of WPI on the bioaccessibility of pure carotene under different digestive conditions. Micellization of co-digested β-carotene was also measured under insufficient digestion parameters [52].

However, the concentration of soluble proteins decreases and the transfer of β-carotene to oil increases if the pH decreases [20]. β-carotene's solubilization increases when the gastric emulsion's surface charge decreases, since it allows a higher adherence of oil to the carotene-containing matrix [20]. In addition to that, the oil, and also the amount of the carotenoid present, affect the solubility of β-carotene in the oily phase, which determines the extension of the carotenoid transfer to the digesta [20].

4.3. Micelle Formation

During the passage through the small intestine, the release of bile salts occurs that promotes the formation of mixed micelles [20]. These micelles are the result of the action of bile salts as surfactants that reduce the size of the gastric emulsion, composed of free fatty acids, monoglycerides, phospholipids, and the carotenoid [53], to micelles with an 80 Å diameter, approximately [53]. The micelles have an amphiphilic structure that allows the lipophilic nutrients to be remain soluble in the aqueous digesta [36].

The carotenoids' absorption only occurs if they are in mixed micelles, since the factors that affect the micelle formation also affect the bioavailability of carotenoids in the digestion process [20]. Dietary fat is a factor that influences the formation of micelles, since lipids are necessary to stimulate the release of bile and for the incorporation of the gastric emulsion into micelles [20]. However, Roodenburg et al. [54] indicated that increasing dietary fat is only beneficial to the formation of micelles until an optimal threshold. In addition to this, the fat type affects the micelle formation, since the longer the fatty acyl chain, the more extensive the micelle formation and the bioavailability of the carotenoids [20].

In addition to lipids, fibres such as alginate, guar, and pectin are also limiting factors, as in the presence of carotenoids they inhibit the formation of micelles and decrease the bioavailability of carotenoids [20].

4.4. Absorption

The final step of the carotenoids' absorption starts when the micelles containing the carotenoid come into contact with the apical side of the enterocytes [20], then enter the enterocytes, are incorporated into chylomicrons with other dietary lipids, and are transported across the basolateral membrane [55]. Then, the carotenoid enters the lymphatic system and is released into the circulation, being distributed throughout the body [36,55].

Although it was believed that the absorption of carotenoids occured in the same way as dietary lipids, through passive diffusion, it has been discovered that the absorption of carotenoids can be facilitated by transporters present in the membrane, such as the scavenger receptor class B type 1 (SR-BI), the cluster determinant 36 (CD36), and NPC1-like transporter 1 (NPC1L1) [9,55]. SR-B1 is a class B receptor found in different tissues, particularly in the intestine, and is involved in the cellular uptake of a wide range of lipid molecules (e.g., cholesterol and liposoluble vitamins) [56] and of the non-provitamin A carotenoids lutein [57] and lycopene [58]. CD36 is present in various tissues, namely in the intestine, and has ligands for carotenoids, long-chain fatty acids, and lipoproteins [56]. CD36 and SR-B1 are glycosylated transmembrane proteins with a large extracellular domain [59]; it has been predicted that CD36 has a large cavity traversing its entire length that allows lipid transfer from extracellular to cellular compartments [60,61]. NPC1L1 is a major sterol transporter in the intestine [62,63] and is involved in the uptake of carotenoids such as α-carotene, β-carotene, β-cryptoxanthin, and lutein [64,65]. Although some studies proved that these proteins have facilitated carotenoid absorption [59], the mechanisms behind these effects are still unknown [56].

In addition, absorption can be affected by the individual's genetic susceptibility, the dose ingested [1], and by viscosity, since it inhibits the formation of micelles and consequently decreases the amount of carotenoid available in a form capable of absorption [20].

5. Intestinal Microbiota

The intestinal microbiota consists of a complex community of microorganisms [30], including bacteria, viruses, and some eukaryotes that live in the digestive tracts of humans and animals [66]. The number of bacterial cells in the human intestinal microbiota is approximately 10^{14}, which is 10 times higher than the number of human cells [67].

The intestinal microbiota composition is different along the GIT [30]. The stomach and small digestive tract are colonized by only a few species of bacteria, and in the colon are present approximately 10^{12} bacterial cells/g of gut content [30]. Almost 99% of the bacteria that colonize the intestine are anaerobes, but in the connection between the small intestine and the colon (cecum), a high density of aerobic microorganisms can be found [30].

In Figure 6 are presented the main microbial species and their respective density present in the different parts of the GIT.

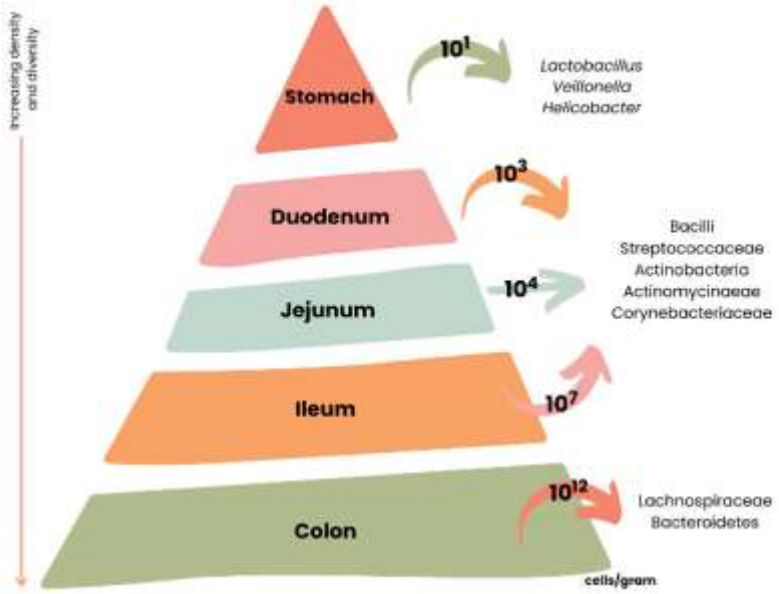

Figure 6. Variations across the length of the gastrointestinal tract (GIT) according to microbial cell number and composition. Adapted from [67].

The relationship between the intestine and its microorganisms is mutualistic, since the host intestine supplies the bacteria with the conditions for their survival and reproduction and the microbiota has important functions such as digestion, nutrient processing, protection against pathogens, production of different antimicrobial substances [30], production of micronutrients such as vitamins, immune cell growth and response [68], and the control of epithelial cell proliferation and differentiation [69].

The intestinal microbiota has a spatial limitation, which consists of its enclosure within the gastrointestinal lumen, allowing the gut bacteria to translocate and generate a local or/and systemic inflammation [70,71]. To overcome this limitation, intestinal microbiota release a large number of different metabolites [71], including bile acids, vitamins, amino acids such as tryptophan, and short-chain fatty acids (SCFAs) [72]. These metabolites will have extensive effects on a host's organs near or far from the gastrointestinal lumen, such as regulating local and systemic immune response, nutrient absorption, host metabolism, and gut microbiota composition to maintain health or develop diseases [71].

Nonetheless, the composition and function of the intestinal microbiota can be affected [30] by individual intrinsic factors such as age, ethnicity, and genetic markers, or by environmental factors such as geographic area, lifestyle, diet, and drugs [73,74]. These factors, particularly diet, can lead to useful or harmful modifications in the production of the metabolites that could change the composition of the microbiota, increasing or decreasing some species present [30,75].

6. Intestinal Microbiota Metabolites

Dietary compounds with low bioavailability or that cannot be absorbed directly [76], pass the small intestine and enter the colon, where they will interact with live gut bacteria [31]. The colon is the ideal place for the interactions between the intestinal microbiota and the dietary compounds since, in addition to a high level and diversity of the microorganisms present, it supplies the suitable pH and time for direct contact between microbes and food [67]. Once there, these compounds can induce functional and compositional modifications of the microbiota or can be transformed into new compounds [31].

The intestinal microbiota contains millions of microbial genes [77] that enable the production of a large number of enzymes, which can ferment the dietary compounds that are not digested by human enzymes, such as fibre or primary bile acids [31]. In consequence, the intestinal microbiota are capable of synthesising and releasing a variety of different metabolites that can be produced directly from dietary compounds, produced by hosts and transformed chemically, or synthesized de novo by gut microbiota [78].

These plant-derived compounds must be absorbed, transferred to the circulating system, delivered to the site of action in the body, and metabolically converted to the vitamin active form to be biologically effective [79]. The carotenoid cleavage products (e.g., apocarotenoids) [80] are generated through the action of specific enzymes, such as the carotenoid cleavage dioxygenase (CCD) in plants [81] and the β,β-carotene-15,15′-oxygenase (BCO) in vertebrates [79]. However, the BCO enzymatic activity in the gut still needs to be clarified [79].

After biological activation, these metabolites can promote a wide range of activities in the host, such as regulating the composition, function, intestinal barrier and motility of the intestinal microbiota, modulating host metabolism, and influencing nutrient absorption, among others [71]. On the other hand, these metabolites, dependent on their chemical nature, can play important roles in the development and progression of diseases such as cancer, hypertension, Parkinson's, and non-alcoholic fatty liver diseases [82–85].

In addition to that, dietary compounds can also indirectly interact with the intestinal microbiota through the modulation of gastrointestinal transit time, pH, and the synthesis and release of antimicrobial peptides and secretory immunoglobulins [86].

The typical metabolites generated by the intestinal microbiota and their respective functions and associated diseases are presented in Table 2.

Table 2. Typical intestinal microbiota metabolites and their roles in health and diseases. Adapted from [71].

Groups	Typical Metabolites	Specific Function	Associated Diseases	References
Short-chain fatty acids	Acetate, propionate, butyrate, hexanoate, isovalerate, isobutyrate.	Regulation of intestinal microbiota composition, barrier integrity, and hormone production.	Diabetes, obesity, colorectal cancer, Crohn's and Parkinson's diseases.	[87–93]
Bile acids	Cholate, hyocholate, deoxycholate, glycocholate, hyodeoxycholate.	Regulation of intestinal microbiota composition, hormones, immunity, and motility.	Amyotrophic lateral sclerosis, cancer, Alzheimer's, and Parkinson's diseases.	[94–98]

Table 2. *Cont.*

Groups	Typical Metabolites	Specific Function	Associated Diseases	References
Gases	H_2S, H_2, CO_2, CH_2, CH_4, NO.	CH_4 slows intestinal motility; H_2S regulates intestinal inflammation and motility; NO mediates gastric mucosal protection.	Parkinson's disease, colitis, ulcer.	[85,99–102]
Vitamins	Vitamins B2, B3, B5, B6, B9, B12, and K.	Involved in cellular metabolism, modulate immune function and cell proliferation, supply vitamins for hosts.	Vitamin-associated diseases such as schizophrenia and dementia.	[103,104]
Lipids	Conjugated fatty acids, cholesterol, lipopolysaccharides (LPS).	Conjugated fatty acids regulate the immune system; cholesterol acts as a material base for bile acid synthesis; LPS triggers systemic inflammation.	Non-alcoholic fatty liver disease, hyperinsulinemia, hypercholesterolemia.	[105,106]
Neurotransmitters	Dopamine, catecholamines, 5-HT, GABA.	Regulate intestinal motility, memory, and stress responses.	Parkinson's disease, autism.	[85,107,108]
Choline metabolites	Dimethylglycine, methylamine, dimethylamine.	Inhibit bile acid synthesis; promote inflammation; exacerbate mitochondrial dysfunction.	Obesity, diabetes, heart failure, hypertension.	[109–111]
Tryptophan and indole derivatives	Indole-3-lactic acid, indole acetic acid, indole-3-acetamide, indole, serotonin.	Influence the intestinal microbial drug resistance and virulence; regulate intestinal barrier functions, hormone secretion, and motility.	Ulcerative colitis, Crohn's, Alzheimer's, and Parkinson's diseases, stroke, irritable bowel syndrome.	[112–116]
Others	Ethanol, triphosadenine, ruminococcin A, cytolysin, microcin B17, benzoate, hippurate, cadaverine.	Regulate intestinal response, act as antibiotics to modulate intestinal microbiota composition, supply nutrients, toxic to host cells.	*C. difficile* and *H. pylori* infections, irritable bowel syndrome, ulcerative colitis.	[105,117–119]

7. Interaction between Carotenoids and the Intestinal Microbiota

The interaction between carotenoids and the intestinal microbiota is a topic that still lacks associated information and clear evidence. However, some studies have indicated that the intestinal microbiota may be the main factor behind the effectiveness of carotenoids' action [120].

Jalal et al. [121] showed that the excessive growth of Proteobacteria, harmful bacteria, led to the damage of the mucosal epithelial cells and an increase in the permeability of the intestine, which provoked a decrease in the absorption of carotenoids. Another study, that used colonic fecal samples, showed that new compounds were generated during the fermentation of carotenoids by the intestinal microbiota, indicating that carotenoids were metabolized [122]. Although the absorption of carotenoids can be different depending on the individual [123], the studies mentioned before indicated that the composition of the intestinal microbiota has an important influence on the absorption and metabolism of carotenoids [120].

Other studies revealed that the composition of the intestinal microbiota can be regulated through dietary carotenoid supplementation such as lycopene, which inhibits the reproduction of Proteobacteria and promotes the growth of Bifidobacterium and Lactobacil-

lus, maintaining the harmony of intestinal immunity and mitigating the symptoms caused by anxiety and dextran sulfate sodium-induced colitis and depression [124]. Astaxanthin has been associated with a relief of inflammation and a decrease in lipid accumulation, through a decrease in Bacteroidetes and Proteobacteria abundance and an increase in the population density of Verrucomicrobiota and Akkermansia sp. [125].

Supplementation with β-carotene also increased the abundance of Bacteroidetes and Proteobacteria and decreased the abundance of harmful bacteria such as Dialister and Enterobacter, which corroborates the positive effects of this carotenoid in intestinal health [120]. In addition, a dose of administered β-carotene also influenced the composition of the intestinal microbiota, since low and medium doses increased the abundance of *Bifidobacterium* and *Collinsella* strains and high doses increased the abundance of *Lactobacillus* strains [120].

In addition to this, the results obtained by Dai et al. [27] suggested that xanthophylls such as lutein and zeaxanthin have a higher impact on the modification of intestinal microbiota composition than carotenes. This demonstrates that carotenoids are structurally distinct and can affect differently the composition of the intestinal microbiota [27].

Therefore, the results obtained from these studies indicate that carotenoids and the intestinal microbiota have a structure–activity relationship and the latter can be a potential target for carotenoids' utilization [27]. However, a comprehensive understanding of the direct interaction between carotenoids and the intestinal microbiota and their relationship is still lacking [126].

8. Carotenoid Metabolites from Microbiota and Activation/Deactivation of Gene Potentiation in Bowel Diseases

Carotenoids have been associated with various health benefits, mainly due to their anti-inflammatory and antioxidant properties that provide protection against lipid peroxidation and damage caused by ROS [127].

In addition to carotenoids' scavenging function, it is believed that these natural pigments can also act indirectly [13]. This indirect pathway may include interactions with cellular signalling cascades, such as nuclear factor κB (NF-κB), mitogen-activated protein kinase (MAPK), or nuclear factor erythroid 2–related factor 2 (Nrf2) [51,52]. Some studies showed that carotenoids can be key players in NF-κB regulation, since they contain electrophilic groups that can interact with the cysteine residues of IκB kinase (IKK) and NF-κB subunits (p65), inactivating the NF-κB pathway and consequently decreasing the transcription of pro-inflammatory cytokine genes (e.g., TNF-α) [128,129]. In a recent study, Li et al. [130] showed that astaxanthin was able to protect retinal epithelial cells from H_2O_2-induced oxidative stress by inducing nuclear localization of Nrf2 and reducing intracellular ROS. However, in other studies, the Nrf2 translocation has been inhibited under different concentrations [13]. The effect of carotenoids in the MAPK pathway has been studied only in a few investigations and the results are very contradictory [13].

Despite the scarce number of investigations and the conflicting results obtained, there is also a major research gap related to carotenoid metabolism along the GIT and interactions with the gut microbiota [131]. Carotenoids are unstable molecules and are very susceptible to undergo various modifications such as hydrogenation, dehydrogenation, double-bond migration, chain shortening or extension, rearrangement, isomerization, oxidation, or combinations of these processes under different conditions [132].

Some studies showed that some unknown metabolites were produced during the carotenoids' pathway through the GIT. These metabolites may include apocarotenoids, that have a shorter chain length and oxygen modification, which increases their aqueous solubility and electrophilicity, and consequently, improves the target of some transcription factors such as NF-κB, giving these metabolites some biological effects [127,128]. Furthermore, it was reported that certain microbes produce carotenoids in the colon, demonstrating a prebiotic-like effect that results in bacterial shifts with health-associated properties [127].

Since it has been proved that carotenoids are important for human health, it would be important to understand the mechanisms used by carotenoids to become available for

absorption in the host colon, how they are utilized by microbes, and how carotenoids and their metabolites are processed to bring so many human health benefits [127]. This information will provide guidance to develop strategies for cell function manipulation through diet/nutraceuticals, impacting positively human health [132].

9. Conclusions

Carotenoids are natural pigments with important bioactive properties that promote health, becoming more studied and used over the last few years. Carotenoids' bioavailability in nature is low and little is known about their pathway through the GIT and consequent processes occurring during digestion, as well as the role of the intestinal microbiota and their metabolites on the metabolism and absorption of carotenoids.

Traditional methodologies such as SFE and HHP are used to increase carotenoids' solubility and bioavailability, but the organic solvents used are toxic to human health, necessitating the purification of carotenoids that are to be used in the food industry. More recently, OH has been proposed as a more advantageous methodology that can replace the traditional methods since it is selective, enabling bioactive compounds to be extracted without organic solvents.

This review set out to give an overview of the carotenoids' absorption mechanisms, mentioning the variables that can affect the stability and functionality of carotenoids. The four steps of such a mechanism were explained and the limiting factors that affect the bioavailability of these natural pigments in each phase were indicated.

In addition, it was highlighted that the intestinal microbiota can have an important influence on the absorption and metabolism of carotenoids. The change in the composition of the intestinal microbiota can enhance or inhibit the reproduction of some microbial species that can have defensive or damaging effects. The physicochemical structure of these natural pigments, the co-consumption with other compounds, the host variables, and the presence and type of food matrix are examples of other factors that can play important roles in carotenoid efficiency.

The intestinal microbiota can synthesise and release a variety of different metabolites that can be produced directly from dietary compounds (e.g., cellulose), produced by hosts and transformed chemically by gut bacteria (e.g., bile acids), or synthesized de novo by gut microbiota (e.g., ATP). These metabolites are absorbed and transferred into the circulating system and can regulate the composition and function of the host's intestinal microbiota, as well as play important roles in the development and progression of some pathologies.

However, just a few studies have been performed to understand the metabolism and absorption of these bioactive compounds along the GIT and further information and details about the mechanisms they use, as well as their metabolites, that contribute to human health benefits are still lacking. For this reason, more studies are required, since carotenoids have many important biological functions in the human organism, including the prevention of some of the most fatal diseases worldwide.

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PART VII

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