Effects of structural differences of biflavonoids from fruits of the Brazilian peppertree (*Schinus terebinthifolius* Raddi) on food-associated microorganisms

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Abstract

Economic, health, or sensory aspects are affected by microbial contamination of food. The preservation of food is crucial for ensuring food safety and reducing economic losses. With increasing resistance, allergies, and intolerances to conventional preservatives, the interest of consumers in natural preservatives is growing. Plants, such as *Schinus terebinthifolius*, which belongs to the Anacardiaceae family, are known to contain a diverse range of phytochemicals with bioactive properties. *Schinus* fruits contain several phenolic compounds, including gallotannins and flavonoids. Flavonoids have a wide range of chemical structures that greatly affect their biological activity, e.g., as antioxidants, anti-inflammatory or antibacterial agents.

The present thesis aimed to investigate the effects of structural differences of flavonoids, including tetrahydroamentoflavone, amentoflavone, agathisflavone, and their monomers apigenin and naringenin, on planktonic cells, biofilm formation, bacterial membrane properties, and components of pigment-forming food-associated microorganisms.

Our investigations showed that the antibacterial efficacy of flavonoids is influenced by their degree of oxidation and dimerization. Tetrahydroamentoflavone, a biflavonoid with a reduced C-ring at positions I-2,3 and II-2,3, exhibited the highest antibacterial activity, particularly against Gram-positive microorganisms. A minimum bactericidal concentration of 0.063 mg/ml was observed against *Bacillus subtilis*. Tetrahydroamentoflavone also exhibited remarkable inhibitory effects of biofilm formation, reducing biofilms by up to 99% in *Listeria innocua* and *Staphylococcus carnosus*.

Analysis of the effects of flavonoids on bacterial lipids and membrane properties showed a significant influence on carotenoids, fatty acids, menaquinones, and membrane fluidity and suggested a stress response of the microorganisms to the envelope or adaptive mechanisms.

The addition of naringenin led to an increase in carotenoid content and a simultaneous decrease in membrane fluidity, causing changes in the fatty acid composition of the bacteria, such as an increase in longer-chain fatty acids. This led to the assumption of membrane stabilization and alteration of the penetration barrier. An adjustment of the permeability barrier can also be inferred from the addition of amentoflavone, as it reduces the total carotenoid content of *Micrococcus luteus* J3.

Tetrahydroamentoflavone addition mainly reduced the staphyloxanthin content in *Staphylococcus xylosus* strains and showed similar trends to naringenin exposure. Considering

the reduction of staphyloxanthin, the impact on biofilm formation, and the CCS value of 220.99 ± 0.27 $Å^2$, which suggests steric hindrance, interactions with external cell components, particularly cell wall interactions, are conceivable and might be crucial for the higher antibacterial efficacy of the biflavonoid tetrahydroamentoflavone as a promising antibacterial agent.

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Preliminary remarks

List of abbreviations

%III/II	ratio of peak III compared to peak II (carotenoid spectral fine structure)
$\%A_B/A_{II}$	(*Z*)-peak intensity
ACP	acyl carrier protein
AGF	agathisflavone
AMF	amentoflavone
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
BHT	butylated hydroxytoluene
CAS	chrome azurol S
CCS	collision cross section
CE	collision energy
cfu	colony forming unit
DAD	diode array detector
DHAF	dihydroamentoflavone
DMSO	dimethyl sulfoxide
dw	dry weight
EDTA	ethylenediaminetetraacetic acid
EI	electron impact
EPS	extracellular polymeric substances

FA	fatty acid
GP	generalized polarization
HDTMA	hexadecyltrimethylammonium bromide
HPLC	high-performance liquid chromatography
IMS	ion mobility spectrometry
λ_{max}	maximum absorption
LC	liquid chromatography
MBC	minimum bactericidal concentration
MES	2-morpholinoethanesulfonic acid
MIC	minimum inhibitory concentration
MS	mass spectrometry
m/z	mass-to-charge ratio
n	number of samples
NAR	naringenin
nd	not detected
n.i.	not identified
PDA	photodiode array detector
ppGpp	guanosine-3',5'-bispyrophosphate
Q	quadrupole
QS	quorum sensing
rpm	revolutions per minute
THAF	tetrahydroamentoflavone
ToF	Time-of-Flight
t_R	retention time
TSB	tryptic soy broth

UHPLC	ultra high-performance liquid chromatography
UPLC	ultra-performance liquid chromatography
UV	ultraviolet
Vis	visible
v/v	volume fraction of two liquids
v/v/v	volume fraction of three liquids

Chapter 1

General introduction

1 Microbial spoilage of foods

Ensuring food safety is a constant challenge, triggered by modifications in food production and processing, as well as by the ever-changing dietary habits of consumers. Food contamination by microorganisms remains problematic and leads to food spoilage by microorganisms, alterations in flavor, odor, color, or foodborne disease outbreaks.

In 2019, 902 potentially foodborne outbreaks with 1213 illnesses were reported to the Robert Koch Institute in Germany. Included pathogens were *Campylobacter* spp., *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* (Robert Koch-Institut, 2020). In the United States, 800 foodborne multistate outbreaks during 2017-2019 were reported. A majority of the identified agents were bacterial agents with 46.8%, e. g. *Salmonella spp.*, *Shiga toxin-producing Escherichia coli*, or *Staphylococcus aureus*. (Centers for Disease Control and Prevention, 2023)

Across Europe, the food vehicles most commonly associated with outbreaks include composite foods, multi-ingredient foods, eggs and egg products, and meat and meat products (European Food Safety Authority, 2023). In foodborne outbreaks, specific microorganisms are related to food products. Meat products were the primary reported food vehicle for *Campylobacter enteritis* outbreaks, followed by dairy products. Eggs or egg products and meat products are associated with salmonellosis outbreaks. (Robert Koch-Institut, 2020)

A significant source of contamination or cross-contamination is the existence and dispersion of biofilms. The Federal Institute for Risk Assessment stated that cross-contamination was a crucial factor in 6 of 33 foodborne outbreaks in 2014 (Bundesinstitut für Risikobewertung, 2015). The ability of pathogenic microorganisms to bind to and form biofilms on various food contact surfaces has been described in numerous publications. Included are *Listeria monocytogenes*, *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus*, and *Campylobacter jejuni*. Similarly, spoilage organisms of the genera *Pseudomonas*, *Acinetobacter*, *Micrococcus*, *Streptococcus,* and *Lactobacillus* are capable of biofilm formation. (Linden et al., 2021; Jahid & Ha, 2014; Srey et al., 2013)

The need for natural preservatives is driven by a number of factors, in addition to an increase in resistance to conventional additives: An increase in consumer intolerances and allergies and their preference for foods produced as "naturally" as possible, or the growing demand for natural preservatives, which may be particularly desirable for use in organic products. (Zühlsdorf & Spiller, 2012)

Spices are valued as food ingredients for their preservative properties and their favorable sensory characteristics. The antimicrobial properties of spices in general are caused by a large variety of phytochemicals. Essential oils, isothiocyanates and other organosulfur compounds, alkaloids, and phenolic compounds are an integral part of current research activities. (Cushnie & Lamb, 2005; Aziman et al., 2014; Zhang et al., 2014a; Zhang et al., 2014b; Gottardi et al., 2016)

Plants from the Anacardiaceae (sumac) family, such as *Mangifera indica* and *Schinopsis brasiliensis* are known to contain a wide variety of phytochemicals with bioactive properties (Engels et al., 2009; Saraiva et al., 2011; Kabuki, 2000; Degáspari et al., 2005; Agedah et al., 2010; Engels et al., 2011; Marčetić et al., 2013; Sarkar et al., 2014; Schulze-Kaysers et al., 2015). The Brazilian pepper tree (*Schinus terebinthifolius* Raddi), which belongs to the Anacardiaceae family, is best known for its spicy fruits and also contains several promising bioactive compounds, including gallotannins and biflavonoids (Skopp, 1986; Feuereisen et al., 2014; Engels et al., 2011; Feuereisen et al., 2017b).

Engels et al. (2009; 2010, 2012a; 2012b) have already demonstrated the efficacy of the gallotannins, but knowledge of the biflavonoids is limited.

2 *Schinus terebinthifolius* Raddi

Schinus terebinthifolius Raddi is known as the Brazilian pepper tree and belongs to the economically important species of the Anacardiaceae family, along with *Mangifera indica* and *Pistacia vera*. The small, evergreen tree is native to Brazil and surrounding regions of South America, but is becoming increasingly widespread. Due to its high resistance and crowding out of native species, the plant is classified as highly invasive in some regions, such as Florida, and is therefore undesirable. (Morgan & Overholt, 2005; Morton, 1978)

The fruits of *Schinus terebinthifolius* Raddi belong to the drupe family, with a brittle exocarp, a parenchymatous mesocarp, and a ligneous endocarp (Figure 1). They are called pink pepper or pink berries and are used primarily as a seasoning. Their flavor is described as sweet-spicy and tart, and slightly astringent. Similarities to juniper are also mentioned. (Carmello-Guerreiro & Paoli, 2002; Skopp, 1986)

Figure 1: *Schinus terebinthifolius* fruits (Feuereisen et al., 2014).

Several studies focus on describing the phytochemicals in *Schinus terebinthifolius* fruits (Feuereisen et al., 2017b; Schulze-Kaysers et al., 2015; Skopp & Schwenker, 1986; de Araujo Gomes et al., 2020). In the essential oil of the fruit, mainly monoterpenes, such as α- and β-phellandrene, α- and β-pinene, and in low concentration sesquiterpenes were identified, while according to Skopp & Schwenker (1986), the ethanolic extract of the fruit is composed of gallic acids and the biflavones tetrahydroamentoflavone, II-2,3-dihydroamentoflavone, and amentoflavone. In addition, the biflavones agathisflavone and robustoflavone were detected in the fruit (Kassem et al., 2004).

The presence of the biflavones agathisflavone (I6,II8-biapigenin), amentoflavone (I3',II8-biapigenin), and II-2,3-dihydroamentoflavone in the fruit exocarp were also demonstrated by Feuereisen et al. (2014; 2017a). The hydrolyzable gallotannins, galloyl glucoses, and galloyl shikimic acids, and the anthocyanins, pelargonidin-3-*O*-galactoside, 7-*O*-methylcyanidin-3-*O*-galactoside, and 7-*O*-methylpelargonidin-3-*O*-galactoside, were identified in the fruit in addition to the biflavones (Feuereisen et al., 2014).

Various parts of the Brazilian pepper tree are used in traditional Brazilian medicine for wound healing, ulcer treatment, and respiratory diseases. Extracts are used as antibacterial, antiseptic, and analgesic medicines. Treatment of oral infections was also described as a potential use of components of the fruits. (Skopp, 1986; Bendaoud et al., 2010; da Silva et al., 2018; Carvalho et al., 2013; de Melo Júnior et al., 2002)

Studies have already confirmed the antibacterial activity of leaf, bark, and fruit extracts against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, and *Streptococci* (Barbieri et al., 2014; de Melo Júnior et al., 2002; Martínez et al., 1996; El-Massry et al., 2009; Muhs et al., 2017; Degáspari et al., 2005; da Silva et al., 2018). De Araujo Gomes et al. (2020) attributed the antimicrobial activity mainly to the extract enriched in phenolic compounds.

3 Flavonoids

In plants, flavonoids are ubiquitous. With the basic structure of benzo-γ-pyrone, they are part of the group of polyphenols, which are organic compounds biosynthesized from phenylalanine or tyrosine with one or more hydroxyl groups attached to a phenyl ring.

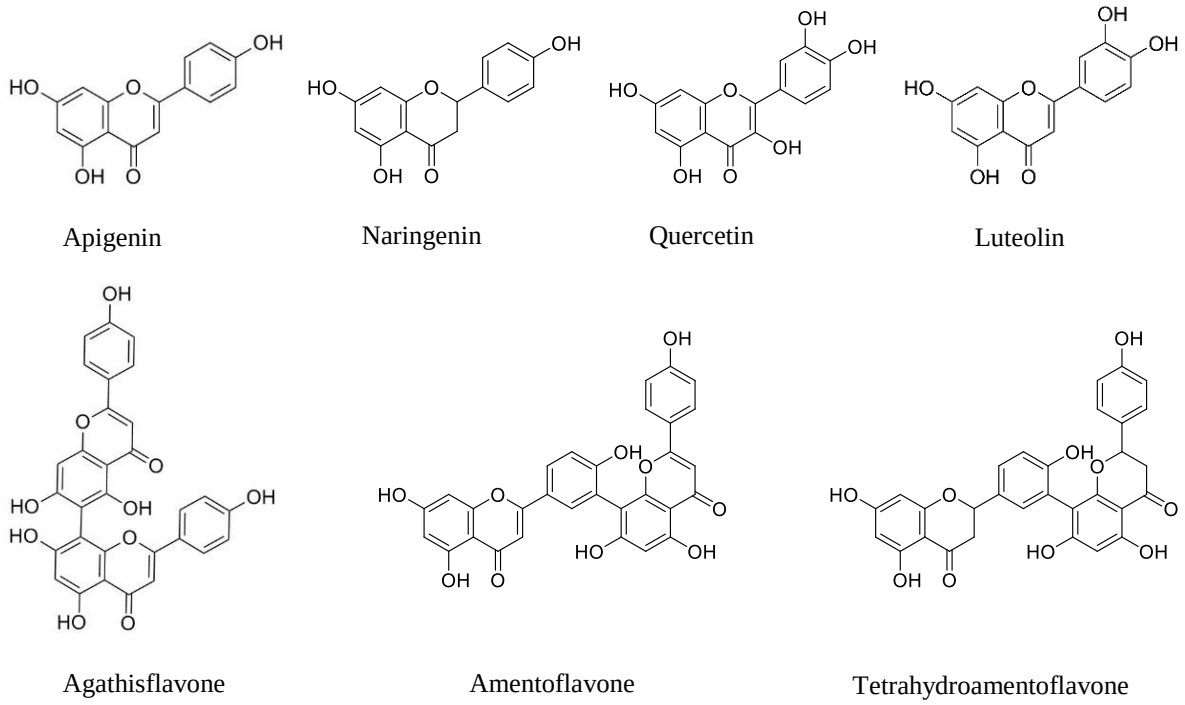


Figure 2: Structures of select flavonoids.

Depending on the degree of unsaturation and oxidation, flavonoids can be classified into various subclasses, such as anthocyanins, flavones (e. g. apigenin and luteolin), flavonols (e. g. quercetin), flavanols, flavanones (e. g. naringenin), flavanonols, and biflavonoids (e. g. agathisflavone, amentoflavone, tetrahydroamentoflavone) (Figure 2).

Biflavonoids are dimers of two flavonoid units and are the product of an oxidative coupling (Geiger & Quinn, 1975). The first biflavonoid was isolated from *Ginkgo biloba* L. in 1929 and later named ginkgetin (Gontijo et al., 2017; Locksley, 1973). Harborne (1967) first described biflavonoids as dimers of apigenin. Geiger & Quinn (1988) stated that biflavonoids are mostly composed of either two flavones, two flavanones, or one flavanone and one flavone. Ferreira et al. (2005) documented further biflavonoid formations with flavonols, dihydroflavonols, isoflavones, aurones, auronols, and chalcones. Tri-, tetra-, penta-, and even hexa-flavonoids can be formed by binding other monomers to biflavonoids.

Consequently, there is a large number of heterogeneous compounds belonging to the biflavonoid group. In 1989, about 90 naturally occurring biflavonoids were counted. By the early 2000s, 200 compounds were identified as biflavonoids and new ones are constantly being added as research progresses. (Gontijo et al., 2017)

Biflavonoids have been found especially in gymnosperms and in a few angiosperm families like Anacardiaceae. Within the Anacardiaceae, biflavonoids have been found in several genera, such as *Anacardium*, *Mangifera*, *Rhus*, *Schinus*, *Semecarpus*, and *Toxicodendron*. (Geiger & Quinn, 1975; Gontijo et al., 2017; Geiger & Quinn, 1988)

Flavonoids have a variety of functions in plant organs. Anthocyanins, together with other flavonoids, contribute to the coloration of flowers and fruits and attract pollinators. In vegetative organs, anthocyanins, or colorless flavonoids, such as flavones and flavonols act as a defense system against a range of biotic and abiotic stresses, such as herbivores, UV radiation, cold, heat, drought, and salinity. (Dixon et al., 1983; Agati et al., 2012; Falcone Ferreyra et al., 2012) Furthermore, studies suggest that flavonoids are important antimicrobial agents in plants and are involved in plant defense responses (Piasecka et al., 2015).

4 Antibacterial effect of flavonoids

Different studies evaluated various antibacterial activities and mechanisms of flavonoid-rich plant extracts and pure flavonoids for Gram-positive and Gram-negative bacteria. These include membrane disruption and inhibition of the cell envelope, nucleic acid, and ATP synthesis. In addition, inhibition of the electron transport chain, bacterial toxins, and efflux pumps were demonstrated and mechanisms influencing biofilm formation were reported. (Farhadi et al., 2019; Cushnie & Lamb, 2005; Vikram et al., 2010; Gontijo et al., 2017; Górniak et al., 2019) Flavonoids exhibit considerable chemical diversity, a factor that strongly influences their biological activity (Dixon, 2001). Hydroxylation, methylation, glycosylation, or reduced C-rings affect the bioactivity (Basile et al., 1999; Bernard et al., 1997; Dixon, 2001; Farhadi et al., 2019; Rauha et al., 2000; Shamsudin et al., 2022; Chen et al., 2018; Alcaráz et al., 2000; Cushnie & Lamb, 2005; Mori et al., 1987; Wu et al., 2013; Kaikabo et al., 2009; Moawad et al., 2010; Songca et al., 2012).

Antibacterial potential against the Gram-negative microorganisms *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* and the Gram-positive microorganisms *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus pyogenes* was demonstrated for the flavone monomer apigenin with an unsaturated C-ring (Basile et al., 1999; Oksüz et al., 1984; Bashir et al., 1994; Mamadalieva et al., 2011; Lucarini et al., 2015). The main targets of the antibacterial effect of apigenin include the nucleic acid processing enzymes, the cell wall, and cell membrane (Wang et al., 2017; Wu et al., 2008; Sato et al., 2000). The D-alanine:D-alanine ligase and the type II fatty acid synthesis pathway, which are involved in cell wall and cell membrane synthesis, were affected by apigenin. In addition, the expression of stress response genes and protein chaperone genes was found to be up-regulated in apigenin-treated *Enterococcus caccae*, suggesting an overall detrimental effect of apigenin. (Wang et al., 2017)

The addition of biapigenin amentoflavone has been shown to inhibit the growth of Gram-positive species, such as *Bacillus cereus*, *Enterococcus faecium*, *Streptococcus mutans*, and *Staphylococcus aureus* and the Gram-negative microorganisms *Escherichia coli* and *Pseudomonas aeruginosa* (Hwang et al., 2013; Kaikabo et al., 2009; Mbaveng et al., 2008). Cowan (1999) attributed the antibacterial effect to the formation of complexes with cell wall components that inhibit further adhesion and microbial growth. Bajpai et al. (2019) showed a significant change in cell morphology in both *Staphylococcus aureus* and *Escherichia coli*, resulting in a damaged cell wall and cell lysis.

Further studies have reported the inhibition of bacterial enzymes by flavones and their influence on biofilm formation by downregulation of quorum sensing regulators and intercellular adhesin gene expression (Chen et al., 2016; Jamil et al., 2014).

The flavanone naringenin showed antibacterial activity against *Staphylococcus aureus*, *Streptococcus mutans*, *Escherichia coli*, *Salmonella enterica,* or *Pseudomonas aeruginosa* (Yue et al., 2018; Vikram et al., 2010; Mundlia et al., 2019; Wang et al., 2018). It was shown that naringenin affects biofilm formation and cell-cell signaling (Vandeputte et al., 2011; Vikram et al., 2010; Yue et al., 2018; Song et al., 2020). Zhang et al. (2013) reported the inhibition of α-toxin production, one of the *Staphylococcus aureus* cytotoxins, by naringenin. In addition, naringenin caused a morphological change in the cell membrane due to a change in the composition of membrane fatty acids (Wang et al., 2018).

Only a few studies have demonstrated an antibacterial effect of binaringenins (Djoufack et al., 2010; Sagrera & Seoane, 2010). Furthermore, data on the antimicrobial properties and mechanisms of action of biflavonoids remain inconsistent or lacking, partly due to their chemical diversity.

5 Bacterial defense and resistance mechanisms

Three lines of defense can be described based on the site of action of antimicrobial agents: biofilm formation, cell envelope with cell wall and cell membrane, and cellular alterations with production of antagonistic agents or regulation of gene expression (Zhou et al., 2015).

5.1 Biofilm

A biofilm is a collection of microorganisms, including different species, attached to biotic and abiotic surfaces or interfaces and embedded in a matrix of extracellular polymeric substances (EPS) (Donlan, 2002). The microorganisms within these complex structures exhibit altered phenotypic and genotypic characteristics in terms of growth rate or gene transcription (Donlan & Costerton, 2002).

Microbial biofilm formation is accompanied by increased resistance, which is attributed to reduced disinfectant penetration, the presence of abiotic particles in the biofilm, or physiological changes, such as limited diffusion, due to EPS production, a reduction in metabolic activity, increased enzyme production, or the formation of efflux systems (Ma et al., 2009; Gilbert et al., 2002; Srinivasan et al., 1995; Wang et al., 2020). The latter allows

microorganisms to transport metabolites, quorum-sensing molecules, or even antimicrobial substances out of the cell (Pearson et al., 1999; Alav et al., 2018; Das & Mehta, 2018).

Biofilms lead to serious hygiene problems and economic losses due to food contamination or spoilage and affect sensory properties (Bremer et al., 2009; Brent et al., 2015). Genera of bacteria that are able to form biofilms and that are associated with food spoilage in the dairy, fish, poultry, meat, and ice cream industries are *Streptococcus*, *Lactobacillus*, *Pseudomonas*, *Moraxella*, and *Shewanella* (Ejaz et al., 2022; Chen et al., 2013; Ibekwe & Murinda, 2019; Bagge et al., 2001; Bagge-Ravn et al., 2003; Sharma & Anand, 2002; Bremer et al., 2009; Hanning et al., 2008; Zadernowska & Chajęcka-Wierzchowska, 2017; Marin et al., 2009; Wagner et al., 2020; Gunduz & Tuncel, 2006).

5.1.1 Biofilm formation

Starting from the planktonic state of microorganisms, the dynamic process of biofilm formation is divided into five phases (Figure 3).

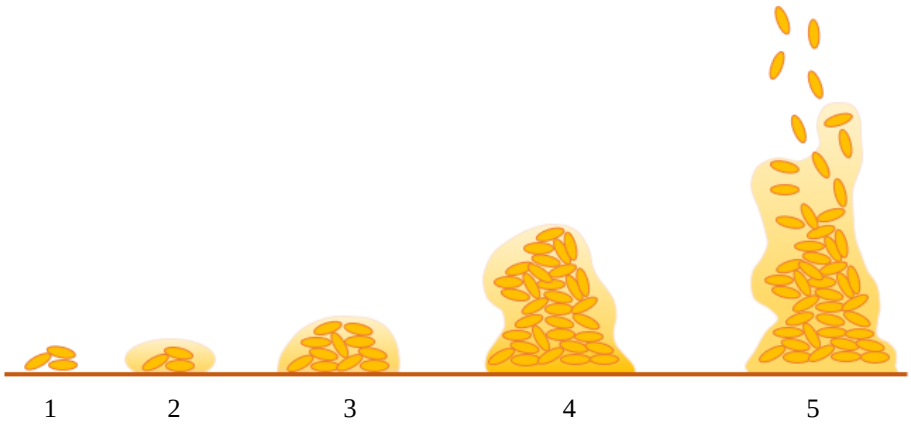


Figure 3: Biofilm formation. 1, initial reversible attachment; 2, irreversible attachment; 3, microcolony formation; 4, maturation; 5, dispersion.

First, there is the initial reversible attachment (1). The production of EPS and quorum sensing molecules promotes irreversible attachment (2) and microcolony formation (3). After maturation (4), there is the dispersion (5) of the biofilm community and ,thus, detachment of the planktonic cells: (van Houdt & Michiels, 2010)

Exposure to antimicrobial substances and other environmental factors, such as pH, temperature, osmolarity, oxygen availability, and nutrient composition, influence the phenotypic change from planktonic to sessile cells (Stepanović et al., 2003). Material and cell

surface properties play an important role in the process of biofilm formation. The negative charge on the surface of many microorganisms is initially a disadvantage for attachment due to electrostatic repulsion from the material surface. However, fimbriae, flagella, and lipopolysaccharides increase the hydrophobicity of the cell surface and allow hydrophobic interactions with contact surfaces. (Takahashi et al., 2010)

The irreversible attachment of pili, flagella, or adhesin proteins to a surface, which occurs within a few hours, involves water-bridging, covalent and ionic bonds or hydrophobic interactions (Kumar & Anand, 1998; Lundén et al., 2000). In *Staphylococcus aureus*, for example, attachment is mediated by surface adhesins, which were described as 'microbial surface components recognizing adhesive matrix molecules' (Foster & Höök, 1998). In addition, the secretion of quorum sensing molecules and production of EPS are involved in the irreversible attachment.

Communication between cells through quorum sensing can influence growth, pathogenicity, genetic factors, and biofilm formation (Davies et al., 1998; Annous et al., 2009). Quorum sensing is the emission of chemical signals, called autoinducers, that enable communication between individual microorganisms and the perception of cell density. The characteristic signaling molecules of Gram-negative bacteria are acylhomoserine lactones, which allow communication within and between species. Gram-positive organisms mostly produce short-chain peptides with chemical modifications as signaling molecules. At a certain concentration, these signaling molecules alter the expression of specific genes and enable the formation of a cell association. In *Pseudomonas aeruginosa*, mutants unable to synthesize the signaling molecules formed a more sensitive biofilm with an altered structure. (Davies et al., 1998; Bassler & Losick, 2006)

The production of EPS is activated in response to population density or certain environmental factors, such as osmotic pressure, pH, temperature, and nutrient availability. These substances determine the living conditions of the adherent cells by influencing porosity, hydrophobicity, absorption properties, and mechanical stability. EPS are composed of polysaccharides, proteins, phospholipids, teichoic and nucleic acids, and other polymeric substances. (Chmielewski & Frank, 2003)

The growth of irreversible adherent microorganisms and their aggregation leads to microcolony formation. This is accompanied by the production of EPS, which strengthens the bond between the bacteria and the surface. (Chmielewski & Frank, 2003; Donlan, 2002)

Biofilm maturation results in the development of organized structures that vary depending on the bacterial composition, age of the biofilm, and available nutrients. *Pseudomonas aeruginosa*

growing with glucose used as carbon source shows a mushroom-shaped structure after 5 days, with citrate as carbon source a flat structure was observed. (Klausen et al., 2003; Bridier et al., 2010; Doiron et al., 2012) In biofilms containing different bacterial species, EPS secretion was shown to vary with nutrient source, influencing biofilm maturation and structure (Xiao et al., 2012). Genetic changes also occur during biofilm maturation. Whiteley et al. (2001) showed that more than 70 genes are altered during maturation in *Pseudomonas aeruginosa*, including genes encoding translation, metabolic, membrane transport, or secretion proteins.

With increasing layer thickness, gradients form within the biofilm and nutrient availability decreases (Dunne, 2002; Characklis & Marshall, 1990). The organisms within the biofilm receive the minimal amount of nutrients needed to survive, resulting in cells low in metabolic activity. The stress response is an upregulation of enzyme synthesis and proliferation is stopped. (Carpentier & Cerf, 1993; Korber et al., 2009) Oxygen transport to the interior of the biofilm is also restricted. Consequently, anaerobic bacteria can proliferate in an initially aerobic system. (Flemming et al., 1992) Differentiation occurs within a heterogeneous biofilm, with different species occupying niches (Donlan, 2002; Costerton et al., 1995; Fuchs et al., 2007).

If energy-providing substrates do not reach the lower layers of the biofilm or if the removal of waste products is severely restricted, a dispersion of single cells or clusters occurs. The dispersion of planktonic cells serves to colonize further environmental niches, allowing growth or biofilm formation on more nutrient-rich surfaces and, thus, bacterial survival. Erosion, sloughing, and seeding are three different types of biofilm dispersal. Erosion is the continuous release of single cells or small clusters. Sloughing is the term used for the sudden detachment of large parts of the biofilm. Seeding is the release of single cells or small clusters of cells from hollow cavities in the biofilm. Erosion and sloughing can be active or passive processes, whereas seeding dispersal is always an active process. Passive processes include external disturbances, such as increased shear forces or human intervention. Internal or active biofilm processes may involve enzymatic degradation of the biofilm matrix or the release of EPS. (Choi & Morgenroth, 2003; Kaplan, 2010)

5.2 Cell envelope

The cell envelope, a complex multi-layered structure consisting of the cell membrane and the cell wall, acts as an external protective barrier against environmental changes and serves as a basic defense mechanism.

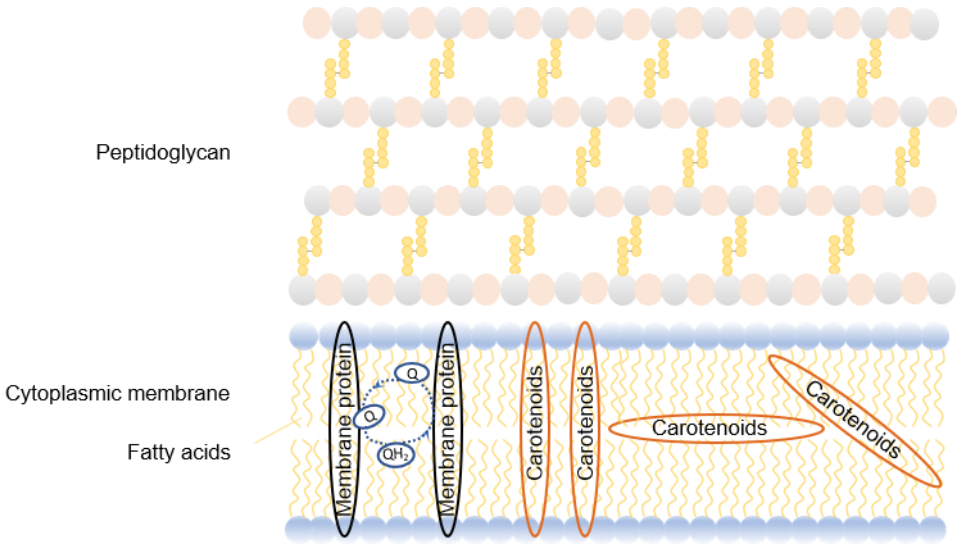


Figure 4: Schematic drawing of a Gram-positive cell envelope.

Bacterial cell walls are composed of a rigid layer called peptidoglycan, which is mainly responsible for the strength of the wall to withstand considerable internal osmotic pressure and to protect the cell from lysis. The Gram-positive cell wall is usually much thicker and peptide chains are highly cross-linked with bridges, whereas the Gram-negative cell wall is chemically complex, consists of at least two layers, and is partially cross-linked.

The cytoplasmic membrane (Figure 4) is a thin barrier surrounding the cell, separating the cytoplasm from the cell's environment. The general structure of the cytoplasmic membrane is a phospholipid bilayer, where the phospholipids contain both hydrophobic (fatty acid) and hydrophilic (glycerol phosphate) components. The internal fatty acids form a hydrophobic environment, whereas the hydrophilic parts remain exposed to the external environment or cytoplasm. The cytoplasmic membrane plays a crucial role as a permeability barrier, in controlling the transport of substances, in information flow, and serves as an anchor for many proteins. It is also the site of energy conservation in the cell and is, therefore, involved in some forms of transport, motility, and ATP biosynthesis. Crucially, the functional efficiency of the membrane is closely linked to its physical properties, in particular membrane fluidity.

Preserving the integrity of the cell envelope in response to stressors, such as flavonoids, is of considerable importance. Bacteria use adaptive mechanisms, such as the cell envelope stress response to effectively protect cellular structural integrity and functionality. These are triggered by two-component systems and sigma factors that are part of the bacterial envelope stress response and are characterized by changes in the cell envelope, such as reprogramming of cell wall biosynthesis to have alternative peptide endings of peptidoglycan. Changes in the cell envelope also include membrane modifications, for example, changes in fatty acid acyl chain properties, such as length, branching, and saturation, changes in the composition of minor membrane lipids, including carotenoid and menaquinone contents, or the synthesis of membrane protective proteins that serve to strengthen the membrane and affect the biophysical properties of the membrane. (Jordan et al., 2008; Bleul et al., 2022; Mishra et al., 2011; Brown et al., 2013; Willdigg & Helmann, 2021)

5.2.1 Fatty acids

The biophysical properties of the membrane are largely determined by the structure of the fatty acids. Fatty acids vary in chain length and many contain a single *cis* double bond or iso- or anteiso-methyl branches.

The type II fatty acid synthesis pathway is the most important pathway for the production of phospholipid acyl chains in bacteria and plants. Fatty acid biosynthesis is initiated by acetyl-CoA carboxylase converting acetyl-CoA to malonyl-CoA. The conversion of malonyl-CoA with acyl-CoA by β-ketoacyl-CoA synthase initiates elongation. Subsequent elongation processes are initiated by the elongation-condensing enzyme FabF. In Gram-positive bacteria, FapR and FabT are global transcriptional repressors. They regulate the expression of a number of genes involved in fatty acid synthesis. (Albanesi & de Mendoza, 2016; Schujman et al., 2003; Lu & Rock, 2006) However, other regulators are also involved. The global regulator guanosine 3',5'-bispyrophosphate influences the regulation of fatty acid synthesis (Heath et al., 1994; Pathania et al., 2021). Induction of guanosine 3',5'-bispyrophosphate inhibits the synthesis of malonyl-CoA (Pathania et al., 2021). Guanosine 3',5'-bispyrophosphate is also known as a signaling molecule of the bacterial stress response and induces pathogen virulence, persistence, and antibiotic resistance in bacteria (Wu et al., 2010).

Bacteria can grow in different physical environments by modeling the fatty acid composition. An essential component of the bacterial defense response is the alteration of biophysical properties, such as fluidity and permeability by adapting the fatty acid composition by changing

the chain length and the ratio of saturated to unsaturated fatty acids. (Murínová & Dercová, 2014; Sen et al., 2016; Zhang & Rock, 2008; Yoon et al., 2015)

5.2.2 Menaquinones

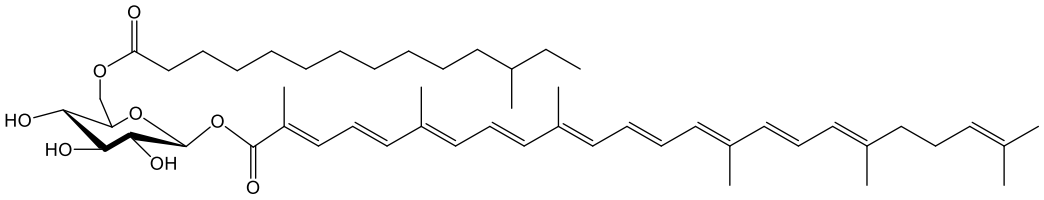
Menaquinones are redox-active small molecules. Menaquinones are known to be involved in the electron transport chain in bacteria and are, therefore, essential for energy production. In recent years, menaquinones have been reported to be involved in the detection of environmental changes, particularly those related to oxidative stress, in the regulation of ion permeability, membrane fluidity, biofilm formation, and persistent infection in *Staphylococcus aureus*. (Jordan et al., 2008; Kellermann et al., 2016; Johnston & Bulloch, 2020; Flegler et al., 2021)

Menaquinones are characterized by a naphthoquinone head group and a polyisoprenyl tail of variable length (3 to 13 repeats) which contributes to membrane anchoring. The classical Men pathway of menaquinone synthesis in bacteria begins with the formation of the naphthoquinone head group precursor 1,4-dihydroxy-2-naphthoic acid in the cytosol. The polyisoprenyl tail is synthesized by sequential condensation of isopentenyl diphosphate to form polyisoprenoid diphosphate products of various lengths, which are finally attached to the head group in the membrane. In response to changing environmental conditions, the composition of menaquinone in the membrane can be adjusted. (Johnston & Bulloch, 2020)

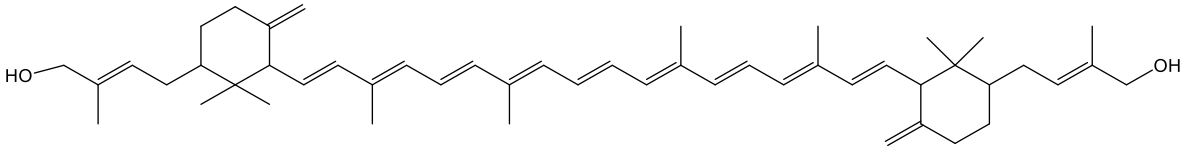
5.2.3 Carotenoids

Carotenoids, naturally occurring pigments, are synthesized by both eukaryotic cells, such as plants, algae, and fungi, and prokaryotic cells, such as bacteria. These lipophilic pigments contribute to the yellow, orange, and red colors that result from the presence of at least six conjugated double bonds in a polyene chain. The carotenoids, which are derivatives of the isoprenoids, can be classified into two main groups: non-oxygenated carotenes (e.g. phytoene, lycopene, and neurosporene) and oxygen-containing carotenoids, also known as xanthophylls. Xanthophylls, which are characterized by oxygen functional groups, include carotenoids, such as staphyloxanthin, sarcinaxanthin, zeaxanthin, astaxanthin, and carotenoid acids or carotenoid glycosides. Xanthophylls have a high structural diversity and are, therefore, the more complex group. They can be found either in free form or esterified with fatty acids. (López et al., 2023)

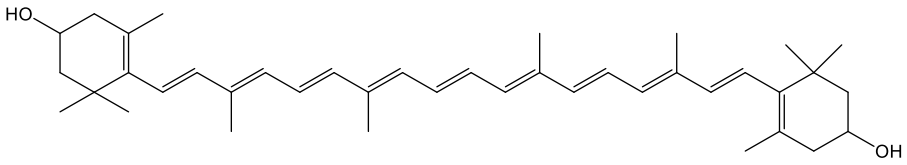
The carotenoids of most organisms are based on a symmetric C_{40} phytoene backbone. There are only a few carotenoids, such as staphyloxanthin or sarcinaxanthin, which are based on a C_{30} or C_{50} backbone. (Tobias & Arnold, 2006)



Staphyloxanthin



Sarcinaxanthin



Zeaxanthin

Figure 5: Structures of selected carotenoids.

Bacterial membranes contain carotenoids as lipid phase components and the mevalonate or methylerithritol phosphate pathway is responsible for the production of the isoprenoid precursor. The mevalonate pathway is found in many Gram-positive bacteria, such as *Staphylococcus aureus*. (Balibar et al., 2009) The precursor for carotenoid biosynthesis of staphyloxanthin, as shown in Figure 5, is isopentenyl pyrophosphate. Dehydrosqualene synthase converts farnesyl diphosphate to dehydrosqualene, which is colorless. Dehydrosqualene desaturase leads to the formation of the first yellow C_{30} carotenoid, 4,4'-diaponeurosporene, with absorption maxima at 415, 438, and 468 nm. The oxidation of the terminal methyl side group of 4,4'-diaponeurosporene to 4,4'-diaponeurosporenoate is the next biosynthetic step and is catalyzed by diaponeurosporene oxidase. The carboxylic acid carotenoid (absorption maxima: 455 and 483 nm) is formed via the aldehyde 4,4'-diaponeurosporenal (absorption maxima: 466 and 492 nm). The next proposed step, the

glycosylation of 4,4'-diaponeurosporenoate to glycosyl-4,4'-diaponeurosporenoate, is catalyzed by a glycosyltransferase. The final step in the pathway is the acylation of glycosyl-4,4'-diaponeurosporenoate with a fatty acid, catalyzed by an acyltransferase, to form staphyloxanthin or staphyloxanthin-like compounds with an absorption maximum at 462 nm. (Pelz et al., 2005; Marshall & Wilmoth, 1981; Takaichi, 2000; Kim & Lee, 2012)

Staphyloxanthin formation is controlled by several regulators within the alternative sigma factor σ^B (Kullik et al., 1998; Lan et al., 2010; Fey et al., 2013). Under unstressed conditions, σ^B is bound in an inactive state with its anti-sigma factor as σ^B-RsbW complex (Senn et al., 2005). Katzif et al. (2005) reported a positive correlation between σ^B activity and *csp*A, a cold shock gene. The level of *csp*A is repressed by Spx, a redox-sensitive transcription factor (Nakano et al., 2005). Donegan et al. (2019) described a post-translational control of the transcription factor Spx through its degradation. Activation of the cold shock protein CspA via Spx degradation would lead to increased pigmentation via increased σ^B activity and, thus, to increased resistance to oxidants (Liu et al., 2005; Donegan et al., 2019).

The carotenoid sarcinaxanthin and its glucosides are produced, for example, by *Micrococcus luteus*. The formation of sarcinaxanthin involves the initial conversion of farnesyl diphosphate to lycopene. Lycopene elongase leads to the formation of nonaflavuxanthin and flavuxanthin with absorption maxima of 445, 470, and 501nm. Finally, sarcinaxanthin with absorption maxima at 414, 438, and 467 nm is formed by carotenoid γ-cyclase. Carotenoid glycosyltransferase catalyzes the glycosylation of sarcinaxanthin. (Netzer et al., 2010)

In addition to providing color, carotenoids also strengthen the bacterial membranes and act as antioxidants, whereby the conjugated double bond system of the carotenoids is of fundamental importance. The interactions with both hydrocarbon chains and polar head groups of the membrane are influenced by structural properties, such as the stereochemistry of the carotenoids or their end groups. This affects biophysical membrane properties and determines their localization and orientation within the membrane (Figure 4). (Gruszecki, 2004; Gruszecki & Strzałka, 2005; Britton, 2008)

Numerous research studies have used model membrane bilayers (liposomes) to investigate the effect of structural properties of carotenoids. Carotenoids with polar substituents, such as the dihydroxycarotenoid zeaxanthin associate with the polar headgroup region of the membrane. Zeaxanthin increases the rigidity of the membrane in its fluid state by restricting the molecular movement of lipids in both the headgroup region and the hydrophobic core. The polar carotenoid alters hydrophobic barriers in model membranes, influencing the permeation of both polar and nonpolar molecules. In addition, zeaxanthin limits oxygen penetration into

the lipid bilayer and affects the permeability to protons and glucose in membrane vesicles. (Britton, 2008; Subczynski et al., 1991; Wisniewska et al., 2006; Subczynski et al., 1992; Berglund et al., 1999; Yamano et al., 2002; Hara et al., 2008)

In contrast to polar carotenoids, non-polar carotenoids, such as β-carotene increase the motional freedom of lipids in the headgroup region. β-Carotene or lycopene remain in the hydrocarbon interior of the bilayer and are almost perpendicular to the membrane lipid acyl chains. (Britton, 2008) Compared to the effects induced by polar carotenoids, the impact of non-polar β-carotene on the membrane is significantly less pronounced. The main effect observed is the fluidization of the well-ordered phase of phosphatidylcholine membranes. Furthermore, β-carotene reduces the membrane headgroup permeation barrier for small molecules. (Strzałka & Gruszecki, 1994)

While studies on model membranes were conducted, there was comparatively less research into the influence of the structural properties of bacterial carotenoids on bacterial membrane properties.

Studies related to the carotenoid content have shown that staphyloxanthin affects membrane permeability and rigidity and, thus, the susceptibility and stress response of microorganisms. This was attributed mainly to the carotenoid content and to a lesser extent to the carotenoid profiles of the microorganisms. (Mishra et al., 2011; Liu et al., 2005; Seel et al., 2020; Chia et al., 2021)

6 Aims of the thesis

Ensuring the shelf life of food, and therefore the edibility and health of the consumer, is limited by microbiological or chemical aspects. Preservation is necessary to reduce economic losses and to ensure food quality and safety. However, contamination or cross-contamination can occur during food production and processing, resulting in food spoilage or foodborne disease outbreaks. The high resistance and adaptability of the biofilm and other resistance mechanisms limit the effectiveness of conventional disinfectants and increase the need for new methods or antimicrobial agents.

In recent years, polyphenols have become increasingly important due to their beneficial effects on human health and represent a promising source of natural preservatives. Many aspects of their chemical and biological activities were identified and evaluated. However, the structural diversity of polyphenols results in a large number of different effects, and the mechanisms of action associated with the structure are still poorly understood. For the targeted and safe use of bioactive substances, such as flavonoids to preserve food quality, it is important to increase the understanding of their influence on bacterial growth, the mechanism of action, and the impact of structural differences.

Therefore, the present thesis aims to characterize the antibacterial activity of poorly studied *Schinus terebinthifolius* fruit extracts and biflavonoids and to investigate effects on the bacterial membrane of food-associated bacteria, considering the influence of specific structural differences of the compounds (Appendix 1 and 2).

Two individual studies had the following specific aims:

- Extraction of biflavonoids from the exocarp (biflavonoid extract) and the isolation of agathisflavone and tetrahydroamentoflavone from *Schinus terebinthifolius* fruits using liquid-liquid extraction and semi-preparative HPLC (Appendix 1)
- Determination of the antibacterial activity of the exocarp extract, drupe, and biflavonoid extract and specific flavonoids (the monomers apigenin and naringenin and the biflavonoids agathisflavone, amentoflavone, and tetrahydroamentoflavone) against planktonic cells of Gram-positive and Gram-negative food-associated bacteria (Appendix 1 and 2)
- Investigation of biofilm inhibition and characterization of biofilm after incubation with tetrahydroamentoflavone (Appendix 1)

- Characterization of membrane carotenoids from pigment-forming food-associated microorganisms (Appendix 2)
- Investigation of the effect of naringenin, amentoflavone, and tetrahydroamentoflavone on membrane components and properties of Gram-positive carotenoid-building microorganisms (Appendix 2)
- Investigation of the impact of the structural differences of flavonoids on their antibacterial activity and effect on microbial membrane components (Appendix 1 and 2)

Chapter 2

Effects of structural differences on the antibacterial activity of biflavonoids from fruits of the Brazilian peppertree (*Schinus terebinthifolius* Raddi)

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Clara Brinckmann: Investigation.
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Andreas Schieber: Supervision, Writing - review & editing.

Microbial contamination of food is a significant economic and public health issue. Natural preservatives are of increasing interest due to resistance, allergies, and intolerances. Plants of the Anacardiaceae family, which include well-known food products, such as mango, pistachio, and Brazilian pepper, are rich in bioactive compounds. The fruits of the Brazilian pepper tree, often used as a spice, contain anthocyanins, gallotannins, and the less studied biflavonoids. Mainly four biflavonoids are reported in the fruits: agathisflavone, amentoflavone, dihydroamentoflavone, and tetrahydroamentoflavone. Structural differences include linkage to the A-ring or B-ring and the degree of saturation of the C-ring.

The inconsistency or lack of studies on the antibacterial activity of the biflavonoids agathisflavone, amentoflavone, dihydroamentoflavone, and tetrahydroamentoflavone and their monomers apigenin and naringenin leave questions about their potential use as a natural food preservative. This study focused on understanding the impact of structural differences on the activity of agathisflavone, amentoflavone, tetrahydroamentoflavone, and their monomers against both Gram-positive and Gram-negative food-associated microorganisms, in addition to the identification and extraction of biflavonoids.

The biflavonoids were identified based on chromatographic, UV spectra, and mass spectrometric data. A biflavonoid-rich extract and the biflavonoids agathisflavone and tetrahydroamentoflavone were extracted from the exocarp and drupe extract of the *Schinus terebinthifolius* fruit. The exocarp extract contained 3.5% biflavonoids, which were significantly enriched by liquid-liquid extraction to 33.5%, consisting of 82.3% agathisflavone, 10.3% amentoflavone, 6.2% dihydroamentoflavone, and 1.2% tetrahydroamentoflavone. The predominant agathisflavone was isolated from this biflavonoid extract with a chromatographic purity of 99%. The drupe extract contained 4.0% tetrahydroamentoflavone, which was obtained with a chromatographic purity of 96%.

Antibacterial efficacy studies showed a high activity of the flavonoid dimer tetrahydroamentoflavone with reduced C-ring at position I-2,3 and II-2,3, particularly against Gram-positive microorganisms, with a minimum bactericidal concentration of 0.063 mg/ml against *Bacillus subtilis*. In the context of biofilm formation, tetrahydroamentoflavone showed remarkable inhibitory capabilities, leading to a reduction of biofilms by up to 99% in *Listeria innocua* and *Staphylococcus carnosus*. Biofilm formation of *Pseudomonas putida* was partially suppressed, while *Escherichia coli* and *Serratia ficaria* remained unaffected.

In addition, tetrahydroamentoflavone was observed to be embedded in the biofilm of *Listeria innocua* and *Staphylococcus carnosus*. Tetrahydroamentoflavone within the biofilm shows an influence on the biofilm structure and, in combination with the inhibitory effect of tetrahydroamentoflavone, indicates an inhibition of the protective function and, thus, an influence on the EPS matrix as one key factor of biofilm resistance.

In contrast, amentoflavone with an oxidized C-ring showed no discernible effect on bacterial growth, whereas the constitutional isomer agathisflavone showed a slight inhibitory effect on *Staphylococcus carnosus*.

The monomeric compounds apigenin and naringenin exhibited negligible antibacterial activity.

Studies have already shown a strong structural dependence of natural products on their bioactivity, including that hydroxyl and methoxy groups are particularly effective at certain positions (Farhadi et al., 2019). Our research findings indicate that the antibacterial effect of biflavonoids is significantly influenced by their dimerization and oxidation state. Among the biflavonoids and their monomers, the flavonoid dimer tetrahydroamentoflavone with the saturated C-ring shows the highest antibacterial activity on planktonic and sessile cells. There is a 15-fold increase in antibacterial activity on planktonic cells compared to the monomers

studied. In general, the saturated C-rings of flavonoids influence the conformation of the molecule. This might contribute significantly to the antibacterial effect.

Tetrahydroamentoflavone, a potential antibacterial found in the fruit of *Schinus terebinthifolius*, could provide a valuable natural alternative to synthetic food additives. The research showed that the antibacterial properties of biflavonoids, such as tetrahydroamentoflavone depend significantly on their specific structural features. These results are important for further research into active ingredients to improve food safety and meet the demand for natural antibacterial agents in the food industry.

Chapter 3

Effects of flavonoids on membrane adaptation of food-associated bacteria

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Brazilian peppertree fruits contain bioactive compounds, such as gallotannins and biflavonoids with known antimicrobial properties, which are of growing interest as natural food additives derived from plants, especially phytochemicals.

Our previous research has shown that the efficacy of flavonoids, including tetrahydroamentoflavone, depends on their structural properties, such as dimerization and oxidation state. Flavonoids are thought to interact with bacterial cell membranes, although the exact mechanisms, particularly for biflavonoids, remain unclear. Cell membranes play a crucial role in bacterial defense, serving as a permeability barrier and energy conservation site. Their composition can be influenced by factors like fatty acid profiles, carotenoids, and menaquinones, affecting properties like fluidity and permeability, key to microbial survival and stress response. This study examines the identification of carotenoids in pigment-forming food-associated microorganisms *Staphylococcus xylosus* (DSM 20266^T and J70), *Staphylococcus carnosus* DSM 20501^T, and *Micrococcus luteus* (ATCC 9341 and J3) and the modification of

their membrane composition by the monomer naringenin and the dimers tetrahydroamentoflavone and amentoflavone focusing on bacterial lipids (carotenoids, fatty acids, and menaquinones) and membrane fluidity based on Laurdan generalized polarization (GP).

In this study, LC-MS^n analysis revealed the presence of 15 carotenoids in the *Staphylococcus xylosus* and *Staphylococcus carnosus* strains, while a total of 19 carotenoids were identified in the *Micrococcus luteus* strains.

For *Staphylococcus xylosus* and *Staphylococcus carnosus* a (*Z*)-staphyloxanthin isomer was tentatively identified in addition to the high concentration of (all-*E*)-staphyloxanthin. Furthermore, 4,4'-diaponeurosporenoic acid and 4,4'-diaponeurosporene were detected. Other compounds with spectral properties identical to those of staphyloxanthin, tentatively identified by mass spectrometry as glucosyl-4,4'-diaponeurosporenoate fatty acid esters, were present in the carotenoid profiles of *Staphylococcus* spp.

Sarcinaxanthin diglucoside, sarcinaxanthin monoglucoside, and sarcinaxanthin were the main carotenoids of *Micrococcus luteus* ATCC 9341. Based on the relative retention time shift, the same UV/Vis spectra and parent ions of the carotenoids produced by *Micrococcus luteus* J3, those identified as decaprenoxanthin diglucoside, decaprenoxanthin monoglucoside, and decaprenoxanthin.

The antibacterial activity determined in the structure-related studies of our initial work was confirmed by the results of the antibacterial effect of flavonoids on selected pigment-forming food-associated microorganisms.

Analysis of the influence of flavonoids on bacterial lipids and membrane properties revealed a remarkable effect on carotenoids, fatty acids, menaquinones, and membrane fluidity of the microorganisms studied. The increased carotenoid content observed with the addition of naringenin was accompanied by an increase in Laurdan GP, i.e. a decrease in membrane fluidity. Naringenin altered the carotenoid profile with an eightfold increase in the 4,4'-diaponeurosporenoic acid proportion and a corresponding reduction in staphyloxanthin and staphyloxanthin-like compounds in *Staphylococcus xylosus*. In addition, naringenin caused changes in the fatty acid composition of the bacteria, with a decrease in specific fatty acids, particularly the C15:0 iso fatty acid, and an increase in longer chain fatty acids. The resulting membrane stabilization and change in the penetration barrier could be attributed to a reduced susceptibility of the microorganism (Wang et al., 2018; Berglund et al., 1999; Britton, 2008).

Amentoflavone addition mainly decreased total carotenoid content in *Micrococcus luteus* J3, with the most pronounced decrease in decaprenoxanthin accompanied by a relatively

proportional increase in polar xanthophyll glucoside, suggesting adaptation by altering the permeability barrier (Berglund et al., 1999). In addition, amentoflavone caused a decrease in menaquinone content in *Staphylococcus xylosus* DSM 20266^T, *Staphylococcus carnosus* DSM 20501^T, *Micrococcus luteus* ATCC 9341, and *M. luteus* J3.

The addition of tetrahydroamentoflavone had effects similar to the exposure to naringenin and mainly affected the carotenoids in the strains studied. Incubation with tetrahydroamentoflavone resulted in a decrease in staphyloxanthin in *Staphylococcus xylosus* DSM 20266^T and *Staphylococcus xylosus* J70, which was accompanied by a reduction in total carotenoid content. The significant difference between the CCS values of tetrahydroamentoflavone (220.99 ± 0.27 Å^2), naringenin (161.96 ± 0.11 Å^2), and amentoflavone (178.58 ± 0.84 Å^2) indicates a steric hindrance and a lower potential to interact with cell membranes. The reduced staphyloxanthin content and the CCS value of tetrahydroamentoflavone suggest interactions with the cell wall and might be crucial for the promising antibacterial activity in carotenoid-producing microorganisms.

The effects of flavonoids on bacterial lipids were structure related and strongly strain dependent. Changes in their profile and contents with addition of flavonoids, especially carotenoids, suggest a flavonoid-induced stress response of microorganisms and the use of adaptive mechanisms. Different mechanisms of action, targets, and membrane interactions can be associated based on the size of the flavonoid, its three-dimensional structure, and the degree of oxidation. This study provides valuable insights into the response of pigment-forming food-associated microorganisms to naringenin, amentoflavone, and tetrahydroamentoflavone, which is important for the targeted and safe application of these as natural preservatives.

Chapter 4

Concluding summary

Natural antimicrobials have gained attention due to the increasing demand for natural preservation of less processed foods. Given the promising results obtained from polyphenol-rich extracts of Anacardiaceae plants, which contain several bioactive compounds, it is desirable to demonstrate the suitability of bioactive compounds as natural food preservatives. Furthermore, it is important to understand the underlying resistance or adaptation mechanisms of microorganisms for subsequent control and preservation of food quality.

In this work, the antibacterial activity of the biflavonoids contained in the fruit of *Schinus terebinthifolius* was studied, considering their structural differences (Appendix 1 and 2). Furthermore, the effects on components of the microbial membrane were characterized (Appendix 2).

1 Effects of extract and flavonoids on the growth of food-associated microorganisms

The antibacterial activity of *Schinus terebinthifolius* extracts was promising against Gram-positive microorganisms. Among the investigated biflavonoids tetrahydroamentoflavone, agathisflavone, and amentoflavone and their monomers naringenin and apigenin, tetrahydroamentoflavone isolated from the drupe extract was found to be the most effective antibacterial flavonoid. Incubation of Gram-positive pigmented and non-pigmented food-associated microorganisms with tetrahydroamentoflavone resulted in a minimum inhibitory concentration and a minimum bactericidal concentration of up to 0.063 mg/mL.

In addition to effects on planktonic cells, we observed a significant biofilm inhibition and reduced cell number for Gram-positive and Gram-negative bacteria by tetrahydroamentoflavone in the range of 0.016 mg/mL to 0.5 mg/mL.

Tetrahydroamentoflavone, as a constituent of *Schinus terebinthifolius* fruits, is therefore a promising antibacterial agent due to its inhibitory and bactericidal effect on planktonic cells and inhibition of biofilm formation, a persistent source of contamination. (Appendix 1 and 2)

2 Antibacterial effect related to the flavonoid structure

Molecular properties, such as molecular weight, galloylation, hydroxylation, and double bond in the C-ring have been observed to influence the antibacterial activity of flavonoids (Leopoldini et al., 2004; Johnson et al., 2011; Liu et al., 2020).

The biflavonoid tetrahydroamentoflavone is characterized by a reduced C-ring at position I-2,3 and II-2,3. At these positions, the less effective amentoflavone is oxidized. The monomer naringenin also shows no inhibitory effect at concentrations below 1 mg/mL in both studies. (Appendix 1 and 2)

Studies on the antibacterial activity have provided a variety of results. According to Echeverría et al. (2017), the double bond in the C-ring might be essential for bacterial growth inhibition. However, Shamsudin et al. (2022) showed an increased antibacterial activity of flavonoids due to the saturated C-ring. Naringenin has been shown to have stronger antibacterial activity than the corresponding flavone apigenin (Xie et al., 2017; Vikram et al., 2010). An increasing antibacterial effect with the degree of C-ring saturation was also demonstrated by Moawad et al. (2010).

The absence of the double bond in the C-ring restricts electronic delocalization and affects the conformation and biological activity of flavonoids (Leopoldini et al., 2004). Johnson et al. (2011) investigated the effect of flavonoids on a health-relevant human enzyme and attributed the differential inhibition to structural differences. Flavonoids with an unsaturated C-ring, such as luteolin, apigenin, and quercetin, allow them to remain planar in the binding cavity of the enzyme, avoiding steric interactions. The C-ring of naringenin is saturated, which leads to bulging in the middle of this molecule and steric interactions in the narrow binding site.

Based on the observations of Johnson et al. (2011), steric factors might be relevant in the effects of tetrahydroamentoflavone due to its higher CCS value of 220.99 ± 0.27 $Å^2$ compared to naringenin with a CCS value of 161.96 ± 0.11 $Å^2$. Nevertheless, a significant antibacterial effect of tetrahydroamentoflavone was observed. (Appendix 1 and 2)

Based on a docking study between amentoflavone and the tyrosine phosphatase 1B in combination with a receptor-oriented in silico screening of biflavonoids by Lee et al. (2008), tetrahydroamentoflavone might be classified as a potent allosteric inhibitor of the protein, with the linkage at the I-3', II-8-position and the hydroxyl group at the 4'-position as critical factors. The conformational change of the enzyme was attributed to hydrogen bonding of the biflavonoid amentoflavone or tetrahydroamentoflavone with, for example, amino acid residues of glutamic acid or phenylalanine. Strong hydrogen bonds between amino acid residues of enzymes and amentoflavone, with associated conformational changes of the enzymes, were

confirmed by further studies (Bai et al., 2019; Li et al., 2023). Furthermore, Bajpai et al. (2019) observed a deformation of bacterial cell walls treated with amentoflavone, which resulted in cell wall damage and cell lysis. Considering the conformational changes of an enzyme that are caused by hydrogen bonds, the cell wall deformation might be attributed to non-covalent interactions with cell wall components. Hydrogen bonding between the peptidoglycan of the bacterial cell wall, particularly the amino acid residues of the cell wall peptides, and biflavonoids might influence the strength and function of the cell wall.

In our study, we showed that tetrahydroamentoflavone effectively inhibits biofilm formation. Further, we detected tetrahydroamentoflavone in the biofilm or microcolonies of *Listeria innocua* and *Staphylococcus carnosus*. We suspected an effect on the EPS matrix or quorum sensing signaling and, thus, on the secreted cyclic peptides of Gram-positive bacteria. (Appendix 1) Biofilm inhibition might result from interactions between cellular components and the biflavonoid, and associated alteration of characteristics in the conformational or functional properties of cell components.

The observed stronger antibacterial activity of tetrahydroamentoflavone might result from its different three-dimensional structure due to the unsaturated C-rings compared to amentoflavone (CCS value of 178.58 ± 0.84 $Å^2$). The conformation of tetrahydroamentoflavone or different binding conformations in the formation of hydrogen bonds might have a greater effect on the functionality of the cell components, such as peptidoglycan, secreted cyclic peptide, or EPS amino acid residues, resulting in outstanding inhibition of bacterial growth or biofilm formation.

3 **Impact of flavonoids on adaptation mechanisms**

Laurdan GP, carotenoid, fatty acid, and menaquinone contents and profiles were significantly altered by the flavonoids naringenin, amentoflavone, and tetrahydroamentoflavone, indicating changes in membrane properties (Appendix 2). In addition, the biofilm composition was strongly influenced by tetrahydroamentoflavone (Appendix 1).

3.1 Characterization of membrane carotenoids

Carotenoids were characterized according to their retention time, absorption spectra, and mass spectrometric data in comparison with published data (Marshall & Wilmoth, 1981; Kim & Lee, 2012; Takaichi, 2000; Pelz et al., 2005; Netzer et al., 2010; Osawa et al., 2010; Arpin

et al., 1973; Vila et al., 2019; Xie et al., 2021). In *Staphylococcus xylosus* and *Staphylococcus carnosus* strains, 15 carotenoids and in *Micrococcus luteus* strains, 19 carotenoids were detected by LC-MS^n analysis. (Appendix 2)

The carotenoid profiles of *Staphylococcus* spp. are characterized by a high concentration of (all-*E*)-staphyloxanthin with *m/z* 819. A (*Z*)-staphyloxanthin isomer was tentatively identified and the precursors 4,4'-diaponeurosporene and 4,4'-diaponeurosporenoic acid were detected. Further compounds were tentatively identified by mass spectrometry as glucosyl-4,4'-diaponeurosporenoate fatty acid esters or staphyloxanthin-like compounds, with spectral properties identical to staphyloxanthin.

The main carotenoids in *Micrococcus luteus* ATCC 9341 were identified as sarcinaxanthin diglucoside (*m/z* value of the parent ion: 1029), sarcinaxanthin monoglucoside (*m/z* value of the parent ion 1029), (all-*E*)-sarcinaxanthin *(m/z* value of the parent ion: 705, *m/z* values of the fragments: 687, 669, and 497), and (*Z*)-sarcinaxanthin isomer based on the presence of a (*Z*)-peak at λ_{cis} = 327 nm and a $\%A_B/A_{II}$ of 52%. In addition to free xanthophylls and their glucosides, the carotenoid profile includes a considerable number of tentatively identified esterified carotenoids. The strain *Micrococcus luteus* J3 produced carotenoids with the same UV/Vis spectra and parent ions. Based on the relative retention time shift of these carotenoids and ion fragments they were identified as decaprenoxanthin diglucoside, decaprenoxanthin monoglucoside, and decaprenoxanthin.

The detailed characterization of the carotenoid profile provided insight into the changes caused by the addition of flavonoids.

3.2 Membrane fluidity

The monomer naringenin, the biflavonoid tetrahydroamentoflavone, and amentoflavone affected Laurdan GP in certain microorganisms. The incubation of the pigment-forming microorganisms with naringenin caused an increase in carotenoid content in *Staphylococcus xylosus* DSM 20266^T and *Staphylococcus carnosus* DSM 20501^T, tetrahydroamentoflavone in *Staphylococcus carnosus* DSM 20501^T. This was associated with an increase in Laurdan GP and, thus, a decrease in membrane fluidity, consistent with previous studies on the effect of carotenoids on membrane fluidity (Tiwari et al., 2018; Gruszecki & Strzałka, 2005; Strahl & Errington, 2017). The resulting stabilization of the membrane implies reduced microbial susceptibility (Britton, 2008; Mishra et al., 2011; Bessa et al., 2018).

Laurdan GP of *Micrococcus luteus* J3 was also increased by amentoflavone, although amentoflavone reduced carotenoid contents. However, the polar predominant carotenoids

decaprenoxanthin glucosides with a content of 62% in *Micrococcus luteus* J3 increased with the addition of amentoflavone. This could result in a reduced freedom of movement of the membrane (Wisniewska & Subczynski, 1998). However, similar results were observed for the carotenoid profile altered by tetrahydroamentoflavone and no change was detected for Laurdan GP. Therefore, the change in fluidity could not be attributed solely to the carotenoid content of *Micrococcus luteus* J3, compared to *Staphylococcus* spp. Furthermore, no correlation was identified between the observed change in fluidity and the changes in lipid profile and menaquinone content, which can also affect membrane fluidity (Kellermann et al., 2016; Sen et al., 2016; Zhang & Rock, 2008; Seel et al., 2018).

In liposomal model membranes, Lenne-Gouverneur et al. (1999) observed a decrease in membrane fluidity caused by amentoflavone membrane interactions. Thus, interactions between amentoflavone and the membrane might affect the membrane fluidity in *Micrococcus luteus* J3.

3.3 Membrane permeability

In addition to the effect on the membrane fluidity, modifications in the carotenoid profile and menaquinone content indicate changes in membrane permeability. A twofold increase in the proportion of the polar 4,4'-diaponeurosporenoic acid and non-polar 4,4'-diaponeurosporene and a decrease in the relative proportion of staphyloxanthin in the membrane was induced by naringenin in *Staphylococcus* xylosus DSM 20266^{T}. In *Staphylococcus xylosus* J70 we observed an eightfold increase in the 4,4'-diaponeurosporenoic acid proportion and a corresponding decrease in staphyloxanthin and staphyloxanthin-like compounds. In addition, incubation of *Staphylococcus xylosus* strains with naringenin resulted in a significant increase in menaquinone content.

In *Staphylococcus carnosus* DSM 20501^{T}, the carotenoid profile showed a similar alteration compared to *Staphylococcus xylosus* strains with a reduced staphyloxanthin content due to naringenin and tetrahydroamentoflavone. There is also an increase in longer-chain staphyloxanthin-like compounds, mainly esterified with $C_{17:0}$, $C_{19:0}$, and $C_{20:0}$, especially in the presence of naringenin.

Amentoflavone reduced the menaquinone content of *Staphylococcus xylosus* DSM 20266^{T} and *Staphylococcus carnosus* DSM 20501^{T}.

In *Micrococcus luteus* J3, tetrahydroamentoflavone and amentoflavone caused a decrease in total carotenoid content accompanied by a relatively proportional increase in polar xanthophyll glucosides. Amentoflavone additionally causes a reduction in the menaquinone content.

Previous studies have reported the influence of structural properties, such as the stereochemistry of the carotenoids or their end groups, which determine the localization and orientation in the membrane, on the permeability of membrane vesicles (Britton, 2008; Gruszecki & Strzałka, 2005). Polar carotenoids were reported to limit oxygen penetration into the lipid bilayer and to affect the permeability of membrane vesicles to protons and glucose. Non-polar carotenoids were found to reduce the permeation barrier for small molecules. (Wisniewska & Subczynski, 1998) In addition, Kellermann et al. (2016) observed a reduction in membrane permeability with increased menaquinone content.

In conclusion, the changes observed in the carotenoid profile and menaquinone content indicate a specific adaptation of the permeability barrier to naringenin, tetrahydroamentoflavone, and amentoflavone.

3.4 Interactions with cell components

Amentoflavone showed evidence of cell envelope interactions by reducing membrane fluidity in *Micrococcus luteus* J3. The effect of tetrahydroamentoflavone on carotenoid content also indicates cell envelope, specifically cell wall interactions. Tetrahydramentoflavone reduced staphyloxanthin by 26% in *Staphylococcus xylosus* DSM 20266^{T} and 35% in *Staphylococcus xylosus* J70, accompanied by a decrease in total carotenoid content. Carotenoid production is controlled in part by the sigma factor σ^{B}, cold chock gene *csp*A, and the redox-sensitive transcription factor Spx (Nakano et al., 2005; Donegan et al., 2019; Kullik et al., 1998; Lan et al., 2010; Fey et al., 2013). Rojas-Tapias & Helmann (2018) demonstrated an induction of the Spx regulon by cell wall stress. According to the pathway proposed by Donegan et al. (2019), increased expression of Spx represses *csp*A expression, leading to a reduction in σ^{B} activity and, consequently, to a reduction in staphyloxanthin content (Donegan et al., 2019; Nakano et al., 2005).

Furthermore, with an initial tetrahydroamentoflavone concentration of 0.5 mg/mL, concentrations of 33.38 ± 7 µg/mL and 30.02 ± 6 µg/mL tetrahydroamentoflavone were detected in the biofilm of Gram-positive microorganisms *Listeria innocua* and *Staphylococcus carnosus* after incubation, with corresponding inhibition of biofilm formation. Biofilm inhibition was also observed in the Gram-negative microorganism *Pseudomonas putida*, but no tetrahydroamentoflavone was detected in the biofilm. The global regulator Spx also affects biofilm formation. Pamp et al. (2006) observed reduced biofilm formation in Spx-producing *Staphylococcus aureus* strains compared to cells without Spx.

In conclusion, tetrahydroamentoflavone decreased staphyloxanthin content and biofilm formation and is incorporated into the biofilm. Furthermore, a potential steric hindrance can be assumed due to the significantly higher CCS value of 220.99 ± 0.27 $Å^2$ of tetrahydroamentoflavone compared to naringenin (161.96 ± 0.11 $Å^2$) and amentoflavone (178.58 ± 0.84 $Å^2$). Based on this, interactions with external cell components, in particular cell wall interactions and the associated activation of the global regulator Spx, are reasonable and could be crucial for the higher antibacterial efficacy of tetrahydroamentoflavone.

4 Preservation of food quality

Tetrahydroamentoflavone has been identified as a promising antibacterial compound. The biflavonoid was obtained from the drupe extract of *Schinus terebinthifolius* fruit with a chromatographic purity of more than 96% by liquid-liquid extraction. Based on the initial weight of 5.0 g of dried drupe extract, 131 ± 11 mg of tetrahydroamentoflavone was isolated.

The addition of tetrahydroamentoflavone to food-associated microorganisms resulted in an antibacterial effect in Gram-positive planktonic cells at 0.063 mg/mL and in biofilms at 0.016 mg/mL. The complex extraction from the plant includes the potential cost increase of adding natural preservatives to food. In this context, extraction yield and antibacterial efficacy play an important role. The yield in relation to the antibacterial activity of tetrahydroamentoflavone is promising for food applications.

Given the complexity of the food matrix, future studies are expected to show changes in the antibacterial efficacy of the biflavonoid in food depending on the composition and structure of the food matrix. The food matrix consists of several regions with very different physical properties that affect the distribution of substances. An added antimicrobial agent may only be effective in one of the regions. Interactions with proteins, carbohydrates, and lipids, such as electrostatic interactions, which are strongly dependent on environmental conditions (pH, temperature, ionic strength), or van der Waals forces might influence the antibacterial efficacy of flavonoids or flavonoid-rich extracts. The hydrogen-bonding groups and the non-polar, polarizable aromatic rings of flavonoids are key chemical structures that facilitate these interactions and might contribute to reduced antibacterial activity. (Weiss et al., 2015; Bordenave et al., 2014; Wu et al., 2024)

In addition to reducing the antibacterial activity in food compared to the model system, these interactions can cause astringency or haze formation, which are often considered undesirable in food and beverage systems (Bordenave et al., 2014).

Non-covalent interactions with proteins, such as van der Waals forces and hydrogen bonding, were observed for amentoflavone (Bai et al., 2019; Li et al., 2023). The interactions between proteins and flavonoids exhibit high specificity, with flavonols, isoflavones, and flavones showing stronger binding affinity to proteins compared to flavanones or flavanols (Piparo et al., 2008). Similar observations have been reported for the interactions between flavonoids and polysaccharides, where hydrogen bonding also appears to play an important role (Wang et al., 2013; Bordenave et al., 2014). Furthermore, interactions between lipids and non-polar, polarisable aromatic rings were higher for isoflavones and flavonols than for flavanones or flavanols (Dangles & Dufour, 2006; Bordenave et al., 2014). Based on these observations, interactions between the flavanone tetrahydroamentoflavone and proteins, polysaccharides, or lipids are conceivable. Given the specific interactions and the dependence on structural properties, further research is required to examine how the food matrix affects the efficacy of tetrahydroamentoflavone.

Therefore, its efficacy within the food matrix, along with the impact on color, odor, and taste, must be considered during application, as these factors influence the subsequent practical application strategy. The application of flavonoids in food products includes strategies, such as direct addition, films, or coatings. Encapsulation is also an application strategy that can have a positive impact on the antimicrobial efficacy of flavonoids in food products. (Wu et al., 2024; Martinengo et al., 2021; Garavand et al., 2021; Shimul et al., 2023)

Direct application of amentoflavone significantly inhibited *Staphylococcus aureus* and *Escherichia coli* in ground chicken and apple juice (Bajpai et al., 2019). Given the sensory properties of the sweet and spicy *Schinus terebinthifolius* fruit, future studies could consider its direct application in savory foods, such as chicken, or sweet desserts.

5 Conclusions

The use of polyphenols as natural preservatives showed significant progress. Flavonoids with promising bioactive properties are increasing. Due to the wide range of structural characteristics, the spectrum of activity is very diverse.

Characterizing the effect of structural properties and, thus, expanding the understanding of effective structures could play a major role in identifying effective antibacterial agents to overcome the challenges associated with resistant bacteria. The interactions with bacterial cell components could be further investigated in future studies. Microscopic studies, such as transmission electron microscopy or confocal laser scanning microscopy might be used to identify the site of action, including changes in the cell wall. With further details on the mode of action, tetrahydroamentoflavone could be combined with preservatives of other cellular targets to investigate beneficial synergistic effects.

However, complex defense mechanisms and strain-specific responses to flavonoids also need to be considered in the investigation of antibacterial activity. Individual cell or membrane adaptations influence the efficacy of flavonoids.

The present studies provide a comprehensive insight into the effects of flavonoids, particularly on Gram-positive microorganisms, considering changes in individual cellular structures. Fundamental information for the application of flavonoids as preservatives was presented, showing different mechanisms of action, targets, and interactions with cell components depending on their size, three-dimensional structure, and degree of oxidation.

The application in different food matrices requires further studies, as it can be assumed that the antibacterial efficacy of the biflavonoids varies in the matrices. In addition to direct application, encapsulation is an alternative strategy that could improve the functionality of the antimicrobial agents. Combining flavonoids with other preservatives to affect multiple cellular targets simultaneously might also be promising for further studies.

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Appendix 1

Effects of structural differences on the antibacterial activity of biflavonoids from fruits of the Brazilian peppertree (*Schinus terebinthifolius* Raddi)

Flavonoids, synthesized by plants across all families and therefore found in a huge variety, possess a diverse range of pharmacological properties. Direct antibacterial and synergistic activities as well as the inhibition of several bacterial virulence factors are known. Besides the mode of action, it is important to understand the structure-activity relationship to identify key structural characteristics.

This study aimed to identify biflavonoids with antibacterial activity from *Schinus terebinthifolius* Raddi fruits. The purified biflavonoids were characterized in terms of their antibacterial effects. We found that the activity of biflavonoids, including agathisflavone (AGF), amentoflavone (AMF), and tetrahydroamentoflavone (THAF), was dependent on their chemical configuration and degree of oxidation, with THAF showing the highest activity on planktonic cells. Additionally, biofilm formation and composition were strongly influenced by THAF.

Even slight differences in the chemical structure have fundamental effects on the activity of isolated biflavonoids. This suggests a specific binding of these substances in bacteria and thus enables detailed investigations of the mode of action in the future.

Keywords: biflavonoids, *Schinus terebinthifolius*, antibacterial activity, biofilm, tetrahydroamentoflavone, structure-activity relationship

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1 Introduction

Microbial contamination of food remains a major problem for public health, consumers, and the food industry around the world. Spoilage microorganisms cause food losses for manufacturers, traders, and consumers (Lipinski et al., 2013). The genera *Pseudomonas*, *Lactobacillus*, *Listeria*, and *Bacillus* are known as spoilage organisms in animal products, fruits and high-sugar foods (Madigan, Martinko, Stahl, & Clark, 2012). Pathogenic microorganisms, such as *Campylobacter* sp., *Escherichia coli*, *Bacillus cereus* or *Yersinia enterocolitica*, have been responsible for foodborne outbreaks (CDC, 2019; Robert Koch-Institut, 2019). Chemical preservatives are used to inhibit the growth of microorganisms to prevent foodborne diseases or to protect food from microbial quality loss. In recent years, resistances to the current chemical preservatives have increased. The efficacy of chemical additives is also affected by the formation of biofilms, which may enhance resistances (Srey, Jahid, & Ha, 2013). Additionally, intolerances and allergies have increasingly been observed among consumers. Besides the lack of effective conventional preservatives, there is also a growing demand for natural preservatives, which may be particularly desirable for use in organic products. For these reasons, food manufacturers are interested in new antibacterial compounds from natural sources.

Plants from the Anacardiaceae family (sumac family) are known to contain a large diversity of phytochemicals with bioactive properties (Agedah, Bawo, & Nyananyo, 2010; Engels, Schieber, & Gänzle, 2011; Marčetić et al., 2013; Sarkar et al., 2014; Schulze-Kaysers, Feuereisen, & Schieber, 2015). This family includes members well known for food use, for example, cashew (*Anacardium occidentale* L.), sumac (*Rhus* sp.), pistachio (*Pistacia vera* L.), marula (*Sclerocarya birrea* A. Rich.), mango (*Mangifera indica* L.), and jocote (*Spondias purpurea* L.). Our investigations on mango kernel polyphenols revealed a high antibacterial activity of hydrolyzable tannins, especially against gram-positive bacteria except lactic acid bacteria (Engels et al., 2009, 2011; Engels, Gänzle, & Schieber, 2010). The Brazilian peppertree (*Schinus terebinthifolius* Raddi) is another member of the Anacardiaceae, and its fruits are widely used as a spice. Spices have been appreciated as food ingredients not only for their favorable sensory characteristics but also because of their preserving effects. The antimicrobial properties of spices in general are caused by a large variety of phytochemicals, such as essential oils, isothiocyanates and other organosulfur compounds, alkaloids, and phenolic compounds (Gottardi, Bukvicki, Prasad, & Tyagi, 2016). In *Schinus* fruits, a number of phenolic compounds have been detected, for example, anthocyanins, gallotannins, and the less well characterized biflavonoids (Skopp & Schwenker, 1986; Feuereisen et al., 2014; Feuereisen et

al., 2017b). Flavonoids act as a constitutive defense against abiotic stresses and as a growth regulator (Agati, Azzarello, Pollastri, & Tattini, 2012; Dixon, Dey, & Lamb, 1983; Kumar & Pandey, 2013). Biflavonoids are known to be involved in the response to microbial infections (Gontijo, Dos Santos, & Viegas, 2017; Williams & Harborne, 1989).

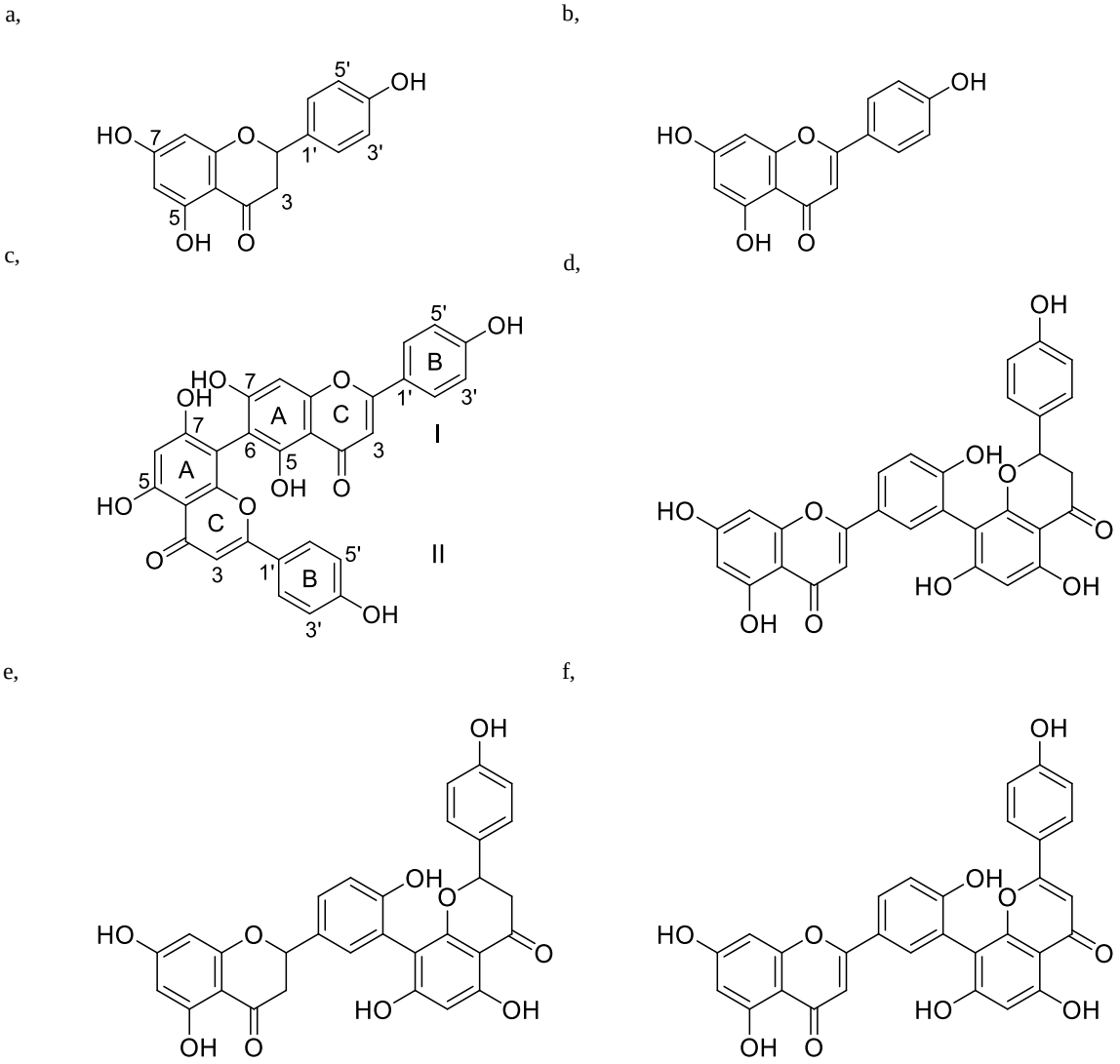

Fig. 1. Structures of flavonoids and biflavonoids: a, naringenin; b, apigenin; c, agathisflavone (AGF); d, amentoflavone (AMF); e, dihydroamentoflavone (DHAF); f, tetrahydroamentoflavone (THAF).

The fruits of the Brazilian peppertree have been reported to contain mainly four biflavonoids: agathisflavone (AGF), amentoflavone (AMF), dihydroamentoflavone (DHAF), and tetrahydroamentoflavone (THAF) (Fig. 1). Because they are composed of two apigenin or naringenin monomers, the degree of oxidation at the C-ring and also the linkage of the single units differ. (Feuereisen et al., 2014; Skopp & Schwenker, 1986) The monomers of AMF, DHAF, and THAF are connected via their A- and B-rings. AGF monomers are linked through their A-rings. The C-rings of AGF and AMF are unsaturated, in DHAF only one is unsaturated, and in THAF both C-rings are saturated. Data published on the antimicrobial activity especially

of biflavonoids have remained inconsistent (Ajileye, Obuotor, Akinkunmi, & Aderogba, 2015; Hwang, Choi, Woo, & Lee, 2013; Moawad et al., 2010; Songca, Sebothoma, Samuel, & Eloff, 2012; Xu, Mughal, Taiwo, & Lee, 2013), and structure-activity relationships have not yet been addressed. However, a deeper knowledge would be desirable to draw conclusions concerning the applicability of biflavonoids as natural food preservatives.

In view of the subtle structural differences of biflavonoids present in *Schinus* fruits, and in continuation of our previous investigations into the antimicrobial properties of phenolic compounds from mango kernel extracts (Engels et al., 2011, 2009, 2010, 2012b; Engels, Gänzle, & Schieber, 2012a), this study aimed at the characterization of the antibacterial activity including structure-activity relationships of biflavonoids.

2 Material and methods

2.1 *Reagents*

Acetonitrile (HPLC-MS grade), ethyl acetate (HPLC grade) and chloroform (HPLC grade) used for the isolation of biflavonoids were purchased from VWR International (Darmstadt, Germany). Methanol was obtained from Avantor Performance Materials (LC-MS grade, Gliwice, Poland). Formic acid (98%) was from Honeywell Riedel-de Haën (Seelze, Germany). Ultrapure water was purified with Purelab flex purification system (Veolia, Celle, Germany). Demineralized water was used for the microbiological experiments. Amentoflavone (≥ 99.0%, Phytoplan, Heidelberg, Germany) was used as a standard substance for UHPLC analyses and for microbiological assays. Apigenin (≥ 97.0%, HPLC grade, Phytoplan, Heidelberg, Germany), naringenin (95%, Sigma-Aldrich, Steinheim, Germany), and chloramphenicol (≥ 98.5%, Applichem, Darmstadt, Germany) were also employed for the determination of the antibacterial activity. Dimethylsulfoxide (DMSO, 99.8%), peptone from casein, peptone from soymeal, tryptic soy broth, dipotassium phosphate and D(+)-glucose monohydrate were purchased from Carl Roth (Karlsruhe, Germany). Furthermore, Gram's crystal violet solution from Merck (Darmstadt, Germany) was used for the microbiological investigations. Sodium chloride was supplied from Labochem International (Heidelberg, Germany). For the chrome azurol S (CAS) assay, and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich (Steinheim, Germany), 2-morpholinoethanesulfonic acid (MES) from Merck (Darmstadt, Germany), hexadecyltrimethylammonium bromide (HDTMA) from VWR Chemicals (Leuven, Belgium) and iron(III) chloride hexahydrate (97%) from AlfaAesar (Kandel, Germany).

2.2 *Isolation of biflavonoids*

2.2.1 *Plant material*

Dried fruits from *Schinus terebinthifolius* (Fuchs, Dissen, Germany) were separated into exocarp and drupe (the residual pericarp and seed) to allow for a more efficient disintegration and extraction. The exocarp and drupe extracts were prepared according to a previously described method (Feuereisen et al., 2017a) and evaporated to dryness. AGF was isolated from the exocarp extract, whereas THAF was obtained from the drupe extract.

2.2.2 *Liquid-liquid extraction*

The recovery of the biflavonoids from the exocarp and drupe extracts was performed by two successive liquid-liquid extractions with different solvent systems. The extracts were processed into a fine powder for the first extraction, which separated anthocyanins and gallotannins from biflavonoids. The solvent system consisted of formic acid/water/ethyl acetate (0.5/50/50, v/v/v). (Feuereisen et al., 2014) In each case, 5 g of extracts was dissolved in 600 mL water with formic acid and the biflavonoids were extracted three times with ethyl acetate. The biflavonoid fractions were evaporated for lyophilization.

The second solvent system was based on the work of Eloff (1998) and was used for the separation of the less polar gallotannins from the biflavonoids. The solvent composition was optimized and contained formic acid/water/chloroform/methanol (0.3/33/33/33, v/v/v/v). The ethyl acetate fractions from the exocarp and drupe extracts were dissolved in 400 mL methanol. Biflavonoids from the exocarp (biflavonoid extract) and THAF were extracted three times with 400 mL chloroform. After evaporation of the biflavonoids, they were lyophilized and stored at –80 °C.

2.2.3 *Semi-preparative HPLC*

The separation of AGF from the biflavonoid extract was performed with a semi-preparative HPLC equipped with a pump model Smartline 1050, a solvent manager model Smartline 5050, an auto-sampling unit model Smartline 3950, a UV/Vis detector model Smartline 2550 from Knauer GmbH (Berlin, Germany), and a fraction collecting unit model Foxy® R1 from Teledyne ISCO (Lincoln, NE, USA). The column was a Eurospher II 5 µm C-18 (250 mm × 16 mm) from Knauer GmbH (Berlin, Germany) with a pre-column (30 mm × 16 mm) of the same material kept at room temperature. The software EZChrome Elite (Agilent Technologies Germany GmbH, Waldbronn, Germany) was applied to control the system. Eluent A was chloroform/water (0.5/99.5, v/v), eluent B was chloroform/methanol (0.5/99.5, v/v). The flow rate was set at 6 mL/min. The gradient started at 40% B and was linearly increased to 68%

during 18 min, where it was kept for 16 min. Subsequently, B was increased to 100% during 2 min, kept at 100% B for 4 min, and reduced to 40% over 2 min, followed by the start of the equilibration period for 2 min. The biflavonoid extract was dissolved in a mixture of mobile phases (1/1, v/v), membrane-filtered and injected into the system. The chromatographic separation was monitored at the absorption maximum of AGF (271 nm). AGF fractions were evaporated under vacuum, lyophilized in sterile tubes and stored at −80 °C for microbiological assays. The chromatographic purity was determined using a UHPLC system (Section 2.2.4).

2.2.4 *Identification and quantification of biflavonoids*

UHPLC-MS/MS analyses for the identification of the biflavonoids AGF, AMF, DHAF, and THAF were performed on an Acquity UHPLC I-Class system (Waters, Milford, MA, USA) consisting of a binary pump, an autosampler cooled to 10 °C, a column oven set at 40 °C, and a diode array detector scanning from 190 nm to 620 nm. An Acquity HSS-T3 RP18 column (150 × 2.1 mm; 1.8 µm) combined with a precolumn (Acquity UPLC HSS T3 VanGuard, 100 Å, 2.1 × 5 mm, 1.8 µm), both from Waters (Milford, MA, USA), was used for chromatographic separation with formic acid/water (1:99, v/v, eluent A) and formic acid/acetonitrile (1:99, v/v, eluent B). The flow rate was set at 0.4 mL/min. Gradient elution was the same as described for the quantification of the biflavonoids. The injection volume was 5 µL. For MS analysis, the UHPLC was coupled with a LTQ-XL ion trap mass spectrometer (Thermo Scientific, Inc., Waltham, MA, USA) equipped with an electrospray interface operating in positive ion mode. MS scan was done in the fullscan mode with a range of *m/z* 250–1500. The capillary temperature was set at 325 °C with a voltage of 1 V. The source voltage was maintained at 4 kV at a current of 100 µA. The tube lens was adjusted to 55 V. Nitrogen was used as sheath, auxiliary and sweep gas at a flow of 70, 10, and 1 arb, respectively. Two consecutive scans were conducted: a full mass scan, a MS/MS scan of the most abundant ion of the first scan using normalized collision energy (CE) of 35%.

The quantification of the biflavonoids in the exocarp, drupe, and biflavonoid extracts and the analysis of the chromatographic purity of AGF and THAF were performed using a Nexera X2 UHPLC system from Shimadzu Corporation (Kyoto, Japan). The system was composed of two high pressure pumps model LC-30AD, a degasser model DGU20A5R, an auto-sampling unit model SIL-30AC (10 °C), a column oven model CTO-20AC, and a diode array detector model SPD-M20A scanning from 190 nm to 600 nm. An HSST3 1.8 µm C18 column (150 mm × 2.1 mm) from Waters Corporation (Milford, MA, USA) equipped with a security guard (5 mm × 2.1 mm) of the same material was used for chromatographic separation. The software LabSolutions (Shimadzu Corporation, Kyoto, Japan) was used to control the system. The

mobile phases were formic acid/water (1:99, v/v, eluent A) and formic acid/acetonitrile (1:99, v/v, eluent B). The flow rate was set at 0.4 mL/min. The gradient started at 20% B and increased linearly to 30% during 5 min, then to 39% during 13 min. B was further increased to 100% over 2 min, where it was kept for 3 min, and subsequently reduced to 20% during 2 min, followed by an equilibration period for 5 min. The column temperature was maintained at 35 °C and the injection volume was 5 µL. The biflavonoids were dissolved in a mixture of the mobile phases (1/1, v/v) and membrane-filtered before UHPLC analysis. Chromatographic purity was measured at 280 nm.

2.3 *Characterization of the antimicrobial activity*

2.3.1 *Strains and culture conditions*

Bacillus subtilis DSM 10, *Listeria innocua* ATCC 14298, *Lactobacillus rhamnosus* DSM 20711, *Staphylococcus carnosus* DSM 20501, *Escherichia coli* K-12 DSM 498, *Pseudomonas putida* DSM 291, *Serratia ficaria* DSM 4569, and *Yersinia rohdei* DSM 18270 were used to determine the antibacterial activity of biflavonoids. The bacterial cultures were incubated in tryptic soy broth (TSB) overnight under shaking at 30 °C. The TSB was prepared with 17 g/L peptone from casein, 3 g/L peptone from soymeal, 2.5 g/L glucose, 2.5 g/L dipotassium phosphate, and 5 g/L sodium chloride in water. The secondary cultures required for the experiments were obtained by inoculation of 5 mL of the precultures in 100 mL TSB and renewed incubation for 24 h.

2.3.2 *Broth microdilution assay*

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to a modified broth microdilution method (DIN 58940-7, 2009).

The fractions and compounds to be tested were the exocarp and drupe extracts, the flavonoid monomers apigenin and naringenin, and the dimeric biflavonoids AGF, AMF, and THAF. The extracts and standard compounds were first dissolved in DMSO to increase their solubility (100 mg/mL). The DMSO concentration present during incubation was set at 1% (v/v), as no significant influence on the bacterial growth of the microorganisms was observed. The dissolved compounds were diluted with sterile water to a concentration of 8 mg/mL for the exocarp and drupe extracts, and to 2 mg/mL for the flavonoids. Afterwards, the solution was adjusted to pH 6.5. Two-fold serial dilutions of extracts and flavonoids were prepared in multiple determinations (n = 6) to a concentration of 0.002 mg/mL with 2% DMSO solution (v/v) in 96-well microtiter plates. In each case, 50 µL of the sample suspension was inoculated with $1 \cdot 10^6 \pm 6 \cdot 10^5$ cfu/mL (50 µL) in double concentrated TSB with 2 g/L agar. The agar was

added to stabilize the suspensions. The positive control was the bacteria suspension in TSB with agar with agar and 1% DMSO. After shaking for 20 min, the absorption was measured to determine blank values. Measurements were performed with a Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany) at a wavelength of 700 nm, which is not affected by polyphenols. Plates were incubated aerobically for 24 h at 30 °C and 75% humidity under shaking (300 rpm), and the growth of microorganisms was also measured at 700 nm. The colonyforming units were determined using the absorption values obtained and via the regression lines of each microorganism. Below the limit of determination of the regression lines, the cell count of the microorganisms was determined using a spiral plater (Interscience, Saint Nom, France). The minimum inhibitory concentration (mg/mL) was defined according to the DIN standard as the lowest concentration of the substances that inhibited bacterial growth. The reduction of the initial colony-forming units by 99.9% was defined as the minimum bactericidal concentration. The initial cell concentration served as a reference. A reduction in growth by one log unit and the reduction of the cell number in relation to the initial cell number was also recorded to determine slight differences between the antibacterial effects of biflavonoids.

2.3.3 *Inhibition of biofilm formation*

The inhibition of biofilm formation by THAF was investigated using the crystal violet assay (Sandasi, Leonard, & Viljoen, 2010; Stepanović, Vuković, Dakić, Savić, & Švabić-Vlahović, 2000). The assay was performed with the microorganisms forming biofilms, that is, *S. carnosus*, *L. innocua*, *P. putida*, *S. ficaria*, and *E. coli*. The conditions of incubation were as described for the broth microdilution assay. A 50 mg/mL stock solution of THAF in DMSO was used. Two-fold serial dilutions of flavonoids were prepared sevenfold to a concentration of 0.008 mg/mL in 96-well microtiter plates. In each case, 50 µL of the sample suspension was inoculated with 50 µL of a bacterial suspension prepared in double concentrated TSB with $1 \cdot 10^6 \pm 6 \cdot 10^5$ cfu/mL. The positive control was the bacteria suspension in TSB with 1% DMSO. The plates were incubated aerobically for 24 h at 30 °C and 75% humidity without shaking. Afterwards, the microtiter plates were rinsed three times under flowing physiological saline solution. The remaining attached bacteria were fixed at 60 °C for 20 min in an oven (Binder FB 56, Tuttlingen, Germany) and subsequently stained with 125 µL of a 1% crystal violet solution for 15 min. The plates were rinsed sevenfold with physiological saline to remove the unabsorbed dye. This was followed by drying at room temperature. The bound crystal violet was dissolved under shaking for 5 min (300 rpm) with 150 µL 95% ethanol; for the strong biofilm producers, *S. carnosus* and *L. innocua*, 270 µL 95% ethanol was used. The crystal violet solution (100 µL)

was transferred to a new plate, and its absorbance determined at 590 nm. The biofilm inhibition assay was performed in duplicate and percentage inhibition was determined (Sandasi et al., 2010). Differences in the degree of biofilm formation were examined by a non-parametric Kruskal-Wallis test. P-values of < 0.05 were considered significant.

2.3.4 *Characterization of the biofilm*

The number of culturable cells and the possible inclusion of THAF were examined for a more detailed investigation of the biofilm. After application of the broth microdilution assay and the subsequent washing step, the attached cells were suspended with 100 µL sterile physiological saline through repeated release and uptake of the solution. Also the biofilm was mechanically removed through the pipette tip. The suspension and the tip were placed in 4.9 mL sterile physiological saline solution and mixed vigorously. The cell count was determined with a detection limit of 10^3 cfu/mL using the spiral plater at THAF concentrations from 0.5 mg/mL to 0.125 mg/mL for *P. putida* and from 0.5 mg/mL to 0.0625 mg/mL for *S. carnosus* and *L. innocua*. The positive control served as a reference. Significant differences in cell counts in relation to the positive control were examined by a nonparametric Kruskal-Wallis test. P-values of < 0.05 were considered significant.

For the quantification of THAF in the biofilms, these were suspended in 200 µL methanol. The suspensions were transferred to caps and the methanol was evaporated at room temperature. The dry material was dissolved, membrane-filtered and analyzed (section 2.2.4). The quantification of THAF in the biofilm was performed using the extracted THAF as a standard compound. These analyses for the investigation of the biofilm were carried out in four replicates.

2.4 *Chrome azurol S assay*

The CAS assay was used for the determination of the capability of flavonoids to bind iron (Engels et al., 2010; Schwyn & Neilands, 1987). The blue CAS-iron(III)-HDTMA complex has a low stability and can therefore be utilized as an indicator of iron-binding compounds.

By removing the iron from the CAS-iron(III)-HDTMA complex due to strong iron-binding compounds, the orange-colored iron-free system is formed. A mixture of 1.5 mL $FeCl_3 \cdot H_2O$ in 10 mM HCl and 7.5 mL 2 mM aqueous CAS solution was slowly added under stirring to 6 mL of 10 mM HDTMA. Then, 50 mL MES-buffer solution (0.5 M NaCl and 0.5 M MES, pH = 5.6) was added and the solution was made up with water to obtain 100 mL CAS solution. (Schwyn & Neilands, 1987)

The flavonoids were first dissolved in DMSO (100 mg/mL) and subsequently diluted with demineralized water to a concentration of 2 mg/mL. Two-fold serial dilutions of flavonoids were prepared fourfold to a concentration of 0.004 mg/mL with 2% DMSO solution in 96-well microtiter plates. Then, 50 µL of CAS solution was added to 50 µL of flavonoid solution. An aqueous EDTA solution (1 mg/mL) was also tested for comparison. After 60 min, the precipitate was centrifuged at 4680 rpm (5430R, Eppendorf, Wesseling-Berzdorf, Germany), and 70 µL of the supernatant was transferred to a microtiter plate for absorption measurement at 630 nm. A visible color change was observed with a change in absorption of more than 50% of the maximum. Thus, the flavonoid concentration exceeding half of the maximum absorption was used to describe the iron binding capacity compared to EDTA. (Engels et al., 2010) This CAS assay was performed in duplicate.

3 Results

3.1 *Separation of biflavonoids*

The structures of the biflavonoids AGF, AMF, DHAF, and THAF in the fruit of *Schinus terebinthifolius* have already been characterized in our previous investigations (Feuereisen et al., 2017b, 2014, 2017a). Based on chromatographic, UV, and mass spectrometric data, the biflavonoids were identified. In the present study, a total content of 3.5% of the biflavonoids in exocarp extract was found. Liquid-liquid extraction led to a biflavonoid concentration of 33.5%, composed of 82.3% AGF, 10.3% AMF, 6.2% DHAF, and 1.2% THAF. The predominant compound AGF was isolated with a chromatographic purity of more than 99% by semi-preparative HPLC. The drupe extract contained 4.0% THAF, which was obtained with a chromatographic purity of more than 96% by liquid-liquid extraction. Based on the initial weight of 5.0 g dried exocarp extract and drupe extract, the yields of the isolated biflavonoids AGF and THAF were 37% and 65%, respectively.

3.2 *Antibacterial activity of extracts and individual compounds*

The antibacterial activity of the exocarp extract, the drupe extract, the biflavonoid extract, and of THAF isolated from the drupe extract is shown in Table 1. Gram-positive microorganisms were susceptible to the crude extracts and MICs of up to 0.25 mg/mL were detected. MICs of up to 0.25 mg/mL were shown for the biflavonoid extract containing AGF, AMF, DHAF, and THAF against the gram-positive microorganisms *B. subtilis* and *S. carnosus*. The growth of *S. carnosus* was inhibited by one log unit at a concentration of 0.5 mg/mL. *L. rhamnosus* was inhibited by one log unit at a concentration of 1 mg/mL (results not shown).

Table 1: Antibacterial activity of extracts and biflavonoids from *Schinus terebinthifolius*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in mg/mL of exocarp extract, drupe extract, biflavonoid extract, tetrahydroamentoflavone (THAF), and standard antibiotic chloramphenicol (CAP) against gram-negative and gram-positive microorganisms (n = 6).

organism	exocarp extract		drupe extract		biflavonoid extract		THAF		CAP
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
E. coli	>4	>4	>4	>4	>1	>1	>1	>1	0.016
P. putida	>4	>4	>4	>4	>1	>1	>1	>1	0.25
S. ficaria	>4	>4	>4	>4	>1	>1	>1	>1	0.031-0.063
Y. rohdei	>4	>4	>4	>4	>1	>1	>1	>1	0.016
B. subtilis	1	1-2	0.25	0.25	0.25-0.5	0.25-0.5	0.063	0.063	0.008
L. innocua	4	>4	2	>4	>1	>1	0.25-0.5	>1	0.031-0.125
L. rhamnosus	>4	>4	3-4	>4	>1	>1	0.125-0.25	>1	0.031
S. carnosus	0.5	2	1	2	1	>1	0.063	>1	0.125-0.25

Our investigation of the antibacterial effect of biflavonoids revealed a strong influence of a reduced C-ring at position I-2,3 and II-2,3. The biflavonoid THAF showed high antibacterial activity against gram-positive microorganisms, among which *B. subtilis* and *S. carnosus* were the most susceptible strains. THAF caused a MIC and MBC of 0.063 mg/ mL for *B. subtilis*.

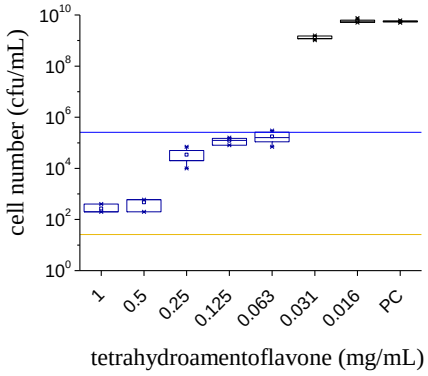

Fig. 2. Antibacterial activity of tetrahydroamentoflavone against *S. carnosus*. ▬ reference for minimal inhibitory concentration; ▬ reference of cell number for minimal bactericidal concentration. PC: positive control (n = 6).

Furthermore, a MIC of 0.063 mg/mL as well as a bactericidal effect at a concentration of 0.125 mg/mL were determined for *S. carnosus* (Fig. 2). Growth inhibition by one log unit was detected for *L. innocua* and *L. rhamnosus* at concentrations of 0.125 mg/mL or 0.063 mg/mL, respectively. In contrast, AMF bearing an oxidized C-ring, did not inhibit the growth of the microorganisms investigated in the concentration range from 0.016 mg/mL to 1 mg/mL. AGF, which is the constitutional isomer of AMF, reduced bacterial growth of *S. carnosus* at a concentration of 1 mg/mL by one log unit. No antibacterial effect was observed for the corresponding monomers apigenin and naringenin. Thus, the saturated C-rings of THAF and the dimerization of naringenin resulted in a 15-fold increase in the antibacterial activity. The

stereochemistry of the examined flavonoids appears to have a minor influence on the antibacterial effect.

3.3 *Effects of biflavonoids on biofilm formation*

THAF, which was found to be the most efficient biflavonoid in this study, was also investigated for its effect on biofilm formation. The biofilm formation of *L. innocua* decreased by up to 96% in the range from 0.016 mg/mL to 0.5 mg/mL THAF (Fig. 3); a biofilm inhibition of up to 99% was observed for *S. carnosus*. An increase in the absorption of gram-positive microorganisms at concentrations of 0.5 mg/mL THAF was also found. In case of *P. putida,* the biofilm production was inhibited up to 62%. In contrast, the biofilms of *E. coli* and *S. ficaria* were not susceptible to THAF.

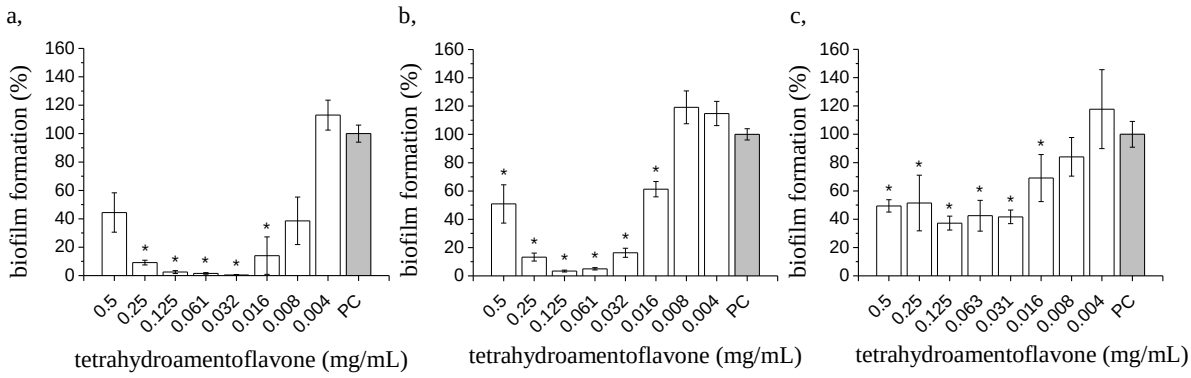

Fig. 3. Biofilm formation in the presence of tetrahydroamentoflavone of a, *L. innocua*; b, *S. carnosus*, and c, *P. putida*. PC: positive control. Data are mean ± SD, n = 14. Statistical analysis: Kruskal-Wallis test: p-value of < 0.05; * significant difference to positive control.

The cell number of *L. innocua* decreased by four log units through the addition of THAF in the range of 0.063 mg/mL to 0.5 mg/mL (Fig. 4). In the case of *S. carnosus*, THAF led to cell counts below the detection limit and thus to an inhibition of at least four log units. For *P. putida*, THAF reduced the number of cells by one log unit.

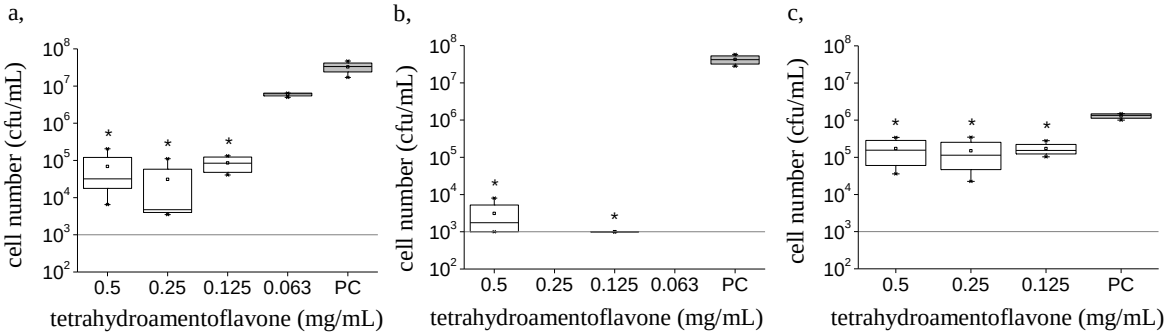

Fig. 4. Inhibition of the culturable cells in the biofilms by tetrahydroamentoflavone of a, *L. innocua*, b, *S. carnosus*, and c, *P. putida*. ▬ detection limit; PC: positive control. Data are mean ± SD, n = 4. Statistical analysis: Kruskal-Wallis test: p-value of < 0.05; * significant difference to positive control.

Quantification of THAF in the biofilms revealed an inclusion of THAF in biofilms or microcolonies of *L. innocua* and *S. carnosus*. At a THAF concentration of 0.5 mg/mL, the biofilm of *L. innocua* and *S. carnosus* contained 33.38 ± 7 µg/mL and 30.02 ± 6 µg/mL THAF, respectively. Addition of 0.25 mg/mL, 0.125 mg/mL and 0.06 mg/mL THAF resulted in THAF concentrations of 1.9 ± 0.3 µg/mL, 0.51 ± 0.03 µg/mL, and 0.15 ± 0.05 µg/mL in the biofilm of *L. innocua*. Accordingly, 2.2 ± 0.2 µg/mL, 0.76 ± 0.06 µg/mL, and 0.30 ± 0.1 µg/mL were detected in the biofilm of *S. carnosus*. In contrast to the gram-positive microorganisms, no THAF was detected in the biofilm of *P. putida*. The inclusion of THAF in the biofilm of grampositive microorganisms causes an increase in the biofilm matrix. This explains the increased absorptions determined in the crystal violet assay at THAF concentrations of 0.5 mg/mL and 0.25 mg/mL for grampositive microorganisms.

3.4 *Iron-binding capacity of flavonoids and biflavonoids*

The CAS assay compares the affinity of polyphenols towards iron (III). The assay indicated an equilibrium concentration between 1.15 M and 2.3 M for THAF and 0.58 M for AMF and AGF. The monomers apigenin and naringenin did not decolorize the blue CAS-complex. EDTA showed the lowest equilibrium concentration of 0.13 M. Consequently, the monomers have the lowest affinity towards iron, while AMF and AGF revealed a higher iron affinity than THAF.

4 Discussion

4.1 *Antibacterial activity of Schinus terebinthifolius fruits and their biflavonoids*

Plant extracts of the Anacardiaceae family, in particular extracts from the fruits of the Brazilian peppertree, are known for their strong antimicrobial activity (Barbieri et al., 2014; de Araujo Gomes et al., 2020; Muhs et al., 2017). However, there is still a gap in our knowledge with respect to the bioactive compounds, their structure-activity relationships, and modes of action. In this study, we could show that fruit extracts of *Schinus terebinthifolius* inhibit the growth of gram-positive food-associated microorganisms. Similar effects have been observed for other plant extracts of the Anacardiaceae family. The antibacterial effects of mango and *Schinopsis brasiliensis* were mainly attributed to tannins (Engels et al., 2009; Saraiva et al., 2011). In addition to the bioactive gallotannins of the *Schinus terebinthifolius* fruit, first studies also demonstrated an antibacterial effect of biflavonoids present in Anacardiaceae. In cashew, the biflavonoid AGF was identified as a moderately antibacterial substance (Ajileye et al., 2015). We isolated two biflavonoids AGF and THAF from *Schinus terebinthifolius* fruits and analyzed their antimicrobial capacity, together with AMF and their monomers. AMF has been

shown to inhibit the growth of gram-positive species such as *E. faecium*, *S. mutans*, *S. aureus*, and the gram-negative microorganisms *E. coli* and *P. aeruginosa* in the range of 0.004 mg/mL to 0.032 mg/mL (Hwang et al., 2013; Kaikabo, Samuel, & Eloff, 2009). In contrast, an earlier study has demonstrated no growth inhibition by AMF below 0.100 mg/mL (Xu & Lee, 2001). Further studies did not observe any inhibitory effects of AGF (da Silva et al., 2018). In accordance with these results, we detected no or only weak antibacterial effects of the above mentioned biflavonoids between 0.001 mg/mL and 1 mg/mL.

An antibacterial effect of THAF against gram-positive food-associated microorganisms was investigated for the first time. We found a remarkably strong activity of THAF, present in the drupe of *Schinus terebinthifolius* fruits, against gram-positive microorganisms such as *B. subtilis* and *S. carnosus*. These are promising antibacterial effects because pathogenic members of the genera *Bacillus* and *Staphylococcus* have become a matter of increasing concern in food safety and health in recent years (CDC, 2019; Robert Koch-Institut, 2019). The constitutional isomers of THAF, 3,8" binaringenin, 3,6" binaringenin and 3',3'" binaringenin have also antibacterial effects (Djoufack et al., 2010; Sagrera & Seoane, 2010). The gram-negative planktonic cells were not susceptible to THAF as previous studies have shown (da Silva et al., 2018).

In addition to effects on planktonic cells, we further observed biofilm inhibition in gram-positive as well as gram-negative microorganisms below the MIC. A considerable biofilm inhibition and reduction in cell number was achieved for *L. innocua*, *S. carnosus*, and *P. putida*. A biofilm inhibition of *E. coli* and *P. putida* by the monomer naringenin has already been reported (Vandeputte et al., 2011; Vikram, Jayaprakasha, Jesudhasan, Pillai, & Patil, 2010). Muhs et al. (2017) showed that biflavonoid-rich extracts of *Schinus terebinthifolius* exhibited an effect against biofilm forming methicillin-resistant *S. aureus* (MRSA). Taking together these findings and the results of our studies, THAF is a promising candidate for the application as an antimicrobial agent.

4.2 *Structure-activity relationship*

Plant-derived natural products exhibit a pronounced chemical diversity, which influences their biological activity (Dixon, 2001). The effects of hydroxylation, methylation, and glycosylation on a common chemical backbone have already been demonstrated for flavonoids (Basile, Giodano, López-Sáez, & Cobianchi, 1999; Bernard et al., 1997; Dixon, 2001; Farhadi, Khameneh, Iranshahi, & Iranshahy, 2018; Rauha et al., 2000). For example, the position of hydroxyl groups plays an important role in the growth inhibition of microorganisms and also

influences hydrophobicity, which is a crucial factor determining membrane permeability (Alcaráz, Blanco, Puig, Tomás, & Ferretti, 2000; Cushnie & Lamb, 2005; Mori, Nishino, Enoki, & Tawata, 1987). Prenyl, hydroxyl and especially methoxy groups at positions 5 and 7 of the A-ring increase the antibacterial effect of flavanes and flavones. Furthermore, different substitutions on position 3 of the C-ring are important because a hydroxyl or an *O*-glycoside group can increase the activity (Bitchagno et al., 2015; Farhadi et al., 2018). The deficiency of antibacterial activity against planktonic cells of the monomers apigenin and naringenin can be attributed to a missing methoxy or hydroxyl group at these positions.

For the biflavonoid AMF, only the influence of methylation has been studied so far (Kaikabo et al., 2009; Moawad et al., 2010). In our study, we investigated the effect of dimerization and oxidative state of the C-ring of flavonoids on the antibacterial activity. Our results demonstrated for the first time that dimerization and a reduced C-ring, such as in THAF, leads to the highest activity. In general, saturated C-rings of flavonoids have a high influence on the conformation of the molecule, which might significantly contribute to the antibacterial effect. Other studies demonstrated a high antibacterial effect of DHAF, confirming an increasing antibacterial effect due to the degree of oxidation (Moawad et al., 2010; Songca et al., 2012). Given the structural diversity of phytochemical components in general and biflavonoids in particular, and the diversity of microbial targets, the necessity of conducting systematic studies becomes very evident.

4.3 *Mechanisms of action*

Polyphenols may protect plants by depriving pathogens from iron (Mila, Scalbert, & Expert, 1996). Chelation of metal ions by biflavonoids may result in iron deficiency for microorganisms and, for example, in an inhibition of essential metalloenzymes (Raza et al., 2016; Smith, Zoetendal, & Mackie, 2005). In our experiments with CAS, biflavonoids exhibited iron complexation abilities. AMF and AGF possess a higher binding capacity towards iron than THAF. It has already been shown that iron is bound by flavonoids at positions 3 and 5 of the A and C-ring (Engelmann, Hutcheson, & Cheng, 2005; Leopoldini, Russo, Chiodo, & Toscano, 2006). This may also be possible with biflavonoids. However, in regards to the results obtained for the MIC values, iron complexation seems to have less influence on the antibacterial effect. Supplementation of TSB medium with iron(III) also showed minor effects on MIC values (data not shown).

This study also showed that the effective inhibition of biofilm formation is an outstanding property of THAF. We detected THAF in the biofilm or microcolonies of *L. innocua* and

S. carnosus but it was below the detection limit in the biofilm of the gram-negative microorganism *P. putida*. These observations indicate the loss of the protective function or at least a reduced formation of the exopolysaccharide (EPS) matrix in gram-positive microorganisms (Das & Mehta, 2018; Drenkard, 2003; Nandu et al., 2018). Quorum sensing (QS) signaling is an important factor related to the composition and formation of the EPS matrix (AlShabib, Husain, Ahmad, & Baig, 2017; Tan et al., 2014). Because of its participation in cell-to-cell communication, QS is essential, especially in establishing biofilms. The inhibition of QS signaling and the direct interruption of cell-to-cell communication has already been demonstrated in extracts from plants of the Anacardiaceae family and for naringenin (Muhs et al., 2017; Sarkar et al., 2014; Vandeputte et al., 2011; Vikram et al., 2010). The inhibition of QS signaling might be the basis for the prevention of biofilm formation of food-associated microorganisms. Moreover, the inhibitory effect of flavonoids can be caused on multilayered mechanisms (Cushnie & Lamb, 2011; Wu et al., 2013). Our results provide indications of the mechanisms of action of biflavonoids and thus constitute the basis of further investigations.

5 Conclusions

THAF has been identified as a promising antibacterial constituent of the fruits of *Schinus terebinthifolius*. Our investigations demonstrated that the antibacterial effects of biflavonoids are strongly dependent on their specific structural properties. Thus, THAF significantly inhibits the growth of planktonic cells and biofilm formation of selected bacteria. In view of the consumer expectations to replace synthetic food additives, these results are of great importance in the search for new antibacterial agents from natural sources. Future work needs to determine the efficacy of THAF in real food matrices.

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Appendix 2

Effects of flavonoids on membrane adaptation of food-associated bacteria

The effects of naringenin and the biflavonoids amentoflavone and tetrahydroamentoflavone on select bacterial lipids (carotenoids, fatty acids, and menaquinones) and membrane fluidity based on Laurdan generalized polarization were investigated. For this purpose, the pigment-forming food-associated microorganisms *Staphylococcus xylosus* (DSM 20266^T and J70), *Staphylococcus carnosus* DSM 20501^T, and *Micrococcus luteus* (ATCC 9341 and J3) were studied.

The results suggest an envelope stress response by microorganisms due to flavonoids and an employment of adaptive mechanisms using carotenoids, fatty acids, and menaquinones. The flavonoid monomer naringenin impacted carotenoids, fatty acids, menaquinones, and membrane fluidity. Naringenin significantly influenced the carotenoid profile, particularly by an increase in the relative proportion of 4,4' -diaponeurosporenoic acid in *Staphylococcus xylosus*. Amentoflavone caused changes mainly in the membrane of *Micrococcus luteus* and decreased the menaquinone content. Tetrahydroamentoflavone mainly affected the carotenoids in the investigated strains.

The noticeably different CCS value of tetrahydroamentoflavone compared to naringenin and amentoflavone revealed further insights into the structure-dependent effects of flavonoids.

This study provides valuable insights into the response of pigment-forming food-associated microorganisms to naringenin, amentoflavone, and tetrahydroamentoflavone, which is important for the targeted and safe application of the latter as natural preservatives and useful for further research on the mechanisms of action.

Keywords: Biflavonoids; Carotenoids.; Staphyloxanthin; Sarcinaxanthin; Menaquinone; Laurdan generalized polarization

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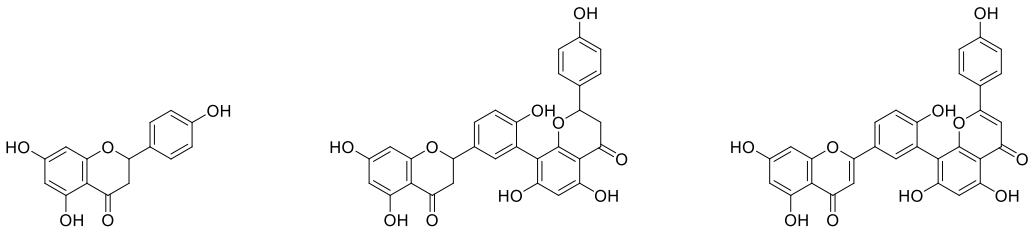
1 Introduction

Food contamination by bacteria, yeasts, and fungi can cause undesirable reactions that may lead to food spoilage and foodborne diseaseoutbreaks, and impair flavor, odor, color, and other sensory properties. The preservation of food is challenged by increased globalization, consumer demand for minimally processed foods, or aversion to synthetic food additives, leading to increased interest in efficient, alternative food additives that preferably originate from plants. Phytochemicals have been an integral part of current research activities because they have been shown to possess a wide range of antimicrobial properties. A number of these bioactive compounds have been detected in the fruits of the Brazilian peppertree (*Schinus terebinthifolius* Raddi), e.g., gallotannins and biflavonoids [1–5].

Previous studies demonstrated strong dependence of the antimicrobial effect on specific structural properties of bioactive flavonoids such as the position of the hydroxy groups, the degree of oxidation of the C-ring, and also the presence of inter-flavonoid bond leading to dimeric structures [4,6–8]. The antimicrobial properties of flavonoids are attributed mainly to membrane interactions, which however are not yet fully understood, especially for biflavonoids such as tetrahydroamentoflavone [6,7,9]. The cell membrane, together with the cell wall, forms the envelope of bacteria and plays a functional role in this first and most important line of defense, as a permeability barrier, in information flow, and in controlling the transport of substances. It is also the site of energy conservation in the cell. Membrane functionality is directly related to physical properties such as membrane fluidity [10–13]. The adaptation of membrane composition to the environment to protect the cell is induced by two-component systems and sigma factors, which are parts of the bacterial envelope stress response [13,14]. Modulations in the structure of a membrane, e.g., by fatty acid profiles, carotenoids, or menaquinones, can therefore affect biophysical and, thus, physiological properties of the membrane [15–18]. The changes in fatty acid composition regarding chain length, isomer profile, and presence of alkyl side chains are an essential defense mechanism of bacteria against membrane-active substances and lead to an adjustment of fluidity and permeability [19]. Further modifications that frame the structure and properties of membranes are caused by carotenoids, which fit into or span the lipid bilayer of membranes by their lipophilic nature and rigid rod-like structure [15]. The impact of carotenoids on membrane permeability and rigidity affects the susceptibility and stress response of microorganisms. So far, studies on bacterial susceptibility and stress response have focused on total carotenoids and, to a lesser extent, on carotenoid profiles of the microorganisms [16,20–22]. Besides fatty acids and carotenoids, menaquinones are also involved in the cell envelope stress response. Menaquinones are

redox-active compounds commonly associated with energy generation, but also act as ion permeability barriers and might regulate membrane fluidity in bacteria [18,23].



Naringenin Tetrahydroamentoflavone Amentoflavone

Fig. 1. Structures of the flavonoids investigated in this study.

The objective of this study was to characterize membrane modifications of food-associated bacteria caused by exposure to the structurally related flavonoids naringenin (NAR), tetrahydroamentoflavone (THAF), and amentoflavone (AMF) (Fig. 1).

2 Material and methods

2.1 *Chemicals and reference compounds*

Valencia Ultrapure water was obtained from Purelab flex purification system (Veolia, Celle, Germany). Acetonitrile (MS grade), ethyl acetate (HPLC grade), chloroform (HPLC grade), methanol (MS grade), *n*-hexane (HPLC grade), acetone (HPLC grade), and hydrochloric acid (37%) were purchased from VWR International (Darmstadt, Germany). Ethanol (HPLC grade) and MS grade water were from Fisher Chemical (Schwerte, Germany). Formic acid (98%, Sigma-Aldrich, Darmstadt, Germany), zinc acetate dihydrate (Merck, Darmstadt, Germany), ethyl acetate (MS grade), potassium hydroxide, sodium hydroxide, and sodium chloride were supplied by Th. Geyer (Renningen, Germany). 2-Methoxy-2-methylpropane, butylated hydroxytoluene (BHT), phosphate buffered saline (10×, pH 7.3–7.5), dimethyl sulfoxide (DMSO, 99.8%), peptone from casein, peptone from soymeal, tryptic soy broth, dipotassium phosphate, D(+)-glucose monohydrate, for the preparation of TE-buffer tris-(hydroxymethyl)-aminomethane (99.9%) and ethylenediaminetetraacetic acid (99%) were obtained from Carl Roth (Karlsruhe, Germany). Lipase from *Aspergillus niger* (200 U/g) and lysozyme from hen egg white (85,400 U/mg) (Sigma-Aldrich, Darmstadt, Germany) were used for cell lysis. Laurdan was from Cayman Chemical (Ann Arbor, MI, USA). β-Carotene (97%), nonadecanoic acid methyl ester (98%), and naringenin (95%) from Sigma-Aldrich (Darmstadt, Germany), menaquinone-7 (98%, Cayman Chemical, Ann Arbor, MI, USA), and amentoflavone (99.7%,

BLDpharm, Kaiserslautern, Germany) were used for microbiological assays or as standards for quantification of carotenoids, fatty acids, and menaquinones.

2.2 *Isolation of tetrahydroamentoflavone*

The drupe extract of *S. terebinthifolius* (Fuchs, Dissen, Germany) used for THAF isolation was prepared according to Feuereisen et al. [5]. The extraction of THAF was performed by two successive liquid-liquid extractions using formic acid/water/ethyl acetate (0.5/50/50, *v/v/v*) and formic acid/water/chloroform/methanol (0.3/33/33/33, *v/v/v/v*) solvent systems [3,4].

2.3 *Analysis of flavonoids*

Analyses were performed on an Acquity UPLC I Class system (Waters, Milford, MA, USA) consisting of a binary pump, a sample manager cooled at 10 °C, a column oven, and a diode array detector scanning from 250 nm to 650 nm. An Acquity HSS-T3 RP18 column (1.8 µm particle size, 100 mm × 2.1 mm) from Waters (Milford, MA, USA) equipped with a pre-column (Acquity UPLC HSS T3 VanGuard, 100 Å, 1.8 µm, 2.1 mm × 5 mm) was used for separation. Formic acid/water (1:99, *v/v*, eluent A) and formic acid/acetonitrile (1:99, *v/v*, eluent B) served as eluents. The flow rate was set at 0.4 mL/min. The gradient program was as follows: 0 min, 20% B; 5 min, 30% B; 13 min, 39% B; 15 min, 100% B; 18 min, 100% B; 20 min, 20% B, followed by an equilibration time of 5 min. The column temperature was maintained at 35 °C, and the injection volume was 5 µL. The flavonoids were dissolved in a mixture of the mobile phases (1/1, *v/v*) and membrane-filtered.

UHPLC coupled to a Vion IMS QTOF mass spectrometer (Waters, Milford, MA, USA) was used to investigate collision cross section (CCS) values of NAR, THAF, and AMF. The latter is a characteristic of an ion in the gas phase which is related to its chemical structure and three-dimensional conformation. Ion mobility separates the flavonoid ions according to their size and shape and reports the separation as the CCS value. The capillary voltage of the Vion IMS QTOF mass spectrometer was 2.00 kV, the source temperature was set at 120 °C, the desolvation gas temperature was 550 °C, and the desolvation gas flow was 600 L/h. The measurements were performed with automatic lock correction every 5 min with leucine enkephalin as the lock mass at a concentration of 100 pg/µL. Nitrogen was used as the drift gas. The MS mode was set to high definition with a low collision energy of 6 eV and a high collision energy ramp of 20–60 eV. Data were acquired and processed using UNIFI v1.9.2.045 (Waters, Milford, MA, USA).

2.4 *Strains and culture conditions*

Micrococcus luteus ATCC 9341 (later reclassified as *Kocuria rhizophila* [24]), *Micrococcus luteus* J3 (isolated from the inner wall of a fridge) [25], *Staphylococcus xylosus* DSM 20266^T, *Staphylococcus xylosus* J70 (isolated from raw milk) [25], and *Staphylococcus carnosus* DSM 20501^T were used to study the effects of NAR, AMF, and THAF on the membrane structures considering their characteristic pigments. The bacteria were cultured under aerobic conditions at 30 °C and 100 rpm in tryptic soy broth (TSB) composed of peptone from casein (17 g/L), peptone from soymeal (3 g/L), glucose (2.5 g/L), dipotassium phosphate (2.5 g/L), and sodium chloride (5 g/L). The secondary cultures required for the experiments were obtained by inoculation of 5 mL of the precultures in 100 mL TSB followed by another incubation for 24 h.

2.5 *Broth microdilution assay*

Minimum inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) were determined according to Linden et al. [4]. After incubation of the cells with the flavonoids the cell count of the microorganisms was determined using a spiral plater (Interscience, Saint Nom, France). The MIC was defined as the lowest concentration of the compounds that inhibited bacterial growth. The initial cell concentration served as a reference. The 99.9% reduction in initial colony forming units was defined as the minimal bactericidal concentration.

2.6 *Characterization of membrane*

2.6.1 *Cultivation of the strains*

The microorganisms were cultivated in flavonoid-enriched TSB to study the effects of NAR, AMF, and THAF on the profiles of carotenoids, fatty acids, and menaquinones, and to detect Laurdan generalized polarization (GP). The method is based on the broth microdilution assay and was performed in triplicate. The flavonoids were first dissolved in DMSO (100 mg/mL). The DMSO concentration during incubation was set at 1%.

Using a geometric dilution, the concentration of flavonoids was adjusted based on dry biomass to a minimal, non-growth inhibitory concentration to minimize growth effects on bacterial lipids, especially on carotenoids [17,26]. The non-growth inhibitory concentration was the highest concentration of flavonoids which showed no significant differences in dry biomass between the incubated bacterial cultures with and without flavonoids.

Stock solutions of NAR and AMF had a concentration of 0.125 mg/mL. THAF stock solutions were diluted with sterile water to 0.01 mg/mL for *S. carnosus*, 0.04 mg/mL for *S. xylosus*

strains, and 0.005 mg/mL for *M. luteus* strains. After dilution, the solutions were adjusted to pH 6.5.

An aliquot of 5 mL of the flavonoid solution was inoculated with $1 \cdot 10^6 \pm 6 \cdot 10^5$ cfu/mL (5 mL) in double concentrated TSB. Bacterial cultures in TSB with 1% DMSO served as a control. After 24 h of incubation at 30 °C and 100 rpm, cultures were harvested by centrifugation at 4 °C, 10,947*g* for 10 min (Heraeus Megafuge 40R centrifuge, Thermo Fisher Scientific, Braunschweig, Germany). The bacterial biomass was washed twice with PBS, lyophilized, and stored at −80 °C for carotenoid, menaquinone, and fatty acid analyses. For Laurdan GP measurements, the biomass was washed twice with TE buffer (pH 7.4).

2.6.2 *Analysis of carotenoids and menaquinones*

2.6.2.1. Cell lysis. Cell disruption was performed according to Kaiser et al. [27]. Initially, 10 mg or 15 mg of the lyophilized samples was resuspended in 750 µL PBS buffer solution. Enzymatic digestion was performed by adding 375 µL of a lysozyme solution (80 KU) and 375 µL of a lipase solution (0.4 U). The samples were incubated for 5 h at 37 °C and 300 rpm in the dark followed by ultrasound treatment for 1 h. This mechanical disruption was enhanced by subsequent freeze-thaw cycles, during which the samples were frozen three times at −80 °C and thawed at 35 °C. After addition of 400 µL of zinc acetate solution, which reduces the loss of glycosylated xanthophylls, samples were centrifugated at 16,260*g* (centrifuge 5430 R, Eppendorf, Hamburg, Germany) and 4 °C for 10 min.

2.6.2.2. Extraction. Carotenoids and menaquinones were extracted using 1.6 mL of methanol/chloroform (7/3, *v/v*) containing 0.1% BHT. The mixture was shaken and centrifuged at 16,260g and 4 °C for 5 min. The extraction step was repeated twice for staphylococci and three times for micrococci. NaCl solution (10%) was added (1/1, *v/v*) to the organic phase. After centrifugation at 2737*g* and 4 °C for 7 min (Heraeus Megafuge 40R centrifuge, Thermo Fisher Scientific, Braunschweig, Germany), the organic phase was transferred to a 4 mL vial. Finally, 1 mL of chloroform containing 0.1% BHT was used to recover compounds remaining in the aqueous phase. This extraction process was repeated using 1.6 mL of hexane/ethanol/acetone (50/25/25, *v/v/v*) containing 0.1% BHT.

The organic phases were evaporated to dryness with nitrogen and stored under argon at −80 °C until analysis. The samples were dissolved in methanol/ethyl acetate (50/50, *v/v*) containing 0.1% BHT to a final concentration of 10 µL/mg dry weight, filtered through 0.2 µm Chromafil RC-20/15 MS filters (Macherey-Nagel, Düren, Germany), and used for quantification by HPLC-diode array detector and HPLC–DAD-APCI MS^n analysis [18,27–29].

2.6.2.3. Quantification. UHPLC-DAD quantification was performed on a Nexera X2 UFLC system from Shimadzu (Kyoto, Japan) equipped with two high pressure pumps model LC-30AD, a degasser model DGU-20A5R, an auto-sampling unit model SIL-30 AC (10 °C), a column oven model CTO-20 AC (27 °C), and an SPD-M20A diode array detector. The data were acquired and processed with LabSolutions software version 5.85 (Shimadzu, Kyoto, Japan). The separation of carotenoids and menaquinones was carried out on an Accucore C30 column (2.6 µm, 150 mm × 2.1 mm) (Thermo Fisher Scientific, Braunschweig, Germany) using methanol/ethyl acetate/water as eluent A (85/5/10, *v/v/v*) and as eluent B (10/85/5, *v/v/v*). The flow rate was set at 0.4 mL/min and the injection volume was 10 µL. The gradient program for the analysis of carotenoids and menaquinones in staphylococci was as follows: 0 min, 0% B; 2 min, 5% B; 9 min, 30% B; 24 min, 30% B; 26 min, 70% B; 28 min, 70% B; 29 min, 100% B; 31 min, 100% B; 32 min, 0% B; 35 min, 100% B. For micrococci, the following gradient program was used: 0 min, 0% B; 2 min, 5% B; 14 min, 25% B; 29 min, 37% B; 36 min, 6 % B; 39 min, 68% B; 40 min, 100% B; 42 min, 100% B; 43 min, 0% B; 46 min, 0% B.

Chromatograms were recorded at 450 nm, and carotenoids were quantified as β-carotene equivalents using an external calibration curve. A stock solution of β-carotene was prepared by dissolving the standard in methanol/ethyl acetate (50:50, *v/v*) containing 0.1% BHT. The concentration of the stock solution was measured spectrophotometrically at 450 nm and calculated with the molar absorption coefficient of β-carotene in ethanol (140,400 L mol−1 cm−1) [30]. The total carotenoid content was calculated as the sum of carotenoids and expressed as µg β-carotene equivalents/mg cell dry weight (dw).

Menaquinones were quantified at 270 nm by a menaquinone-7 standard in methanol/ethyl acetate (50:50, *v/v*) containing 0.1% BHT using an external calibration curve.

2.6.2.4. Identification. The bacterial carotenoids were identified on an Acquity UPLC I-Class system (Waters, Milford, MA, USA) consisting of a binary pump, an autosampler cooled at 10 °C, a column oven set at 27 °C, a PDA eλ detector scanning from 190 nm to 620 nm coupled to an LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Braunschweig, Germany) equipped with an APCI ionization source operating in positive ion mode.

The chromatographic conditions (column, gradient program, flow rate, injection volume) were the same as described for the quantification. Both eluents A and B were spiked with 5 mmol/L ammonium formate to improve ionization. Data were acquired over a range of *m/z* 200–1300. The capillary temperature was set at 275 °C with 15 V. The source voltage was maintained at 6 kV at a current of 5 µA. The tube lens was adjusted to 65 V.

Data analysis was performed using Xcalibur 2.2 SP1.48 software from Thermo Fisher Scientific (Braunschweig, Germany). Carotenoids were identified according to the following parameters: retention time and elution order on Accucore C30 column, UV/Vis spectrum (positions of absorption maxima λ_{max}, fine spectral structure expressed as %III/II, and (*Z*)-peak intensity expressed as $\%A_B/A_{II}$), and mass compared with literature data [31–40]. A sample of each cultivated and lysed microorganism was saponified to facilitate the identification of esterified carotenoids [41].

2.6.3 *Fatty acid analysis*

Extraction, saponification, and methylation of fatty acids (5–13 mg of lyophilized samples) were performed as described by Sasser [42]. The fatty acid methyl esters were separated, identified, and quantified using an Agilent 7890A gas chromatograph equipped with an HP-5ms column (0.25 µm, 30 m × 0.25 mm) and coupled to an Agilent 5975 mass spectrometer (Santa Clara, CA, USA) in electron impact ionization (EI) mode with −70 eV [43]. Helium as the carrier gas was set at 1.2 mL/min. The column temperature started at 120 °C, was held for 5 min, increased to 240 °C at a rate of 5 °C/min, and was kept for 10 min. The temperature of the injector was set at 250 °C and was operated in the split mode with a ratio of 1:13. The transfer line temperature was adjusted to 250 °C, the ion source to 230 °C, and the quadrupole to 150 °C. Mass spectra were recorded in full scan mode with a mass range of *m/z* 30–400.

Data analysis was carried out using ChemStation (Agilent Technologies Germany GmbH). Fatty acid methyl esters were identified by their retention times, equivalent chain lengths, and mass spectra. The total fatty acid content was calculated with the internal standard nonadecanoic acid methyl ester.

2.6.4 *Laurdan generalized polarization analysis*

The Laurdan fluorescent membrane probe was used to investigate the effects of flavonoids on the membrane properties in acyl chain region [44,45]. Laurdan is a lipophilic, polarity-sensitive dye that is tightly anchored in the hydrophobic core of the membrane. The fluorescence spectrum depends on the amount and dynamics of water molecules in the membrane. Variations cause shifts in the Laurdan emission spectrum. Thus, Laurdan fluoresces with higher intensity at green wavelengths in a more fluid membrane, whereas the emission maximum shifts to blue wavelengths in more rigid regions [46–49].

Steady-state fluorescence was measured in a LS 55 spectrofluorometer combined with a Peltier element PTP-1 (Perkin Elmer LAS GmbH, Rodgau, Germany) for sample temperature

regulation. Sample preparation and staining were performed as described by Seel et al. [18]. The cells were washed with TE-buffer (pH 7.4) and diluted to an optical density (OD620) of 0.2. For Laurdan GP analyses, samples were excited at 360 nm (slit 10.0nm) at 30 °C. The emission spectra (slit 9.0 nm) were recorded from 380 nm to 600 nm. GP values were calculated according to Parasassi et al. [47] (Eq. (1)).

$$GP_{Laurdan} = \frac{I_{435} - I_{500}}{I_{435} + I_{500}}, \quad (1)$$

where *I* refers to the fluorescence intensity measured at the respective wavelength (nm); unlabeled cells were used as the blank reference. Each data point was calculated from 4 to 6 single measurements. Data are reported as means with standard deviations from independent biological triplicates.

2.7 *Statistical analyses*

Statistical analyses were conducted using the XLSTAT software version 2014.4.06 (Addinsoft, Paris, France). An ANOVA with Tukey post hoc test was performed to determine significant differences. The level of significance was defined as $p \leq 0.05$.

3 Results and discussion

3.1 *Characterization of membrane carotenoids*

In this study, LC-MS^n analysis revealed the presence of 15 carotenoids in *S. xylosus* and *S. carnosus* strains and of 19 carotenoids in *M. luteus* strains. Characteristic chromatograms obtained after separation of carotenoids from *Staphylococcus* spp. and *Micrococcus* spp. on a C30 column are presented in Figs. 2 and 3, respectively. Carotenoids were characterized based on their retention time, absorption spectra, and mass spectrometric data in comparison with published data (Tables 1 and 2) [31–40].

Staphyloxanthin is formed in pigmented *Staphylococcus* spp. via the precursors 4,4'-diapophytofluene, 4,4'-diaponeurosporene, 4,4'-diaponeurosporenoic acid, and glucosyl-4,4'-diaponeurosporenoic acid [32]. In *S. xylosus* and *S. carnosus*, the molecular ion $[M + H]^+$ of staphyloxanthin (5) was detected at *m/z* 819. Besides the high concentration of (all-*E*)-staphyloxanthin (Fig. 4), also a (*Z*)-staphyloxanthin isomer (4) was tentatively identified, based on a (*Z*)-peak at λ_{cis} = 346 nm, elution before (all-*E*)-staphyloxanthin, and a $\%A_B/A_{II}$ of 44%, describing the (*Z*)-peak intensity as the ratio between the height of the (*Z*)-peak and the main absorption peak (λ_{II}).

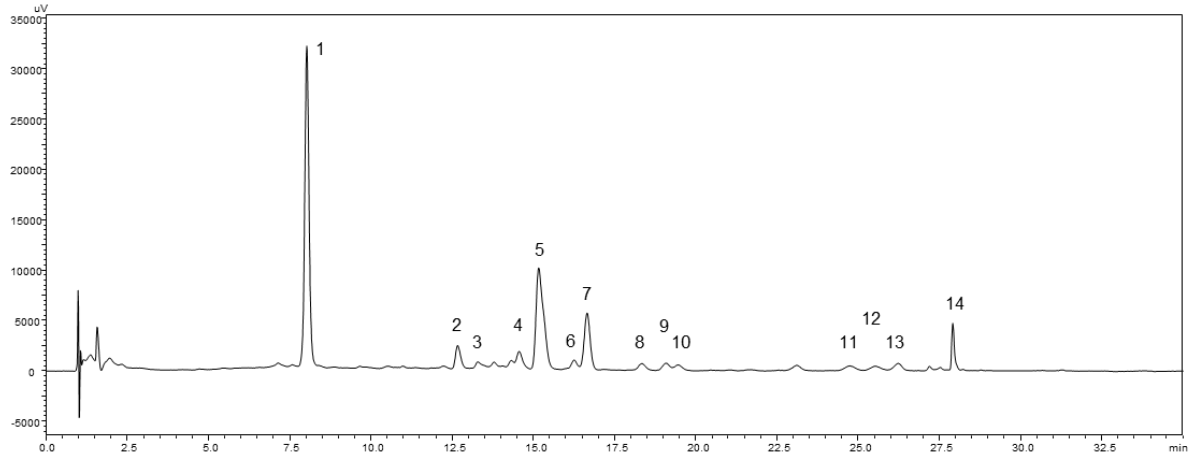


Fig. 2. Separation of carotenoids from *S. xylosus* DSM 20266^T incubated with naringenin by HPLC-DAD (450 nm). Peak assignment is given in Table 1.

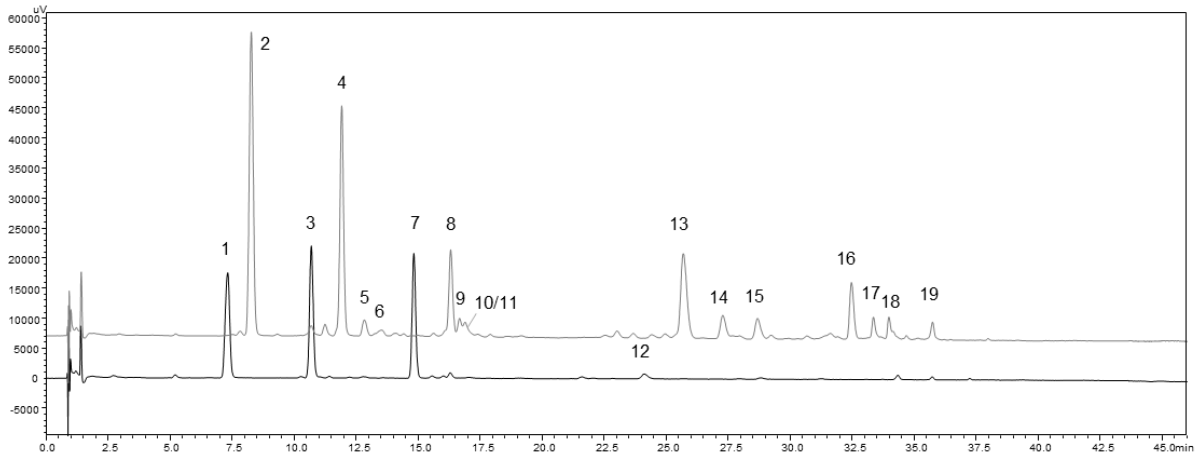


Fig. 3. Separation of carotenoids from (–) *M. luteus* ATCC 9341 and (–) *M. luteus* J3 by HPLC-DAD (450 nm). Peak assignment is given in Table 2.

The $[M + H]^+$ ion of 4,4'-diaponeurosporenoic acid (1) was detected at *m/z* 433, and fragments were found at *m/z* 415, 387, and 309 formed by the loss of water $[M + H-18]^+$, by the elimination of carbonic acid $[M + H-46]^+$, and by the loss of 2,4-methylhepta-1,3-dien $[M + H-124]^+$ from the protonated molecule, respectively.

The carotenoid profiles of *Staphylococcus* spp. included further compounds that exhibited spectral properties identical to staphyloxanthin and were hence tentatively identified by mass spectrometry as glucosyl-4,4'-diaponeurosporenoate fatty acid esters or staphyloxanthin-like compounds (Table 1). The carbon chain length of the fatty acids ranged from $C_{13:0}$ to $C_{20:0}$. The elution order corresponds to the polarity and the fatty acid chain length. Due to the presence of the carboxylic acid group, the more polar 4,4'-diaponeurosporenoic acid elutes earlier than the glucosyl-4,4'-diaponeurosporenoate fatty acid esters.

Table 1: UHPLC-DAD-MS^n identification of carotenoids from *S. xylosus* and *S. carnosus* (Fig. 2).

Peak	Compound	t_R[a](min)	λ_{max} (nm)	%III/II	$\%A_B/A_{II}$	$[M + H]^+$ (*m/z*)
1	4,4'-diaponeurosporenoic acid	8.02	452, 474	17	nd[b]	633
2	staphyloxanthin-like ($C_{13:0}$)	12.67	462	nd[b]	nd[b]	791
3a	staphyloxanthin-like ($C_{14:0}$)	13.28	462	nd[b]	nd[b]	805
3b	4,4'-diapophytofluene	13.28	332, 348, 368	102	nd[b]	nd[b]
4	(*Z*)-staphyloxanthin	14.56	346, 459	nd[b]	44	819
5	staphyloxanthin	15.16	464	nd[b]	nd[b]	819
6	(*Z*)-4,4'-diaponeurosporene	16.24	416, 439, 467	93	nd[b]	403
7	4,4'-diaponeurosporene	16.65	416, 438, 468	92	nd[b]	403
8	(*Z*)-staphyloxanthin-like ($C_{17:0}$)	18.31	346, 459	nd[b]	14	847
9	staphyloxanthin-like ($C_{17:0}$)	19.06	466	nd[b]	nd[b]	847
10	staphyloxanthin-like ($C_{17:0}$)	19.43	466	nd[b]	nd[b]	847
11	staphyloxanthin-like	24.73	467	nd[b]	nd[b]	861
12	staphyloxanthin-like ($C_{19:0}$)	25.52	467	nd[b]	nd[b]	875
13	staphyloxanthin-like ($C_{19:0}$)	26.22	465	nd[b]	nd[b]	875
14	staphyloxanthin-like ($C_{20:0}$)	27.92	465	nd[b]	nd[b]	889

[a]Retention time on Accucore C30 column. nd, not detected.
[b]%III/II, $\%A_B/A_{II}$ or $[M + H]^+$ was not detected or could not be calculated due to poor resolution of the UV/Vis spectrum.

Table 2: UHPLC-DAD-MS^n identification of carotenoids from *M. luteus* (Fig. 3).

Peak	Compound	t_R[a](min)	λ_{max} (nm)	%III/II	$\%A_B/A_{II}$	$[M + H]^+$ (*m/z*)
1	decaprenoxanthin diglucoside	7.33	415, 439, 468	95	nd[b]	1029
2	sarcinaxanthin diglucoside	8.26	416, 440, 469	94	nd	1029
3	decaprenoxanthin monoglucoside	10.70	415, 439, 468	95	nd[b]	867
4	sarcinaxanthin monoglucoside	11.91	416, 440, 469	94	nd[b]	867
5	sarcinaxanthin monoglucoside ester	12.82	417, 440, 470	47	nd[b]	nd[b]
6	(*Z*)-sarcinaxanthin monoglucoside	13.45	329, 410, 435, 462	66	51	867
7	decaprenoxanthin	14.83	415, 439, 468	95	nd[b]	705
8	sarcinaxanthin	16.31	416,440,469	92	nd[b]	705
9	sarcinaxanthin monoglucoside ester	16.67	417, 440, 470	62	nd[b]	nd[b]
10	dihydrosarcinaxanthin	16.89	400, 420, 448	97	nd[b]	nd[b]
11	(*Z*)-sarcinaxanthin	17.04	327, 411, 433, 463	67	52	705
12	sarcinaxanthin ester	24.13	416,438,469	97	nd[b]	nd[b]
13	sarcinaxanthin ester	25.69	417, 440, 470	94	nd[b]	nd[b]
14	sarcinaxanthin ester	27.29	416, 440, 470	92	nd[b]	nd[b]
15	sarcinaxanthin ester	28.69	417, 440,470	89	nd[b]	nd[b]
16	sarcinaxanthin ester	32.48	417, 440, 470	92	nd[b]	nd[b]
17	sarcinaxanthin ester	33.36	418, 440, 470	89	nd[b]	nd[b]
18	sarcinaxanthin ester	33.99	417, 440, 470	90	nd[b]	nd[b]
19	nonaflavuxanthin	35.75	448, 474, 501	75	nd[b]	621

[a]Retention time on Accucore C30 column. nd, not detected.
[b]%III/II, $\%A_B/A_{II}$ or $[M + H]^+$ was not detected or could not be calculated due to poor resolution of the UV/Vis spectrum.

Table 3: Carotenoid concentration in bacterial membranes incubated with naringenin (NAR), tetrahydroamentoflavone (THAF), and amentoflavone (AMF) in µg β-carotene equivalents/mg cell dry weight.

organism	control	NAR	THAF	AMF	F-Value
S. xylosus DSM 20266^T	0.060 ± 0.002 BC	0.079 ± 0.004 A	0.053 ± 0.004 C	0.064 ± 0.002 B	43.05
S. xylosus J70	0.054 ± 0.003 A	0.056 ± 0.002 A	0.041 ± 0.005 B	0.054 ± 0.004 A	12.53
S. carnosus DSM 20501^T	0.137 ± 0.003 D	0.336 ± 0.015 B	0.393 ± 0.011 A	0.225 ± 0.000 C	450.6
M. luteus ATCC 9341	0.226 ± 0.007 A	0.224 ± 0.009 A	0.177 ± 0.002 B	0.166 ± 0.016 B	35.04
M. luteus J3	0.076 ± 0.001 A	0.076 ± 0.003 A	0.063 ± 0.002 B	0.061 ± 0.004 B	28.47

Values are mean ± standard deviation (n = 3). Different letters indicate significant differences within each strain ($p \leq 0.05$).

The total carotenoid content in *S. xylosus* DSM 20266^T was 0.060 ± 0.002 µg/mg cell dw (Table 3). *S. xylosus* DSM 20266^T was characterized by a high relative proportion of precursors 4,4'-diaponeurosporenoic acid and 4,4'-diaponeurosporene at 25%. Staphyloxanthin and other esters accounted for 44% and 16%, respectively (Fig. 4A).

S. xylosus J70 exhibited a total carotenoid content of 0.054 ± 0.003 µg/mg cell dw with relative proportions of 6% 4,4'-diaponeurosporenoic acid, 60% staphyloxanthin, and 22% staphyloxanthin-like compounds (Fig. 4B).

S. carnosus DSM 20501^T possessed the highest total carotenoid content of 0.137 ± 0.003 µg/mg cell dw compared with *S. xylosus* DSM 20266^T and *S. xylosus* J70. In *S. carnosus* DSM 20501^T, 7% 4,4'-diaponeurosporenoic acid, 65% staphyloxanthin, and 13% other carotenoid esters were detected (Fig. 4C).

In *Micrococcaceae*, the carotenoids sarcinaxanthin, decaprenoxanthin, and their glucosides formed from phytofluene, neurosporene, and nonaflavuxanthin have been described [35,38,39]. The carotenoids identified in *M. luteus* ATCC 9341 are listed in Table 2, with the main carotenoids being sarcinaxanthin diglucoside (2), sarcinaxanthin monoglucoside (4), and sarcinaxanthin (8). Besides (all-*E*)-sarcinaxanthin, a (*Z*)-sarcinaxanthin isomer (11) was detected based on the presence of a (*Z*)-peak at λ_{cis} = 327 nm and a $\%A_B/A_{II}$ of 52%. The mass spectrum of sarcinaxanthin showed ions of *m/z* 687, 669, and 497 formed by the loss of one ($[M + H\text{-}18]^+$) or two ($[M + H\text{-}36]^+$) water molecules, and by the elimination of 1-(3-methylbut-2-enyl)-2,2-dimethyl-4-methylenecyclohexane $[M + H\text{-}36\text{-}172]^+$ from the protonated molecule, respectively [36].

The strain *M. luteus* J3 produced carotenoids with the same UV/Vis spectra and parent ions at *m/z* values of 1029, *m/z* 867, or *m/z* 705, respectively. Based on the relative retention time shift of these carotenoids and ion fragments of *m/z* 687 ($[M + H\text{-}18]^+$), 669 ($[M + H\text{-}36]^+$), and 595 ($[M + H\text{-}18\text{-}92]^+$) of peak 7, they were identified as decaprenoxanthin diglucoside (1), decaprenoxanthin monoglucoside (3), and decaprenoxanthin [38–40].

The carotenoid profile of *M. luteus* ATCC 9341 includes a considerable amount of esterified carotenoids in addition to free xanthophylls and their glucosides, which were tentatively identified. The carotenoid fatty acid esters showed UV/Vis spectra identical to sarcinaxanthin but eluted after the polar glucosylated derivatives and sarcinaxanthin. *M. luteus* ATCC 9341 revealed a carotenoid content of 0.226 ± 0.007 µg/mg cell dw with relative proportions of 47% sarcinaxanthin glucosides, 8% free sarcinaxanthin, and 31% esterified carotenoids (Table 3, Fig. 5A).

In comparison with *M. luteus* ATCC 9341, *M. luteus* J3 showed a notably lower total carotenoid content of 0.076 ± 0.001 µg/mg cell dw and obvious differences in carotenoid profile. In M. luteus J3, decaprenoxanthin glucosides are represented by 62% and free xanthophylls by 28%. The relative proportion of xanthophyll esters was 2% (Fig. 5B).

3.2 *Antibacterial activity*

Investigations into the antibacterial activity of THAF against Gram-positive, carotenoid-forming, food-associated microorganisms revealed a MIC of 1 mg/mL or less (Table 4) and MBCs higher than 1 mg/mL. MICs of NAR and AMF were higher than 1 mg/mL for *S. xylosus* (DSM 20266^T and J70), *S. carnosus* DSM 20501^T, and *M. luteus* (ATCC 9341 and J3). The antibacterial effect of NAR, THAF, and AMF verify the dependence of dimerization and oxidative state of the C-ring of flavonoids on the antibacterial effect we demonstrated in our previous study [4].

The interactions of flavonoids with bacterial membranes play an important role in their antibacterial activity [6,7,9]. Hydrophobic interactions are crucial due to the rather hydrophobic nature of flavonoids. In contrast, polar interactions of the flavonoids with membrane head groups have been shown to exert an additional effect due to the pH-dependent deprotonation of these molecules [50].

Table 4: Minimal inhibitory concentration (mg/mL) of naringenin (NAR), tetrahydroamentoflavone (THAF), and amentoflavone (AMF).

organism	NAR	THAF	AMF
S. xylosus DSM 20266^T	>1	1	>1
S. xylosus J70	>1	1	>1
S. carnosus DSM 20501^T	>1	0.063	>1
M. luteus ATCC 9341	>1	1	>1
M. luteus J3	>1	1	>1

3.3 *Impact of flavonoid addition on membrane carotenoids and Laurdan GP*

In addition to the antibacterial effect, the response of the microorganisms to flavonoids is vital to understand their mode of action and the resistance mechanisms. Membrane carotenoids are associated with membrane functions and are considered adaptation mechanisms of bacteria [17,21]. The flavonoids caused significant changes in the carotenoid content (Table 3), profiles (Figs. 4 and 5), and Laurdan GP (Fig. 6), as exemplified by naringenin in *S. xylosus* DSM 20266^T and *S. carnosus* DSM 20501^T. However, no clear trend can be observed that applies to all strains. Furthermore, interactions of the membrane with the flavonoids seem to have a minor role because no correlation was observed.

S. xylosus DSM 20266^T treated with NAR showed an increased carotenoid content (Table 3) accompanied by an increase in Laurdan GP, i.e., a decrease in membrane fluidity (Fig. 6). The effect of carotenoids on membrane fluidity is consistent with previous studies [17,51,52]. This relationship might result from a decreased molecular motion of the acyl tails due to Van der Waals and hydrophobic interactions with carotenoids [44,51,53–55]. The inferred stabilization of the membrane and alteration of the penetration barrier imply reduced susceptibility of the microorganism [15,16,54,56–58].

The modifications in the carotenoid profile due to NAR in the membrane of *S. xylosus* DSM 20266^T were characterized by a twofold increase in the precursors 4,4'-diaponeurosporenoic acid and 4,4'-diaponeurosporene proportion and a decrease in the relative proportion of staphyloxanthin in the membrane (Fig. 4A).

In contrast to the results obtained for *S. xylosus* DSM 20266^T, there were no significant differences in carotenoid content and Laurdan GP values between control and NAR-treated *S. xylosus* J70. However, incubation of *S. xylosus* J70 with NAR resulted in an eightfold increase in the 4,4'-diaponeurosporenoic acid proportion and a corresponding reduction in staphyloxanthin and staphyloxanthin-like compounds (Fig. 4B). Although there was no change in carotenoid content, alterations of the carotenoid profiles very likely impact membrane properties. Structural properties, such as stereochemistry of the carotenoids or their end groups, alter the interactions with both hydrocarbon chains and polar head groups of the membrane. This determines their localization and orientation within the membrane and influences fundamental biophysical properties [15,51,59]. Polar carotenoids have been shown to modify hydrophobic barriers in model membranes and affect the nonspecific permeation of polar and nonpolar molecules. Limitation of oxygen penetration into the lipid bilayer was demonstrated for the polar zeaxanthin [15,60–65]. The non-polar carotenoid β-carotene decreases the penetration barrier for small molecules in the headgroup region of the membrane [66]. The

observed modifications of the carotenoid profile in *S. xylosus*, such as an increase in the polar 4,4'-diaponeurosporenoic acid and the nonpolar 4,4'-diaponeurosporene, suggest a specific adjustment of the penetration barrier by carotenoids as an adaptive mechanism against NAR.

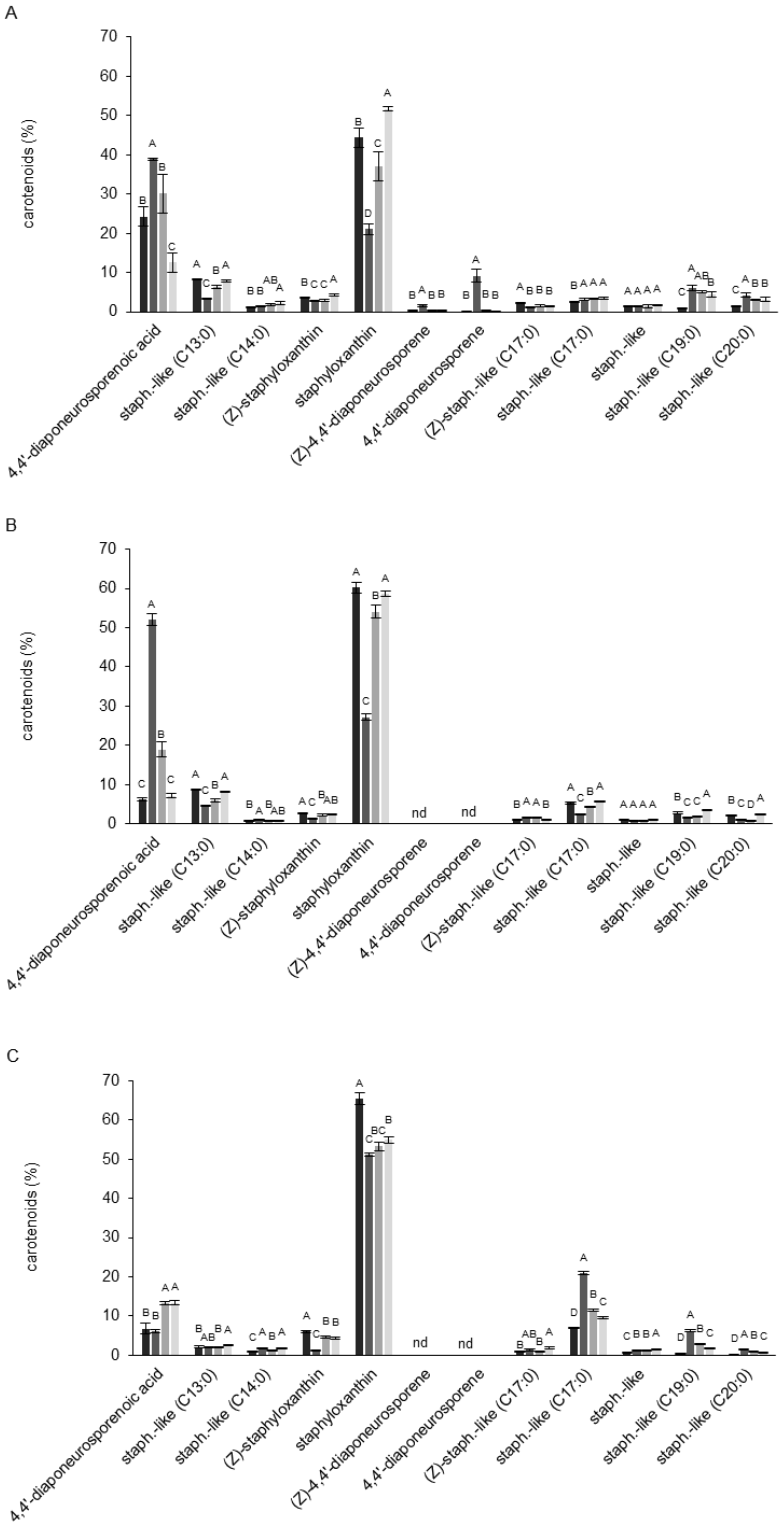


Fig. 4. Relative carotenoid content in A, *S. xylosus* DSM 20266^{T}; B, *S. xylosus* J70; and C, *S. carnosus* DSM 20501^{T}. Control (■); 24 h incubation with naringenin (■); tetrahydroamentoflavone (■); and amentoflavone (■). Values are mean ± standard deviation (n = 3). Statistical analysis: ANOVA with Tukey post hoc test, different letters indicate significant differences ($p \leq 0.05$). nd, not detected.

The changes in the carotenoid profile in *S. xylosus* DSM 20266^T in response to THAF treatment showed similar trends as after exposure to NAR. In particular, incubation of *S. xylosus* DSM 20266^T with THAF resulted in a 26% reduction in staphyloxanthin (from 0.027 ± 0.002 µg/mg to 0.02 ± 0.000 µg/mg), whereas in case of *S. xylosus* J70, a reduction of 35% was observed (from 0.034 ± 0.002 µg/mg to 0.022 ± 0.002 µg/mg), which was accompanied by a decrease in total carotenoid content.

Carotenoid production is controlled by the sigma factor σ^B activity, which plays a role in response to specific stress signals [67,68]. Rojas-Tapias and Helmann [69] showed that cell wall stress, but not membrane stress, induced the Spx transcription factor, a widely disseminated group of regulators found throughout the Firmicutes. Increased expression of *spx* could repress *cspA* expression, which controls σ^B activity [70]. Thus, a reduced staphyloxanthin content might be associated with cell wall stress responses.

Moreover, the noticeably different CCS value of 220.99 ± 0.27 Å^2 of THAF from NAR (161.96 ± 0.11 Å^2) and AMF (178.58 ± 0.84 Å^2) indicated steric hindrance and less potential for membrane interactions. Both the CCS value and the reduced staphyloxanthin content suggest that THAF interacts with the cell wall rather than the membrane. This might be crucial for its higher antibacterial effect in comparison with NAR and AMF in the carotenoid-forming microorganisms.

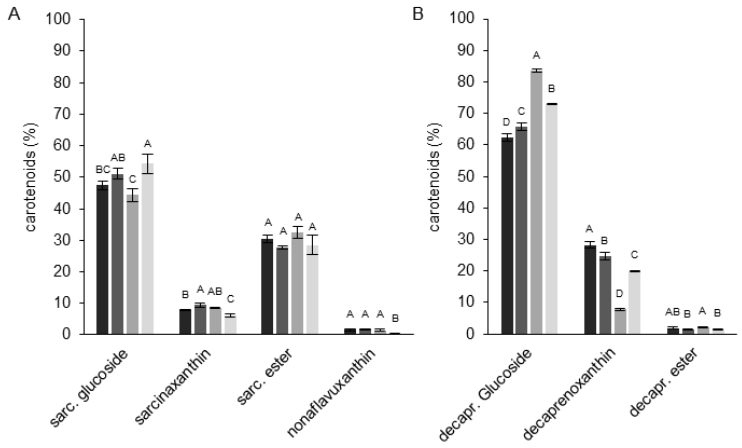


Fig. 5. Relative carotenoid content in A, *M. luteus* ATCC 9341 and B, *M. luteus* J3. Control (■); 24 h incubation with naringenin (■); tetrahydroamentoflavone (■); and amentoflavone (■). Values are mean ± standard deviation (n = 3). Statistical analysis: ANOVA with Tukey post hoc test, different letters indicate significant differences ($p \leq 0.05$).

AMF had a negligible effect on the carotenoid profile on *S. xylosus* membranes.

In *S. carnosus* DSM 20501^T, flavonoids caused a significant increase in carotenoid content, which was related to an increase in Laurdan GP (Fig. 6). The change in the carotenoid profile is similar to the alteration observed in *S. xylosus* strains. The carotenoid profile shows a

decreased staphyloxanthin proportion, whereas longer-chain esters, mainly esterified with $C_{17:0}$, $C_{19:0}$, and $C_{20:0}$, increased, particularly in the presence of NAR (Fig. 4C).

In *M. luteus* ATCC 9341, NAR did not notably affect the carotenoid content and profile (Table 3, Fig. 5A). THAF and AMF exhibited minor influences on the profile but reduced the carotenoid content by 22% and 27%, respectively. *In M. luteus* ATCC 9341, the changes in carotenoid content had no effects on Laurdan GP.

Similar to *M. luteus* ATCC 9341, carotenoid content and profile in *M. luteus* J3 are less affected by NAR (Fig. 5B). THAF and AMF decrease the total carotenoid content in *M. luteus* J3, whereby the decrease in decaprenoxanthin was most pronounced. Decaprenoxanthin content was reduced from 0.022 ± 0.001 µg/mg cell dw to 0.005 ± 0.000 µg/mg cell dw for THAF and to 0.013 ± 0.001 µg/mg cell dw for AMF, respectively. This is associated with a relative proportional increase in polar xanthophyll glucosides. The polar carotenoid zeaxanthin was shown to decrease the permeability barrier to protons and increase the permeability of glucose in membrane vesicles [63]. The modification of carotenoid proportions in *M. luteus* J3 indicates an adaptation to THAF and AMF stressors by alteration of the permeability barrier. Laurdan GP of *M. luteus* J3 was decreased only by AMF, although THAF and AMF had similar effects regarding carotenoids (Fig. 6). Thus, the fluidity seems to be unaffected by membrane carotenoids in *M. luteus* strains.

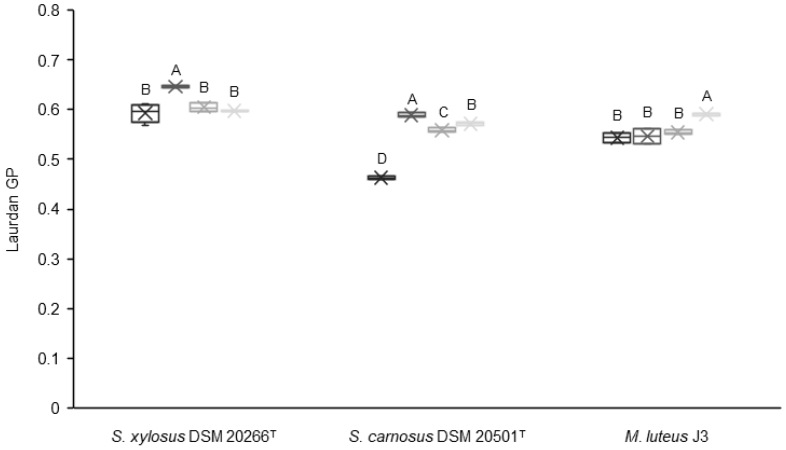


Fig. 6. Laurdan GP value in A, *S. xylosus* DSM 20266^T; B, *S. carnosus* DSM 20501^T; and C, *M. luteus* J3. Control (–); 24 h incubation with naringenin (–); tetrahydroamentoflavone (–); and amentoflavone (–). Mean value (x), median (–) (n = 3). Statistical analysis: ANOVA with Tukey post hoc test, different letters indicate significant differences ($p \leq 0.05$).

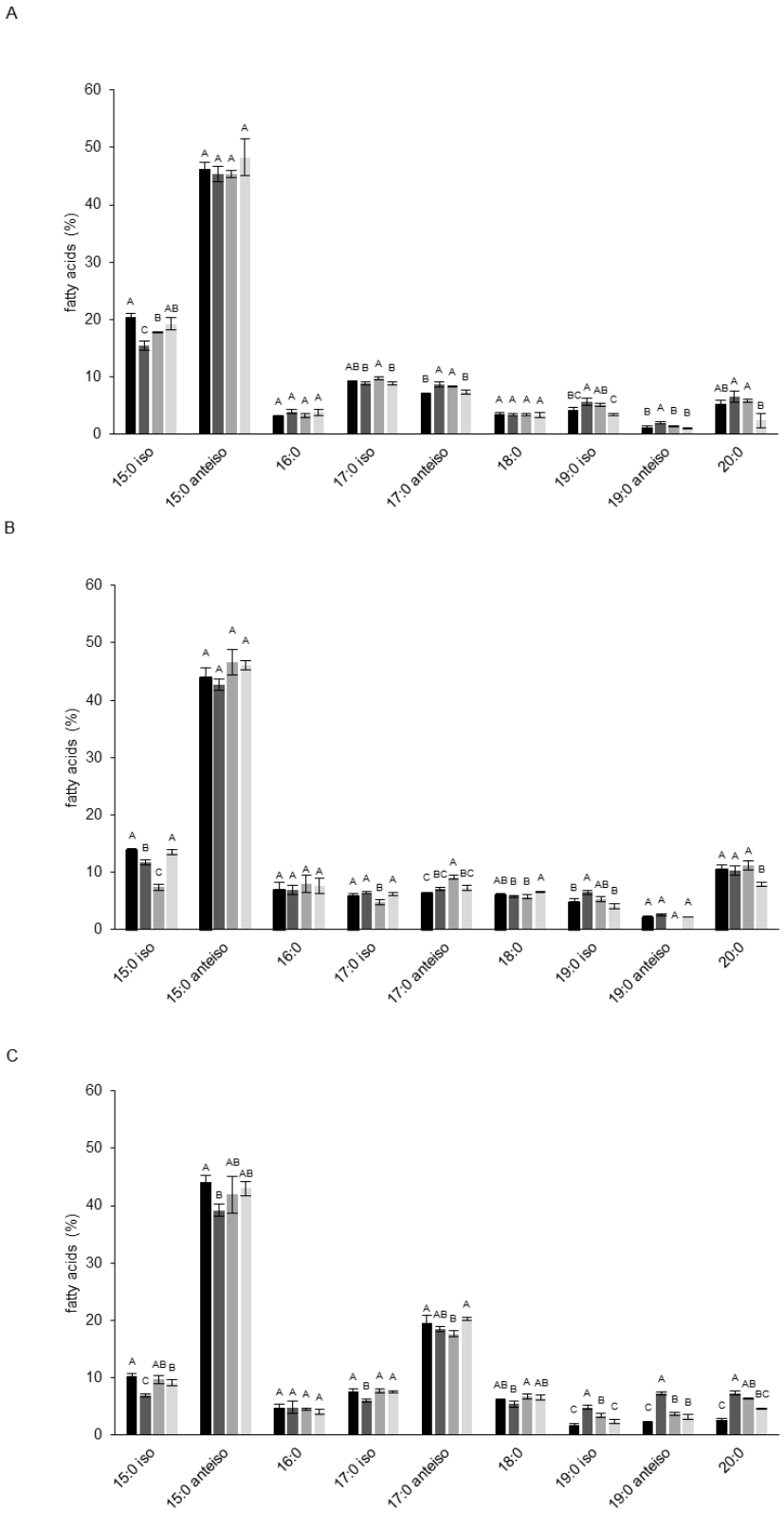


Fig. 7. Relative fatty acid content in A, *S. xylosus* DSM 20266^{T}; B, *S. xylosus* J70; and C, *S. carnosus* DSM 20501^{T}. Control (■); 24 h incubation with naringenin (■); tetrahydroamentoflavone (■); and amentoflavone (■). Values are mean ± standard deviation (n = 3). Statistical analysis: ANOVA with Tukey post hoc test, different letters indicate significant differences ($p \leq 0.05$).

3.4 *Fatty acid profile*

The fatty acid composition is a major determinant of biophysical properties of membranes that directly impacts membrane fluidity, passive permeability to hydrophobic molecules, active transport, susceptibility to membrane-active antimicrobials, and the ability of bacteria to adapt to changing environments [57,71,72].

In *S. xylosus* (DSM 20266^T and J70), *S. carnosus* DSM 20501^T, and *M. luteus* (ATCC 9341 and J3), NAR and biflavonoids mainly caused a reduction in specific fatty acids, especially $C_{15:0,iso}$ fatty acid, and a slight increase in the relative proportion of longer chain fatty acids (Figs. 7 and 8). Wang et al. [12,73,74] showed a decrease in $C_{15:0,iso}$ fatty acid in *S. aureus* and an increase in longer chain fatty acids under antimicrobial stress. Modification of acyl chain lengths and, in particular, the increase in the relative proportion of longer chain fatty acids imply a strengthening of the hydrophobic membrane barrier of the microorganisms studied [19,75,76].

In addition to the changes in fatty acid profile, a significant reduction in the fatty acid concentration of 32% and 39% was observed in *M. luteus* ATCC 9341 caused by THAF and AMF, respectively (Table 5). Heath et al. [77] showed a relationship between guanosine-3',5'-bispyrophosphate (ppGpp) accumulation and reduction of the fatty acid content. The global regulator ppGpp is associated with stress responses and linked to the resistance against antimicrobials [72,78,79]. The reduction of fatty acid concentration by the stressors AMF and THAF might involve in resistance mechanism and implies further changes in membrane structure to reduce susceptibility of *M. luteus* ATCC 9341.

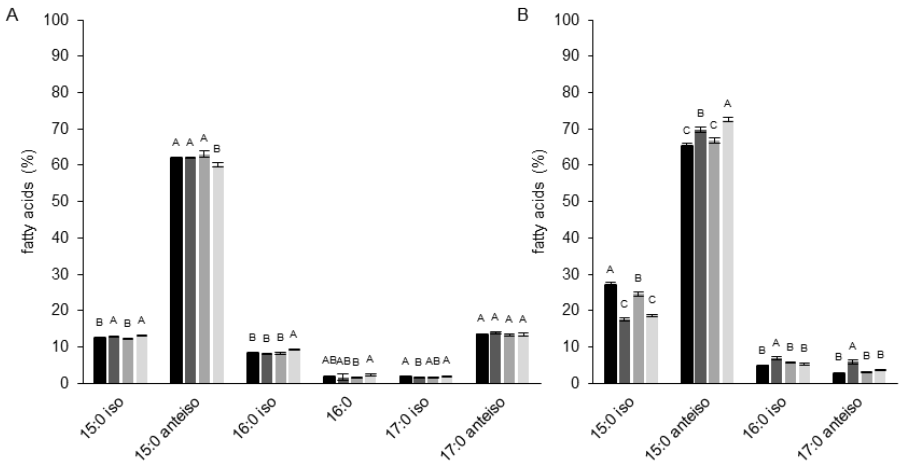


Fig. 8. Relative fatty acid content in A, *M. luteus* ATCC 9341 and B, *M. luteus* J3. Control (■); 24 h incubation with naringenin (■); tetrahydroamentoflavone (■); and amentoflavone (■). Values are mean ± standard deviation (n = 3). Statistical analysis: ANOVA with Tukey post hoc test, different letters indicate significant differences ($p \leq 0.05$).

Table 5: Fatty acid concentration in bacterial membranes incubated with naringenin (NAR), tetrahydroamentoflavone (THAF), and amentoflavone (AMF) in µg nonadecanoic acid methyl ester equivalents/mg cell dry weight.

organism	control	NAR	THAF	AMF	F-Value
S. xylosus DSM 20266^T	87.07 ± 3.79 AB	110.32 ± 8.36 A	70.80 ± 4.04 B	88.28 ± 28.37 AB	5.49
S. xylosus J70	22.72 ± 4.78 A	25.54 ± 1.43 A	20.51 ± 2.53 A	20.29 ± 1.16 A	
S. carnosus DSM 20501^T	58.86 ± 2.64 A	57.89 ± 3.00 A	63.75 ± 3.75 A	49.97 ± 10.06 A	
M. luteus ATCC 9341	22.05 ± 0.27 A	22.29 ± 4.85 A	14.98 ± 1.16 B	13.40 ± 1.21 B	14.06
M. luteus J3	18.58 ± 1.49 A	18.29 ± 3.34 A	17.39 ± 0.92 A	16.12 ± 0.80 A	

Values are mean ± standard deviation (n = 3). Different letters indicate significant differences within each strain ($p \leq 0.05$).

Table 6: Menaquinone concentration in bacterial membranes incubated with naringenin (NAR), tetrahydroamentoflavone (THAF), and amentoflavone (AMF) in µg/mg cell dry weight.

organism	control	NAR	THAF	AMF	F-Value
S. xylosus DSM 20266^T	98.19 ± 1.14 B	116.22 ± 8.36 A	87.43 ± 2.44 C	84.22 ± 1.51 C	95.83
S. xylosus J70	71.77 ± 1.23 B	102.10 ± 5.81 A	77.91 ± 2.75 B	77.72 ± 2.73 B	43.21
S. carnosus DSM 20501^T	206.70 ± 9.23 A	143.81 ± 7.79 C	215.18 ± 4.39 A	179.09 ± 5.81 B	62.47
M. luteus ATCC 9341	72.86 ± 0.67 AB	77.36 ± 4.74 A	60.80 ± 4.52 BC	52.05 ± 5.77 C	17.38
M. luteus J3	109.27 ± 1.85 A	98.26 ± 3.13 B	109.61 ± 0.88 A	89.71 ± 0.66 C	76.37

Values are mean ± standard deviation (n = 3). Different letters indicate significant differences within each strain ($p \leq 0.05$).

3.5 *Menaquinone*

Menaquinones in bacteria are known for their participation in electron transport chains and, therefore, are crucial for energy generation. In addition, menaquinones are part of the adaption to environmental changes, particularly oxygen deficiency, act as ion permeability barriers, and regulate membrane fluidity [18,23,80,81].

The flavonoids caused significant changes in the menaquinone content (Table 6), whereby no clear trend was observed. Both a reduction and an increase in menaquinone content were found.

In both *S. xylosus* strains (DSM 20266^T and J70), NAR led to a significant increase in menaquinone content (Table 6), suggesting a decrease in membrane permeability [23]. In the context of significant changes in the carotenoid profile in *S. xylosus* strains triggered by NAR, a specific adaptation of membrane permeability apparently results [15,51,59].

AMF cause a decrease in menaquinone content in *S. xylosus* DSM 20266^T, *S. carnosus* DSM 20501^T, *M. luteus* ATCC 9341, and *M. luteus* J3. Bentley et al. [82] demonstrated the dependence of menaquinone biosynthesis on many factors including antibacterial agents. Further studies attributed a reduction in menaquinone content due to antibacterial compounds [83–86]. Flavonoids, in particularly AMF, might inhibit menaquinone biosynthesis due to the considerable reduction of menaquinone content in carotenoid-forming food-associated bacteria.

4 Conclusion

This study contributes to a comprehensive knowledge of the structure related effects of flavonoids and their strain dependency by analyzing bacterial lipids, especially carotenoids, and CCS levels. Our investigations demonstrated varying reactions of microorganisms and, thus, different mechanisms of action, targets, and interactions with membrane depending on the size of the flavonoid, its three-dimensional structure, and the degree of oxidation.

These insights are of considerable importance in the elucidation the mechanisms of action of flavonoids and might be useful for targeting combination of preservatives to study beneficial synergistic effects.

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