Maillard reaction in cocoa

The use of fluidized bed roasting to modulate aroma compounds and physiological aspects

Ruth Fabiola Peña Correa

Propositions

1. Porosity is an underestimated quality control parameter of roasted food. (this thesis)
2. Cocoa melanoidins are used by gut bacteria as prebiotics. (this thesis)
3. Drafts, trials, and failed experiments are the submerged part of the iceberg supporting the thesis.
4. Research projects written in stone make PhD candidates less happy.
5. Celebrations and awards honoring women reinforce gender inequality.
6. One of the biggest challenges for PhD students is conveying the essence of their thesis to an ordinary audience.

Propositions belonging to the thesis, entitled

Maillard reaction in cocoa. The use of fluidized bed roasting to modulate aromatic compounds and physiological aspects

Ruth Fabiola Peña Correa
Wageningen, 26 May 2023

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Thesis

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To Bernardo and Gabriel

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Chapter 1

General introduction

1.1 *Theobroma cacao* L.

Cocoa beans are the dried and fermented seeds of *Theobroma cacao* L. The tree is about 6 to 12 m, and its fruits grow from the trunk and thicker branches, as observed in Fig. 1.1, panels A and B. Cacao trees best grow within 20° north and south of the equator, in areas with a suitable environment (e.g., rainfall, soil type) like west Africa, Southeast Asia, and South and Central America.[1] There are four main varieties of the cacao plant: Forastero, Criollo, Trinitario, and Nacional, from which Forastero represents more than 80% of the world's cocoa production.[2]

FIGURE 1.1 *Theobroma cacao* L. tree (A), flowers (B), and pods and seeds (C).

The upper Amazon region is generally believed to be the center of origin of cacao.[3] Since its domestication in Mesoamerica around 3.000 years ago, cacao has been mainly cultivated for elaborating edible products. Nowadays, the confectionery industry has the highest demand for cocoa beans and their primary derivatives

(i.e., cocoa liquor or cocoa mass, cocoa powder, and cocoa butter). Cocoa beans and primary derivatives are traded as commodities around the globe.[4]

1.2 Key aspects of chocolate processing and quality

Among all edible cocoa derivatives, chocolate is the most popular one. The roasting process determines their quality. Insufficient roasting produces cocoa nibs with a poor chocolate aroma and does not reduce their acidic off-flavors. Moreover, if the water content is not sufficiently removed during roasting, a cascade of defects occurs like excess hardness in the nibs (which makes the grinding process difficult), low flowability in the produced cocoa liquor (due to the high viscosity), and 'sugar bloom' in chocolate bars.[2]

Besides roasting, there are other critical steps across the elaboration of chocolate, as shown in Fig. 1.2. In the field, as soon as the cocoa pods are harvested, the seeds are extracted for fermentation, as observed in Fig. 1.1 Panel C. This stage is of particular interest due to the generation of aroma precursors (e.g., amino acids, monosaccharides, and free fatty acids)[5,6] and the reduction in bitterness and astringency.[7] After fermentation, cocoa beans are dried to reduce their moisture content sufficiently for safe storage and transportation. Deficient fermentation and over-fermentation generate unpleasant tastes, and incomplete drying favors the proliferation of microorganisms.[8]

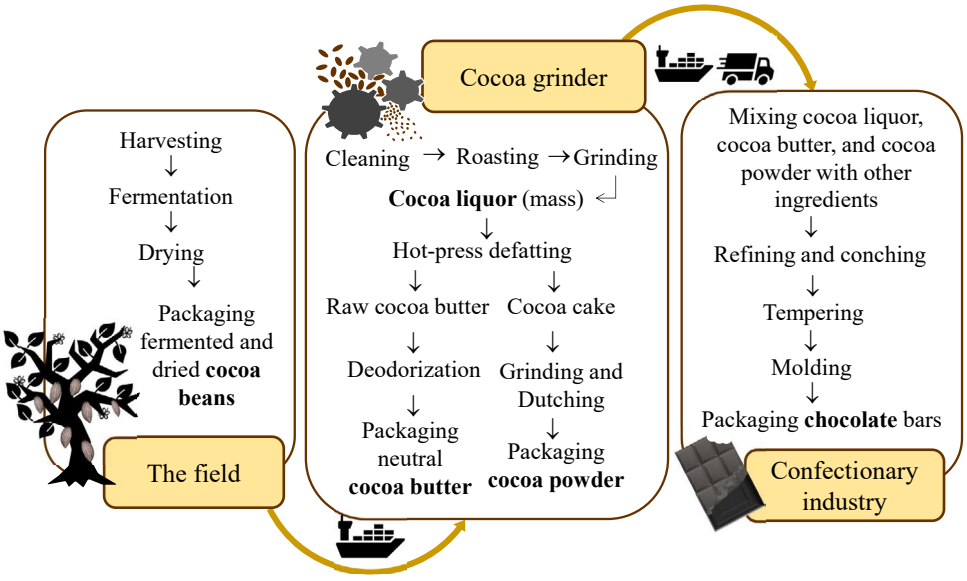

FIGURE 1.2 The actors and the processing steps for the production of chocolate bars.

Chocolate manufacturing can continue in one or more different locations. Large cocoa processors convert cocoa beans into cocoa liquor, cocoa butter, and cocoa powder, which they distribute to the confectionery, food, cosmetic, and pharmaceutical industries. These kinds of processing plants are known as 'Cocoa grinders' (Fig. 1.2). Cocoa grinders receive cocoa beans. The first activity they do is to clean cocoa beans, which can also carry stones and steams from the field. Then, cocoa nibs are roasted. To obtain cocoa liquor, roasted cocoa nibs are finely ground (50 °C approx.). Consequently, cocoa butter is released from parenchyma cells, and the mass turns liquid. Cocoa liquor is subjected to hot-press to leak out the lipid fraction (i.e., raw cocoa butter) from the solid moiety of cocoa nibs, namely the cocoa cake. Raw cocoa butter is further deodorized, while cocoa cake (which is not 100% defatted) is ground to produce natural cocoa powder. Natural cocoa powder is alkalized to enhance its brown color and improve its dispersion in water. This process is known as Dutching.[8]

Some cocoa grinders also manufacture end products to supply directly to the retail or food service sector.[9] However, most of the chocolate producers in the world depend on them from whom they purchase cocoa butter, cocoa powder, and cocoa liquor. In the confectionery industry, these cocoa ingredients, plus sugar and milk powder, among other minor ingredients, are mixed, refined, and conched to finely disperse the solid ingredients into cocoa butter, thus producing a smooth chocolate mass (Fig. 1.2).[8] If the particle size of the solid ingredients is not reduced accordingly, a gritty sensation can be perceived in the mouth when melting a chocolate bite. The chocolate mass follows three more basic steps: Tempering, molding, and packaging. Tempering is critical as it stabilizes the crystallization of cocoa butter to slightly shrink the mass during molding, thus conferring the proper hardness to chocolate bars and the typical 'snap' sound when breaking them. Non-well-tempered chocolate is soft, lacks shine, and partially melts in the package during transportation and commercialization.[8]

1.3 The Netherlands, an important cocoa trade hub in the world

World cocoa beans production has increased steadily over the past 40 years. About 95 % of them are traded on international commodity markets. According to the data supplied by the Food and Agriculture Organization of the United Nations (2020),[10] the year 2020 closed with about 5 million tonnes of cocoa beans produced worldwide. Ivory Coast is by far the largest cocoa-producer country, accounting for over 40% of global cocoa production, as observed in Fig. 1.3.

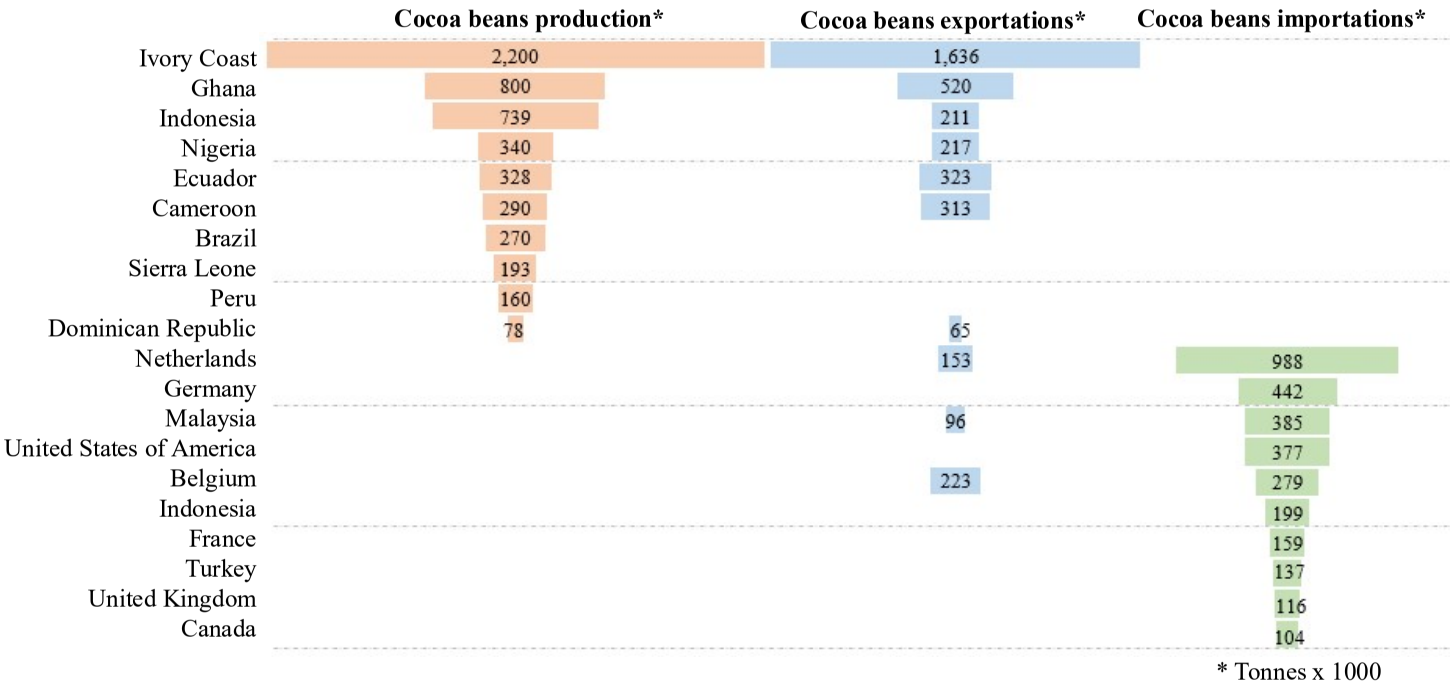

FIGURE 1.3 Cocoa beans trading dynamics within the world's top ten producing, exporting, and importing countries in 2020. Source of the data: FAO (2020)[10]

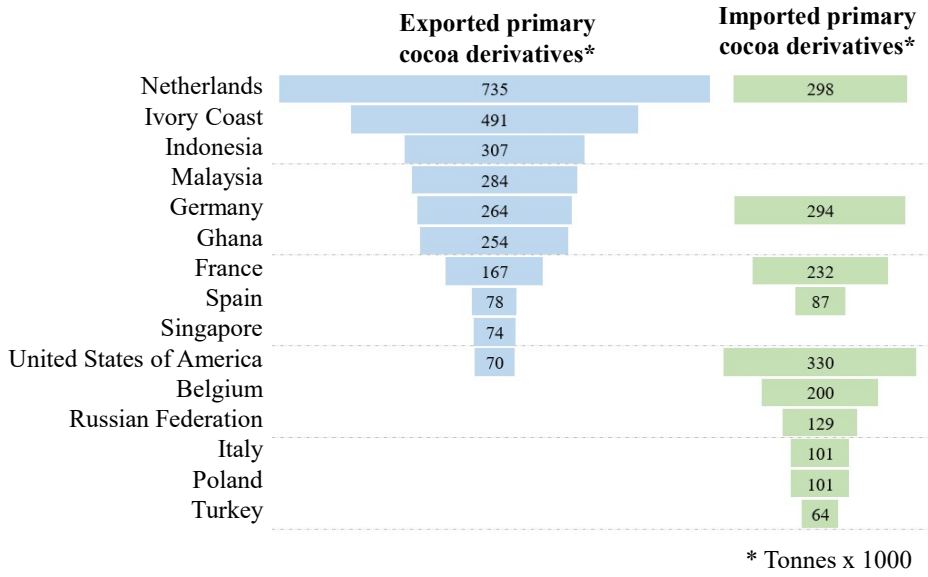

FIGURE 1.4 Trading dynamics of cocoa beans' primary derivatives (i.e., the sum of cocoa mass, cocoa powder, and cocoa butter) within the world's top ten exporting and importing countries in 2020. Source of the data: FAO (2020)[10]

TABLE 1.1 List of the top 20 confectionery companies in the world in 2020.

Top	Company Name	Country	Net Sales (Mill.)
1	Mars Wrigley Confectionery Mars Inc.	USA	$20,000
2	Ferrero Group	Italy	$13,566
3	Mondelez International	USA	$11,467
4	Meiji Co. Ltd.	Japan	$10,075
5	Hershey Co.	USA	$8,066
6	Nestle SA	Switzerland	$7,636
7	Pladis	UK	$4,655
8	Lindt & Sprungli AG	Switzerland	$4,331
9	Ezaki Glico Co. Ltd.	Japan	$3,311*
10	Haribo GmbH & Co. K.G.	Germany	$3,300
11	Perfetti Van Melle Spa	Italy and The Netherlands	$3,150
12	General Mills	USA	$2,221
13	August Storck KG	Germany	$2,000
14	Orion Corp.	Korea	$1,736
15	Kellogg Co.	USA	$1,323
16	Morinaga & Co. Ltd.	Japan	$1,229
17	Valeo Foods Group	Ireland	$1,190*
18	Bourbon Corp.	Japan	$1,134*
19	Chocolat Frey AB	Switzerland	$1,100
20	United Confectionary Manufacturers	Russia	$1,055

* Includes non-confectionery items. Reference: Candy Industry (2021)[13]

Even though cocoa beans' producers do not elaborate a substantial amount of cocoa derivatives, the global trading of cocoa is of significant economic importance, as it generates export revenues, income, and employment. Although the global economy is facing inflationary pressures that originated during the COVID-19 period, and chocolate is considered a non-essential good, the demand for confectionery products is still growing as consumers find chocolate a timeless product and indulgent food, and especially see dark cocoa as a healthy choice.[14]

1.4 Adopting the fluidized bed coffee roaster for cocoa nibs processing

Since ancient times, the roasting process of cocoa beans has been predominantly performed with conductive heat transfer (Fig. 1.5 panel A). Due to the importance of this step, various improvements have been implemented, especially during the industrialization times,[15] for example:

- More homogeneous heating of the beans was obtained with the injection of hot air (convective heat transfer), plus the implementation of the rotating drum system (Fig. 1.5 panel B).
- The increase of the batch size.
- The separation of cocoa cotyledons (i.e., cocoa nibs) from cocoa husks before roasting.
- Less batch processing and more continuous processing.

To date, no disruptive modifications have been put forward on the roasting process. Conductive heat transfer (from the hot steel drum, tray, or pan) still predominates over convective (from the hot air) and over radiative heat transfer (from the superheated steel or from the burner); therefore, the process is still slow and deals with overroasted edges and under-roasted cores.

Since 1960, the coffee industry started to consider an almost exclusive convective heat transfer roasting technique: the fluidized bed.[16] Fluidized bed roasting consists in blowing a strong flow of hot air from the bottom of the roasting chamber, thus making the solid particles to constantly move, resembling so a stirring fluid.[17] Fig. 1.4 panel C shows a basic schema of this roaster. This simple, efficient, and low-carbon footprint technique[18] demonstrated a higher generation of desirable aroma compounds in coffee with respect to traditional drum roasting.[19] Nowadays, this technique is only available for small batches like those handled by small coffee companies and coffee shops. Likely, the most trusted

manufacturers have preferred to improve the open-flame drum roasters instead of scaling the fluidized bed roaster. It is probably that they found it less easy to manufacture huge chambers supplied with enough vertical high-pressurized hot air than horizontal drum roasters. Moreover, there was a perception that convection went with poor roasting results, as coffee edges did not burn.[16]

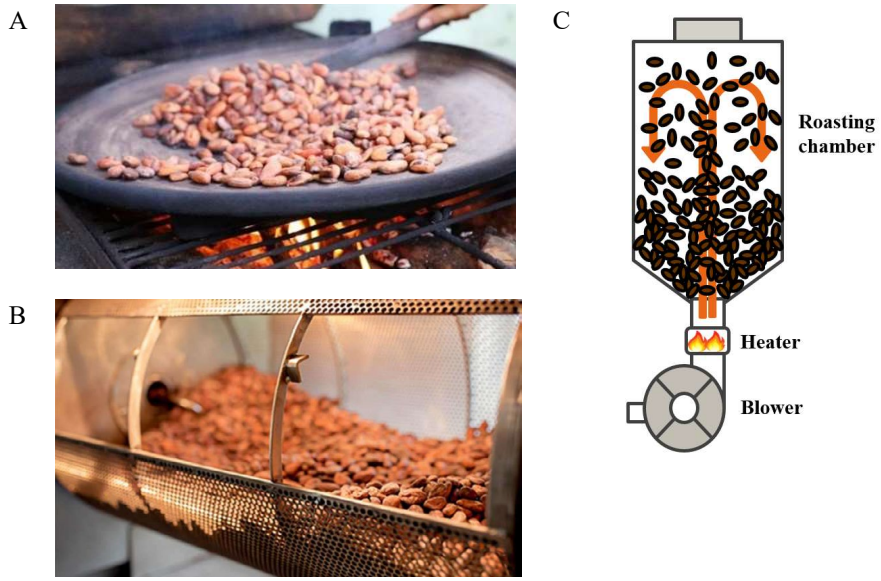

FIGURE 1.5 Cocoa roasting technologies: Ancient cocoa roasting method (Panel A) (Source: https://www.nationalgeographic.com/travel/article/anitgua-maya-chocolate-making), current drum cocoa roasting method (Panel B) Source: (https://www.chocovira.in/cocoa-bean-roasting/), and a schema of a fluidized bed roaster (C)

In this thesis, a fluidized bed roaster is re-examined for cocoa nibs processing and compared with roasting cocoa over aluminum trays inside a forced convective oven (which resembles the traditional horizontal roasting process in terms of heat transfer) from various quality points of view.

1.5 Roasting and Maillard reaction

Maillard reaction (MR) spontaneously takes place in food wherein the reactive carbonyl groups of reducing sugars react with the nucleophilic amino group of amino acids, peptides, or proteins to form a large variety of compounds. An increase in temperature leads to an increase in their reactivity.[20] Lipid-derived carbonyl compounds[21, 22] and polyphenols[23] are also involved in thermoactivated reactions producing volatile organic compounds (VOC) and melanoidins, respectively.

The Maillard reaction transforms a great variety of food and raw ingredients into palatable dishes with exquisite aromas, colors, and texture.[24] During the roasting process of cocoa, desirable VOC like pyrazines and Strecker aldehydes are produced. They are intermediate products of the MR. Pyrazines mainly display nutty, earthy, roasty, typical chocolate, potato, and green aromas, while aldehydes exert sweet and malty notes.[25, 26] Melanoidins are produced in the advanced stages of MR. They are heterogeneous high molecular weight compounds (ranging from about 30 to 70 kDa) capable of exerting brown color.[27] The brown color of melanoidins is not much appreciated in cocoa due to the deep brownness of fermented and dried cocoa nibs. Melanoidins have a negative charge, which confers functional properties like metal chelating[28] and emulsifying[29]; the latter modifies the texture of melanoidin-containing food.[24]

Although the MR improves food acceptance, it is also associated with losses of nutritive value, such as the decrease in bioavailability of the essential amino acids, as a consequence of amino acid consumption in the formation of MR products, and the reduction in mineral absorption due to the chelation properties above mentioned.[28] The cocoa roasting process also comes with other concerns, like the formation of thermal contaminants and the reduction in polyphenols. The formation of melanoidins is partially responsible for the latter.[23] Nevertheless, when polyphenols undergo condensation reactions, their bitterness and astringency are mitigated, thus improving cocoa flavor. All in all, the roasting process of cocoa undoubtedly comes with more positive changes than adverse situations, as summarized in Table 1.2. Interestingly, when a fluidized bed roaster is used, the balance is turned more in favor of the pros, as reported throughout this thesis.

TABLE 1.2 Pros and cons of the roasting process of cocoa .

Pros	Cons
• The microbial load is reduced.[8] • The husks of the nibs detach with ease.[8] • The microstructure of the nibs turns more porous (4th chapter of this thesis); consequently, the hardness of the nibs is reduced.[30] This condition facilitates further processing steps such as grinding, defatting (4th chapter of this thesis), and conching. • The chocolate aroma is fully developed[26] due to the generation of Strecker aldehydes and pyrazines via MR (3rd and 4th chapters of this thesis) and the reduction of acidic volatiles, especially acetic acid (4th chapter of this thesis) • The bitterness and astringency of cocoa nibs are reduced due to the reduction of free polyphenols.[7] • The *in vitro* digestibility and gut microbiota fermentability of water-soluble compounds > 14 kDa are significantly improved due to the formation of melanoidins (6th chapter of this thesis) • Polyphenols bound to cocoa melanoidins are released upon *in vitro* microbiota fermentation, thus improving its bioavailability.[31]	• Thermal processing contaminants such as HMF (5th chapter of this thesis) and acrylamide[32, 33] are generated. • Free polyphenols content is reduced (5th chapter of this thesis). As a consequence, the antioxidant capacity of cocoa decreases.[34]

1.6 Health effects of chocolate and cocoa

Roasted cocoa nibs contain approximately 50% fat-free cocoa powder and 50% cocoa butter. Cocoa powder concentrates the most healthy cocoa factors, such as polyphenols, which may help to prevent heart diseases,[35, 36] and melanoidins, which may improve gut health.[37] Cocoa butter mainly comprises saturated fatty acids (36% stearic acid and 26% palmitic acid), and a moiety of about 37% corresponds to unsaturated fatty acids, mainly represented by oleic acid.[38] Though it is generally considered that saturated fats adversely increase total cholesterol and low-density lipoprotein cholesterol levels, clinical studies have demonstrated that consuming cocoa products like chocolate significantly improves blood lipid profiles.[39] That benefit would be because stearic and oleic acids, both comprising about 70% of the cocoa fatty acid composition, do not have cholesterol-raising effects.[40]

The utilization of cocoa butter and cocoa powder (i.e., cocoa solids) within the diverse kinds of cocoa-based edible products diverges notoriously. The purest expression of cocoa solids in confectionary products is found in chocolate bars, followed by sweet chocolate, milk chocolate, and white chocolate bars. Their essential composition factors are regulated around the globe. Table 1.3 shows the minimum cocoa solids content for them, according to the Codex Alimentarius regulation No. 87-1981.[41] The rest of the composition corresponds to sugar, milk powder, and other minor ingredients.

Dark chocolate is typically 60%-70% cocoa solids, making it a healthier choice than sweet, milk, or white chocolate. This product is very appreciated for its antioxidant compounds, which have proven positive effects on cardiovascular health.[42] Even though regular cocoa processing significantly reduces the total amount of polyphenols (from which epicatechin and procyanidin B2 are the most abundant ones), the relative amount of these compounds that reach a bar of dark chocolate is exceptionally higher than that in other foods like apple, cranberry juice, red wine, and brewed black tea.[43]

TABLE 1.3 Cocoa solids requirements for chocolate types.

Product	Cocoa butter (%)	Cocoa powder* (%)	Total cocoa solids (%)**
Chocolate	≥18	≥14	≥35
Sweet chocolate	≥18	≥12	≥30
Milk chocolate		≥2.5	≥25
White chocolate	≥20		

* Fat-free

** Sum of cocoa butter and fat-free cocoa powder

Reference: Codex Alimentarius regulation No. 87-1981[41]

The exact mechanisms by which cocoa flavonols improve vascular function and prevent cardiovascular diseases are not clear at all. However, it has been proven that cocoa polyphenols promote the production of endothelial nitric oxide (NO),[35, 36] which is essential for the control of the vascular system. A reduction in the bioavailability of NO is associated with the development of atherosclerosis and type-2 diabetes condition.[43]

There is other promising evidence of cocoa intake: the prevention of neuronal injury,[44] skin protection,[45] positive psychoactive effects on mood and cognitive performance,[46] and gut health improvement via prebiotic mechanisms.[47] The latter is perhaps the most recently studied health effect of cocoa, in which cocoa melanoidins exert an important role.

Melanoidins are heterogenous polymers mainly formed by aldol condensations of highly reactive α-dicarbonyl compounds (a carbohydrate-based skeleton) and partially branched by amino compounds.[27] Other compounds like epicatechin, catechin, and procyanidin B2, among other phenolic compounds, have been found to be covalently bound to cocoa melanoidins.[23] Gut microbiota found melanoidins as a carbon source, as demonstrated by Pérez-Burillo & coworkers (2020)[37] via *in vitro* experiments with cocoa melanoidins extracted from commercial chocolate bars. As a result, the microbial community changed by promoting the genera of bacteria responsible for forming short-chain fatty acids (e.g., *Bifidobacterium* and *Faecalibacterium*). Moreover, bound polyphenols were released, thus making them potentially bioavailable at the level of the colon.[37]

Many details of the digestion and utilization of cocoa melanoidins by gut microbiota have not been elucidated. In Chapter 6, native high molecular weight compounds present in unroasted cocoa and cocoa melanoidins extracted from fluidized-bed-roasted and oven-roasted cocoa nibs were subjected to *in vitro* digestion and fermentation. Interestingly, the melanoidins obtained from fluidized-bed-roasted cocoa exerted the higher relative release of polyphenols and best interaction with gut microbiota. The *in vitro* batch fermentation of this study discriminated proximal and distal colon. We found that cocoa melanoidins are used by gut bacteria in both colon sections, while native high molecular weight compounds (from unroasted cocoa) are more selectively used by distal colon bacteria. Last but not least, the potential of melanoidins to release polyphenols in the upper gut was proven against plenty of studies demonstrating that food melanoidins are poorly digestible.[48-50]

1.7 Thesis outline

This thesis aimed to compare diverse physicochemical parameters of cocoa nibs roasted with a fluidized bed coffee roaster and a forced-convective oven with aluminum trays, and to evaluate the influence of cocoa melanoidins on gut microbiota communities. Fig. 1.6 shows a graphic explanation of the workflow of this thesis. The fluidized bed roasting was referred to as fast roasting (FR), and the oven roasting with trays as slow roasting (SR). Before the experiments, an investigation of the state of the art of cocoa roasting was performed. As a result, a comprehensive review was presented in Chapter 2. In Chapter 3, the thermodynamics of formation of nitrogen-heterocycles and the effective water diffusivity in cocoa nibs were calculated for FR and SR cocoa nibs.

The microstructure of cocoa nibs and the cocoa butter extractability were assessed in Chapter 4. In Chapter 5, the Maillard reaction (MR) was monitored from the reduction of the main precursors (amino acids, sugars, and polyphenols) and the formation of some MR products such as HMF, acrylamide, and melanoidins. The *in vitro* digestibility and gut microbiota fermentability of cocoa melanoidins and the effects on the microbiota profile were evaluated in Chapter 6. Lastly, a general discussion is presented in Chapter 7.

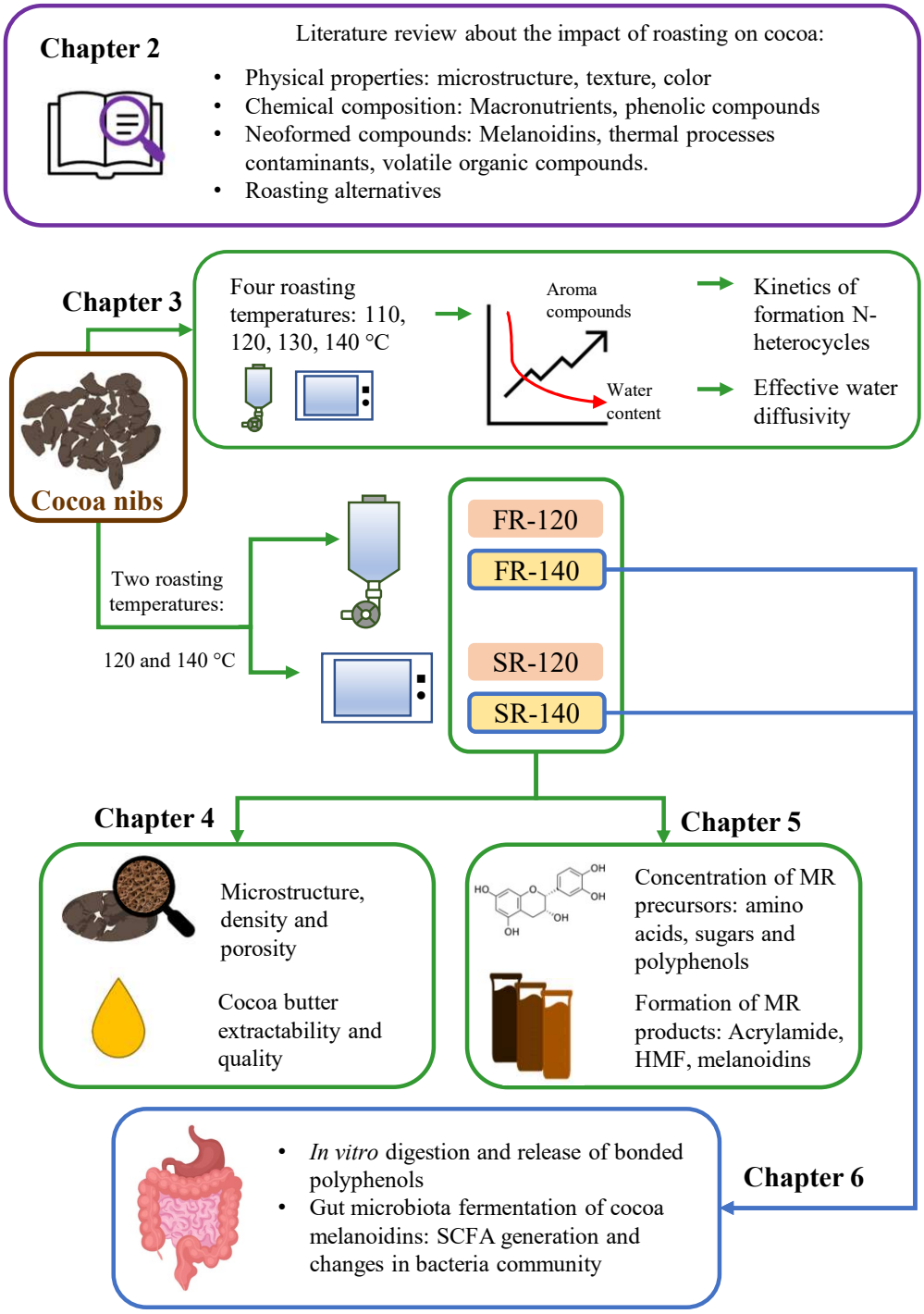

FIGURE 1.6 Schematic overview of the framework of this thesis.

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Chapter 2

The impact of roasting on cocoa quality parameters

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Abstract

Roasting is an essential process in cocoa industry involving high temperatures that causes several physicochemical and microstructural changes in cocoa beans that ensure their quality and further processability. The versatility in roasting temperatures (100 – 150 °C) has attracted the attention of researchers toward the exploration of the effects of different roasting conditions on the color, proximal composition, cocoa butter quality, concentration of thermolabile compounds, formation of odor-active volatile organic compounds, generation of melanoidins, production of thermal processes contaminants in cocoa nibs, among others. Some researchers have drowned in exploring new roasting parameters (e.g., the concentration of water steam in the roasting chamber), whilst others have adapted novel heat-transfer techniques to cocoa nibs (e.g., fluidized bed roasting and microwaves). A detailed investigation of the physicochemical phenomena occurring under different cocoa roasting scenarios is lacking. Therefore, this review provides a comprehensive analysis of the state of the art of cocoa roasting, identifies weak and mistaken points, presents research gaps, and gives recommendations to be considered for future cocoa studies.

Keywords: Proximal composition, Maillard reaction, melanoidins, polyphenols, volatile aroma compounds, cocoa butter.

2.1 Introduction

Several steps are required in processing cocoa beans (fermented and dried seeds of *Theobroma cacao* L.) before they are consumed or used as ingredients. Roasting particularly stands out due to the generation of authentic chocolate flavor via Maillard reaction (MR) (Minifie 1999). Other essential and desirable transformations that occur during roasting are the proper dehydration of the cocoa nibs, the significant reduction in microbial load, and the decrease in acidity, bitterness, and astringency (Gutiérrez 2017). Comprehensive reviews and books covering the whole cocoa processing chain are available (Rojas et al. 2022; Gutiérrez 2017; Beckett 2008). However, a detailed analysis of the physicochemical phenomena occurring during cocoa roasting process is missing.

Most of cocoa manufacturers roast cocoa beans in large rotating drum equipment, also known as Sirocco-type batch roasters, through which hot air of 110 to 150 °C is blown for 20 min to 2 h until the moisture content and water activity of cocoa beans decrease to about 1% and 0.2, respectively (Rojas et al. 2022). After that, the cocoa bean's testas (the coats of the seeds, commonly named shells or husks) are removed by winnowing (Gutiérrez 2017; Beckett 2008). Some continuous roasters are provided with a pre-heating chamber that removes the shells before roasting (Varnam and Sutherland 1994). In that unit, cocoa beans are exposed to a high temperature produced by infrared radiant heat for a short time. Consequently, water from the shells and the edges of the cotyledons (also known as cocoa nibs) quickly evaporates and presses the shells outwards; thus, shells detach from the nibs and can be easily separated (Gutiérrez 2017). Pictures and synonyms of these cocoa beans' fragments are shown in Fig. 2.1.

Essential physical, chemical, and organoleptic changes occur in cocoa nibs during roasting, for example: (i) Micropores are formed, leading to reduced hardness (Abo-Bakr and Shekib 1987; Hoskin and Dimick 1980); thus facilitating grinding processes. (ii) The concentrations of some macro (Oracz and Nebesny 2018; Zzaman et al. 2017; Redgwell, Trovato, and Curti 2003) and micronutrients (Oracz and Nebesny 2018; de Taeye et al. 2017) decrease due to volatilization or to the formation of Maillard reaction products. (iii) High molecular weight compounds such as melanoidins (Quiroz-Reyes and Fogliano 2018; Sacchetti et al. 2016; Oracz and Nebesny 2018), and low molecular weight compounds with odor activity (Tan and Kerr 2018; Mohamadi Alasti et al. 2019; Zzaman et al. 2017; Diab et al. 2014; Huang and Barringer 2011) are formed via MR. (iv) Thermal process contaminants, such as 5-hydroxymethylfurfural (HMF) (Quiroz-Reyes

and Fogliano 2018; Sacchetti et al. 2016) and acrylamide (Żyżelewicz et al. 2017; Granvogl and Schieberle 2007) are formed. Finally, (v) the acidity in cocoa butter, which is the most abundant and expensive cocoa derivative (International Cocoa Organization, 2021), is reduced.

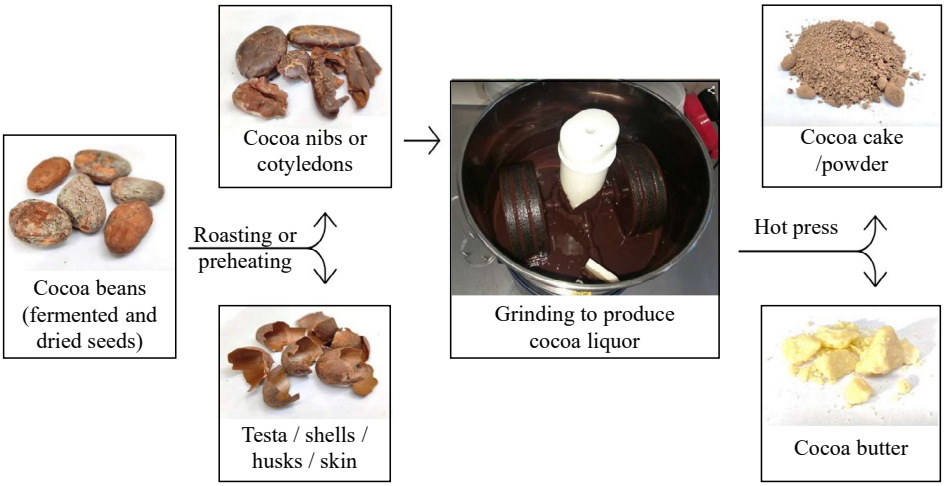

FIGURE 2.1 Obtention of the primary cocoa components.

Those valuable findings needed to be collated and compared to provide a holistic approach of the physicochemical changes of cocoa upon roasting. Thus, in this review, the current state of knowledge on the effect of different roasting conditions on microstructure, color, proximate composition, polyphenols content, formation of melanoidins, generation of thermal processes contaminants, and production of volatile organic compounds in cocoa nibs; as well as the quality of cocoa butter, are presented. Roasting alternatives and research gaps are also discussed.

2.2 Literature search

The following databases were used: WUR library search, Web of Science, Google books, Google Patents, and Google Scholar. For each section of this review, studies performed with various roasting conditions (e.g., different roasting temperatures) were highly preferred over those holding only one. Recent studies (last 10 years) were also prioritized over the older ones; nonetheless, we extended our search up to 50 years in some sections due to the abovementioned filter (more than one roasting condition).

2.3 Effect of roasting on the microstructure and texture of cocoa nibs

Typical cocoa-roasting temperatures trigger the evaporation of water and low-boiling-point organic compounds present in the food matrix. This physical phenomenon implicates the formation of large amounts of gases that generate high internal pressure. This in turn causes noticeable changes in the structure, such as breakdown of the cell walls, microporosity formation, increase in volume, and reduction in density (Massini et al. 1990) and hardness (Zzaman and Yang 2013).

A couple of papers have explored the microstructure of cocoa nibs before and after roasting. Observations of stained samples through polarized light microscopy demonstrated that drying and roasting processes cause the disruption of parenchymal cells (de Brito et al. 2000). Abo-Bakr and Shekib (1987) and Hoskin and Dimick (1980), who made a structural and topographical examination of cocoa nibs through scanning electron microscopy, found that more pores and pits were present on the surface of the cotyledons of roasted cocoa than on unroasted nibs. The typical brittleness of roasted cocoa nibs is attributed to these microscopic modifications.

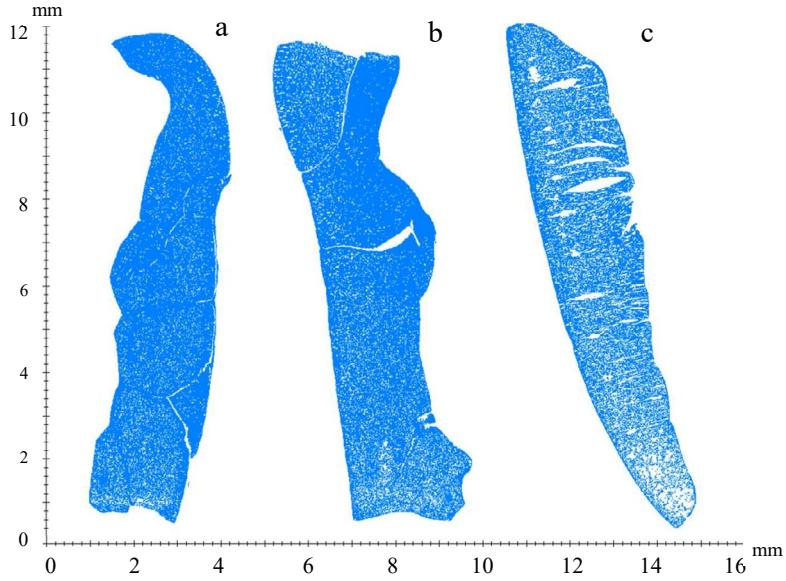

FIGURE 2.2 2-D projections of virtual cross-sections of the solid fraction obtained by X-ray tomography from (a) unroasted cocoa nibs, (b) cocoa nibs roasted in a conventional oven at 120 °C, and (c) cocoa nibs roasted in a fluidized bed roaster at 120 °C.

Despite those insights, there is still a lack of knowledge on how the main roasting parameters (i.e., temperature and time) affect the microstructure of cocoa nibs. Further studies might go beyond superficial observations by providing quantitative data on porosity and correlating it with the fat bloom phenomenon. Micro X-ray tomography technique may assist this gap since it can reconstruct the three-dimensional density distribution within an object, providing qualitative and quantitative information about the solid and the empty (or airy) sections (Maire and Withers 2014). Examples of virtual bidimensional projections of cocoa nibs obtained by X-ray tomography are presented in Fig. 2.2 (authors' unpublished data).

2.4 Effect of roasting on the color of cocoa nibs

The initial color of cocoa nibs before roasting is already deep brown. Most of their pigments are formed during fermentation and drying via complexation of amino acids and/or proteins with quinines (an enzymatic oxidation product of polyphenols) or with condensed tannins (high molecular weight product of flavonoid polymerization) via hydrogen bonding (Misnawi et al. 2003). This naturally-dyed matrix hinders visual-color analysis (Peña-Correa et al. 2022). Moreover, the water content changes upon roasting. It is well known that water affects the color perception of materials by reducing light absorption; as a consequence, materials look darker when they are wet. So, finding the sole effect of roasting on the formation of non-enzymatic brown chromophores in cocoa is challenging.

Nevertheless, numerous studies have investigated the effect of different roasting conditions on color changes of cocoa nibs. The color measurement has been done directly on the surface of the nibs (Zzaman and Yang 2013) and in different processed forms, for example: ground cocoa nibs by Zyzelewicz et al. (2014) and Sacchetti et al. (2016); cocoa liquor (cocoa nibs sufficiently minced into a smoothy pulpy mass -See Fig. 2.1-) by Nurhayati et al. (2019); cocoa powder (defatted cocoa liquor - See Fig. 2.1-) by Quiroz-Reyes and Fogliano (2018), and high molecular weight water-soluble extracts (HMW-WSE) by Quiroz-Reyes and Fogliano (2018), Oracz and Nebesny (2018), and Sacchetti et al. (2016). Most researchers used colorimeters to obtain the CIE L*a*b* color attributes regardless of the aliquot shape. Some other studies have drawn on UV/VIS-spectrophotometry or fluorescence techniques. In our recent study, we showed that computer vision-based image analysis was an effective technique for measuring color changes of cocoa nibs (Peña-Correa et al. 2022). Table 2.1 summarizes the findings of the mentioned studies.

TABLE 2.1. Changes in color attributes in cocoa beans upon roasting.

Color attribute	Sample form	Roasting technique and temperature(s)	Changes after roasting**	References
L*	Cocoa nibs	Convective oven and fluidized bed roaster. 110 - 140 °C	No difference	Peña-Correa et al. (2022)
	Cocoa nibs	Convective oven capable of changing the airflow speed and the relative humidity. 135 - 150 °C	No difference	Zyzelewicz et al. (2014)
	Cocoa nibs	Superheated steam oven. 150 - 250 °C	Significant decrease	Zzaman and Yang (2013)
	Cocoa powder	Convective oven. 130 - 150 °C	Significant decrease	Quiroz-Reyes and Fogliano (2018)
	Unfermented cocoa liquor	Oil bath roaster. 170 °C	Significant decrease	Nurhayati et al. (2019)
	HMW-WSE	Convective tunnel with humid air. 110 - 150 °C	Significant decrease	Oracz and Nebesny (2018)
a*	Cocoa nibs	Convective oven. 110 - 140 °C	No difference	Peña-Correa et al. (2022)
	Cocoa nibs	Fluidized bed roaster. 110 - 140 °C	Significant increase	Peña-Correa et al. (2022)
	Cocoa nibs	Convective oven capable of changing the airflow speed and the relative humidity. 135 - 150 °C	Significant increase	Zyzelewicz et al. (2014)
	Cocoa powder	Convective oven. 130 - 150 °C	Significant increase	Quiroz-Reyes and Fogliano (2018)
	HMW-WSE	Convective tunnel with humid air circulating. 110 - 150 °C	Significant decrease	Oracz and Nebesny (2018)
b*	Cocoa nibs	Convective oven. 110 - 140 °C	No differences	Peña-Correa et al. (2022)
	Cocoa nibs	Fluidized bed roaster. 110 - 140 °C	Significant increase	Peña-Correa et al. (2022)
	Cocoa nibs	Convective oven capable of changing the airflow speed and the relative humidity. 135 - 150 °C	Significant increase	Zyzelewicz et al. (2014)
	Cocoa powder	Convective oven. 130 - 150 °C	Significant increase	Quiroz-Reyes and Fogliano (2018)
	HMW-WSE	Convective tunnel with humid air. 110 - 150 °C	Significant decrease	Oracz and Nebesny (2018)
°Hue	Unfermented cocoa liquor	Oil bath roaster. 170 °C	Significant increase	Nurhayati et al. (2019)

** 95% confidence interval; HMW-WSE stands for High molecular weight water-soluble extract

The majority of the studies quoted in Table 2.1 agree that roasting increases a* and b* values, especially the latter. Their findings on L* value are diverse; they are influenced by the roasting temperature and sample shape: When the typical cocoa-roasting temperatures (110 – 150 °C) were applied, L* values of cocoa nibs' surface did not significantly change upon roasting. However, a darkening effect was evident when roasting between 150 °C – 250 °C, or when processing the nibs into cocoa powder, cocoa liquor, or HMW-WSE. Probably, the very high roasting temperatures removed significant amounts of water, so the interference of water with L* was mitigated. Moreover, deep-brown and black compounds were generated.

Neither the efforts in transforming cocoa nibs into other forms nor the use of a robust method like computer vision-based picture analysis seem to sharpen the color analysis of cocoa roasted under regular roasting temperatures. The native pigments seem to hinder any color change. The development of a robust sample preparation would be of great assistance.

2.5 Chemical changes and neo-formed compounds

During heating treatments of food matrixes, the MR occurs with the formation of various products of diverse molecular weights. The reactants are mainly reactive carbonyl compounds (e.g., monosaccharides and lipid-oxidation products) and amino acids. Fig. 2.3 represents the global dynamics of the main chemical reactions involved during the roasting process of cocoa. The entire pathways of the MR have not been unraveled at all. However, due to its great importance on the quality and final organoleptic characteristics of diverse thermally processed food, it has been the subject of investigation, as reviewed Ruan, Wang, and Cheng (2018), Somoza and Fogliano (2013), Echavarría, Pagán, and Ibarz (2012), and Martins, Jongen, and van Boekel (2000).

In contrast to other typically thermally-processed foods like coffee and bread, cocoa beans are particularly rich in fat and phenolic compounds, and contain little quantities of sugars and water. Moreover, the cocoa beans' roasting temperature range is not as high as for coffee roasting (180 – 280 °C) or dough baking (180 – 250 °C). Nonetheless, the MR occurs in cocoa nibs and cocoa model systems (Oliviero et al. 2009), as addressed in the coming subsections.

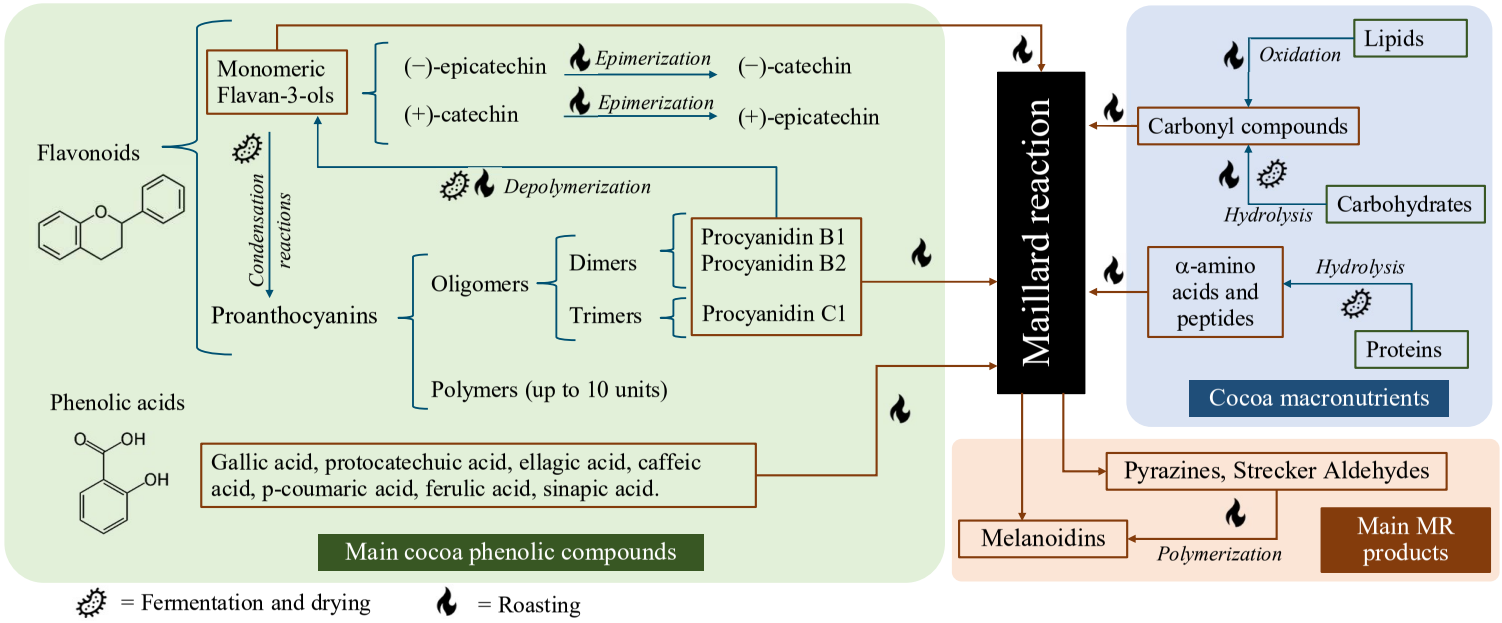

FIGURE 2.3 Classification and transformation of the main cocoa polyphenols (green box) during fermentation and roasting. Formation of aroma precursors (carbonyl compounds and amino compounds) during fermentation and roasting (blue box) and its interaction with phenolic compounds via Maillard reaction (black box) toward the formation of Maillard reaction (MR) products such as desirable volatile organic compounds (pyrazines and Strecker aldehydes) and Melanoidins (brown box).

2.5.1 The effect of roasting on the proximate composition of cocoa

The roasting process leads to two critical physical phenomena that might interfere with the interpretation of the chemical analysis if they are not carefully taken into account. They are the loss of water and the migration of lipid fractions from cocoa nibs toward the shells. The second one causes losses of as much as 0.5% of cocoa butter during deshelling (Gutiérrez 2017). Both physical phenomena might cause misinterpretations of the results of the proximal composition of cocoa when: (i) the outcomes are not expressed either in dry basis (d.b.) or in dry and defatted basis (cocoa powder); and (ii) the authors do not specify whether they removed or not the shells before analyzing their samples. The shells could account from 10 to 17% of the total cocoa bean weight (Rojo-Poveda et al. 2020). Table 2.2 summarizes the proximal composition of cocoa from various studies and includes the basis of the units according to the roasting conditions. Most of the researchers declared using deshelled beans during sample preparation; however, their results showed typical features of cocoa beans instead of cocoa nibs. Thus, the proper names of the various cocoa derivatives, as expressed in Fig. 2.1, need to be constantly adopted by scientific communities.

As summarized in Table 2.2, more than half of the macronutrients in cocoa nibs are fat. Proteins are in second place, comprising about one-fourth. The carbohydrate content of cocoa beans is less than 7 % (w/w), including a wide variety of monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Sugars account for less than 2 % (w/w). The order of predominance of sugars in fermented and unroasted cocoa nibs is not even among studies (Oracz and Nebesny 2018; Redgwell, Trovato, and Curti 2003). It seems not to be a matter of the variety; the heterogeneous fermentative conditions markedly affect the final amount of sugars in cocoa beans (Redgwell, Trovato, and Curti 2003).

When the roasting time and the roasting temperature of cocoa beans increase, the concentration of sugars is significantly reduced. Fructose is the most affected one, followed by glucose, and then sucrose (Zzaman et al. 2017; Oracz and Nebesny 2018). Fructose and glucose are reducing sugars, and they are directly involved in the reaction with an amine source, while sucrose first needs to be hydrolyzed into glucose and fructose (Rufian-Henares 2016). This explains the higher reactivity of monosaccharides. The higher decrease in fructose compared to glucose is in line with model systems experiments (Brands 2002). The hydroxyl group orientation at C3 and C4 positions of fructose may confer such a higher reactivity (van Putten et al. 2013). Concerning the oligosaccharides, they seem to be steady after roasting (Redgwell, Trovato, and Curti 2003).

TABLE 2.2 Effect of roasting in proximate composition of cocoa nibs.

Component	Content in unroasted cocoa sample	Basis for the units	Effect of roasting	References
Total carbohydrates	5.5 - 6.7%	Cocoa beans d.b.	Significantly reduced, especially at temperatures above 135 °C (by >10%)	Oracz and Nebesny (2018)
Fructose	0.70 - 0.75%	Cocoa beans d.b.	Significantly reduced, especially at temperatures above 135 °C (by 90% approx.)	Oracz and Nebesny (2018)
Glucose	0.16 - 0.22%	Cocoa beans d.b.	Significantly reduced, especially at temperatures above 135 °C (by 60% approx.)	Oracz and Nebesny (2018)
Sucrose	0.08 - 0.18%	Cocoa beans d.b.	Significantly reduced, especially at temperatures above 135 °C (by 50% approx.)	Oracz and Nebesny (2018)
	0.15 - 0.45%	Cocoa nibs*	No significant effect of roasting	Redgwell, Trovato, and Curti (2003)
Raffinose	< 0.02%	Cocoa nibs*	No significant effect of roasting	Redgwell, Trovato, and Curti (2003)
Stachyose	< 0.1%	Cocoa nibs*	No significant effect of roasting	Redgwell, Trovato, and Curti (2003)
Verbascose	< 0.01%	Cocoa nibs*	No significant effect of roasting	Redgwell, Trovato, and Curti (2003)
Crude fiber	4.0 - 4.3%	Cocoa nibs*	Increases by two folds due to melanoidins formation	Redgwell, Trovato, and Curti (2003)
Total lipids	53.2 - 54.5%	Cocoa nibs d.b.	No significant effect of roasting	Unpublished data by Ruth Fabiola Pena Correa †
Total proteins	24.4 - 24.8%	Cocoa powder d.b.	No significant effect of roasting	Unpublished data by Ruth Fabiola Pena Correa †
Total amino-terminal groups	32.6 mg/g approx.	Cocoa powder d.b.	Significantly reduced (by 32% approx.)	de Brito et al. (2000)
Total amino acid content	35.3 mg/g approx.	Cocoa powder d.b.	Significantly reduced (by 32% approx.)	de Brito et al. (2000)
	674.6 - 719.7 mg/100g	Cocoa beans*	Significantly reduced (by 26 to 55%)	Reineccius, Keeney, and Weissberger (1972)
	- -	Cocoa nibs*	Significantly reduced (by 50% approx.)	Rohan and Stewart (1966)
	16.36 g/kg	Cocoa nibs*	Significantly reduced (by 28 to 59%)	Zzaman et al. (2017)
	838.8 mg/100g	Cocoa nibs*	Significantly reduced (by 15 to 24%)	Bonvehí and Coll (2002)

*There is no information concerning the conversion of the data into dry bases (d.b)

† Cocoa nibs roasted at 120 and 140 °C for the time needed to reach a moisture content of about 1%.

In many plant-based products, the insoluble material remaining after specific chemical treatments is referred to as crude fiber and mainly corresponds to lignin. The crude fiber content increases after roasting cocoa beans (Redgwell, Trovato, and Curti 2003). Some other compounds, such as tannin/protein complexes and HMW Maillard reaction products, formed during roasting may also contribute to that moiety. The higher content of crude fiber in roasted cocoa compared with unroasted might be related to the formation of non-water-soluble melanoidins (Redgwell, Trovato, and Curti 2003).

Cocoa butter is considered a stable fat due to its fatty acid composition. Nevertheless, transformations may occur to some extent during roasting, as addressed in the section 'Cocoa butter quality.' According to the tolerance limits of some lipid-oxidation products established by Codex Alimentarius (1981), the concentrations of those products in cocoa butter do not represent significant gravimetric amounts under regular processing conditions. Thus, their formation should not significantly reduce the initial amount of cocoa butter. The authors of this review confirmed it, as observed in Table 2.2. Considering this rationale, fat losses upon roasting can be hardly attributed to the generation of neoformed lipid-derived products, as Oracz and Nebesny (2018) explained for the 3.8% fat loss. It is unclear whether the study considered the fat migration toward the shells.

Drawbacks also happen when comparing the total protein content before and after roasting. When using nitrogen-determination methods to calculate protein content by using conversion factors (e.g., Kjeldahl and Dumas), the results from unroasted cocoa *vs.* roasted cocoa should not significantly differ. In principle, roasting makes some nitrogen atoms to move from one place (e.g., proteins) to other (e.g., volatile organic compounds and melanoidins) within the same matrix, so no significant changes in 'total protein content' are obviously expected. The authors of this review confirmed it (unpublished data), as observed in Table 2.2. Nevertheless, some researchers (Oracz and Nebesny 2018; de Brito et al. 2000) reported protein reductions of 27.1 % and 37 % upon roasting, respectively. The latter used Bradford's protein assay method, which also lacks specificity.

Investigating the effect of roasting in changing the concentration of specific proteins or amino acids makes more sense than total protein content, as shown in Table 2.2. The free amino acid content in cocoa is significantly reduced by roasting time and temperature (Zzaman et al. 2017; Bonvehí and Coll 2002; de Brito et al. 2000; Reineccius, Keeney, and Weissberger 1972; Rohan and Stewart

1966), most probably due to their involvement in MR (Fig. 2.3). The percentage of reduction of total amino acids roughly ranged from 25 to 50% (Table 2.2). However, some amino acids were more affected than others: In the study of Zzaman et al. (2017), the amount of glutamic acid, tyrosine, glycine, and histidine was the most reduced, in contrast to other tested amino acids. According to the data published by de Brito et al. (2000) and Bonvehí and Coll (2002), the most affected amino acids were histidine, cysteine, and methionine.

It is worth to mention that the composition of cocoa nibs fluctuates among cocoa varieties and origins (Febrianto, Wang, and Zhu 2021); furthermore, harvesting activities, fermentation, and drying contribute to those differences.

2.5.2 Effect of roasting on polyphenols content in cocoa

The classification of the most abundant polyphenols present in the seeds of *Theobroma cacao* L. and their dynamics during roasting are shown in Fig. 2.3. Monomeric flavan-3-ols such as (-)-epicatechin and (+)-catechin, as well as the proanthocyanidins B1, B2, and C1 (abbreviated as P-B1, P-B2, and P-C1, respectively), are the most abundant classes of polyphenols identified in cocoa beans and derivatives (Melania et al. 2019; Mazor Jolić et al. 2011). With minor participation, phenolic acids such as gallic acid, protocatechuic acid, ellagic acid, caffeic acid, p-coumaric acid, ferulic acid, and sinapic acid, have been identified in cocoa beans (Oracz and Nebesny 2018).

The concentration of polyphenols differs among cocoa varieties, being that epicatechin the most predominant one, followed by P-B2, which is made of two epicatechin units. Both account for about half of the total polyphenols, according to the data supplied by Oracz and Nebesny (2018) and Quiroz-Reyes and Fogliano (2018). Lower but significant concentrations of P-B1 (made of one unit of epicatechin and one unit of catechin) and P-C1 (comprising three units of epicatechin) in cocoa beans have been reported (Oracz and Nebesny 2018)

The concentration of phytochemicals in cocoa seeds is exceptionally high: they account for 15 to 20% of the dried fat-free mass of fresh cocoa seeds. Their content usually drops to about 5% upon fermentation and drying, as poliphenols oxidize to condensed high molecular compounds, mostly insoluble tannins (Wollgast and Anklam 2000). When this reduction is above 10%, it is considered a bad fermentation (Wollgast and Anklam 2000) due to the excess bitterness and astringency that may persist in cocoa derivatives (Stark, Bareuther, and Hofmann 2005). About 50% of the remaining polyphenols are lost upon roasting (Wollgast

and Anklam 2000). Some of them become part of the melanoidins' polymeric structure (Quiroz-Reyes and Fogliano 2018; Oracz, Nebesny, and Zyzelewicz 2019), as shown in Fig. 2.3. These enzymatic and non-enzymatic transformations of polyphenols have been negatively perceived due to the imminent reduction in antioxidant capacity of cocoa (Urbánska and Kowalska 2019). However, thanks to these transformations, the organoleptic characteristics of cocoa are considerably improved (Stark, Bareuther, and Hofmann 2005).

Various researchers have evaluated or reviewed the changes in concentration of cocoa polyphenols from postharvest until the preparation of chocolate bars (Gil et al. 2021; Urbańska et al. 2019; Bordiga et al. 2015; Payne et al. 2010; Wollgast and Anklam 2000). So, this section focuses on the specific changes by different roasting conditions.

During heating treatments, proanthocyanidins experiment depolymerization through the cleavage of the interflavanic $(4\beta \rightarrow 8)$ linkages; consequently, monomeric-flavan-3-ols are released (Fig. 2.3) (de Taeye et al. 2014). Simultaneously, (-)-epicatechin and (+)-catechin undergo epimerization reactions to produce (-)-catechin and (+)-epicatechin, respectively. The epimerization of (+)-catechin into (+)-epicatechin is usually less favored (de Taeye et al. 2014). These changes mainly result in: (i) the significant reduction of the most predominant phenolic compounds, namely epicatechin and P-B2; and (ii) a significant increase of (-)-catechin. The higher the roasting temperature, the more pronounced the changes (Stanley et al. 2018; Quiroz-Reyes and Fogliano 2018; Żyżelewicz et al. 2016; Kothe, Zimmermann, and Galensa 2013). Payne et al. (2010) proposed calculating the epicatechin/catechin ratio as an indicator for the processing history of cocoa beans. However, other studies have reported other dynamics in catechin by roasting. Catechin content increases during the first 15 min, and then decreases to original or lower concentrations (Żyżelewicz et al. 2016); or just decreases (Oracz and Nebesny 2018; Ioannone et al. 2015). Changes in the concentration of catechin are determined by cocoa variety (de Taeye et al. 2017).

The fate of the missed cocoa polyphenols upon roasting has been scarcely studied. As shown in Fig. 2.3. they are involved in the formation of soluble (Oracz, Nebesny, and Zyzelewicz 2019; Quiroz-Reyes and Fogliano 2018) and insoluble (Fogliano et al. 2011) melanoidins via MR, mitigating so the bitterness and astringency. However, the extent of participation of free phenolic compounds in that chemistry is unknown. More detailed information about the kind of polyphenols bound to cocoa melanoidins is given in the following section.

2.5.3 Melanoidins in cocoa as an advanced MR product

Cocoa melanoidins are high molecular weight heterogeneous compounds with brown-chromophoric properties that are formed in the advanced stages of the Maillard reaction. As shown in Fig. 2.3, they result from the interaction of the amino groups of peptides and free amino acids with carbonyl compounds and polyphenols. Melanoidins can be either soluble or insoluble. The insoluble fraction has been scarcely studied, while the soluble one has drained most of the attention.

Different techniques have been presented to estimate the formation of soluble cocoa melanoidins. Quiroz-Reyes and Fogliano (2018) and Oracz and Nebesny (2018) weighed the dry fraction of high molecular weight water-soluble extracts (HMW-WSE) obtained by ultrafiltration (Quiroz-Reyes and Fogliano 2018) and dialysis (Oracz and Nebesny 2018). Sacchetti et al. (2016) also used dialysis to separate HMW-WSE. They determined cocoa melanoidins via fluorescence detection using quinine sulfate as standard. Different from Quiroz-Reyes and Fogliano (2018) and Oracz and Nebesny (2018), the extraction was not carried out by using 100% of polar solvent; a mix of solvents with a predominant presence of acetone was used instead. With this in turn, Sacchetti et al. (2016) demonstrated that the formation of melanoidins in cocoa beans depends on the roasting temperature and increases exponentially at temperatures above 135 °C. In contrast, the gravimetric method leads to some divergences between studies: On the one hand, Quiroz-Reyes and Fogliano (2018) found that the dry mass of the HMW-WSE (> 20 kDa) of unroasted cocoa was around 6.4 – 7.7% of the cocoa powder. Roasting increased it to about two folds; however, the roasting temperature did not lead to any trend. On the other hand, Oracz and Nebesny (2018) found 12.9 - 14.3% w/w of HMW-WSE (> 12.4 kDa) in cocoa powders of unroasted cocoa. The roasting experiments performed at 110 - 120 °C slightly reduced that yield, while the highest temperatures (135 – 150 °C) did not significantly change it. Differences between research may be explained by the composition of the samples themselves, as well as the roasting conditions and sample preparations.

Estimating the formation of melanoidins by fluorescent or gravimetric methods is still out of focus. One reason is that fluorescent methods are specific to the standard being used; therefore, other neo-formed compounds might be underestimated. Another point to be considered is that gravimetric methods retain everything above the molecular weight cut-off of the membrane used. Consequently, other high molecular weight compounds, such as proteins,

polysaccharides, and tannins, are retained in the HMW fraction, which might lead to overestimating the amount of melanoidins.

The exploration of the physical and chemical properties of cocoa melanoidins, as studied by Oracz and Nebesny (2018), Oracz, Nebesny, and Zyzelewicz (2019), Quiroz-Reyes and Fogliano (2018), and Summa et al. (2008) might assist in designing a more accurate quantification method. These are their laudable contributions:

- The molecular weight distribution of the HMW-WSE above 12.4 kDa obtained from unroasted cocoa differs from that of roasted cocoa: in unroasted cocoa, the weight of these compounds was almost equally distributed between 12.4 and 150 kDa, while in roasted cocoa, there was a Gaussian bell distribution that peaked at about 50 kDa. The peak height increased when the roasting temperature increased; thus, it could probably correspond to the formation of cocoa melanoidins (Oracz & Nebesny, 2019). Isolating a narrower fraction of the HMW-WSE, perhaps between 30 and 70 kDa, may sharpen cocoa melanoidis' analysis.
- Aligned results were presented by Quiroz-Reyes and Fogliano (2018). The water-soluble compounds were extracted from unroasted and roasted cocoa by consecutive ultrafiltration through membranes of 20, 10, and 2 kDa cut-off. Gravimetric measurement of the fraction indicated that the fraction containing compounds above 20 kDa represented about 65 to 85% of the total HMW-WSE, while the fractions of 20 to 10 and 10 to 2 kDa hold about 10 to 20% each.
- According to the data presented by Quiroz-Reyes and Fogliano (2018), 50% of the antioxidant activity of the whole water-soluble extract of unroasted cocoa relies on compounds above 20 kDa. In roasted cocoa, this fraction was responsible for 60 to 80% of the antioxidant activity. The evident increment in the antioxidant activity upon roasting suggested the effective incorporation of phenolic compounds in cocoa melanoidins (Quiroz-Reyes and Fogliano 2018).
- The HMW-WSE of unroasted cocoa is mainly composed of proteins (15 to 20% w/w), carbohydrates (8 to 16% w/w), and water (≈5 %) (Oracz and Nebesny 2018), and a fraction of at least 0.2% w/w d.b. correspond to phenolic compounds according to the data published by (Oracz, Nebesny, and Zyzelewicz 2019). So far, more than half of the composition of cocoa melanoidins is unknown. Nevertheless, the current information is enough to conclude that cocoa melanoidins may differ from other roasted products

like coffee beans: Extracts containing coffee melanoidins have more carbohydrates (40 - 60% w/w) and a wider range of protein content (2.3 - 28% w/w), according to the data published by Coelho et al. (2014).

- Roasting slightly decreases the protein content in cocoa HMW-WSE, significantly reduces their carbohydrate content (Oracz & Nebesny, 2019), and significantly increases the polyphenols content. Interestingly, the increment in bound phenolic compounds increases when roasting temperature increases (Oracz, Nebesny, and Zyzelewicz 2019). This rise is also aligned with the antioxidant activity mentioned above.
- The aforementioned phenolic compounds present in HMW-WSE of cocoa diverge between cocoa varieties and polyphenols' hydrolysis methods (Oracz, Nebesny, and Zyzelewicz 2019). The acid hydrolysis released more phenolic compounds from Criollo's HMW-WSE than alkaline hydrolysis, while HMW-WSE of Forastero beans showed the opposite trend.
- The most predominant phenolic compound released upon alkaline hydrolysis of Forastero's and Criollo's HMW-WSE was epicatechin (60 % approx.), followed by catechin (20% approx.). The acid hydrolysis revealed catechin (45 % approx.) as the most abundant polyphenol bound to HMW-WSE, followed by epicatechin (20 % approx.). The rest of the polyphenols in both hydrolysis methods corresponded to P-B2, gallic acid, ellagic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, and protocatechuic acid (Oracz, Nebesny, and Zyzelewicz 2019). Phenolic compounds could be linked to diverse matrix components through either ester, ether, or acetal bonds. This diversity makes it difficult the development of one definitive method for hydrolysis (Robbins 2003).

2.5.4 Thermal processes contaminants formed by the MR during roasting

Heating treatments like roasting have food safety concerns caused by some neoformed compounds like HMF and acrylamide. There are many doubts about HMF's health effects on humans. In rats, the LD50 is between 2.5 and 5.0 g/kg. Those values are away from the estimated dietary HMF intake of 1.6 mg/person, as Capuano and Fogliano (2011) thoroughly reviewed. Nevertheless, the major concern for HMF is related to its *in vivo* conversion to 5-sulfoxymethyfurfural (Surh et al. 1994). Acrylamide is a well-known carcinogenic and neurotoxic molecule (International Agency for Research on Cancer 1994). Similar to HMF, the toxicological levels of acrylamide estimated in rats and mice ranged far above dietary exposure (World Health Organization 2006). Although the long-term consequences of the dietary intake of those compounds are still under

investigation, several strategies for their mitigation have been put forward (Kolek, Simko, and Simon 2006; Claeys, De Vleeschouwer, and Hendrickx 2005).

Both HMF and acrylamide can be formed by MR in foods during thermal treatment. HMF can also be formed via sugar dehydration. The amino acid asparagine forms the backbone of acrylamide. Free asparagine and reducing sugars are needed to form acrylamide via MR. Consequently, both thermal process contaminants mainly occur in carbohydrate-rich products (Capuano and Fogliano 2011). As shown in Table 2.2, this is not the situation of cocoa nibs; nevertheless, limited formation of these contaminants in cocoa ranging from 15 to 75 μg HMF/g cocoa powder (Quiroz-Reyes and Fogliano 2018), 1.2 to 7.3 nmol HMF/g cocoa beans (Sacchetti et al. 2016), 222 to 922 μg acrylamide/kg cocoa beans (Granvogl and Schieberle 2007), and 20 to 270 μg acrylamide/kg cocoa beans (Żyżelewicz et al. 2017), has been reported. The analysis of acrylamide in various cocoa-based products, including unroasted cocoa beans, roasted cocoa nibs, cocoa butter, cocoa powder, and several end-consumer chocolate products, demonstrated that the level of acrylamide is below the general German signal value of 1000 μg/ kg (Raters and Matissek 2018). A similar analysis for HMF would provide valuable information for cocoa manufacturers and customers.

HMF formation could vary within cocoa varieties, being Criollo more prone to produce HMF than Forastero (Quiroz-Reyes and Fogliano 2018). The generation of this compound in cocoa increases exponentially at temperatures above 135 °C (Sacchetti et al. 2016); however, at an equal moisture content of 1.9% w/w (almost the end of roasting), the concentration of HMF in cocoa beans roasted at the highest roasting temperatures led to the lowest HMF formation. It means that not only the roasting temperature but also the roasting time plays an essential role in HMF formation, as the lower temperatures demand longer roasting times.

Even though acrylamide formation is highly favored under thermal processes, this compound was detected in unfermented and fermented cocoa beans (Granvogl and Schieberle 2007). When roasting both the unfermented and the fermented beans, the acrylamide content significantly rose, especially when roasting the fermented beans. The results suggest that fermentation generates precursors and creates a favored environment for acrylamide formation (Granvogl and Schieberle 2007). Żyżelewicz et al. (2017) did not find acrylamide in unroasted cocoa, but in line with Granvogl and Schieberle (2007), they observed a sharp increase of this compound upon roasting.

2.5.5 Volatile organic compounds

The fermentation process holds diverse enzymatic-catalyzed reactions within the freshly harvested seeds of cocoa, such as proteolysis, hydrolysis of carbohydrates (Santander Munoz et al. 2020), and lipolysis (Afoakwa et al. 2014). As a result, amino acids, monosaccharides, and free fatty acids, among other compounds, are produced (Santander Munoz et al. 2020), as briefly shown in Fig. 2.3. For several years, the first two have been referred to as 'aroma precursors' because of their proven participation in the formation of chocolate flavor via MR. Recently, lipid-derived carbonyl compounds were found to participate in that chemistry (Zamora, Lavado-Tena, and Hidalgo 2020; Hidalgo and Zamora 2019); therefore, they also deserve to be called aroma precursors.

More than 600 volatile organic compounds (VOC), including organic acids, alcohols, aldehydes, esters, fatty acids, furans, hydrocarbons, ketones, phenols, pyrazines, pyrroles, and sulfur compounds, have been identified along with cocoa processing. The flavor chemistry in cocoa processing has been reviewed by a number of authors (Rojas et al. 2022; Santander Munoz et al. 2020; Aprotosoaie, Luca, and Miron 2016; Afoakwa et al. 2008).

During cocoa roasting, two relevant phenomena in the matter of VOC profile occur: (i) the amount of acetic acid is significantly reduced. It is the highest odor-active compound in unroasted cocoa beans and is responsible for the typical vinegar-like off-flavor (Michel et al. 2021). (ii) Several nitrogen-heterocycles (e.g., pyrazines) and Strecker Aldehydes are produced in the intermediate stages of the Maillard reaction. They display nutty, earthy, roasty, typical chocolate, potato, and green aromas (Owusu, Petersen, and Heimdal 2012; Michel et al. 2021). Both phenomena lead to improved chocolate flavor.

The formation of pyrazines (the main class of nitrogen-heterocyclics), especially 2-ethyl-5-methylpyrazine, is temperature-dependent under typical roasting temperatures, as demonstrated in our recent study (Peña-Correa et al. 2022). However, when roasting at temperatures above 150 °C, their concentration peaks at intermediate-roasting times, and then drops through the final times of roasting (Huang and Barringer 2011; Zzaman et al. 2017). That decrease might correspond to the contribution of those valuable aroma compounds in forming advanced Maillard reaction products like melanoidins.

Different roasting techniques change the VOC profile of the cocoa beans. Our recent study (Peña-Correa et al. 2022) compared the formation of nitrogen-heterocyclics under two roasting techniques, fluidized bed roasting (FBR) and

convective oven roasting (CO), at four roasting temperatures (110 – 140 °C). By using the data of the final roasting time (published in the supplementary material of that study), we elaborated Fig. 2.4. The nitrogen-heterocyclics are positively correlated with FBR cocoa, especially with the highest roasting temperature (FBR-140), meaning that this roasting condition highly favors the formation of pyrazines (Fig. 2.4). The aldehydes were correlated to the highest roasting temperatures of convective-oven-roasted cocoa nibs, while acetic acid was correlated with the lower roasting temperatures of FBR.

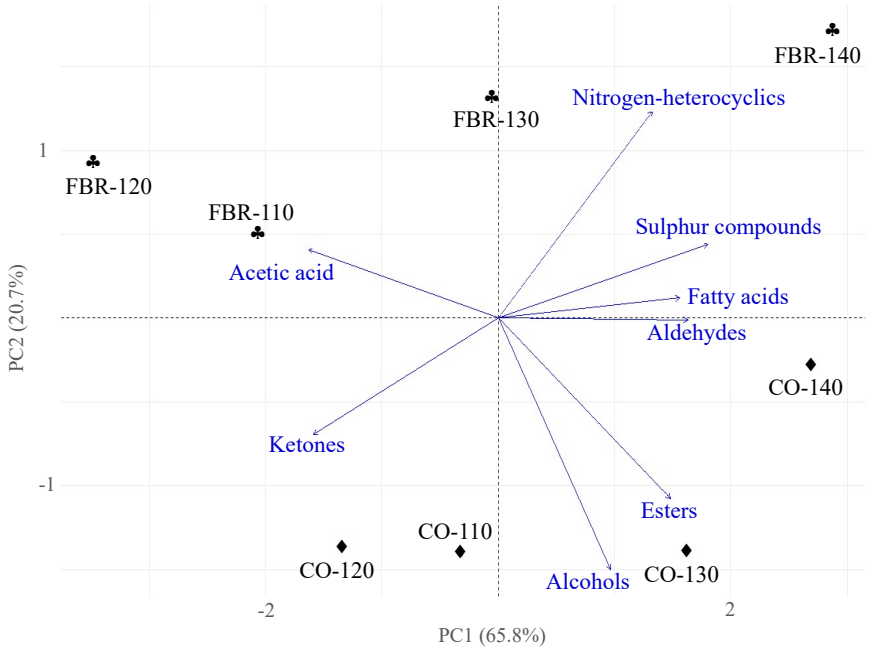

FIGURE 2.4 PCA of the volatile organic compounds grouped by chemical groups present in cocoa beans roasted at four different temperatures (110, 120, 130, and 140 °C) in two different equipment: a fluidized bed roaster (FRB) and a convective oven (CO). The data correspond to the final roasting times. At those points, the water content ranged from 1 to 2% w/w. Source of the data: Peña-Correa et al. (2022)

The differences between VOC profile of cocoa nibs roasted under different techniques are probably due to the final water activity. The a_w value of FBR roasted cocoa nibs is close to 0.3, whilst cocoa nibs roasted in CO had an a_w close to 0.2. The formation of pyrazines peaks at a_w values nearby 0.3, and is strongly reduced when approaching to 0.2 or 0.38, as confirmed by Scalone et al. (2015) in a model system. It is well known that the performance of chemical reactions depends on the water activity of the matrix, and this factor has been poorly considered in understanding the chemistry behind cocoa roasting.

With these findings, the scientific community and cocoa manufacturers may consider pursuing an a_w of 0.3 in cocoa nibs to intensify the chocolate aroma by naturally increasing nitrogen heterocyclics without carrying burnt flavors (Peña-Correa et al. 2022).

2.6 Cocoa butter quality

Even though cocoa butter is the largest and most expensive fraction of cocoa nibs, few studies focus on the effect of roasting on the quality of cocoa butter. It might be because: (i) cocoa butter mainly consists of saturated fatty acids; thus conferring high thermal stability (Żyżelewicz et al. 2014; Oracz, Nebesny, and Żyżelewicz 2014). (ii) No much primary-oxidation products (e.g., hydroperoxides) are expected during roasting, as the solubility of oxygen is limited at high temperatures (Velasco and Dobarganes 2002); stable secondary-oxidation products such as polymeric compounds and VOC are favored instead (Velasco and Dobarganes 2002; Tena et al. 2019; Carlinl et al. 1985).

The hydrolysis of triacylglycerols into free fatty acids (FFA), diacylglycerols, and monoacylglycerols in cocoa beans mainly occur during postharvest and fermentation via enzymatic catalyzed reactions (Afoakwa et al. 2014). At such low-moderate temperatures, the solubility of oxygen is high; consequently, the lipid-hydrolysis products (especially the FAA) easily interact with oxygen to produce hydroperoxides (Velasco and Dobarganes 2002). This phenomenon explains the initial load of FFA and the peroxide value (PV) in fermented and dried cocoa beans. As expected, roasting at conventional temperatures like 120 °C (Afoakwa et al. 2014) and 135 °C (Żyżelewicz et al. 2014) leads to a marginal effect on FFA and PV content. However, the stability of cocoa starts to fade at 150 °C (Żyżelewicz et al. 2014); and at 180 °C, the increase in FFA and PV is significantly evident (Djikeng et al. 2018).

The changes in the quality of cocoa butter upon roasting at regular temperatures can be better assessed through the analysis of their VOC. Hashim, Hudiyono, and Chaveron (1997) intentionally oxidized cocoa butter and found relevant changes in the VOC profile: They reported a significant increase in the aldehydes hexanal, heptanal, octanal, nonanal, and decanal. Those aldehydes are oxidative decomposition products formed from free fatty acids (Grebenteuch et al. 2021). An extension of this study involving different roasting temperatures and/or some mathematical modeling might provide valuable tools for cocoa butter producers to understand and predict the limits of this 'stable' fat.

It is worth mentioning that solvent extraction is the most common method used in the lab to obtain cocoa butter; however, this is not the method used in cocoa industry. A horizontal press is mainly used to extract the butter from cocoa liquor. In principle, hot cocoa liquor is introduced into a container where pressing is carried out by applying a sequence of increasing pressures that leaks out the melted fat from the rest of the cocoa (the cocoa cake, see Fig. 2.1) (Gutiérrez 2017). The technological parameters of this treatment may affect the quality of cocoa butter; however, this step has not been deeply considered by scientific community.

2.7 Conventional roasting and roasting alternatives

As mentioned in the introduction of this review, the roasting process in cocoa industry mainly starts from the whole cocoa beans, which are thermally processed in continuous drum roasters for long periods ranging from 20 min to 2 hours (Gutiérrez 2017). Some modifications of the conventional roasting process have been proposed either to improve efficiency or to preserve thermolabile compounds. Table 2.3 gathers those valuable studies.

Even though it is well known that heat transfer is more efficient when the particle size is reduced to cocoa mass (Goerling and Ernst-Zuercher 1975; Schmitt and Birkeneck 1983) or cocoa nibs (Peña-Correa et al. 2022), many cocoa manufacturers and scientists still practice whole-cocoa bean roasting because it makes shells-winnowing easier, and pre-roasting equipment is not needed. Moreover, large companies deal with tons of cocoa per day; thus, continuous processes are more convenient than batch processes. These considerations explain why fast roasting processes such as thin-layer roasting (Table 2.3, entries 1 and 2) and fluidized bed roasting (Table 2.3, entry 3) are not popular, although they were invented more than 40 years ago. We recently demonstrated that fluidizing bed roasting boosted the formation of pyrazines (chocolate-aroma compounds) in cocoa nibs without carrying the formation of typical over-roasting off-flavors (Peña-Correa et al. 2022); thus, this technique deserves reconsideration.

The injection of superheated steam during convective oven roasting preserves some lipid-soluble antioxidant compounds in cocoa butter like the tocopherol isomers α, γ, and δ, and the phytosterols β-sitosterol, stigmasterol, campesterol, and Δ^5-avenasterol (Table 2.3, entry 4). The significant losses of those lipophilic phytochemicals were detected from the very mild roasting temperature of 110 °C. The losses were higher when increasing the temperature. The degradation of

tocopherols and phytosterols was less advanced when roasting under the relative humidity of 5% than during dry roasting (Oracz, Nebesny, and Zyzelewicz 2014). The researchers claimed that the formation of a steam barrier around the beans protects them from contact with oxygen.

The protective effect of water steam on tocopherols and phytosterols seems not to work for the most appreciated phytochemicals in cocoa, the hydrophilic ones: epicatechin, epigallocatechin, procyanidins B1, B2, B5, and C1 (Table 2.3, entry 5). In the study presented by Oracz and Nebesny (2018), all the flavan-3-ols decreased significantly after thermal treatment, with or without steam inside the roasting chamber. Catechin was the only flavan-3-ol that increased upon roasting; however, its occurrence was not affected by the hot steam. Furthermore, the presence of steam slowed the roasting process, making this technique less attractive for further industrial scaling.

Infrared radiation is a common alternative in food processing (Aboud et al. 2019). However, its application in cocoa has been limited to the deshelling process. It might be due to the low wavelength of the infrared spectrum, which is not enough to penetrate the center of the kernels; therefore, it only causes vibrating movements of the surface-located water molecules (Aboud et al. 2019). Radiation energy with higher wavelengths, e.g., microwaves (MW), gets better penetration to the inside of solid foods. Only one study was found to use MW in the roasting process of cocoa (Table 2.3, entry 6). The study of Krysiak (2011) only included one MW roasting condition (700 W, 12.5 min), which resulted in significant reductions of total acidity and volatile acidity in cocoa nibs, and fewer losses of cocoa butter towards the shells, in contrast to convective-roasted cocoa. The FFA, SV, PV, refractive index, and viscosity of the cocoa butter obtained from MW- and convective-roasted beans were not significantly different. Only the IV showed lower values for MW-roasted beans, which was reported to be probably due to the degradation of triacylglycerols.

Krysiak (2011) provided important insights into the use of microwaves in the cocoa roasting process. However, only one power condition was tested among the broad microwave spectrum. Pramudita et al. (2017) roasted peanuts by MW with different power levels. They found that the moisture loss, the browning, and the formation of micropores on the surface of the peanuts tended to increase when increasing MW-power. In addition, they demonstrated a shorter roasting time compared with drum roasting. A similar study with cocoa beans or nibs would provide valuable information for the use of MW as alternative roasting technique for cocoa.

TABLE 2.3 Investigations on roasting alternatives for cocoa.

Entry	Roasting techniques and modifications	Objective	Pros	Cons	References
1	Roasting a thin layer of cocoa liquor under atmospheric or reduced pressure in a conductive-heat transfer cylinder. The roaster is provided with a gaseous flow of air or water.	To improve roasting efficiency. To remove the non-pleasant volatile compounds.	Short roasting time (0.5 to 12 min). This process might reduce or substitute the conching process.	Extra activities required prior roasting: deshelling and grinding.	US Patent number 3904777 (Goerling and Ernst-Zuercher 1975)
2	Roasting a thin layer of cocoa liquor. There are two heating sources: firstly, heat is generated in the paste by friction; then, the paste is lifted out of the layer and sprayed with hot air.	To improve roasting efficiency. To remove the non-pleasant volatile compounds.	Short roasting time (1 to 5 min.).	Extra activities required prior roasting: deshelling and grinding.	US Patent number 4389427 (Schmitt and Birkeneck 1983)
3	Fluidized bed roaster.	To improve roasting efficiency. To improve chocolate flavor.	Roasting time no longer than 4 min (16 times faster than oven roasting). Known as a low-carbon footprint method. Higher production of pyrazines in contrast to oven-roasted cocoa.	Cocoa beans need to be deshelled. Then cocoa nibs have to be cracked into a proper size.	Peña-Correa et al. (2022)
4	A combination of superheated steam with convective oven roasting to raise the chamber's temperature because of the high heat transfer capability of water steam.	To preserve tocopherols, phytosterols, and the antioxidant capacity of the lipid fraction of cocoa beans.	The humid air in the roasting chamber helped to preserve more tocopherols and phytosterols. The loss of cocoa butter migrating toward the shells was reduced.	The roasting time was longer when steam was present in the roasting chamber. Therefore, it might need higher energy.	Oracz, Nebesny, and Zyzelewicz (2014)

Entry	Roasting techniques and modifications	Objective	Pros	Cons	References
5	A combination of superheated steam with convective oven roasting to raise the chamber's temperature because of the high heat transfer capability of water steam.	To test the applicability of the new technology to cocoa beans.	The presence of steam induced similar changes in proximal composition, and similar features in reduction of flavan-3-ols in cocoa beans, as roasting with a dry-atmosphere.	The roasting time was longer when steam was present in the roasting chamber. Therefore, it might need higher energy.	Oracz and Nebesny (2018)
6	Microwaves (MW)	To test the applicability of the new technology to cocoa beans.	Short roasting time (12.5 min). About three times faster than oven roasting. Significant reduction of total acidity and volatile acidity in cocoa nibs. Fewer losses of cocoa butter toward the shells. MW holds the capability of instantaneously applying and removing the heat source.	This technique alone is expensive, and results in the formation of hot spots in some food products. In order to mitigate the costs and the lack of temperature uniformity, this technique might be combined with other conventional heating methods (e.g., hot air, infrared, vacuum).	Krysiak (2011) and Awuah, Ramaswamy, and Tang (2014)

2.8 Recommendations for future research

There are numerous studies in the literature providing insight into cocoa roasting from different aspects. This review aimed to critically evaluate these studies, draw attention to the important points or limitations, and provide recommendations for future work. The first recommendation would be that scientists should make proper use of the words 'beans' and 'nibs.' Despite the differences between them (as described in this review), there are many imprecisions in the published papers.

To properly compare the lab outcomes to real industrial situations, it is necessary to conduct experiments toward an equal water content of cocoa beans/nibs below 2%. This limit is optimal for further processing, like grinding and fat extraction. Values above 2% are considered incomplete roasting. Water excess in cocoa nibs causes rheological problems in cocoa liquor, affecting its flowability. Moreover, when preparing a chocolate mass, a water content higher than 2% causes difficulties in the dispersion of solid ingredients such as sugar and milk powder into the continuous cocoa butter phase.

A considerable amount of literature has published lab-scale investigations, while few research have reported the results of industrial or pilot-scale experiments. This gap might be due to the need to protect industrial know-how. However, scaling lab results up to industrial levels is very important for properly using the generated knowledge.

As reported in this review, the physicochemical properties of cocoa are more affected by roasting time than by roasting temperature. Novel and alternative cocoa roasting techniques should rather focus on accelerating this process. So far, this goal has been achieved by microwaves, fluidized bed roasting, and thin-layer roasting techniques. Finally, energy efficiency and sustainability should also be considered when a new roasting technique is implemented in cocoa processing.

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Chapter 3

Fluidized bed roasting of cocoa nibs speeds up processing and favors the formation of pyrazines

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Abstract

Roasting is an important step in cocoa processing that causes water loss and generates volatile compounds responsible for chocolate aroma like nitrogen-heterocycles. This study compares the roasting process of cocoa nibs in an oven with aluminum trays and in a fluidized bed roaster in terms of effective water diffusivity (De) and activation energies of formation (*Ea*) of nitrogen-heterocycles. Fluidized bed roasting, recognized for its energy efficiency and low-footprint synthesis, was 16 times faster than oven roasting. The order of magnitude of De in fluidized-bed-roasted nibs was -8, while it was -9 in the oven-roasted nibs. Moreover, the a_w was 50% higher in fluidized-bed-roasted nibs than in the oven-roasted ones. The *Ea* of nitrogen-heterocycles ranged roughly between 40 and 80 kJ/mol. Those values were lower under fluidized bed roasting than under oven roasting. The more effortless water mobility within fluidized-bed-roasted cocoa demanded lower *Ea*, and favored the formation of nitrogen-heterocyclics.

Industrial relevance

This study can inspire cocoa manufacturers and equipment designers to pursue the formation of nitrogen-heterocycles during the roasting process of cocoa. It can be done either by adapting and scaling the current fluidized bed coffee roasters to cocoa beans or nibs; or by exploring other alternatives capable of leading to enough water diffusivity and water activity in the cocoa nibs, as reported in this study. These physicochemical conditions undoubtedly boosted the formation of volatile compounds responsible for chocolate aroma, e.g., the pyrazines, without carrying the formation of typical-burn volatile compounds. This natural way of favoring the generation of pyrazines in cocoa nibs could contribute to clean labels by reducing or avoiding the subsequent use of flavorings. The implementation of efficient heat-transfer techniques during roasting, e.g., fluidized bed roasting, could reduce the processing cost and improve sustainability. Studies in sensory profile and energy consumption/conversion can be achieved in future research.

Keywords: Kinetic, Water Diffusivity, Water activity, Activation Energy, Color

3.1 Introduction

The fermented and dried seeds of *Theobroma Cacao* L., commonly known as cocoa beans, are a commodity of huge economic significance as they are the primary raw material for chocolate manufacturing. The cotyledons, also known as cocoa nibs, constitute the edible fraction of cocoa beans. They are encapsulated by testas, commonly called husks. Husks are removed either immediately before or after roasting. The first option involves an additional step aimed to heat the surface of the cocoa beans (Gutiérrez, 2017).

Roasting is an important step that reduces the water content in cocoa nibs and produces desirable aromatic compounds (Rojas, Hommes, Heeres, & Chejne, 2022). Cocoa nibs are mainly roasted in large drum equipment at 110 to 150°C ranging from 20 minutes to 2 hours until the water content and water activity decrease to about 1% and 0.2, respectively (Rojas et al., 2022). This process causes a series of non-enzymatic chemical reactions, well known as the Maillard reaction. They mainly involve reducing sugars and amino compounds to produce compounds of different molecular weights (Hodge, 1953; Martins, Jongen, & van Boekel, 2000). Some of the low-molecular-weight ones are volatile organic compounds (VOC). They are typically produced in the intermediate steps of the Maillard reaction, while the high-molecular-weight ones, known as melanoidins, are formed in the advanced stages. They exert brown color, and contribute to the texture and the antioxidant capacity of cocoa nibs (Aprotosoaie, Luca, & Miron, 2016).

Hundreds of VOC formed during roasting have been identified in cocoa beans of different origins (Diab, Hertz-Schünemann, Streibel, & Zimmermann, 2014; Marseglia, Musci, Rinaldi, Palla, & Caligiani, 2020; Mohamadi Alasti, Asefi, Maleki, & SeiiedlouHeris, 2019; Tan & Kerr, 2018) including nitrogen heterocycles like pyrazines, pyridines, and pyrimidines. Pyrazines, more specifically alkylpyrazines with different substituents (methyl, ethyl, propyl), are the main class of heterocyclic volatiles and the key-odor components in cocoa derivatives by displaying cocoa, nutty, sweet, potato, coffee, chocolate, roasty, and earthy notes (Michel, Franco-Baraka, Ibañez, & Mansurova, 2021). The formation of pyrazines is enhanced with a water activity of 0.33 (Scalone, Cucu, De Kimpe, & De Meulenaer, 2015).

Heat transfer in solid foods is particularly slow, especially when surface heating techniques are applied, as in traditional drum roasting. This technique involves more conductive heat transfer from the steel of the drum than convective heat

transfer from the hot air sucked through the burners. In that sense, traditional roasting is not efficient: the temperature at the core of the roasted particles remains much lower than at the surface (Ling, Tang, Kong, Mitcham, & Wang, 2015)

There are other roasting techniques faster than oven roasting. Fluidized-bed roasting, for instance, has been successfully proven with coffee (Baggenstoss, Poisson, Kaegi, Perren, & Escher, 2008). Unlike traditional roasting, this technique is almost exclusive to convective heat transfer. In principle, fluidized beds operate by balancing the downward gravity forces of the weight of the particles to be roasted with the upward forces created by a stream of heated air (Sewel, 1995). Inside the fluidized bed's chamber, the particles are highly mobile, resulting in relatively uniform bed temperatures. Consequently, dead zones, hot spots, inhomogeneities, and variability in the quality of the products are significantly mitigated, if not completely avoided (Di Renzo, Scala, & Heinrich, 2021). Moreover, sustainable energy production/conversion and low-carbon footprint also occur (Di Renzo et al., 2021). Even though the fluidized bed technique was introduced in the first half of the 1900s, there is no any published report on its use with cocoa beans. It could be because this technique does not resemble industrial practices. Another reason is the weight of the whole cocoa beans, which is too high to be suspended in the air in regular fluidized-bed coffee roasters; therefore, they need to be deshelled, cracked, and sieved.

Kinetic studies are frequently applied in food science and industry to understand the relations between product and processing variables (M.A.J.S. van Boekel, 2021). Several studies have investigated the kinetics of cocoa roasting processes in terms of water loss. Domínguez-Pérez et al. (2019) found that the water loss was represented with a pseudo-first-order equation. They used the Arrhenius equation to find the temperature dependence. Hii, Menon, Chiang, and Sharif (2017) observed the typical exponential decay of cocoa's moisture content and reported the moisture diffusivities as a function of temperature. Focused on color and neo-formed compounds, Sacchetti et al. (2016) found that the lightness of cocoa decreased following first-order kinetics.

However, the exploration of different roasting techniques in cocoa, e.g., fluidized bed *vs.* oven roasting, has not been reported in kinetic studies. Thus, this research compares the water diffusivity in cocoa nibs, and the temperature dependence of formation of some VOC during the roasting process of cocoa nibs in two different equipment, a conventional oven provided with aluminum trays, and a fluidized

bed roaster. Water activity and color properties were also evaluated at final roasting times.

3.2 Material and methods

3.2.1 Chemicals and consumables

Fermented and dried Forastero cocoa beans (*Theobroma cacao* L.) from Ivory Coast, with a water content of 6.25 ± 0.03 % w/w, were supplied by Barry Callebaut (Wieze, Belgium). Solid-phase microextraction fibers (75 µm Carboxen/ Polydimethylsiloxane) were purchased from Sigma Aldrich (St. Luis, USA).

3.2.2 Preparation of cocoa nibs

Batches of cocoa beans (180g) were heated in a pre-heated pan using an induction stove (ATAG BV, The Netherlands) with constant stirring (4 mins) and then cooled (40°C). The husks were manually removed and winnowed with an air gun. Finally, cocoa nibs were softly cracked with mortar and pestle and sieved to retain only the cocoa nibs with a size between 4.0 and 7.0 mm. This size was suitable for balancing the weight of the cocoa particles with the power of the air blower of the fluidized bed roaster. The sieved cocoa nibs were kept in vacuum bags at -20 °C until the roasting experiments.

3.2.3 Roasting of cocoa nibs

Batches of 70 g of cocoa nibs with an initial water content of 4.10 ± 0.18 % w/w and a water activity of 0.481 ± 0.05 were roasted in a pre-heated electric fluidized bed coffee roaster (Toper Optical Roaster, Izmir, Turkey) for 2'15", 2'45", 3'15", and 3'45" (min's"), at 110, 120, 130, and 140°C. This roaster has a maximum capacity of about 70 g of cocoa nibs, and the fan holds a constant speed of 2100 rpm. At the end of the thermal treatment, the fluidized bed roaster cooled the roasted nibs to 40 °C, in approximately 5 min. This study referred to this technique as fast roasting (FR).

Batches of 70g of cocoa nibs were dispersed in aluminum trays and then placed in a pre-heated electric convective oven (VWR International B.V., Breda, the Netherlands) for 30', 40', 50', and 60' at 110, 120, 130 and 140°C. These experiments are referred to as Slow Roasting (SR). A fan with a constant speed of 1400 rpm injected hot air into the oven's chamber. The inner volume of the oven is 115 L. At

the end of the process, the roasted nibs were immediately removed from the oven and cooled to 40 °C with an air gun, in approximately 5 min.

In both roasting systems, the temperature was measured with thermocouples located inside the roasting chambers. The two roasting techniques and the four roasting temperatures ended up with eight different roasting conditions abbreviated as FR-110 (i.e., Fast roasting at 110°C), FR-120, FR-130, FR-140, and SR-110 (i.e., Slow Roasting at 110°C), SR-120, SR-130, and SR-140. Each roasting condition was carried out four different times; thus, 32 independent preparations were performed, each in duplicate. Unroasted cocoa nibs were used as control, i.e., time zero. Twenty grams of each roasting procedure, and 20 g of unroasted cocoa were ground using a screw-juicer (Vital Max Oscar 900, Hurom, Korea). Ground and non-ground cocoa nibs were vacuum-packed. The first ones were stored at -20°C until usage, while the second ones, which were addressed to color analysis, were stored at 4ºC, and used the day after.

The second roasting times of each roasting technique, i.e., 2'15" for FR, and 30' for SR, were the average times needed for the chambers to reach the corresponding roasting temperatures once the load was introduced. The final roasting times, i.e., 3'45" for FR, and 60' for SR, corresponded to the average-times that the cocoa nibs needed to lose about 5% of their weight.

3.2.4 Measurement of Water Content (*W*)

Approximately 1 g of ground cocoa was placed in a small aluminum cup and dehydrated in an oven (VWR International B.V., Breda, the Netherlands) at 105°C for 18 hours in triplicate. The water content was determined by gravimetric difference as shown in Eq. 3.1, with m_i being the initial mass (g) of the nibs, and m_d the dry mass (g).

$$W\left(\%w/w\right) = \frac{m_i - m_d}{m_i} x100 \qquad \text{EQUATION 3.1}$$

3.2.5 Measurement of Water Activity (a_w)

The water activity of 1.5 g of ground cocoa nibs was measured using a LabMaster-a_w instrument (Novasina, Horsham, UK) at 25°C.

3.2.6 Color properties

The color analysis of cocoa nibs was performed in triplicate by using an Iris Visual Analyser 400 (Alpha M.O.S., Toulouse, France) equipped with a charge-coupled

device camera. Ten grams of cocoa nibs were placed over a white background of the chamber, and high-resolution pictures (2588 x 1942 pixels) were taken under identical controlled light conditions. Examples of the pictures can be seen in Fig. 3.2. The proportion of the pixels present in each image was obtained with the software AlphaSoft V14.1 (Alpha M.O.S., Toulouse, France). Data mining excluded the pixels whose contribution was below 0.01%. The estimation of darkness (reduction of L*), redness (increase of a*), and yellowness (increase of b*) of every picture was calculated from the individual L*a*b* values of every pixel and their contribution to the entire picture.

3.2.7 Volatile organic compounds (VOC)

The VOC of the headspace of the cocoa nibs was measured by Gas Chromatography-Mass Spectrometry (GC-MS) with a Thermo Scientific™ Trace GC Ultra equipment coupled to DSQ II mass spectrometer connected to a Thermo Scientific™ TriPlus RSH™ Autosampler (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.). Vials with 1.00 g of ground cocoa were heated (10 min, 40 °C). Then, a 75 µm Carboxen/Polydimethylsiloxane Solid Phase Microextraction (SPME) fiber was inserted into the vials, exposed to the headspace (10 min), and transferred into the GC injection port for desorption of the trapped volatiles at 200 °C for 10 min. The extracted volatiles were separated in a Stabilwax®-DA 30 m:0.25 mm column (Restek Corporation, U.S., Bellefonte, PA, USA) by heating from 40 to 200 °C (10 °C/min) and then holding 200 °C for 5 min. Helium was used as carrier gas (2.0 mL/min). The separated compounds were eluted through 200 °C mass-transfer lines, and the mass spectrum of the individual compound was detected by the mass detector operating in an electron ionization mode (70 eV) with a scan range from 33 to 250 m/z (GC Clarus 500, PerkinElmer, Norwalk, CT, USA). Each sample was measured in duplicate. The instrument setup and the data acquisition were made with the software Xcalibur 3.0 (Thermo Fisher Scientific Inc., U.S.). The mass spectrum of individual compounds was compared with the mass-spectral library of the National Institute of Standards and Technology (NIST) with the software Chromeleon 7.2 (Thermo Fisher Scientific Inc., U.S.). Compounds with a similarity index above 75% were selected to ensure high conformity of the detected compounds. The retention time and the analyte peak area of each identified VOC were obtained for every sample. The peak area was interpreted as the abundance of the compound. The VOC profile of each sample was obtained by normalizing the data: ratios of single VOC were calculated by dividing their peak area by the sum of the total VOC.

3.2.8 Kinetics of water diffusivity and nitrogen-heterocycles formation during the roasting process of cocoa nibs

Effective water diffusivity and temperature dependency

The rates of water loss k (s^{-1}) during roasting time t (s) for the eight roasting conditions described in Section 3.2.3 were obtained with the first-order exponential model expressed in Eq. 3.2.

$$WR = \frac{(W - W_e)}{(W_0 - W_e)} = \exp(-k^t) \qquad \text{EQUATION 3.2}$$

Afterward, the solution of Fick's second law of diffusion (Eq. 3.3) was used in determining the effective water diffusivity De (m^2s^{-1}) by considering the rate of water loss calculated in Eq. 3.2 as the slope of Eq. 3.3. This calculation assumed three conditions in the cocoa nibs: their shape resembles a slab, the most widely used geometry in modeling the drying process of fruits and vegetables (Onwude, Hashim, Janius, Nawi, & Abdan, 2016); they have an average thickness of 5.5 mm, which corresponds to the arithmetic mean of the major and the minor sieve mesh size, i.e., 4 and 7 mm; and that the samples retain their compact shape during the roasting experiment.

$$WR = \frac{8}{\pi^2} \exp\left(\frac{-\pi^2 \mathrm{De}\ t}{4(h^2)}\right) \qquad \text{EQUATION 3.3}$$

WR stands for the water ratio; W is the water content (g / 100 g cocoa nibs) at any time t; W_0 is the water content of unroasted cocoa (g / 100 g cocoa nibs) or initial water content; W_e is the equilibrium water content (g / 100 g cocoa nibs); and h is the half average thickness (m).

The relationship between effective water diffusivity De and temperature T (°C) is assumed to be an Arrhenius function (Onwude et al., 2016). However, the Arrhenius-like equation (Eq. 3.4) suits those physical phenomena that do not have activation energy, e.g., the molecular mobility of water during roasting (Martinus A.J.S. van Boekel, 2009).

$$\mathrm{De} = \mathrm{De}_0 \exp\left(\frac{-B}{T}\right) \qquad \text{EQUATION 3.4}$$

where De_0 represents the pre-exponential factor (m^2s^{-1}), and B the temperature dependency factor (°C).

Temperature dependence in the formation of nitrogen-heterocycles

A Global fitting can be achieved to calculate the activation energy of formation of organic compounds when measurements of their concentration are taken at various temperatures (M.A.J.S. van Boekel, 2021), as is the case of the current study. Equation 3.5 assumes that the temperature effect on the production of VOC can be described by Arrhenius' law:

$$C_i = C_0 + k_{ref} \exp\left(\frac{E_a}{R T_{ref}}\left(1 - \frac{T_{ref}}{T}\right)\right) t \qquad \text{EQUATION 3.5}$$

where C_i is the relative concentration of a specific VOC at any time t (min); C_0 is the initial concentration of the same VOC; Ea is the activation energy (J/mol); R is the ideal gas constant 8.3143 $J \cdot mol^{-1} \cdot K^{-1}$; T is the absolute temperature in K; k_{ref} is a reference rate of formation of the VOC; and T_{ref} is a reference temperature.

3.2.9 Statistical analysis and mathematical models

Data were statistically analyzed by ANOVA. The least Significant Differences (LSD) method with 95% significance was applied using the statistic software StatGraphics Centurion XVIII (StatGraphics Technologies Inc., USA). Principal Component Analysis (PCA) was performed using R commander 3.6.1 (R Foundation, Austria) and R studio (RStudio Team, USA). The aforementioned equations were executed in Microsoft Excel 365 (Microsoft Corporation, USA).

3.3 Results and discussion

3.3.1 Water content and water activity at the final roasting time

Expectedly, the water content and the a_w of cocoa nibs at the final roasting times (i.e., 3'45" for FR and 60' for SR) were significantly lower ($p<0.05$) than that in unroasted cocoa, as given in Table 3.1. The water content decreased slightly with increasing roasting temperature. Interestingly, there was no significant effect of the roasting technique on the water content at the same temperature; therefore, we considered that they ended up in an "equal roasting degree." This little water interference makes them comparable from physical and chemical points of view. In other words, reaching similar water content in cocoa nibs at 110, 120, 130, or 140°C in the fluidized bed roaster was 16 times faster than in the convective oven.

TABLE 3.1 Water content and a_w of cocoa nibs roasted at different temperatures at the final roasting time, i.e. 3'45" for FR, and 30" for SR

Roasting technique	Temperature (°C)	W ± Std.Dev (g / 100 g cocoa nibs)	a_w ± St.Dev.
Unroasted		4.10 ± 0.1^{a}a	0.481 ± 0.005^{a}
FR	110	1.94 ± 0.28^{b}	0.308 ± 0.016^{b}
	120	1.72 ± 0.28^{bc}	0.284 ± 0.007^{bcd}
	130	1.16 ± 0.43^{d}	0.288 ± 0.060^{bc}
	140	0.89 ± 0.34^{d}	0.312 ± 0.028^{b}
SR	110	1.74 ± 0.18^{bc}	0.206 ± 0.010^{e}
	120	1.47 ± 0.17^{c}	0.213 ± 0.022^{de}
	130	1.14 ± 0.17^{d}	0.186 ± 0.020^{e}
	140	0.94 ± 0.04^{d}	0.223 ± 0.004^{cde}

Values with different superscript letters within the same column are significantly different ($p < 0.05$).

A water activity below 0.33 is desired in cocoa derivatives. It corresponds to typically crispy and brittle nibs or chocolate products (Hanselmann, 2008). This criterion was met in the eight roasting conditions, being the FR cocoa nibs closer to the limit than SR cocoa. Table 3.1 indicates two aspects in a_w: i) roasting technique made a significant difference ($p < 0.05$); ii) no statistical differences ($p > 0.05$) were found when changing the temperature within the same roasting technique. Thus, the remaining water in FR cocoa could be more available for chemical activities than that in SR cocoa at equal roasting degrees.

Consistent with our results, Collazos-Escobar, Gutiérrez-Guzmán, Váquiro-Herrera, and Amorocho-Cruz (2020) found a_w values of 0.20 in cocoa beans roasted in a rotatory system at 120°C for 20 min (2%W); and Rojas, Chejne, Ciro, and Montoya (2020), who roasted cocoa nibs in an infrared moisture analyzer (100 to 200°C) for about 20 min, reported water activity values about 0.35. Moreover, the latter found an effect of roasting temperature in reducing the a_w. Not aligned with our results, García-Alamilla, Lagunes-Gálvez, Barajas-Fernández, and García-Alamilla (2017), reported a_w values ranging from 0.31 to 0.54 in cocoa beans roasted in a rotatory drum system (110 to 150°C). In addition, some of their data were not significantly different from that of unroasted cocoa.

3.3.2 Changes in water diffusion during roasting

Roasting is necessary for cocoa processing because it reduces the moisture content of the kernels up to optimal levels for their further processing, such as grinding and fat pressing (Minifie, 1999). During roasting, the water present near the surface is initially evaporated; subsequently, the water from the inner layers of parenchyma cells moves toward the surface (Kashaninejad, Mortazavi, Safekordi, & Tabil, 2007). This dynamic process, which is favored when the

temperature increases, can be described by Fick's Law (Crank, 1975; Onwude et al., 2016). Fig. 3.1 shows the decrease of the effective water diffusivity De in both roasting systems in function of the inverse of the temperature. In other words, the De increases when the temperature increases, as expected. As expressed by the *B* factor (Fig. 3.1), the rise of De was higher when FR was applied, meaning that the water diffusivity in FR cocoa nibs was more temperature-dependent than in SR cocoa. The order of magnitude of De in FR cocoa was higher than that of SR cocoa at the same temperature. It implies that the FR technique allowed faster mobility of water molecules in cocoa nibs than SR.

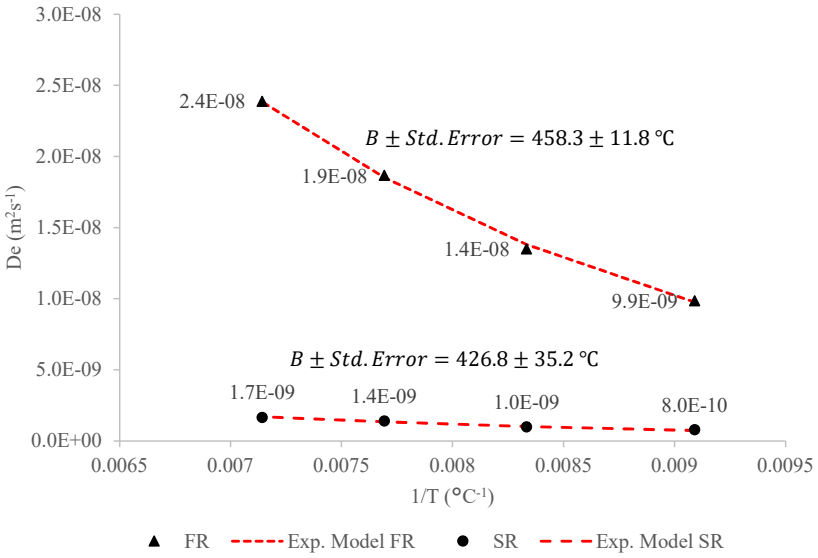


FIGURE 3.1 Temperature dependency of the effective water diffusivity (De) in Fast Roasted (FR) and Slow Roasted (SR) cocoa nibs.

Consistent with our SR results, Domínguez-Pérez et al. (2019) reported De with orders of magnitude of -9 in whole cocoa beans (W_0 = 7% w/w) roasted in a rotating cylindrical structure at different temperatures ranging from 100 to 220 °C. Hii et al. (2017) found orders of magnitude of -10 roasting cocoa nibs (W_0 = 15% w/w) of a thickness between 3.4 to 4.0 mm in a hot air convective oven at 120, 130 and 140 °C; and Sacchetti et al. (2016) also reported orders of -10 in whole cocoa nibs (W_0 = 6% w/w) roasted in an electric oven at 125 to 145 °C. At room temperature, the De of pure water is 1.7E-9 (Walstra, 2002), which is not distinctly different from the results of this study at roasting temperatures. The estimation of diffusion coefficients is far from simple; these differences could be due to the thickness of cocoa, the presence of the husks, the initial moisture content, the range of roasting

temperatures, the equipment used for roasting, the ambient relative humidity, among other systematic factors. Moreover, the diffusivity of molecules depends quite strongly on the food matrix composition. The presence of macromolecules and networks, for instance, hinders their motion. Consequently, the diffusion coefficient can fluctuate in orders of magnitude (Martinus A.J.S. van Boekel, 2009).

The effect of the roasting technique on De is also in line with the results of water activity.

3.3.3 Modification of color attributes by roasting technique

According to the color analysis, most of the 30 pixels identified in roasted and unroasted cocoa nibs correspond to brown colors. Nevertheless, some deep-red, deep-purple, grey, orange, deep-green, and yellow colors were present as well, and can be appreciated in Appendix 3.1. Although no noticeable difference can be detected by naked eye among the eight different kinds of cocoa nibs roasted up to the final roasting time, the PCA analysis presented in Fig. 3.2 clearly differentiates the two roasting techniques. PC1 and PC2, explaining 76.8% of the data variability, showed that FR cocoa nibs form a distinct cluster to the right side, while SR and unroasted nibs are mostly placed to the left and are highly overlapping.

The clusters' separation in Fig. 3.2 is mainly explained with the b* color attribute, as observed in Table 3.2. Three relevant insights came from Table 3.2: (i) the FR cocoa nibs had lower L*, higher a*, and higher b* than SR nibs, having b* the more distinctive differences. (ii) The samples did not turn significantly darker (reduction in L*) upon any roasting condition. (iii) there was no significant effect of roasting temperature in any color attribute. The results reported by Zyzelewicz, Krysiak, Nebesny, and Budryn (2014) are strongly aligned with our FR findings.

The initial color of cocoa nibs before roasting is usually deep-brown, as can be observed in Picture 53, inserted in Fig. 3.2. This condition makes cocoa a challenging product for visual color inspection. During roasting, several heat-induced chemical and physical phenomena take place in cocoa nibs, some of them working in favor of browning or darkening, and some others the other way round: (i) The hydrolysis of tannins (Perkebunan, 2009), for instance, hypothetically reduce the brownness; (ii) the formation brown color pigments via sugar caramelization and or Maillard reaction, should increase the brown color intensity; and (iii) the evaporation of water reduces the darkness of the surface of cocoa (water darkens the color of materials by increasing light absorption

(Jacson, 2014)). A predominance of the second situation may explain the increase in b* and a*. This trend also happens when roasting cocoa at temperatures far above the most common roasting procedures (Zzaman & Yang, 2013).

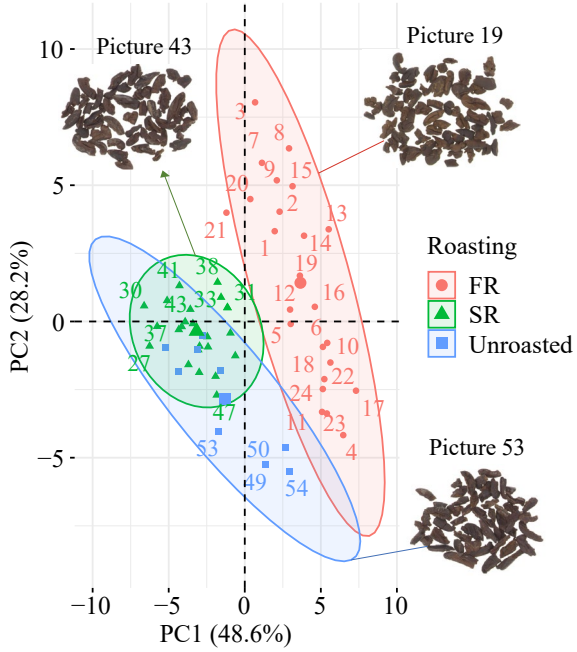


FIGURE 3.2 PCA analysis of the pixel's color data of FR and SR cocoa nibs at the final roasting time (3'45" for FR and 60' for SR). Numbers 1-6 in the PCA correspond to 6 different pictures of Fast Roasted cocoa at 110°C (FR-110), 7-12 to FR-120, 12-18 to FR-130, 19-24 to FR-140; 25-30 to Slow Roasted cocoa at 110°C (SR-110), 31-36 to SR-120, 37-42 to SR-130, 43-48 to SR-140; and numbers 49 to 57 to unroasted cocoa.

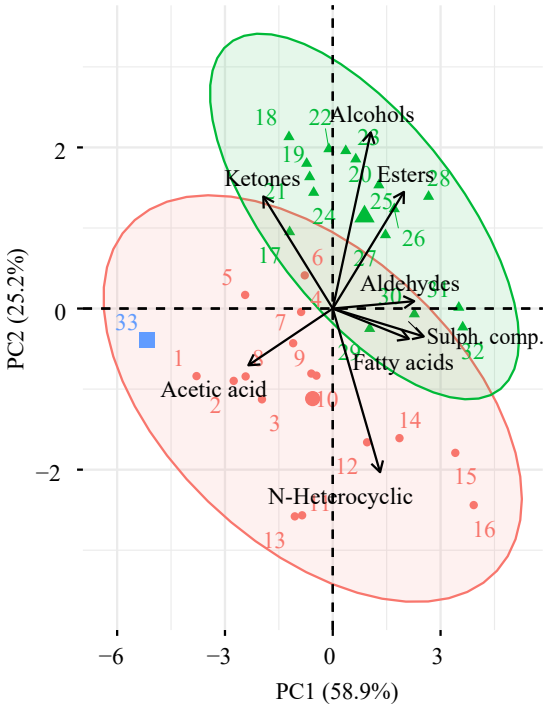
TABLE 3.2 L*a*b* color attributes of cocoa beans at the final roasting time, 3'45" for fast roasting (FR), and 60' for slow roasting (SR).

Roasting condition	Temperature (°C)	L* ± St.Dev.	a*± St.Dev.	b*± St.Dev.
Unroasted		35.18 ± 1.37^{abc}	5.76 ± 0.24^{a}	5.55 ± 0.35^{a}
FR	110	34.97 ± 1.47^{ab}	6.54 ± 0.50^{de}	8.29 ± 0.88^{b}
	120	35.10 ± 1.29^{abc}	6.48 ± 0.38^{cde}	8.33 ± 0.98^{b}
	130	34.40 ± 0.94^{a}	6.63 ± 0.51^{e}	8.62 ± 0.65^{b}
	140	34.66 ± 1.46^{a}	6.17 ± 0.50^{abcd}	7.91 ± 0.64^{b}
SR	110	36.57 ± 0.82^{d}	5.93 ± 0.22^{ab}	5.82 ± 0.26^{a}
	120	36.24 ± 0.34^{bcd}	6.05 ± 0.18^{abc}	6.15 ± 0.48^{a}
	130	37.05 ± 0.37^{d}	6.08 ± 0.25^{abc}	6.22 ± 0.39^{a}
	140	36.41 ± 0.75^{cd}	6.18 ± 0.14^{bcde}	6.20 ± 0.45^{a}

Values with different superscript letters within the same column are significantly different ($p < 0.05$)

3.3.4 Kinetics of formation of volatile organic compounds (VOC)

Forty-three VOC were identified in ground cocoa by SPME GC-MS, including acetic acid, alcohols, aldehydes, fatty acids, esters, ketones, nitrogen heterocyclics, and sulphur-compounds (see Appendix 3.2). In order to have an overview of the dynamics of the eight roasting conditions in terms of VOC formation, the normalized data were grouped by chemical functions and then analyzed via PCA. As shown in Fig. 3.3, the PC2-axis separated the roasting techniques and clustered FR and unroasted on the negative side, whereas the PC1-axis divided the lowest temperatures from the highest. Alcohols and N-heterocycles showed the most extended vectors, meaning that they strongly contributed to the formation of those clusters. Acetic acid was positively correlated with unroasted cocoa and the lowest roasting temperatures of the FR conditions. Ketones, alcohols, esters, aldehydes, and sulphur compounds were positively correlated to SR cocoa. Interestingly, the scores of both FR and SR cocoa were located along PC2 from the negative side toward the positive one in increasing order, also representing the increase in temperature. This tendency is towards the loadings of the N-heterocyclics, which suggests that their formation could depend on roasting time and temperature, especially under FR conditions.



Roasting technique

FR
SR
Unroasted

Description of the scores:

Roasting time	Temperature (°C)			
	110	120	130	140
FR-2'15"	1	5	9	13
FR-2'45"	2	6	10	14
FR-3'15"	3	7	11	15
FR-3'45"	4	8	12	16
SR-30'	17	21	25	29
SR-40'	18	22	26	30
SR-50'	19	23	27	31
SR-60'	20	24	28	32
Unroasted	33			

FIGURE 3.3 PCA biplot of the chemical groups of VOC identified in unroasted, fast roasted (FR), and slow-roasted (SR) cocoa nibs at different roasting times and temperatures.

As predicted by the PCA, the formation of the identified N-heterocyclics, e.g., 2,3-Dimethylpyrazine (2,3-DMP), 2,5-Dimethylpyrazine (2,5-DMP), 2,6-Dimethylpyrazine (2,6-DMP), 2-Ethyl-5-methylpyrazine (2-Et-5-MP), Tetramethylpyrazine (TtMP), Trimethylpyrazine (TrMP), 2-Aminopyridine (2-AP), and 4-Methylpyrimidine (4-MP) displayed temperature dependence as can be appreciated with the activation energies in Table 3.3. The values ranged roughly between 40 and 80 kJ/mol, which are in line with the *Ea* calculated for some Maillard reactions in model systems reviewed by Martinus A.J.S. van Boekel (2001).

TABLE 3.3 Activation energies of formation of N-heterocyclics in cocoa nibs under different roasting conditions.

N-heterocyclics	Fast roasting	Slow roasting
	Ea **± Std.Error (kJ/mol)**	***Ea.*** **± Std.Error (kJ/mol)**
2,3-Dimethylpyrazine	55.3 ± 3.7	67.7 ± 7.6
2,5-Dimethylpyrazine	58.5 ± 4.8	89.9 ± 35.9
2,6-Dimethylpyrazine	56.5 ± 4.6	87.0 ± 32.9
2-Ethyl-5-methylpyrazine	67.2 ± 5.5	94.5 ± 30.0
Tetramethylpyrazine	43.4 ± 5.5	18.3 ± 10.9
Trimethylpyrazine	62.1 ± 4.3	76.0 ± 8.8
4-Methylpyrimidine	64.3 ± 5.4	86.9 ± 30.8
2-Aminopyridine	36.6 ± 4.3	43.0 ± 6.0

In principle, reactants have to overcome an energy barrier (i.e., *Ea*) before reacting and generating new compounds (Walstra, 2002). The formation of all the N-heterocycles, except for tetramethylpyrazine, demanded higher *Ea* in convective-oven roasting than in fluidized bed roaster. In other words, FR allowed a more effective collision of the reactant molecules contained in unroasted cocoa nibs, such as reducing sugars and amino acids, to produce those VOC, probably due to the higher water activity and water diffusivity of FR cocoa nibs. 2-Ethyl-5-methylpyrazine was the compound that showed the highest temperature dependency under both FR and SR conditions; this pyrazine can serve as an indicator compound to monitor roasting cocoa under typical roasting temperatures. The decreasing order of the other *Ea* values diverges between the roasting techniques. For instance, the formation of tetramethylpyrazine registered the lowest *Ea* in SR, but its temperature dependence was not that low in FR cocoa. The differences in *Ea* could be due to the specificity of the amino acids. For example, various amino acids can act as amino-group substrates to produce 2,3-DMP, 2,5-DMP, 2,6-DMP, 2-Et-5-MP, and TrMP. In contrast, only the amino acid Leucine can produce tetramethylpyrazine. Because leucine is also involved in the formation of 2,5-DMP, 2-Et-5-MP, and TrMP (Arnoldi, Arnoldi,

Baldi, & Griffini, 1988), there could have been a higher competition for Leucine in FR nibs than in SR nibs.

The authors acknowledge the following limitations: i) The formation of VOC may diverge within cocoa varieties (Aprotosoaie et al., 2016) and origin (Marseglia et al., 2020); therefore, extending this investigation to other kinds of cocoa beans might provide extra support or no correlation with our findings. ii) N-heterocycles are not final products. They are intermediate products of the Maillard Reaction capable of further reacting to produce advanced products such as melanoidins. Consequently, the net amount of N-heterocycles produced during roasting was not detected. Notwithstanding those points, the results of this study provided valuable insights into the formation of aroma compounds under two roasting systems at regular cocoa roasting temperatures. To the best of our knowledge, this is the first study that assessed the kinetics of formation and temperature dependence of N-heterocyclics during cocoa roasting.

The high correlation of the vector "N heterocyclics" with the cluster of FR cocoa (Fig. 3.3) is also reflected in Fig. 3.4. The data shows the sums of the eight N-heterocycles detected in cocoa nibs subjected to FR-120, FR-130, FR-140, and SR-140 were significantly higher ($p < 0.05$) than that in unroasted cocoa. At the same roasting temperatures, FR trended to produce a higher amount of N-heterocycles than SR. The boost of pyrazines under FR certainly intensified the chocolate aroma of cocoa nibs. However, a sensory analysis is needed to correlate these findings. The presence of the N-heterocyclics in unroasted cocoa, specially tetrametylpyrazine, was totally expected since they are also formed during the fermentation and drying processes of cocoa beans (Mohamadi Alasti et al., 2019). In addition, the surface-heating process addressed to remove the husks could have also initiated some Maillard reactions in the edges of the beans.

The concentration of VOC in cocoa during the roasting process was also reported by Huang and Barringer (2011). They also found that the concentration of 2,3-DMP, 2,5-DMP, 2,6-DMP, and TrMP increases during roasting. In addition, they reported that the higher the temperature, the higher the concentration of those pyrazines, as demonstrated in our kinetic study. Marseglia et al. (2020), Tan and Kerr (2018), and Diab et al. (2014) also found aligned results. The exploration of very high roasting temperatures (150 to 250°C) performed by Zzaman, Bhat, Yang, and Easa (2017) demonstrated that the concentration of pyrazines peaks at the roasting time of 10 min, and then decreases.

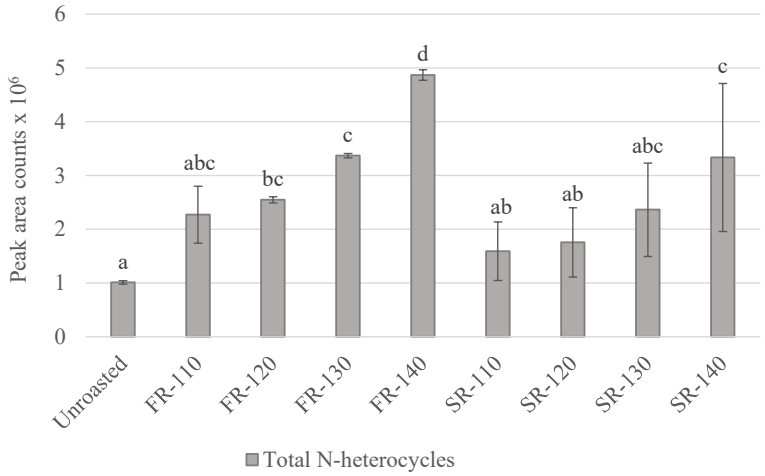


FIGURE 3.4 Total peak area counts of N-heterocycles detected in unroasted cocoa and cocoa roasted under different conditions: Fast Roasted at 110°C (FR-110), Fast Roasted at 120°C (FR-120), Fast Roasted at 130°C (FR-130), Fast Roasted at 140°C (FR-140), Slow Roasted at 110°C (SR-110), Slow Roasted at 120°C (SR-120), Slow Roasted at 130°C (SR-130), and Slow Roasted at 140°C (SR-140), for 3'45" in FR, and 30' in SR. Different letters above the bars represent significant differences ($p<0.05$) in the sum of the N-heterocycles. The error bars correspond to the standard deviation.

Considering the roasting technique, Baggenstoss et al. (2008) found that the formation of pyrazines in coffee was higher in a fluidized bed roaster than in a drum roaster, as we found in cocoa; however, they did not report water activity nor water diffusivity. Aligned to our results, the effect of a_w in the formation of pyrazines in model systems was reported by Scalone et al. (2015). The system representing a water activity of 0.33 produced more pyrazines than the other systems holding a_w values of 0.16, 0.38, and above 0.38.

The zero-order global model (Eq. 5) applied to the data of one of the N-heterocycles was plotted in Fig. 3.5. As observed in Fig. 3.5A and 3.5B, the model strongly fitted the data. Zero-order reactions are rather frequently reported for changes in foods, especially for formation reactions when the amount of product formed is only a small fraction of the amount of precursors present in the matrix (Martinus A.J.S. van Boekel, 2009). It would be the case of intermediate Maillard Reaction products, like the aforementioned N-heterocyclics, which are typically produced by Amadori rearrangement and Strecker degradation pathways (Gibis, 2016).

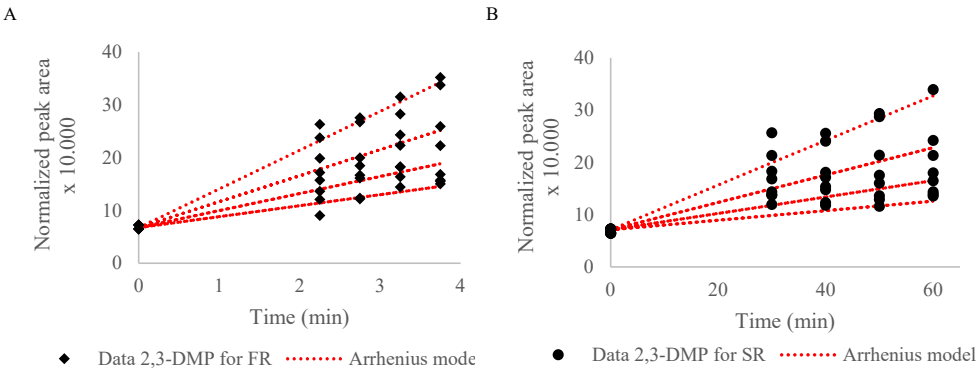


FIGURE 3.5 Global fit for all temperatures using a zero-order kinetic model for 2,3-Dimethylpyrazine (2,3-DMP) under Fast Roasting (FR) (Panel A) and Slow Roasting (SR) (Panel B).

3.4 Conclusions

At conventional cocoa-roasting temperatures (from 110 to 140°C), the fluidized bed roasting technique (Fast roasting) allowed faster mobility and higher availability of water molecules within cocoa nibs than oven roasting (Slow roasting) as it was expressed in the higher effective water diffusivity (De) and a_w values of its roasted cocoa nibs. The De in FR cocoa was more temperature-dependent than in SR cocoa.

It is probably that the higher water diffusivity and water activity in FR cocoa favored the interaction of the reactants intricated in the formation of 2,3-Dimethylpyrazine, 2,5-Dimethylpyrazine, 2,6-Dimethylpyrazine, 2-Ethyl-5-methylpyrazine, trimethylpyrazine, 2-Aminopyridine, and 4-Methylpyrimidine. As a consequence: (i) the activation energy of those chemical reactions was lower in FR cocoa than in SR cocoa; and (ii) the concentration of those N-heterocyclics (expressed as peak area counts) was higher in FR cocoa than in SR cocoa at the same roasting temperatures.

The formation of 2-Ethyl-5-methylpyrazine under both FR and SR conditions showed higher temperature dependency than the other N-heterocyclics. The temperature-sensitivity of 2-Ethyl-5-methylpyrazine could serve as an indicator to monitor the progress of roasting cocoa at typical roasting temperatures.

The initial color of cocoa nibs before roasting is usually deep-brown; therefore, finding significant changes ($p < 0.05$) in L* was not possible. Interestingly the b* parameter stood out and displayed significant differences ($p < 0.05$), being the FR cocoa higher in yellowness than SR cocoa.

Data of this study highlighted the advantages of using fluidized bed roasting as an alternative roasting technique for cocoa nibs, being sixteen times shorter than conventional roasting. The efficiency of energy production/conversion of fluidized bed roasting and its low-carbon footprint can undoubtedly reduce the processing cost, improve sustainability, and ensure the cocoa aroma quality with high pyrazine contents.

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Appendices

APPENDIX 3.1 L*a*b* color attributes of the pixels detected in pictures taken at roasted and unroasted cocoa nibs.

Pixel code	Color	L*	a*	b*
802		18.05	7.73	3.07
819		23.52	0.00	0.00
1074		24.97	5.44	12.86
1075		25.31	7.32	2.84
1076		25.77	9.77	-7.07
1091		30.22	-2.36	9.66
1092		30.59	0.00	0.00
1331		27.43	14.68	6.17
1347		31.94	5.06	12.31
1348		32.28	7.01	2.68
1349		32.72	9.41	-6.85
1364		37.04	-2.32	9.33
1365		37.41	0.00	0.00
1603		33.91	12.49	15.35
1604		34.23	14.10	5.76
1619		38.40	3.21	21.09
1620		38.67	4.78	11.87
1621		39.02	6.76	2.56
1637		43.65	-2.28	9.06
1638		44.01	0.00	0.00
1876		40.52	11.94	14.74
1877		40.84	13.62	5.45
1892		44.94	2.92	20.52
1893		45.21	4.57	11.51
1894		45.56	6.55	2.46
2148		46.70	10.06	23.20
2149		46.97	11.49	14.24
2165		51.31	2.69	20.01
2166		51.59	4.39	11.21
2167		51.93	6.38	2.38

APPENDIX 3.2 Volatile organic compounds identified in roasted and unroasted cocoa nibs by GC-MS.

Chemical group	Identified compound	Retention time (min)
Alcohol	1-Pentanol	9.8
	2-Butanol	11.7
	3-Methyl-1-butanol	9.4
	3-Methyl-1-buten-3-ol	6.3
	3-Methyl-2-heptanol	11.0
	Furfuryl alcohol	15.7
Aldehyde	2-Methylpentanal	4.5
	2-Methylpropanal	3.1
	Benzaldehyde	14.0
	Furfural	13.1
Ester	1-Methylbutyl acetate	7.0
	2-Pentyl acetate	10.0
	3-Methyl-1-butanol acetate	7.9
	Ethyl acetate	4.1
	Isobutyl acetate	6.0
	Methyl acetate	3.4
Fatty acid	2-Methylpropanoic acid	14.2
	3-Methylbutanoic acid	15.4
	4-Methyloctanoic acid	16.9
	Butanoic acid	14.9
	Hexanoic acid	17.4
	Pentanoic acid	16.2
	Propanoic acid	13.8
	Propionic acid	6.8
Ketone	1-Hydroxy-2-propanone	10.9
	2-Heptanone	8.9
	2-Hydroxy-3-butanone	14.5
	2-Nonanone	12.0
	3-Hydroxy-2-butanone	10.6
	Cyclopropylethanone	8.1
	Dihydro-5-methyl-2(3H)-furanone	15.2
Organic acid	Acetic acid	12.7
Pyrazines	2,3-Dimethylpyrazine	11.5
	2,5-Dimethylpyrazine	11.1
	2,6-Dimethylpyrazine	11.1
	2-Ethyl-5-methylpyrazine	12.1
	Tetramethylpyrazine	13.2
	Trimethylpyrazine	12.2
Pyridine	2-Aminopyridine	19.2
Pyrimidine	4-Methylpyrimidine	10.3
Sulphur compounds	Dimethyl disulfide	2.6
	Methyl disulfide	7.2

Chapter 4

Fluidized bed roasting modifying the microstructure of cocoa nibs and improving cocoa butter quality

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Abstract

The extraction of butter from cocoa seeds involves various processing steps that weaken the lipid-storing cell walls of cocoa cotyledons. Roasting is particularly critical, making cocoa nibs porous and brittle. In this study, the degree of disruption of the microstructure of cocoa nibs, and the quality and aroma profile of cocoa butter, were evaluated using two roasting systems: a forced convective oven with aluminum trays and a fluidized bed roaster. Fluidized bed roasting, recognized for its energy efficiency and low-carbon footprint, was 12 times faster than oven roasting. This technique allowed a rapid release of steam when parenchyma cell walls were still in a glassy state, while oven roasting caused gradual physical modification allowing the cell wall to become more elastic. Consequently, small pores expanded and coalesced to produce larger pores. X-Ray tomographic analysis showed a total porosity in unroasted cocoa nibs of 8.5 ± 2.0% (v/v), which was doubled upon oven roasting and triplicated upon fluidized bed roasting. The higher porosity in fluidized-bed-roasted nibs was reflected in the lowest densities and highest cocoa butter yield. Cocoa butter obtained from fluidized-bed roasted cocoa showed a higher presence of pyrazines and 3-methylbutanal, and a lower concentration of hydroperoxides, thus enhancing the chocolate flavor and quality. In this paper, we proved that the pore structure of cocoa nibs is a key quality descriptor of roasting processing, and we concluded by quality considerations that fluidized bed roasting technique should be preferred for cocoa nibs roasting over conventional roasting.

Keywords: *Theobroma cacao* L., microscopy, X-ray tomography, porosity, pyrazines, peroxide value.

4.1 Introduction

Cocoa butter is the major component of the seeds of *Theobroma cacao* L. (53 to 59% d.b.) (Oracz et al., 2014) and their most valuable derivative (International Cocoa Organization, 2021). Its extraction involves various typical processing steps to soften, brittle, and break the cell wall of the reserve cells of cocoa seeds that enclose the fat crystals. The loss of quality of cocoa butter across the processing chain is a concrete challenge, and technological parameters must be optimized to minimize it.

Fermented and dried cocoa beans are composed of testa and cotyledons, known as shells and nibs, respectively. During fermentation and drying, secondary metabolites (e.g., acetic acid) produced around the beans penetrate the cotyledons. As a consequence, the parenchyma cells' structure turns smooth, water leaks (Camu et al., 2007), and some cells get disrupted (de Brito et al., 2000). In addition, lipases hydrolyze triacylglycerols with the release of fatty acids, compounds that easily follow the formation of primary oxidation products (i.e., hydroperoxides) (Hammond, 2003) thanks to the high solubility of oxygen at the mild temperatures of fermentation (Tena et al., 2019).

The typical roasting temperatures cause the evaporation of a significant part of the remaining water and some other low-boiling-point organic compounds like acetic acid and fatty acids. This physical phenomenon implicates the formation of large amounts of gases inside the matrix that generate high internal pressure. Thus, roasting modifies the microstructure of the nibs with the appearance of more disrupted cells (de Brito et al., 2000) and the increase in porosity (Schenker et al., 2000). Those changes are responsible for the friability of roasted cocoa nibs, which facilitate the grinding process. Moreover, the solubility of oxygen is remarkedly reduced by the high roasting temperatures, leading to the decrease of primary oxidation products, and the formation of the secondary oxidation products (e.g., volatile compounds and polymeric structures) (Tena et al., 2019).

When roasted cocoa nibs are ground, abundant cocoa butter is released. This leads to a homogenous cocoa mass, also named cocoa liquor (Hoskin and Dimick, 1980). A horizontal hot press extracts the cocoa butter by gradually increasing the pressure on cocoa liquor. This process separates a rigid solid cake containing mainly proteins, polysaccharides, and polyphenols (Hoskin and Dimick, 1980) from the lipid phase named cocoa butter. At this point, the aroma of cocoa butter is very unpleasant due to the high concentration of acidic volatiles, especially acetic acid (Carlin et al., 1986); thus, deodorization is required before use.

Unfortunately, this process also removes desirable chocolate notes, leading to a neutral aroma (Gutiérrez, 2017).

The fatty acid composition of cocoa butter consists of about 62% of saturated and 38% of unsaturated fatty acids (Dorota et al., 2014). These chemical characteristics make cocoa fat more resistant to oxidation than other edible oils and fats. Nonetheless, roasting can be optimized to avoid the formation of undesirable secondary oxidation products like some aldehydes (e.g., pentanal, hexanal, and nonanal), which exert undesirable rancid flavors (Grebenteuch et al., 2021).

Different roasting techniques and temperatures might affect the microstructure of cocoa nibs, as well as the quality and aroma profile of their cocoa butter. To date, few studies have studied the microstructure of cocoa nibs before and after roasting; however, none have neither compared different roasting conditions (techniques/temperatures) nor quantified the degree of disruption, namely porosity formation. The effect of roasting conditions on cocoa butter quality parameters and volatile organic compounds formation has also been poorly assessed.

The fluidized bed apparatus was proposed as an alternative roasting technique for its energy efficiency and low-footprint synthesis (Di Renzo et al., 2021). In our previous study (Peña-Correa et al., 2022), we compared fluidized bed roasting *vs.* forced-convective oven roasting by means of kinetics of formation of volatile organic compounds and effective water diffusivity. This study demonstrated that fluidized bed-roasted cocoa nibs have a higher water activity and diffusivity than oven-roasted cocoa, favoring the formation of pyrazines without carrying typical overroasting aroma compounds. Pyrazines are desirable aroma compounds displaying nutty, earthy, roasty, and chocolate notes (Michel et al., 2021); their presence in the lipidic fraction of cocoa is a factor deserving further investigation. Different heat transfers methods involved in the roasting processes determined those results: Fluidized bed roasting is an almost exclusive convective heat transfer technique (from the high pressurized hot air), while in convective oven, a combination of conductive (from the metallic surface containing the cocoa nibs), convective and radiation heat transfers occur. The heat transfer efficiency of fluidized bed roasting resulted in speeding up the process to about 16 times, according to that study.

Fluidized bed roasting could release the steam in a considerably shorter time than oven roasting. As a consequence, a higher disruption of the microstructure

of cocoa nibs could occur. Moreover, the further migration of cocoa butter along the pores may expose it to oxygen interaction. Thus, higher exposition may come with higher lipid oxidation.

This study aimed to evaluate the effect of fluidized bed roasting and forced-convective oven roasting at two roasting temperatures (120 °C and 140 °C) on the microstructure, density, and porosity of cocoa nibs; and on the yield of extraction, volatile organic compounds (VOC) profile, and quality of their cocoa butter.

4.2 Experimental procedures

4.2.1 Materials

Fermented and dried Forastero cocoa beans (*Theobroma cacao* L.) from Ivory Coast, with a water content of 6.14 ± 0.02 % w/w, were supplied by Olam International (Koog aan de Zaan, The Netherlands). Wijs solution, 9-(2-Carboxyphenyl)-6-(diethylamino)-N,N-diethyl-3H-xanthen-3-iminium chloride (Rhodamine B), 4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a,4a-Diaza-s-Indacene (Bodipy™ 505/515), soluble starch, acetic acid (99.7%), anhydrous sodium thiosulfate ($Na_2S_2O_3$), ethyl alcohol (96%), sodium hydroxide, potassium iodide, and solid-phase microextraction fibers (75 µm Carboxen/ Polydimethylsiloxane) were purchased from Sigma Aldrich (St. Luis, USA). Petroleum ether with a boiling point between 40 – 60 °C, and iso-octane were purchased from Actu-All Chemicals (Oss, The Netherlands). Whatman 595 ½ filter papers were purchased from GE Healthcare Life Sciences, UK.

4.2.2 Roasting of cocoa nibs

The remotion of cocoa beans' shells to get cocoa nibs of 4.0 to 7.0 mm was performed as described in Chapter 3, Section 3.2.2.

Batches of cocoa nibs (70g) with an initial water content of 4.31 ± 0.11 (w/w) were roasted in a pre-heated electric fluidized bed coffee roaster (Toper Optical Roaster, Izmir, Turkey) at 120 and 140 °C, for 6 min and 27 s, and 3 min and 43 s, respectively. This study referred to this technique as fast roasting (FR). Batches of 70 g of cocoa nibs were dispersed on aluminum trays and then placed in a pre-heated electric convective oven (VWR International B.V., Breda, the Netherlands) at 120 °C for 73 min and 18 s, and at 140 °C for 45 min and 16 s. This roasting procedure was referred to as slow roasting (SR) in this study. The roasting times were determined by a water content of 1.0 ± 0.1% w/w in roasted cocoa nibs.

More detailed information of the roasting equipment is provided in Chapter 3, Section 3.2.3.

The two roasting techniques and the two roasting temperatures resulted in four different roasting conditions abbreviated as FR-120 (i.e., Fast roasting/roasted at 120°C), FR-140, SR-120 (i.e., Slow Roasting/roasted at 120°C), and SR-140. Every roasting condition was performed nine times. The batches were pooled in this way to be handed as triplicates: [1st, 2nd, and 3rd], [4th, 5th, and 6th], and [7th, 8th, and 9th]. Unroasted cocoa nibs (200 g) were separated as a control. All the samples were vacuum-packed and stored at -20°C until usage.

4.2.3 Microscopy of cross-sections of cocoa nibs

Observations through a confocal laser microscope (Type 510, Zeiss, Oberkochen, Germany) were performed based on the methodology described by Zahir et al. (2018) with some modifications. Based on the fluorochrome binding affinity to cell microstructures, the fluorescent dyes rhodamine B and Bodipy 505/515 were used to stain protein and oil bodies, respectively. A solution containing 0.001% of both was prepared in demineralized water. Three cocoa nibs from each roasting batch and control were selected and transversely cut with a scalpel. Dye-mix solution (100 µl) was added to a watch glass. The cut-side of the two halves was dept into this solution for 15 min and then placed on a glass microscope slide with the cut-side touching it. The microscopic observations were done using a 543 nm HeNe laser for rhodamine B, and a 488 nm argon laser for Bodipy. The images were acquired using a 10/20 EC Plan-Neofluar/0.5A lens, and analyzed with the software ZEN blue edition (Carl Zeiss Microscopy).

4.2.4 X-ray tomography exploration of cocoa nibs and porosity calculation

Three cocoa nibs were selected from each roasting batch and control. A tomographer (GE Phoenix v|tome|x, General Electric, Wunstorf, Germany) and its software GE (Wunstorf, Germany) were used to get non-invasively and non-destructively 2D and 3D imaging. Each cocoa nib was placed 28.55 mm away from the X-ray source (240 kV microfocus tube with tungsten). X-rays were generated with a voltage of 80 kV and a current of 120 µA. A 0.3 mm Cu filter was used to avoid beam hardening. A full scan of 1500 projections over 360° (recorded by a GE DXR detector array) allowed to obtain single images of 2024 × 2024 pixels (pixel size 200µm) with a spatial resolution of 7.0 µm. Bidimensional projections representing virtual cross-sections were obtained from the average of 3 images obtained over 250 ms exposure time. The software Avizo (Thermo Fisher, version 2019.1, Germany) was further used to obtain the voxels (the volume elements,

like the pixels of a picture) to make the 3D reconstructions of the whole nib and to calculate the volumes of its continuous solid structure and the enclosed air sections, namely porosity. Because the cracks were not enclosed air sections, they were not considered pores. Voxels of air sections below $6.85x10^{-7}$ mm^3 were not considered for volume calculations either, as they are likely to be imaging artifacts.

4.2.5 The density of cocoa nibs

Representative samples of cocoa nibs were weighed (10 grams approx.) on an analytical balance and then transferred into a glass-volumetric cylinder containing 50 mL of ethanol (96% v/v, 0.799 g/mL at 20 °C). The volume displacement was immediately registered and used to calculate the density of cocoa nibs.

4.2.6 Cocoa butter extraction

Superficial extraction

A superficial-solvent extraction was performed to estimate to what extent a non-polar solvent can access cocoa nibs' pores to withdraw cocoa butter. Briefly, 20 mL of petroleum ether was added to 5 g of cocoa nibs and placed over a shaking plate (Universal Shaker SM30, Edmund Bühler, Germany) at 75 rpm (15 min, room temperature (RT)). Then, the mixture was filtered through a Whatman 595 ½ filter paper. The solvent was evaporated (55 °C, 300 mbar) using a rotary evaporator (R215, Buchi, Flawil, Switzerland). The weight (g) of the cocoa butter obtained (w_{CB}) was used to estimate the yield of extraction of cocoa butter by using Eq. 4.1, where (w_{CN}) stands for the weight (g) of the dry fraction of cocoa nibs.

$$Yield\ of\ cocoa\ butter\ (\%\ w/w\ d.b.) = \frac{w_{CB}}{w_{CN}} * 100 \qquad \textbf{EQUATION 4.1}$$

Hot-press extraction

Unroasted cocoa nibs and cocoa nibs processed under FR-140 and SR-140 conditions were transformed into cocoa liquor for further hot-press defatting: Cocoa nibs (1 kg) were ground at room temperature with a continuous screw-juicer (Vital Max Oscar 900, Hurom, Korea), and then finely ground with a cocoa grinder (Premier Wonder, Chennai, India) (60 min, 50-53 °C). Cocoa liquors were heated in an incubator (90 °C), and batches of approximately 250 g were subjected to hot-press (95 °C, 250 bar, 600 s) in a lab-scale cocoa machinery press (GRS 1/1400, Calozicorte, Italy, recommended load: 0.20 to 0.55 L). The yield of cocoa butter was calculated with Eq. 4.1, corresponding w_{CN} to the dry weight (g) of the cocoa liquor.

Chemical extraction of cocoa butter for chemical analysis

Cocoa butter was exclusively extracted from ground cocoa with a non-polar-organic solvent for further chemical analysis. Briefly, cocoa nibs were twice-ground using a screw juicer (Vital Max Oscar 900, Hurom, Korea). Ground cocoa (100g) was defatted with petroleum ether (4 x 200 mL). After each defatting step, the mix was stirred (15 min, RT) and then allowed to precipitate for 1 h. The upper layers containing the solvent with the cocoa butter were pipetted off, pooled, and centrifuged (4500 rpm,10 min, RT). The solvent was evaporated (55 °C, 300 mbar) in a rotary evaporator. The obtained cocoa butter was stored at -20 °C until analysis. The pellets and the sediments (cocoa powder) were reserved for Chapters 5 and 6.

4.2.7 Volatile organic compounds (VOC) in cocoa butter

The VOC of the headspace of 1.0 g of cocoa butter was measured by Gas Chromatography-Mass Spectrometry (GC-MS) with a Thermo Scientific™ Trace GC Ultra equipment coupled to DSQ II mass spectrometer connected to a Thermo Scientific™ TriPlus RSH™ Autosampler (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.), as described in Chapter 3, Section 3.2.7.

4.2.8 Determination of cocoa butter quality

Free fatty acids (FFA) content

The FFA content in the cocoa butter was measured according to the AOCS Official Method Ca5a-40 (American Oil Chemists' Society, 1989) with some modifications. A melted cocoa butter sample (7 g, 50 °C) was added with warm ethyl alcohol (75 mL, 50 °C, 96% v/v) and immediately titrated with 0.25 N NaOH in the presence of phenolphthalein under constant stirring. The FFA content, expressed as a percentage of oleic acid, was calculated with Eq. 4.2, where V stands for the volume of NaOH (mL), N is the normality of the NaOH solution, and W corresponds to the mass of cocoa butter (g).

$$FFA\left(Oleic\ acid\ \%\right) = \frac{V*N*28.2}{W} \qquad \text{EQUATION 4.2}$$

Peroxide value (PV)

The peroxide value is defined as the milliequivalents of peroxide in one kilogram of fat. It was determined for cocoa butter with the official method 965.33 (Association of Official Analytical Chemists, 2005) with some modifications. A starch indicator solution was prepared by first dissolving 0.5 g of soluble starch

in 30 mL of water. Then, 70 mL of boiling water was added, and the solution was boiled for 3 min. Finally, the starch indicator solution was cooled at room temperature. Separately, 5 grams of cocoa butter were melted at 50 °C, then 25 mL of a solution of iso-octane and acetic acid 2:3 (v/v), 0.5 mL of saturated KI aqueous solution, and 30 mL of water were added. A similar preparation was done without cocoa butter, i.e., the blank. The solutions were titrated with 0.01 M $Na_2S_2O_3$ in the presence of 0.5 mL of starch indicator solution until a white color was detected. PV was calculated using Eq. 4.3 where V_0 and V are the titration volumes (mL) of the blank and the sample respectively, N is the normality of the $Na_2S_2O_3$ solution, and W represents the mass of cocoa butter (g).

$$PV\left(meq\ of\ peroxide/1\ kg\ Cocoa\ butter\right)=\frac{\left(V-V_0\right)*N*1000}{W} \qquad \textbf{EQUATION 4.3}$$

4.2.9 Statistical analysis

Data were statistically analyzed by ANOVA. The Least Significant Differences (LSD) method with 95% significance was applied using the statistic software StatGraphics Centurion XVIII (StatGraphics Technologies Inc., USA). The Heatmap with dendrogram was performed using R commander 3.6.1 (R Foundation, Austria) and R studio (RStudio Team, USA).

4.3 Results and discussion

4.3.1 Microstructure of cocoa nibs

The microstructure of cocoa nibs roasted in different conditions was explored via confocal laser microscopy and X-ray tomography. Fig. 4.1 to 4.4 shows some of the obtained images. In the confocal-laser-obtained images (Fig. 4.1 and 4.2), the protein bodies are stained in red, and the lipids in greenish-yellow color.

Fig. 4.1A shows that cocoa nibs contain lipid/protein storage cells of different shapes and sizes with a diameter (Φ) ranging between 15 to 25 μm. Those sizes are in line with the observations reported by de Brito et al. (2000); Lopez et al. (1987); Abo-Bakr and Shekib, (1987); and Alean et al. (2019). The organelles of the cells (Φ ≈ 2 to 10 μm) can also be appreciated. Small and big pores (Φ ≈ 5 to 100 μm), as well as the fission of cytoplasms of storage cells due to cell wall disruption, were detected in both roasted and unroasted nibs. The fission of cytoplasms was more frequent in roasted cocoa, as observed in Fig. 4.1B.

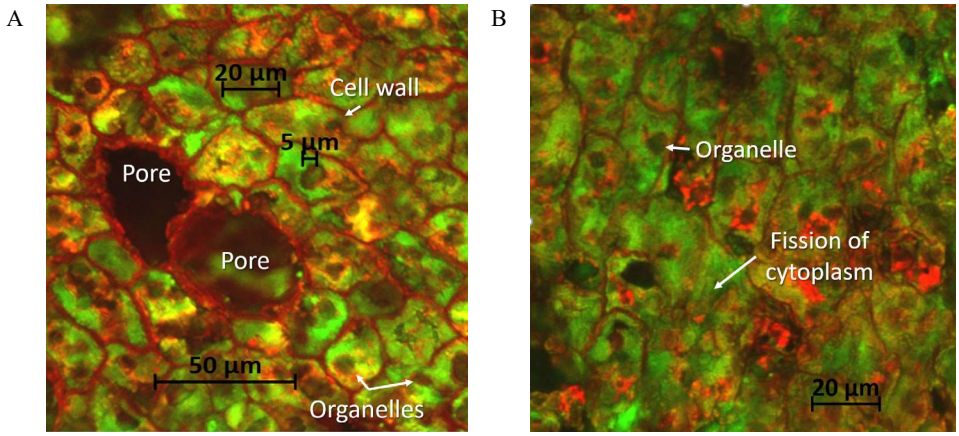

FIGURE 4.1 Confocal microscopy images of stained cross-sections of unroasted cocoa nibs (Panel A) and cocoa nibs fast roasted at 140°C (Panel B).

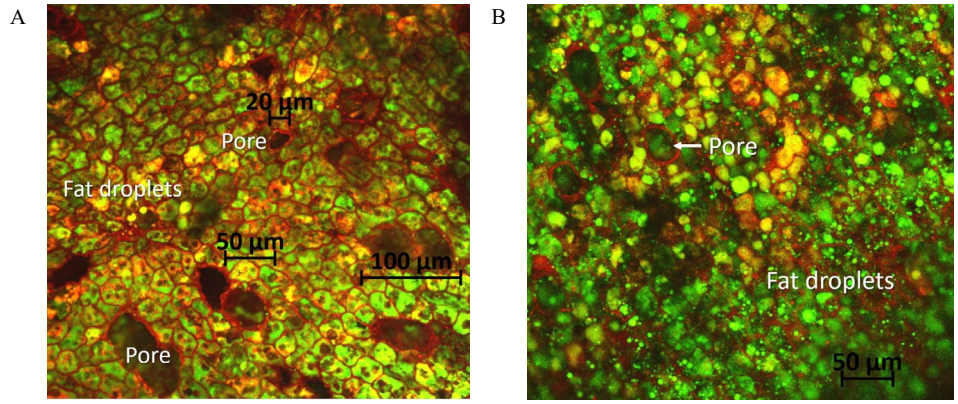

FIGURE 4.2 Confocal microscopy images of stained cross-sections of unroasted cocoa nibs (Panel A) and cocoa nibs fast roasted at 140°C (Panel B).

Another change revealed by confocal microscopy was the presence of more fat droplets in roasted cocoa than in unroasted, as shown in Fig. 4.2, panels A and B. However, no notorious differences were appreciated among the four roasting conditions. The typical high roasting temperatures mobilized cocoa butter (fat bloom) to fill up the empty sections left by evaporated water.

The phenomena behind the pore-formation in cocoa nibs are unclear. Certainly, they originated during the drying process of fermented cocoa beans. As demonstrated in the SEM-microscopy observations performed by Lopez et al. (1987), the parenchyma cells of unfermented and fermented cocoa seeds are irregularly packed with no spaces between them. During the fermentation, the microflora stays over the testa, and does not penetrate it to invade the cotyledons

(Lopez et al., 1987); therefore, the pores are not caused by bacterial damage. The first sign of cell damage (de Brito et al., 2000) and pores and pits formation (Abo-Bakr and Shekib, 1987) were observed in dried cocoa cotyledons, probably due to water leaking.

The 3D reconstructions of unroasted and roasted cocoa nibs (Fig. 4.3) demonstrated that those pores are microchannels instead of bubbles. So, cocoa nibs structure resembles a micro-honeycomb. Schenker et al. (2000) found a similar microstructure in roasted coffee beans. The micropores formed in coffee could correspond to modified cell-to-cell plasmodesmata microchannels or a 3-D intercellular permeable wad-like network of polysaccharides. The latter is partially degraded and removed during roasting, giving way to oil droplets to migrate toward the surface of roasted coffee beans (Schenker et al., 2000). A similar phenomenon is probably occurring in cocoa nibs; however, a more detailed observation of the intracellular structure is needed.

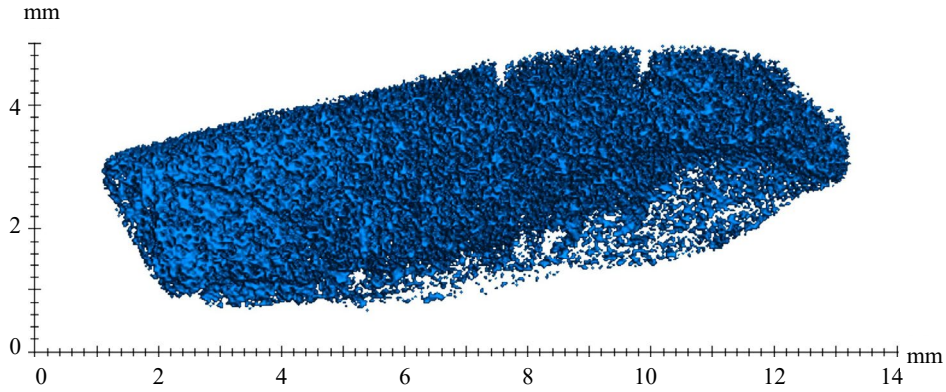

FIGURE 4.3 X-ray tomography reconstruction of a cocoa nib slow-roasted at 120°C. The deeper the blue color, the deeper the location of the solid mass in the 3-D space.

4.3.2 Density and porosity of cocoa nibs

Table 4.1 shows the density of unroasted and roasted cocoa nibs. As expected, the roasting process significantly reduced it. Such effect was higher in FR nibs than in SR nibs. Data also shows the effect of roasting temperature on FR cocoa density: the higher the temperature, the lower the density of cocoa nibs. The reduction in density in cocoa nibs could be a consequence of losing water with a minor shrinking effect or because cocoa nibs expanded. As the four kinds of roasted cocoa lost the same amount of water, we attribute their differences to the second phenomenon.

TABLE 4.1 Physical properties of unroasted and roasted cocoa nibs.

Roasting condition	Density (g/cm^3)	Total porosity (%, v/v)	Surface-removable fat (g cocoa butter/100 g cocoa nibs d.b.)	Fat extractability by hot-press (g cocoa butter/100 g cocoa liquor d.b.)
Unroasted	0.996 ± 0.020^{c}	8.5 ± 2.0^{a}	0.44 ± 0.07^{a}	49.60 ± 0.01^{a}
FR-120	0.913 ± 0.003^{b}	25.3 ± 3.7^{b}	1.08 ± 0.17^{b}	-
FR-140	0.836 ± 0.020^{a}	24.2 ± 5.1^{b}	1.11 ± 0.14^{b}	49.90 ± 0.16^{a}
SR-120	0.927 ± 0.022^{b}	14.6 ± 4.8^{a}	0.58 ± 0.12^{a}	-
SR-140	0.943 ± 0.047^{b}	14.3 ± 6.6^{a}	0.51 ± 0.04^{a}	50.30 ± 0.79^{a}

FR-120 and FR-140 stand for fast-roasted cocoa nibs at 120 and 140 °C, and SR-120 and SR-140 for slow-roasted cocoa nibs at 120 and 140 °C, respectively. Superscripts in the same column indicate significant differences at the 95.0% confidence level. The values correspond to means ± standard deviation.

Widyotomo et al. (2006) also reported the effect of roasting in reducing the density of cocoa. The values they reported ranged from 0.36 to 0.44 g/cm^3, which were lower than those obtained in this study. These differences are due to sample preparation, as they worked with whole cocoa beans (shells included).

The porosity was defined as the volume ratio of the empty enclosed sections, calculated with X-ray tomography in unroasted and roasted cocoa nibs. The results are presented in Table 4.1. The porosity of unroasted cocoa nibs was almost duplicated upon SR ($p > 0.05$) and triplicated upon FR ($p < 0.05$). There was no significant effect of roasting temperature on porosity formation. The changes in porosity are aligned with the reduction in density. Fig. 4.4 showing the 2-D projections of virtual cross-sections of cocoa nibs, supports these quantitative data. The glass transition phenomena may play an important role in microstructure resistance (Schenker et al., 2000). A possible explanation for those differences is that during fluidized bed roasting, most of the steam was quickly generated (due to the higher heat-transfer efficiency of the convection) when the cocoa nibs' cell walls were still in a glassy state. Glassy cell walls were easily broken, leading to the formation of large pores. In contrast, slow roasting allowed cell walls to become more elastic during the release of steam due to the low conductive-heat transfer efficiency, thus causing less disruption of the original structure. It is worth to mention that in this study, fluidized bed roasting was about 12 times faster than oven roasting, while in our previous study (Peña-Correa et al., 2022), it was about 16 times. Those differences would be due to cocoa beans' features (e.g., the initial water content and porosity) and seasonal environmental conditions in the lab.

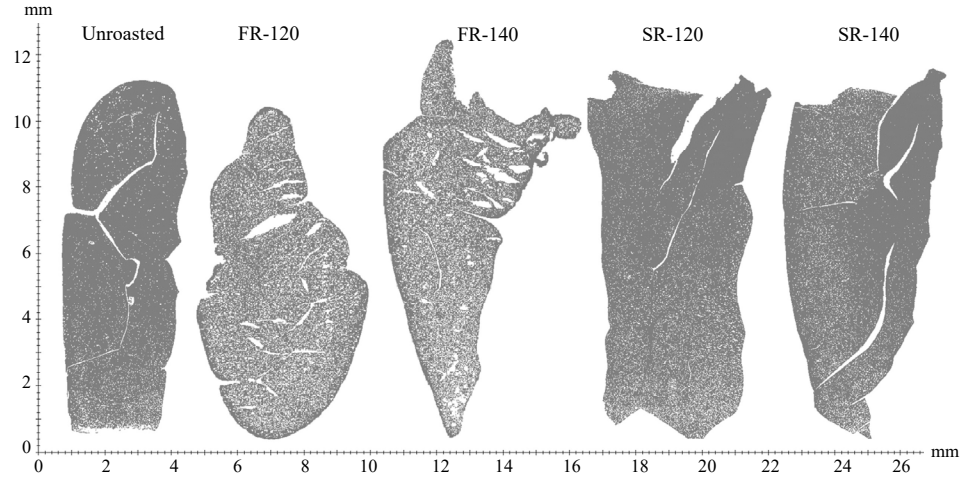

FIGURE 4.4 X-Ray tomography projections of virtual 2-D cross-sections of cocoa nibs. From left to right: unroasted cocoa nibs, cocoa nibs fast roasted at 120°C (FR-120), cocoa nibs fast roasted at 140°C (FR-140), cocoa nibs slow roasted at 120°C (SR-120), and cocoa nibs slow roasted at 140°C (SR-140)

Each material has different capacities to absorb and scatter X-rays. Air, for example, scatters the majority of them, while dense-solid structures tend to absorb them (Burbridge and Mah, 2017). Those differences allow the reconstruction of objects and the quantification of the different components through the X-ray tomography technique, which is a non-invasive method (Maire and Withers, 2014). To the best of our knowledge, this is the first study presenting 3-D reconstructions, 2-D projections, and quantified porosity of unroasted and roasted cocoa nibs.

The X-ray tomography analysis quantified around 130.000 to 590.000 enclosed air sections per 100 mm^3 of cocoa nib. Their volume ranged from 1.0×10^{-6} mm^3 to 20 mm^3. To understand to what extent the pore size influenced the total porosity of cocoa nibs, the range mentioned above was divided into three pore-size groups:

- Small: 1.0×10^{-6} $mm^3 \leq x < 1.0 \times 10^{-4}$ mm^3
- Medium: 1.0×10^{-4} $mm^3 \leq x < 1.0 \times 10^{-1}$ mm^3
- Large: 1.0×10^{-1} $mm^3 \leq x < 2.0 \times 10^{2}$ mm^3

For each sample, the volumes of the pores (x) falling into those groups were summed. Then the data were normalized by calculating the percentage of each volume-group in the total porosity. The results are presented in Fig. 4.5.

Unroasted cocoa nibs showed equal porosity for small and large pores ($p > 0.05$). Together, they represent approximately 80% of the total porosity. It means that unroasted cocoa has plenty of tiny pores but not many large and medium-sized

pores. The small and medium-sized pores expanded and probably coalesced to generate larger pores during the roasting processes, especially when the cell walls of the nibs were still in their glassy state, as it probably happened during FR. In both FR-120 and FR-140 nibs, the large-sized pores represented around 90% of the total porosity, while in SR nibs, it was about 65%. These results support the differences in density and the possible volume expansion in cocoa nibs, as described above. The roasting temperature did not cause significant effects in changing the porosity profiles of cocoa nibs ($p > 0.05$).

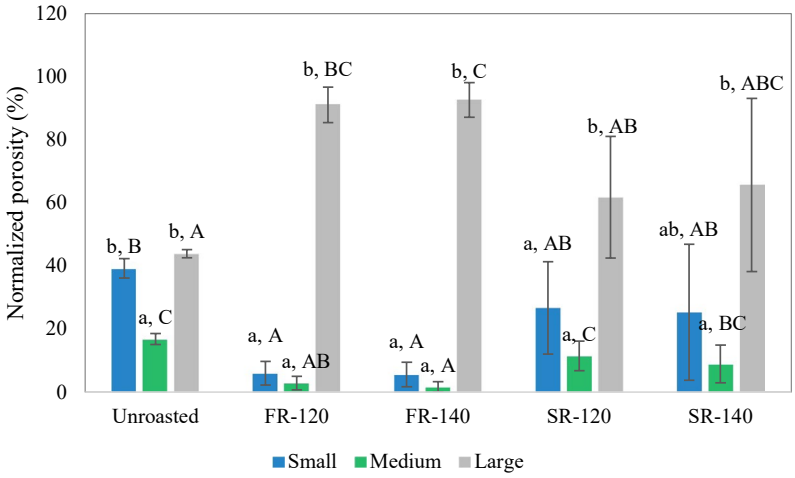

FIGURE 4.5 Normalized porosity of unroasted and roasted cocoa nibs explained in three groups of pore-volume: Small (1.0×10^{-6} mm³ ≤ x < 1.0×10^{-4} mm³), Medium (1.0×10^{-4} mm³ ≤ x < 1.0×10^{-1} mm³), and Large (1.0×10^{-1} mm³ ≤ x < 2.0×10^{2} mm³). FR-120 and FR-140 stand for Fast Roasted cocoa nibs at 120 and 140 °C, and SR-120 and SR-140 for Slow Roasted cocoa nibs at 120 and 140 °C, respectively. Different lowercase letters represent significant differences ($p < 0.05$) among pore sizes. Different uppercase letters correspond to differences ($p < 0.05$) among treatments.

Our results are partially in line with the estimation of porosity in roasted and unroasted cocoa performed by Rojas et al. (2020) with the Brunauer-Emmett-Teller method. They found that the pore distribution shifts from smaller to larger pores upon roasting. However, in their case, the specific internal volume resulted in a diminution effect. The authors acknowledge the interference of fats in clogging small pores, thus affecting their results.

4.3.3 Cocoa butter extractability

Yield of surface-fat extraction

Differences in density and porosity might influence cocoa butter extractability. The role of microchannels in defatting cocoa nibs was explored by superficial solvent extraction. As shown in Table 4.1, the surface-cocoa-butter yield was two folds higher in FR cocoa than in SR cocoa nibs ($p < 0.05$). The SR's yield was not statistically different from that of unroasted cocoa nibs ($p > 0.05$); however, the means were slightly higher. The roasting temperature did not significantly affect surface-cocoa butter remotion ($p > 0.05$). These results confirmed the pore-structure differences between FR and SR cocoa described above. Changing the structure and pore formation in cocoa nibs upon fluidized bed roasting result in a higher surface-solvent interaction and a higher fat bloom; this in turn favors cocoa butter extraction.

Yield of fat extraction by hot-press

Considering the role of porosity in cocoa butter accessibility, we decided to investigate if the different microstructures of fast and slow-roasted cocoa are appreciated in the harsh conditions used in industrial processing. In cocoa industry, a hot press is usually adopted to squeeze out the cocoa butter from cocoa liquors by gradually applying pressure until obtaining an almost defatted cocoa cake. Because roasting temperature did not significantly affect pore formation and surface-cocoa butter extraction in cocoa nibs under both roasting techniques, only cocoa nibs roasted at 140 °C were chosen for the hot-press process. Fig. 4.6 shows the progress of cocoa butter extraction and the increasing pressure applied to cocoa liquors during hot-press defatting. The cocoa butter extraction and pressure growth were similar for unroasted, FR-140, and SR-140 cocoa liquor. The final cocoa butter yield did not differ significantly either ($p > 0.05$), as observed in Table 4.1. It can be concluded that under the grinding conditions applied to prepare the cocoa liquors, the differences in sample microstructure are not relevant for the cocoa butter separation.

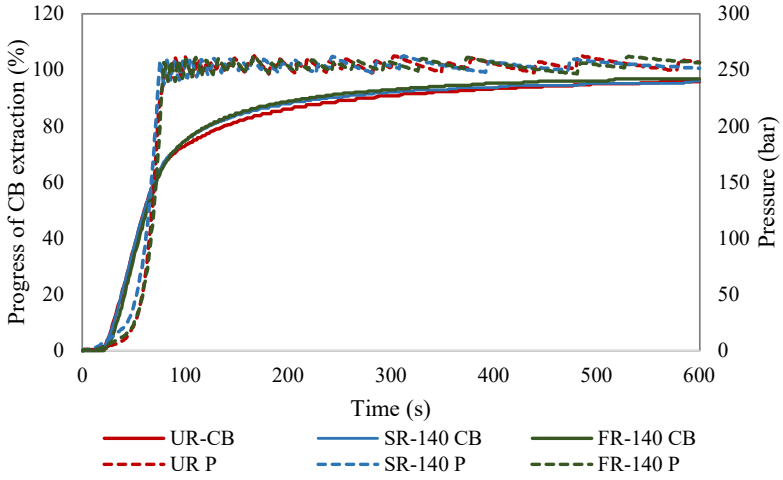

FIGURE 4.6 Hot-press cocoa butter (CB) extraction % (left axis) from cocoa liquors obtained from unroasted cocoa nibs (UR-CB), cocoa nibs slow roasted at 140 °C (SR-140 CB), and cocoa nibs fast roasted at 140 °C (FR-140 CB). UR P, SR-140 P, and FR-140 P correspond to their respective operating pressures (right axis).

4.3.4 Volatile organic compounds (VOC) in cocoa butter

The roasting process of cocoa is responsible for removing and generating desirable and undesirable VOC. A final balance of these dynamics might result in polishing the chocolate aroma. Table 4.2 presents the total VOC peak area counts for each kind of cocoa butter, which were interpreted as flavor 'abundance.' The data shows that roasting significantly increased the total flavor abundance in cocoa butter ($p < 0.05$) by 80% upon FR and by 50% upon SR, but the temperature did not cause a significant change ($p > 0.05$).

TABLE 4.2 Total volatile organic compounds (VOC) and quality parameters of cocoa butter (CB) extracted from cocoa nibs roasted under different conditions

Cocoa butter	Total VOC peak area counts x10^7	Free fatty acids (g oleic acid/100g fat)	Peroxide value (mEq/kg fat)
UR-CB	2.08 ± 0.03^a	1.33 ±0.22^b	1.45 ±0.09^b
FR-120 CB	3.57 ± 0.70^{bc}	1.34 ±0.13^b	1.01 ±0.12^a
FR-140 CB	3.91 ± 0.62^c	1.21 ±0.14^b	1.08 ±0.16^a
SR-120 CB	3.28 ± 0.62^{bc}	0.92 ±0.13^a	1.36 ±0.22^b
SR-140 CB	3.09 ± 0.63^b	0.78 ±0.09^a	1.24 ±0.19^{ab}

UR-CB means unroasted cocoa butter, FR-120 and FR-140 stand for fast-roasted cocoa nibs at 120 and 140 °C, and SR-120 and SR-140 for slow-roasted cocoa nibs at 120 and 140 °C, respectively. Superscripts in the same column indicate significant differences at the 95.0% confidence level. The values correspond to means ± standard deviation.

The total VOC corresponded to 24 compounds belonging to these chemical groups: Organic acids, alcohols, aldehydes, fatty acids, esters, ketones, and pyrazines. Their relative abundance, namely VOC profile, is presented in Fig. 4.7. The horizontal dendrogram separated unroasted cocoa butter (UR-CB) from the treatments, thus demonstrating that both FR and SR significantly changed the VOC profile of cocoa nibs. The dendrogram clustered FR-120 CB with FR-140 CB and SR-120 CB with SR-140 CB. This demonstrates similarities between roasting temperatures and differences between roasting techniques.

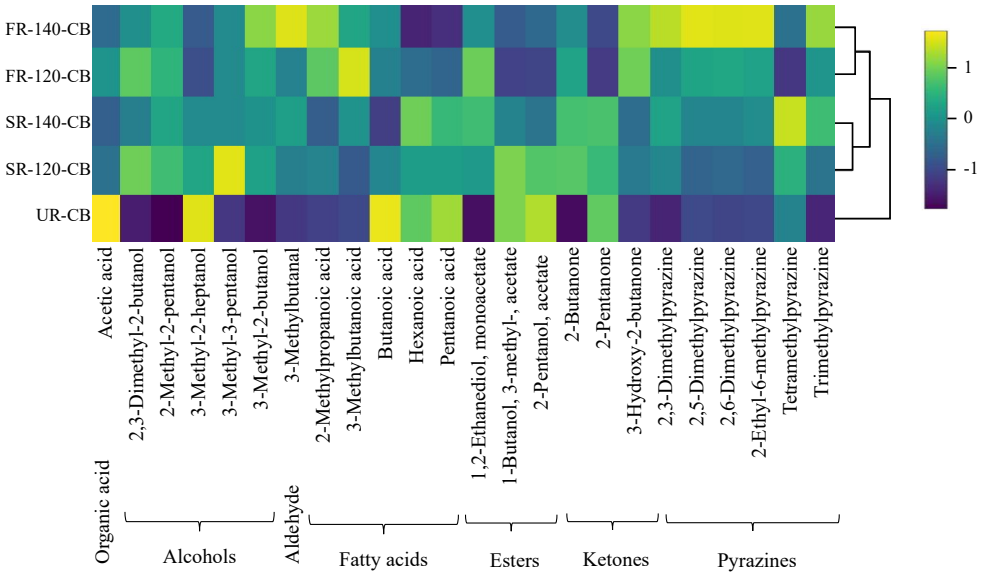

FIGURE 4.7 Heatmap of averaged and normalized peak area counts of the VOC detected by GC-MS in cocoa butter (CB) extracted from: unroasted cocoa nibs (UR-CB), cocoa nibs fast roasted at 120 °C (FR-120-CB) and 140 °C (FR-140-CB), and cocoa nibs slow roasted at 120 °C (SR-120-CB) and 140 °C (SR-140-CB). The data was scaled per column, being zero the mean with a standard deviation of 1. Data were accompanied by color: the mean is represented by green color; values above the mean fade to yellow color, and values below the mean to deep purple color.

As expected, the presence of acetic acid in the four kinds of cocoa butter extracted from the roasted cocoa nibs was not as relevant as in UR-CB. Roasting evaporated a significant part of this organic acid. Another notorious change upon roasting was the increase of pyrazines such as 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethyl-6-methylpyrazine, and trimethylpyrazine; and the aldehyde 3-methylbutanal. Similar findings were reported by Carlin et al. (1986) in cocoa butter. Pyrazines are intermediate Maillard reaction products (Arnoldi et al., 1988; Adams et al., 2008) responsible for cocoa, roasted, earthy, nutty, and sweet notes (Michel et al., 2021; Adams et al., 2008); and 3-methylbutanal is a

Strecker aldehyde that exerts malty flavor (Hofmann and Schieberle, 2000). All these single notes contribute to the whole chocolate aroma in cocoa derivatives. The relative concentration of the mentioned pyrazines and 3-methylbutanal was especially higher in cocoa butter obtained from FR cocoa nibs, particularly at 140 °C; so fluidized bed roasting is likely to produce cocoa butter with a potentially high-naturally-developed chocolate aroma. This same pattern was found in cocoa nibs, as we recently reported (Peña-Correa et al., 2022). Thus, the VOC profile of cocoa nibs is well retained in cocoa butter.

The increase in total flavor abundance and relative abundance of pyrazines and 3-methylbutanal in fluidized-bed-roasted cocoa nibs could be explained by the higher efficiency of the convective heat transfer method: the pressurized hot-air was capable of heating more evenly cocoa nibs from edges to centers, thus harnessing the Maillard reaction reactants (i.e., free amino acids, reducing sugars) present in the whole nib to produce more VOC. The formation of pores across the nibs (Fig. 4.4) demonstrates such heat penetration. In contrast, conductive heat transfer favored the Maillard reaction only in the edges of the nibs and sub-utilized the cores.

Pyrazines are not exclusively formed during roasting. They can also be generated in cocoa beans during fermentation and drying, being the tetramethylpyrazine the most abundant one (Fang et al., 2020). It explains the initial load of pyrazines in UR-CB. The predominance of tetramethylpyrazine over the rest of the pyrazines in UR-CB was preserved upon slow roasting, as shown in Fig. 4.7. A similar tendency was reported by Hashim et al. (1997) and Carlin et al. (1986). Fast roasting reduced the relative concentration of tetramethylpyrazine. It could be explained by (i) a hindering effect of the increased relative abundance of the other VOC, especially the other pyrazines; (ii) higher utilization of tetramethylpyrazine to generate advanced Maillard reaction products (e.g., melanoidins); and (iii) the physicochemical characteristics of FR nibs: as demonstrated in our recent study (Peña-Correa et al., 2022), to ignite the formation of tetramethylpyrazine, there is a higher energy barrier in fast-roasted cocoa nibs than in slow-roasted cocoa; therefore, its generation is more favored in slow roasted cocoa nibs.

Alcohols, esters, and ketones are produced in cocoa beans during fermentation (Fang et al., 2020). Their concentration is exceptionally high in fine-aroma cocoa beans, displaying floral, fruity, and green notes (Mohamadi Alasti et al., 2019; Michel et al., 2021). The VOC profile of the cocoa butter obtained from our four roasting treatments showed a higher relative abundance of most of these compounds than that in UR-CB. Even though roasting evaporated of

some of them, the considerable evaporation of acetic acid enhances the relative abundance of the remaining VOC.

Fatty acids are more related to fatty, waxy, rancid, sweet, and cheesy flavors (Mohamadi Alasti et al., 2019; Frauendorfer and Schieberle, 2006). Its occurrence is attributed to enzymatic activities during harvesting and fermentation processes (Afoakwa et al., 2014). On the one hand, the presence of butanoic, hexanoic, and pentanoic acids was especially relevant in UR-CB, and their relative abundance was reduced upon roasting. These changes could be due to their volatilization during roasting and the formation of secondary lipid-oxidation products. On the other hand, roasting increased the relative abundance of 2-methylpropanoic acid and 3-methylbutanoic acid, especially upon FR. Aligned with our results, Carlin et al. (1986) reported a significant decrease in most of the volatile acids in cocoa butter upon roasting.

It is worth to mention that no typical lipid-oxidative aldehydes responsible for rancid flavors like pentanal, hexanal, and nonanal (Hashim et al., 1997; Grebenteuch et al., 2021) were detected in this study. Our results indicate that fluidized bed roasting is as safe as oven roasting under traditional cocoa roasting temperatures.

The findings given in this section are particularly relevant for white chocolate manufacturing. The unpleasant flavor of unrefined cocoa butter (also known as raw or natural cocoa butter) has been an issue for cocoa industry; therefore, cocoa butter needs to be thoroughly deodorized before use (Gutiérrez, 2017). Consequently, cocoa-derived products containing only cocoa butter as cocoa solid (e.g., white chocolate) lack natural chocolate-like flavors (Beckett, 2008). Future studies are needed to correlate our findings with sensory studies and/or odor threshold assessments, as well as partial deodorization experiments of FR-140-CB for industrial applications.

4.3.5 Cocoa butter quality

Due to the triacylglycerol composition, cocoa butter exhibits unique crystal packing properties responsible for surface brightness, solid consistency, and snap, in well-tempered chocolate bars (Masuchi Buscato et al., 2017). These physical properties and the flavor are negatively affected by oxidation products like FFA and hydroperoxides (determined with the PV). FFA in cocoa butter should not exceed 1.75% w/w, expressed as oleic acid (Codex Alimentarius, 1981), and PV must be below 10 meq/kg fat (Kong and Singh, 2011).

The FFA and PV of unroasted and roasted cocoa butter were under the safe threshold values, as observed in Table 4.2. As expected, neither SR nor FR increased FFA and PV. Fluidized bed roasting did not significantly reduce FFA ($p > 0.05$) but significantly reduced PV ($p < 0.05$). Aligned (Afoakwa et al., 2014; Dorota et al., 2014), partially aligned (Krysiak, 2011), and no aligned results (Djikeng et al., 2018) were reported in previous studies. The use of uncommon roasting temperatures (e.g., 180°C), as well as the origin and postharvest practicals of the cocoa beans (Chaiseri and Dimick, 1989; Marseglia et al., 2020), would determine those differences.

Roasting is also performed to relieve the initial load of primary oxidation products towards the formation of stable oxidation products like VOC. The fluidized bed roasting technique was more efficient than oven roasting due to the more effective heat penetration across the cocoa nibs. A model system study could demonstrate the straight relation between the reduction of hydroperoxides and the formation of specific VOC under cocoa-roasting conditions.

4.4 Conclusions

This study demonstrated that the microstructure of fluidized-bed-roasted cocoa nibs strongly differs from traditional oven-roasted nibs: higher volume expansion and porosity occur, with the predominance of large-sized pores. This phenomenon would be explained by the inability of the parenchyma cell walls to quickly turn from a glassy state to a more elastic condition during the fluidized bed roasting process; consequently, higher disruption of the internal structure and higher release of fat crystals occur.

Compared to oven roasting, the fluidized bed roasting process leaves a higher relative concentration of pyrazines and 3-methylbutanal in cocoa butter. Thus, this technique produces cocoa butter richer in Maillard reaction-derived chocolate aroma compounds than that of oven-roasted cocoa. Moreover, the quality of cocoa butter was not affected by the fast roasting technique; it rather helped to reduce the initial load of hydroperoxides, probably toward the formation of stable compounds like VOC. The high penetration of convective heat (from the pressurized hot-air of the fluidized bed roaster) towards the center of cocoa nibs may have favored the occurrence of the Maillard reaction across the whole nib.

We showed in this study that the pore structure of cocoa nibs is a key quality parameter determined by roasting processing. We concluded by quality and potentially energy-saving considerations that fluidized bed roasting should be preferred over conventional roasting.

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Chapter 5

Fluidized bed roasted cocoa has different chemical characteristics than conventional roasted cocoa

This chapter is based on:

Peña-Correa RF, Ataç Mogol B, Fryganas C, and Fogliano V (**2023**). Fluidized bed roasted cocoa has different chemical characteristics than conventional roasted cocoa. Submitted for publication in Journal of Agricultural and Food Chemistry.

Abstract

The roasting process can modulate cocoa's desired sensory and physicochemical characteristics. This study compared the chemical characteristics of cocoa nibs roasted over aluminum trays and placed inside a convective oven (slow roasting - SR) *vs.* cocoa nibs roasted in a fluidized bed roaster (fast roasting - FR), at two temperatures (120 and 140 °C). The content of sugars, free amino acids (FAAs), polyphenols, acrylamide, 5-hydroxymethylfurfural, and melanoidins was monitored. Roasting reduced the concentration of fructose, glucose, and sucrose by 95, 70, and 55%, respectively, with FR reducing them more than SR. The concentrations of total FAAs were reduced by about 40% at 140 °C, and the FAAs profile revealed that FR favored the consumption of amino acids (Leu, Lys, Phe, and Val) that are relevant in the formation of Maillard reaction aroma compounds and melanoidins. An increase of catechin and the reduction of 5-hydroxymethylfurfural concentrations, together with the generation of more intense brown melanoidins, were more profound in FR cocoa than in SR cocoa.

Keywords: *Theobroma cacao* L., reducing sugars, amino acids, polyphenols, acrylamide, 5-hydroxymethylfurfural, melanoidins

5.1 Introduction

A number of chemical reactions occur during the roasting process of cocoa beans (the fermented and dried seeds of *Theobroma cacao* L.) with a prominent role played by Maillard reaction (MR). In a well-designed manufacturing process, MR mainly leads to improvement in texture,[1] the generation of chocolate aroma,[2] and the reduction in bitterness.[3] Changes in browning can also take place in cocoa due to the generation of melanoidins; however, they are less evident than in coffee due to the natural brown color of unroasted cocoa.[4]

Monosaccharides, free amino acids, peptides, proteins, and polyphenols are the main reactants of MR in cocoa.[5] The complexity of the interactions that these compounds undergo under heating treatments has been thoroughly investigated.[6–14] Recent studies have suggested that the presence of phenolic compounds such as epicatechin and catechin in cocoa melanoidins is also connected to MR development.[15, 16] However, the precise pathway of phenolic incorporation to melanoidins has not been described yet.

The most relevant MR products in cocoa are:

- Volatile odor-active compounds such as Strecker aldehydes and pyrazines.[9] Strecker degradation aldehydes exert sweet aromas, while pyrazines display more chocolate-type and roasted flavors.[2]
- Melanoidins, which are heterogeneous high molecular weight compounds (HMWC) with diverse yellow to brown hues and antioxidant properties.[10, 12]
- Acrylamide and 5-hydroxymethylfurfural (HMF). Both are intermediate-MR products; however, HMF may also be formed via sugar caramelization reactions. HMF and acrylamide, especially the latter, have been considered thermal process contaminants due to their potential adverse effects on humans.[17]

Different roasting conditions can modulate MR by modifying the concentration of the above-mentioned reactants and the generation of MR products; however, relatively little innovation in cocoa roasting has been proposed in the last 50 years.[5]

The fluidized bed is a roasting technique originally developed for coffee processing. It consists in blowing a strong flow of hot air from the bottom of the roasting chamber, thus making the solid particles to constantly move, resembling a stirring fluid.[18] This technique involves almost 100% convective heat transfer and is recognized for its low-carbon footprint.[19] In contrast, oven roasting is

highly based on conductive heat transfer from the metallic surface of the trays, thus resembling the traditional cocoa roasting. In our previous studies, we found that the heat transfer efficiency of fluidized bed roasting reduced the roasting time by a factor of 12 compared to roasting on trays inside a forced-convective oven. As a consequence, fluidized bed roasting led to (i) a higher porosity, thus suggesting a deeper heat penetration within cocoa nibs (as discussed in Chapter 4, Section 4.3.2), and (ii) a lower reduction in water activity,[4] which favored the formation of nitrogen-heterocyclics such as pyrazines. The changes in the chemical composition that fluidized bed roasting leads to precursors and MR products in cocoa deserve investigation.

The objective of the current study is to compare the effect of two different roasting techniques (fluidized bed roasting and convective oven roasting with aluminum trays) and two temperatures (120 °C and 140 °C) on the formation of MR-related compounds by monitoring the content of sugars, amino acids, polyphenols, HMF, and acrylamide, and the formation of melanoidins in cocoa nibs.

5.2 Materials and methods

5.2.1 Chemicals and samples

Fermented and dried Forastero cocoa beans (*Theobroma cacao* L.) from Ivory Coast, with a water content of 6.14 ± 0.02 % w/w, were supplied by Olam International (Koog aan de Zaan, The Netherlands). Petroleum ether with a boiling point between 40-60°C, methanol (99.9%), and acetonitrile (HPLC grade) were purchased from Actu-All Chemicals (Oss, The Netherlands). Formic acid (99%) was purchased from Sigma Aldrich (St. Luis, USA). Carrez I (potassium hexacyanoferrate (II) trihydrate 10.6%m/v) and Carrez II (zinc sulfate 22%m/v) solutions were purchased from Chem-Lab (Zedelgem, Belgium). Cellulose acetate (CA) and polytetrafluoroethylene (PTFE) syringe filters were purchased from Phenomenex (Niederlassung, Germany). Oasis HLB solid phase extraction cartridges were purchased from Waters (Milford, MA, USA). The following standards were obtained in analytical standard quality (purity ≥ 97%) from Sigma Aldrich (St. Luis, USA): D-(-)-fructose, D-(+)-glucose, sucrose, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, (-)-epicatechin, (+)-catechin, procyanidin B2, chlorogenic acid, gallic

acid, caffeic acid, ferulic acid, acrylamide, and 5-hydroxymethylfurfural. The system of PureLab Ultra (ELGA LabWater, UK) prepared MilliQ water.

5.2.2 Roasting process of cocoa nibs

The remotion of cocoa beans' husks and the roasting process of cocoa nibs at 120 and 140°C (initial water content of 4.31 ± 0.11 % w/w) in a fluidized bed roaster and a convective oven provided with aluminum trays were performed as described in Chapter 4, Section 4.2.2. Because the time needed to roast cocoa nibs in a fluidized bed roaster was about one-twelfth of the time needed to reach the same water content (1.0 ± 0.1% w/w) in a convective oven, the fluidized bed roasting technique was referred to as fast roasting (FR), and the convective oven as slow roasting (SR). Unroasted cocoa nibs (UR) were separated as a control. All the samples were vacuum-packed and stored at -20°C until usage.

5.2.3 Preparation of cocoa powder (CP)

A defatting procedure with petroleum ether was performed to obtain cocoa powder (defatted ground cocoa nibs), as described in Chapter 4, Section 4.2.6 (Chemical extraction of cocoa butter for chemical analysis). The solvent containing cocoa butter was discarded, and the pellets and the sediments (cocoa powder) were air-dried (48 h, RT) in a fume hood. They were gently stirred with a spatula every 12 h. The fat-free and solvent-free cocoa samples, namely cocoa powder (CP), were put into airtight plastic containers and stored at -20 °C until used for chemical analysis.

5.2.4 Measurement of pH in cocoa powders

Solutions of 0.1g CP/mL were prepared in MilliQ water. They were vortexed (1 min, RT) and centrifuged (4700 rpm, 5 min, RT). The supernatants were used to measure the pH with a calibrated digital pH-meter (Serial Nr. 11110295, UWR International, Germany).

5.2.5 Determination of free sugars content in cocoa powders

Fructose, glucose, and sucrose were determined on 2.5 g of CP extracted with 10.5 mL of MilliQ water, 12.5 mL of ethanol, and 1 mL of each Carrez solution I and II. First, the mixture was vortexed for one minute, then heated for 60 min in a water bath at 50 °C, and centrifuged at 10.000 rpm for 5 min (5430 R, Eppendorf, Hamburg, Germany). The supernatant was filtrated through a 0.2 μm CA syringe filter (Φ 28mm) and collected into HPLC vials. The extraction process

was performed in duplicate. The samples were immediately analyzed using an HPLC Ultimate 3000 system 1 (Thermo Fischer Scientiefic™, Massachusetts, USA) with a detector RI-501 (Shodex, Munchen, Germany) and an evaporative light scattering detector (ELSD) (Polymer Labs, Washington, US) under these settings: evaporation temperature 90 °C, nebulizer temperature 50 °C, and carrier flow 1.60 slm. A Grace prevail carbohydrate ES column (5 µm, 250 mm x ID 4,6 mm) was used. The elution was achieved using 75% acetonitrile and 25% Milli-Q water. Calibration curves of fructose, glucose, and sucrose (0.25-2.00 mM) were prepared with their respective standards. Finally, the results were processed using the software Chromeleon™ version 7.2.6 (Thermo Fisher Scientific™, Massachusetts, USA). The limits of detection and quantification for fructose, glucose, and sucrose, calculated from the signal noise area, were 0.01 and 0.03 mM, respectively.

5.2.6 Determination of free amino acids content in cocoa powders

For the determination of the free amino acids (FAAs) alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), and valine (Val) in cocoa powder, on 1 g of sample was extracted three times with 20 mL of water (10-5-5 mL) and vortexed (5 min) in each stage. After centrifugation (4650 g × 3 min), the supernatants were collected and combined. A sample of the combined extract (200 µL) was mixed with acetonitrile (1:4, v/v), centrifuged (4650 g × 3 min), and filtered through a 0.45 µm syringe filter into an HPLC vial. The FAAs were analyzed using an Agilent Ultivo system consisting of an Agilent 1260 Infinity II LC system coupled to an Agilent 6465 triple quadrupole mass spectrometer according to the method described by Salman and coworkers (2021).[20] The extraction was performed in duplicate.

5.2.7 Determination of polyphenols in cocoa powders

The flavan-3-ols epicatechin, catechin, and Procyanidin B2 (P-B2), and the phenolic acids chlorogenic acid, gallic acid, caffeic acid, and ferulic acid were determined on 100 mg of cocoa powder. The sample was vortexed with 10 mL of MilliQ (10 min) and centrifuged (4700 rpm for 8 min, RT). A sample of 200 µL of supernatant was added with 800 µL of a solution of methanol with water (1:1, v/v). The mixture was vortexed (2 min) and centrifuged (12.000 rpm for 10 min, RT). The supernatant was filtrated through 0.2 µm PTFE Φ 15 mm filters and collected into HPLC amber vials.

The extracts were analyzed by a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The UPLC unit consisted of a SIL-30AC autosampler, a LC-20ADXR solvent delivery module, DGU-20ASR degassing unit, a CTO-20AC column oven and a FCV-20AH$_2$ valve unit. Five µL of each sample contained in vials were injected into a Kinetex Evo C18 column (2.6 µm, 2.1 × 100 mm, 100 Å, Phenomenex, Torrance, CA, USA). The flow rate was set at 0.5 mL/min and the column temperature at 40 °C. The mobile phases consisted of 0.2% formic acid (solvent A), acetonitrile with 0.2% formic acid (solvent B) with the following elution profile (t in [min]/[%B]): (0.0/5), (0.5/5), (2.0/25), (5.0/50), (7.0/95), (8.5/95), (8.60/5) and (12.5/5). The voltage of the turbo ion-spray ionization was 4.0 kV. The temperature of the electrospray ionization probe, desolvation line, and heat block were set at 300 °C, 250 °C, and 400 °C, respectively. The pressure of the gas for the collision-induced dissociation was 4 kPa, whereas the flow rates of the drying gas, nebulizer gas, and heating gas were set at 10 mL/min, 3 mL/min, and 10 mL/min, respectively. The electrode voltage of Q1 pre bias (collision cell energy entrance potential), collision cell Q2 (collision energy), Q3 pre bias (collision cell energy exit potential), parent and fragment ion m/z of the multiple reaction monitoring transitions were optimized using standard solutions of the target analytes (concentration 10-20 mg/L) and the support software (Shimadzu Corporation, Kyoto, Japan). The dwell time and the time window for MS data acquisition in the negative mode were also optimized for single reaction monitoring (SRM). The most abundant fragment ion was selected for quantitation. The second and third fragments in ion yield were selected for structural confirmation based on the optimized SRM transition (Appendix 5.1). Finally, the data was processed with the software LabSolutions (Shimadzu Corporation, Kyoto, Japan). The concentration of each phenolic compound in samples was calculated by means of a calibration curve built in the range between 25 µg/L and 15 mg/L. The limits of detection and quantification, based on the standard deviation of the blank response, can be observed in Appendix 5.1.

5.2.8 Determination of acrylamide and 5-hydroxymethyl furfural (HMF) in cocoa powders

A triple extraction procedure was carried out in duplicate for the quantification of acrylamide and HMF in 1 g of CP. The first extraction was done with 9 mL of 10 mM formic acid, 0.5 mL of Carrez I, and 0.5 mL of Carrez II solutions by vortexing (3 min) and then centrifuging (3000 rpm for 5 min). The second and third extractions were performed with 5 mL of 10 mM formic acid each. All

supernatants were combined, centrifuged (10.000 rpm, 5 min, RT), and kept at -20 °C until analysis.

For acrylamide analysis, 1 mL of supernatant was passed through a preconditioned Oasis MCX solid phase extraction cartridge, and the pure extract was analyzed using the LC-MS/MS according to the method described by Žilić and coworkers (2020).[21] The limits of detection and quantitation of acrylamide, calculated from the signal noise area, were 3 and 10 ng/g cocoa, respectively.

For HMF analysis, 1 mL of supernatant was passed through a preconditioned HLB solid-phase extraction cartridge. The eluent was discarded, and HMF was eluted with 1 mL of methanol and collected into HPLC glass vials for further HPLC analysis in an HPLC Ultimate 3000 Dionex (ThermoScientific, Germany) equipped with a Diode Array Detector and a temperature-controlled column oven. The chromatographic separations were performed on a C18-A 4.6x150 mm 5µm Polaris column (Agilent, Santa Clara, USA) using the mixture of 10 mM aqueous formic acid solution and acetonitrile (95:5, v/v) at a flow rate of 1.0 mL/min for 28 min at 20 °C. Data acquisition was performed by recording chromatograms at 283.5 nm using the software Chromeleon 7.2 (ThermoFisher Scientific, USA). The concentration of HMF was calculated through a calibration curve built in a range between 0.1 and 10 µg/mL. The limits of detection and quantifications of HMF, calculated from the signal noise area, were 0.03 and 0.10 µg/mL, respectively.

5.2.9 Determination of water-soluble compounds above 20 kDa

To obtain native high molecular-weight compounds and high molecular-weight melanoidins, an extractive procedure was performed based on a previous study.[16] Briefly, 10 g of CP was thoroughly mixed with 80 mL of Milli-Q water using a homogenizer (Ultra-Turrax®, T25 digital, Probe T4, IKA, Staufen, Germany) for 4 min at 9000 rpm. The mixture was capped and placed in a shaking hot water bath (70 °C, 20 min, 80 rpm) and ultracentrifuged (15000 rpm for 15 min) (Avanti ultra-centrifuge, Rotor ID 16250, Beckman Coulter, USA). The pellet was discarded, and the supernatant was successively vacuum-filtrated through Whatman filter papers Nr 4, 44, and 602.

The last filtrate was ultrafiltrated in a stirring cell unit (Amicon®, model 8400, max. volume 400 mL, Millipore, Billerica, USA) equipped with a 20 kDa membrane (Mycrodyn Nadir, Nadir FM UP020Pes, Sterlitech, USA) under a positive pressure of 4.5 bar generated by nitrogen supply. Three washing steps with 30 mL of Milli-Q were done on the retentate when its volume was about 20 mL. The filtrates were discarded, and the retentate (about 20 mL) was freeze-dried at -80 °C (Christ,

Alpha 2-4 LDplus, Osterode am Harz, Germany). The weight of the dry fraction (W_{HMWC}), and the weight of the cocoa powder (W_{CP}) were used to calculate the relative content of water-soluble high molecular weight compounds (HMWC) > 20 kDa present in cocoa powder, according to Eq. 5.1.

$$HMWC\left(\%\, w/w \text{ d.b.}\right) = \frac{W_{HMWC}}{W_{CP}} \times 100 \qquad \text{EQUATION 5.1}$$

5.2.10 Analysis of brown compounds

Solutions of 3.3 mg /mL were prepared with the freeze-dried HMWC extracts and filtrated with 0.45 nm CA filters. The browning intensity was determined by measuring their absorbance (420 nm, RT) with a spectrophotometer (Cary® 50 UV-Vis Spectrophotometer,Varian, Austrialia). MilliQ water was used as blank.

5.2.11 Statistical analysis

Data were statistically analyzed by ANOVA. The Least Significant Differences (LSD) method with 95% significance was applied using the statistic software StatGraphics Centurion XVIII (StatGraphics Technologies Inc., USA). The heat map analysis with dendrogram clustering was performed using R commander 3.6.1 (R Foundation, Austria) and R studio (RStudio Team, USA).

5.3 Results and discussion

5.3.1 pH in cocoa powder

The pH of cocoa powders was measured to confirm their acidity and to know to what extent the pH was affected by roasting conditions. The pH of unroasted CP was 5.44 ± 0.13, and it was significantly different from the four roasting conditions ($p < 0.05$), which ranged from 5.29 to 5.32 without significant differences among them ($p > 0.05$); therefore, changes found in the coming subsections cannot be attributed to pH.

It is well known that the Maillard reaction is dependent on the pH of the food. At low pH (< 7), the formation of furanic compounds (e.g., HMF) from Amadori rearrangement products is favored, whereas the routes to reductones and fission products, which are highly responsible for the formation of volatile aromatic compounds, are preferred at pH >7.[22] In addition, the non-enzymatic browning reactions are accelerated in neutral or alkaline conditions.[23]

The effect of roasting on decreasing the pH of cocoa powder was also reported.[16] Non-enzymatic browning reaction in model systems also demonstrated a gradual decrease in pH.[23]

5.3.2 Decrease in sugars' content during roasting

The MR is the main reaction occurring during the roasting process of cocoa nibs. Reducing sugars, such as glucose and fructose, are important reactants of MR.[24] Fructose, glucose, and sucrose contents in unroasted cocoa powder were 667.59 ± 4.25, 569.56 ± 9.38, and 1691.26 ± 59.74 mg/100g d.b., respectively. These values were significantly reduced ($p < 0.05$) during the four roasting conditions, as shown in Fig. 5.1. The decreases in fructose (by approximately 95%) and glucose (by approximately 70%) were more pronounced than in sucrose (by approximately 55%). These results were expected, as fructose and glucose are reducing sugars that directly react with an amine source, while sucrose first needs to be hydrolyzed into its monomers.[25]

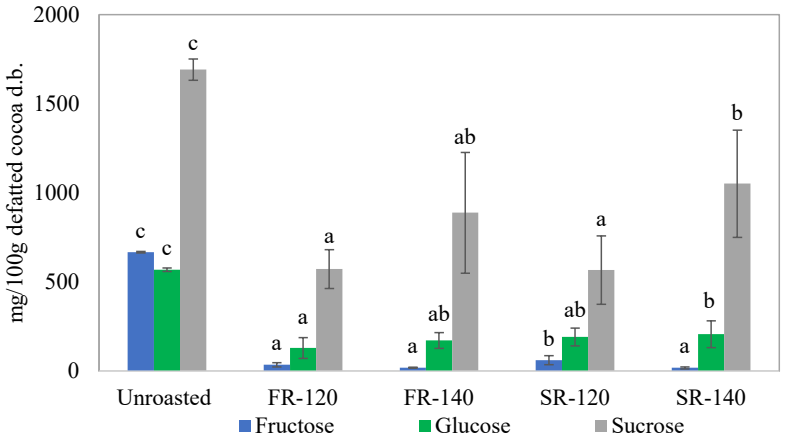

FIGURE 5.1 Fructose, glucose, and sucrose content in unroasted cocoa and cocoa roasted under different conditions: Fast roasting at 120 °C (FR-120) and 140 °C (FR-140), and slow roasting at 120 °C (SR-120) and 140 °C (SR-140). The results are expressed in mg / g of cocoa powder (CP) d.b. The error bars correspond to the standard deviations, and the lowercase letters represent significant differences ($p < 0.05$) among roasting conditions for each kind of sugar.

Fructose content in SR-120 cocoa was significantly higher ($p < 0.05$) than that in cocoa roasted under the other three roasting conditions, which were not significantly different ($p > 0.05$). There was a significant effect of roasting temperature on fructose content only by slow roasting, with the highest reduction at the highest temperature. Neither the roasting technique nor the roasting temperature significantly affected the glucose content in cocoa nibs ($p >$

0.05); however, there was a slight tendency in FR cocoa to have the lowest glucose content at equal roasting temperatures. Regarding the sucrose content, it was not significantly affected by the roasting techniques ($p > 0.05$), but it was influenced by roasting temperature under SR ($p < 0.05$) and FR ($p > 0.05$) conditions: the lowest the temperature, the highest the reduction of sucrose. For each roasting technique, this inverse effect of roasting temperature in sucrose reduction might be a time effect instead: the experiments performed at 120 °C required about 40% extra time to reach the same water content as those executed at 140 °C. Thus, our data suggested that the conversion of sucrose into its monomers might be more time-dependent than temperature-dependent.

Our results of fructose, glucose, and sucrose content in unroasted cocoa powder cannot be easily compared with previous studies, as the initial content of sugars is affected by heterogeneous conditions of fermentation.[26] Despite the initial differences, the general effect of roasting in the reduction of fructose, glucose, and sucrose is aligned with other studies.[26, 27]

5.3.3 Reduction of free amino acids concentration during roasting

During heating treatments, two opposite phenomena influence the FAAs concentration in cocoa: amino acids are released via degradation of proteins and peptides,[28] and FAAs are consumed to produce volatile odor-active compounds,[1,29] and melanoidins.[30] At the roasting conditions of cocoa between 120 to 140 °C, the consumption of FAA is predominant over the formation: we found a significant reduction ($p < 0.05$) in total FAAs content under the four conditions, as shown in Fig. 5.2. There was not a significant effect of the roasting technique, but roasting temperature determined the extent of reduction of FAAs by about 40% at 140 °C and 30% at 120 °C. In other words, the higher temperature (140 °C) had a more profound effect on decreasing total FAAs content than on producing. These phenomena can be explained by the temperature dependence of formation of volatile compounds such as pyrazines[4] and melanoidins.[31]

Comparing the total FAAs content in unroasted cocoa with other studies is far from simple, as it comes from the sum of the single FAAs. The 20 common amino acids have not been covered in one cocoa study. Most of the cocoa studies reporting total FAAs analyzed 15 to 18 amino acids.[29, 32-36] Also our study misses one amino acid: cysteine; nevertheless, it is perhaps the most complete FAA study of cocoa reported to date. Although FAAs content in unroasted cocoa is also affected by fermentation,[36] the reduction of total FAAs upon roasting is aligned with other studies.[29, 32-35]

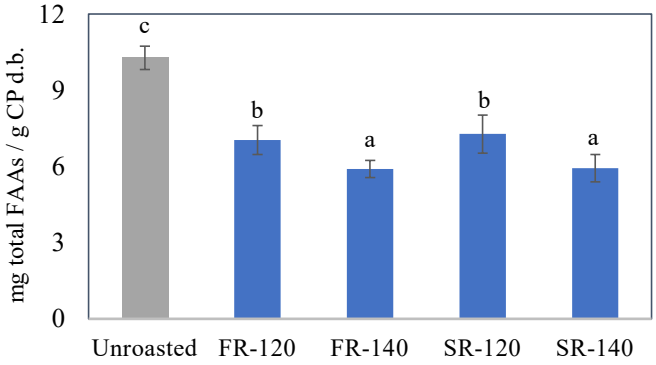

FIGURE 5.2 Total free amino acids (FAAs) content in unroasted cocoa and cocoa roasted under different conditions: Fast roasting at 120 °C (FR-120) and 140 °C (FR-140), and slow roasting at 120 °C (SR-120) and 140 °C (SR-140). The results are expressed in mg / g of cocoa powder (CP) d.b. The error bars correspond to the standard deviations, and the lowercase letters represent significant differences ($p < 0.05$).

The concentration of each amino acid decreased upon the four roasting conditions ($p < 0.05$); however, some amino acids were more affected than others under specific treatments, as reflected in the heatmap presented in Fig. 5.3. The heatmap was generated from the normalized data previously converted to mmol / g CP d.b. (Appendix 5.2). The vertical dendrogram of the heatmap separates two main groups of amino acids: Group 1 gathers the amino acids Gln, Met, Phe, Ile, Leu, Lys, Tyr, Val, Arg, and Pro, and Group 2 Ala, Asp, Gly, Ser, Thr, Asn, Glu, Trp, and His. The roasting technique determined them: The FAA concentration of Group 1 was more reduced under fluidized bed roasting, while the concentration of FAAs of Group 2 was more affected by oven roasting, as observed in the purple areas in Fig. 5.3. The roasting temperature determined the subdivision of Group 2: SR-120 reduced the concentration of Ala, Asp, Gly, and Ser, while SR-140 decreased the concentration of Thr, Asn, Glu, Trp, and His. Group 1 is also subdivided into two groups (Fig. 5.3); however, the separation was not determined by roasting temperature. FR-140 reduced the concentration of Met, Phe, Ile, Leu, Lys, and Tyr, while FR-120 decreased the concentration of Val, Arg, and Pro.

Appendix 5.2 shows that the five most relatively abundant FAAs (in terms of moles) in our unroasted cocoa were Ala, Leu, Asn, Phe, and Tyr (decreasing order). They covered half of the total FAAs moles. Leucine, phenylalanine, and tyrosine were more reduced upon FR-140, while alanine was more affected by SR-120 and asparagine by SR-140 conditions. Model system studies showed these five amino acids have a prominent role in the formation of volatile organic compounds with odor activity[9, 37] and melanoidins[8, 10, 38]. Following the fate of the FAAs in a real-

food experiment is challenging as their initial concentration is not even, and their contribution to the formation of MR products can differ.

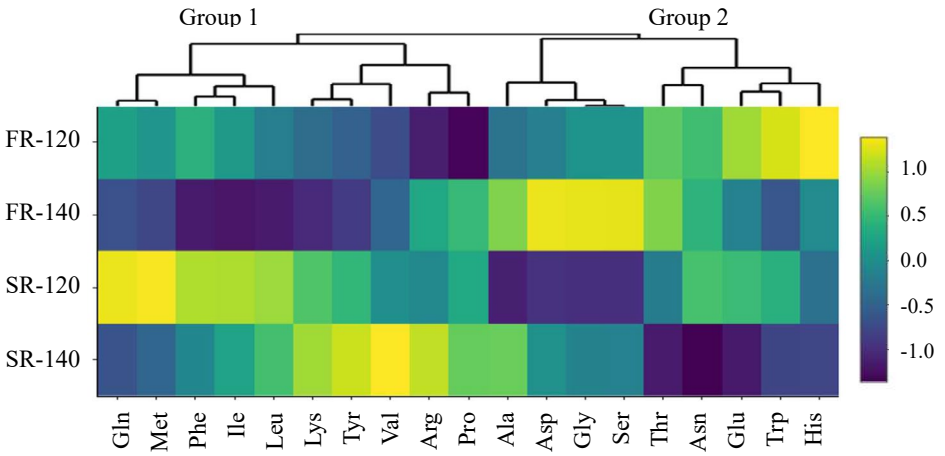

FIGURE 5.3 Heatmap of the relative abundance of free amino acids content (based on mmol) in cocoa powder (d.b.) obtained from cocoa nibs roasted under different conditions: Fast roasting at 120 °C (FR-120) and 140 °C (FR-140) and slow roasting at 120 °C (SR-120) and 140 °C (SR-140). Data were scaled per column.

The MR chemistry is influenced by the FAA concentration and the degree of versatility of the amino acids to produce volatile compounds. According to the cocoa-model systems performed by Arnoldi & coworkers (1988),[9] Leu, Lys, Phe, Val, Thr, Glu, Ala, and Asp are amino acids involved in generating diverse pleasant odor-active volatile compounds via MR like pyrazines and Strecker degradation aldehydes. However, some of them were more specific than others. Leucine was the most versatile amino acid; it produced 11 pyrazines out of 22, followed by threonine with 9, and valine and phenylalanine with 8 each. Ala, Asp, and Glu were more specific amino acids generating 4, 3, and 2 pyrazines, respectively.[9] Cha & coworkers (2019)[37] confirmed leucine's ability to produce various pyrazines.

On the one hand, the ability of leucine to produce various pyrazines is exceptional. It was the second most relatively abundant amino acid in our unroasted cocoa, and fluidized bed roasting, specially FR-140, reduced it significantly. Phe and Val, which were the fourth and the sixth more relatively abundant FAAs in unroasted cocoa (Appendix 5.2), are also very versatile amino acids in terms of formation of pyrazines. Their consumption was also favored by FR. These results are aligned with the greater formation of pyrazines in fast-roasted cocoa nibs compared with the slow-roasted nibs, as reported in our recent study.[4]

On the other hand, alanine was the most abundant amino acid in our unroasted cocoa; however, its relative concentration did not decrease upon roasting; it rather increased. The absolute value of the concentration of Ala was reduced by about 15%, while Leu was degraded by about 50%. The higher affectation of Leu, among other amino acids, determined the increase in relative concentration of the less affected amino acids like Ala. The sub-utilization of Ala could be due to its high specificity in producing volatile MR products.[9] SR did not favor the utilization of Leu, Val, and Phe as FR did, but it rather favored the degradation of Thr, another versatile amino acid, but not an abundant one in our cocoa nibs (twelfth position).

Other factors, like water activity, have essential roles in the performance of MR.[8, 39] As reported in one of our previous studies,[4] the a_w of fluidized bed roasted cocoa nibs was 0.1 a_w-units above oven-roasted cocoa ($a_w \approx 0.2$). This difference might have also determined the FAAs profile. Moreover, the efficiency of the pressurized hot air to penetrate the cores of the nibs might have harnessed the amino acids towards the formation of MR products, while the conductive heat transfer from the trays of oven roasting worked mainly at the edges, as evidenced by porosity formation, as discussed in Chapter 4, Section 4.3.2.

In summary, these results demonstrate that the availability of the amino acids can be modulated by roasting technique and roasting temperature, and that fluidized bed roasting, especially at 140 °C, optimizes the utilization of amino acids like Leu, Phe, and Val, which have proven to be highly involved in the formation of desirable MR aroma compounds.

5.3.4 Changes in polyphenols concentration during roasting

Polyphenols undergo variable chemical reactions during heat treatments, such as depolymerization, epimerization, and condensation.[40] When polyphenols undergo condensation reactions, probably via the formation of melanoidins, their bitterness and astringency are mitigated, thus improving cocoa flavor.[3] The changes in epicatechin, catechin, procyanidin B2 (P-B2), and ferulic acid content in cocoa under the four roasting conditions are presented in Fig. 5.4. Chlorogenic acid, caffeic acid, and gallic acid were also analyzed, as they have been found in cocoa in free form and bound to melanoidins;[15] however, we did not detect them in our samples.

The effect of the four roasting conditions on the concentration of epicatechin, catechin, and P-B2 is presented in Fig. 5.4. As expected, the four roasting processes significantly reduced ($p < 0.05$) the concentration of epicatechin and P-B2, the

two more abundant phenolic compounds in cocoa. SR-120 proved to be the less severe treatment with the lowest reduction in epicatechin and P-B2, and no significant modification in catechin content ($p > 0.05$). The concentration of epicatechin and P-B2 upon FR-120, FR-140, and SR-140 was not significantly different ($p > 0.05$). Catechin content increased significantly under SR-140, FR-120, and FR-140 conditions, and was also determined by roasting temperature: the highest the temperature (140 °C), the highest the catechin content. Catechin content was also influenced by roasting technique ($p < 0.05$): at equal roasting temperatures, fluidized bed roasted cocoa had the highest catechin content. The rise in catechin could be due to epimerization reactions of epicatechin,[40] which seems to be favored by FR. The concentration of ferulic acid in cocoa was not significantly affected by any roasting condition.

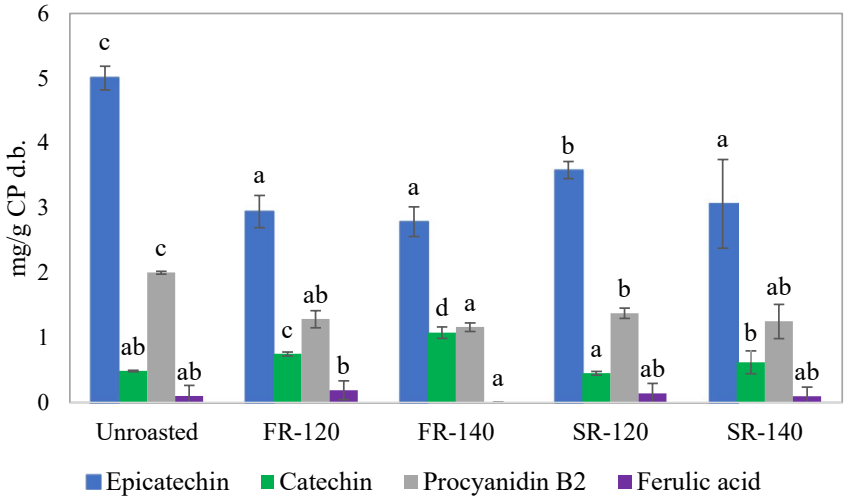

FIGURE 5.4 Epicatechin, catechin, procyanidin B2, and ferulic acid content in cocoa powder (CP) d.b. obtained from unroasted cocoa nibs, and cocoa nibs roasted under different conditions: Fast roasting at 120 °C (FR-120), fast roasting at 140 °C (FR-140), slow roasting at 120 °C (SR-120), and slow roasting at 140 °C (SR-140). The error bars correspond to the standard deviations, and the lowercase letters represent significant differences ($p < 0.05$) among roasting conditions for each phenolic compound.

The sum of epicatechin, catechin, P-B2, and ferulic acid in unroasted, FR-120, FR-140, SR-120, and SR-140 cocoa, accounted for 7.59 ± 0.30, 5.17 ± 0.45, 5.03 ± 0.36, 5.55 ± 0.35, and 5.02 ±1.46 mg / g CP d.b, respectively. The four roasting conditions significantly reduced the sum of these compounds ($p < 0.05$). SR-120 had the highest total polyphenol content of the four kinds of roasted cocoa; however, there were no statistical differences among them ($p > 0.05$). Aligned

results on epicatechin, P-B2, and catechin contents in unroasted cocoa, and the effect of roasting on the decrease of epicatechin and P-B2 have been reported.[27, 41-44] Looking at the literature, there are several discrepancies in the dynamics of the trends of catechin concentration during roasting. Żyżelewicz & coworkers (2016)[44] demonstrated that catechin increases steadily during the first 15 min of roasting (135 and 150 °C) and then gradually drops to its initial content. According to De Taeyte & coworkers (2017),[41] the epimerization of epicatechin into catechin during roasting occurred only in Criollo cocoa beans but not in Forastero and Trinitario, which showed a decrease in catechin content. In contrast, Oracz and Nebesny (2018)[27] reported a similar decrease in catechin content in the same three cocoa varieties.

The results presented in this study demonstrate that roasting leads to a decrease in the content of free polyphenols in cocoa, and also suggest that the increase in catechin content, probably via epimerization reactions of epicatechin, was more pronounced during fluidized bed roasting.

5.3.5 Formation of acrylamide and HMF during roasting

The development of the MR under the different roasting conditions was assessed by measuring the concentration of acrylamide and HMF in cocoa. The analysis of acrylamide in cocoa is in progress. Cocoa is a complex matrix providing many interferences, thus making the analysis cumbersome. HMF was successfully analyzed. It was formed up to 18 mg/kg CP d.b., as observed in Table 5.1.

TABLE 5.1 HMF content in cocoa powder (CP) obtained from cocoa nibs roasted under different conditions.

Roasting condition	mg HMF / kg CP d.b.
Unroasted	Not detected
FR-120	3.56 ± 0.06^{a}
FR-140	4.43 ± 0.34^{b}
SR-120	12.48 ± 0.22^{c}
SR-140	18.01 ± 0.14^{d}

The results are expressed as means ± standard deviations. Lowercase letters in the same column represent significant differences ($p < 0.05$).

HMF was not detected in unroasted cocoa despite the thermal process involved in the deshelling process. Table 5.1 shows that the formation of HMF in cocoa was significantly higher under SR than under FR ($p < 0.05$). Data also demonstrated that the higher the roasting temperature, the higher the HMF content, having SR

the most remarkedly effect. Two aspects of FR cocoa could have mitigated the formation of HMF: (i) The formation of HMF strongly increases when reducing a_w.[45] As demonstrated in our previous study,[4] fluidized bed roasting led to a lower reduction in a_w in cocoa nibs than oven roasting. (ii) The higher content of free catechin and other phenolic compounds present in FR cocoa could have trapped carbonyl compounds, thus hindering the formation of HMF.[11, 46]

Our study demonstrates that, although unroasted cocoa had a pH < 7 and contained the reactants to produce HMF either via sugar dehydration or MR,[17] the formation of this compound was reduced when using a fluidized bed roaster, even at high roasting temperatures. HMF content in our roasted cocoa was aligned with the results reported by Quiroz and Fogliano (2018)[16] (17 – 75 mg / kg CP), while Sacchetti (2016)[31] reported lower values (0.1 – 0.8 mg / kg CP) and Maldonado-Mateus, et al. (2021)[47] higher values (3000 to 8000 mg/ kg CP). Several factors can account for these differences, e.g., the pH, the chemical composition of the raw material, the roasting procedure (s), and the analytical method. Despite the differences in concentration of HMF in roasted cocoa, these studies and ours have something in common: the increase in HMF content when roasting temperature increases.

Acrylamide can be formed from sugars and free asparagine during MR.[17] Free asparagine content was significantly degraded by both roasting techniques, especially under SR-140 conditions (Fig. 5.3), probably due to the formation of Strecker aldehydes like acrylamide. Other studies have reported the formation of acrylamide when roasting whole cocoa beans.[48, 49]

5.3.6 Amount of water-soluble high molecular weight compounds (HMWC) and their brown color intensity

Cocoa melanoidins are brown-colored heterogeneous HMWC roughly ranging from 30 to 70 kDa.[27] The content of water-soluble HMWC (> 20 kDa) in unroasted and roasted cocoa is presented in Table 5.2. Data show that soluble HMWC (likely polysaccharides, proteins, and condensed polyphenols) constitute 3% of unroasted CP. The amount of compounds > 20 kDa in FR-120 and FR-140 cocoa was slightly lower ($p > 0.05$) than that of unroasted cocoa. In contrast, the weight of the matter > 20 kDa in SR cocoa was significantly higher ($p < 0.05$) than in unroasted CP: it was duplicated upon SR-120 and increased by about 50% upon SR-140.

TABLE 5.2 Content and absorbance of water-soluble high molecular weight compounds (HMWC) of cocoa roasted under different conditions.

Roasting condition	g HMWC / 100g CP d.b.	Absorbance of HMWC solutions (3.3 mg / mL) at 420 nm
Unroasted	2.99 ± 0.05[a]	0.42 ± 0.00[a]
FR-120	2.80 ± 0.66[a]	0.55 ± 0.02[bc]
FR-140	2.80 ± 0.04[a]	0.63 ± 0.07[d]
SR-120	6.52 ± 0.33[c]	0.51 ± 0.06[b]
SR-140	4.43 ± 1.16[b]	0.62 ± 0.04[cd]

The data correspond to means ± standard deviations. Lowercase letters in the same column represent significant differences ($p < 0.05$)

These data suggest that during roasting, native HMWC undergo depolymerization reactions giving rise to low molecular weight compounds. At the same time, due to condensation reactions of reducing sugars and FAAs, brown melanoidins were generated.[5] Unfortunately, the ratio of the native HMWC and neoformed melanoidins is unknown, and we can appreciate melanoidins formation only thanks to the increase of browning of the high molecular weight fraction. Quiroz and Fogliano (2018)[16] also found a significant increase in compounds > 20 kDa in cocoa roasted over metallic trays inside a convective oven. Their values were higher than ours: their unroasted cocoa powder had about 7% HMWC, and roasted cocoa rose up to 17.2%. The lack of fermentation of their cocoa is probably responsible for such a difference, as plenty of sugars could have been present in their cocoa beans. Oracz and Nebesny (2018)[27] did not find significant changes in the content of high molecular weight material (>12.4 kDa) by oven roasting. They reported minor reductions of HMWC in some roasting experiments, as we found in our fluidized bed roasted cocoa. However, the content of high molecular weight material in cocoa powder was higher than in ours. In their study, it ranged from 12.5-15.0 % d.b of CP. The lower cut-off of their membrane would explain it, as it retained more matter.

The amount of HMWC did not significantly change upon FR, as presented in Table 5.2. However, data in Table 5.2 shows that the HMWC of the FR samples had a higher brown color compared to unroasted, thus confirming the formation of cocoa melanoidins. Roasting temperature led to significant differences ($p < 0.05$) in both roasting techniques: the temperature increase led to higher brown color intensity. These results support the temperature-dependency of formation of cocoa melanoidins previously reported.[31] The HMWC fraction of FR-140 had the most intense browning of the four roasting conditions ($p < 0.05$).

The brown color intensity is not only determined by the concentration of the melanoidins but also by their composition. The kind of amino acids involved in the formation of melanoidins determines their brownness, as demonstrated in various model systems experiments. [8, 10, 38] As presented in Table 5.3, lysine is exceptionally recognized for its intense brown color formation. Ala, Gly, Ile, Leu, Met, Phe, Ser, Trp, Tyr, and Val are also capable of generating intense to medium brown colors. In contrast, the amino acids Arg, Asn, Asp, Cys, Gln, Glu, His, and Pro have been classified as medium to low brown color producers.

By comparing Table 5.3 with Fig. 5.3, interesting considerations can be made. Fluidized bed roasted cocoa, especially FR-140, optimized the utilization of 7 of the 11 high-medium brown producers (i.e., Ile, Leu, Lys, Met, Phe, Tyr, and Val), while slow roasting favored the relative reduction of the other four (i.e., Asp, Glu, His, and Thr). This observation suggests that the differences in brownness among HMWC could have been determined by the kind of amino acids that were used to build up melanoidins. Fluidized bed roasting caused the preferential decrease of more high-medium brown amino acid producers than SR, forming darker melanoidins.

TABLE 5.3 Amino acids with the highest capacity of formation of brown melanoidins .

Amino acid	Brown color intensity	
	High	Medium
Ala	z	x
Gly	x, z	
Ile	z	x
Leu	z	x, y
Lys	x, y, z	
Met	z	x
Ser	z	x
Trp	x	
Tyr	x, z	
Val	z	x

The letters indicate the source of information: (x) Ashoor and Zent (1984),[8] (y) Lamberts et al. (2008),[38] and (z) Wong et al. (2008),[10] respectively.

Other factors like the polyphenol composition of the HMWC may affect their browning color capacity, as degraded polyphenolic compounds generate brown chromophoric products such as quinone derivatives.[50] In addition, the formation of melanoidins and the color intensity of HMWC extracts may diverge within cocoa varieties.[16] Therefore, our findings would be complemented by extending this investigation to other cocoa bean varieties, and analyzing quinone derivatives present in the water-soluble HMWC fraction.

In conclusion, our study provides insights to modulate MR during the roasting process of cocoa nibs. The possibility to change the sensory, chemical, and physical characteristics of cocoa by changing the roasting conditions offers the opportunity to design different cocoa-based ingredients for diverse final products.

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Appendices

APPENDIX 5.1 Single Reaction Monitoring (SRM) conditions of the phenolic compounds in negative mode determined by LC-MS/MS, and their limits of detection (LOD) and quantification (LOQ).

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Q1 Pre Bias (V)	Collision energy (V)	Q3 Pre Bias (V)	LOD (µg/L)	LOQ (µg/L)
Epicatechin	289.1	245.05	4	15	15	12	8.1	24.6
		109.00	4	21	27	11		
		122.85	4	15	31	24		
		202.95	4	15	19	14		
Catechin	289.1	244.95	4	15	15	17	2.3	7.0
		109.00	4	15	25	11		
		122.90	4	15	30	13		
		203.00	4	15	19	14		
Procyanidin B2	577.1	407.10	4	30	24	15	2.9	8.9
		288.95	4	30	26	22		
		424.95	4	30	17	16		
		125.10	4	30	36	13		
Chlorogenic acid	353.1	191.00	4	26	20	13	0.6	1.9
		85.00	4	18	42	13		
		93.05	4	25	42	10		
		126.95	4	26	36	25		
Gallic acid	169.1	125.05	13	20	16	13	2.7	8.2
		78.95	13	10	22	13		
		80.95	13	11	18	13		
		124.55	13	19	25	12		
Caffeic acid	179.1	135.05	5	20	17	14	4.8	14.5
		134.60	5	20	25	13		
		107.05	5	20	22	11		
		88.95	5	21	32	14		
Ferulic acid	193.2	133.95	11	12	17	14	3.2	9.7
		178.00	11	28	15	12		

APPENDIX 5.2 Free amino acids (mg/g cocoa powder d.b.) in unroasted and roasted cocoa powder obtained from cocoa nibs roasted under different conditions: Fast roasting at 120 °C (FR-120) and 140 °C (FR-140), and slow roasting at 120 °C (SR-120) and 140 °C (SR-140).

Amino Acid	Unroasted	FR-120	FR-140	SR-120	SR-140
Ala	0.89 ± 0.04^{c}	0.78 ± 0.02^{b}	0.71 ± 0.04^{a}	0.76 ± 0.05^{ab}	0.71 ± 0.04^{a}
Arg	0.69 ± 0.08^{c}	0.47 ± 0.01^{ab}	0.46 ± 0.02^{a}	0.54 ± 0.05^{b}	0.49 ± 0.04^{ab}
Asn	1.10 ± 0.05^{c}	0.81 ± 0.09^{b}	0.68 ± 0.03^{a}	0.83 ± 0.09^{b}	0.62 ± 0.08^{a}
Asp	0.35 ± 0.05^{b}	0.32 ± 0.02^{ab}	0.32 ± 0.01^{ab}	0.30 ± 0.03^{a}	0.28 ± 0.01^{a}
Gln	0.08 ± 0.0^{d}	0.01 ± 0.00^{b}	0.00 ± 0.00^{a}	0.01 ± 0.00^{c}	0.00 ± 0.00^{a}
Glu	0.71 ± 0.03^{d}	0.38 ± 0.05^{c}	0.25 ± 0.01^{b}	0.35 ± 0.06^{c}	0.18 ± 0.04^{a}
Gly	0.12 ± 0.00^{c}	0.11 ± 0.00^{b}	0.10 ± 0.00^{b}	0.10 ± 0.01^{ab}	0.09 ± 0.00^{a}
His	0.20 ± 0.01^{c}	0.17 ± 0.06^{bc}	0.12 ± 0.02^{ab}	0.14 ± 0.03^{ab}	0.10 ± 0.02^{a}
Ile	0.46 ± 0.03^{c}	0.37 ± 0.02^{b}	0.30 ± 0.01^{a}	0.40 ± 0.03^{b}	0.32 ± 0.02^{a}
Leu	1.19 ± 0.06^{d}	0.68 ± 0.05^{bc}	0.53 ± 0.03^{a}	0.76 ± 0.06^{c}	0.61 ± 0.06^{ab}
Lys	0.44 ± 0.03^{d}	0.29 ± 0.02^{bc}	0.24 ± 0.01^{a}	0.31 ± 0.03^{c}	0.26 ± 0.02^{ab}
Met	0.04 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Phe	1.06 ± 0.01^{c}	0.64 ± 0.06^{b}	0.46 ± 0.02^{a}	0.69 ± 0.06^{b}	0.51 ± 0.05^{a}
Pro	0.29 ± 0.01^{c}	0.23 ± 0.01^{ab}	0.21 ± 0.01^{a}	0.26 ± 0.02^{b}	0.21 ± 0.01^{a}
Ser	0.37 ± 0.03^{c}	0.29 ± 0.01^{b}	0.27 ± 0.01^{ab}	0.28 ± 0.02^{b}	0.24 ± 0.01^{a}
Thr	0.32 ± 0.02^{c}	0.23 ± 0.01^{b}	0.20 ± 0.01^{a}	0.23 ± 0.02^{b}	0.18 ± 0.01^{a}
Trp	0.13 ± 0.01^{c}	0.07 ± 0.00^{b}	0.05 ± 0.00^{a}	0.07 ± 0.00^{b}	0.05 ± 0.00^{a}
Tyr	1.09 ± 0.18^{b}	0.56 ± 0.03^{a}	0.47 ± 0.02^{a}	0.61 ± 0.06^{a}	0.52 ± 0.03^{a}
Val	0.65 ± 0.01^{c}	0.52 ± 0.03^{b}	0.44 ± 0.02^{a}	0.54 ± 0.04^{b}	0.46 ± 0.03^{a}
Total	10.27 ± 0.46^{c}	7.03 ± 0.56^{b}	5.89 ± 0.34^{a}	7.27 ± 0.75^{b}	5.92 ± 0.54^{a}

The results are expressed as means ± standard deviations. Lowercase letters in the same row represent significant differences ($p < 0.05$).

Chapter 6

Digestion and gut-microbiota fermentation of cocoa melanoidins: an *in vitro* study

This chapter is based on:

Peña-Correa RF, Wang Z, Mesa V, Ataç Mogol B, Martínez-Galán JP, and Fogliano V (**2023**). Digestion and gut-microbiota fermentation of cocoa melanoidins: an *in vitro* study. To be submitted for publication in Journal of Functional Foods.

Abstract

This research aimed to compare the *in vitro* digestibility and fermentability of melanoidins extracted from cocoa nibs fast-roasted with a fluidized bed roaster (FR-melanoidins), melanoidins from cocoa nibs slow-roasted over aluminum trays inside a forced convective oven (SR-melanoidins), and native high molecular weight compounds extracted from unroasted cocoa nibs (UR-HMWC). Data showed FR-melanoidins better release polyphenols during digestion while both melanoidins types induced a higher production of short-chain fatty acids during *in vitro* batch fermentation *vs.* the unroasted cocoa nibs. Both melanoidins and the native HMWC increased the relative abundance of nonpathogenic bacteria of the genera *Bacteroides* and *Megasphera* after 24 h of fermentation. The effect showed this order: FR-melanoidins > SR-melanoidins > UR-HMWC. The catabolism of UR-HMWC resembled the one of dense fiber-like cellulose, whilst FR- and SR-melanoidins resembled oligosaccharides. This study demonstrated the *in vitro* digestibility and the potential prebiotic activity of cocoa melanoidins.

Keywords: *Theobroma cacao* L., roasting, Maillard Reaction, polyphenols, short-chain fatty acids, *Bacteroides* sp.

6.1 Introduction

During the roasting process of cocoa, native high molecular weight compounds (HMWC) ranging from 12 to 150 kDa (Oracz & Nebesny, 2019), such as polysaccharides, proteins, and condensed polyphenols, may undergo depolymerization reactions, thus producing compounds with lower molecular weight. Simultaneously, water-soluble cocoa melanoidins, which are heterogeneous polymers (30 to 70 kDa), are produced via Maillard reaction (MR) involving polyphenols in their structure (Oracz & Nebesny, 2019). Therefore, the composition of the soluble fraction of cocoa above 12 kDa changes upon roasting.

As reviewed by Echavarría, Pagán, and Ibarz (2012); Tagliazucchi and Bellesia (2015); and H. Y. Wang, Qian, and Yao (2011), food melanoidins are poorly-digestible compounds with low ability to release low molecular mass products, thus passing by the gastrointestinal tract to be mainly recovered in feces. So far, this simulation has not been performed with cocoa melanoidins. According to Pérez-Burillo, Rajakarunab, Pastoriza, Paliy, & Rufián-Henares, (2020), the content of released polyphenols upon *in vitro* gut microbiota fermentation is exceptionally higher than that from other dietary melanoidins. Therefore, cocoa melanoidins can be a very efficient vehicle to promote the enzymatic polyphenols release throughout their transit in the upper digestive system.

The molecular structure of cocoa melanoidins is not fully defined. It is well known that they are heterogenous polymers mainly formed by aldol condensations of highly reactive α-dicarbonyl compounds (a carbohydrate-based skeleton) and partially branched by amino compounds (Echavarría, Pagán, & Ibarz, 2012). Melanoidins also contain covalently bound polyphenols (Oracz, Nebesny, and Zyzelewicz, 2019). The water-soluble cocoa extracts containing melanoidins are mainly composed of proteins (15 to 20% w/w), carbohydrates (8 to 16% w/w), and water (≈5 %) (Oracz & Nebesny, 2018). The exact amount of polyphenol is difficult to assess. Upon chemical hydrolysis, a moiety of 200 to 500 mg of total polyphenols is released from 100g of HMWC extract d.b. They are mainly represented by epicatechin and catechin, as reported by Oracz, Nebesny, and Zyzelewicz (2019).

The microbiota's capacity to use food melanoidins as a source of carbon is well established (Pérez-Burillo et al., 2020; van Boekel et al., 2010). Pérez-Burillo et al. (2020) demonstrated via *in vitro* experiments that the growth of microorganisms of the genera *Bifidobacterium,* the formation of short-chain fatty acids (SCFAs), and the release of polyphenols were favored with the presence of cocoa

melanoidins with respect to the control; however, many details of the utilization of cocoa melanoidins have not been assessed.

The discrimination of the sections of the colon on the *in vitro* fermentation of cocoa melanoidins by gut microbiota is one of the queries to be elucidated. It is well known that the microbiota profile of the ascendent, transversal, and descendent parts of the colon strongly differ, as the pH gradually increases from about 5.5 in the cecum to about 6.9 in the rectum. The change in pH along the colon reflects the bacterial fermentative production of acids upon the breakdown of carbohydrates by bacteria under anaerobic conditions. Thus, the greatest fermentative activity is associated with the highest availability of carbohydrate substrates in the cecum and proximal colon (Tannock, 2017), while the fermentation of proteins has been reported to be the highest in the distal large intestine (van der Wielen, Moughan, & Mensink, 2017). In this framework, it is worth to investigate how a heterogeneous polymer like the melanoidins is utilized across the colon.

In the 4th chapter of this thesis, we demonstrated via X-ray tomography analysis of cocoa nibs that an almost 100% convective heat transfer technique (represented by a fluidized bed roaster) had a superior capacity to heat the cores of cocoa nibs than a combination of conductive and convective heat transfer (represented by an oven with aluminum trays). The optimization of the roasting process via fluidized bed roasting was reflected in the higher generation of intermediate MR-volatile organic compounds like pyrazines, as demonstrated in the 3rd Chapter of this thesis (Peña-Correa, Ataç Mogol, Van Boekel, & Fogliano, 2022). Therefore, the formation of advanced MR products in cocoa, e.g., melanoidins, and the depolymerization of native HMWC (e.g., proteins and polysaccharides) may change between heat transfer techniques. Consequently, the ratio of melanoidins / native-HMWC in water-soluble melanoidins extracts may differ, affecting so their interaction with digestive enzymes and gut microbiota.

The objective of this study is to compare the native HMWC extracted from unroasted cocoa nibs and the extracts containing melanoidins obtained from fluidized-bed roasted cocoa and oven-roasted cocoa, on: (i) their potential to store and release polyphenolic compounds via alkaline hydrolysis and simulated *in vitro* digestion; (ii) the release of polyphenols and the production of SCFAs in *in vitro* simulated proximal colon (PC) and distal colon (DC), via batch fermentation experiments; and (iii) the modification of the bacteria communities of the PC and the DC, due to the use of melanoidins as carbon source.

6.2 Materials and methods

6.2.1 Materials and consumables

Fermented and dried Forastero cocoa beans (*Theobroma cacao* L.) from Ivory Coast, with a water content of 6.14 ± 0.02 % w/w, were supplied by Olam International (Koog aan de Zaan, The Netherlands). Pepsin, bile salt, pancreatin, K_2HPO_4, KH_2PO_4, $NaHCO_3$, yeast extract, peptone, mucin, L-Cysteine HCL, and Tween-80 were purchased from Sigma Aldrich (St. Luis, USA). Polytetrafluoroethylene (PTFE) filters were purchased from Phenomenex (Niederlassung, Germany). The following standards were obtained in analytical standard quality (purity ≥ 97%) from Sigma Aldrich (St. Luis, USA): (-)-epicatechin, (+)-catechin, procyanidin B2, chlorogenic acid, gallic acid, caffeic acid, ferulic acid, acetic acid, propionic acid, isobutyric, butyric acid, isovaleric acid. The system of PureLab Ultra (ELGA LabWater, UK) prepared the MilliQ water.

6.2.2 Roasting process of cocoa nibs

The remotion of cocoa beans' husks and the roasting process of cocoa nibs (140 °C, initial water content of 4.31 ± 0.11 % w/w) in a fluidized bed roaster and a convective oven provided with aluminum trays were performed as described in Chapter 4, Section 4.2.2. Because of the time needed to roast cocoa nibs in a fluidized bed roaster was about one-twelfth (3 min 43 s) of the time needed to reach the same water content (1.0 ± 0.1% w/w) in a convective oven with aluminum trays (45 min 16 s), the fluidized bed roasting technique was referred to as fast roasting (FR), and the convective oven as slow roasting (SR). Unroasted cocoa nibs (UR) were separated as a control. All the samples were vacuum-packed and stored at -20°C until usage.

6.2.3 Preparation of cocoa powder and extraction of water-soluble cocoa extracts

A defatting procedure with petroleum ether was performed to obtain cocoa powder (defatted ground cocoa nibs), as described in Chapter 4, Section 4.2.6 (Chemical extraction of cocoa butter for chemical analysis)

An extractive procedure was performed based on a previous study (Quiroz-Reyes & Fogliano, 2018) to obtain the water-soluble moiety > 14 kDa of each kind of cocoa powder. Briefly, 10 g of cocoa powder was thoroughly mixed with 80 mL of Milli-Q water using a homogenizer (Ultra-Turrax®, T25 digital, Probe T4,

IKA, Staufen, Germany) for 4 min at 9000 rpm at room temperature (RT). The mixture was capped and placed in a shaking hot water bath (70 °C, 20 min, 80 rpm) and ultracentrifuged (15000 rpm for 15 min) (Avanti ultra-centrifuge, Rotor ID 16250, Beckman Coulter, USA). The pellet was discarded, and the supernatant was successively vacuum-filtrated through Whatman filter papers Nr 4, 44, and 602. The last filtrate was dialyzed with a dialysis tubing cellulose membrane (14 kDa cut-off, 76 mm flat-width, Sigma Aldrich, Darmstadt, Germany) for 24 h at 4 °C against 4 L of MilliQ water. The water was changed 3 times. The dialyzed part (the permeate) was discarded, and the retentate, which corresponded to cocoa compounds >14 kDa, was freeze-dried (Alpha 1-2 LDplus, Christ, Germany) at -82 °C and 1.0 mbar. The workflow of the obtention of cocoa extracts >14 kDa and their usage throughout this study are represented in Fig. 6.1.

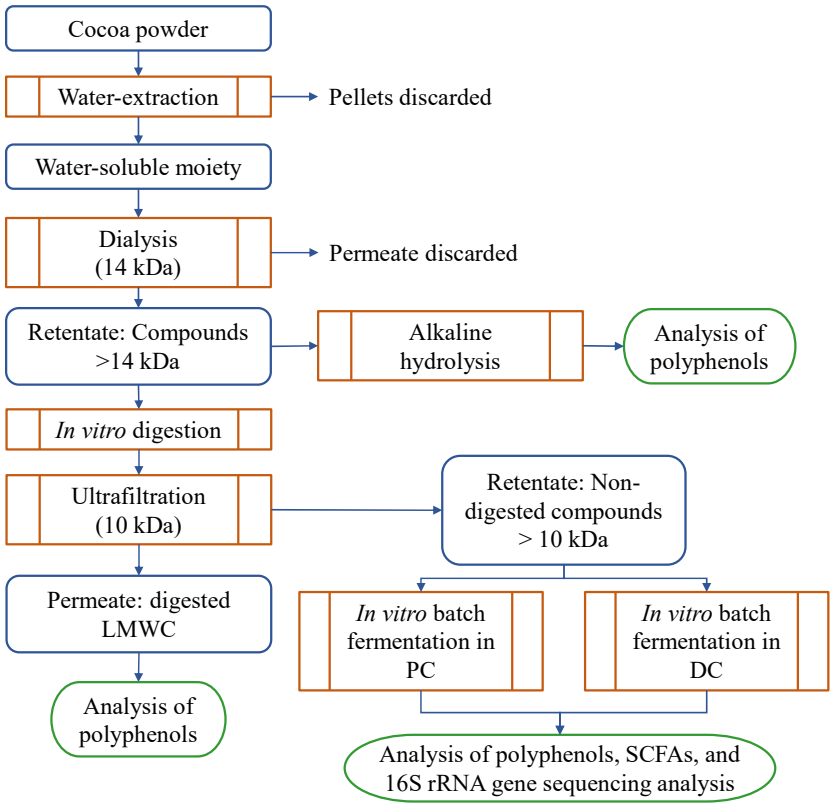

FIGURE 6.1 Workflow of the experiments performed in this study from the obtention of water-soluble cocoa extracts > 14 kDa, followed by their alkaline hydrolysis and *in vitro* digestion, and finishing with the *in vitro* batch fermentation experiments. LMWC stands for low molecular weight compounds, PC for proximal colon, DC for distal colon, and SCFAs for short-chain fatty acids.

6.2.4 Alkaline hydrolysis of cocoa melanoidins

The alkaline hydrolysis method described by (Oracz et al., 2019) was adapted to estimate the content of covalently-bound phenolic compounds in native cocoa HMWC extracted from unroasted cocoa (UR) and cocoa melanoidins obtained from FR and SR cocoa nibs. Briefly, 20 mg of freeze-dried cocoa extracts were hydrolyzed (90 min, 30 °C) with 2 mL of a solution containing 2 M NaOH, 2% (w/w) ascorbic acid, and 20 mM EDTA. Then, the mixture was adjusted to pH 1.0 with 5 M HCl and filled up to 10 mL with MilliQ water. Finally, the mixture was centrifuged (4700 rpm, 10 min, RT), and the supernatant was stored at -20 °C for further analysis. The pellet was discarded.

6.2.5 *In vitro* digestion of cocoa melanoidins

To evaluate the content of polyphenols that can be released from UR-HMWC, FR-melanoidins, and SR-melanoidins in the upper gastrointestinal tract, the INFOGEST® static *in vitro* method (Brodkorb et al., 2019) was adapted. Simulated digestion fluids of the oral (SSF), gastric (SGF), and intestinal (SIF) phases were prepared as described by Brodkorb et al. (2019). All the solutions involved in this method were kept in a water bath at 37 °C. To simulate the formation of a bolus, a sample of 1.2 g of HMWC was added with 6.8 mL SSF, 120 µL 0.3 M $CaCl_2$, and 31.2 mL of Milli-Q water. The mixture was vortexed (20 s). Then, it was added with 36 mL of SGF, and the pH was adjusted to 3.0 (1 M HCl). Then 24 µL of 0.3 M $CaCl_2$, and 7.68 mL of pepsin solution (25000 U/mL) were added. Milli-Q water was added to reach a final volume of 100 mL. The mixture was placed in an incubator (VENTI-Line®, Avantor, USA) provided with a rotating unit (37 °C, 2 h, 120 rpm). The enzymatic hydrolysis of the gastric phase was terminated by adjusting the pH to 7.0 (1 M NaOH), thus producing a simulated chyme. To mimic the intestinal phase (the formation of a chyle), 52.8 mL SIF, 12 mL of bile salt solution (28.8 mg/mL), 192 µL 0.3 M $CaCl_2$, and 24 mL pancreatin solution (amylase activity 1600 U/mL) were immediately added. Milli-Q water was added to fill up to 190 mL approximately. The samples were incubated (37 °C, 120 rpm, 2 h). Finally, the enzymatic activity was halted by immersing the bottles in an ice bath for 30 min. To remove the precipitated material of the chyle, the samples were centrifuged (5000 rpm, 10 min). The supernatant was ultrafiltrated in an ultrafiltration stirring cell unit (Amicon®, model 8400, max. volume 400 mL, Millipore, Billerica, USA) provided with a 10 kDa membrane (Mycrodyn Nadir, Nadir FM UP020Pes, Sterlitech, USA) under a positive pressure of 4.5 bar generated by nitrogen supply. Three washing steps with 30 mL of Milli-Q water were done on the retentate when its

volume was about 20 mL. The retentate containing non-digested water-soluble cocoa compounds > 10 kDa was filled up to 50 mL, and the permeate containing digested low molecular weight compounds (Fig. 6.1) was filled up to 250 mL. Both were stored at -20 for further usage. The method was performed in duplicate for each kind of cocoa extract.

6.2.6 *In vitro* batch fermentation of non-digested cocoa HMWC

Preparation of fecal inoculum

The SHIME® system (PRODIGEST, Belgium) is a reactor capable of mimicking the continuous human large intestinal fermentation throughout two or three gut sections. By following the method described by Rovalino-Córdova, Fogliano, and Capuano (2021), we used this system for separately and simultaneously conditioning the fecal material of two donors (healthy volunteers of Caucasian ethnicity, non-smokers, aged between 25 and 35 years old with no history of antibiotic treatment for at least 6 months before stool collection) in bioreactors mimicking the conditions of the PC and the DC.

In vitro batch fermentation

The *in vitro* batch fermentation of the non-digested compounds > 10 kDa was performed as previously described by Rovalino-Córdova et al. (2021), with some modifications. Briefly, buffered colon mediums were carefully prepared to simulate the pH conditions of the proximal (pH 5.8 ± 0.1) and distal colon (6.5 ± 0.1) (See composition Appendix 6.1). Then, sterile penicillin bottles were added with 7.7 mL of sterilized buffered colon medium and 14.8 mL of solution transporting the non-digested compounds > 10 kDa (8.4 mg/mL). The bottles were closed with rubber and aluminum caps. To create anaerobic conditions, the headspace of the bottles was replaced by flushing with nitrogen. Then, 2.5 mL of the fecal inoculums prepared in the SHIME® system was injected. Negative control (NC) and blank control (BC) were prepared by respectively replacing the solution transporting non-digested cocoa extracts and the fecal inoculum with sterile MilliQ water. Immediately after the inoculation, 1.5 mL of biomass was taken (corresponding to time zero sampling) and immersed in an ice bath. Right after, the bottles, namely batch bioreactors, were incubated over a rotating shaker (37 °C, 120 rpm). Samples of 1.5 mL were taken again after 2, 5, and 24 h of fermentation. This experiment was performed in duplicate for each biological donor.

The samples of biomasses were centrifuged (4°C, 15.000 rpm, 10 min). The supernatants were reserved for further chemical analysis, and the pellets for DNA extraction and sequencing. Both were kept at -20 °C.

6.2.7 Analysis of polyphenols

To determine the release of epicatechin, catechin, procyanidin B2 (P-B2), chlorogenic acid, gallic acid, caffeic acid, and ferulic acid, the supernatants obtained in Sections 6.2.4 and 6.2.6, and the permeates of Section 6.2.5 were thawed and then filtrated with PTFE filters. By duplicate, 80 µL of the filtrated sample and 80 µL of methanol were transferred into amber vials, and then vortexed for 30 s.

The extracts were analyzed by a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan), as described in Chapter 5, Section 5.2.7.

6.2.8 Analysis of short-chain fatty acids (SCFAs)

The supernatants obtained in Section 6.2.6 were thawed and then filtrated with PTFE filters. By duplicate, 100 µL of the samples were transferred into GC-amber vials. The analysis was performed using a Shimadzu GC-2014 gas chromatograph (Kyoto, Japan) equipped with a flame-ionization detector (FID), a capillary fatty acid-free Stabilwax-DA column (1 µm × 0.32 mm × 30 m) (Restek, Bellefonte, PA, USA), and a split injector, according to the setting conditions described by Guo et al. (2020). Nitrogen was used as the carrier gas, and standard solutions of acetic, propionic, butyric, isovaleric, and isobutyric acids in concentrations of 5 to 500 mg/L were prepared and used for identification and quantification. The limits of detection and quantification are presented in Appendix 6.2.

6.2.9 DNA extraction, quantification, and sequencing of barcoded amplicons

The cell pellets of bacterial cultures of fermentative times 0 h and 24 h of donor 1 obtained in section 6.2.6 were used for DNA isolation using QIAamp PowerFecal Pro DNA Kit (Qiagen). DNA yield was quantified using Qubit HS Fluorescence (Invitrogen). Of each DNA sample, 2.5 uL was used for bacterial 16S rRNA gene amplification by PCR targeting the variable V3 and V4 region using the primers Pro341F CCTACGGGNBGCASCAG and Pro805R GACTACNVGGGTATCTAATCC, both with universal extension as recommended by 16S Metagenomic Sequencing Library. After initial amplification of the 16S target region, amplicons were purified and analyzed on a Bioanalyzer using DNA1000 chips. After that, indexing PCR was done using Nextera UD indexes adapters (Illumina). Barcoded amplicons were quantified using Qubit HS Fluorescence and equimolar pooled. Sequencing was performed on the Illumina MiSeq instrument using v2 flowcell and chemistry with 4 pM library loading concentration. Paired-end reads sequencing was

obtained by 2 x 251 cycles. Base-calling and subsequent data demultiplexing were performed using bcl2fastq v2.20.0.422

6.2.10 Data processing

The bioinformatics analysis of sequences was performed in the QIIME2 program (Quantitative Insights Into Microbial Ecology) version 2022.2.0 (Bolyen et al., 2019). The samples were demultiplexed, thus eliminating the associated primer and barcode sequences. The DADA2 method was implemented to detect and correct sequencing noises and remove chimeric sequences. The sequences were grouped in amplicon sequence variants (ASV) with 99% similarity. To classify the sequences according to their taxonomic information, the *q2-feature-classifier* plugin was used based on the Vsearch alignment method (Rognes, Flouri, Nichols, Quince, & Mahé, 2016) with the SILVA v132 database (Quast et al., 2013).

6.2.11 Analysis of microbiota data

The 16S rRNA gene sequencing analyses were conducted in RStudio, version 3.2.4, utilizing the Phyloseq (McMurdie & Holmes, 2013) and Microbiome packages (Lahti & Shetty, 2012-2019) to import sample data and calculate alpha and beta diversity metrics. Alpha diversity was estimated with four indexes: Shannon, Chao 1, Inverted Simpson, and Phylogenetic distance (PD). The significance of categorical variables was determined using the nonparametric Wilcoxon test for two category comparisons or the Kruskal-Wallis test when comparing three or more categories. For beta diversity, principal coordinate analysis (PCoA) was performed to identify a clustering pattern of microbial composition as a function of the variables of interest using permutation-based methods (PERMANOVA, permuted multivariate analysis of variance, using the Adonis2 library) for weighted and unweighted UniFrac distances (Fierer et al., 2010).

6.2.12 Statistical analysis of parametric data

Polyphenols and SCFAs data were statistically analyzed via ANOVA. The Least Significant Differences (LSD) method with 95% significance was applied using the statistic software StatGraphics Centurion XVIII (StatGraphics Technologies Inc., USA). Principal Component Analysis (PCA) was performed using R commander 3.6.1 (R Foundation, Austria) and R studio (RStudio Team, USA).

6.3 Results and discussion

6.3.1 Phenolic compounds bound to cocoa melanoidins, and their bioavailability upon *in vitro* digestion

Phenolic compounds present in plants mainly include those free polyphenols that can be extracted by organic solvents and the ones bound by ester and C–C covalent linkages to native HMWC like cellulose, hemicellulose, pectin, protein, and arabinoxylans (Zhu, Li, Deng , Li, & Zhang, 2020). Thermo-processed food like cocoa contains another source of bound polyphenols, i.e., melanoidins (Oracz et al., 2019). Alkaline hydrolysis (AH) and enzymatic hydrolysis (like in the simulated *in vitro* digestion) can cleavage covalent bonds and release low molecular weight compounds. The content of released polyphenols upon AH and *in vitro* digestion of native cocoa HMWC and cocoa melanoidins are presented in Table 6.1.

TABLE 6.1 Phenolic compounds released from native cocoa HMWC and cocoa melanoidins upon alkaline hydrolysis and *in vitro* digestion

Treatment	Sample	Epicatechin†	Catechin†	P- B2†	Sum†
Alkaline hydrolysis	UR-HMWC	286.1 ± 27.3^{c}	232.2 ± 31.3^{c}	99.1 ± 13.9^{b}	626.8 ± 52.3^{c}
	FR-melanoidins	109.4 ± 10.9^{a}	97.8 ± 10.2^{a}	42.3 ± 5.4^{a}	268.2 ± 28.5^{a}
	SR-melanoidins	179.2 ± 68.7^{b}	155.4 ± 59.8^{b}	56.7 ± 18.2^{a}	400.7 ± 151.9^{b}
In vitro digestion	UR-HMWC	195 ± 6.0^{C} (68 %)	21.8 ± 1.1^{A} (9 %)	47.8 ± 4^{B} (48 %)	264.7 ± 6.1^{B} (42 %)
	FR-melanoidins	113.5 ± 11.9^{A} (104 %)	53.3 ± 10.7^{C} (54 %)	37.2 ± 8.4^{AB} (88 %)	204.1 ± 14.9^{A} (76 %)
	SR-melanoidins	133.3 ± 5.9^{B} (74 %)	35.4 ± 3.4^{B} (23 %)	30.6 ± 6.9^{A} (54 %)	210.5 ± 9.6^{A} (53 %)

† mg/100g cocoa HMWC d.b.
The results correspond to mean ± standard deviation. The values in parenthesis correspond to the percentage of digestion with respect to alkaline hydrolysis. Lowercase and uppercase superscript letters in the same column represent significant differences ($p < 0.05$) for alkaline hydrolysis and *in vitro* digestion data, respectively.

The most predominant phenolic compound integrated to UR-HMWC and cocoa melanoidins was epicatechin, followed by catechin and procyanidin B2 (P-B2). Ferulic, chlorogenic, caffeic, and gallic acids were found below the LOQ.

As presented in Table 6.1, the AH treatment demonstrated that UR-HMWC holds a significantly higher content of epicatechin, catechin, and P-B2 than FR- and SR-extracts containing melanoidins. The content of released polyphenols from FR- and SR-melanoidins is about 40 and 60% of the total polyphenols of UR-HMWC, respectively. The depolymerization of native HMWC, and the formation of melanoidins determined the polyphenols content in FR- and SR-extracts; however, the final ratio of native HMWC and melanoidins (both carrying polyphenols) is unknown. Our results of AH-released polyphenols are highly aligned with those reported by Oracz et al. (2019), except for two main differences: they detected other kinds of bound phenolic acids, and reported the effect of roasting in increasing the number of bound polyphenols. The conditions of the roasting experiments could mainly explain these divergences (e.g., they roasted whole beans).

Looking at the polyphenols content released upon *in vitro* digestion, the picture is different. The total amount released from UR-HMWC was higher than in FR- and SR-extracts containing melanoidins (Table 6.1); however, there were differences in the single phenolic compounds, especially in catechin, which was higher in FR-melanoidins, followed by SR-melanoidins. Catechin is a special phenolic compound in cocoa. Its native form is (+)-catechin. During roasting, free (-)-epicatechin epimerizes to (-)-catechin (De Taeye, Kankolongo Cibaka, Jerkovic, & Collin, 2014). Although both catechin enantiomers have different features, they produce the same fragment ions under the LC-MS analysis. Consequently, they are treated as a whole, and are generally referred to as catechin. This explains the increase in free catechin content during the roasting process of cocoa reported in various studies (De Taeye, Bodart, Caullet, & Collin, 2017; Żyżelewicz a et al., 2016) and in the 5th Chapter of this thesis. As free catechin content increases in cocoa during roasting, bound catechin to melanoidins may increase too. This hypothesis suggests that more melanoidins were present in FR-extracts than in SR-extracts, and that catechin may be the key to further unraveling the gray area between native HMWC and melanoidins.

The differences in digestibility of UR-HMWC *vs.* cocoa extracts containing melanoidins can be appreciated by considering the AH-released polyphenols data as 100% (Table 6.1). FR- and SR-melanoidins released the highest percentage of epicatechin, catechin, and P-B2 than UR-HMWC, especially epicatechin, which was fully digested from FR-melanoidins. These results suggest that the compactness of melanoidins and/or the strength of the covalent bonds of polyphenols within melanoidins are lower than in native HMWC. Differences in the percentage of digestibility between FR- and SR-cocoa extracts containing

melanoidins suggest that the ratio of generated melanoidins / native HMWC is lower in SR-extracts due to a higher presence of native HMWC explained by the lower capacity of the oven-roasting process to heat the cores of cocoa nibs.

In line with our results, the *in vitro* digestion of coffee extracts containing melanoidins led to an increase in antioxidant activity in the fraction containing compounds < 10 kDa. This result suggests that digestive enzymes modify water-soluble high molecular-weight molecules, including melanoidins, and increase the bioavailability of low molecular-weight structures (Rufián-Henares & Morales, 2007). In contrast, the *in vitro* digestion of melanoidins elaborated in a model system resulted in no formation of degradation products with nominal molecular masses below 3000 Da (Ames, Wynne, Hofmann, Plos, & Gibson, 1999). This difference may be explained by the limited kind of reactants of the model systems (lysine and glucose), in contrast to the large variety of amino acids, peptides, carbonyl compounds (e.g., reducing sugars and lipid oxidation products), and polyphenols present in unroasted cocoa and green coffee.

This investigation showed for the first time the potential of cocoa melanoidins to release bound polyphenols before entering the gut, and the modulation of the bioavailability of bound polyphenols via the formation of cocoa melanoidins.

6.3.2 Production of short-chain fatty acids (SCFAs) in *in vitro* batch fermentation experiments

The catabolism of cocoa melanoidins by gut microbiota via *in vitro* batch fermentation may be reflected in the formation of the SCFAs acetic, propionic, butyric, and the branched-chain SCFAs (BCFAs) isobutyric and isovaleric acids. The data obtained from the blank control (BC) (absence of bacteria inoculum) showed negligible amounts of these compounds, thus confirming the clear performance of the experiment and the absence of SCFAs in our cocoa extracts. The means of concentration of SCFAs in biomasses containing UR-HMWC, FR-melanoidins, SR-melanoidins, and in negative controls (absence of test product) were normalized to plot the PCAs (81.8% variability) shown in Fig. 6.2 and Appendix 6.3. The formation of SCFAs was strongly correlated with the presence of cocoa extracts in the fermentative mediums (Fig. 6.2A) and with the DC (Fig. 6.2B). It means that gut microorganisms used UR-HMWC and cocoa melanoidins as a carbon source, being the DC the more active site for their catabolism.

The PCA of Appendix 6.3 showed that there was not much effect of the biological donors in the production of SCFAs. For that reason, only the data of one donor (donor 1) was used for further data analysis.

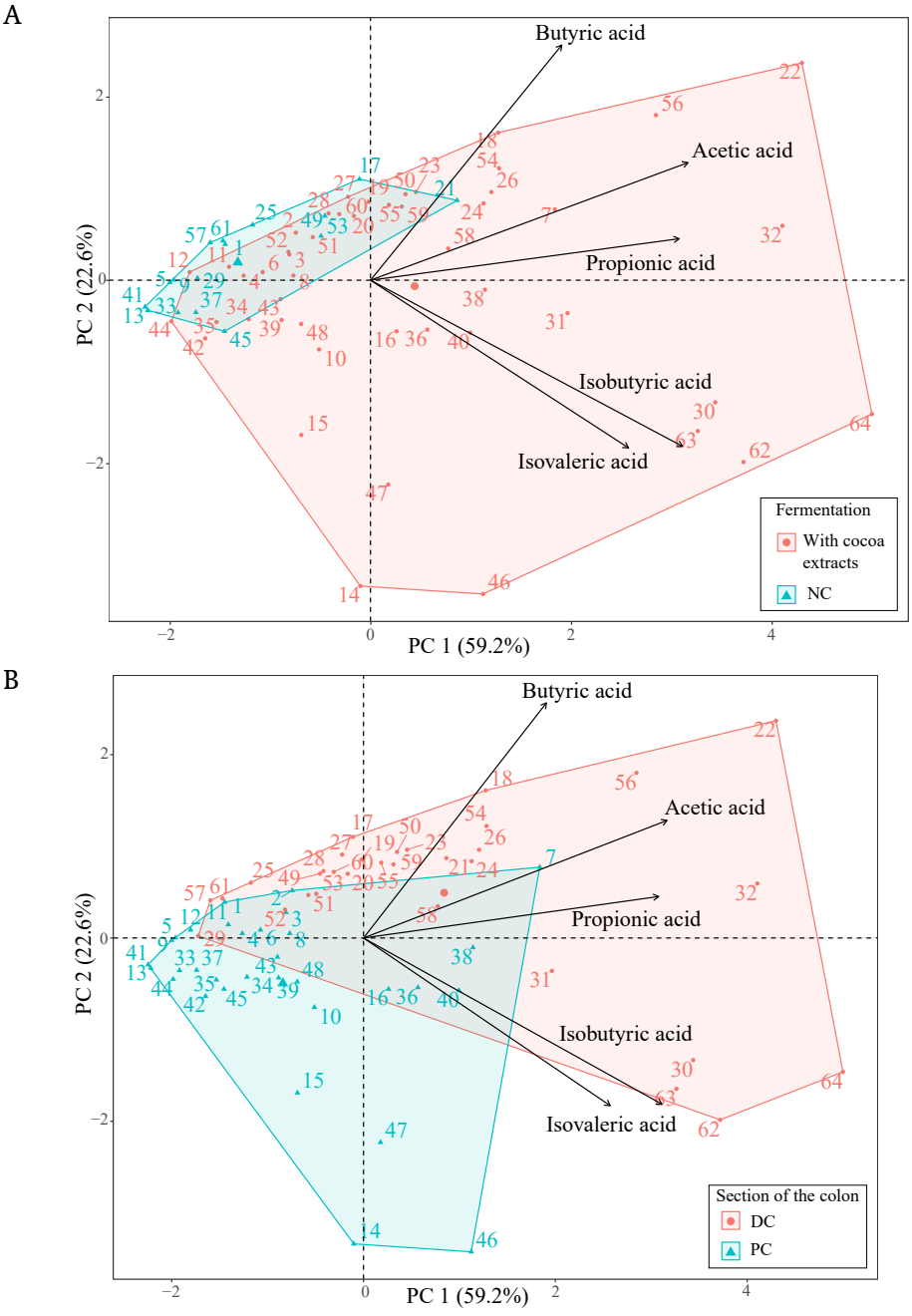

FIGURE 6.2 PCA-cluster analysis of the means of SCFAs data of *in vitro* batch fermentation experiments containing microbiota of donors 1 and 2 by clustering: (A) the presence of cocoa extracts and the negative control (NC); and (B) the section of the colon. Scores 1 to 16 correspond to proximal colon (PC), and 17 to 32 to distal colon (DC) batch fermentations with biological material of donor 1; scores 33 to 48 correspond to PC, and 49 to 64 to DC batch fermentations with biological material of donor 2.

The dynamics of generation of SCFAs during *in vitro* batch fermentation of native cocoa HMWC and cocoa melanoidins in PC- and DC-simulated bioreactors with gut microbiota of donor 1 are presented in Fig. 6.3. Data of isobutyric and isovaleric acids were summed and represented as BCFAs. At first glance, it is evident that DC-bacteria produced higher content of acetic, propionic acid, and butyric acids than PC-bacteria. This trend was reflected in the PCA (Fig. 6.2B). Fig. 6.3 also demonstrates that the most abundant metabolite was acetic acid, as regularly reported in other studies (Cher & Yassour, 2021; Y. Li, Faden, & Zhu, 2020; Pérez-Burillo et al., 2020).

The inoculums taken from the SHIME® continuous system carried SCFAs, thus explaining the load of these compounds in batch fermentation biomasses at time 0 h. However, the abundance of these metabolites was not even within the treatments and control. In general, the concentration of SCFAs was higher in biomasses containing our test products than in the NC-biomasses. As discussed before, the blank controls proved that cocoa extracts were SCFAs free; therefore, these differences might be due to the fast production of SCFAs from the moment of the inoculation of the SHIME®-stabilized bacteria in the batch bioreactors until the cooling process of the extracted sample.

The data of SCFAs corresponding to 2 hours of fermentation showed a high standard deviation. Likely, the adaptation of bacteria to the new fermentative environments was not yet even within the replicates; therefore, we do not consider time 2 h a good time-point to discuss the formation of SCFAs.

The formation of acetic acid after 5 and 24 h of fermentation of UR-HMWC, FR-, and SR-cocoa melanoidins notoriously increased compared with the NCs (Fig. 6.3, panels A and B). In both PC and DC systems, the biomasses containing FR-melanoidins showed the highest concentration of acetic acid after 5 hours of incubation, followed by SR-melanoidins ($p < 0.05$). After 24 h of fermentation in PC-systems, the acetic acid content in biomasses containing FR- and SR-melanoidins was significantly reduced. In contrast, the DC fermentative medium with FR-melanoidins showed a significant increase of acetic acid, while it was steady in that of SR-melanoidins. In both PC- and DC-biomasses containing UR-HMWC, the content of acetic acid increased significantly from 5 to 24 h of incubation ($p < 0.05$). The delayed usage of UR-HMWC by gut bacteria to produce acetic acid may reflect the complexity of native polymeric compounds.

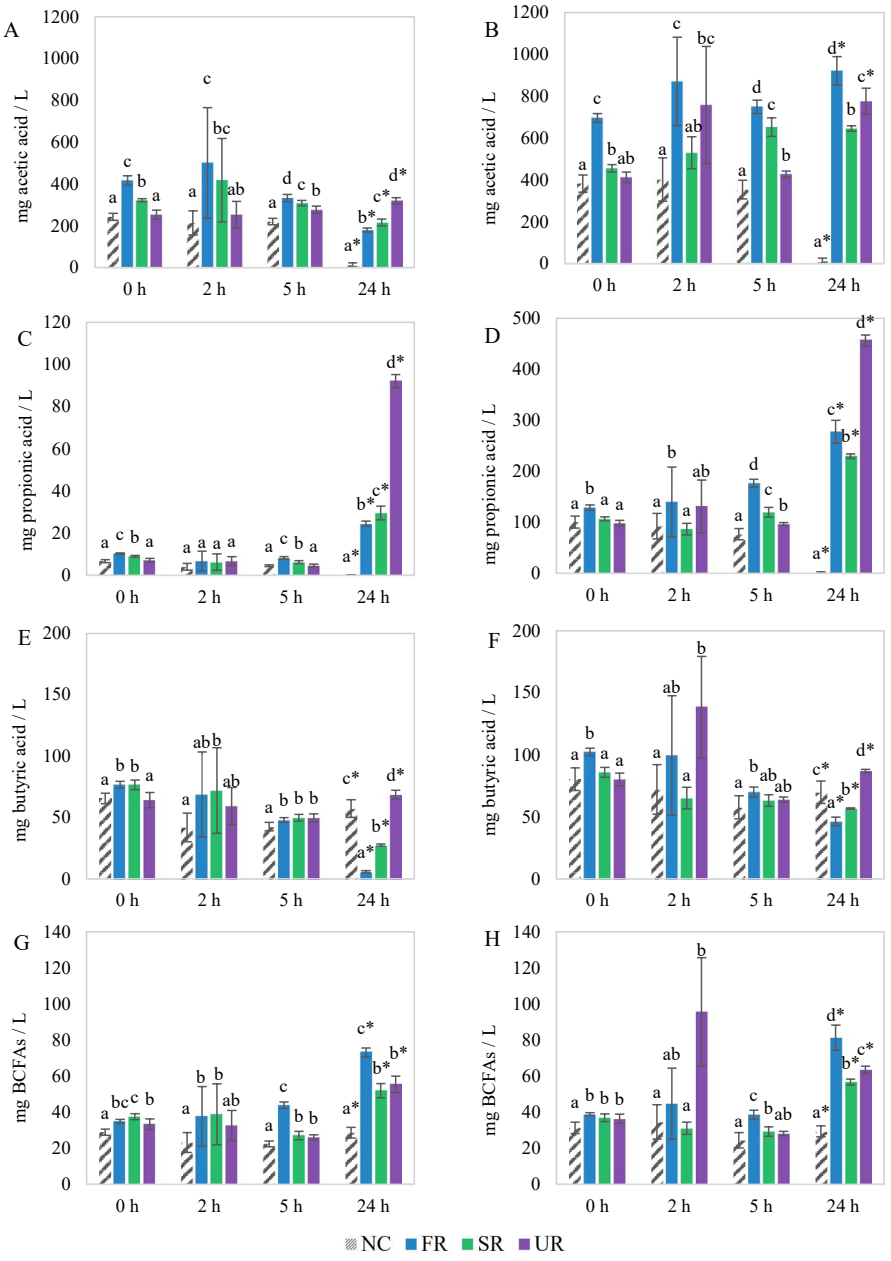

FIGURE 6.3 Formation of acetic acid, propionic acid, butyric acid, and branched short-chain fatty acids (BCFAs) (sum of isobutyric and isovaleric acids) during *in vitro* the batch fermentation of non-digested cocoa compounds > 10 kDa with the gut microbiota of donor 1. The cocoa extracts were obtained from fast roasted (FR), slow roasted (SR), and unroasted (UR) cocoa. NC stands for negative control. Panels A, C, E, and G correspond to bioreactors mimicking the proximal colon, and panels B, D, F, H, to the distal colon. The error bars show the standard deviation. Different lowercase letters identify significant differences ($p < 0.05$) among the source of cocoa HMWC within each fermentation time. Asterisks * at time 24 represent statistical differences ($p < 0.05$) with respect to time 5 h for the same kind of cocoa extract.

Bacteria responsible for the transformation of dietary macronutrients into propionic acid were very active in the DC (Fig. 6.3D). The formation of this metabolite was about 5 folds of that in the PC (Fig. 6.3C). In both kinds of colonic systems, the formation of propionic acid after 5 h of fermentation followed this order: FR-melanoidins > SR-melanoidins > UR-HMWC > NC ($p < 0.05$), meaning that bacteria showed a higher preference for melanoidins. After 24 h of incubation, the fermentation of UR-HMWC showed a considerable increase in propionic acid, higher than that of FR- and SR-melanoidins ($p < 0.05$), thus demonstrating again the delayed usage of this substrate. Maldonado-Mateus et al. (2021) found aligned results when fermenting the unsoluble fraction of unroasted cocoa powder with gut microbiota via *in vitro* experiments.

The formation of butyric acid in PC and DC bioreactors (Fig. 6.3, panels E and F) displayed similar trends. After 5 h of fermentation, the generation of butyric acid was significantly higher with the presence of cocoa extracts. There were no statistical differences among them. After 24 h, the production of butyric acid decreased in biomasses containing FR- and SR-melanoidins and increased in those containing UR-HMWC and in the NC ($p < 0.05$). The similarity between UR-HMWC and NC after 24 h of incubation suggests that such a rise is not caused by a delayed use of the cocoa substrate to generate butyric acid. It could be a cross-feeding effect. Butyrate, as a crucial energy source for diverse metabolic functions in the gut (Mirzaei et al., 2022), could have been used by specific microorganisms present in biomasses containing FR- and SR-melanoidins, while it was not significantly consumed in NC and UR-HMWC biomasses.

Branched short-chain fatty acids (BCFAs) are considerably produced when proteins are present (Rios-Covian et al., 2020), and a considerable moiety of cocoa extracts containing melanoidins (15 to 20% w/w) correspond to proteins (Oracz & Nebesny, 2018). According to the literature, the fermentation of proteins better occurs in the distal colon (van der Wielen, Moughan, & Mensink, 2017). However, our batch *in vitro* experiment showed similar trends in formation of BCFAs in both kinds of *in vitro* colon bioreactors (Fig. 6.3, panels G and H). Continuous *in vitro* systems and *in vivo* experiments can better mimic the saccharolytic and proteolytic activity across the colon.

BCFAs content in PC and DC systems ranged from 20 to 40 mg/L after 5 h of fermentation and from 20 to 80 mg/L after 24 h. In both time-points, biomasses containing FR-melanoidins showed the highest BCFAs production ($p < 0.05$), while those containing SR-melanoidins and UR-HMWC showed similar BCFAs content. These results strongly suggest that FR-melanoidins contain a higher

protein moiety than SR-melanoidins and UR-HMWC, thus supporting the hypothesis abovementioned that the ratio of melanoidins / native-HMWC in FR-extracts is higher than that in SR-extracts. The formation of BCFAs was boosted from 5 to 24 h of fermentation for the three kinds of cocoa extracts ($p < 0.05$), probably due to the complexity of their protein part.

In general, SCFAs data demonstrated that FR- and SR-melanoidins were well accepted by gut bacteria from 5 h onwards, while UR-HMWC took more time to be catabolized. This demonstrates that cocoa melanoidins are more usable by gut bacteria than native HMWC. These results are aligned with the *in vitro* digestibility described in Section 6.3.1. The delayed usage of UR-HMWC resembles the fermentation of slowly breakdown fiber like cellulose (Bai et al., 2021) and lemon pectin (van Trijp et al., 2020), while the advanced fermentation of FR- and SR-melanoidins by gut microbiota resembles the degradation of galactooligosaccharides and fructooligosaccharides (van Trijp et al., 2020).

Unlike our results, the *in vitro* fermentation of high molecular weight material extracted from roasted coffee showed significantly higher total SCFAs generation after 24 h of incubation than at previous fermentation times (Reichardt, Gniechwitz, Steinhart, Bunzel, & Blaut, 2009). The proximal composition of coffee extracts showed a higher content of carbohydrates (25 - 50% w/w) than that of cocoa extracts (> 12.4 kDa) (8 to 16% w/w (Oracz & Nebesny, 2018)). Complex water-soluble carbohydrates present in coffee beans with a molecular weight up to 200 kDa (e.g., arabinogalactan type II and galactomannans) determined the fermentability of coffee extracts (Oosterveld, Harmsen, Voragen, & Schols, 2003).

6.3.3 Release of phenolic compounds in the colon

The catabolisms of food melanoidins by gut microbiota can generate free polyphenols, among other low molecular weight compounds (Pérez-Burillo et al., 2020). During the *in vitro* fermentation of UR-HMWC, FR-, and SR-melanoidins, we found a release of phenolic compounds; however, they did not show significant differences compared to the control without bacteria (data not shown). In principle, this indicates the absence of a specific fermentation capacity by the gut bacteria. In contrast, Pérez-Burillo et al. (2020) found a significant release of polyphenols from pre-digested cocoa melanoidins by gut microorganisms via *in vitro* batch fermentation experiments. Two relevant differences between our experiments and theirs could explain this divergence: (i) after centrifuging the *in vitro*-produced chyle, Pérez-Burillo & colleagues used the pellet and 10% of the supernatant for further *in vitro* batch fermentations. In our case, we discarded the

pellet, ultrafiltrated the supernatant, and used the retentate for gut microbiota fermentation, as described in Fig. 6.1. (ii) Pérez-Burillo & colleagues used a ten-times higher concentration of digested fraction in their bioreactors (500 mg /10 mL), respect to our study.

6.3.4 Changes in gut microbiota diversity and profile

The human gut microbiome is shaped by factors such as diet, environment, and genetic background. The ethnic background, for instance, may change alpha diversity (the richness and diversity within the community) (Liu et al., 2020), while different diets may affect beta diversity (the degree of change in composition between treatments) (H. Li et al., 2016). The Shannon, Chao1, inverted (Inv.) Simpson, and phylogenetic distance (PD) indexes are indicators of microbial alpha diversity, and Unweighted UniFrac and Weighted UniFrac are qualitative and quantitative estimators of beta diversity, respectively (Lozupone, Hamady, Kelley, & Knight, 2007).

Alpha diversity analysis was performed for the factors fermentation time, section of the colon, and kind of cocoa extract. Shannon index is presented in Fig. 6.4, while Chao 1, Inv. Simpson, and PD are shown in Appendix 6.4. The fermentation time did not affect the richness and diversity of the microbial community (Shannon, $p = 0.164$ (Fig. 6.4A); Chao 1, $p = 0.781$; Inv. Simpson, $p = 0.220$; and PD, $p = 0.146$ (Appendix 6.4)). The alpha diversity analysis of the factors section of the colon and kind of cocoa extract was performed only with the data of time 24 h. According to Shannon ($p = 3.7$ e-5) (Fig. 6.4B), Chao 1 ($p = 3.6$ e-5), Inv. Simpson ($p = 3.7$ e-5), and PD ($p = 3.7$ e-5) indexes (Appendix 6.4), the richness of the taxa of DC-biomasses was significantly higher than that of PC, thus explaining the huge formation of SCFAs in those bioreactors. The alpha diversity was not affected by the presence of the different kinds of cocoa extracts (Shannon, $p = 0.470$ (Fig. 6.4C); Chao 1, $p = 0.370$; Inv. Simpson, $p = 0.275$; and PD, $p = 0.929$ (Appendix 6.4)). These results demonstrate that native cocoa HMWC and cocoa extracts containing melanoidins (FR and SR) do not compromise gut microbial diversity.

The beta diversity analysis based on Weighted and Unweighted UniFrac distances of the factors section of the colon and kind of cocoa extract was performed only with the data of time 24 h, as observed in Fig. 6.5. The PCoAs of Weighted UniFrac and Unweighted UniFrac were explained by 91.4% and 60.2% of the variability, respectively. The beta diversity analysis of the factor section of the colon revealed notorious differences ($p < 0.001$) between PC and DC (Fig. 6.5 panels A and B). Both alpha and beta diversity differences in the sections of the colon were expected as buffered pH conditions strongly determine the kinds and the number

of microorganisms (Mailhe et al., 2018). Aligned results were reported by Faria Duque et al. (2021) *in vitro* experiments.

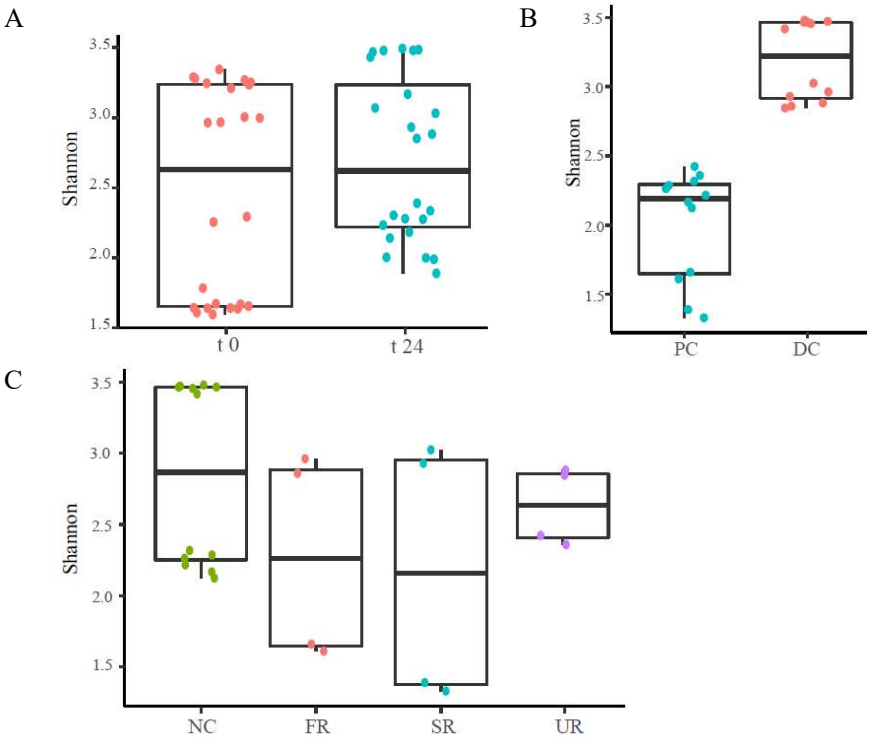

FIGURE 6.4 Alpha diversity using Shannon Index for gut microbiota community of donor 1 from different perspectives. Panel A shows the fermentation time, representing t0 and t24 the time zero and 24 h of fermentation, respectively. Panel B discriminates the sections of the colon, corresponding PC and DC to proximal and distal colon, respectively. Panel C shows the kinds of cocoa extract, where NC, UR, FR, and SR stand for negative control, unroasted, fast roasted, and slow roasted cocoa extracts, respectively.

The incorporation of native cocoa HMWC and cocoa melanoidins did not significantly change the beta diversity of the bacterial community (Weighted, $p = 0.123$; and Unweighted, $p = 0.423$) (Fig. 6.5 panels C and D). However, the clusters of Fig. 6.5C were not fully overlapping: the NC is almost separated, and UR-HMWC shifted slightly from FR- and SR-melanoidins. It means that microbial community tends to change when they are fed with cocoa extracts, and some particular differences occur in bacteria fed with cocoa melanoidins. These divergences can be appreciated in detail in the microbiota profile in Fig. 6.6.

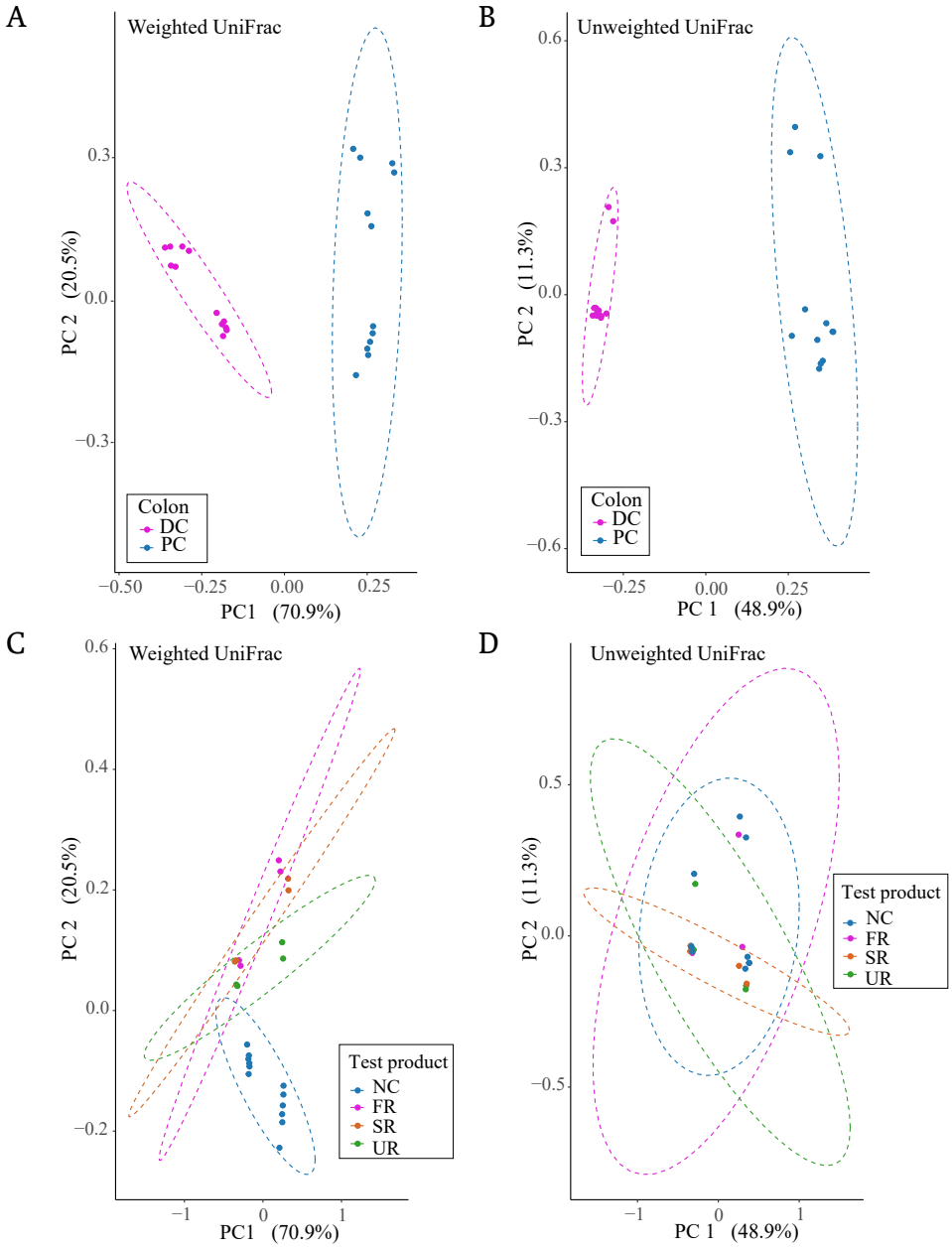

FIGURE 6.5 Beta diversity of gut microbiota community of donor 1 explained by Weighted (A and C) and Unweighted (B and D) UniFrac distances from different perspectives: Section of the colon (A and B), corresponding PC and DC to proximal and distal colon, respectively; and kind of cocoa extract (C and D), where NC, UR, FR, and SR stand for negative control, unroasted, fast roasted, and slow roasted cocoa, respectively.

The taxonomic classification at the genus level revealed that the microbiota profile of donor 1 was affected by the factors fermentation time, kind of cocoa extract, and section of the colon, as presented in Fig. 6.6. The most abundant microorganisms in PC-biomasses at time zero belonged to the genus *Megasphaera* (33 to 60%) (Fig. 6.6A). *Megasphaera* sp. have demonstrated a preference for acidic environments (Firrman et al., 2022). The relative abundance of *Megasphaera* sp. in both kinds of colonic systems notoriously decreased in NC-biomasses after 24 h of fermentation. The incorporation of UR-HMWC in PC and DC bioreactors, and SR-melanoidins in DC bioreactors, did not change this trend. In contrast, when feeding with FR-melanoidins in both colonic systems and with SR-melanoidins in PC, the relative abundance of *Megasphaera* sp. increased. These results demonstrate that microorganisms of the genera *Megasphaera* highly preferred cocoa melanoidins over native HMWC.

Species belonging to the genera *Bacteroides* are equipped with a wide range of carbohydrate-depolymerizing enzymes, which confer them the ability to degrade complex carbohydrates (Reichardt et al., 2009). *Bacteroides*-related bacteria may dominate the more distal parts of the large intestine, as the luminal pH increases to 6.5 (den Besten et al., 2013). It explains their almost exclusivity to DC-systems in our study. *Bacteroides* sp. were the most abundant microorganisms in DC-biomasses by covering 40 to 50% of the relative abundance of the detected genus at time zero (Fig. 6.6B). The relative abundance of *Bacteroides* sp. in DC-biomasses of the NC did not change notoriously after 24 h of fermentation. In contrast, the presence of cocoa extracts increased it. That growth was not different among the kinds of cocoa extracts. These results, and the notable increase in acetic and propionic acids in DC bioreactors containing cocoa extracts after 5 and 24 h of fermentation (Fig. 6.3 panels B and D), are aligned, as butyrate and acetate are typical fermentation products of the microorganisms of the genus *Bacteroides* (Macy & Probst, 1979). In line with these results, *in vitro* batch fermentation of melanoidins elaborated in a model system (Ames et al., 1999) and coffee melanoidins (Reichardt et al., 2009) showed a significant increase in *Bacteroides* after 24 h of incubation.

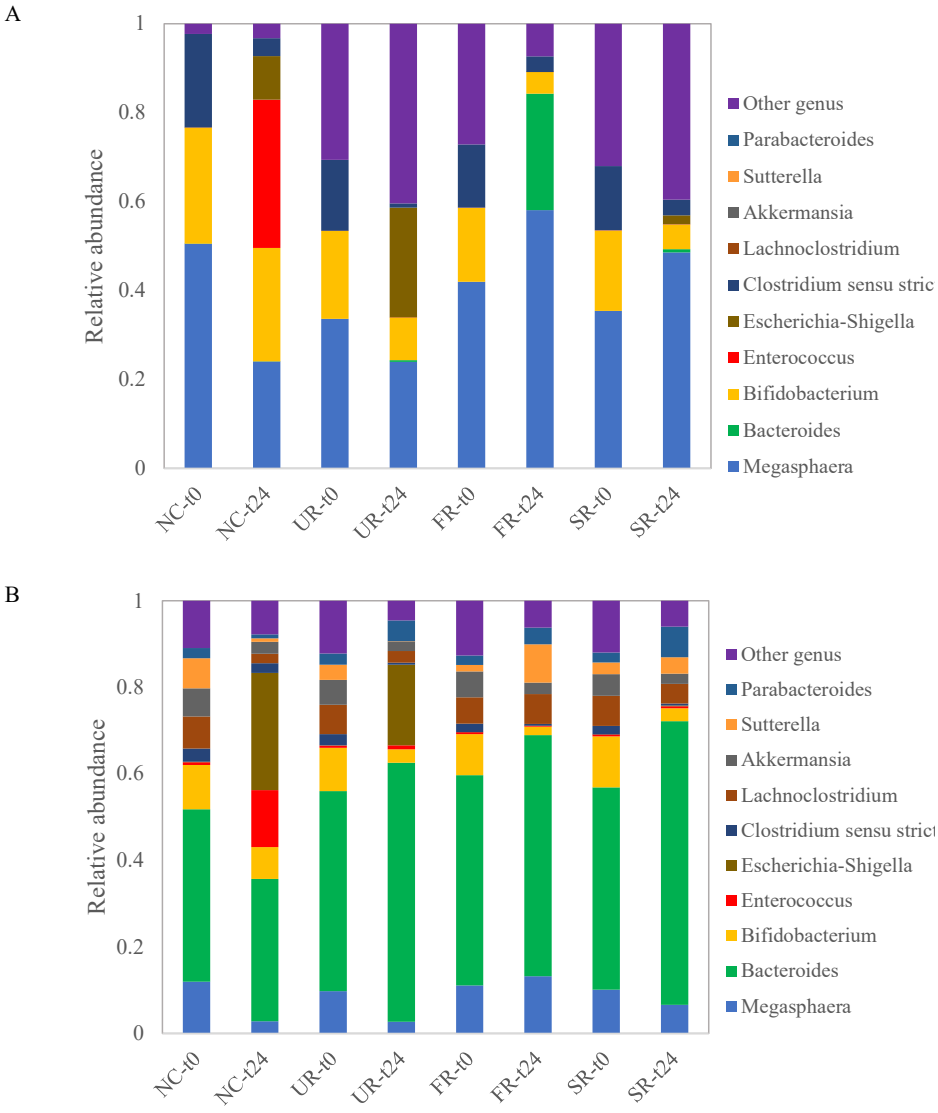

FIGURE 6.6 Relative abundance of different genus in the microbiota of donor 1 pre-stabilized in the SHIME® continuous system, and inoculated *in vitro* batch fermentations experiments mimicking the conditions of the proximal colon (Panel A) and the distal colon (Panel B) at the initial time of fermentation (-t0) and after 24 h (-t24). The first two bars correspond to the negative control (NC), and the rest to the fermentations containing different kinds of cocoa extracts obtained from unroasted (UR), fast roasted (FR), and slow roasted (SR) cocoa nibs.

Bacteroides sp. were almost absent in PC bioreactors at time zero of fermentation (Fig. 6.6A). Interestingly, the PC-fermentative systems led to differences among cocoa extracts after 24 h of fermentation. The presence of FR-melanoidins showed a remarkable relative increase of species belonging to genera *Bacteroides*.

At that point, it turned the second most abundant genus, thus changing the bacteria community profile notoriously. The PC-biomasses containing UR-HMWC and SR-melanoidins showed a slight increase in *Bacteroides* sp., but did not lead to drastic changes in bacteria profiles. The advanced formation of acetic and propionic acid in PC bioreactors containing FR-melanoidins (Fig. 6.3, panels A and C) may explain such a proliferation. Because the systems were buffered and closed, *Bacteroides* sp. that grew after 5 hours did not die during the next 19 h. Considering that FR-melanoidins' extract contains more melanoidins than SR-melanoidins' extract, we conclude that cocoa melanoidins have a potential prebiotic effect along the whole large intestine, while UR-HMWC is more selectively used in the distal region.

Species of the genera *Bifidobacterium* were the second most abundant in the PC systems at time zero, ranging from 17 to 26% (Fig. 6.6A). *Bifidobacterium* sp. are acid-tolerant, and their proliferation is affected when fecal pH increases (Henrick et al., 2018). However, they competed with *Megasphaera* sp. for the second place in relative genera abundance in DC systems (about 10%). The relative abundance of microorganisms of the genera *Bifidobacterium* in the NC-PC systems was not affected after 24 h of fermentation. In contrast, it was reduced in the DC, thus demonstrating its low resistance to pH close to neutrality. The reduction was more profound when adding cocoa extracts in both PC and DC bioreactors, especially in those containing FR- and SR-melanoidins. These results may initially suggest that cocoa extracts inhibit the relative growth of microorganisms of the genera *Bifidobacterium*. However, *Bacteroides*- and *Bifidobacterium*-related bacteria metabolize dietary fibers as carbon sources; therefore, they compete for similar substrates, especially short-chain fructooligosaccharides (Biavati, Vescovo, Torriani, & Bottazzi, 2000). As a consequence, the growth of *Bacteroides* and the decrease of *Bifidobacterium*, or vice versa, may simultaneously occur (Cher & Yassour, 2021). This rationale explains the differences with previous studies that demonstrated that chocolate-obtained melanoidins (Pérez-Burillo et al., 2020) and bread-crust-extracted melanoidins (Borrelli & Fogliano, 2005) favored the growth of *Bifidobacterium* sp, and reduced the proliferation of microorganisms of the genera *Bacteroides*. In other words, the microbiota profile of the donor determines the results of *in vitro* studies.

The relative abundance of species belonging to the genera *Enterococcus* at time zero was negligible (< 1%) in all the fermentative systems (Fig. 6.6). After 24 h of fermentation, the relative abundance of *Enterococcus* sp. increased outstandingly in the NC, especially in PC biorreactors, in which it occupied one-third of the

total genera's relative abundance. As a consequence, the microbiota profile of the NC had a profound change. The incorporation of UR-HMWC, FR-, and SR-melanoidins mitigated the growth of *Enterococcus*-related bacteria in all the fermentative systems.

A similar situation happened with *Escherichia-Shigella* sp. in the DC systems (Fig. 6.6B), with the difference that the relative reduction of microorganisms of the genera *Escherichia-Shigella* led to notorious differences among kinds of cocoa extracts. Fermentations with FR- and SR-melanoidins were more effective in mitigating the proliferation of *Escherichia-Shigella* sp. than UR-HMWC. Microorganisms of the genera *Escherichia-Shigella* are affected by the presence of SFCA (Mirzaei et al., 2022); therefore, the higher production of SCFAs in bioreactors containing FR- and SR-melanoidins may explain their relative decrease.

Species of the genera *Clostridium sensu stricto 1* were the third most abundant in the PC systems at time zero, ranging from 14 to 21% (Fig. 6.6A). Their relative abundance dropped after 24 h of fermentation, with and without the incorporation of cocoa extracts. A similar trend occurred in DC; however, the addition of UR-HMWC, FR-, and SR-melanoidins accelerated their reduction. This situation may also be due to the formation of SCFAs (den Besten et al., 2013).

Microorganisms of the genus *Lachnoclostridium*, *Akkermansia*, *Sutterella*, and *Parabacteroides* were exclusive of the DC fermentative systems (Fig. 6.6B). Their relative abundance in the NC decreased after 24 h of fermentation; however, the presence of cocoa extracts slightly mitigated this effect, especially when cocoa FR- and SR-melanoidins were present. Other microorganisms belonging to different genera out of the top ten were summed and presented in Fig. 6.6 (purple bar). The list is shown in Appendix 6.5.

The tendency of the NC to form a separated cluster in the Weighted UniFrac analysis (Fig. 6.5C) could be explained by the notorious abundance of microorganisms of the genus *Enterococcus*, *Escherichia-Shigella*, and *Clostridium sensu stricto 1* after 24 h of fermentation. The relative abundance of species of these genera was significantly reduced or even eliminated when incorporating cocoa extracts in the batch *in vitro* fermentation of both colonic systems. Species of the genus *Enterococcus*, *Escherichia-Shigella*, and *Clostridium sensu stricto 1* cover several known pathogenic bacteria for humans (Gorbach, 1996), thus suggesting an antibacterial effect of cocoa extracts, especially cocoa FR-melanoidins, by stimulating the proliferation of SCFAs-producers.

The slight separation of the cluster UR from FR and SR in the Weighted UniFrac analysis (Fig. 6.5C) was driven by the exceptional growth of species belonging to the genus *Bacteroides, Megasphaera*, *Sutterella*, and *Parabacteroides* (especially *Bacteroides*) fed with FR- and SR-melanoidins. *Megasphaera* species are nonpathogenic; they have been negatively associated with diarrhea disease (Carey et al., 2021) and related to butyrate and propionate production from lactic acid (Camargo Restrepo, 2021). The increase in the *Sutterella* sp. (Wang et al., 2020) and *Parabacteroides* sp. (Martínez, Kim, Duffy, Schlegel, & Walter, 2010) have been related to the intake of prebiotics and resistant starches.

Both FR- and SR-melanoidins showed similar effects on changing the gut microbiota profile; however, FR-melanoidins outstood with the highest rise in the relative abundance of *Bacteroides* sp. in the PC, and *Sutterella* sp. in the DC. *Bacteroides* species are beneficial as they can establish stable and long-term contact with the host and can degrade dietary fiber into SCFAs, thus providing energy for cells, promoting barrier function, and reducing the occurrence of inflammatory reactions (Deng et al., 2020).

This study demonstrated that the roasting process of cocoa, and the consequent formation of melanoidins are not harmful to gut microbiota; they instead improve the balance of pathogenic and nonpathogenic genera by increasing the relative abundance of the latter, especially the *Bacteroides* sp. We speculate that such a positive effect was determined by the structure of cocoa melanoidins, which resemble prebiotic fibers. As discussed in Section 6.3.1, it is possible that FR-extracts contained more melanoidins than SR-extracts, thus explaining the most profound positive effects of FR-melanoidins in gut microbiota communities.

The effect of roasting on the interaction of the unsoluble fraction of cocoa powder with gut microbiota via *in vitro* experiments also revealed differences in microbiota profile (Maldonado-Mateus et al., 2021). Unroasted cocoa powder favored the increase in the relative abundance of *Veillonella* genera, while roasted cocoa favored *Faecalibacterium* genera. The genera *Veillonella* was found in the microbiota community of donor 1 of this study; however, its abundance was not significant (Appendix 6.5).

6.4 Conclusions

We proved via *in vitro* digestion experiments that the roasting process of cocoa improves the digestibility of polyphenols bound to water-soluble compounds > 14 kDa via the formation of melanoidins. A higher relative content of polyphenols was digested from melanoidins extracted from fluidized-bed-roasted cocoa nibs (FR-melanoidins), followed by melanoidins extracted from convective-oven-roasted (or slow-roasted) cocoa nibs (SR-melanoidins). The lowest relative digestion of polyphenols was found in native high molecular weight compounds obtained from unroasted cocoa (UR-HMWC). The molecular structure of native HMWC would be more compact than that of cocoa melanoidins. The higher digestibility of FR-melanoidins over SR-melanoidins suggests this extract contains more melanoidins and less native HMWC. As the pressurized hot air of the fluidized bed roaster could reach the cores of the nibs, more melanoidins were generated, and more native HMWC were hydrolyzed.

The content of digested catechin was particularly higher in cocoa melanoidins (especially FR-melanoidins) than in UR-HMWC. As free catechin content tends to increase during the roasting process of cocoa, its integration into cocoa melanoidins may increase too. Therefore, catechin may be the key to further understanding the gray area between native HMWC and melanoidins present in water-soluble extracts obtained from roasted cocoa.

The response of gut microbiota fed with cocoa extracts revealed that just after 5 h of fermentation, FR- and SR-melanoidins were highly accepted by gut bacteria, as demonstrated with the formation of SCFAs, while UR-HMWC delayed up to 24 h. The content of SCFAs was higher when fermenting FR-melanoidins compared to SR-melanoidins. Native HMWC resembled the fermentation of a dense fiber like cellulose, while both extracts containing cocoa melanoidins were fastly metabolized as if they were oligosaccharides, especially FR-melanoidins.

Cocoa melanoidins demonstrated higher potential antibacterial activity than native HMWC with a higher reduction of the pathogenic bacteria of the genus *Enterococcus*, *Escherichia-Shigella,* and *Clostridium sensu stricto 1*, and the increase in abundance of nonpathogenic microorganisms of the genus *Bacteroides*, *Megasphaera*, *Sutterella*, and *Parabacteroides*. The proliferation of the nonpathogenic microorganisms was aligned with the production of SCFAs, and most of the species belonging to the pathogenic genus are sensitive to those metabolites, thus explaining the antibacterial effect of cocoa extracts, especially melanoidins.

The results of this investigation strongly suggest that roasting changes the structure of cocoa water-soluble compounds > 14 kDa by degrading more native HMWC and producing cocoa melanoidins. This in turn improves the accessibility of digestive enzymes and gut microbiota to catabolize polymeric structures. Fluidized bed roasting, a fast and low-carbon footprint technique, outstood over traditional oven roasting by showing more profound effects on the Maillardization of native HMWC. The modulation of the formation of MR products like melanoidins by roasting process could allow the formulation of healthier cocoa-based products, more friendly to the human digestive system and health.

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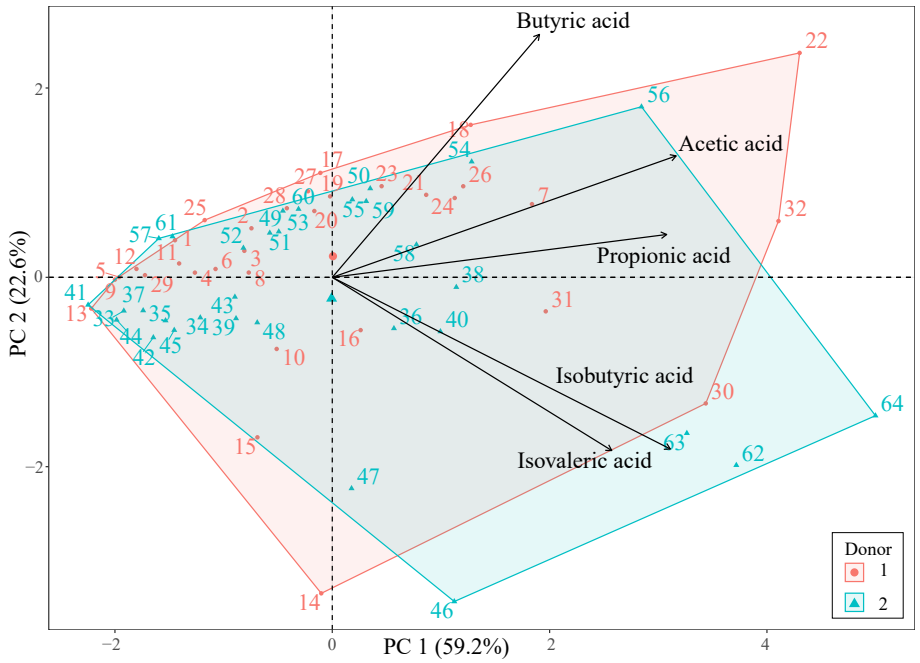
Appendices

APPENDIX 6.1 Formulation of buffer colon medium for *in vitro* batch fermentation systems mimicking the proximal colon (PC) and the distal colon (DC).

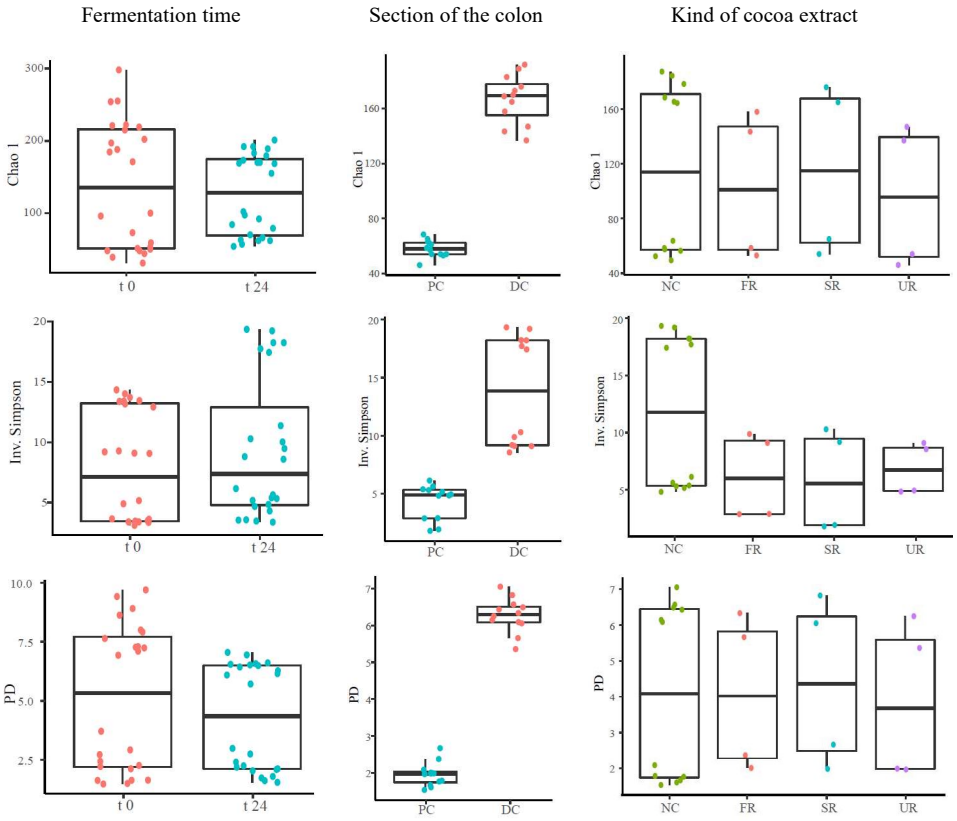
Compounds	PC pH 5.8 ± 0.1	DC pH 6.5 ± 0.1
K_2HPO_4 (g)	3.05	17.62
KH_2PO_4 (g)	38.44	27.06
$NaHCO_3$ (g)	2	2
Yeast extract (g)	0.4	0.4
Peptone (g)	0.4	0.4
Mucin (g)	1	1
L-Cysteine HCL (g)	0.07	0.07
Tween-80 (mL)	2	2
Milli Q Water (L)	1	1

APPENDIX 6.2 Limits of detection (LOD) and limits of quantification (LOQ) of SCFAs analyzed by GC-FID.

Analyte	LOD (ppm)	LOQ (ppm)
Acetic acid	0.168	0.508
Propionic acid	0.142	0.429
Butyric acid	0.068	0.207
Isobutyric	0.079	0.239
Isovaleric acid	0.063	0.191



APPENDIX 6.3 PCA-cluster analysis of the means of SCFAs data of *in vitro* batch fermentation experiments containing microbiota of donors 1 and 2 by clustering the donor of fecal material. Scores 1 to 32 correspond to donor 1, and scores 33 to 64 to donor 2.



APPENDIX 6.4 Alpha diversity using Chao 1, Inv. Simpson, and Phylogenetic Distance (PD) indexes for gut microbiota community of donor 1 from different perspectives: Fermentation time (First column), representing t 0 and t 24 the time zero and 24 h of fermentation, respectively; section of the colon (Second column), corresponding PC and DC to proximal and distal colon, respectively; and kind of cocoa extract (Third column), where NC, UR, FR, and SR stand for negative control, unroasted, fast roasted, and slow roasted cocoa extracts, respectively.

APPENDIX 6.5 List of the genera out of the top ten detected in batch fermentation experiments with pre-stabilized fecal microbiota of donor 1 in the SHIME® continuous system.

Achromobacter	*Flavonifractor*	*Pedosphaeraceae*
Acinetobacter	*Fournierella*	*Phascolarctobacterium*
Alistipes	*Fusobacterium*	*Pirellulaceae*
Alloprevotella	*Gaiella*	*Porphyromonas*
Anaerofilum	*GCA-900066225*	*Pseudocitrobacter*
Anaerofustis	*GCA-900066575*	*Rhodospirillales*
Anaerotruncus	*Klebsiella*	*Rubinisphaeraceae*
Arthrobacter	*Kluyvera*	*Ruminiclostridium 5*
Bacillus	*Lachnospiraceae*	*Ruminococcaceae*
Bilophila	*Lachnospiraceae FCS020 group*	*Ruminococcus 1*
Blautia	*Lactobacillus*	*Salmonella*
Brevundimonas	*Lactonifactor*	*Sphingobium*
Butyricicoccus	*Latescibacteria*	*Sphingomonas*
Butyricimonas	*Lysinibacillus*	*Stenotrophomonas*
Candidatus Soleaferrea	*Megamonas*	*Streptococcus*
Caproiciproducens	*Mesorhizobium*	*Streptomyces*
Caulobacter	*Methylobacterium*	*Subgroup 6*
Caulobacteraceae	*Methyloligellaceae*	*Terrabacter*
Clostridium sensu stricto 12	*Olsenella*	*Terrisporobacter*
Coprococcus 3	*Oscillibacter*	*UBA1819*
Desulfovibrionaceae	*Paenibacillus*	*Veillonella*
Dialister	*Parasutterella*	*Victivallis*
Enhydrobacter	*Pediococcus*	*WS6 (Dojkabacteria)*
Enterobacter	*Pedomicrobium*	*Xanthobacter*

Chapter 7

General discussion

7.1 The need for a study on cocoa roasting

The intensity of thermal food processing is guided by heat transfer efficiency. Heat transfer is slow in solid foods, and the central temperature is difficult to raise when conventional surface heating methods are used.[1] This is a crucial issue for cocoa industry: the fermented and dried seeds of *Theobroma cacao* L., namely cocoa beans, are traditionally roasted in large rotating drum equipment (110 to 150 °C, 20 min to 2 h) where the metallic walls of the drum slowly transfer heat to the edges of the beans via conductive heat transfer mechanism.[2] Convective heat transfer from surrounding air and radiation heat transfers also occur, but they contribute less. As a consequence, the edges of the beans may burn, and the centers may not be completely roasted.[3] This issue is moderately mitigated when reducing the particle size, as it occurs when roasting cocoa nibs (pieces of cocoa without husks).[4] However, a temperature gradient from edges of the nibs touching the metallic surface to the centers may still lead to some unroasted mass, as represented in Fig. 7.1B.

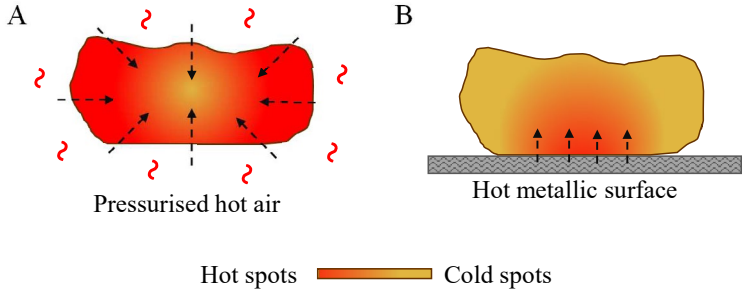


FIGURE 7.1 Schematic representation of the influence of the convective heating method of fluidized bed roasting technique (Panel A) and the conductive heating method (Panel B) on the temperature gradient in cocoa nibs.

The cocoa industry is sub-utilizing the cores of cocoa material, which could be used by improving heat penetration during roasting. The good news is that an almost 100% convective heat transfer and low carbon footprint[5] equipment already exists for roasting small batches of green coffee (< 5 kg); this is the fluidized bed roaster (See Fig. 1.5C and 7.1A). The use of a fluidized bed roaster led to a higher generation of desirable aroma compounds in coffee with respect to traditional drum roasting.[6] This technique has been poorly studied with coffee and was first explored with cocoa nibs in this thesis, leading to more positive results in aroma and chemical quality compared with a predominantly-conductive heat transfer technique: oven roasting with metallic trays. Table 7.1 shows a summary of the findings of this thesis.

TABLE 7.1 Summary of the main results of this thesis.

Chapter	Objectives	Main findings
3	To compare the water diffusivity, the kinetics of formation of volatile organic compounds (VOC), the water activity, and changes in the color of cocoa nibs roasted in a fluidized bed roaster and in oven roasting supplied with aluminum trays at regular cocoa processing temperatures (110 – 140 °C)	• FR was sixteen times faster than SR, reaching equal moisture content. • The water activity was less reduced with FR than with SR. • The water diffusivity in FR nibs was dependent on the temperature more than that in SR nibs. • The color of FR nibs was significantly different from SR, especially the b* color parameter. • The formation of pyrazines was higher in FR cocoa nibs than in SR nibs, which was associated with their lower activation energy under the FR process.
4	To explore microstructural, density, and porosity changes of FR and SR cocoa nibs. To compare the yield of extraction of cocoa butter from FR and SR cocoa nibs and to evaluate the quality and volatile organic compounds (VOC) profile of the extracted cocoa butter.	• FR nibs were more porous and less dense than SR nibs. • The yield of superficial cocoa butter extraction from FR was higher than that from SR nibs. • The quality of cocoa butter was improved with the FR technique, as it reduced the peroxide value of unroasted nibs (UR) compared to SR. • Cocoa butter obtained from FR cocoa nibs contained more pyrazines than that from SR nibs.
5	To compare the Maillard reaction progress (MR) in FR and SR nibs by monitoring the concentration reactants (i.e., sugars, amino acids, and polyphenols) and products such as acrylamide, 5-hydroxymethylfurfural (HMF), and melanoidins.	• The roasting technique did not influence the reduction of total amino acids, but the roasting temperature did: the higher the temperature, the lower the concentration of total amino acids in cocoa. • The amino acids profile diverged between FR and SR nibs: FR favored the use of the most MR-reactive amino acids (i.e., Leu, Lys, Phe, and Val) • FR favored epimerization reactions of epicatechin to catechin. • FR mitigated the formation of HMF. • Water-soluble extracts of FR nibs displayed higher brownness than those of SR nibs.

Chapter	Objectives	Main findings
6	To compare the *in vitro* digestibility and the *in vitro* gut-microbiota fermentability of cocoa melanoidins extracted from FR and SR nibs and native cocoa high molecular weight compounds obtained from unroasted cocoa nibs (UR-HMWC), and to evaluate the changes in the bacteria communities of the proximal colon (PC) and the distal colon (DC).	• FR-cocoa extracts containing melanoidins were more digestible than SR-extracts containing melanoidins • An unknown moiety of FR- and SR- extracts corresponds to melanoidins; however, FR extract has more melanoidins and less native HMWC than SR-extracts. • Gut microbiota preferred cocoa melanoidins over UR-HMWC, as they produced more short-chain fatty acids (SCFA) in more early stages of *in vitro* fermentation. • The fermentation in the PC revealed the differences between FR- and SR-melanoidins' extracts: both significantly modified the bacterial community of the DC by increasing the relative abundance of *Bacteroidetes* sp. and reducing species belonging to pathogenic genera, but only FR did it in PC.

The main objective of this thesis was to compare various quality parameters of cocoa nibs roasted over aluminum trays inside a convective oven *vs.* nibs roasted with a fluidized bed roaster. The first scenario may resemble the industrial roasting process in terms of heat transfer, and was referred to as slow roasting (SR), while the fluidized bed roasting was referred to as fast roasting (FR) across this thesis.

7.2 The effect of fluidized bed roasting efficiency on physical and chemical changes in cocoa

Various physical and chemical analyses were performed in FR and SR cocoa in this thesis. To understand the association between selected variables, a correlation analysis of the data of effective water diffusivity, water activity, formation of nitrogen-heterocycles, brown color developing in cocoa extracts (formation of melanoidins), generation of HMF, and porosity was achieved, as shown in Fig. 7.2.

During roasting, the water present near the surface is initially evaporated; subsequently, the water from the inner layers of parenchyma cells moves toward the surface. This dynamic process, known as effective water diffusivity (De), can be described by Fick's second law of diffusion by determining the water content of cocoa samples at different roasting times.[7]

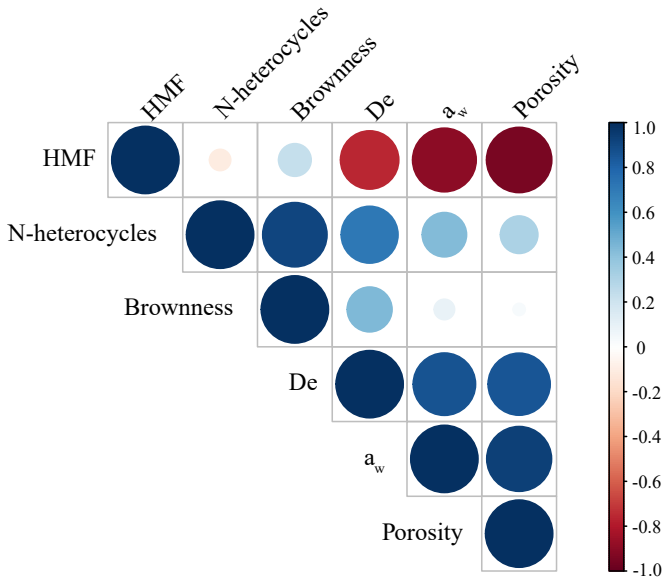


FIGURE 7.2 Pearson's correlation analysis of the outcomes of HMF formation, nitrogen-heterocycles generation, brown color intensity of cocoa extracts, effective water diffusivity (De), water activity (a_w), and porosity of cocoa nibs obtained in Chapters 3, 4, and 5 of this thesis. Positive correlations are displayed in blue and negative correlations in red color. The color intensity and the size of the circle are proportional to the correlation coefficients (*r*).

As presented in Chapter 3, FR led to a De in cocoa nibs one exponential unit higher than in SR cocoa. The higher De in FR cocoa obviously reflected the efficiency of the fluidized bed roasting technique: SR cocoa required 60 min to reach the moisture content that FR cocoa reached in 3 min and 45 s (16 times faster).

The X-ray microscopy image analysis and the quantified porosity of roasted cocoa (Ch. 4) showed traces of heat penetration. The effective heat transfer by the pressurized hot air of the fluidized bed roaster allowed the cores of the nibs to reach higher temperatures than those roasted in an oven with aluminum trays, as represented in Fig. 7.1. Consequently, water evaporated within FR nibs generated internal pressure; then, steam trying to escape through the layers of parenchyma cells produced large pores. In SR cocoa, the heat was mainly transferred in one direction from the edges in contact with the aluminum trays towards the opposite side (Fig. 7.1B). Steam was slowly released, leading to less cell disruption and lower porosity generation than the fluidized bed roasting technique. Interestingly, De was highly correlated ($p < 0.05$) with porosity formation ($r = 0.85$), as shown in Fig. 7.2. Thus, the porosity evidences the water (or steam) diffusion within cocoa nibs, explaining so the efficiency of FR over SR.

De was also highly correlated with a_w (Fig. 7.2, $r = 0.86$). Both are typically obtained to estimate the availability of water for transporting reactants; however, most of the studies have reported the generation of chemical products in the form of a_w. In the third chapter of this thesis, it was reported that the a_w in unroasted cocoa (≈ 0.48) was less affected by FR (≈ 0.30) than by SR (≈ 0.20). It is uncertain how a_w in FR nibs was not reduced as in SR nibs when applying the same roasting temperature and reaching the same moisture content. It could also be related to the microstructure. The increase in porosity probably increased the specific water adsorption sites during FR, thus creating more capillary water and leaving less bound and immobilized water than SR. Figure 7.3 represents this possible phenomenon. This hypothesis could be validated with an analysis of adsorption isotherms.

As observed in the correlation analysis in Fig. 7.2, the formation of N-heterocycles, and the brown color intensity of cocoa extracts were positively correlated with a_w (r = 0.44 and 0.09, respectively), while the generation of HMF was negatively correlated with a_w (r = -0.90). In principle, the higher a_w in FR cocoa should mean this roasting technique favors higher collisions of the reactants of all possible reactions. However, not all thermoactivated reactions are favored with a moderated presence of water. For instance, the generation of pyrazines peaks at a water activity of 0.3,[8] while the formation of melanoidins[9] and HMF[10] starts to increase exponentially only at a_w <0.3, according to model system studies. FR cocoa having a a_w ≈ 0.3, indeed favored the generation of pyrazines (Ch. 3 and 4), but also favored the brown color intensity of cocoa extracts containing melanoidins (Ch. 5), thus differing from the model system study.[9]

As expected, the a_w of FR mitigated the generation of HMF (Ch. 5). This explains the negative correlation of HMF with a_w. However, there is a possibility that very high amounts of HMF were generated during FR and were directly involved in the formation of melanoidins. An HMF analysis on hydrolyzed FR-melanoidins and SR-melanoidins could prove whether there was significant production of HMF during FR. Moreover, it could be a marker of cocoa melanoidins within cocoa extracts.

Another factor that could have favored the mitigation of HMF in FR cocoa was the boosted formation of N-heterocyclics (Ch. 3). These compounds would compete for the reactants with HMF (Ch. 5), especially the reducing sugars, which were substantially degraded.

7.3 Melanoidins are formed at a higher rate in FR cocoa, and they are darker than in SR cocoa

In the 5th chapter of this thesis, it was found that the mass of water-soluble high molecular weight material of SR cocoa increased upon roasting ($p < 0.05$), while FR did not change it significantly ($p > 0.05$). However, the increase in brown color intensity was higher in FR extracts than in SR extracts.

It is difficult to assess with the gravimetric data of dry extracts to what extent melanoidins were generated, as degradation of polysaccharides and proteins also occurs during heating treatments.[11, 12] It is well known that melanoidins are heterogenous polymers mainly formed by aldol condensations of highly reactive α-dicarbonyl compounds (a carbohydrate-based skeleton) and partially branched by amino compounds.[13] Although numerous attempts have been undertaken to isolate and purify them from food and model systems, little is known about their overall structure.[14]

Data of this thesis allowed us to formulate the following hypothesis: "Cocoa extracts obtained from FR nibs have a higher ratio of melanoidins / native HMWC than SR cocoa extracts." In other words, the Maillardization of the cocoa high molecular weight reactants (protein and polysaccharides) occurred to a more considerable extent using FR conditions.[15] The brownness of high molecular weight cocoa extracts is the first evidence supporting this conclusion; however, these additional considerations support the hypothesis:

i. Due to the higher heat-penetration capacity of fluidized bed roasting (reflected in porosity formation), the Maillard reaction was more pronounced in the whole cocoa particles than in SR cocoa (Fig. 7.1A). As a consequence, more melanoidins were produced, and more hydrolytic activity over native HMWC occurred. The generated melanoidins compensated the losses of native HMWC. SR probably favored the preservation of native HMWC located in the coldest spots of cocoa nibs (Fig. 7.1B), while the generation of melanoidins was probably concentrated in the hot spots.
ii. In the 6th chapter of this thesis, UR-HMWC, FR- extracts containing melanoidins, and SR- extracts containing melanoidins were subjected to *in vitro* digestion and *in vitro* fermentation experiments. Interestingly, the relative release of bound polyphenols under *in vitro* digestion showed this order FR-melanoidins extracts > SR- melanoidins extracts > UR-HMWC. In the same order, the *in vitro* fermentation showed a higher and earlier formation of SCFAs. These results indicated that the molecular structure of

native HMWC could be more compact than that of melanoidins; therefore, the higher the digestibility and the fermentability of cocoa extracts, the higher the presence of melanoidins.

iii. According to model system studies, the amino acids leucine, phenylalanine, and valine, among other amino acids, produce deep-brown melanoidins, while other amino acids produce middle to light-brown colors.[16-18] Free amino acids (FAAs) are important building blocks of melanoidins, and their content was determined in cocoa powders (Ch 5). Leucine, phenylalanine, and valine were the second, fourth, and sixth most abundant FAAs in unroasted cocoa powder (Ch. 5), and FR utilized them more than SR.

iv. Leucine, phenylalanine, and valine can also produce various pyrazines, being that Leu the more versatile amino acid.[19, 20] As demonstrated in the third chapter of this thesis, the generation of pyrazines was boosted with the fluidized bed roasting technique. As pyrazines are intermediate MR products capable of generating melanoidins, a higher concentration of pyrazines could have produced more melanoidins. This hypothesis is supported by the positive correlation of N-heterocycles formation in cocoa nibs and brown color development in cocoa extracts (Fig. 7.2, $r = 0.91$).

v. Branched short-chain fatty acids (BCFA) are produced in the gut when proteins reach the colon.[21] Cocoa extracts containing melanoidins are mainly composed of proteins (15 to 20% w/w).[22] The generation of these metabolites during *in vitro* fermentation (Ch. 6) followed this order FR-melanoidins extracts > SR-melanoidins extracts > UR-HMWC.

vi. Among the polyphenols in cocoa, catechin shows a different trend during roasting. Catechin concentration tends to increase during roasting (especially upon FR), as demonstrated in the 5th chapter of this thesis. This phenomenon is explained by epimerization reactions that free epicatechin undergoes under heating treatments.[23] As free catechin content increases in cocoa, catechin bound to melanoidins could also increase. The *in vitro* digestion of cocoa extracts (Ch 6) revealed a higher release of bound catechin from FR-melanoidins extracts > SR-melanoidins extracts > UR-HMWC. A higher release of catechin suggests a higher presence of melanoidins in cocoa extracts.

All this evidence demonstrated that fluidized bed roasting leads to more profound changes in the water-soluble high molecular weight moiety of cocoa nibs by increasing the ratio of melanoidins / native HMWC. The FR-melanoidins show several interesting technological and physiological features that might be explored in future experiments.

7.4 The effect of fluidized bed roasting in efficiency and quality: a comparison between coffee and cocoa

Coffee beans are perhaps the most similar food to roasted cocoa nibs, as they involve fermentation of the freshly harvested seeds of the fruit, followed by drying and roasting processes. Unlike cocoa, coffee is mainly processed for the beverage industry,[24] and its water-soluble moiety (≈ 30%), which mainly contains native polysaccharides and melanoidins, is the most valuable part.[25]

Baggenstoss & coworkers (2008)[6] reported positive results in the use of a fluidized bed roaster compared with a conventional drum coffee roaster. In their study, the FR process was 2 to 7 times faster than drum roasting in terms of reaching the same darkness (L* = 21) and similar water content (1.3 to 2% w/w). Two main differences may explain the lower efficiency of FR in coffee compared to cocoa nibs (12 to 16 times faster): (i) The water content of green coffee (10% w/w) was more than two folds of that in the cocoa nibs of this thesis. (ii) They compared a production-scale drum roaster (20 kg load) with a lab-scale fluidized bed roaster (100 g load). Nevertheless, these important insights inspired us to use the FR technology for cocoa nib roasting:

- During FR, the core temperature of coffee beans rapidly converged to air temperature. Even though they did not install thermocouples in drum-roasted beans, they speculate that the rise of bean core temperature under this technique was slower since the process demanded a longer time to reach similar physicochemical properties in coffee. Their speculation was aligned with ours: the higher porosity in FR cocoa nibs reflected the higher heat penetrability of the FR technique.
- The density of FR coffee beans was lower compared to drum-roasted coffee beans. We found the same phenomenon in FR cocoa vs. SR cocoa nibs (Ch. 4). The reduction in density is a consequence of porosity formation.
- The concentration of abundant aroma compounds in coffee, including sulfur compounds, Strecker aldehydes, ketones, and pyrazines, at L* = 21 was higher in FR coffee than in drum-roasted coffee. One of the two detected pyrazines in coffee was found in cocoa in this thesis: 2,3,5-trimethylpyrazine. Though pyrazines are not the most relevant kind of volatile compound in coffee, the higher abundance of 2,3,5-trimethylpyrazine in FR coffee was in line with the results in cocoa nibs (Ch. 3) and cocoa butter (Ch. 4).

The roasting process of coffee also comes with the reduction of phenolic compounds, more specifically chlorogenic acid, which is the most abundant

one.[26] In another coffee roasting study,[27] a spouted bed roaster, a variant of the fluidized bed roaster, was used at different temperatures. Two relevant trends were reported. The higher the roasting temperature, (i) the higher the chlorogenic acid content in coffee beans, and (ii) the higher the total soluble solids. According to the authors, the shorter roasting time of the higher roasting temperatures permitted more chlorogenic acid to remain in the beans. Under this consideration, we though cocoa polyphenols could also be preserved when the roasting process is accelerated. However, the results showed a different trend (Ch. 5). In general, FR reduced more polyphenols in cocoa than SR ($p > 0.05$); moreovoer, the higher roasting temperatures, which also demanded shorter roasting times, showed higher loss of polyphenols ($p > 0.05$). Different thermal resistance of the phenolic compounds may explain these differences.

The roasting process determines the quality of cocoa and coffee and their consumer acceptance. The use of the fluidized bed technique improved the aroma profile of these food products. Moreover, thanks to the higher heat transfer efficiency of the pressurized hot air, the fluidized bed roasting would save more energy than roasting with conductive heat transfer from metallic surfaces.

7.5 Roasting improves the gastrointestinal release of cocoa components

The beneficial effects of cooking in terms of digestibility are well established.[28] On one hand, denatured proteins and hydrolyzed polysaccharides are more accessible for digestive and gut-microbiota enzymes than their original forms. As mentioned above, we believe that SR and FR degraded native HMWC, having fluidized bed roasting the more profound effects. On the other hand, cooking comes with the formation of melanoidins, which are potential prebiotic material, as gut microbiota catabolize them towards the generation of SCFAs, and favor the growth of beneficial microorganisms.[28, 29]

According to review studies,[13, 14, 30] food melanoidins are poorly digestible in the upper gut. In our study, cocoa melanoidins' extracts released a considerable amount of bound polyphenols upon *in vitro* digestion compared to extracts containing native HMWC. It means that cocoa melanoidins are not complex structures; digestive enzymes penetrate them with ease to produce low molecular weight compounds. The *in vitro* digestion of coffee extracts containing melanoidins led to an increase in antioxidant activity in the fraction containing compounds < 10 kDa. This result also suggests that digestive enzymes modify

extracts containing coffee melanoidins and increase the bioavailability of low molecular weight structures.[31] The *in vitro* digestion of melanoidins elaborated in a model system[32] resulted in no formation of degradation products with nominal molecular masses below 3000 Da. Model-system-elaborated melanoidins lack native HMWC, thus reducing the interference of those polymeric compounds. However, their structure is hardly comparable with real-food melanoidins, as the richness in reactants considerably changes: real food has a wide variety of amino acids and peptides, different sources of carbonyl compounds like reducing sugars and lipid oxidation products, and might contain polyphenols.

The extraction of melanoidins from coffee via consecutive water filtrations carries a significant moiety of native HMWC, probably higher than in cocoa. Green arabica coffee beans consist of 50-60% of polysaccharides, from which arabinogalactan type II and galactomannans play an important role in the water-soluble fraction providing foam stability in espresso coffee.[11] As water-soluble polysaccharides are the preferred substrate of some species inhabiting the large intestine, a high presence of these structures determined the results of *in vitro* fermentation of extracts containing high molecular weight material, as reported Reichardt & teamwork (2009).[25]

It is worth to mention that Reichardt & coworkers (2009),[25] performed a laudable attempt to localize coffee melanoidins by fractioning coffee extracts > 3 kDa into four groups of different molecular weight ranges. Though the carbohydrate content changed in the different fractions (25 to 50% w/w), the *in vitro* fermentation showed the same trend: the SCFAs content gradually increased during 24 h of incubation. Likely, the fractionation was not enough to better localize coffee melanoidins, and the fermentation time was not sufficient to catabolize all the carbohydrates and to allow bacteria to digest the melanoidins moiety. The results of Reichardt & colleagues (2009)[25] resemble the fermentation of our UR-HMWC, which also showed the highest formation of SCFAs after 24 h of fermentation. In contrast, the higher formation of SCFAs from the catabolism of FR- and SR- extracts containing melanoidins occurred at 5 h of incubation (Ch. 6). The roasting process of cocoa dealt with the hydrolysis of fewer polysaccharides than coffee roasting; thus, the interference of these native HMWC was lower in our experiments.

According to Ames & teamwork (1999),[32] the fermentation of melanoidins elaborated in a model system did not show melanoidins' degradation products (e.g., free lysine, SCFAs), but changed the bacteria community. Bacterial counts corresponding to *Bacteroides, Clostridia, and Lactobacilli* sp. significantly

increased after 24 h of incubation. The no detection of SCFAs, as explained by the authors, could be because SCFAs were metabolized by the bacteria as soon as they were formed or because the chosen HPLC conditions were not suitable for the analysis of these components. As SCFAs are volatile compounds, they are better separated via gas chromatography (GC) rather than liquid chromatography, so we believe that the fermentation experiments of Ames & coworkers produced SCFAs that could have been detected via GC techniques. Nevertheless, this study is aligned with our results of fermentation of FR- and SR- extracts containing melanoidins, in terms of modification of bacterial communities, especially by favoring the growth of microorganisms of the genera *Bacteroides.*

The fermentability of coffee brews prepared with green coffee and roasted coffee of different origins showed the effect of roasting. In the study of Perez-Burillo & colleagues (2019),[33] coffee brews were pre-digested *in vitro*. The solid fraction obtained after centrifugation, plus 10% of the supernatant, was subjected to *in vitro* batch fermentation. Their results showed that green coffee had a tendency to produce higher amounts of SCFAs than roasted coffee brew and favored the growth of *Bacteroides* sp. In contrast, our study (Ch. 6) reported a higher formation of SCFAs and a more notorious increase in the relative abundance of *Bacteroides* sp. when fermenting extracts containing cocoa melanoidins than when fermenting UR-HMWC. The exclusion of low molecular weight compounds in our cocoa extracts before digestion and fermentation, which contained a significant concentration of sugars in unroasted cocoa nibs compared to roasted cocoa (as demonstrated in Ch. 5), determined these differences. Even though our study showed opposite results compared with the study of Perez-Burillo & coworkers (2019),[33] both investigations demonstrated the importance of using untreated material as a control, and analyzing the changes in the microbiota community to unravel the overall effect of Maillard reaction products.

The majority of *in vitro* batch-fermentation studies with cocoa/coffee melanoidins represented only the distal colon conditions. The proximal colon could be a harsh environment for some low-pH-sensible species like *Bacteroides* sp.[34] This in turn affects the alpha and beta diversity of bacterial communities, as reported in this thesis (Ch. 6). It is worth to recap that microorganisms of the genera *Bacteroides* in the simulated proximal colon revealed the effect of cocoa melanoidins: they adapted and notoriously grew when fed with FR-cocoa extracts, and slightly grew when fed with SR-cocoa extracts, but they were absent in bioreactors containing UR-HMWC. These results demonstrate that cocoa melanoidins have a potential prebiotic effect along the entire large intestine, while UR-HMWC can be more selectively used in the distal region.

The potential of melanoidins as prebiotic material has been proven with different food and model systems extracts. Although other high molecular weight compounds interfered the experiments, the presence of melanoidins has not been harmful to gut microbiota; it rather intensified the prebiotic effects. This thesis demonstrated that extracts containing cocoa melanoidins improved the balance of pathogenic and nonpathogenic genera by increasing the relative abundance of the latter, especially the *Bacteroides* sp. The species of this genera are beneficial as they can establish stable and long-term contact with the host and can degrade dietary fiber into SCFAs.[35] We speculate that such an antibacterial effect was determined by the structure of cocoa melanoidins, which resemble prebiotic fibers, thus favoring the proliferation of SCFAs producers.

The importance of dietary fiber in the prevention of several non-communicable diseases like colorectal cancer is well known. Colorectal cancer is the third most common cancer type worldwide and the second most common cause of cancer death.[36] Epidemiological studies have demonstrated the association between a higher prebiotic intake and the reduced risk of bowel disorders and colon cancer.[34] The positive balance of pathogenic and nonpathogenic genera mentioned above, was more profound when fermenting cocoa extracts obtained from fluidized bed roasted cocoa than from SR cocoa. We demonstrated in this thesis the importance of optimizing the roasting process by improving the heat transfer efficiency to favor the formation of MR products like melanoidins and to increase the hydrolysis of native HMWC. In this way, the formulation of healthier cocoa-based products, more friendly to the human digestive system and health, are possible.

Last but not least, the catabolism of melanoidins in the gut could trigger a cascade of beneficial effects on human health. Walker & teamwork (2020)[37] conducted a clinical study to evaluate whether coffee and bread crust melanoidins incorporated in bread influence appetite sensations and energy intake. Interestingly, after 180 min of eating bread with coffee melanoidins and bread with bread melanoidins, the energy intake was reduced by 26% and 18%, respectively. The postprandial metabolic responses showed a generally lower glucose and insulin concentration in participants consuming bread with coffee melanoidins. The authors attributed these results to a concomitant effect of the delayed digestion of melanoidins, and the absorption of metabolites (e.g., polyphenols) produced by gut microbiota fermentation. These metabolites can elicit the secretion of anorexigenic hormones. Cocoa melanoidins having an important amount of epicatechin, catechin, and procyanidin B2 (Ch. 5) and delivering them in the lower gut[29] could also trigger positive effects on human health.

7.6 Knowledge utilization and future research

Across the development of this thesis, various research questions emerged. Hereby, we want to give a list of the possible challenges, and especially the actions that can be taken in future research:

- *Would fluidized bed roasting be good for cocoa 'fine flavor' varieties?*

The present study showed promising results of using the fluidized bed roasting technique to roast the most common cocoa variety in the world, i.e., Forastero. However, it is worth to evaluate whether the fluidized bed roasting is good for some Criollo cultivars, especially for those recognized as cocoa 'fine flavor.'

Fine flavor cocoa has strong fruity and floral aroma expressions exerted by organic chemical compounds of esters and alcohols.[38] In cocoa industry, they are carefully roasted at the lowest temperatures in order to keep those volatile organic compounds (VOC). The PCA analysis of the VOC of Forastero cocoa presented in Chapter 2, Fig. 2.4 showed a positive correlation of alcohols and esters with SR cocoa. Finding the same trend with fine flavor cocoa would disfavor the fluidized bed roasting technique. The VOC profile that fluidized bed roasting leads to these unique cocoa cultivars deserves investigation. This thesis suggests to explore FR with very low roasting temperatures, such as 90 – 120 °C.

- *The a_w in FR nibs is higher than that in SR nibs. Moreover, the fat bloom turned more evident. How does it affect their shelf life and further usability?*

Although cocoa nibs are not massively traded as cocoa powder, cocoa mass, or cocoa butter, their commercialization demands shelf life studies, as any other cocoa derivative. The higher porosity of fluidized-bed-roasted cocoa nibs might come with higher water adsorption and/or higher fat bloom during storage. The second situation is an aesthetic phenomenon that might not be an issue if the roasted nibs are addressed to produce cocoa masses. However, the water adsorption in the pores (as represented in Fig. 7.3) could compromise the further processability of cocoa nibs if they are stored for an extended period. Quality analyses of FR cocoa nibs combined with suitable packaging are needed to determine their shelf life.

As the presence of water in cocoa liquors reduces their flowability and affects the texture of elaborated chocolate bars, the shelf life experiment of cocoa nibs shall include rheological analysis of elaborated chocolate mass (≈80% cocoa solids) and texture analysis of elaborated chocolate bars.

- *Before analyzing the VOC of our samples, we noticed a more intense cocoa aroma in FR respect to SR cocoa. Can this be confirmed by a trained cocoa panel? Would these VOC intensity last until the preparation of a chocolate bar?*

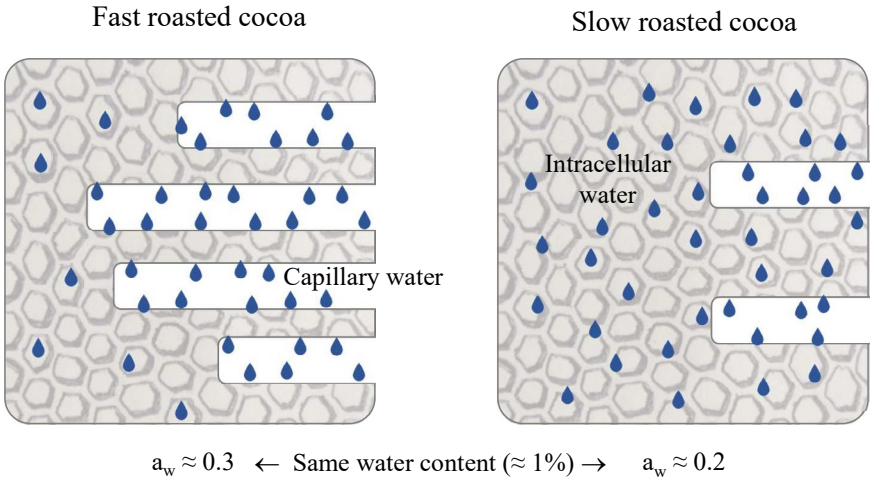


FIGURE 7.3 Schematic representation of the influence of porosity formation on the mobilization of water towards the pores (capillary water) in fast and slow-roasted cocoa, and its effect on water activity (a_w).

So far, this thesis reported promising results in the formation of pyrazines via the fluidized bed roasting technique; however, the correlation of the chemical findings with sensory perception is missing. It would be of great assistance to evaluate the sensory profile of FR and SR cocoa nibs with the support of many panelists (> 10). The chocolate bars above suggested to be prepared for texture analysis, would also be used for sensory analysis.

- *Can FR-obtained cocoa butter be directly used to elaborate white chocolate?*

In the fourth chapter of this thesis, the quality and VOC profile of cocoa butter were evaluated. The results were promising due to the higher relative concentration of pyrazines in cocoa butter obtained from fluidized bed roasted nibs and the reduced peroxide value. As suggested in Chapter 4, future research may apply the cocoa butter obtained from FR cocoa in the elaboration of white chocolate. If a sensory analysis finds excess off-flavors (especially acidic flavors), further gradual deodorization can be assessed. The deodorization reduces the concentration of all VOC; however, the extent to which some may be more affected than others deserves investigation. The development of cocoa butter with natural chocolate aroma may contribute to clean labels.

7.7 The interfacial properties of cocoa melanoidins deserve investigation

It is worth to study the physicochemical and functional properties of the obtained cocoa extracts, especially of those obtained upon fluidized bed roasting processes, as they could have more melanoidins and less native HMWC. Feng & colleagues[39] demonstrated that coffee melanoidins are excellent emulsifiers, as their protein structure leads to the exposure of hydrophobic groups of proteins that have an affinity with oil-water interfaces. The higher digestibility and fermentability of FR-melanoidins strongly suggest that melanoidin's structure is less compact than native HMWC, which may favor their interfacial functional properties. Exploring the overall charge of FR-cocoa melanoidins and correlating this data with some functional properties like foaming or emulsifying o/w systems may provide valuable information about their contribution to stabilizing cocoa derivatives.

The preparation of a chocolate mass regularly requires the addition of food emulsifiers like soy lecithin and polyglycerol polyricinoleate (PGPR) to lubricate the interaction of hydrophilic solid ingredients like sugar, defatted cocoa powder, and milk powder with the continuous cocoa butter phase, as represented in Fig. 7.4. When lubricants are not added, the solid ingredients do not disperse appropriately within the cocoa butter; consequently, the chocolate mass becomes thick and clogs pipes of industrial chocolate manufacturing.[2] Soy lecithin and PGPR are incorporated into chocolate masses according to the fate of the chocolate mass. Both lecithin and PGPR reduce the viscosity of chocolate masses, but PGPR usually affects more the yield value than soy lecithin. It is worth to explore the lubricating properties of cocoa melanoidins and their effect in reducing viscosity and yield value in a chocolate mass. Positive findings may contribute to clean labels and could substitute a soy-derived allergen.

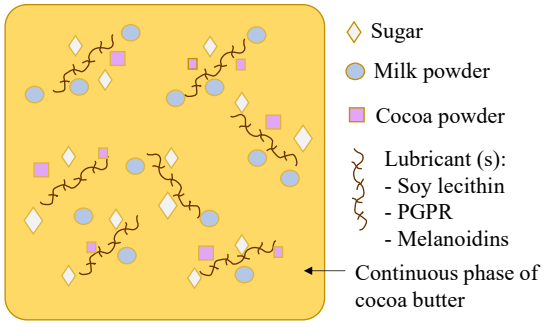


FIGURE 7.4 Structure of a chocolate mass and the role of lubricants in stabilizing the dispersion of the solid hydrophilic ingredients (i.e., sugar, milk powder, and cocoa powder) in the cocoa butter continuous phase.

7.8 The potential of FR to replace the cocoa alkalinization process

The optimization of MR to produce more melanoidins via fluidized bed roasting technique could compete with the Dutching process of cocoa. This process is aimed to increase the pH of cocoa products, such as cocoa beans, cocoa nibs, cocoa mass, or cocoa cakes, with solutions of alkaline compounds (e.g., potassium carbonate).[40] This in turn intensifies the brown color of cocoa derivatives (which is concentrated in the cocoa powder fraction) and improves the dispersion of cocoa powder in cocoa-based beverages. The color changes caused by alkalization are not entirely understood. It has been associated with different phenomena like the oxidation of catechin and epicatechin via enzymatic activity of polyphenol oxidase (whose activity is optimal at ph 8), the combination of anthocyanins and sugar, and the formation of melanoidins via MR.[41]

The Dutching process also comes with concerns. It increments energy demand, as the water added to transport the alkali needs to be further evaporated. Moreover, alkaline additives may negatively interact with the cocoa butter leading to hydrolysis of triacylglycerols and saponification reactions.[42] Future research may compare the cocoa powders obtained from fluidized bed roasted nibs with different Dutching levels of traditionally roasted cocoa powder in terms of brownness and stability in water-based preparations. Positive results would deliver valuable information for the cocoa-beverage industry.

7.9 The future of roasting in cocoa industry

The roasting process of cocoa needs disruptive changes. They should combine the improvement of cocoa quality, the increase in efficiency of the process, and the reduction of carbon footprint. The second and the third are typically related. As cocoa industry is aware of the energy costs and pollution, studies demonstrating the sole cocoa quality improvement (e.g., pursuing a specific aroma profile, preserving phytochemicals) could be rejected for further pilot scaling.

We showed in this thesis promising lab-scale results of roasting cocoa nibs with a low-carbon footprint technique: the fluidized bed roasting. The next step is pilot-scale experiments. A pilot scale machinery of the fluidized bed roasting of about 50 to 100 kg capacity should be closer to real industrial scale in terms of batch or continuous processing necessities and source of energy, and closer to lab-scale equipment in terms of heat transfer technique, proper fluidization of nibs' particles, and porosity development, a_w, and effective water diffusivity in cocoa nibs.

Scaling the fluidized bed roasting is not far from reality. Some large cocoa manufacturing plants have a piece of equipment known as Micronizer® that transforms cocoa beans into cocoa nibs. The Micronizer® heats the surface of the beans with infrared radiation energy. It allows to release the shells of cocoa and weakens the cocoa cotyledons. In this way, cocoa nibs are ready for further continuous traditional roasting.[3] It would be of great assistance if the Micronizer® is adapted to a large-batch size or continuous version of the fluidized bed roasting. Fluidized bed roasters capable of processing 1000 tons per day have been built for metallurgic industry,[43] so scaling up, in principle, is possible.

Changing the kind of heat transfer of the roasting machinery is another alternative. It could be challenging but feasible. Further experiments may consider different sources of heating, e.g., radiation energy from microwave and radio frequency spectrum. The wavelength of both microwaves and radio frequency better penetrate inside the solids than infrared radiation (as used by the Micronizer®). Microwave and radio frequency have been widely used in the food industry for many decades in baking, pasteurization, sterilization, and food drying processes,[44] but they have not been implemented in roasting processes. The main reasons could be the formation of hot spots in solid products and the cost of these techniques. Both situations can be mitigated by combining them with conventional heating methods.[44]

Whatever pathway is taken in the future of roasting, this thesis advises considering a proper heat penetration (reflected in porosity formation) and a $a_w \approx 0.3$ in cocoa nibs. As demonstrated in this thesis, both parameters were highly associated with positive changes performed via Maillard reaction in cocoa nibs, like the formation of more desirable volatile aroma compounds (pyrazines), the generation of more cocoa melanoidins, the mitigation of HMF, and the improvement in digestibility and fermentability of the high molecular weight moiety.

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Summary

Civilizations from all continents consume cocoa derivatives in thousands of different ways, being the confectionery products the most popular ones. Several steps are required in processing cocoa beans (fermented and dried seeds of *Theobroma cacao* L.) before they are consumed or used. Roasting outstands because of its essential role in dehydrating the nibs to insurance proper further processability and also because of the formation of authentic chocolate flavor. Although the importance of roasting cocoa has been evident since ancient times, not much effort has been put forward to harness it. Moreover, the heat transfer technique has not drastically changed: It has been mainly founded on the conductive heat transfer from the metallic surface in touch with the beans.

More than 50 years ago, an almost exclusive convective-heat transfer equipment was invented for processing small batches of green coffee beans: the fluidized bed roaster. Even though it demonstrated advantages in simplicity, costs, and coffee aroma formation, it has been poorly studied. This thesis adopted this technique for cocoa nibs roasting. The general aim of this thesis was to compare diverse quality parameters of cocoa nibs roasted over aluminum trays inside a convective oven *vs.* nibs roasted with a fluidized bed roaster. The first scenario may resemble the industrial roasting process in terms of heat transfer. It was referred to as slow roasting (SR) across this thesis, and the fluidized bed roasting as fast roasting (FR).

Before the experimental part, an extensive investigation of the state of the art of cocoa roasting was achieved. As a result, a comprehensive review was elaborated and presented in Chapter 2. This investigation compared the results of different studies assessing the effect of roasting on the physicochemical phenomena occurring under different cocoa roasting scenarios. Moreover, this review identified weak and mistaken points, presented research gaps, and gave recommendations to be considered for future cocoa studies.

The exploratory experiments comparing SR with FR started with Chapter 3. Chapter 3 is a thermodynamic study of the water diffusivity and the formation of volatile organic aroma compounds. The estimation of water dynamics during the roasting processes was the basis for understanding the efficiency of FR compared with SR: water (or perhaps steam) moves one exponential unit faster in FR nibs than in SR nibs. This explains the short names 'Fast roasting' and 'slow roasting.' The kinetic study of the formation of volatile organic compounds showed for the first time the temperature dependence of generation of nitrogen-heterocycles, mainly represented by pyrazines, during the roasting process of cocoa. The

activation energy N-heterocycles, was lower under fluidized bed roasting than under oven roasting, thus explaining the higher formation of nitrogen-heterocyclics in FR nibs.

Chapter 4 gives a microstructural analysis of FR and SR cocoa and the relation of porosity with cocoa butter extractability and quality. This study quantified the porosity and ranked the size of the pores in cocoa nibs for the first time. Considerable differences were found between the two roasting techniques. The porosity of FR cocoa nibs was higher than that of SR nibs. It is possible that FR allowed a rapid release of steam when parenchyma cell walls were still in a glassy state, while oven roasting caused gradual physical modification allowing the cell wall to become more elastic. Consequently, small pores expanded and coalesced to produce larger pores. The porosity could also explain the heat penetrability capacity, being that of the pressurized hot air of the fluidized bed roaster higher than that of the conductive heat transfer of SR. The higher porosity in fluidized-bed-roasted nibs was reflected in the lowest densities and highest cocoa butter yield. Cocoa butter obtained from fluidized-bed roasted cocoa showed a higher presence of pyrazines and 3-methylbutanal, and a lower concentration of hydroperoxides, thus enhancing the chocolate flavor and quality.

Chapter 5 focused on Maillard reaction (MR). This study compared the chemical characteristics of FR and SR at two roasting temperatures (120 and 140 °C) by monitoring the content of sugars, free amino acids (FAAs), polyphenols, acrylamide, 5-hydroxymethylfurfural, and melanoidins. Both roasting techniques almost eradicated fructose, and significantly reduced the concentration of glucose (by 70%) and sucrose (by 55%). Total FAAs content was affected by roasting temperature, but not by roasting technique. The FAAs profile revealed that FR favored the reactivity of various amino acids (Leu, Lys, Phe, and Val) that are relevant for generating Maillard-related aroma compounds and melanoidins. An increase of catechin and the reduction of 5-hydroxymethylfurfural concentrations, together with the generation of more intense brown melanoidins, were more profound in FR cocoa than in SR cocoa.

Chapter 6 starts with the exploration of the phenolic compounds covalently bound to extracts containing water-soluble high molecular weight material of unroasted cocoa (UR-HMWC), FR cocoa (FR-melanoidins), and SR cocoa (SR-melanoidins), and their release upon *in vitro* digestion. A higher relative content of polyphenols was digested FR-melanoidins, followed by SR-melanoidins. The lowest relative digestion of polyphenols was found in UR-HMWC. This finding suggests that (i) the molecular structure of native HMWC is more compact than that of cocoa

melanoidins; and (ii) FR-melanoidin's extract contains more melanoidins and less native HMWC than SR extract. The second consideration could be explained by the exceptional capacity of the pressurized hot air of the fluidized bed roaster to reach the cores of the nibs, thus generating more melanoidins and hydrolyzing more native HMWC.

The less compact structure of cocoa melanoidins was also demonstrated in the *in vitro* fermentation experiments of Chapter 6. Data showed that cocoa extracts containing melanoidins induced a higher and earlier production of short-chain fatty acids (SCFAs) during *in vitro* batch fermentation than UR-HMWC. The relative increase of microorganisms of the genera *Bacteroides* was in line with the production of SCFAs. The catabolism of UR-HMWC resembled the one of dense fiber-like cellulose, whilst FR- and SR-melanoidins resembled oligosaccharides.

A general discussion of this thesis is presented in Chapter 7. A correlation analysis of some of the observations of Chapters 3 to 5 was achieved. Interestingly, we found a high correlation of porosity formation (Ch. 4) with water diffusivity data (Ch. 3). The formation of nitrogen-heterocycles (Ch. 3) was also highly correlated with the brownness of high molecular weight material containing melanoidins (Ch. 5).

In Chapter 7 we formulated this hypothesis: "Cocoa extracts obtained from FR nibs have a higher ratio of melanoidins / native HMWC than SR cocoa extracts." In other words, the Maillardization of the cocoa high molecular weight reactants (protein and polysaccharides) occurred to a more considerable extent using FR conditions. We supported that conclusion with different considerations taken from this thesis.

Finally, we compared our findings with coffee studies, as this food is perhaps the most similar to cocoa nibs in terms of processability. We also provided in Ch. 7 a list of possible challenges for future cocoa researchers, and presented potential positive cascade effects of the fluidized bed roasting technique in other cocoa processing scenarios like alkalization, stabilization of chocolate masses, and the improvement of chocolate aroma, especially in white chocolate.

Acknowledgements

I am facing a blank page again, but this time the rational brain is taking a break, and the emotional one is going to write. This is an enjoyable experience!

I am becoming a PhD in Food Science at Wageningen University. This important achievement couldn't be possible without the great support of many actors, one of them my beloved husband **Bernardo**. *Amor*, thank you for sponsoring my dreams and for being my twin soul. I cannot be more placed with your companionship.

A very magical experience happened during my PhD: I became a mother. There are no words to describe this. This situation may sound like a speed reducer to reaching my goal; however, it was not. My little one was my mindfulness coach. **Gabriel,** thank you for teaching me to slow down, to be stuck in the present and enjoy the moment, and to play with my inner child. You made a more efficient and less overthinking person out of me!

My heart is full of gratitude to my parents **Álvaro** and **Gloria**, especially my mother, who has been my model to follow. Thank you for setting the bar very high for me. That's why I came here [*Mamá, gracias por ponerme la vara muy alta. Por eso llegue a aquí*]. Many thanks to my brothers **Orlando**, **Alexander**, and **David**, to my sisters-in-law **Yady** and **Mónica**, and my nieces **Stephanie** and **Alicia** for supporting me during these 4 years and 9 months of being away from home, and for taking care of my cat Romeo. How I wish you all (Romeo included) were here!

One of the best things my husband has is his family, which is my family too! **Don Bernardo** (my father-in-law), thank you for keeping an eye on my PhD progress and for your wise advice. Many thanks also to **Toño**, **Oriana**, **Michelle**, **Lucas** (my cover thesis designer, *me encantó tu trabajo!*), and **Martina**.

Vincenzo, there are many things I want to thank you for. First, for believing in me and adopting me to the FQD family. This action changed my life completely. Second, for guiding me from the beginning til the end of my PhD, and taking care of every little detail. And third, for always being there to listen to my personal situations and taking action as if they were yours. That is so empathetic! FQD is privileged to have you as the leader!

Having **Burçe** as a second supervisor was a gift. What a bunch of theoretical and practical things I learn from you! Thank you for being there 24/7 to support me, and many thanks for your friendship!

The experimental part of a PhD thesis brings a lot of 'lab workouts' for training an army. Luckily, at Wageningen University, PhD candidates may spare these activities with BSc and MSc students. This is the best example of a win-win relationship I have experimented in my life: students do part of the experiments and prepare their thesis, and PhD candidates are their thesis supervisors in charge of teaching them and giving them feedback on their manuscripts. I want to thank **Benjamin, Jisca, Menika, Susan, Pippa, Andronikos, Mirjam**, and **Zixuan**, for your tidy work, responsibility, proactivity, energy, and friendship. It was a pleasure to work with you.

The cooperation also took place during the writing part. Besides my excellent supervisors, **Vincenzo** and **Burçe**, I had the privilege to write Chapter 3 with **Tiny**, Chapter 5 with **Christos**, and Chapter 6 with **Zixuan, Victoria**, and **Julián**. Many thanks for your clever contributions and detailed review. Your points of view lead to improving my writing skills and analytical thinking.

The big 'FQD learning package' included the great support of the lab technicians, **Frans, Charlotte, Erik, Jelle, Anastasia, Geert, Nienke**, and **Xandra**; the wonder-women secretaries and educative & research support **Corine, Kimberley, Lisa, Trang Anh**, **Sjhoera** and **Ciska**; among other staff members like **Edoardo**, and **Josep**. Thank you all for your help!

The friendly environment of FQD made this PhD journey more pleasant. This chair group has changed a lot since I arrived in it. People came, and people left, especially international PhDs. The contrast was huge after COVID. Before COVID, we were **Sara** (my Colombian friend! *Gracias Sari por tu orietación y paciencia desde antes de iniciar este doctorado*), **Lucia** (unconditional friend supporting me with drawing tools and parenting tips. We were pregnant at the same time!), **Femke**, **Pieter** (great support in Dutch language and R), **Ayusta, Evita, Jonna, Ana María, Mostafa, Ita, Annelies** (we were roommates at PhD week!), **Domenico, Andrijana, Dario, Ornella, Julie, Jonna, Yao, Diana, Zongyao, Yifan** (male), **Araceli, Fiametta, Jiaying, Mohammed, Alim, Ranti, Zhijun, Lijiao, Folake, Naomi, Mike, Umi** (unconditional friend since PhD week!), **Onu, Sharon, James, Sine, Teresa, Edoardo, Dieuwerke, Matthijs, Kasper, Tamara, Catriona, Anita, Pieternel, Nicoletta, Blerina, Ebru, Jenneke, Bea, Ruud,** and many more that I cannot remember at this moment. Some of you graduated or finished your activities within FQD, and some of you are still there doing laudable scientific and educative work.

After COVID, I met in person the new generation of PhDs and staff members: **Peter B**. (my funny Brazilian friend, we always had a topic to talk about during lunch), **Shiksha, Cristina, Nayara, Abbey** (my native-English spoken support), **Ruth B., Ruth N., Renske, Swantje, Rutger, Lintianxiang, Sofia, Marialena, Andrea, Zekun, Tomer, Quing, Laura, Michelle, Pengfei, Yifan** (female), **Xiangnam, Thisum, Bei, Peiheng, Feilong, Jingje, Seren, Ajeng, David, Xiang, Luigi, Mariana, Melania, Arnau, Josep, David I., David de P., Mei, Fabio**, and **Juliana**. I want to express my gratitude to the former and the current FQDers for the wonderful companionship, potlucks, chats in our flexible offices, birthday celebrations, brainstorming, and for sharing your culture. Living in this multicultural team is perhaps one of the most valuable presents I got from this PhD journey.

As planned, my maternity happened just in the middle of my PhD. What was not in the plan was the pandemic situation, which made parenting more difficult. Luckily, my beloved Colombian friends and **Collin** were there to support us. Thank you **Lina, Sara, Dani Ch., Dani P., Laura A., Collin, Laura J., Cata, Mauro, Natalia,** and **Estefania**, for softening one of the hardest moments of my life. I will never forget what you did for me!

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During my life as an R&D specialist in **'Compañía Nacional de Chocolates**,' I had the privilege of learning the art of chocolate making. I want to express my gratitude to this amazing corporation, which inspired me to do this thesis and gave me knowledge in cocoa processing and many choco-friends.

Lastly, I want to thank my Paranimphs **Lina** and **Ruth** for the fantastic activities they are doing to make this last PhD-stretch so memorable. **Lina**, you were the first person I met in Wageningen. Since that day, our friendship has grown and grown. Thanks for your unconditional support. **Ruth**, thanks for listening to me, for advising me wisely, and for your sweet friendship. It was a great pleasure to have you as an FQD colleague!

The list of actors who made my life better in the Netherlands is endless. Perhaps I missed some of you here, but for sure, you won't be vanished from my heart.

About the author

Ruth Fabiola Peña Correa was born in Duitama, Boyacá, Colombia, on the 3rd of January 1983. She obtained her BSc in Food Chemistry at Universidad Pedagógica y Tecnológica de Colombia, Tunja (Colombia), in 2006. There, she got to be the best student on 5 out of 10 occasions (semesters), and graduated with a 'Meritory thesis,' which she performed as an exchange student at Universidad Autónoma de Chiapas, Mexico. She moved to Medellín, Colombia, to study a Master in Food Science and Technology at Universidad Nacional de Colombia. She graduated in 2010, also with a Meritory thesis. After that, she worked as a cathedra teacher at two Universities, one of them her former one. In 2012, she moved from academic life to the food industry, more specifically, the chocolate industry at Compañía Nacional de Chocolates (Medellín, Colombia), where she worked for 6 years (2012 - 2018) as an R&D specialist. There, she developed plenty of confectionary products and learned the art of chocolate making. In 2016, she graduated as a Specialist in Technology management and innovation at Universidad Pontificia Bolivariana (Medellín, Colombia). In 2017, ICETEX (*Instituto Colombiano de Crédito y Estudios Técnicos en el Exterior*) granted her a scholarship for PhD education. In August 2018, she arrived in the Netherlands to study PhD in Food Science within the chair group Food Quality and Design at Wageningen University and Research. Her research was inspired by her work at the chocolate company: She was curious about the performance of Maillard reaction in cocoa when changing the roasting technique to a more sustainable method. Her PhD thesis was supervised by Prof. Dr. Vincenzo Fogliano, and Dr. Burçe Ataç Mogol. She presented her PhD results and defender her thesis with propositions on the 26th of May, 2023.

Fabiola is also the wife of Bernardo, and the mother of Gabriel. Her hobbies are traveling, watching movies, dancing, cooking, and cycling. She is also a cat lover. She can be reached at this email fabiolapc46@gmail.com.

List of publications

Peña-Correa RF, Ataç Mogol B, and Fogliano V. (2022) The impact of roasting on cocoa quality parameters. Critical Reviews in Food Science and Nutrition. Doi: 10.1080/10408398.2022.2141191

Peña-Correa RF, Ataç Mogol B, van Boekel M.A.J.S., and Fogliano V (2022). Fluidized bed roasting of cocoa nibs speeds up processing and favors the formation of pyrazines. Innovative Food Science and Emerging Technologies 79 (103062). Doi: 10.1016/j.ifset.2022.103062

Peña-Correa RF, Ataç Mogol B, and Fogliano V (2023). Fluidized bed roasting modifying the microstructure of cocoa nibs and improving cocoa butter quality. Journal of the American Oil Chemists' Society, 12696 (1-13) https://doi.org/10.1002/aocs.12696

Peña-Correa RF, Ataç Mogol B, Fryganas C, and Fogliano V (2023). Fluidized bed roasted cocoa has different chemical characteristics than conventional roasted cocoa. Submitted for publication in Journal of Agricultural and Food Chemistry.

Peña-Correa RF, Wang Z, Mesa V, Ataç Mogol B, Martínez-Galán JP, and Fogliano V (2023). Digestion and gut-microbiota fermentation of cocoa melanoidins: an *in vitro* study. To be submitted for publication in Journal of Functional Foods.

Overview of complete training activities

Category A: Discipline specific activities

2019	Advanced Food Analysis (VLAG)	Wageningen, NL
	Applied Biocatalysis (VLAG)	Wageningen, NL
	Intestinal microbiome of humans and animals (VLAG)	Wageningen, NL
	15th International Postgraduate Course on the Production and Use of Food Composition Data in Nutrition (VLAG)	Wageningen, NL
	Recent insights into the role of the Maillard Reaction (Wageningen Academy)	Wageningen, NL
	5th international conference on Cocoa, Coffee and Tea (Jacobs University)*	Bremen, Germany
	6th WPS "Science with Impact" (Wageningen PhD Council)*	Wageningen, NL
	Chemometrics (VLAG)	Wageningen, NL
2021	Healthy food design (VLAG)	Wageningen, NL
	Reaction kinetics in food science (VLAG)	Wageningen, NL
	Rmarkdown (VLAG)	Wageningen, NL
	Fourteenth triennial virtual conference of the International Maillard Reaction Society (IMARS-14) (International Maillard Reaction Society)**	Doha, Quatar

Category B: General courses

2018	PhD week (VLAG)	Baarlo, NL
	Searching and organising literature (WGS)	Wageningen, NL
2019	Reviewing a Scientific paper (WGS)	Wageningen, NL
	Introduction to R (VLAG)	Wageningen, NL
	Scientific publishing (WGS)	Wageningen, NL
	Applied Statistics (VLAG)	Wageningen, NL
	Competence Assessment (WGS)	Wageningen, NL
	Project and time management (WGS)	Wageningen, NL
2020	The essentials of Scientific Writing & presenting (W in'to Languages)	Wageningen, NL
2021	Scientific Writing (W in'to Languages)	Wageningen, NL
2022	Presenting with impact (W in'to Languages)	Wageningen, NL
	Adobe InDesign - from dissertation layout to poster design (WUR Library)	Wageningen, NL

Category C: Other activities

2018	Preparation of research proposal. WUR, Chair group: FQD	Wageningen, NL
2019-2022	PhD representative at FQD Group meetings. WUR, Chair group: FQD	Wageningen, NL
	Supervison of MSc- and BSc-students. WUR, Chair group: FQD	Wageningen, NL

* Poster presentation
** Oral presentation
Abbreviations:
WGS: Wageningen Graduate School
FQD: Food Quality and Design

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