KU LEUVEN

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

Cadmium distribution in Ecuadorian cacao beans during post-harvest processes

Cadmium verdeling in Ecuadoraanse cacaobonen tijdens na-oogst processen

Promotor:
Prof. Erik Smolders
Departement Aard- en Omgevingswetenschappen
Afdeling Bodem- en Waterbeheer

Masterproef voorgedragen
tot het behalen van het diploma van
Master of Science in de bio-ingenieurswetenschappen:
levensmiddelenwetenschappen en voeding

Vincent De Mesmaeker

juni 2019

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Abstract (English)

In January 2019, a new regulation on the maximum cadmium (Cd) concentration in chocolate products was imposed by the European Commission (EC). This regulation threatens the export position of many South American countries where elevated levels of cadmium in cacao have been found.

As part of our aim to develop a possible mitigation strategy to lower Cd in cacao nibs to meet these new EC regulations, we first investigated the spatial distribution of Cd in Ecuadorian cacao beans during post-harvest processes. Moreover, the extent to which Cd is redistributed during fermentation, was investigated.

Unfermented cacao tissues (from outside to inside: husk, placenta, mucilage, testa and nib) from five different locations in Ecuador were analyzed. The Cd content in those tissues generally decreased from testa > nib > mucilage. Husk and placenta Cd were measured in two samples. Cadmium concentration in husk and placenta tends to range between those of mucilage and nib. The cadmium concentration in the testa was generally twofold higher compared to Cd concentration of the nib. Those observations could be ascribed to preferential binding of Cd to compounds present in cacao such as phytate, organic acids, polyphenols, ….

During fermentation, a change in Cd concentration in different tissues could be observed. Fermentation of two cacao cultivars (CCN-51 and Nacional) was investigated in two different fermentation set-ups: *'cascade'* (CCN-51 and Nacional) and *'one-box'* (Nacional). Based on two out of four large-scale fermentations (each executed in duplicate), nib Cd decreased while testa and mucilage Cd increased with fermentation time. Factors that influence this metal mobility include temperature of the fermenting mass (around 45 °C) and changes in pH of cacao tissues (nib pH roughly decreased from 6.3 to 4.5). Finally, drying - as a post-fermentation processing step - did not significantly alter Cd content in the cacao nibs. Also, a pre-drying treatment (prior to fermentation) performed on one fermentation (in duplicate), did not significantly affect nib Cd. Finally, washing of unfermented cacao beans with ethylenediaminetetraacetic acid (EDTA) as a possible mitigation strategy to lower Cd in the nibs, did not yield significantly lower nib Cd concentrations. This is possibly due to a limited nib pH decrease from 6.3 to 5.4. Washing could have caused significant alterations in fermentation performance. Moreover, penetration of EDTA into the cacao nib could be limited through the larger size of the molecule.

Abstract (Nederlands)

In januari 2019 werd een nieuwe verordening betreffende de maximale concentratie aan cadmium (Cd) in chocoladeproducten opgelegd door de Europese Commissie (EC). Deze verordening bedreigt de exportpositie van veel Zuid-Amerikaanse landen waar hoge Cd concentraties in cacaoproducten werden teruggevonden.

Als onderdeel van ons doel om een mogelijke strategie te ontwikkelen om Cd in cacao nibs te verlagen om aan deze nieuwe EC-voorschriften te voldoen, hebben we eerst de spreiding van Cd in Ecuadoraanse cacaobonen onderzocht tijdens naoogst processen. Bovendien werd de mate waarin Cd zich herverdeeld tijdens de fermentatie onderzocht.

Ongefermenteerde cacao weefsels (van buiten naar binnen: peul, placenta, mucilage, testa en nib) van cacaobonen uit vijf verschillende locaties in Ecuador werden geanalyseerd. Algemeen daalde de Cd inhoud van testa > nibs > mucilage. Peul en placenta Cd van twee locaties werden gemeten. Beide concentraties liggen tussen deze van nibs en mucilage. De Cd concentratie in testa was over het algemeen tweemaal hoger in vergelijking met de Cd concentratie van de nibs. Deze observaties kunnen mogelijks wijzen op een preferentiële binding van Cd met cacaocomponenten zoals fytaat, organische zuren, polyfenolen,…

Tijdens de fermentatie werd er een verandering in Cd concentratie van verschillende weefsels waargenomen. Fermentatie van twee cacao cultivars (CCN-51 en Nacional) werd onderzocht in twee verschillende opstellingen: de '*cascade'* methode (CCN-51 en Nacional) en de '*een-box'* methode (Nacional). In twee van de vier uitgevoerde fermentaties (elke fermentatie werd uitgevoerd in duplicaat) werd een daling in nib Cd en een stijging in mucilage en testa Cd geobserveerd. Mogelijke factoren die deze metaalmobiliteit beïnvloeden, zijn temperatuur van de fermentatiemassa (ongeveer 45°C) en veranderingen in pH van de verschillende weefsels (nib pH daalt van 6.3 tot 4.5). Ten slotte werd er geen invloed van drogen op Cd concentratie van nibs vastgesteld. Bovendien werd er ook geen verandering in Cd concentratie van nibs vastgesteld door cacaobonen te drogen voor aanvang van de fermentatie (uitgevoerd bij één fermentatie).

Ten slotte bleek het wassen van ongefermenteerde cacaobonen met ethylenediaminetetraazijnzuur (EDTA) geen effectieve mitigatiestrategie om Cd te verlagen in de nibs. Dit is mogelijks te wijten aan de beperkte verlaging in nib pH van 6.3 naar 5.4. Het wasproces veroorzaakte mogelijks veranderingen in het fermentatieproces. Bovendien is de penetratie van EDTA in de cacaobonen beperkt door de grotere omvang van het molecule.

List of abbreviations

AAB	Acetic acid bacteria
ANOVA	Analysis of variance
CCN-51	Colección castro naranjal 51
Cd	Cadmium
CFU	Colony forming units
CONTAM	Scientific panel of contaminants in the food chain
CV	Coefficient of variation
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
IARC	International Agency for Research in Cancer
ICGT	International Cacao Genebank in Trinidad
ICP - MS	Inductively coupled plasma mass spectroscopy
LAB	Lactic acid bacteria
LOQ	Limit of quantification
MF	Mass fraction
Micro-PIXE	Micro-particle induced X-ray emission
NIST	National Institute of Standards and Technology
PVC	Polyvinyl chloride
RASFF	Rapid Alert System for Food and Feed
TDF	Total dietary fiber
TWI	Tolerable weekly intake
WHO	World Health Organization

1 Literature Review

1.1 Research Objectives

Due to a new regulation of the European Commission (EC) that went into force on the 1st of January 2019, cadmium (Cd) in cacao has gained interest. Limits between 0.1 and 0.8 mg kg^{-1} have been imposed on chocolate products (milk- and dark chocolate and cacao powder). Since cacao beans from South American countries naturally contain higher concentrations of Cd compared to cacao from other origins (e.g. Africa), this new legislation forced (and will force) research and industry to take action in mitigating this threat to their economy. Especially Ecuador (which represents 6% of worldwide production), one of the biggest cacao producers in South America, will lose significant market share in Europe. According to The International Cocoa Organization (ICCO), the European Union (EU) is responsible for 30% of their cacao export value (ICCO, 2018). Cacao beans from South American countries display elevated concentrations of Cd. However, no closing reason behind this phenomenon has been given so far. Possible explanations can be found in the weathering of volcanic parent material and high bio-availability of soil Cd as compared to other cacao growing countries.

There is a knowledge gap concerning Cd distribution in fresh cacao fruits and beans. Therefore, in this study, the Cd content of different unfermented cacao tissues will be analyzed. Furthermore, there is little information concerning Cd distribution during post-harvest processing of cacao. In particular, this study focusses on fermentation as a crucial post-harvest processing step. During the fermentation of two cacao cultivars, Cd content in different cacao tissues will be measured on a daily base. Moreover, fermentation temperature and tissue pH will be measured as those factors could possibly explain metal mobility. The influence of drying as a post-fermentation processing step on the Cd content of cacao nibs and testa will also be investigated. Ultimately, a better understanding of those distribution patterns could lead to mitigation strategies to lower Cd concentration in the nib to meet the new EC regulations. It is of primordial importance to lower Cd concentration in the nib as this tissue is the raw material for chocolate products. To this end, unfermented cacao beans will be washed with ethylenediaminetetraacetic acid (EDTA) as a possible mitigation strategy.

1.2 Cadmium

1.2.1 Cadmium biochemistry

Cadmium is a non-essential heavy metal from group 12 and period 5 of the periodic table, therefore, it has an atomic number of 48. With a filled d-shell of electrons, cadmium(II) biology resembles zinc(II) biology. Even though the crystal ionic radius (109 pm) of Cd^{2+} is larger than that of Zn^{2+} (88 pm), it still follows similar behavior as zinc in complexing with a wide range of biological compounds (Sigel et al., 2013). According to Smolders and Mertens (2013), this heavy metal naturally occurs in almost every soil and it can be mainly found as a divalent cation (Cd^{2+} at concentrations typically ranging 0.1 – 1.0 mg kg^{-1} soil; Smolders & Mertens, 2013).

1.2.2 Cadmium toxicity to human health

The toxicity, bio-availability and retention of Cd are dependent on both dietary and physiological characteristics including nutritional status such as low iron (Il’ yasova & Schwartz, 2005; Järup & Åkesson, 2009) or diabetes (Buchet et al., 1990). Sigel et al. (2013) attributed approximately 85% of the total Cd body burden to that of the kidney and liver (Sigel et al., 2013). Because Cd is efficiently retained in the kidney (half-life 10-30 years) and poorly excreted by the liver, it can cause nephrotoxic effects like kidney tubular damage. Severe toxicity effects start to appear at an estimated daily intake via food of 300 μg Cd (Smolders & Mertens, 2013). If exposure is high and prolonged, this could eventually progress further to end stage renal disease (Hellström et al., 2001; Järup & Åkesson, 2009; Järup et al., 1998; Järup et al., 2000; Smolders & Mertens, 2013).

Besides, Cd could also cause bone damage via direct effect on the bone tissue or indirectly as a result of kidney failure. This effect was first reported in the 1950s in the Jinzu river basin near Toyama, Japan. People who were exposed to high Cd levels for a prolonged time period, mainly due to consumption of locally grown rice, suffered from ‘itai-itai’ disease. Primary traits included fractures, distortions and malformations of bones (Järup et al., 1998; Matsuda et al., 2003).

Furthermore, increased cancer risks and mortality can be ascribed to populations exposed to elevated Cd concentrations (Järup & Åkesson, 2009). The International Agency for Research on Cancer (IARC, 1993) classified Cd as a human carcinogen (group I) on the basis of sufficient evidence for carcinogenicity in both humans and experimental animals. The EU classified some

Cd compounds as possibly carcinogenic (Carcinogen Category 2; European Union, 2007). Cadmium exposure has been associated with renal cancer (Il’yasova & Schwartz, 2005), breast cancer (McElroy et al., 2006) and lung cancer (Jones et al., 2007).

1.2.3 Exposure of cadmium to humans

Exposure of Cd to humans is mainly caused by the consumption of food with elevated Cd concentrations. This contributes to an intake rate between 8-25 µg Cd per day on average (Olsson et al., 2002). Other sources include drinking water (Olsson et al., 2002), tobacco smoke (Arora, et al., 2008; Järup et al., 1998; McElroy et al., 2007) and ambient air (Hogervorst et al., 2007; Vahter et al., 1991). To ensure sufficient protection to all consumers, the European Food Safety Authority (EFSA) Scientific Panel on Contaminants in the Food Chain (CONTAM) recommended a tolerable weekly intake of 2.5 µg Cd kg^{-1} body weight (EFSA, 2011).

Cadmium can be found in a wide range of foodstuff. However, the concentrations range extremely, depending on the type of food and possible environmental contamination. Elevated concentrations of Cd can be found in mollusks, crustaceans, cephalopods and crabs. Furthermore, high levels can be found in offal (e.g. liver and kidney), oil seeds, wild mushrooms and cacao (EFSA, 2012). Cacao products only contribute to 4.3% of the total dietary Cd exposure of European people (EFSA, 2012). Thus, often, food consumption quantity, rather than Cd concentration, is the major deterministic factor in assessing Cd risk to human health. Olsson et al. (2002) estimated that 80% of the total dietary Cd contamination could be ascribed to cereal, vegetable and potato consumption (Olsson et al., 2002).

1.2.4 Sources of cadmium in environment

1.2.4.1 Natural cadmium occurrence

Smolders and Mertens (2013) stated that soil Cd concentrations typically range between 0.1 – 1.0 mg Cd kg^{-1}. By combining different large-scale surveys, they found average and median values in the range of 0.1 – 0.3 mg Cd kg^{-1} soil. All Cd in the environment could be traced back to weathering of parent rock material such as zink minerals (e.g. ZnO), greenockite (CdS) and otavite (CdCO3; Smolders & Mertens, 2013).

In 1990, Nriagu reported worldwide emissions of trace metals from natural sources to the atmosphere. According to this research, natural mobilization of Cd can occur through volcanic eruptions (0.82 tons per year), the production of marine biogenic aerosols (0.24 tons per year), physical and chemical weathering of the parent rock material or derived soils (wind-borne particles; 0.21 tons per year), burning of vegetation (0.11 tons per year) and sea salt spray (0.06 tons per year; Nriagu, 1990, 1996; Nriagu & Pacyna, 1988; Sigel et al., 2013). According to Nriagu and Pacyna (1988), 60% of the total natural atmospheric emission of Cd can be ascribed to volcanic eruptions (Nriagu & Pacyna, 1988).

1.2.4.2 Anthropogenic sources of Cd in the environment

Cadmium is generally recovered from zinc ores (as sphalerite) and concentrates. Global refinery production of Cd in 2018 was 23 000 metric tons, with China (8 200 tons) and the Republic of Korea (3 600 tons) as leading producers (Tolcin, 2018). A smaller amount of secondary Cd metal was recovered from recycling nickel-cadmium (NiCd) batteries (Tolcin, 2018). Over 80% of total available Cd is used for the production of rechargeable NiCd batteries. End-use of Cd and Cd compounds also includes alloys, anticorrosive coatings, pigments and plating, polyvinyl chloride (PVC) stabilizers, and semiconductors for solar cells (Nriagu, 1989; Nriagu & Pacyna, 1988; Pacyna & Pacyna, 2001; Smolders & Mertens, 2013; Tolcin, 2018).

During the process of Cd mining and recycling, contamination to the environment can occur. Besides, soil Cd could be elevated because of other anthropogenic activities, e.g. applications of P-fertilizers, atmospheric deposition (mainly through oil combustion) and sewage sludge (Smolders & Mertens, 2013). Phosphorus fertilizers are thought to cause the highest level of Cd contamination. However, its impact heavily depends on the source of phosphate rock used to produce the fertilizer, as the Cd concentration of phosphate rock can vary from 2 to more than 100 mg kg^{-1} (Roberts, 2014).

The major anthropogenic sources to the atmosphere from non-ferrous metal production (2171 tons per year) and fossil fuel combustion (691 tons per year) exceed major natural sources like volcanic outgassing (0.82 tons per year), and wind borne dust (0.21 tons per year), by nearly a factor 2 (Nriagu, 1990; Nriagu & Pacyna, 1988; Pacyna & Pacyna, 2001).

1.3 Raw materials: the cacao fruit

Chocolate is made from fermented and dried cacao beans, which are the seeds of the cacao tree (*Theobroma cacao L.*). The cacao plant is a perennial tree, originating from the rainforests of South and Central America. Raw cacao beans are fermented, dried and roasted before consumption (De Vuyst & Weckx, 2016). *Theobroma cacao* is the only species in the *Theobroma* genus that is cultivated on a global scale. It is planted in about 50 countries in the tropics between latitudes 15 °S and 18 °N. It can only be grown in those areas since it requires a total annual rainfall between 1250 and 2800 mm and a temperature varying between 18 °C (mean min.) and 32 °C (mean max.) with an absolute minimum of 10 °C (Wood & Lass, 1985). The main cacao cultivars are Forastero and Criollo. Those cultivars have been used to make higher yielding hybrids such as Colección castro naranjal 51 (CCN-51) and Nacional. (De Vuyst & Weckx, 2016; Wood & Lass, 1985). Both CCN-51 and Nacional yield fine flavor cacao (in comparison with African cacao). The cacao genotype determines harvest yields, resistance to diseases, and composition, flavor and quality of the cacao beans (De Vuyst & Weckx, 2016).

The cacao fruit consists of an outer husk, a placenta and 30 to 50 beans surrounded by a viscous mucilage. The placenta is attached to the pod husk at one connecting point and connects the beans to each other. It is discarded before fermentation since it is more difficult to remove afterwards and it could lead to beans sticking together (Wood & Lass, 1985). Each cacao bean consists of two cotyledons and a radicle enveloped in a seed coat (the cacao testa). The two cotyledons and radicle form the cacao nib, which is the raw material for chocolate processing. The mucilage that surrounds the cacao beans makes up 30 - 40 % of the wet bean total mass. This mucilage consists of water (85 % total wet weight), sugars like glucose, fructose and sucrose (10 - 15 %), citric acid (1 - 3 %), pectin and other polysaccharides (1 - 2 %) and proteins (0.6 %). Because of the citric acid content, the mucilage is acidic (pH 3 – 4). The cacao beans (nibs and testa) on the other hand contain approximately 32 – 39 % water, 30 – 32 % fat, 8 – 10 % proteins, 2 – 3 % cellulose, 4 – 6 % starch, 4 – 6 % pentosanes, 2 – 3 % sucrose, 5 – 6 % polyphenols, 1 % organic acids (mainly citric, oxalic, and malic acids), 1 – 3 % theobromine, and 0.2 – 1 % caffeine. The testa comprises 10 – 15 % of the total dry bean weight. Because of the lower organic acid content compared to the mucilage, the nibs are less acidic with a pH value between 6.3 and 7. The initial color of the nibs

mainly depends on the cultivar. Nacional and CCN-51, which are used in this research, have a purple color (Schwan & Fleet, 2012).

1.4 Postharvest processing of cacao

In order to make chocolate, raw (freshly harvested) cacao beans first need to be fermented by environmental inoculation to improve flavor and color formation and to eliminate embryonic growth. Afterwards, drying and roasting ensure proper aroma development. Furthermore, pathogenic and toxicogenic microorganisms are reduced to a minimum, leading to a safe food product. Before or after roasting, whole cacao beans are cracked with subsequent testa removal by air (a processed referred to as breaking and winnowing). Finally, to obtain chocolate or derivatives, cacao nibs are ground and further processed. In figure 1, an overview of the post-harvest cacao process is given.

1. Raw material: fresh cacao beans

- Cacao bean consists of mucilage, nib and testa
- Cultivars used in this thesis: CCN-51 and Nacional
- Mucilage pH: 3 - 4
- Nib pH: 6.3 - 7

2. Fermentation

- Improve color and flavor formation
- Environmental inoculation
- Metabolite conversion by yeast, lactic acid bacteria and acetic acid bacteria
- Three consecutive phases (anaerobic, micro aerobic and aerobic) characterised by oxygen availability, metabolite concentration, temperature and pH
- 2 - 10 days
- 50 - 250 kg at once in heap or wooden box
- Temperature increase to 45 - 50 °C, nib pH decreases to 4.0 - 5.5 and mucilage pH increases to 4.5

3. Drying

- Reduce moisture content, enhance flavor development and brown color
- Moisture content reduced from 40 - 55 % to 4 - 8 % (total wet weight base)
- Sun-drying OR artificial drying

4. Testa removal

- Bean cracking and removal of extraneous material
- Removal by air (winnowing)
- Max 5 % remaining on fat-free dry weight base
- 10 - 15 % of total dry bean weight

5. Roasting

- 10 - 30 min. at 90 - 170 °C
- Darkening of nib color, development of flavor, reduction in number of microorganisms, reduction in amount of bitter- and acid compounds (Maillard reactions)
- Moisture reduction to 2 % on total dry weight basis
- Flavor formation and decrease in micorbial load

6. Further processing into chocolate (or other derivates)

- Grinding into low viscosity paste (= cacao liquor)
- Addition of other ingredients e.g. cacao butter, sugar, milk powder
- Agitation of mixture to remove off-flavours (= conching)
- (alkalinization)
- Crystallization of fat in appropriate polymorphic structure for shiny appearance, good texture (= tempering)

Figure 1: Post-harvest cacao processing.

1.4.1 Fermentation

De Vuyst and Weckx (2016) ascribed several purposes to fermentation. First of all, it is a post-harvest processing step that facilitates the removal of mucilage around the cacao beans and subsequent drying of those beans. Secondly, fermentation improves color and flavor development of the non-germinating cacao beans, as it avoids embryonic growth and activates hydrolytic bean enzymes. Lastly, fermentation reduces bitterness and astringency of the cacao nibs, in particular by the exchange of compounds through diffusion between the cacao bean cotyledons and the environment (De Vuyst & Weckx, 2016).

1.4.1.1 Fermentation techniques

There are two major traditional set-ups for fermentation: heap and box fermentation. Cacao beans fermented according to those methods undergo similar chemical and biological changes. However, due to different fermentation sizes, changes in duration, temperature and oxygen availability; significant differences in final product quality can be found. It should be noted that current fermentation techniques are not standardized since most cacao is fermented on small farms (often 1 – 5 ha; Wood & Lass, 1985). Box fermentation method is mostly used in South American and West Indian countries, and involves the use of rectangular wooden boxes with dimensions ranging from 50 cm to 1.20 m, leading to a total fermenting mass of 50 - 250 kg. Holes in the floor of the box are provided for drainage and aeration. The fermenting mass is covered with jute bags or banana leafs to limit temperature fluctuations and is mixed every day or every other day for 2 - 10 days depending on the cacao cultivar and local practices. Boxes are often set up in cascades to facilitate mixing of the fermenting mass, by transferring it to the next box (Wood & Lass, 1985). In the heap method, cacao beans are deposited on plantain or banana leaves in heaps of 200 to 500 kg and topped with more banana leaves to prevent heat loss. Fermentation generally lasts for 6 days and the heaps are turned manually once or twice. However, some farmers also ferment for shorter periods of time (3 - 5 days) with one turning, or no turning at all (Wood & Lass, 1985). Heap fermentation is mostly used by smallholder farmers in West-Africa since it is inexpensive, easy to carry out, and does not require any permanent structures (Schwan & Fleet, 2012).

1.4.1.2 Chemical and microbiological changes during fermentation

Fermentation involves a succession of microbial populations, namely yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). Lefeber et al. (2012) identified that these consecutive

activities depend on several factors: microbial load, species diversity, temperature, pH, oxygen tension and available substrates and metabolites (Lefeber et al., 2012). Under optimal conditions, these microbial activities should not exceed four days (De Vuyst & Weckx, 2016; Lefeber et al., 2012).

Mucilage and cacao nibs from ripe pods are a rich medium for microbial growth. Once these tissues get inoculated with microorganisms from the environment, spontaneous fermentations starts (Camu et al., 2007; Nielsen et al., 2007). The initial load of microorganisms ranges between 10^4 and 10^8 colony forming units per gram (CFU/g), depending on the main inoculating source. Factors affecting this initial load include the quality and integrity of the pods, the hygiene of the processes used to remove the beans from the pods, and the time between bean removal and start of the fermentation process (Schwan & Fleet, 2012).

1.4.1.3 Anaerobic yeast phase (24-48 h into fermentation)

The first major event involves the mucilage being hydrolyzed and degraded by yeasts, which are the dominant microorganisms at the onset of fermentation. As a result, up to 20 % of the mucilage can be removed (referred to as *'mucilage sweatings'*; Schwan & Fleet, 2012). To this end, fermentation boxes are provided with holes in the bottom. In total, between 10 and 50 kg of mucilage can be lost per fermentation box. Growth of yeasts is favored by the acidity of the mucilage (pH 3-4), together with low oxygen levels. This reducing environment is created by the tightly packed cacao mucilage - bean mass and the production of carbon dioxide by yeasts and hetero-fermentative LAB. The large volume of carbon dioxide formed during the first stage of fermentation will move downwards from a box and outwards from a heap. Therefore, the oxidational changes will start at the top of a box and on the surface of a heap. During the course of fermentation, the oxygen level falls rapidly during the first day and then recovers until the beans are mixed, when there is another sharp fall followed by a steady rise (Wood & Lass, 1985).

Yeasts (e.g. *Candida, Pichia, Saccharomyces, Kloeckera* and *Trichosporon*) are able to ferment carbohydrates from the mucilage (mainly glucose and fructose). According to Schwan and Wheals (2004), the total yeast population increases from 10^7 CFU/g mucilage to 10^8 CFU/g mucilage during the first 12 h, after which it remains constant for 12 h. Finally, after one day of fermentation, the yeast population declines to 10^4 CFU/g mucilage, followed by a slower decrease leading to a

final population at the end of fermentation of 10 viable cells per gram mucilage (Figure 2). After 132 - 160 hours of fermentation, there is an increase in the number of yeast cells. This is due to growth of thermo-tolerant yeasts (e.g. *Kluyveromyces thermotolerans*) that utilize some of the acids and are able to grow in an aerobic environment (Schwan & Wheals, 2004).

Ethanol, CO_2, organic acids, pectinolytic enzymes, glycerol and volatiles are produced by yeasts in the first 2-3 days of fermentation. As a result, temperature of the fermenting mass increases from ambient temperature (25-30 °C) to 35 – 40 °C within de first 48 h of fermentation. Furthermore, yeasts from *Candida* and *Pichia* genera break down the citric acid in the mucilage leading to an increase in pH from 3.5 to 4 - 4.5. However, as those yeasts also produce organic acids like acetic, oxalic, phosphoric, succinic and malic acid, pH fluctuations tend to be reduced by the buffering capacity of those metabolites (Figure 2; Schwan & Fleet, 2012).

1.4.1.4 Micro aerobic enterobacterial and lactic acid bacteria phase (24-72 h into fermentation)

The second major phase of fermentation occurs when mucilage drains away and air enters into the fermentation cacao mass. This creates ideal conditions for the growth of bacteria, in particular enterobacteria and lactic acid bacteria. After 24 to 36 h, there is no more run-off of mucilage, indicating that the majority of pectinolytic reactions have seized since not all mucilage is removed at this stage (Gálvez, Loiseau, Paredes, Barel, & Guiraud, 2007).

The LAB count (e.g. *Lactobacillus fermentum, Lb. plantarum* and *Leuconostoc mesenteroides*) reaches a peak of 10^7-10^8 CFU/g mucilage around 36 hours after the fermentation process begins (Figure 2; Schwan & Wheals, 2004). The LAB exhibit the fastest growth rate during the 16 – 48 h period of fermentation and are present in greater numbers, but not necessarily in greater biomass, than yeasts (Schwan & Fleet, 2012; Schwan & Wheals, 2004).

Growth of LAB is coincident with a decline of the yeast population. This is due to a slight increase in pH of the mucilage from to 3 - 4 to 4.5 and an increase in oxygen availability. As a result of biochemical conversions of metabolites, temperature increases from 35 - 40 °C to 45 - 50 °C after 48 h (De Vuyst & Weckx, 2016; Gálvez et al., 2007; Schwan & Wheals, 2004).

During the micro aerobic phase, lactic acid is produced from glucose by LAB via the Embden-Meyerhof pathway. However, some LAB utilize glucose via the hexose monophosphate pathway. Other metabolites that are produced by LAB during this phase include carbon dioxide, ethanol, mannitol, diacetyl, acetoin and 2,3-butanediol (De Vuyst & Weckx, 2016; Schwan & Wheals, 2004).

1.4.1.5 Aerobic acetic acid Bacteria phase (48-112 h into fermentation)

During the aerobic AAB phase, aeration of the fermenting mass increases and there is no more loss of mucilage. Increased oxygen availability facilitates AAB (e.g. *Acetobacter* and *Gluconobacter)* to become the dominant microorganism as oxygen is required in their reaction to convert ethanol to acetic acid.

The total population of AAB reaches a peak after 72 - 84 hours of fermentation with 10^7 - 10^8 CFU/g mucilage (Figure 2). As the temperature of the fermenting mass further increases to 50 °C, growth of acetic acid bacteria slows down (Schwan & Wheals, 2004). Finally, after 4 to 5 days, bacilli and filamentous fungi can participate in the fermentation too (De Vuyst & Weckx, 2016; Pereira et al., 2012).

During the aerobic AAB phase, ethanol is oxidized to acetic acid and further oxidized to carbon dioxide and water. Schwan (1998) reported maximal concentrations of 6 g acetic acid L^{-1} in the mucilage after 88 hours of fermentation (Schwan, 1998). After 72 h, a decrease in acetic acid concentration can be observed due to its evaporation at high temperature (De Vuyst & Weckx, 2016). During fermentation, acetic acid (and lactic acid) penetrates into the nibs, leading to the destruction of internal cotyledon membranes. Subsequently, two pH-dependent enzymes are released that break down particular bean proteins to produce amino acids and peptides that play a key role in the development of chocolate flavor when the beans are roasted at a later stage of processing (Schwan & Fleet, 2012; Schwan & Wheals, 2004).

As a result of the exothermal reactions, the temperature increases and reaches a maximum of 45 – 50 °C after 4 - 5 days. The increase in temperature resulting from these reactions depends largely on the weight of the cacao beans, the frequency of mixing and the thermal insulation (De Vuyst & Weckx, 2016; Schwan & Fleet, 2012; Schwan & Wheals, 2004). There can also be considerable variation in temperature within the fermenting mass. In a box, the temperature of the cacao beans

at the sides and close to the bottom rises more slowly and may not exceed 45 °C (Wood & Lass, 1985). Oxidation of acetic acid and lactic acid results in a slight pH increase of the mucilage from 4.5 to 5 - 6.

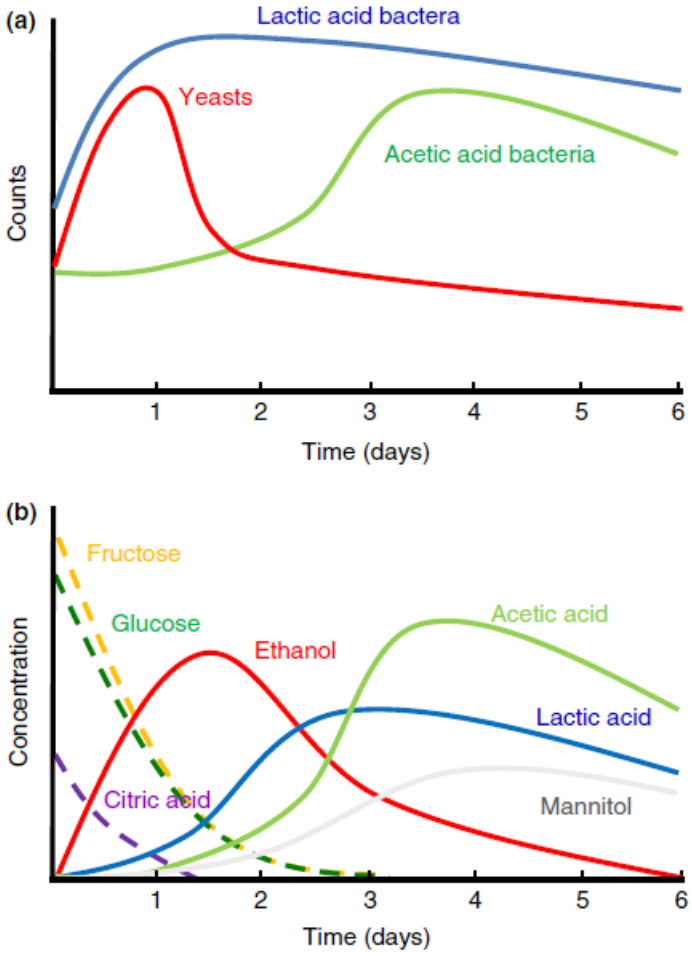

Figure 2: Microbial cell counts (a) and metabolite production kinetics (b) of a spontaneous cacao bean fermentation (De Vuyst & Weckx, 2016).

1.4.1.6 Influence of fermentation on cacao nibs

Metabolites produced by microorganisms during fermentation in the mucilage (lactic acid, acetic acid, ethanol, …) diffuse across the testa into the nib. Conversely, substances (like polyphenols, peptides and proteins) found in the nib can diffuse back into the mucilage. The testa acts as a biological barrier that controls the kinetics of fermentation and concomitant diffusion processes in and out of the beans (De Vuyst & Weckx, 2016).

Ethanol, water, acetic acid, and lactic acid have been identified to be the most important microbial metabolites diffusing into the seed. After penetration into the seed, acetic acid is responsible for

killing the embryo, decreasing pH of the nib (from 6.3 - 7 to 4.0 - 5.5) and forming precursors for Maillard and Strecker reactions during cacao roasting later in the process. Consecutively, this pH decrease causes the internal membranes of the cacao nib to degrade (De Vuyst & Weckx, 2016; Pereira et al., 2012; Schwan & Fleet, 2012; Schwan & Wheals, 2004).

Degradation of internal nib structures and flux of metabolites across the testa, causes biochemical conversions to take place in the nib. These involve vicilin (globulin) storage protein degradation by proteases and anthocyanin, and glycoside-bound terpene splitting by glycosidases during the anaerobic and microaerophilic phases of the cacao bean fermentation process, as well as polyphenol oxidase activity that is taking place during its aerobic phase. These reactions ultimately lead to the production of flavor precursors (e.g. reducing sugars, hydrophilic peptides and hydrophobic amino acids) and alteration of nib color from purple to brown in case of the CCN-51 an Nacional cultivars (De Vuyst & Weckx, 2016).

1.4.2 Drying

Drying of fermented cacao beans has two main purposes. Firstly, moisture content is reduced from 40 - 55 % to 4 - 8 % (on total wet weight basis), making the product safe for storage and shipment. Secondly, drying leads to lower astringency, bitterness, and acidity, while enhancing flavor development and the brown color associated with well-fermented beans (Schwan & Fleet, 2012; Wood & Lass, 1985).

The drying time of cacao beans is determined by two factors: the rate of heat transfer into the bean for moisture evaporation and the movement of the vaporized moisture within the bean to the surrounding drying air. The drying rate is determined by whichever of these two factors is slower and has a crucial effect on the flavor and quality of the final dried beans (Schwan & Fleet, 2012; Wood & Lass, 1985).

The two most commonly used methods to dry cacao beans are natural sun-drying and artificial drying. In some cases, a combination of both is used. In the sun-drying method, fermented beans are spread out on mats, concrete floors or wooden trays and directly exposed to sunlight. Even though it is more labor intensive and time-consuming compared to artificial drying, it is simpler,

cheaper and results in better product quality. To allow uniform drying, cacao beans are turned at least twice a day, regardless of the drying method.

It is important that drying rate and -temperature are controlled to ensure good quality cacao beans. An excessively rapid drying rate may lead to several adverse effects, including high acidity and low browning of the nibs. Final bean acidity is determined by acetic acid, the main volatile acid, and lactic acid, the main nonvolatile acid. Because acetic acid is removed from the cacao nib by oxidative vaporization, drying processes are controlled to maximize the loss of this volatile acid. However, rapid drying may also result in hardening of the testa, limiting acid flux to the environment. Browning of the nibs is also determined by the drying rate. During the browning reaction, polyphenol oxidase will oxidize anthocyanins and catechins resulting in less purple beans. This reaction is mainly controlled by the oxygen transfer rate to the nib. Drying temperatures above 65 °C could lead to hardening of the bean surface (testa and mucilage remainings), ultimately limiting oxygen transport. As a result, cacao beans remain purple and more astringent in taste (Schwan & Fleet, 2012; Wood & Lass, 1985).

1.4.3 Testa Removal

Cacao beans of consistent quality are obtained by two major sanitary procedures: extraneous material removal and cleaning of beans from dirt and infestation. Afterwards, the cacao beans are broken and deshelled by the process of winnowing. This involves blowing away lighter testa particles with a steady air flow. This is needed to obtain pure cacao nibs as testa material will give the final product an unpleasant astringent and bitter taste. According to standards from the Codex Alimentarius, cacao liquor (explained in section 1.4.5) may not contain more than 5 % testa on fat free dry matter (Codex Alimentarius, 2001). Finally, metallic extraneous material is separated with strong magnets.

1.4.4 Roasting

Cacao beans are roasted to further develop flavor and color. According to Schwan and Fleet (2012) several other physicochemical changes take place, including: (i) moisture loss to 2 % water content on dry weight basis, (ii) darkening of nib color, (iii) reduction in the amount of volatile acids and substances contributing to an acidic and bitter taste, (iv) development of flavor compounds through

Maillard reactions and (v) reduction in the number of microorganisms present in beans (Schwan & Fleet, 2012).

The process takes between 10 and 30 min, depending on the applied temperatures (90 - 170 °C) and the initial and desired final moisture content. Well-fermented, dried and roasted cacao beans are characterized by a complex profile of nearly 400 volatile compounds, including thiazoles, pyrazines, oxazoles, furans, pyridines, and pyrrol derivates (De Vuyst & Weckx, 2016; Schwan & Fleet, 2012).

1.4.5 Further processing into the final product

After roasting, nibs are ground into a low-viscosity paste, the cacao liquor. First of all, rough stone, disk or ball mills are used to break fat cells and melt fat crystals by heat generated due to friction. Secondly, grinding of nibs decreases the average particle size to 30 μm or lower, depending on the application of the final product (Schwan & Fleet, 2012).

At this stage of the process, cacao liquor is stored at 90 - 100 °C to avoid microbiological growth and facilitate product handling. This product is than further used as the main raw material for cacao butter extraction, cacao powder production or chocolate manufacturing. In the latter, cacao liquor and butter are mixed with sugar (and skimmed milk powder for milk chocolate). The agglomeration of those ingredients requires additional particle size reduction to obtain a smooth final product. Next, three stage conching is needed to further develop flavor and texture of the chocolate. During this step, other ingredients like surfactants (e.g. lecithin) are added to a heated agitator (conch) to alter the viscosity and the final melting behavior of the chocolate. Conching times and temperatures vary between 16 - 24 h at 60 °C for milk powder products and 8 h at 70 - 82 °C for dark chocolates (Schwan & Fleet, 2012).

Finally, the chocolate is tempered to give it a shiny appearance, good texture, contraction upon crystallization and resistance to fat phase separation (bloom). Tempering is a process where temperature is altered to yield fat crystals of the appropriate polymorphic structure (Schwan & Fleet, 2012). Beckett (2008) described that tempering time and temperature depend on the cacao content of the chocolate (Beckett, 2008).

1.5 Cadmium in cacao

1.5.1 Regulations on cadmium levels in cacao and cacao products

The maximum Cd levels in chocolate are based on recommendations of the Scientific Panel of Contaminants in the Food Chain (CONTAM panel) of the European Food Safety Authority (EFSA). In that recommendation, EFSA established a tolerable weekly intake (TWI) of 2.5 µg Cd kg^{-1} body weight (EFSA, 2011). The Joint Food and Agriculture Organization (FAO) / World Health Organization (WHO) Expert Committee on Food Additives established a provisional tolerable monthly intake of 25 µg kg^{-1} body weight (EFSA, 2012).

For chocolate, three maximum levels for Cd have been established depending on the cacao mass content of the chocolate variety. The strictest maximum levels apply to the chocolate varieties mostly eaten by children. The darker the chocolate, the higher the maximum levels are (Table 1). This is based on the negligible Cd content of non-cacao solids like milk powder and sugar. These maximum levels entered into force on January 1st, 2019 (European Commision, 2014).

Table 1: Maximum allowed Cd levels in specific cacao and chocolate products (European Commision, 2014).

	Max allowed Cd concentration (mg kg^{-1} dry weight)
Milk chocolate with < 30 % total dry cacao solids	0.10
Chocolate with < 50 % total dry cacao solids Milk chocolate with ⩾ 30 % total dry cacao solids	0.30
Chocolate with ⩾ 50 % total dry cacao solids	0.80
Cacao powder sold to the final consumer or as an ingredient in sweetened cacao powder sold to the final consumer	0.60

Even though these maximum levels entered into force on January 1st, 2019, measures have already been taken to avoid high Cd products from entering the European market. According to the Rapid Alert System for Food and Feed (RASFF) there have been four cases in which cacao based products have been refused or taken out of the EU market. All cases involve the presence of elevated levels of Cd (ranging between 0.899 and 2.46 mg kg^{-1}) in cacao products from Latin American countries (Ecuador, Colombia and Peru). Products of concern were either withdrawn

from the market, recalled from customers or destructed at the border (RASFF portal). At the international workshop on Cd in cacao and chocolate products of the ICCO (May 2012) scientist and cacao-producing countries established permissible levels of Cd in cacao and cacao-based products. In whole cacao beans, 0.6 mg Cd kg^{-1} has been proposed as the threshold (Chavez et al., 2015).

1.5.2 Variations of cadmium levels in cacao beans

1.5.2.1 Geographical variation of cadmium levels in cacao beans

The influence of the geographical origin on the total Cd concentration of cacao has been well established. Literature available (Abt et al., 2018; Bertoldi et al., 2016; Mounicou et al., 2003) suggests that Cd content in South American cacao products is generally higher compared to that of other cacao growing regions.

In 2016, Bertoldi et al. reported a multi-elemental fingerprinting and geographic traceability method for cacao beans and products (Bertoldi et al., 2016). They investigated the content of 56 macro-, micro- and trace-elements of 61 cacao beans produced in 23 countries of East and West Africa, Asia and Central and South America using inductively coupled plasma mass spectrophotometry (ICP-MS). Interesting opportunities were pointed out in tracing back the subcontinental origin of 13 commercial samples of dark chocolate. In those chocolates, significantly higher Cd concentrations (mean ± standard deviation) could be found for South American samples (615 ± 398 µg kg^{-1}) compared to East African and Central American samples (201 ± 31 and 142 ± 51 µg kg^{-1} respectively; Bertoldi et al., 2016).

Abt et al. (2018) investigated 76 cacao powder and chocolate products in the United States market. In this research, the greatest influence of geographic variation is observed for cacao powder, which also had the highest Cd concentrations (mean 0.70 mg kg^{-1} and range between 0.01 and 3.15 mg kg^{-1}) relative to the other chocolate products (cacao nibs: mean 0.62 mg kg^{-1} and range between 0.32 and 1.44 mg kg^{-1}; dark chocolate: mean 0.27 mg kg^{-1} and range between 0.02 and 1.29 mg kg^{-1}; milk chocolate: mean 0.06 mg kg^{-1} and range between 0.004 and 0.31 mg kg^{-1}; Abt et al., 2018).

Results from Mounicou et al. (2003) concerning geographic variation of Cd levels in cacao products are less conclusive. Mounicou et al. (2003) reported higher Cd concentrations (mean ± standard deviation) in cacao powder from Ecuador (533 µg kg^{-1}; n=1), Venezuela (1833 ± 20 µg kg^{-1}; n=3) and Malaysia (602 ± 73 µg kg^{-1}; n=7) compared to Brazil (148 ± 32 µg kg^{-1}; n=2), Ivory Coast (94 ± 14 µg kg^{-1}; n=3) and Ghana (133 ± 20 µg kg^{-1}; n=3). Given the limited number of countries investigated and the small sample sizes per investigated country, care should be taken when analyzing these results (Mounicou et al., 2003).

Even though significant positive relationships were identified between Cd in cacao tissues and corresponding total Cd levels in Ecuadorian soils by Argüello in 2019, this variation does not explain the difference in Cd content of cacao from different countries (Argüello et al., 2019). This finding is supported by data in table 2. It can be seen that soil Cd levels are not always higher in Ecuador as compared to other cacao growing regions. However, these findings are not statistically confirmed. Besides, within country variation of Cd levels may exceed the variation between countries (Argüello et al., 2019). One possible explanation for elevated Cd concentrations in South American cacao can be found in the availability of soil Cd. Smolders and Mertens (2013) stated that bio-availability of Cd can vary by approximately a factor 10 at identical total soil Cd concentration. Bio-available Cd generally increases 1.5-fold per unit decrease in soil pH (Smolders & Mertens, 2013). Thus, liming of soil could be considered as a possible Cd remediation strategy. Arguëllo et al. (2019) showed that bean Cd concentrations increased with decreasing soil pH, oxalate-extractable manganese and organic carbon (R^2 = 0.65), suggesting that Cd solubility in soil mainly affects Cd uptake (Argüello et al., 2019).

Table 2: Mean (min – max) Cd concentration in soils of different countries on dry weight base (mg Cd kg^{-1} soil).

Country	Mean Cd concentration	Sample Size	Reference
Cuba	1.2 (0.1 - 6.1)	n = 33	(Alfaro et al., 2015)
China	0.27 (0.01 - 152.95)	n = 486	(Zhang et al., 2015)
New Zealand	0.32 (0.01 - 2.70)	n = 939	(Mcdowell et al., 2013)
Thailand	0.03 (/ - 1.30)	n = 318	(Zarcinas et al., 2004b)
Malaysia	0.12 (0.01 - 2.02)	n = 241	(Zarcinas et al., 2004a)
Ecuador	0.44 (0.03 - 10.0)	n = 560	(Argüello et al., 2019)
	Surface soil: 1.54 (0.88 - 2.45)	n = 19	(Chavez et al., 2015)
	Subsurface soil: 0.85 (0.06 - 2.59)		
Honduras	0.25 (0.02 - 0.6)	n = 55	(Gramlich et al., 2018)
26 EU countries	0.2 (median) (0.01 - 23.6)	n = 1588	(Lado et al., 2008)

1.5.2.2 Variation of cadmium levels in cacao beans due to genetic variation

Lewis et al. (2018) investigated genetic variation in the bioaccumulation of Cd in cacao to mitigate high Cd levels in cacao beans. In this study, a 13-fold variation in bean Cd and a 7-fold variation in leaf Cd was observed between 100 genetic cultivars of the International Cacao Genebank in Trinidad (ICGT), despite the uniform bio-available and total Cd in the soil (Lewis et al., 2018). Similar studies in rice and soybeans have shown a two to four fold variation in grain Cd content between cultivars (Arao & Ae, 2003). These results point to the potential of using a genetic strategy to mitigate Cd in the short-term within cacao beans either through breeding or through the use of low Cd uptake rootstocks in grafting (Arao & Ae, 2003; Kubo et al., 2008; Lewis et al., 2018).

Other attempts have been made to lower Cd uptake in rice cultivars. Ishikawa et al. (2012) identified the gene responsible for low Cd cultivars by sequencing different mutations of the same gene (OsNRAMP5). These mutants (produced by ion-beam radiation) showed decreased Cd uptake by roots, resulting in lower Cd levels in grain (< 0.05 mg Cd kg^{-1}) compared to their parent (1.73 mg Cd kg^{-1}; Ishikawa et al., 2012). However, no similar genetic solutions to lower Cd in cacao cultivars have been reported to date.

Despite succesfull attempts to lower Cd in rice and other crops, literature remains inconsistent concerning the significance of genetic variability of the cacao plant on Cd uptake in cacao beans.

For example, Argüello et al. (2019) found no significant effect of genotype (CCN-51 vs. Nacional) on Cd concentration in cacao beans, despite a very large sample size of 200. None the less, if they took other influencing variables into account such as soil and agronomic factors, a difference in bean Cd concentration was observed between Nacional and CCN-51 (factor 1.3 more in Nacional; Argüello et al., 2019). This difference could be attributed to a dilution effect, in which cacao beans from higher yielding cultivars (such as CCN-51 with 1.0 ton/ ha annual yield) tend to have a lower Cd concentration compared to lower yielding cultivars (such as Nacional with 0.4 ton/ ha annual yield; $p \leq 0.05$; Argüello et al., 2019).

Literature points to the potential of using a genetic strategy to mitigate Cd within cacao beans either through breeding or through the use of low Cd uptake rootstocks in grafting. However, some caution must be given: both in the research of Argüello et al. (2019) and this work, the specific Nacional genotype was not identified and the differences in grafting and rootstock selection were also not recorded. Moreover, it cannot be said with certainty that cacao beans from identical clones were analyzed.

1.5.2.3 Variation of cadmium levels within cacao beans: difference between the cacao tissues

Literature on variation of Cd in the different cacao tissues is inconsistent. In general, Cd concentrations are reported to be higher in the testa compared to the nibs (Table 3). Lee & Low (1985), Lewis et al. (2018) and Ramtahal et al. (2015) reported roughly a two-fold higher testa Cd concentration compared to nib Cd concentration (Lee & Low, 1985; Lewis et al., 2018; Ramtahal et al., 2015). Contradictory to those results, Chavez et al. (2015) reported over a ten-fold lower Cd concentration in testa compared to nibs (Chavez et al., 2015; table 3).

Valiente et al. (1996) investigated Cd binding behavior (capacity) on cacao and isolated total dietary fibre (TDF) under simulated physiological pH conditions. These researchers concluded that Cd was preferentially bound to dietary fiber. Moreover, phytate was also assumed to be an important cacao constituent that binds Cd (Valiente et al., 1996). They found a pronounced effect of pH on the Cd binding behavior. This pH dependent binding to lignin, cellulose, phytate and pectin polysaccharides is mostly due to altered ionization of functional groups such as carboxyl, methoxyl and hydroxyl. Finally, Valiente et al. (1996) reported that mineral binding increases with

increasing pH (Valiente et al., 1996). All findings listed above could help to explain differences in Cd concentration between testa and nib. However, to the best of our knowledge, no literature is available that is able to describe the Cd distribution in cacao testa and nib.

In order to better understand Cd accumulation and distribution, Vogel-Mikus et al. (2007) investigated spatial distribution of Cd within seeds and germinating seedlings of *Thlaspi praecox*, a known hyperaccumulator. Localization by micro-particle induced X-ray emission (Micro-PIXE) revealed preferential storage of Cd in the embryonic axis and in the epidermis of cotyledons. (Vogel-Mikus et al., 2007).

Table 3: Distribution of cadmium [mean (coefficient of variation if reported) in mg kg^{-1}] in cacao nib, testa and whole bean: overview of literature.

Cd in nib	Cd in testa	Cd in whole bean	Sample size	Reference
0.88	1.68	0.99	n = 2	(Lee & Low, 1985)
0.88	1.83	0.99 (28%)	n = 20	(Lewis et al., 2018)
0.98 (46%)	2.09 (52%)	/	n = 45	(Ramtahal et al., 2015)
0.94	0.09	/	n = 19	(Chavez et al., 2015)

1.5.2.4 Variation of cadmium levels during post-harvest processing after fermentation

According to Lee and Low (1985), no significant amount of Cd is present in cacao processing facilities (Lee & Low, 1985). Furthermore, Bertoldi et al. (2016) and Yanus et al. (2014) found a significant positive correlation between cacao solid content and Cd concentration (Bertoldi et al., 2016; Yanus et al., 2014). Moreover, Abt et al. (2018) found a decreasing trend in Cd concentration (mean $\pm$ standard deviation) from milk chocolate (0.06 $\pm$ 0.07 mg kg^{-1}) to dark chocolate (0.27 $\pm$ 0.25 mg kg^{-1}) and cacao powder (0.70 $\pm$ 0.83 mg kg^{-1}). Moreover, Cd content in cacao nibs (0.62 $\pm$ 0.38 mg kg^{-1}) was comparable to that of cacao powder. If processing would have an influence, Cd content of cacao powder and chocolates would be significantly higher than the nib Cd content. Therefore, these results point out that the post-fermentation cacao processing has a negligible influence on final Cd content of the cacao products. It should be also noted that the Cd content of other ingredients (cacao butter, milk powder, sugar or lecithin) that are added to cacao liquor is low compared to the nib Cd (raw material for the cacao liquor). For example, according to Mounicou et al. (2003), Cd concentration in cacao butter (range: 0.007 - 0.010 mg kg^{-1}) can be

a 20-fold lower than Cd concentration in cacao liquor (range: 0.282 - 1.082 mg kg^{-1}; Mounicou et al., 2003).

Processing of fermented cacao beans (e.g. roasting, grinding, removal of the testa) can affect the binding of Cd to various chelating agents. For example, Mounicou et al. (2003) observed a (two- to fivefold) lower solubility of Cd in roasted beans (25 % extractable with SDS) in comparison with unroasted beans (10 % extractable with SDS). This suggests that less soluble species may be formed in chemical reactions during roasting. Note that no differences in total Cd concentration were reported between unroasted and roasted cacao (Mounicou et al., 2003).

1.6 Research questions

The objective of this master thesis was three-fold.
First, the Cd distribution in unfermented cacao beans will be investigated. The research questions are stated as follows: *"How is cacao partitioned in the different tissues of cacao beans and pods? Is there any correlation between Cd content in the different tissues? What could be the cause of this distribution?*" To this end, multiple samples will be tested on Cd content and pH of the composing tissues (mucilage, testa and nib).

Secondly, the Cd distribution in cacao beans will be investigated during post-harvest processing. The research question is stated as follows: "*Is there a change in cadmium concentration in cacao beans during fermentation?"*. The temperature of the fermenting mass and pH of cacao tissues will be measured on a daily base.

Finally, we will work towards a mitigation strategy to ultimately decrease Cd concentration in cacao nibs during post-harvest processing, as this is the raw material for further cacao processing into cacao liquor. More specifically, possible mitigation strategies could include washing unfermented cacao beans with a chelating agent such as ethylenediaminetetraacetic acid (EDTA) prior to fermentation. Therefore, the research question is stated as follows: *"Is chelating agent washing of cacao prior to fermentation a feasible technique to lower the Cd concentration in the final cacao-derived product?"*

2 General materials and methods

This section concerns general materials and methods that are repeatedly used in different experiments (Sections 3, 4 and 5) of this thesis research. Other materials and methods used, will be explained in more detail in sections 3.2, 4.2 and 5.2.

All glassware used in the experiments was soaked overnight in 0.2 M HCl to avoid trace metal contamination. Then, it was rinsed using distilled deionized water and dried in an oven at 70 °C. All reagents used in this study were of analytical grade.

2.1 Digestion and elemental analysis with ICP-MS

Dried and ground cacao samples were weighed out in duplicate (0.1 ± 0.001 g) into glass digestion tubes. Next, 2 mL of concentrated analytical grade Suprapur® nitric acid (HNO3, 65% V/V; Merck, Darmstadt, Germany) was added to each test tube. All tubes were swirled to mix and left overnight in a ventilated fume hood at room temperature. For each batch of analyzed cacao tissues, four sample blanks and three internal lab standards (reference material: NIST 2384 baking chocolate) were prepared identically and treated in the same way as the samples. All samples were digested in a dry-block heater (Digiprep MS) for 2 hours at 90 °C followed by roughly 4 hours at 120 °C until near dry. Then, an extra 1 mL of concentrated analytical grade nitric acid was added and the samples were again digested until near dry at 120 °C. After acid digestion, samples were brought to a 10 mL volume with milli-Q water (18.2 M Ω cm^{-1}), individually covered with parafilm and placed overnight in a ventilated fume hood at room temperature. Samples were then further diluted (10 or 20 times) for ICP-MS analysis. The elemental composition of the digest was determined by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, USA) in the He gas mode, using the 111Cd isotope.

2.2 Quality assurance and control of Cd measurements

The method was validated with National Institute for Standards and Technology (NIST) standard reference material (NIST 2384 baking chocolate), included in triplicate in each digestion. The

Cd^{111} (He) recoveries (in %) were calculated per batch by dividing the actual Cd content by the theoretical Cd content of the reference material (0.0734 ± 0.008 mg kg^{-1}; in triplicate). This yielded a minimum recovery of 87 % and a maximum recovery of 108 %.

The limit of quantification (LOQ) of the ICP-MS analysis was 0.002 µg Cd L^{-1} (mean), with 0.017 µg Cd L^{-1} as maximum value. This limit of quantification (LOQ) on solid weight basis was calculated as the maximum value of either three times the standard deviation of the blanks, the LOQ of the ICP-MS or the maximum Cd in the blanks. This yielded an LOQ on solid base of 0.02 mg kg^{-1}. All Cd^{111} (He) measurements were above the LOQ, and are therefore considered to be reliable. Moreover, for all samples, duplicates were included to evaluate reproducibility. The coefficient of variation between those duplicates ranged between 0.066 % and 77 % and had an average value of 6.11 %.

2.3 Statistical data analysis

Statistical analysis among means was evaluated by using analysis of variance (ANOVA) at P-value = 0.05, with statistical software JMP. Pearson correlation coefficients yielded information about correlation between two variables (at P-value = 0.95).

3 Distribution of cadmium in unfermented cacao beans

3.1 Research question and hypothesis

In this section, we will assess the spatial variability of Cd in unfermented cacao beans after harvesting. Our research question is as follows: "*How is cacao partitioned in the different tissues of cacao beans and pods? Is there any correlation between Cd content in the different tissues?* Answers to those questions could ultimately lead to a hypothesis concerning possible migration patterns of Cd during fermentation and to the development of a strategy to lower Cd in cacao nibs.

Little prior research data concerning metal content in cacao tissues could be found. The available literature suggests a higher Cd concentration in testa compared to nibs but the reported values are inconsistent. Our hypothesis follows what is stated previously in the literature review (Lee & Low,

1985; Lewis et al., 2018; G. Ramtahal et al., 2016): *we expect the Cd concentration to decrease from testa > husk > nibs*. To the best of our knowledge, no research papers are available concerning the Cd content in mucilage and placenta. Therefore, no hypothesis concerning Cd content in those cacao tissues is made.

In this research, measurements on Cd content and pH of unfermented cacao placenta, husk, mucilage, testa and nib will be performed. To account for influencing factors such as cultivar and location, multiple samples were taken. Those results could help to understand the influence of certain pre-fermentation treatments like washing or drying of beans (elaborated in sections 0 and 5).

3.2 Materials and methods

3.2.1 Sample selection

To investigate the distribution pattern of Cd in the cacao fruit, multiple cacao fruit samples were collected across Ecuador between July and September 2019 (Table 4). These samples were specifically selected for high Cd content, based on results reported by Argüello et. al (2019). Note that batch C consisted of pods (6 pods and 4 pods) harvested at two different time intervals on the same cacao farm.

Table 4: Overview of unfermented cacao samples with their genetic and geographical origin and number of biological replicates.

Batch	Cultivar	Province	Number of pods
A	Nacional	Guayas	3
B	CCN-51	El Oro	3
C	Nacional	Guayas	10
E	CCN-51	Los Ríos	4
F	Nacional	Sucumbíos	6

3.2.2 Sample processing

Intact cacao pods were dissected in the lab. Next, beans were separated from placenta and husk. Thereafter, mucilage was removed manually with laboratory gloves, placed in an aluminum tray and oven-dried for 3 days at 65 °C prior to grinding in a coffee grinder (Hamilton Beach® 80335).

Next, since not all mucilage could be removed from the beans, excess mucilage was cleaned away by using rough paper towel. All those beans (per pod) were oven-dried for 3 days at 65 °C. Afterwards, 10-15 of those beans were manually deshelled to separate nibs and testa. Both nibs and testa were ground separately in a coffee grinder. Besides, the Cd content of placenta and pod husk from batches A and B were investigated. To this end, the placenta was dried as a whole for 3 days at 65 °C prior to grinding. To investigate the pod husk, four to five cross-sectional pieces (approx. 2 x 2 cm) were selected and the mucilage attached to the inner surface of these husk sections was removed. Next, the pod husk pieces were dried (3 days at 65 °C) and ground. Finally, dried cacao tissue samples were used for pH measurement (not for mucilage) and ICP-MS analysis.

3.2.3 pH measurements of cacao tissues

The pH of the mucilage was measured in an Erlenmeyer by suspending 50 g (±0.001 g) of whole (undried) cacao beans into 100 mL of distilled water. Next, the Erlenmeyer was covered with parafilm and vigorously shaken (manually) for 30 seconds. Subsequently, pH was measured without removing the cacao beans. The nib pH was measured in an Erlenmeyer by suspending 5 g (±0.001 g) of dry, ground nibs into 100 mL of distilled water. Next, the Erlenmeyer was covered with parafilm and vigorously shaken (manually) for 30 seconds. Subsequently, the suspension was filtered with filter paper (F2040, 125 mm, CHMlab group, Barcelona, Spain) the pH of the filtrate was measured. Finally, also pH of the testa was measured in an Erlenmeyer by suspending 1 g (± 0.001 g) of dry, ground testa in 50 mL distilled water. Next, the Erlenmeyer was covered with parafilm and vigorously shaken (manually) for 30 seconds. Subsequently, pH was measured without removing testa.

3.3 Results and discussion

3.3.1 pH values in different cacao tissues

In Table A.1, an overview of all pH values (mean ± standard deviation) of different unfermented cacao tissues is given. In general (all batches together), mean (± standard deviation) pH values of the composing tissues were: 3.76 ± 0.25 (mucilage, n = 16), 4.49 ± 0.34 (testa, n = 18) and 6.23 ± 0.14 (nib, n = 26). These values are significantly different based on an all pairs Tukey-Kramer test at p = 0.05. Note that not all tissues from each batch were measured (Table A.1).

Broadly speaking, these findings are in agreement with previous reports. For example, Schwan and Fleet (2012) reported mucilage pH values between 3 and 4. This acidic mucilage pH is caused by high concentrations of citric acid (1 – 3 % on total wet weight basis). Nib pH ranges between 6.3 and 7 due to a lower organic acid content like citric, malic and oxalic acid (1% on total wet weight base; Schwan & Fleet, 2012). Romanens et al. (2018) reported pH values (mean ± standard deviation; n = 3) of 3.90 ± 0.03 and 5.50 ± 0.13 for mucilage and nib, respectively (Romanens et al., 2018). To the best of our knowledge, testa pH has not been reported in literature to date.

3.3.2 Cadmium content in different cacao tissues

For all batches, the mean Cd content in composing cacao tissues was measured and statistically tested for significant differences within one batch. Even though those differences were not always significant, Cd content generally decreased from testa > nib > mucilage. Moreover, Cd content of husk and placenta (only measured for batches A and B) was between Cd content of mucilage and testa and was not significantly different compared to nib Cd (Table 5; Tukey-Kramer test $p \leqslant 0.05$).

Note that absence of a significant difference between tissues of the same batch could be ascribed to relatively high standard deviations (and coefficients of variation; Table 5). Even though intact pod samples originated from the same farm, still a large variability in the Cd content of the different cacao tissues could be found. These results are in accordance with data reported by Argüello et al. (2019). These researchers reported a 39 % coefficient of variation (mean value) in bean Cd within one field (1-18 trees/field; Argüello et al., 2019). In our results, coefficients of variation can be interpreted in the same way, as intact pods came from the same field. For nib Cd, the CV varied between 18 % (batch A) and 54 % (batch E), with average CV of 31 %.

These variabilities in Cd content are in agreement with the 129 % CV in bean Cd reported by Argüello et al. (2019) in a nationwide survey of Ecuador (Argüello et al., 2019). Argüello et al. (2019) surveyed 159 cacao farms in 15 provinces, representing 97 % of the total production area. Cadmium concentrations ranged from 0.03 to 10.4 mg Cd kg^{-1} with geometric and arithmetic means of 0.55 and 0.90 mg Cd kg^{-1}, respectively. This provides evidence that there exists high

geographic variation of Cd in cacao beans. Note that Argüello et al. (2009) submerged the beans for 60 s in a 0.01 M Na_2-EDTA solution prior to drying and peeling. This method could possibly influence Cd concentration in the composing tissues, as EDTA is a strong chelating agent for Cd (Argüello et al., 2019). However, this method still allows relative comparison of samples within the survey, as ultimate goal to point out the influence of geographic spread on Cd in cacao. In accordance to these results, Barraza et al. (2017) surveyed 31 small-scale farms in 5 provinces of Ecuador. Here, Cd concentrations ranged between 0.09 and 3.51 mg Cd kg^{-1} (Barraza et al., 2017). Note that the range in Cd concentrations is smaller compared to Argüello et al. (2019), probably due to a lower sampling size and geographic spread. The large influence of spatial aggregation on Cd content in cacao beans is important to keep in mind when analyzing data from different provinces, farms or even different trees on the same farm.

The observations concerning Cd distribution are in accordance with available literature. Lee & Low (1985) reported Cd (mean ± standard deviation) concentrations of 0.88 ± 0.18 mg kg^{-1} (nib) and 1.68 ± 0.52 mg kg^{-1} (testa). However, these researchers only briefly reported materials and methods used to obtain and analyze nib and testa separately (Lee & Low, 1985). Lewis et al. (2018) reported 0.88 mg kg^{-1} (nib) and 1.83 mg kg^{-1} (testa; no standard deviations were reported). Raw beans were removed from the pods and mucilage was removed by washing with deionized water. Therefore, care should be taken when analyzing these results. The average nib Cd content of all samples was found to be 50% of that of the testa, although in individual batches (originating from the same farm) it varied from 13 to 81% (Lewis et al., 2018). Contradictory, Chavez et al. (2015) reported over a ten-fold higher mean Cd concentration in nibs (0.94 mg kg^{-1}; range between 0.02 and 3.00 mg kg^{-1}; n = 19) compared to testa (0.09 mg kg^{-1}; range between 0.02 and 0.46 mg kg^{-1}; n = 19). It should be noted that Chavez et al. (2015) washed the whole cacao beans with a 1% hypochlorite solution and rinsed them with tap water before drying and peeling (Chavez et al., 2015). Therefore, care should be taken when analyzing results from Chavez et al. (2015). It is safe to assume that hypochlorite washing removed Cd from the testa in significant amounts. To the best of our knowledge, Cd content in cacao placenta has not been reported to date. Literature remains inconsistent concerning Cd content in pod husk. On one hand, Gramlich et al. (2018) reported similar Cd concentrations (mean ± standard deviation) of the pod husks (1.1 ± 0.2 mg kg^{-1} dry weight) and nibs (1.1 ± 0.1 mg kg^{-1} dry weight). On the other hand, Gramlich et al. (2017)

reported higher Cd content (mean ± standard deviation) in pod husks (0.54 ± 0.04 mg kg^{-1} dry weight) compared to nibs (0.21 ± 0.02 mg kg^{-1} dry weight).

Table 5: Mean cadmium content of cacao tissues in unfermented cacao beans on dry weight base. Different letters denote significant difference in Cd content between tissues within one batch, based on a Tukey - Kramer test P ⩽ 0.05. Sample size for each tissue corresponds to the one in the 2nd column or is denoted separately.

		Mean Cd concentration ± standard deviation (mg kg^{-1}) in				
Batch	**Sample Size**	**Mucilage**	**Nib**	**Testa**	**Husk**	**Placenta**
A	3	0.09 ± 0.03^{B}	0.39 ± 0.07^{AB}	0.66 ± 0.26^{A}	0.28 ± 0.01^{B}	0.31 ± 0.12^{AB}
B	3	0.07 ± 0.01^{D}	0.51 ± 0.11^{BC}	0.94 ± 0.22^{A}	0.59 ± 0.21^{AB}	0.18 ± 0.01^{CD}
C	10	0.38 ± 0.23^{A}	2.10 ± 0.71^{B}	3.46 ± 1.29^{C}	/	/
E	4	0.14 ± 0.08^{A}	0.97 ± 0.52^{B}	1.35 ± 0.42^{B}	/	/
F	6	1.52 ± 0.32^{A} (n=2)	8.50 ± 2.30^{B}	15.88 ± 4.63^{C}	/	/

3.3.3 Correlation in Cd content between different tissues

Cadmium content in cacao nibs is strongly correlated with Cd content in cacao testa (Pearson correlation, r = 0.85) for all batches (Figure 3). From visual observation (Figure 3), one could expect a linear correlation between these two variables, which is confirmed by a strong linear correlation coefficient (linear fir with P-value ⩽ 0.05, R^2 = 0.72). No correlation in Cd content between other tissues could be found. Similarly, Ramtahal et al. (2016) reported a positive and significant (P ⩽ 0.05) correlation of 0.86 (Pearson correlation) for unfermented, dried cacao nibs and testa (Ramtahal et al., 2016). Moreover, Lewis et al. (2018) reported a correlation coefficient (Pearson correlation) between Cd content in testa and nibs to be significant but moderate in size (r = 0.5; P ⩽ 0.05; Lewis et al.,2018).

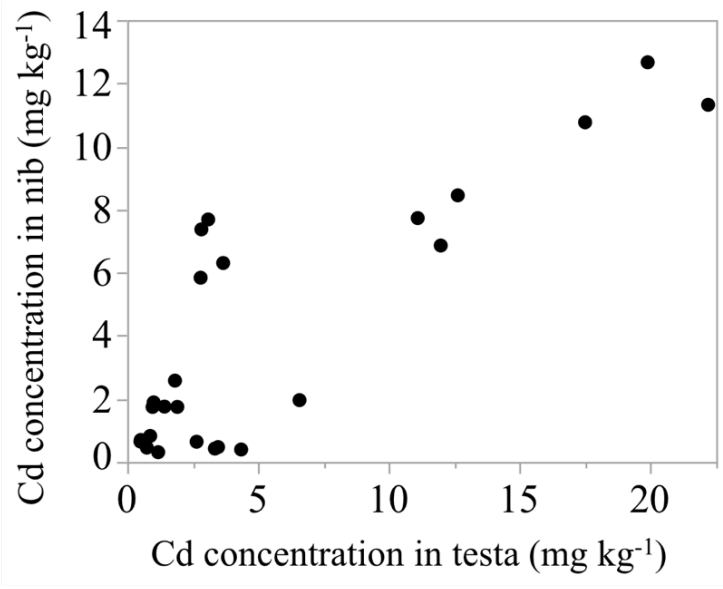

Figure 3: Nib Cd content versus testa Cd content of unfermented cacao beans (in mg kg^{-1}).

3.4 Conclusion

The Cd content in different cacao tissues generally decreased from testa > nib > mucilage. Moreover, the Cd content of husk and placenta (only measured for samples A and B) was between the Cd content of mucilage and testa and was not significantly different compared to the nib Cd. Moreover, Cd content in the cacao nibs is strongly correlated with Cd content in the cacao testa. Note that, even though pod samples from the same batch originated from the same farm, still a large variability in the Cd content of the different cacao tissues could be found.

4 Distribution and migration of Cd in cacao beans during fermentation

4.1 Research question and hypothesis

In this section, we will assess the distribution of Cd in cacao beans during fermentation. Our research question is stated as follows: "*Is there a change in cadmium concentration in cacao beans during fermentation?"* Answers to this question could ultimately lead to strategies to lower Cd in cacao nibs, as this is the major raw material for further cacao processing.

To the best of our knowledge, no research papers are available concerning distribution and migration of Cd during fermentation of cacao beans. As explained previously in the literature review, the pH of the different cacao tissues is acidic (pH 3-5) throughout fermentation and the fermenting mass heats up to 45-50 °C due to microbial activity (De Vuyst & Weckx, 2016; Schwan & Wheals, 2004). This environment may favor metal mobility. Therefore, *we expect a change in cadmium concentration in different cacao bean tissues during fermentation*.

To verify this hypothesis, we will conduct multiple fermentation experiments on both Nacional and CCN-51 cultivars and measure Cd content and pH of cacao tissues (mucilage, testa and nib) throughout fermentation. Besides, temperature of the fermenting mass will be measured on a daily base.

4.2 Materials and methods

4.2.1 Fermentation set-up

To investigate the distribution pattern of Cd in beans during fermentation, four large-scale fermentation experiments were conducted in duplicate. Information regarding the different experimental set-ups is given in Table 6. These batches were specifically selected for high Cd content, based on results reported by Argüello et. al (2019). Note that batches C and D originated from the same farm (just as sample C in section 3).

Table 6: Overview of set-up of fermentation experiments with their genetic and geographical origin and data on general fermentation set-up.

Batch	**Cultivar**	**Origin of the cacao**	**Mixing method**	**Mass of fermenting cacao beans (kg)**	**Box dimensions (h x w x l; cm)**	**Fermentation time (days)**	**Mixing (after onset of fermentation)**
A	Nacional	Guayas	Cascade	280	60 x 60 x 60	5	48 h
B	CCN-51	El Oro	Cascade	280	60 x 60 x 60	7	48 and 96 h
C	Nacional	Guayas	One box	650	61 x 100 x 118	4	24 h
D	Nacional	Guayas	One box	650	61 x 100 x 118	3	24 h

Ripe cacao was harvested by local farmers and cacao beans with mucilage attached were transported in plastic bags (50-70 kg) to the fermentation sites. In this research, two different fermentation locations were used. Fermentation of batches A and B was carried out nearby ESPOL University, in Guayaquil (Ecuador). Fermentation of batches C and D was carried out on a cacao farm in the Guayas province. Before the start of the experiment, all cacao (quantity required for duplicate fermentation boxes, i.e. 560 or 1300 kg) was mixed with a wooden shovel on a plastic foil. Subsequently, 1 kg cacao subsamples (one for each day of fermentation) were taken and placed into permeable mesh bags, which were placed in the center of the cacao mass at the onset of fermentation. Then, the rest of the mass was divided into two equal parts (biological replicates) and left to ferment in wooden fermentation boxes for a predetermined period (Table 6). All fermentation boxes had holes in the bottom (roughly 1 cm diameter) to ensure proper drainage of mucilage and aeration of the fermenting mass. Two mixing methods were used: cascade and one box. In the cascade method, mixing is obtained by transferring fermenting cacao beans from one box into another, lower situated box. In the one box method, cacao beans are homogenized by mixing them with a wooden shovel in the same wooden box.

In fermentation experiment D, cacao beans were pre-dried for approximately 24 h overnight on concrete floors prior to fermentation. During this drying period, beans with surrounding mucilage were turned every half an hour till 24 - 25 % of total water content was reached. This value is an average moisture content obtained from local farmers and is no estimation of the moisture content of batch D before fermentation. After the pre-drying treatment, beans were fermented according to the characteristics listed in Table 6.

After fermentation, beans were dried on concrete floors in open-air (sun-drying method) until 5 - 6 % water content was reached (after 4 - 8 days). The cacao was turned every hour to assure homogeneous drying. The endpoint of fermentation is mostly determined by organoleptic analysis (taste, color, cracking sound) and was assessed by employers of the fermentation facility. Cacao beans from the replicate fermentation boxes were dried separately. After drying, composite samples were taken from the dried cacao mass (one sample per fermentation replicate). Note that batches C and D were piled and covered with plastic bags at night. This prolonged fermentation since temperature at the core of those piles could rise up to 48 °C (batch C) or 46 °C (batch D).

4.2.2 Daily sampling and physicochemical parameters

Each day, temperature at the center of the fermenting mass was measured at 1:00 pm with a digital thermometer (VWR International, Darmstadt, Germany). Next, one mesh bag subsample per replicate was taken out the fermentation box for further sample processing. Those samples were transported to the lab, where the pH of the mucilage was measured (according to section 3.2.3) and the mucilage was removed (until 250g of clean beans was obtained) and dried as described in section 3.2.2. Then, roughly half of those dry beans were deshelled to separate testa and nibs. After grinding, pH of all tissues was measured (Section 3.2.3). Finally, al samples were digested using the same protocol as outlined in section 2.1 prior to ICP-MS analysis.

4.2.3 Mass balance measurements

For the mass balance experiments, three cacao beans per biological replicate per fermentation day (including samples after drying) were pealed for all batches. Next, testa and nib were weighed for each individual replicate. The Cd concentration on mass fraction (MF) base (mg kg^{-1}) in both testa and nib was then calculated by using following formulas:

1) $$\text{nib Cd conc. (MF)} = \frac{\text{weigth nib (g)}}{\text{weight nib (g) + weight testa (g)}} * \text{ average nib Cd conc.}$$

2) $$\text{testa Cd conc. (MF)} = \frac{\text{weigth testa (g)}}{\text{weight nib (g) + weight testa (g)}} * \text{ average testa Cd conc.}$$

Note that the average nib and testa concentrations in the equations above were taken from two biological replicates per fermentation day per experiment. Next, the average Cd content (MF) was calculated in nib and testa separately by averaging data from three cacao beans. Finally, the sum of those two averages was taken to yield the average total cacao bean Cd concentration (mg kg^{-1}) on a mass fraction base.

To check for data quality, the coefficient of variation from three cacao beans per biological replicate per tissue was calculated. As such, there could be evaluated whether enough cacao beans were included and whether data are reliable. Coefficients of variation (CV) of Cd content (on mass fraction base) in nib (min CV = 0.01 %, max CV = 4.32 %, average CV = 1.14 %) differ empirically

from those of testa (min CV = 0.12 %, max CV = 67.07 %, average CV = 13.48 %). Even though large CV are found in testa, with some caution we can assume that these data are reliable.

4.3 Results and discussion

4.3.1 Effect of harvesting practices on Cd in cacao

An overview of Cd concentrations in all tissues of raw cacao beans after harvesting (AH; collected at the harvest location) and before fermentation (BF; collected at the fermentation location) is given in table A.2. Samples after harvest correspond to the intact pod samples that were analyzed in section 3. The BF samples are taken just before the onset of fermentation. For AH, average Cd content and standard error are based on the number of biological replicates (= intact pods; Table A.2). For BF, average Cd content and standard error are based on two biological replicates (Table A.2). Variation between AH samples was large (up to 10-fold higher concentrations within the same tissue). This variation has been described in section 3 and will therefore not be further discussed in this section. The relatively low standard errors of BF samples indicate that biological replicates have been properly executed.

Transport of freshly harvested (without pod) cacao beans to the fermentation site could have an impact on Cd concentration in multiple ways. First, in our experiments, beans were transported in plastic bags that have been used to store and transport fertilizer. Secondly, cacao beans were left in open air during harvest and transport, leaving them vulnerable for environmental contamination (air, insects, other plant material, pesticides, fertilizer, …). Moreover, beans tended to start fermenting during transport. Even though significant difference between AH and BF samples could have been expected in mucilage, this was not statistically confirmed. This hypothesis was based on the fact that mucilage forms the outer layer of a fresh cacao bean and all sources of Cd contamination described above are supposed to affect this tissue in the first place. Besides, mucilage undergoes biochemical changes due to fermentation as it provides optimal conditions for fermentation after environmental inoculation. Therefore, it was expected that the Cd content in mucilage would change significantly after transport. It should be noted that biggest changes between AH and BF Cd of one tissue are found in testa and mucilage material (except for batch A). As those materials form the outer layers of a fresh cacao bean, it is expected that they are more prone to factors (external contamination, early fermentation, …) that alter Cd concentration as

described above. However, there were no significant differences between AH and BF samples from the same tissue in the same batch.

Finally, it should be noted that only testa and nib BF Cd concentrations of batch A were significantly different (roughly 2-fold higher) compared to AH samples. This could be explained by the fact that Nacional cacao beans used in this experiment displayed inferior quality characteristics at the onset of fermentation (BF sampling point). Namely, a brownish color of the mucilage was observed, which may indicate that fermentation had already started during transport. Therefore, care should be taken when analyzing batch A data.

4.3.2 Changes in physicochemical parameters during fermentation

The mean pH of both mucilage and testa from batch A significantly increased during fermentation from 3.9 and 4.3 to a value of 4.6 and 5.1, respectively (Figure 4). Also, the mean pH of the nibs significantly decreased from 6.0 to 4.6 over the course of fermentation. After day three, pH values were relatively stable. The observations concerning pH of cacao tissues are in accordance with the available literature. Schwan & Fleet (2012) and De Vuyst & Weckx (2016) reported an increase in mean mucilage pH from 3.5 to 4 – 4.5 (mainly) due to the breakdown of citric acid. Subsequently, mucilage pH was expected to further increases to 5 – 6 due to the oxidation of acetic acid and lactic acid. Moreover, they also reported mean nib pH to decrease from 6.3 - 7 to 4.0 - 5.5 due to the penetration of acetic acid into the nibs (Schwan and Fleet, 2012; De Vuyst and Weckx, 2016). To the best of our knowledge, no literature is available concerning testa pH during fermentation. Finally, relatively stable mucilage pH values were also reported by Gálvez et al. in 2007. They reported mucilage pH value (mean $\pm$ standard deviation) to increase from 4.00 $\pm$ 0.10 to roughly 4.50 after 24 h of fermentation with relatively little change during subsequent fermentation (end value of 4.48 $\pm$ 0.02 after 144 h; Gálvez et al., 2007).

For batch A, temperature increased rapidly to 48 °C at the onset of fermentation until day five (Figure 4). These results are in agreement with findings from De Vuyst & Weckx (2016). They reported temperature to increase from ambient temperature (25 – 30 °C) to 45 - 50 °C within the first 48 h of fermentation. This temperature increase could be explained by a combination of exothermal reactions. For example, ethanol is oxidized to acetic acid and further oxidized to carbon dioxide and water (De Vuyst and Weckx, 2016).

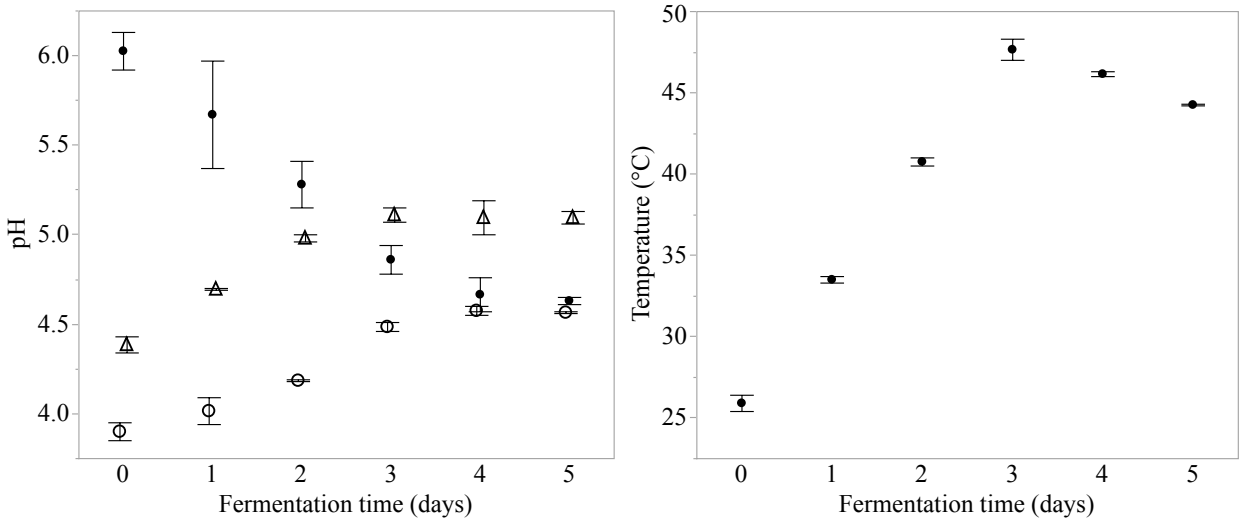

Figure 4: Mean (and standard error) pH in cacao tissues (mucilage, nib and testa; left) and temperature (°C) of fermenting mass (right) during fermentation for batch A: (△) Testa, (●) Nibs, (○) Mucilage.

For batch B, similar observations as those from batch A can be made: the mean pH of both testa and mucilage significantly increased during fermentation from 4.1 and 3.6 on day zero to 5.1 and 4.6 on day seven, respectively (Figure 5). At the onset of fermentation, pH of both the mucilage and the testa increased with roughly 0.5 pH units per day. From day four until day seven, their pH was relatively stable (as was confirmed by results from Gálvez et al. in 2007). The mean pH of the nibs on the other hand decreased significantly from 6.3 to 4.6, with also a period of relatively little change between day four and day seven. During fermentation, the difference between mucilage pH and nib pH diminished in time until they reached approximately the same value at day seven of fermentation. For batch B, temperature increased rapidly to a maximal value of 47 °C at the onset of fermentation until day four (Figure 5). From then, an equilibrium was reached where temperature stayed within a 2 °C range of this maximum. So overall, a lot of similarities in fermentation parameters with batch A can be found, even though the latter concerns another cacao cultivar and was only fermented for five days. Accordingly, pH and temperature results from batch B are also in agreement with literature available (Schwan and Fleet, 2012; De Vuyst and Weckx, 2016; Gálvez et al., 2007).

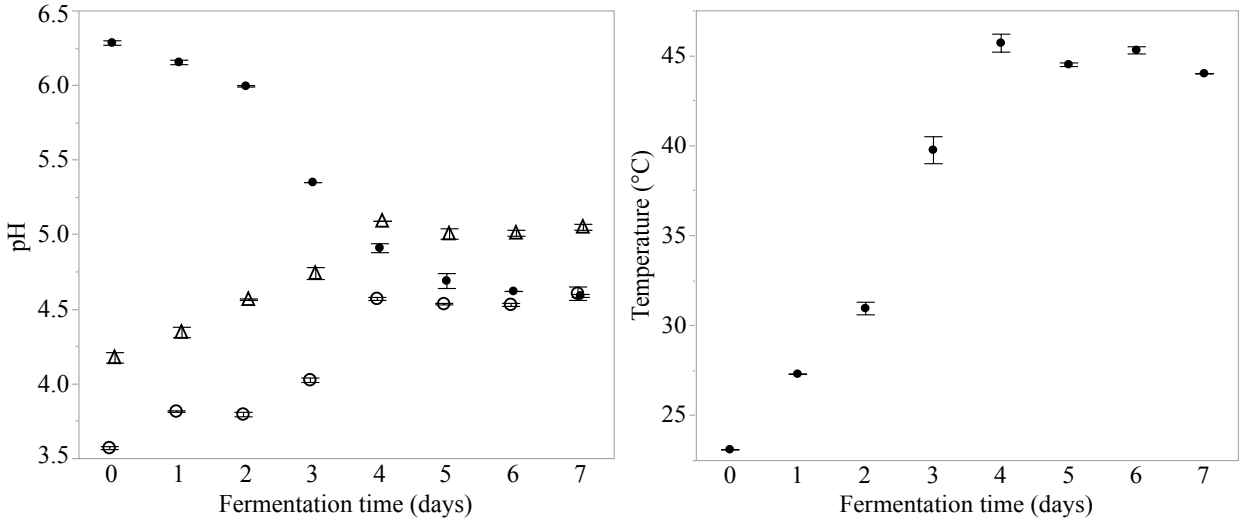

Figure 5: Mean (and standard error) pH in cacao tissues (mucilage, nibs and testa; left) and temperature (°C) of fermenting mass (right) during fermentation for batch B: (△) Testa, (●) Nibs, (○) Mucilage.

For batch C, nib pH and temperature were the only variables that displayed a significant (P-value $\leqslant$ 0.05) correlation with fermentation time (Pearson correlation, r = -0.94 and r = 0.96, respectively; Figure 6). In this experiment, the same cultivar (Nacional) as batch A was used. Therefore, one could expect similar behavior as batch A. For nib pH and temperature of the fermenting mass, this reasoning generally holds. The mean nib pH decreased from 6.2 to 5.2 during fermentation and temperature increased to > 40 °C. Despite a slightly lower maximal temperature of batch C (41 °C for batch C compared to 48 °C for batch A), similar behavior over time was observed compared to batches A and B. Even though an increase in testa and mucilage pH could have been expected based on results of batches A and B, this was not observed for batch C. Mean testa and mucilage pH both increased non-significantly from 4.19 and 3.59 to 4.82 and 3.81 (Pearson correlation, P-value $\leqslant$ 0.05).

Similar observations compared to batch C could be made for batch D (Figure 7). Despite the fact that temperature reached a maximal value of 46 °C, the mean nib pH only slightly decreased from 6.3 to 5.9 (day 0 compared to day 3; Pearson correlation at P-value $\leqslant$ 0.05; r = -0.94). Also changes in testa and mucilage pH were limited. Mean testa pH increased non-significantly from 4.3 to 4.4, while the mean mucilage increased significantly from pH 3.9 to 4.0 (Pearson correlation at P-value $\leqslant$ 0.05; r = 0.72). The data on pH of cacao tissues from batches C and D could point to an incomplete fermentation since they are not in accordance with the literature available. Mean

mucilage pH at the end of fermentation for both samples C and D was to low (3.81 and 4.0, respectively) compared to what could be expected from literature (5 – 6; Schwan and Fleet, 2012). Moreover, the mean nib pH was too high for batch D (5.9 compared 4.0 – 5.5 according to Schwan and Fleet, 2012). Also, temperature of batch C was rather low (41 °C compared to 45 – 50 °C according to Schwan and Fleet, 2012). According to De Vuyst and Weckx (2016), an increase from ambient temperature (25 – 30 °C) to 45 - 50 °C within the first 48 h of fermentation could be expected (De Vuyst and Weckx, 2016). Therefore, it is safe to assume that fermentation of batches C and D was not properly fermented.

As stated in section 4.2.1, beans from batches C and D were mounted on piles at night after four (batch C) and three (batch D) fermentation days. For batch C, nib pH (mean ± standard error) decreased from 5.2 ± 0.01 (day four) to 4.9 ± 0.03 (after drying) and testa pH increased from 4.8 ± 0.01 (day four) to 5.2 ± 0.1 (after drying). For batch D, nib pH (mean ± standard error) decreased from 5.9 ± 0.04 (day four) to 5.6 ± 0.01 (after drying) and testa pH decreased from 4.4 ± 0.04 (day four) to 4.3 ± 0.01 (after drying). No changes in pH due to pre-fermentation drying were significant.

Also note that, even though temperature of batch D rapidly increased to maximum temperatures that were comparable to those measured in the other batches, change in pH of all cacao tissues of D was limited. Bear in mind that batch D was only fermented for three days and underwent a pre-drying treatment. This pre-drying treatment reduced moisture content to 24-25%, according to average values reported by the local farmers. Pre-drying increased mucilage pH significantly from 3.6 to 3.9. Nib and testa pH remained unchanged (Pearson correlation, P-value $\leqslant$ 0.05). These results suggest that a lower water content of the fermenting mass facilitates temperature increase, but impedes movement of pH influencing metabolites, as pH change of mucilage and testa during fermentation is limited. However, this limited change in tissue pH could also be due to the limited fermentation duration. If fermentation would have lasted one or two days longer, pH of nib, testa and mucilage may have reached similar values as those observed in batches A and B.

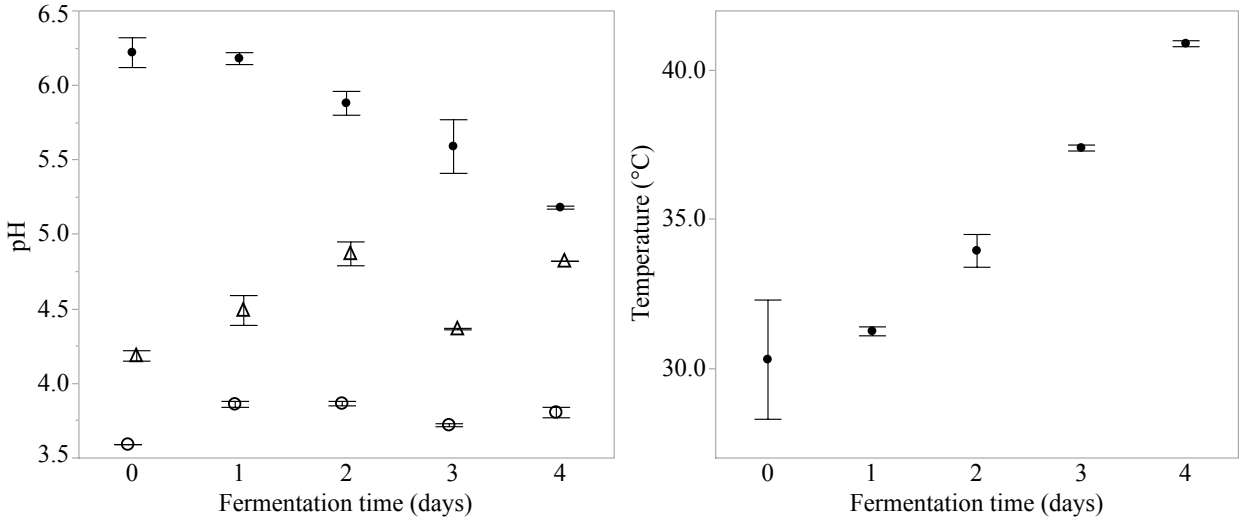

Figure 6: Mean (and standard error) pH in cacao tissues (mucilage, nibs and testa; left) and temperature (°C) of fermenting mass (right) during fermentation for batch C: (△) Testa, (●) Nibs, (○) Mucilage.

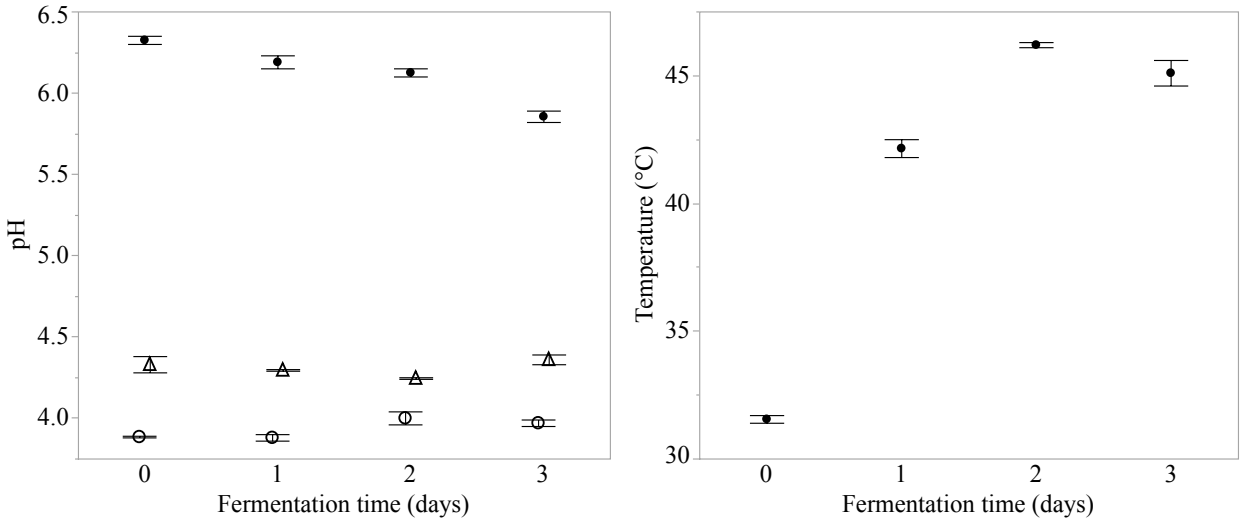

Figure 7: Mean (and standard error) pH in cacao tissues (mucilage, nibs and testa; left) and temperature (°C) of fermenting mass (right) during fermentation for batch D: (△) Testa, (●) Nibs, (○) Mucilage.

4.3.3 Influence of fermentation on Cd content of cacao tissues

In batch A, the fermentation time was significantly correlated with Cd content in mucilage (Pearson correlation r = 0.67 at $P \leqslant 0.05$; Figure 8). The data point corresponding to the biological replicate with average Cd content of 6.61 mg kg^{-1} on day 3 was removed as outlayer for mucilage. For nib, there existed a significant negative correlation (Pearson correlation r = -0.78 at $P \leqslant 0.05$; Figure 8). Finally, no correlation between testa Cd and fermentation time could be found. When the data point corresponding to the biological replicate with average Cd content of 2.2 mg kg^{-1} on

day 3 was removed as outlayer for testa, a pearson correlation coefficient of 0.66 was found ($P \leqslant 0.05$).

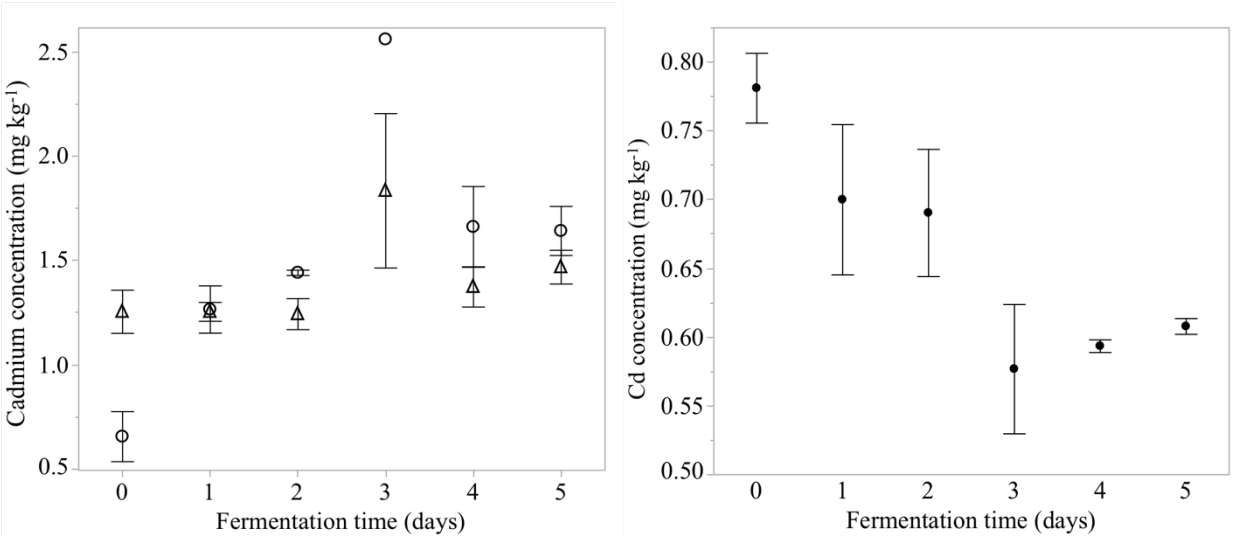

Figure 8: Mean Cd concentration with standard error (mg kg^{-1}) of mucilage (○, left), testa (△, left) and nibs (●, right) versus fermentation time for batch A.

Fermentation time was significantly correlated to Cd content in all cacao tissues of batch B (Pearson correlation at $P \leqslant 0.05$; Figure 9). Starting from day zero, Cd increased in mucilage and testa and decreased in nibs up to the end of fermentation (day seven). The mean Cd concentration of both testa (Pearson correlation at $P \leqslant 0.05$; $r = 0.78$) and mucilage ($r = 0.89$) significantly increased during fermentation (day zero to day seven) from 0.6 and 0.2 mg kg^{-1} to 1.3 and 1.3 mg kg^{-1}, respectively (Figure 9, left). The mean Cd concentration of the nibs on the other hand decreased significantly with time (Pearson correlation at $P \leqslant 0.05$; $r = -0.84$) from 0.5 to 0.4 mg kg^{-1} (Figure 9).

As outlaid in section 4.3.2, the difference between mucilage pH and nib pH for batch B diminished during fermentation until they reached approximately the same value at day seven of fermentation. Based on visual observation of Figure 9, differences in Cd concentration were the greatest between day zero and day four into fermentation. From day four until day seven, Cd concentration in all tissues was relatively unchanged, which corresponds to the behavior of pH in those tissues. This suggests that Cd mobilization might be related to the changes in tissue pH.

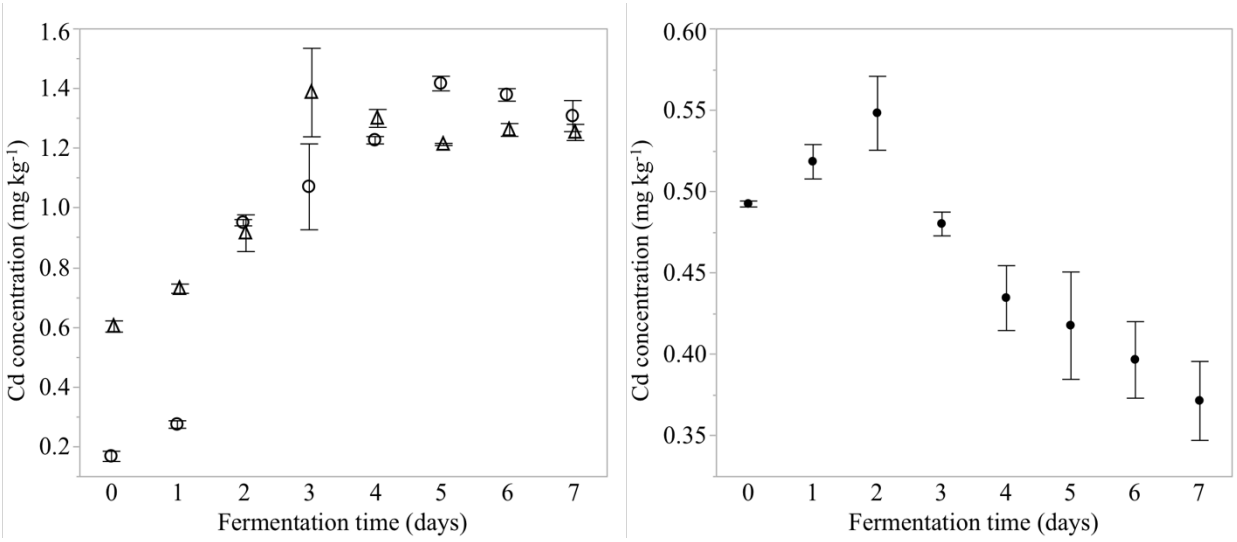

Figure 9: Mean Cd concentration with standard error (mg kg^{-1}) of mucilage (○, left), testa (△, left) and nibs (●, right) versus fermentation time for batch B.

For batch C, fermentation time was significantly correlated with Cd content for testa (Pearson correlation at $P \leqslant 0.05$; r = -0.87) and for mucilage (r = 0.91). For nib material, no significant correlation was found. For all cacao materials, the biggest change in Cd concentration was observed between day one and two. During this 24 h time period, mean testa Cd significantly decreased from 3.0 to 0.6 mg kg^{-1} and mean mucilage Cd significantly increased from 0.9 to 3.1 mg kg^{-1} (Figure 10).

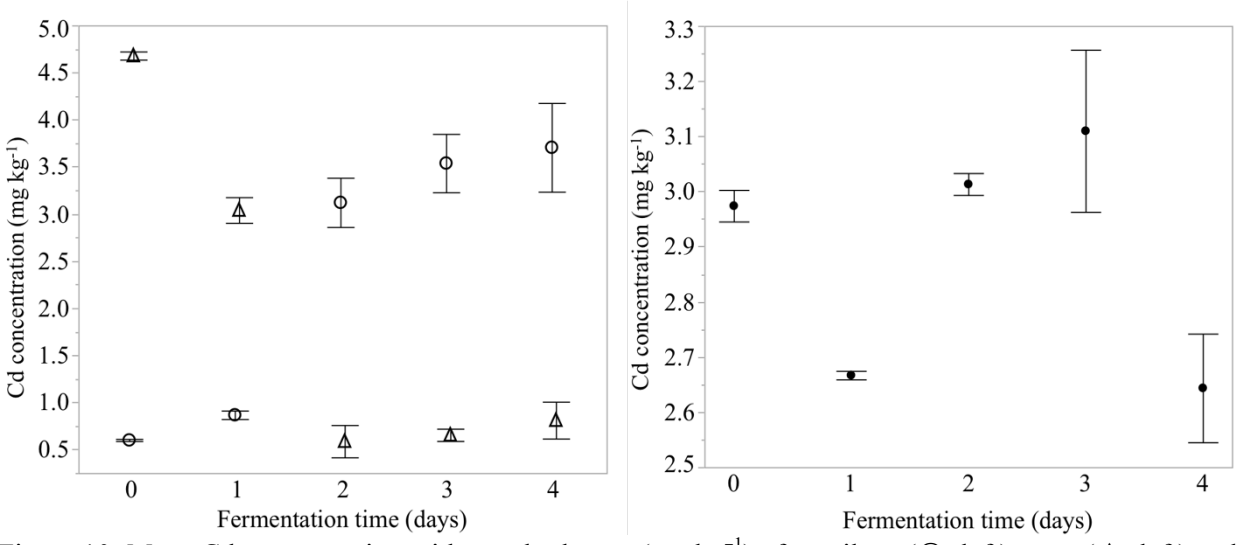

Figure 10: Mean Cd concentration with standard error (mg kg^{-1}) of mucilage (○, left), testa (△, left) and nibs (●, right) versus fermentation time for batch C.

Fermentation time was significantly correlated with Cd content in mucilage of batch D (Pearson correlation at $P \leqslant 0.05$; r = 0.88; Figure 11). Important to note is that the final Cd content in the

testa was lower than Cd content in nib at the end of the fermentation (last 2 - 3 days) for both batches C and D. For batch D, this difference is not significant. It remains unclear what causes this deviant behavior compared to batches A and B. In Table 7, an overview of all correlations (Pearson correlation at $P \leqslant 0.05$) between fermentation time and tissue Cd are given.

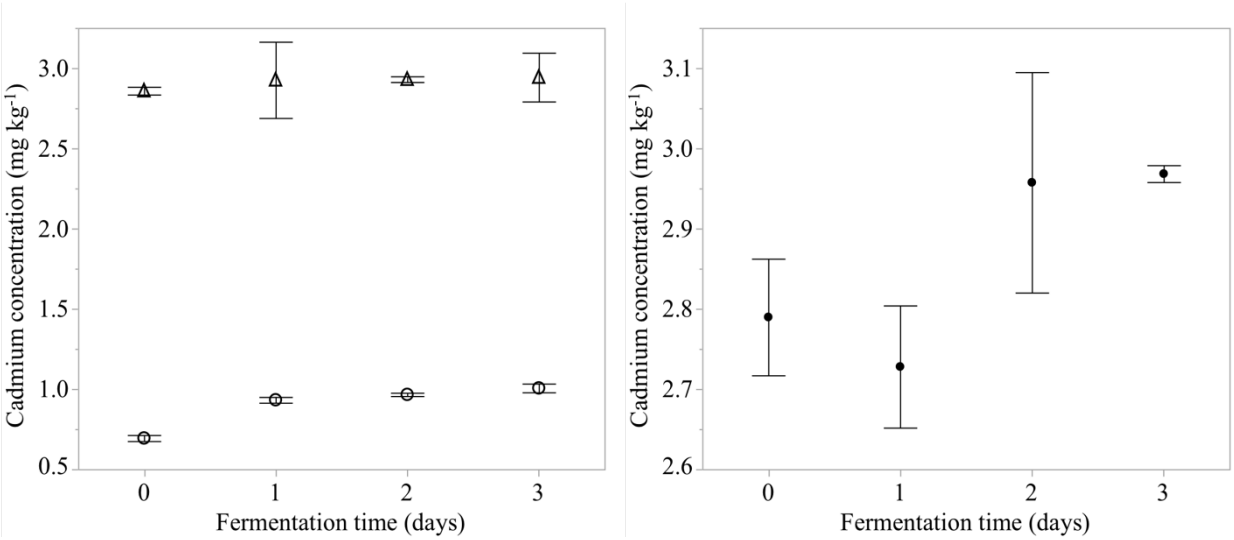

Figure 11: Mean Cd concentration with standard error (mg kg^{-1}) of mucilage (○, left), testa (△, left) and nibs (●, right) versus fermentation time for batch D.

Table 7: Pearson correlation coefficients ($P \leqslant 0.05$) between fermentation time and tissue Cd for different fermentation batches. NS denotes no significant correlation.

Batch	Nibs	Testa	Mucilage
A	-0.78	0.66	0.67
B	-0.84	0.78	0.89
C	NS	-0.87	0.91
D	NS	NS	0.88

To the best of our knowledge, literature on Cd migration during cacao fermentation is not available to date. Argüello et al. (2019) found a significant difference between Cd content (mean ± standard deviation) in fermented nibs (0.90 ± 0.014 mg Cd kg^{-1}) and testa (0.84 ± 0.021 mg Cd kg^{-1}) compared to unfermented nibs (0.73 ± 0.005 mg Cd kg^{-1}) and testa (0.53 ± 0.005 mg Cd kg^{-1}). It should be noted that Argüello et al. (2019) conducted 4-day incubation and subsequent drying on a batch of beans of approx. 40 beans (Argüello et al., 2019). For three reasons care should be taken when discussing results of Argüello et al. (2019). First of all, Cd content was higher in nibs compared to testa, both before and after fermentation. Those results are not in line with our results (section 3) and the majority of literature available for unfermented beans. Secondly, Argüello et

al. (2019) incubated on lab-scale (40 beans), while our research was conducted on a large-scale fermentation (280 or 650 kg), which is used by most farmers in Ecuador. Finally, in all our batches except for batch D, Cd concentration in nibs was lower after fermentation then before. Given these three factors, no direct comparison of our results to those of Argüello et al. (2019) can be made (Argüello et al., 2019).

According to Zhai et al. (2019), fermenting rice by *Lactobacillus plantarum* reduced Cd significantly (over 80% removal). This Cd removal capacity could be ascribed to Cd-binding and acid-producing capacities of different *L. plantarum* strains. Moreover, rice fermentation by these bacteria induced changes in physicochemical properties of rice such as reductions in the protein, lipid, and ash contents; appearance of a porous micro-morphology and alteration of the crystal structure of rice starch granules. This decrease in protein levels also explained the solubilization of Cd during the fermentation process, as most Cd was distributed in the high-protein tissues of rice grains (e.g. endosperm and embryo). Here, it is mostly bound to glutelin and globulin. During the *L. plantarum* fermentation, these proteins are solubilized by lactic acid and taken up by the bacterial strains (Zhai et al., 2019).

These results suggest that fermentation by *L. plantarum* strains is an effective means of removing Cd from rice and could be considered as a strategy for the development of Cd-free rice-based foods. Besides, the addition of hydrochloric acid and lactic acid (without an inoculation of *L. plantarum* strains) also significantly reduced Cd contents in rice (Zhai et al., 2019). It should be noted that this study only concerns rice fermentation with some *L. plantarum* strains. As macromolecule content as well as matrix of rice is different compared to that of cacao beans (e.g. the latter contains more fat instead of starches), care should be taken in comparing results. None the less, an interpretation could be formulated to explain our results. In general, our results displayed highest alterations in Cd content during the first 2-3 days of fermentation. According to De Vuyst and Weckx (2016), alterations in lactic acid bacteria are the highest at roughly the same time point into cacao fermentation (De Vuyst & Weckx, 2016). So, keeping the discussion from Zhai et al. (2019) in mind, one could argue that alterations in cacao Cd could be ascribed to the Cd removal capacity of some lactic acid bacteria (Zhai et al., 2019).

4.3.4 Correlation between cadmium concentrations and physicochemical fermentation parameters

Cadmium concentration was correlated with pH in all tissues for batch A (Pearson correlation coefficients at $P \leqslant 0.05$; Table 8). For batch B, all materials displayed a positive correlation coefficient of 0.87 (Pearson correlation coefficients at $P \leqslant 0.05$; Table 8). Cadmium concentration was negatively correlated with pH in testa of batch C (Pearson correlation coefficients at $P \leqslant 0.05$; Table 8). In other tissues of batch C, no significant correlation could be found. In all tissues of batch D, Cd concentration was not significantly correlated with pH (Table 8). This is probably due to the fact that pH changed relatively little during fermentation. All batches (with one exception for batch C) displayed positive correlations between Cd concentration and pH, indicating that Cd content is higher when pH of those tissues is higher (during fermentation).

A parallel could be found when comparing correlations between Cd content and pH on one hand and Cd content and fermentation time on the other hand. Most likely, this is due to an interdependent relationship between fermentation time, pH and Cd content. Cadmium content in all cacao tissues during fermentation seems to be dependent on pH, even though Cd content in most tissues of sample C and D did not display remarkable correlation with pH (Table 8). However, this could be due to the fact that Cd concentration did not always change during fermentation. In section 4.3.2, it was assumed that physicochemical parameters measured in batches C and D did not always correspond to what could be expected in literature (Schwan and Fleet, 2012; De Vuyst and Weckx, 2016): an increase in mucilage pH to 5 – 6 and a decrease in nib pH to 4 – 5.5 during fermentation. Moreover, temperature of the fermenting mass did not increase as much as expected (See 4.3.2). Therefore, it is safe to assume that fermentation of batches C and D was not properly fermented. This could help to explain the limited changes in Cd content of cacao tissues in batches C and D, as these physicochemical factors (pH and temperature) were expected to influence metal mobility.

Note that similar relationships between Cd content and temperature were investigated for all batches. However, these correlations were not statistically significant and are therefore not further discussed. None the less, temperature is supposed to indirectly affect or correlate with Cd migration: as temperature of the fermenting mass increases, it is a valid indicator (also used in

practice by cacao farmers in Ecuador) for an ongoing or complete fermentation process (De Vuyst & Weckx, 2016; Schwan & Fleet, 2012).

Table 8: Pearson correlation coefficients ($P \leqslant 0.05$) between Cd concentration and pH during fermentation for different batches. NS denotes no significant correlation.

Batch	Nib	Testa	Mucilage
A	0.90	0.63	0.80
B	0.87	0.87	0.87
C	NS	-0.69	NS
D	NS	NS	NS

4.3.5 Influence of drying on cadmium content of cacao tissues

To investigate the effect of drying on Cd content of cacao tissues (nibs and testa), samples from the last day of fermentation were compared with samples after drying (Figure 12). Mucilage Cd measurements are not included since mucilage was too difficult to remove separately after drying. In samples after drying, mucilage was dried out on the testa, ultimately leading to one cacao part that is composed of both testa and mucilage. However, in samples after drying, this cacao part will be further denoted as testa. The last day of fermentation differs for different samples: day 5 (batch A), day 7 (batch B), day 4 (batch C) 3 day 3 (batch D).

In testa of batches B, C and D, a significant difference between Cd content in between samples before and after drying was observed. Changes in nib Cd (for all batches; Figure 12) were not significant. Cacao beans are left in open air for several days during drying, leaving them vulnerable for environmental contamination. Especially fuel combustion for agricultural machinery and misting of pesticides and fertilizers could drastically alter Cd content of the outer layers of cacao beans. These environmental deposits could also be present in soil and dust on the concrete floor where drying takes places. However, those hypotheses are not tested. To fully understand the influence of drying on Cd content of cacao tissues, more controlled experiments (with e.g. artificial driers) should be carried out. However, as those latter drying methods are not commonly used in Ecuador (and will probably not be used due to high capital requirements), it would not give a realistic view on how this processing step is executed in practice.

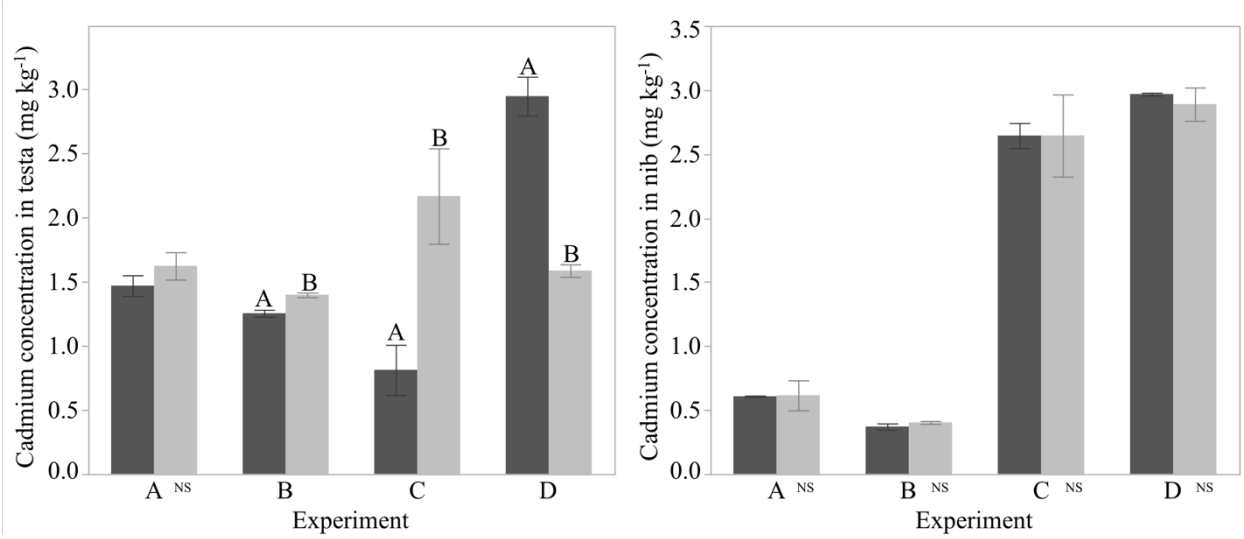

Figure 12: Mean Cd concentration with standard error before (dark grey) and after (light grey) drying for testa (left) and nibs (right). Different letters denote significant difference in Cd content between samples before and after drying, based on a Tukey-Kramer test P $\leqslant$ 0.05. NS denotes no significant difference.

4.3.6 Mass balance for cadmium before and after fermentation

Even though in most tissues of all samples a difference in Cd concentration could be seen before and after fermentation, there cannot be concluded that there was an absolute loss or increase in Cd content of the whole cacao beans. Therefore, the mass balance for Cd will be investigated in this section.

No significant difference in total average cacao bean Cd concentration on mass fraction base (mg kg^{-1}; explanation in section 4.2.3) between samples before fermentation, after fermentation and after drying could be found (Figure A.5) for any experiment (all pairs Tukey-Kraemer test; P-value $\leqslant$ 0.05). Also, when comparing all total average cacao bean Cd concentrations on mass fraction base (based on a Tukey-Kramer test, P-value $\leqslant$ 0.05) during fermentation, no significant difference between any individual days was observed (Figure A.1, Figure A.2, Figure A.3, Figure A.4). When treating fermentation time (days) as a continuous variable, correlations of total average cacao bean Cd concentration with time can be found (Pearson correlation at P-value $\leqslant$ 0.05). For batch A and B those correlations were -0.73 and -0.60, respectively. The mass balance of batches C and D did not display any significant time-dependent behavior. Given those deviant observations, it remains difficult to formulate any conclusion concerning the mass balance of Cd in cacao beans.

Moreover, since mucilage Cd is not taken into account (for practical reasons as described in section 4.3.5), care should be taken into account when analyzing these mass balance data. Mucilage data are not included since it is hard to determine the absolute mucilage weight at any given point in time. None the less, basic observations (Cd migration pattern) concerning Cd mass balance of testa and nibs can be made since dry weight of mucilage at any point in time is low. Schwan and Fleet (2012) reported mucilage weight to be 30-40 % of the total wet bean weight (after harvest). Moreover, this mucilage consists mostly of water (85 % total wet weight) and roughly 20% is removed at the early (anaerobic yeast phase) stages of fermentation (Schwan & Fleet, 2012). Therefore, it is assumed that this tissue only has a marginal contribution to the total dry weight of a cacao bean (at any point in time during fermentation). Since Cd content is expressed on dry weight base, mucilage will also have a marginal contribution to the total Cd content of a cacao bean. However, it remains unclear how much Cd is removed in mucilage draining during those first days of fermentation. Moreover, in figures 8, 9, 10 and 11 of section 4.3.3, there could be seen that mucilage increased during fermentation. Therefore, care should be taken when analyzing these results. In future experiments, the mucilage weight can be taken into account as was reported by Romanens et al. (2018). Here, the mucilage content was determined by weighing ten wet cacao beans before and after the mucilage was removed by rubbing the beans with sawdust and tissue paper.

4.4 Conclusion

First of all, changes in Cd content were observed in cacao beans during fermentation. In general, testa and mucilage Cd content increased and nib Cd content decreased during fermentation. From the mass balance, an outward migration of Cd from nibs to testa, has been empirically established. This migration is contradictory against the concentration gradient of Cd since in section 3, there has been concluded that Cd concentration was higher in testa compared to the nibs after harvest. Note that drying - as a post-fermentation processing step - did not significantly alter Cd content in the cacao nibs. Also, a pre-drying treatment (prior to fermentation), performed on one fermentation (in duplicate), did not significantly affect nib Cd.

At first sight, these changes in tissue Cd during fermentation could be related to alterations in tissue pH, which is also related to fermentation time. Changes in pH during fermentation are most

likely due to breakdown and formation of metabolites (e.g. breakdown of citric acid in the mucilage leads to a pH increase), which are caused by microorganisms. Subsequently, these metabolites could migrate and alter pH in the relevant cacao tissues. Generally speaking, nib pH decreased from 6.3 to 4.5 during fermentation. Accordingly, a decrease in nib Cd with roughly 25% was observed (only for batches A and B). Breakdown and formation of metabolites by microorganisms during cacao fermentation cause the temperature of the fermenting mass to increase (to roughly 45 °C). Both these pH alterations and an increase in temperature of the fermenting mass favor mobility of Cd.

In order to get a better understanding of these factors that govern Cd migration in cacao beans during fermentation, sorption experiments could be conducted. Here, (pH dependent) adsorption of Cd can be investigated to various cacao tissues. These results could give insights into maximal Cd adsorption of tissues and movements of free Cd. Next, there can be investigated which compounds (polyphenols, peptides, proteins, phytate, organic acids, …) drive preferential adsorptions by researching in which tissues those compounds are mostly present. Ultimately, the amount of 'free' (or the number of 'free' binding sites at those complexing compounds) Cd determines the concentration gradient and therefore also the migration pattern during fermentation. As some of these compounds get broken down, more free Cd is available for (outward) migration.

Finally, it should be noted that testa Cd content after fermentation was up to a three-fold higher compared to nibs (for samples A and B). To avoid high Cd contents in cacao liquor, the cacao testa should be removed as much as possible. In the literature review, there could be seen that a maximum of 5 % testa on fat-free dry weight base is tolerated in cacao products.

5 Chelating agent washing as a possible mitigation strategy to lower Cd in cacao

5.1 Research question and hypothesis

In this section, we will assess the potential effect of chelating agent washing treatments, performed on unfermented beans, on the Cd content in different cacao tissues (mucilage, testa, nib). Our

research question is stated as follows: *"Is chelating agent washing of cacao prior to fermentation a feasible technique to lower the Cd concentration in the final cacao-derived product?"*

Our hypothesis is stated as follows: *"Washing with EDTA will have an effect on Cd of unfermented cacao bean tissues in that it will lower Cd content in cacao tissues (more pronounced effect in outer layers)".* This hypothesis is based on the fact that EDTA is a strong chelating agent for Cd. Besides, it is expected that water will decrease the performance of the fermentation process by e.g. lowering fermentation temperature.

To verify this hypothesis, we will conduct multiple lab-scale fermentation experiments where unfermented cacao beans are being washed before fermentation. Before washing, after washing and after fermentation, samples will be taken from each replicate and all tissues (mucilage, testa and nib) will be analyzed for their Cd concentration and pH. Besides, temperature of the fermenting masses will be measured.

5.2 Materials and methods

5.2.1 Experiments to establish appropriate small-scale fermentation size

As is it very costly and potentially hazardous for the environment, washing treatments were not performed in full scale fermentation set-ups (250-500 kg). Instead, research on the proper lab-scale fermenting size was performed prior to conducting washing experiments. We evaluated which small-scale fermentation size was the most effective and representative for the full-scale fermentation set-ups based on the following factors: (i) financial considerations, (ii) big enough for representative sampling, (iii) adequate and proper fermentation.

Different small-scale fermentations were set up in plastic containers containing 2.5, 5, 10 and 15 kg of fresh cacao. Fermentations were performed in triplicate (except the 15 kg size which was performed in duplicate) and fermentation parameters (temperature and nib and mucilage pH) were followed throughout fermentation.

Overall, there can be concluded that the 5 kg fermenting mass provided the most potential to investigate the influence of washing treatments, as temperature and nib pH (data not included) were comparable to those of large-scale fermentations.

5.2.2 Washing experiments

For this experiment, Nacional cacao beans from the Guayas province in Ecuador were fermented for 4 days. This cacao originated from the same farm as batches C and D in Section 4. Cacao pods were harvested and opened at the farm.

Before fermentation, raw cacao beans for all treatments (68.4 kg) were mixed with a wooden shovel on a plastic foil to ensure proper homogenization. Cacao beans were either washed with 0.01M or 0.1M EDTA (Extra pure, Loba Chemie, Mumbai, India), with distilled water or not washed (blank) prior to fermentation. In total, four treatments were obtained: blank (BL), water (W), low EDTA (0.01 M EDTA; L) and high EDTA (0.1 M EDTA; H). The water treatment was included to investigate the influence of washing treatment on fermentation. The comparison between the water treatment and EDTA treatments can give insights into the influence of EDTA on Cd content of cacao tissues, taking the influence of the washing treatment into account.

For each replicate, 5.7 kg of cacao beans was taken from the homogenized mass. For the blank treatment, 5.35 kg of cacao beans was taken since no washing was performed (no after washing sample). Next, one sample of 350 g (denoted as “before washing”) per replicate was taken and processed as outlined in sections 2.1, 3.2.2 and 3.2.3. The remaining 5.35 kg was suspended for 60 seconds in 3 L of washing solution (ether water, 0.01M or 0.1M EDTA) and mixed manually. Afterwards, the washing solution was removed by draining the beans on a plastic foil with holes (1 cm diameter). This plastic foil was placed on a frame (1 m x 1 m) of wire mesh. Next, the procedure was repeated with distilled water to remove excess chemicals. As much liquid as possible was removed by manually pressing the beans together. After 60 s of drainage, 350 g of sample was taken (denoted as “after washing”) and analyzed in the same way as the samples before washing. Finally, the remaining cacao of all treatments (5.0 kg per replicate) were placed in plastic planter pots (flat-cone shaped with 17.5 cm bottom diameter, 23.5 cm top diameter and 22.5 cm height). All fermentation pots had holes in the bottom (roughly 1 cm diameter) to ensure proper drainage of mucilage and aeration of the fermenting mass. Next, beans were fermented for four

days. After fermentation, 350 g samples were taken from the core of the fermenting masses (denoted as “end of fermentation”) and processed and analyzed as outlined in section 2.1, 3.2.2 and 3.2.3.

Each day, temperature was measured for each replicate at the center of the fermenting masses at 9:00 am, 1:00 pm and 5:00 pm. This was done using a digital thermometer (VWR, Radnor, United States of America).

5.3 Results and discussion

5.3.1 Influence of washing on fermentation parameters

To investigate the influence of washing with EDTA or water, temperature of the fermenting mass and pH of the different cacao tissues was compared to the blank treatment. The temperature of the blank was higher than in the washing treatments, with a maximum temperature that was roughly 5 °C more than the other treatments (blank: 45 °C after 70 hours, Figure 13). This indicates that washing influenced the fermentation of cacao beans, as temperature is a good indicator for the completeness of fermentation. According to Schwan and Fleet (2012), the temperature of the fermenting mass should reach 45-50 °C to obtain a good fermentation (Schwan & Fleet, 2012).

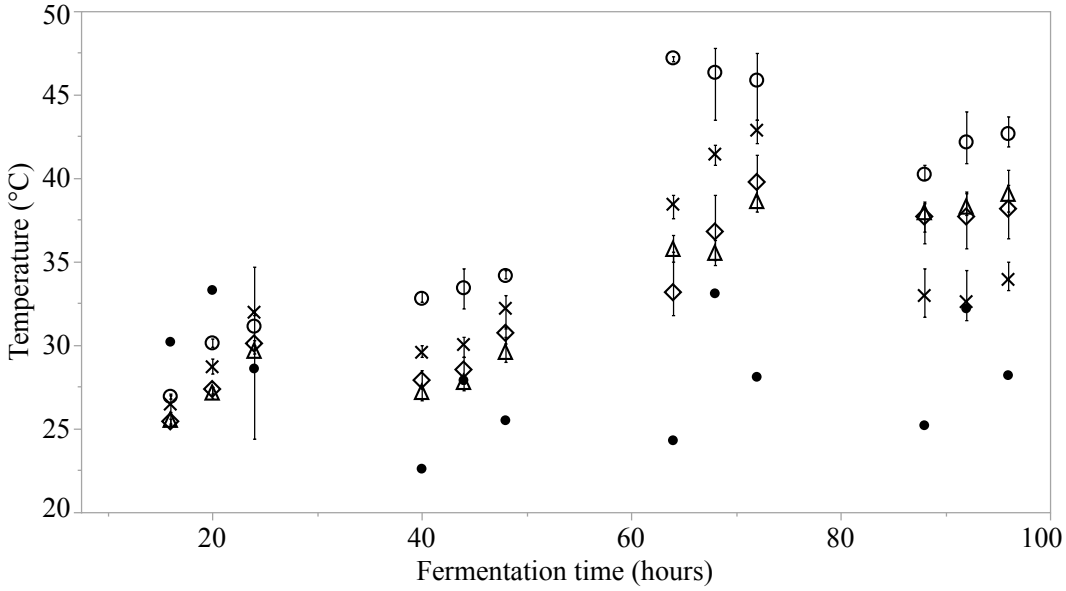

Figure 13: Temperature (°C) of fermenting mass in washing experiment vs fermenting time (in hours) for ambient temperature (dots), BL (circle), W (cross), L (square) and H (triangle).

The initial mucilage pH (before washing) was the same for all treatments. Washing with water and 0.1 M EDTA increased mucilage pH (mean ± standard deviation) significantly from 3.80 ± 0.05 to 4.00 ± 0.03 (water) and from 3.80 ± 0.01 to 4.00 ± 0.02 (high EDTA; t-test with P-value ⩽ 0.05). For the low EDTA treatment, pH did not increase significantly due to washing. These results indicate that washing could have an impact on pH of mucilage. More specifically, washing could increase mucilage pH by a diluting effect. Since 3 L of distilled water was added to 5.35 kg of cacao beans (see section 5.2.2), hydronium ions from the (aqueous) mucilage were diluted causing the mucilage pH to increase (with 0.2 pH units).

The mucilage pH (mean ± standard deviation) of the water treatment at the end of fermentation (7.70 ± 0.10) was significantly larger compared to the other treatments after fermentation (blank: 6.30 ± 0.40; low EDTA: 6.40 ± 0.60; high EDTA: 5.80 ± 0.09; Tukey-Kramer test with P-value ⩽ 0.05; Figure 14). This could be an indication that washing with water had an influence on the fermentation process. This causal relation is not easy to confirm since there are multiple factors that influence the fermentation process. Note also that all final mucilage pH values were larger (roughly 1 to 2 pH units) compared to pH measured in large-scale fermentations (around pH 4.5 for samples A and B in section 4). This could be an indication that fermentation did not occur as it supposed to be.

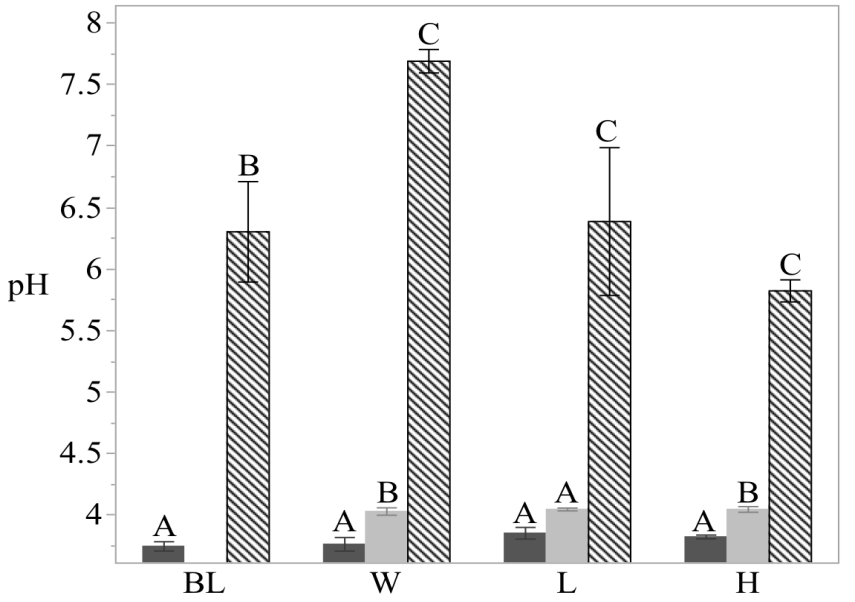

Figure 14: Mean pH with standard error of mucilage before fermentation (dark grey), after washing (light grey) and after fermentation (stripes) for different treatments. Different letters denote significant differences within one treatment, based on a Tukey-Kramer test with P-value ⩽ 0.05.

The initial nib pH of the blank treatment was significantly lower compared to all other treatments. This is a peculiar observation since all samples before washing originated from the same homogenized mass. Washing with water or EDTA did not cause any significant alteration in nib pH when samples before and after washing (t-test with P-value $\leqslant$ 0.05; Figure 15).

Due to fermentation, all nib pH values decreased (Figure 15). However, this change was more pronounced for the blank (not statistically confirmed). Significant difference was observed between nib pH (mean ± standard deviation) value of the blank (4.80 ± 0.03) after fermentation compared to those of other treatments after fermentation (W: 5.40 ± 0.07; low EDTA: 5.39 ± 0.02; high EDTA: 5.44 ± 0.04; Tukey-Kramer test with P-value $\leqslant$ 0.05). Based on the large-scale fermentations (sample A and B in section 4), a nib pH of around 4.5 could be expected. This was the case for the blank treatment. However, the water and EDTA treatments displayed final pH values around 5.4. Based on these results, one could argue that washing had an influence on fermentation. Moreover, the difference in nib pH between the blank treatment and other treatments was larger after fermentation compared to before fermentation. To conclude, based on all data provided concerning temperature and pH of mucilage and nib, there can be concluded that washing had a significant influence on the fermentation process.

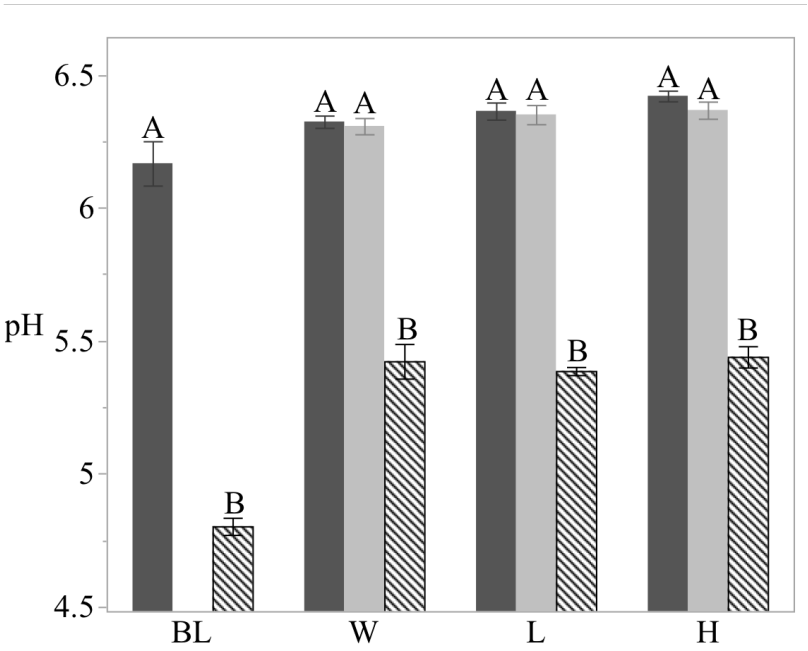

Figure 15: Mean pH with standard error of nib before fermentation (dark grey), after washing (light grey) and after fermentation (stripes) for different treatments. Different letters denote significant differences within one treatment, based on a Tukey-Kramer test with P-value $\leqslant$ 0.05.

5.3.2 Influence of washing on Cd content cacao bean tissues

The initial hypothesis stated that washing with EDTA is expected to lower Cd concentration in all cacao tissues. To verify this hypothesis, Cd content in mucilage, testa and nib were measured before and after washing. The mucilage Cd concentration of the blank treatment before fermentation was not statistically different compared to mucilage Cd content of other treatments before washing ($p = 0.05$; Figure 16). The latter values are also not significantly different from each other. After washing with water, mucilage Cd concentration did not change significantly from (t-test with $P \leqslant 0.05$; 0.60 ± 0.05 mg kg^{-1} before washing to 0.51 ± 0.04 mg kg^{-1} after washing). For both EDTA washing treatments, Cd content in mucilage was up to a 5-fold higher after washing (low EDTA: 2.55 ± 0.25 mg kg^{-1}; high EDTA: 2.50 ± 0.39 mg kg^{-1}) after washing compared to before washing (t-test with P-value $\leqslant 0.05$; low EDTA: 0.62 ± 0.10 mg kg^{-1}; high EDTA: 0.71 ± 0.05 mg kg^{-1}). Contradictory, this refutes the hypothesis that EDTA washing decreases Cd levels in mucilage. It is generally known that EDTA is a strong complexing agent for Cd. One possible explanation for the increase in mucilage Cd after washing (with EDTA) is the complexation of Cd by EDTA in the testa and subsequent removal of this complex via the aqueous phase. During this outward movement, some of these EDTA-Cd complexes could have stayed behind in the mucilage.

After fermentation, the mucilage Cd content was higher compared to samples before fermentation (for all treatments, both before and after washing), which corresponds to the observation in large-scale fermentations (section 4): for all batches, mucilage Cd increased significantly due to fermentation. For the EDTA treatments, this increase in mucilage Cd was less pronounced compared to the blank and water treatments. Based on a Pearson correlation test (P-value $\leqslant 0.05$), mucilage pH is positively correlated with mucilage Cd within each treatment: blank ($r = 0.99$), water ($r = 0.99$), low EDTA ($r = 0.82$) and high EDTA ($r = 0.86$).

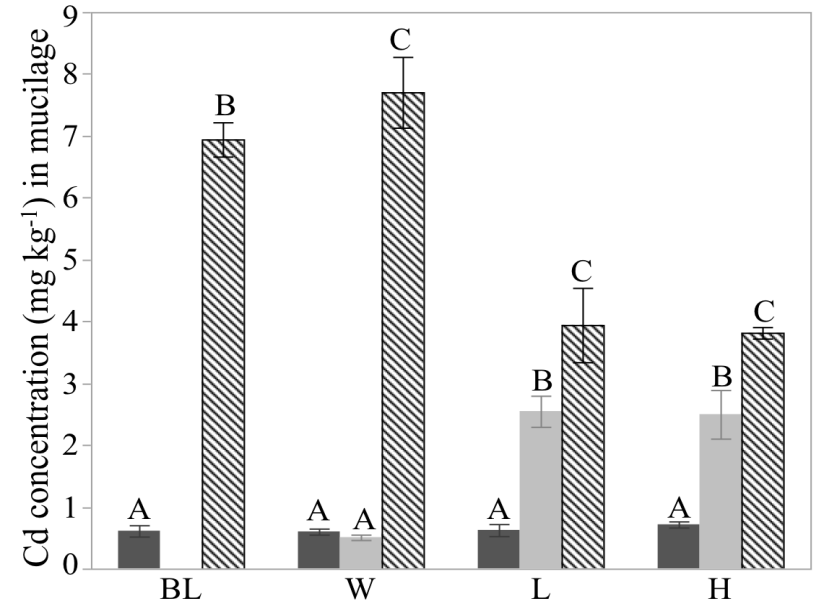

Figure 16: Mean Cd concentration with standard error before washing (dark grey), after washing (light grey) and after fermentation (stripes) in mucilage for different treatments. Different letters denote significant differences within one treatment, based on a Tukey-Kramer test with P-value ⩽ 0.05.

The testa Cd concentration (mean ± standard deviation) of the blank treatment before washing was not statistically different compared to the testa Cd content of the water and low EDTA treatments before washing (Tukey-Kramer test with P-value ⩽ 0.05). The high EDTA washing treatment displayed a significantly lower testa Cd concentration before fermentation of 2.00 ± 0.90 mg kg^{-1} compared to the other treatments before washing (water: 3.63 ± 0.22 mg kg^{-1}; low EDTA: 3.65 ± 0.05 mg kg^{-1}). No exact reason could be found for this difference, as all samples originated from the same homogenized mass and were treated identically (Figure 17).

After washing with water, no significant difference in testa Cd (mean ± standard deviation) was observed (before washing: 3.63 ± 0.22 mg kg^{-1}; after washing: 3.82 ± 0.83 mg kg^{-1}). After washing with EDTA, Cd decreased (t-test with P-value ⩽ 0.05) from 3.70 ± 0.05 mg kg^{-1} (before washing) to 1.20 ± 0.05 mg kg^{-1} (after washing; low EDTA treatment) and from 2.00 ± 0.90 mg kg^{-1} (before washing) to 0.80 ± 0.40 mg kg^{-1} (after washing; high EDTA treatment). Note that the decrease in testa Cd in the high EDTA treatment was not statistically confirmed. The EDTA solutions were thus able to wash out some of the Cd present in the cacao testa (Figure 17).

For the testa, only the low EDTA treatment displayed a significantly different Cd concentration after fermentation. Here, testa Cd (mean ± standard deviation) decreased from 3.70 ± 0.05 mg kg^{-1} to 1.20 ± 0.05 mg kg^{-1} due to washing and subsequently increased back to 2.00 ± 0.2 mg kg^{-1} after fermentation (Figure 17). The same pattern could be observed for the high EDTA treatment. However, no differences were statistically confirmed in the latter. Based on batches A and B in section 4, an increase in testa Cd due to fermentation could be expected. For the EDTA and blank treatment, this was also observed. For the water treatment, a decreasing trend in testa Cd due to fermentation was observed. Therefore, it remains plausible that washing with water influences Cd distribution, as these observations do not correspond to results from large-scale fermentations. Note that the testa Cd of the water treatment after fermentation was not significantly different compared to the testa Cd of the low and high EDTA treatments after fermentation. Contradictory, testa Cd after washing was higher in the water treatment compared to the EDTA treatments. Different Cd migration patterns during fermentation thus seem to influence its final distribution in cacao beans.

It is generally known that EDTA is a strong complexing agent for Cd. After its binding to Cd (during the washing treatment), EDTA removal via the aqueous phase was expected to remove Cd from the testa. This hypothesis was confirmed with results presented in Figure 17 (except for the water treatment). During fermentation, an increase in testa Cd (in EDTA treatments) could be explained by an outward migration of Cd from the nibs to the testa and mucilage. To check this hypothesis, mass balance calculations were performed. Since mass balance data from washing experiments are not present, average nib and testa mass fractions from samples A and C (both Nacional cultivar) in section 4.3.6 were used. The total average cacao bean Cd content on mass fraction base (mean ± standard deviation) decreased from 2.11 ± 0.12 mg kg^{-1} (before fermentation) to 1.82 ± 0.16 mg kg^{-1} (after fermentation) for the low EDTA treatment and from 2.23 ± 0.14 mg kg^{-1} (before fermentation) to 2.17 ± 0.08 mg kg^{-1} (after fermentation). Note that mucilage Cd is not taken into account (for practical reasons) even though mucilage Cd increased significantly due to fermentation (before washing compared to after fermentation). However, with some care, there can be concluded that there was an outward Cd migration in both EDTA treatments since no (or no significant amounts of) Cd is lost from the whole cacao beans.

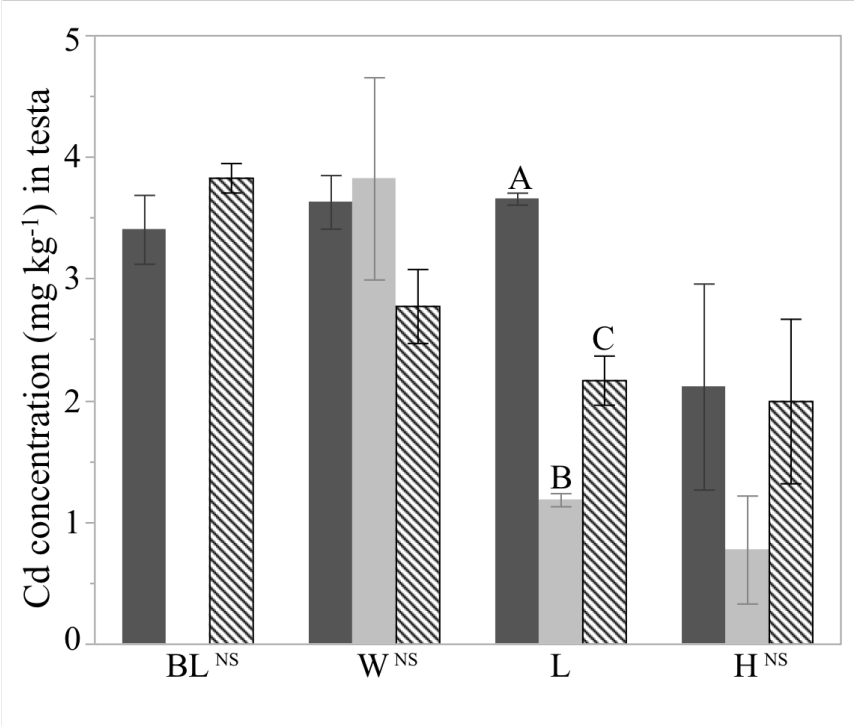

Figure 17: Mean Cd concentration with standard error before washing (dark grey), after washing (light grey) and after fermentation (stripes) in testa for different treatments. Different letters denote significant differences within one treatment. NS denotes non-significant difference in Cd content within samples of the same treatment, based on a Tukey-Kramer test with P-value $\leqslant$ 0.05.

For the nib, Cd concentration of the blank before fermentation was not statistically different compared to Cd content of the other treatments before washing (Tukey-Kramer test, P-value $\leqslant$ 0.05; Figure 18). The latter values are also not significantly different from each other. In Figure 18, it can be seen that there is no significant difference in nib Cd content before and after washing for all washing treatments (water and EDTA treatments). Moreover, there was no difference in nib Cd concentration in samples after washing and after fermentation (for all treatments, Tukey-Kramer test, P-value $\leqslant$ 0.05). This observation does not fall in line with the hypothesis, where a decreasing trend in nib Cd was expected in EDTA treated samples. Two explanations could explain this behavior. First, differences in nib Cd could be low due to a limited contact time of EDTA with the cacao beans in suspension. Longer suspension times could therefore lower nib Cd possibly more. Secondly, the EDTA radius could be too large to penetrate into the cacao nib. In this latter scenario, no influence of EDTA is expected on nib Cd. Smaller organic acids (e.g. lactic acid 90 g mol^{-1} and acetic acid 60 g mol^{-1}) formed during fermentation, can penetrate into the nib (De Vuyst & Weckx, 2016; Schwan & Fleet, 2012). Citric acid (192 g mol^{-1}) however is bigger and naturally present in the mucilage of unfermented beans (1-3 % on wet weight). If citric acid would be able

to penetrate into the nib, nib pH would be lower than values found in unfermented beans (pH: 6.3 - 7; De Vuyst & Weckx, 2016; Schwan & Fleet, 2012). Therefore, it is assumed that penetration of EDTA into the cacao nibs was limited due to its voluminous dimensions.

Based on batches A and B in section 0, a decreasing trend in nib Cd due to fermentation could be expected. Even though a decreasing trend was observed for the blank and both EDTA treatments (before washing and after fermentation), differences due to fermentation could not be statistically confirmed. Moreover, nib Cd (mean ± standard deviation) of the blank treatment after fermentation (1.56 ± 0.15 mg kg^{-1}) was significantly different compared to nib Cd (mean ± standard deviation) of the other treatments after fermentation (water: 2.06 ± 0.12 mg kg^{-1}; low EDTA: 1.80 ± 0.19 mg kg^{-1}; high EDTA: 2.19 ± 0.06 mg kg^{-1}; Tukey-Kramer test with P-value ⩽ 0.05). The nib Cd of the water treatment after fermentation was not significantly different compared to the nib Cd of the EDTA treatments after fermentation. This supports the hypothesis that washing does influence Cd distribution. Washing thus seems to impede Cd migration from inner (nib) to outer (testa and mucilage) cacao tissues, as no influence of fermentation on Cd content in nib could be found.

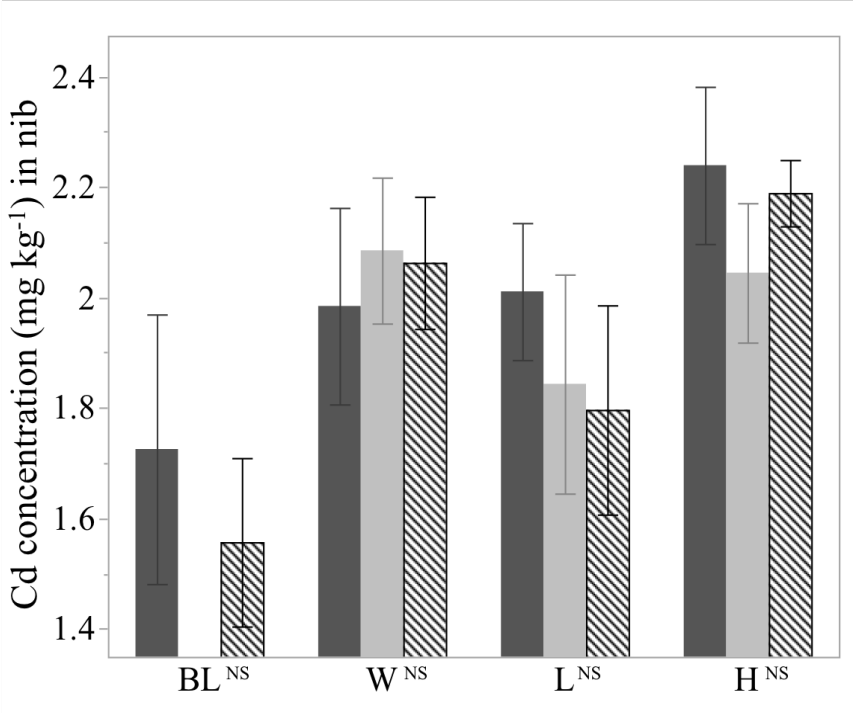

Figure 18: Mean Cd concentration with standard error before washing (dark grey), after washing (light grey) and after fermentation (stripes) in nib for different treatments. NS denotes non-significant difference in Cd content within samples of the same treatment, based on a Tukey-Kramer test with P-value ⩽ 0.05.

For the blank, there could be assumed that a portion of the nib Cd migrates to outer layers (mucilage and testa) during fermentation. However, the slight decrease in nib Cd does not seem to correspond with the increase in testa and mucilage Cd (mucilage Cd increases from 0.60 ± 0.09 mg kg^{-1} before fermentation to 7.00 ± 0.30 mg kg^{-1} after fermentation). It is thus assumed that no Cd was removed during fermentation. To check this hypothesis, mass balance calculations should be performed. Since mass balance data from washing experiments are not present, average nib and testa mass fractions from samples A and C (both Nacional cultivar) in section 4.3.6 were used. The total average cacao bean Cd content on mass fraction base (mean ± standard deviation) decreased from 1.83 ± 0.23 mg kg^{-1} (before fermentation) to 1.72 ± 0.15 mg kg^{-1} (after fermentation). Note that mucilage Cd is not taken into account (due to practical reasons) even though mucilage Cd increased a 10-fold due to fermentation (before washing compared to after fermentation). However, with some care, it can be concluded that no Cd was removed during fermentation in the blank treatment. Moreover, an outward migration of Cd was confirmed.

To summarize, washing cacao beans with EDTA did not significantly affect Cd concentrations in testa and nib (except for testa of low EDTA treatment). Moreover, washing tended to interfere drastically in the fermentation process by altering mucilage and testa pH and lowering fermentation temperature (as could be seen from the water treatment). Therefore, it is safe to assume that fermentation of batches C and D was not properly fermented. This could help to explain the limited changes in Cd content of cacao tissues in batches C and D, as these physicochemical factors (pH and temperature) were expected to influence metal mobility.

We expected an inward movement of Cd since concentrations are generally higher in testa compared to nibs, as could be seen in section 1.5.2.3. Washing out Cd from outer layers (mucilage and testa) before fermentation with EDTA, could possibly lower this Cd migration, ultimately decreasing nib Cd. However, in section 4.3, we saw that Cd migration is contradictory against the concentration gradient and thus that Cd moves from the nib to the testa. There can be concluded that chelating agent washing of cacao prior to fermentation was not a feasible technique to lower the Cd concentration in the final cacao-derived product.

To the best of our knowledge, no literature concerning mitigation strategies to lower Cd in postharvest cacao processing are available. However, Argüello et al. (2019) published some

preliminary results in which washing treatments on approximately 40 beans (per treatment) were conducted. Two lab-scale batches (treatment 1 and 3) underwent a 4-day incubation. Two treatments (1 and 2) were washed with a solution of 0.01 M Na2-EDTA prior to incubation and subsequent (7 day) drying. Argüello et al. (2019) reported no significant difference in nib Cd due to fermentation on EDTA washed cacao beans. This corresponds to our results at the end of fermentation from blank and low EDTA treatments (Argüello et al., 2019)

5.4 Conclusion

Based on all data provided concerning temperature and pH of mucilage, testa and nib, there can be concluded that washing with water had a significant influence on the performance of the fermentation process. Besides, it remains difficult to ascribe changes in temperature, pH and Cd concentrations to the various washing treatments. An elevated mucilage pH compared to the full-scale experiments (the latter being 1 to 2 pH units lower) and a limited temperature increase during the first 3 days of fermentation are indications that fermentation of the blank treatment was not fully in line with what could have been expected based on large-scale fermentations. Therefore, changes in temperature, pH and Cd concentrations of other washing treatments could also be ascribed to the small-scale fermenting size (5 kg). None the less, washing cacao prior to fermentation with chelating agents was not a feasible technique to lower the Cd content in the final cacao-derived product. This conclusion is based on two observations. First, EDTA washing did not significantly decrease nib Cd (before washing compared to after washing). Secondly, in section 4.3, we established that Cd migration during fermentation was contradictory against the concentration gradient and thus that Cd moved from the nib to the testa during fermentation.

6 General conclusion

Overall, three general conclusions could be made from this research.
First, the Cd content in different unfermented cacao tissues generally decreased from testa > nib > mucilage. Husk and placenta Cd were measured in two samples. Cadmium concentration in husk and placenta tends to range between those of mucilage and nib. The Cd concentration in the testa was generally twofold higher compared to Cd concentration of the nib. Those observations could

be ascribed to preferential binding of Cd to compounds present in cacao such as phytate, organic acids, polyphenols, ….

Secondly, we established that there was a change in Cd distribution during fermentation. Nib Cd decreased while testa and mucilage Cd increased with fermentation time. From the mass balance, an outward migration of Cd from nib to testa, has been empirically established. At first sight, these changes seem to relate to alterations in tissue pH during fermentations and an increase in temperature of the fermenting mass. Besides, drying - as a post-fermentation processing step - did not significantly alter Cd content in the cacao nibs.

Finally, washing cacao prior to fermentation with chelating agents (0.01 and 0.1 M EDTA) was not a feasible technique to lower the Cd concentration in the final cacao-derived product. Moreover, washing with water had a significant influence on the performance of the fermentation process.

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8 Appendix

Table A.1: pH of different cacao tissues in unfermented cacao beans (mean ± standard deviation). (*) Denotes no significant difference in Cd content of tissues within one batch, based on a Tukey-Kramer test with P-value ⩽ 0.05.

Batch	mucilage	nib	testa
A	4.12 ± 0.20 (n = 3)	6.23 ± 0.09 (n = 3)	4.52 ± 0.11 (n = 3)
B	3.45 ± 0.03 (n = 3)	6.28 ± 0.04 (n = 3)	4.28 ± 0.08 (n = 3)
C	3.75 ± 0.14 (n = 10)	6.28 ± 0.16 (n = 10)	4.43 ± 0.30 (n = 6)
E	/	6.09 ± 0.09 (n =4)	/
F	/	6.22 ± 0.13 (n = 6)	4.64 ± 0.49 (n = 6)

Table A.2: Cadmium content for different cacao tissues at AH and BF. (*) Denotes significant difference in Cd content between AH and BF sample of the same tissue in one sample.

Sample Code	Cacao tissue	Mean ± standard error Cd in AH sample (mg kg^{-1})	Mean ± standard error Cd in BF sample (mg kg^{-1})	Number of AH biological samples
A	Nib*	0.385 ± 0.042	0.781 ± 0.025	3
	Testa*	0.662 ± 0.152	1.254 ± 0.104	
	Mucilage	0.092 ± 0.019	0.655 ± 0.120	
B	Nib	0.512 ± 0.066	0.492 ± 0.002	3
	Testa	0.935 ± 0.128	0.603 ± 0.019	
	Mucilage	0.068 ± 0.008	0.168 ± 0.017	
C	Nib	2.296 ± 0.364	2.974 ± 0.029	6
	Testa	3.704 ± 0.671	4.680 ± 0.043	
	Mucilage	0.479 ± 0.109	0.601 ± 0.011	
D	Nib	2.296 ± 0.364	2.926 ± 0.020	6
	Testa	3.704 ± 0.671	4.736 ± 0.001	
	Mucilage	0.479 ± 0.109	0.580 ± 0.001	

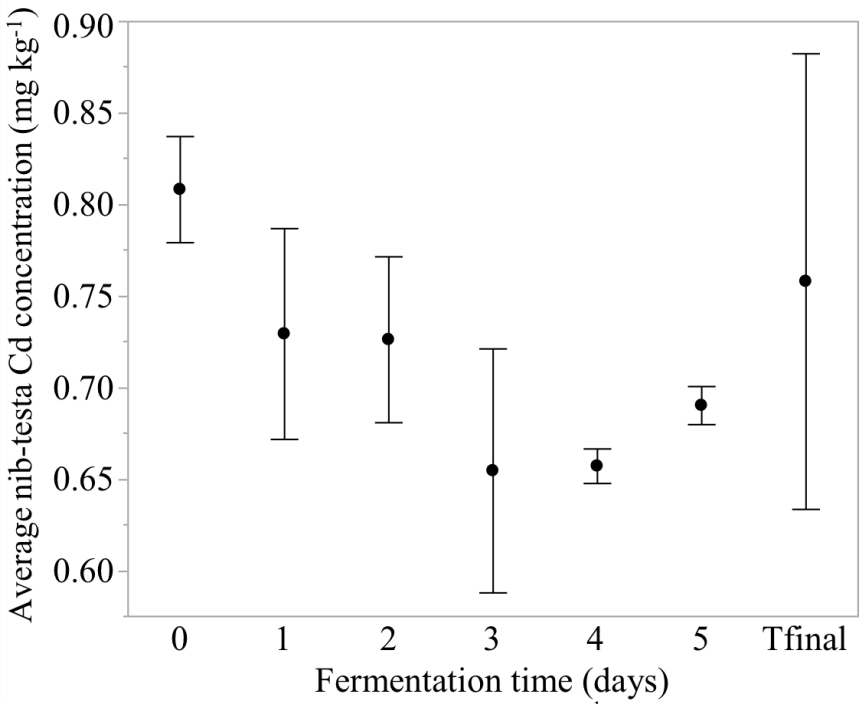

Figure A.1: Average nib-testa cadmium concentration (mg kg^{-1}) during fermentation for batch A.

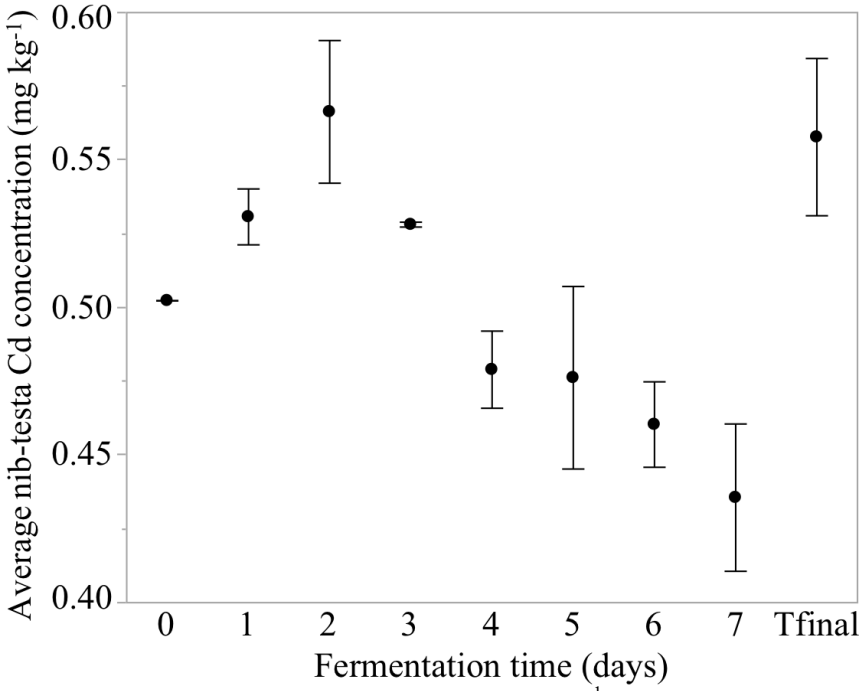

Figure A.2: Average nib-testa cadmium concentration (mg kg^{-1}) during fermentation for batch B.

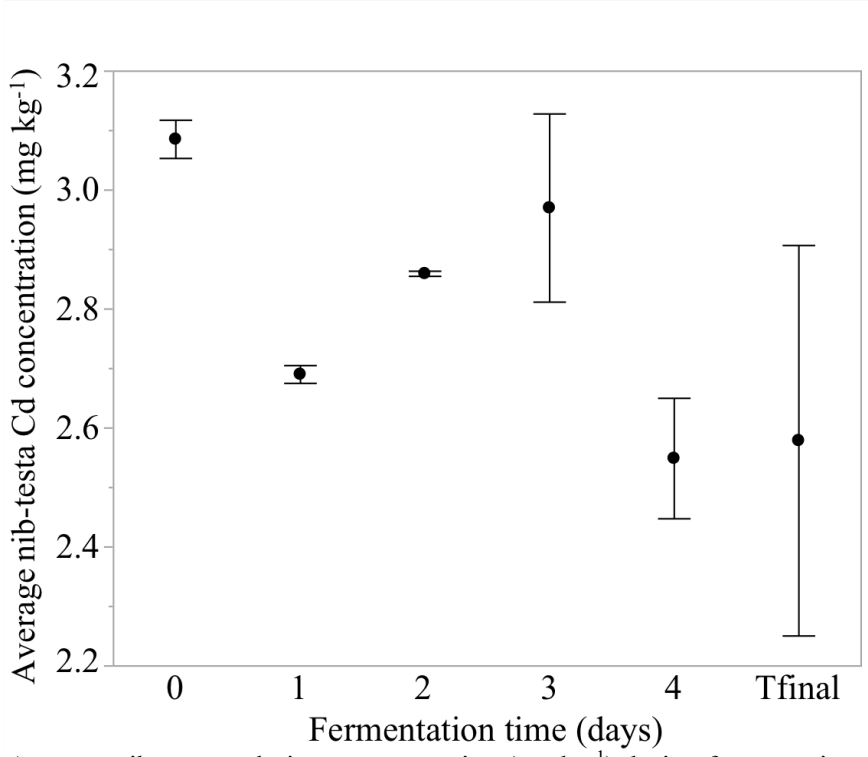

Figure A.3: Average nib-testa cadmium concentration (mg kg^{-1}) during fermentation for batch C.

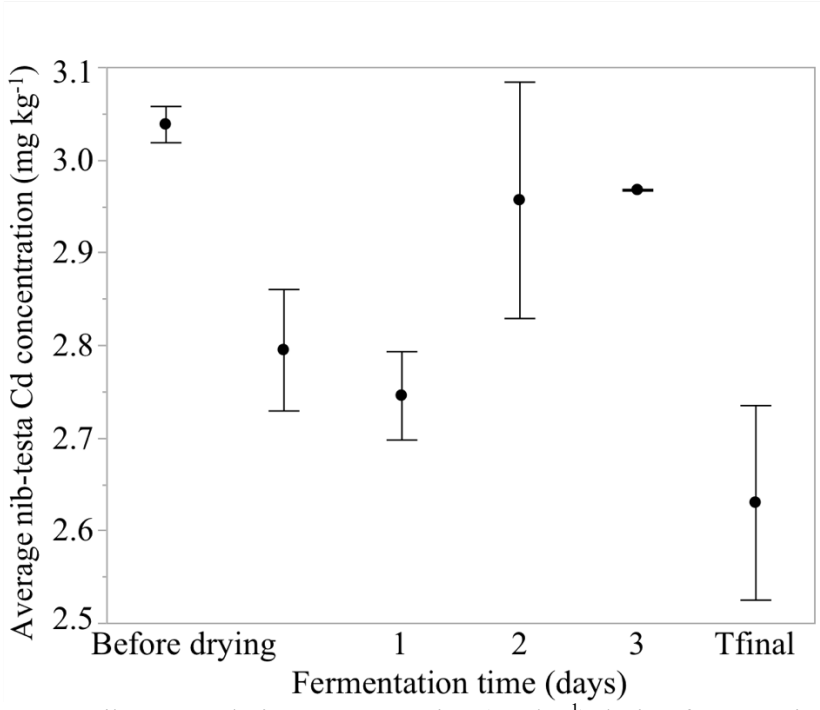

Figure A.4: Average nib-testa cadmium concentration (mg kg^{-1}) during fermentation for batch D.

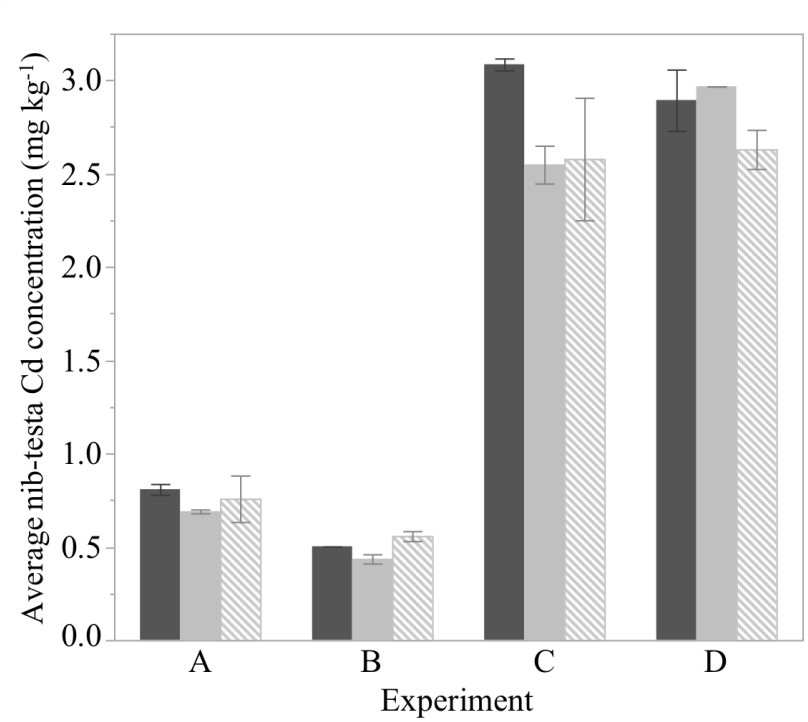

Figure A.5: Average nib-testa cadmium concentration with standard error (mg kg^{-1}) at before fermentation (dark grey), after washing (light grey) and after fermentation (line pattern) for all washing experiments.

9 Summary in Laymans terms

On the 1st of January 2019, a new regulation of the European Commission went into force concerning the cadmium content in cacao. Cadmium is a metal that is present in a wide range of foods and is toxic to humans in high concentrations. Since cacao beans from South American countries naturally contain high concentrations of cadmium, this new legislation will impose a threat to the export cacao industry.

There is little information concerning Cd distribution during post-harvest processing of cacao. After harvest, the content of the cacao fruit (cacao mucilage and cacao beans) is fermented. This fermentation is a crucial step in the post-harvest processing of cacao, as during this step, the development of the unique cacao flavor precursors occurs. This process is accompanied by high temperatures (45°C) and extreme pH changes in cacao tissues. This study is carried out in order to better understand the distribution pattern of Cd within fresh cacao beans and differences in Cd distribution during post-harvest processing. Ultimately, a better understanding of those migration patterns could lead to mitigation strategies to lower Cd concentration in the nib, as this tissue is the raw material for chocolate products.

Unfermented cacao tissues (from outside to inside: husk, placenta, mucilage, testa and nib) were analyzed. The Cd content in those tissues generally decreased from testa > nib > mucilage. Cadmium content in testa was generally 2-fold higher compared to Cd content in nib. Fermentation of two cacao cultivars (CCN-51 and Nacional) was investigated in two different fermentation set-ups. Based on four large-scale fermentations, nib Cd decreased while testa and mucilage Cd increased with fermentation time. Factors that influence this metal mobility include and changes in pH of cacao tissues (nib pH roughly decreased from 6.3 to 4.5). Finally, drying did not significantly alter Cd content in the cacao nibs.

Washing of unfermented cacao beans with ethylenediaminetetraacetic acid (EDTA) as a possible mitigation strategy to lower Cd in the nibs, did not yield significantly lower nib Cd concentrations. This is possibly due to a limited nib pH decrease from 6.3 to 5.4. Washing could have caused significant alterations in fermentation performance.