Cadmium migration from nib to testa during cacao fermentation is driven by nib acidification

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ABSTRACT

Previous work has shown that cacao nib cadmium (Cd) concentrations decrease during fermentation, but only when reaching sufficiently low nib pH. In this work, lab-scale experiments (5 kg units) with lactic and acetic acid amendments were ineffective at reducing the total nib Cd concentration. In contrast, the water-extractable fraction of the nib Cd concentration clearly increased when the pH was decreased. When single pod derived beans were embedded inside a full-scale fermentation box to monitor the effect of the fermentation effect with high precision, nib Cd concentrations decreased by a factor 1.25 (P-value <0.05) after four days of fermentation. Visualisation of the mobile Cd gradient within beans with LA-ICP-MS (using imprints of transversal cuts exposed to a metal binding gel) revealed that fermentation enhances the Cd mobility in the nibs.

KEYWORDS

Cadmium

Cacao

Fermentation

Laser ablation imaging (LA-ICP-MS)

Organic acids

1. INTRODUCTION

In 2019, the European Commission enforced a regulation which sets the maximum allowed cadmium (Cd) concentrations in cacao-derived consumer products (European Commission 2014). Similar limits were also approved by the Codex Alimentarius (Codex Alimentarius Commission 2018). These regulations have a large impact on the cacao industry, especially in Latin America where soils naturally contain elevated Cd concentrations. Larger Cd concentrations have been reported in cacao beans and chocolates from Latin America, compared to other geographical origins such as Africa (Bertoldi et al.2016; Vanderschueren et al. 2019). A recent extensive meta-analysis (Vanderschueren et al. 2021) demonstrated the impact of the Cd regulation on cacao farmers in Latin America. More than 50% of compiled bean Cd data exceeded the unofficial industry threshold for EU export (0.60 mg Cd kg^{-1}). The regulation has prompted researchers to study mitigation strategies to lower the Cd concentrations in cacao-derived products. While research thus far focused mostly on the use of soil amendments (Ramtahal et al. 2019) and selection of cultivars for reduced Cd uptake and translocation (Lewis et al. 2018; Engbersen et al. 2019), the potential of postharvest mitigation has also been pointed out (Meter et al. 2019).

The effect of fermentation on bean Cd concentrations was recently demonstrated by Vanderschueren et al. (2020), their results indicated that Cd migrates from the nib to the testa and the mucilage during fermentation. This migration decreased the nib Cd concentration by up to a factor 1.3, but only occurred with sufficient nib acidification, i.e. nib pH <5. This migration is counterintuitive since Cd concentrations in the testa are typically higher than in the nib Cd (Ramtahal et al. 2016; Lewis et al. 2018; Vanderschueren et al. 2020), and migration thus occurs against the total Cd concentration gradient. The authors speculated that the concentration

gradient of mobile Cd is opposite to the total concentration gradient, especially in more acid, fermented beans.

The objective of this study is to better understand the causes of Cd migration during cacao fermentation. More specifically, this study was set up (i) to reveal whether nib acidification is the driving force behind Cd migration during cacao fermentation; and (ii) to identify whether artificial acidification during fermentation using organic acids typically formed during fermentation (acetic and lactic acid) can be used to lower the Cd concentration in the final product. The work consisted of two lab-scale fermentation experiments with different organic acid treatments, and one micro-fermentation experiment established inside a full-scale commercial fermentation unit. The idea behind the lab-scale fermentation experiments is that the effects of acidity on mobile and total nib Cd concentrations can be disentangled from the other effects in the fermentation process, such as heat-induced cell breakdown or the effects of ethanol penetration. A novel method was adopted to map Cd mobility at sub mm resolution. An imprinting technique was used followed by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) analysis of the imprint. This visualisation method allows to test the hypothesis that the mobile Cd concentration gradient in cacao beans is opposite to the total Cd concentration gradient.

2. MATERIALS AND METHODS

2.1. Lab-scale fermentations with organic acid treatments

2.1.1. Experimental setup and sampling

Two separate lab-scale (5 kg) fermentation experiments were executed in a greenhouse in Guayaquil (Ecuador) with administration of lactic and acetic acid before, during, or at the end of

fermentation (Table 1). Lab-scale fermentations were executed in planter pots with a flat cone shape (small diameter 17.5 cm, large diameter 23.5 cm, height 22.5 cm). Three to five holes in the bottom of each planter pot ensured drainage of fermentation sweatings. Cacao beans (Nacional cultivar) were provided by local farmers in Manabí and Guayas provinces (Ecuador). Prior to setting up each fermentation experiment, 60 kg fresh beans were deposited on a plastic sheet and homogenised manually before dividing them among the planter pots (5 kg each). The planter pots were placed inside Styrofoam boxes to retain heat. The fermentation lasted 5 (lab-scale fermentation A) or 6 days (lab-scale fermentation B). The cacao mass was mixed manually on day 2 in experiment A, and on days 2 and 5 in experiment B. Treatment doses, treatment time and total fermentation time for both experiments are shown in Table 1. To apply the treatments, the cacao beans were spread out in plastic trays and treatment solutions were applied using spray bottles. The application of acids before fermentation and on the second fermentation day logically disturbed the fermentation process and associated temperature increase. Deionised water treatments were included on days 0 and 2 to assess the effect of liquid application on the overall course of fermentation. Those water treatments allowed to disentangle the effect of liquid treatment from the acidification effect. The treated cacao beans were mixed manually to ensure an even distribution of the treatment components and were redeposited in the plastic planter pots. Samples containing 500 g beans were taken after mixing, at the start of fermentation, at the end of fermentation, and before treatment. Samples of the first experiment (A) thus included day 0, day 2, day 4 for the cacao treated that day, and day 5; samples of the second experiment (B) included day 0, day 5 and day 6. The mucilage was removed manually using paper towels, after which the intact beans were oven-dried for 72 hours at 65 °C. After drying, beans were peeled to separate nib and testa, and both fractions were ground using an electric coffee grinder.

105 2.1.2. Water-extractable Cd

106 The Cd mobility was assessed by measuring Cd concentrations in deionised water extracts of the
107 nibs. Ground nib samples were sieved to obtain a more homogenous particle size distribution
108 (800 µm test sieve, VWR International). Sieved material (0.50 g) was incubated with 4.0 mL
109 deionised water in an end-over-end shaker at 20 °C for 5 days. After incubation, samples were
110 centrifuged (15 minutes at 2000 g, Heraeus Multifuge X3R, Thermo Scientific, Waltham, MA,
111 USA). The supernatant was filtered through a 0.45 µm syringe filter and filtered samples were
112 diluted 200 times prior to Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis
113 (described below).

114 Additionally, the effect of artificial acidification on Cd mobility was determined using water-
115 extractable Cd as a proxy. To this end, ground nib and testa material from the blank treatments of
116 experiment A (unfermented samples at day 0 and fermented samples at day 5) were used.
117 Duplicate ground materials (0.50 g for nibs and 0.25 g for testa) were incubated in 12.0 mL
118 deionised water, either or not acidified to pH 4.5 with hydrochloric acid (HCl, 37% w/w). The
119 pH of the contact solutions was adjusted prior to incubation and remained unchanged after
120 incubation (<0.1 pH unit difference). Samples were incubated in an end-over-end shaker,
121 centrifuged, and their supernatant was filtered and diluted prior to ICP-MS analysis, as outlined
122 below.

123 2.1.3. Determining the elemental composition

124 Subsamples containing 100 mg dry nib or testa material were acid digested in an open digestion
125 block for 8 h in 3.0 mL Normatom® nitric acid (HNO_3 67–69% w/w, VWR International,
126 Radnor, PA, USA), reaching a maximum temperature of 130 °C. Digests were brought to a
127 volume of 10.0 mL and diluted ten times with Milli-Q water (18.2 MΩ cm^{-1}) prior to elemental

analysis with ICP-MS (Agilent 7700x, Agilent Technologies, Santa Clara, CA, USA). Cadmium concentrations were measured by monitoring the ^{111}Cd isotope in helium (He) collision cell mode, using ^{103}Rh as an online internal standard. The limit of quantification (LOQ) for the analysis was 0.001 mg Cd kg^{-1} dw. Duplicate samples were included in the digestions at intervals of ten samples and the coefficient of variation (CV) of these duplicates ranged from 0.4 to 13% (average CV 3.5%). Blank samples (in quadruplicate) and certified reference NIST 2384 baking chocolate (in triplicate, certified concentration 0.073 ± 0.008 mg Cd kg^{-1}) were included in all digestions and treated identical to the cacao samples. Recoveries of the certified reference material ranged between 87 and 100%. In addition to Cd, several other elements were also analysed (Al, P, K, Ca, Cr, Mn, Co, Ni, Cu, Zn, As, Mo and Pb). Specific parameters regarding the ICP-MS analysis for those elements and a discussion on the quality control can be found in the Supplementary Information (SI).

2.1.4. Temperature and pH

The temperature (T) in the centre of the fermentation boxes was measured daily using a digital thermometer (VWR International). The pH was determined only at sampling times to avoid excessive disturbance of the fermentation process. The pH of the mucilage, not including the testa, was determined in suspension after shaking 25 g beans with mucilage in 100 mL deionised water on an orbital shaker (KS 130 orbital shaker, IKA laboratory equipment, Staufen, Germany) for 5 minutes at 320 rpm. The nib pH was determined by shaking 5.0 g dried ground material in 20 mL deionised water on an orbital shaker for 5 min and measuring the pH in the supernatant after centrifugation for 25 minutes at 1800 g (MTL-5MS Tabletop Low Speed Centrifuge, Micronlab, Shandong, China).

2.1.5.

2.2. Micro-fermentation

2.2.1. Cacao material and setup

The micro-fermentation experiment was carried out at a plantation in Guayas province (Ecuador), using Nacional cultivar cacao. Fifteen ripe cacao pods were opened using a machete. Half of the beans from each pod were immediately sampled and processed without fermentation. The other half of the beans from each pod were collected in mesh bags with drawstring closing and placed in the centre of a wooden fermentation box (82 x 65 x 65 cm) filled with Nacional cacao from the same field (225 kg). Fermentation lasted four days and the mass was mixed manually on the second day. The mesh sample bags were kept in the centre of the box throughout the process and were sampled when fermentation was complete (fermented samples) (SI, Figure S 1).

Unfermented and fermented beans were cleaned with paper towels to remove the mucilage, oven-dried for 72h at 65 °C, and peeled to separate nib and testa. Due to the small sample size, testa and nibs of three individual beans per sample were digested separately and their elemental composition was analysed with ICP-MS. The temperature in the centre of the fermentation box was measured daily using a digital thermometer (Custom, CT-422WR, Hitchcock, TX, USA). Nib and mucilage pH were determined before and after fermentation in the bulk cacao surrounding the micro-fermentation samples, as described above.

2.2.2. Visualisation of Cd mobility using LA-ICP-MS

A novel visualisation method was developed to map mobile Cd in cacao beans at mm resolution, using the principles of the Diffusive Gradient in Thin film technique (DGT) developed by Santner et al. (2015). In brief, a transversely cut bean was exposed to a gel that contains a metal ion selective binding agent. The mobile metal ions migrate from the source to the gel. After a given deployment time, the gel is removed, dried, and its surface composition is scanned with LA-ICP-MS.

Metal binding gels containing micro-milled Chelex-100 resin were prepared as described by Zhou et al. (2018), with a resin-to-gel ratio of 4.5% (w/w). Due to the small size of the resin beads (<10 µm), this gel can be used for chemical analysis with high spatial resolution. Mobile Cd was visualised for 5 of the 15 micro-fermentation pods and included two unfermented and two fermented beans from each pod. Immediately after sample collection (either before or after fermentation, not dried), beans were transversely cut in half using a clean razor blade and placed on the gel overnight. A Nuclepore® membrane (0.2 µm pore size, Whatman Maidstone, UK) was placed in between the beans and the gel as a barrier to avoid particulate transfer. The micro Chelex gel acts as a zero sink for cationic trace metals. It binds mobile Cd ions present in the contact surface with the bean and thereby creates a print of the mobile Cd in the beans. Imprinted Chelex gels were stored in plastic bags with several drops of ultrapure water to keep the gels hydrated. Gels were dried on 0.45 µm Millipore membrane with Whatman chromatography paper as a support (3 mm CHR), in a slab gel dryer (DrygelSr, Hoefer, Holliston, MA, USA) for at least 48 hours. During drying, gels were covered with clean polyethylene plastic to protect the reactive layer. Dried gels were fixed on glass microscopy slides using double sided tape and submitted to LA-ICP-MS analysis.

For elemental analysis, a quadrupole 7700cs ICP-MS (Agilent Technologies) mounted with platinum cones was used. The sensitivity and operational conditions of the ICP-MS (stability, background and mass calibration) were initially checked using a 1.0 µg L^{-1} Y, Tl, Li, Ba and Ce tuning solution. The ICP-MS was then coupled to a 213-nm laser ablation system equipped with a TV2 cell (NWR213, ESI, Fremont, CA, USA) and coupling was optimised using a NIST 612 glass by monitoring ^{238}U and ^{232}Th for maximal sensitivity, and a U to Th ratio as close as possible to the unit (NIST 612 certified concentrations 37.79 ± 0.08 mg Th kg^{-1} and 37.38 ± 0.08 mg U kg^{-1}). Visualisation of Ca, Cd, K, Ni and Zn fixed on micro Chelex gel was performed by running several ablation line scans with a 20 Hz laser shot repetition rate, fluency maintained between 4.1 and 4.5 J cm^{-2}, a laser beam of 50 µm² and a scan speed of 50 µm s^{-1}. Ablated material was transported with 800 mL min^{-1} He and mixed with Ar gas before the ICP torch inlet. The ICP-MS was used in He mode, allowing monitoring of ^{111}Cd (0.2 s), ^{114}Cd (0.2 s), ^{39}K (0.005 s), ^{44}Ca (0.15 s), ^{60}Ni (0.1 s), and ^{64}Zn (0.1 s as integration time).

Several sets of two or three ablation lines (distance between lines 100 µm) were run on each sample (SI Figure S 6). Signal intensities were averaged for each set of ablation lines. To account for the differences in elemental composition between samples and elements, signal intensities measured for each element (counts per second, cps) were divided by the elemental concentration determined in each sample by acid digestion and ICP-MS analysis as described above. Several gels were lost prior to LA-ICP-MS imaging as they were either compromised during storage and transportation or broken during gel drying. In addition, gels that displayed disproportionally low K intensities, i.e. factor 10 lower than the other samples, were excluded from the discussion below. Even though the affinity of the Chelex resin is lower for K compared to the other elements evaluated here (Cd, Ca, Ni and Zn), K concentrations in the nibs are high and K is

readily water-extractable (see results below). Therefore, low K intensities were considered as an indication for poor contact between gel and bean, or as an indication that the gel was erratically used upside down, as these gels are typically asymmetric because Chelex resin beads settle to the bottom during gel casting.

3. RESULTS AND DISCUSSION

3.1. Lab-scale fermentations with organic acid treatments

3.1.1. Effect on fermentation parameters

Both water and organic acid treatments affected the fermentation temperature. It dropped to ambient temperature (i.e., 30 °C or below) within 24 hours after treatment in both experiments (Figure 1 and SI, Figure S 2). Pre-fermentation treatment (day 0) delayed heat production in the fermenting mass. In fermentation A, this effect was stronger for the acid compared to the corresponding water treatment (SI, Figure S 2). The temperature profiles of fermentation A suggest that the effect of treatment on temperature was smallest when applying the acids after fermentation, as was performed in fermentation B.

The mucilage pH was strongly affected by the treatments as this tissue was in direct contact with the solutions. The final mucilage pH was significantly lower in acid treated cacao compared to control and water treatments in both experiments [Tukey's Honestly Significant Difference (HSD) test, P-value < 0.05, Table 2]. Water treatment on day 0 increased the mucilage pH from 4.0 before fermentation to 6.9 on day 5, indicating suboptimal fermentation. Water treatment prior to fermentation likely delayed and/or impeded the outgrowth of yeasts in the first fermentation phase, which reduced the ethanol production. Ethanol is the main substrate for the acetic acid bacteria in the third and final fermentation phase. During this phase, the mucilage pH

239 decreases as these bacteria convert ethanol to acetic acid, and the fermentation temperature
240 increases to >45 °C because this process is strongly exothermic. Thus, reduced production of
241 ethanol may explain the higher final mucilage pH and lower maximum temperature observed in
242 the pre-fermentation water treatment.

243 At the end of experiment A, the nib pH was significantly lower in the control, and in the acetic
244 acid day 2 and day 4 treatments compared to acetic acid day 0 and water treatments (Tukey's
245 HSD test, P-value < 0.05, Table 2). The acetic acid treatments in experiment A thus did not
246 lower the nib pH below that of the control, and results of the water treatments indicated that
247 liquid application hindered nib acidification with fermentation. This again suggests reduced
248 availability of ethanol substrate for the acetic acid bacteria, as nib acidification mostly results
249 from penetration of acetic acid into the nibs. Acetic acid treatment before fermentation (day 0)
250 immediately decreased the nib pH, but this effect disappeared with time [significant decrease
251 from pH 6.5 ± 0.1 (standard deviation, stdev) to 5.9 ± 0.1 within one hour after treatment,
252 Student's t-test, P-value < 0.05, results not shown). Despite this immediate penetration of the
253 acetic acid into the nibs, pre-fermentation treatments yielded higher final nib pH values
254 compared to control treatments, which was likely related to the effects of the liquid application
255 on the overall fermentation (i.e. less acetic acid production from fermentation activity).

256 Much larger acid doses were applied in experiment B compared to A (35 and 53 g kg^{-1} cacao
257 compared to 13 and 15 g kg^{-1} cacao, Table 1). These treatments were applied only after
258 fermentation and resulted in significantly lower final nib pH values (on average 4.4 for lactic
259 acid and 4.5 for acetic acid treatment) compared to control or water treatments (average nib pH
260 5.2, Tukey's HSD test, P-value < 0.05, Table 2). A detailed overview of the changes in nib pH
261 with fermentation time can be found in SI Figure S 3. Within the first hour after application, the

262 average nib pH decreased significantly from 5.3 ± 0.2 (stdev) to 4.6 ± 0.1 with lactic acid and
263 from 5.3 ± 0.1 to 4.7 ± 0.1 with acetic acid application (Students t-test, P-value <0.05).
264 Penetration of both acids into the nib thus occurs quickly and does not require fermentation, as
265 equally fast acid penetration was observed in the pre-fermentation treatment of experiment A. It
266 should be noted that, while both lactic and acetic acid treatments in experiment B resulted in a
267 lower nib pH compared to the blank and water treatments, final nib pH values were still within
268 the range for fermented cacao reported in literature, i.e. pH 4.0–5.5 (Schwan and Wheals 2004;
269 Belitz et al. 2009; Papalexandratou et al. 2011; De Vuyst and Weckx 2016). The differences in
270 final nib pH between acid treated and untreated cacao (control or water) in experiment B thus
271 may have been related to less extensive fermentation in these small planter pots.

272 3.1.2. Effect on tissue Cd concentrations

273 A detailed overview of nib and testa Cd concentrations as a function of fermentation time can be
274 found in SI Figures S 3 and S 4, a summary is given in Table 2. Nib Cd concentrations were not
275 significantly affected by fermentation (Tukey's HSD test, P-value ≥ 0.05), except for the
276 replicates treated with acetic acid on day 2 in experiment A, where nib Cd decreased from 2.3 ±
277 0.3 mg Cd kg^{-1} before fermentation to 2.0 ± 0.1 mg kg^{-1} after fermentation (day 5) (SI, Figures S
278 3 and S 4). In both fermentation experiments, no significant differences could be observed in
279 final nib Cd concentrations among the different treatments (Tukey's HSD test, P-value ≥ 0.05,
280 Table 2). However, final testa Cd concentrations did differ significantly among treatments in
281 both experiments. Because the testa represents a much smaller weight fraction than the nib, the
282 total Cd stock in the testa is also smaller. For example, considering the weight fractions of nib
283 and testa from which mucilage had been removed [average weight fractions nib 0.93 and testa
284 0.07 (Vanderschueren et al. 2020)], a cacao bean from the control in experiment A with a total

285 weight of 1 g (nib and testa) would have a total stock of 1.8 µg Cd in the nib but only 0.26 µg Cd
286 in the testa. This explains why outward Cd migration from nib to testa can result in a significant
287 increase in the testa Cd concentration but not in a detectable decrease in the nib Cd
288 concentration. However, patterns were not consistent as testa Cd concentrations increased in
289 some treatments but decreased in others (SI, Figures S 3 and S 4). The inconsistency in the effect
290 of fermentation on testa Cd concentrations may have been related to further migration of Cd
291 toward the mucilage. This hypothesis could not be verified as the mucilage was not collected for
292 elemental analysis in this study.

293 While both lactic and acetic acid treatments in experiment B resulted in a similar final nib pH of
294 4.5, testa Cd increased more extensively with acetic acid compared to lactic acid treatment
295 (Table 2). Both acids thus penetrated and acidified the cacao nib, but testa Cd results suggested
296 that only acetic acid treatment resulted in relevant Cd mobilisation. However, this hypothesis
297 was not confirmed by the water extractions, as the water-extractable Cd fractions in the nibs of
298 both lactic and acetic acid treated cacao were similar at the end of fermentation and did not differ
299 from those in blank or water treatments. In experiment A, the total nib and testa Cd
300 concentrations decreased and increased significantly with decreasing nib pH (Pearson correlation
301 test, P-value < 0.05, Figure 2). In experiment B, no significant correlations could be found. Still,
302 a trend of increasing testa Cd concentrations with decreasing nib pH could be discerned, with the
303 acetic acid treated samples standing out in the low pH/high testa Cd area of the graph (Figure
304 2.B, top left). This relation between tissue Cd and nib pH suggests a pH dependent mobilisation
305 of Cd in the nib tissue. Indeed, the water-extractable nib Cd fraction increased significantly with
306 decreasing nib pH in both experiments (Pearson correlation test, P-value < 0.05). The effects of

different fermentation treatments on tissue elemental concentrations and water-extractable nib fractions of Ca, Cu, K, Mn, Ni, P and Zn are reported in SI Table S 1 and Figure S 6.

The water extraction of unfermented bean tissues, that were artificially acidified to pH 4.5, indicated an increase of water-extractable nib Cd at lower pH (Figure 3), again suggesting the importance of acidification for Cd migration. Similar effects of acidification were observed for e.g. Mn and Zn (SI, Figure S 7). Water-extractable Cd fractions were much lower in the testa compared to the nib. The increased water-extractability of Cd in the fermented testa may represent the Cd that migrated from nib to testa during fermentation, while the Cd present in this tissue prior to fermentation was less water-extractable (Figure 3).

3.2. Micro-fermentation

3.2.1. Effect on tissue Cd concentrations

The temperature in the centre of the box increased from ambient temperature (26 °C) to a maximum of 50 °C on the third day, after which it decreased to 46 °C by day 4. The mucilage pH increased from 3.9 (day 0) to 4.4 (day 4), while the nib pH decreased from 6.3 (day 0) to 4.8 (day 4). Even though all pods were collected from a single field, both nib and testa Cd concentrations varied by more than a factor 3 among the pods (CV 37% for nibs and 31% for testa), while variations among beans within a pod were relatively small (average CV 12% for nibs and 10% for testa) (Figure 4). Similar large variations in bean Cd within a single field were found previously by Argüello et al. (2019). They reported an average within-field CV of 39% in a nationwide study in Ecuador. Pairwise analysis of single pod micro-fermentations revealed an effect of fermentation on both nib and testa Cd concentrations. A pairwise t-test indicated a significant decrease in the nib Cd concentration with fermentation by -0.30 mg Cd kg^{-1}, and an increase in the testa Cd concentration by +0.75 mg Cd kg^{-1} (P-value <0.05). In contrast, unpaired

analysis of the ensemble of data did not detect these changes (SI Table S 2). The nib Cd concentration decreased on average by a factor 1.25, which is similar to the factor 1.3 reported previously (Vanderschueren et al. 2020). Significant decreases in nib elemental concentrations were also observed for Cu (factor 1.3), K (factor 1.3), Ni (factor 1.3) and P (factor 1.1), while testa elemental concentrations increased for Cu (factor 2.9), K (factor 2.5), Mn (factor 2.6), Ni (factor 2.2), P (factor 5.5) and Zn (factor 2.1) (SI Table S 2).

Considering the weight fractions of nib and testa from which mucilage had been removed [average weight fractions nib 0.93 and testa 0.07 (Vanderschueren et al. 2020)], the average mass of Cd lost from the nibs (0.28 ± 0.20 mg Cd kg^{-1}_{bean}) was significantly larger than the mass of Cd gained in the testa (0.05 ± 0.03 mg Cd kg^{-1}_{bean}). Similar discrepancies were found in the mass balances of Cu, K, Ni and P, i.e. the decrease in mass of these elements in the nib was significantly larger than the mass gained in the testa. This discrepancy in mass balances is likely related to leaching of elements into the mucilage (the liquid phase surrounding the beans). Indeed, Cd, Cu, K, Ni and P concentrations in the mucilage (among other elements) have been reported to increase with fermentation by a factor 2–8 depending on the element and on the fermentation conditions. It was also reported that fermentation reduced the total bean Cd level by 15%, likely due to leaching of Cd in the mucilage sweatings (Vanderschueren et al. 2020).

3.2.2. Visualisation of elemental mobility

While duplicate mobile elemental prints were originally generated before and after fermentation for five micro-fermentation pods, only eight samples are discussed here because several samples had to be excluded, as explained above. The contact area between the beans and the Chelex resin gels could be clearly discerned on dried gels, as the samples stained the gel (Figure 5 and SI Figure S 8). This imprint allowed targeting the contact area during analysis. Visualisation of the

elemental imprints on the Chelex resin gels by LA-ICP-MS indicated that mobility of Cd, Ni and Zn increased with fermentation, with largest mobile element signals located in the centre of the nib imprints (Figure 5). Mobile K also increased while mobile Ca decreased with fermentation (SI Figure S 9). For Cd and Zn, the observations are in accordance with the increased water extractability of these elements in fermented nibs (Figure 3 and SI Figure S 6). The water extractability of Ni in nibs was not significantly affected by fermentation, more than 70% of nib Ni was water-extractable regardless of the nib pH (SI Figure S 6). However, signal intensities for Ni on the Chelex gels were clearly larger for fermented than for unfermented samples, and nib Ni concentrations were positively correlated to nib pH in both fermentation experiments (SI Table S 2 and Figure S 6). The maps of the mobile element prints obtained here (signal larger for nib than for testa) were markedly different from the LA-ICP-MS images of total element composition in unfermented cacao beans obtained previously, where the total Cd signal was larger for the testa than for the nib (Vanderschueren et al. 2020). This suggests that fermentation induces an increase in Cd mobility in the nibs but not in the testa, resulting in a mobile concentration gradient opposite to the total Cd concentration gradient. The lack of element signal peaks at the edges of the Chelex imprints was in accordance with the lower water-extractable Cd fraction found in the testa compared to the nib. Imaging with LA-ICP-MS using imprints of cacao beans on a metal chelating gel was used to successfully visualise element mobility in unfermented and fermented cacao beans. As such, the visualisation method was used here to better understand the underlying mechanisms of Cd migration during cacao fermentation, while bulk chemical analysis using acid digestion and ICP-MS (see above) allows to quantify the extent of migration occurring. This novel imaging technique can be employed in the future for the visualisation of element mobility in a variety of sample matrices and may be strengthened by

including analysis of chelating gel standards with predetermined Cd concentrations which would allow (semi-)quantitative analysis of the images.

3.3. Impact of the experimental setup

The results of the micro-fermentation experiment revealed a factor 1.25 decrease in nib Cd (Figure 4), but no such effect was found in the lab-scale experiments (Table 2). The lack of effect in the lab-scale experiments was likely not related to insufficient acidification. The final nib pH for the control in lab-scale fermentation A (pH 4.6) was even lower than that reached in the micro-fermentation (pH 4.8). The difference in effects between setups may be related to differences in temperature. While small lab-scale fermentations of 5 kg cacao have practical advantages for experimental work, the small fermentation volume offers less heat retention compared to micro-fermentation experiments performed in a box of >200 kg cacao. The temperature in the centre of the lab-scale vessels reached 45 °C in the controls of both experiments (A and B, Figure 1 and SI Figure S 2), which was on the lower end of maximum temperatures typically reported in literature (Lima et al. 2011) and lower than the temperature reached in the micro-fermentation (50 °C). The lab-scale temperature profiles showed a delay in initial temperature rise, especially in experiment B where the temperature only started to increase after two days (Figure 1). Jespersen et al. (2005) reported that yeast outgrowth in the outer layers of the fermenting cacao mass is delayed in comparison to that in the centre of the mass. They found that the maximum yeast cell count was reached after 24 hours in the centre, while it was only reached after 72 h in the outer layers. The delay in fermentation activity observed in the lab-scale fermentations, indicated by the lag phase in the temperature profile, may be related to the small size and large surface-to-volume ratio of the lab-scale vessels. Fermentation temperature may thus explain the lack of decrease in nib Cd concentrations in the lab-scale experiments.

Sufficient heat may be required to allow acidification-driven mobilisation of Cd in cacao nibs during fermentation. Additional research under controlled temperature conditions is required to verify or reject this hypothesis.

The differences in detected effects between both setups may also be related to the large variation in bean Cd. Results of the micro-fermentation experiment demonstrated how large inter-pod variability (up to a factor 3) can mask the effect of fermentation on bean Cd concentrations (Figure 4). The coefficient of variation of the nib Cd concentration between all samples prior to treatment in lab-scale fermentation B was large (CV 13%). A power test was performed to assess the number of replicates in the lab-scale experiments, using the online tool developed by Rollin Brant [University of British Columbia (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html)]. To account for differences in the initial bean Cd concentrations, the CV was used instead of the standard deviation. Based on that analysis, at least four replicates are required to detect a factor 1.3 effect with statistical significance (CV = 13%, α = 5% and power = 80%). However, the power test also indicated that two replicates per treatment were sufficient to detect the effect in lab-scale fermentation A (CV between all nib samples prior to treatment was only 6%).

3.4. Organic acid fermentation treatments as a mitigation strategy for Cd in cacao

The micro-fermentation experiment indicated that nib acidification is the driver for nib Cd mobilisation, and thus also for reducing the final nib Cd concentration. However, organic acid application in lab-scale fermentations did not reduce the nib Cd concentration, probably because of the lower temperatures compared to commercial fermentations. Organic acid treatments reduced the nib pH, but only when applying large doses, and the final nib pH was still within the range of commercial values (pH 4–5). Thus, nib Cd does decrease with decreasing nib pH but organic acid treatments during fermentation may not be the best option to acidify the nibs.

Optimisation of fermentation conditions can likely yield final nib pH values similar to those resulting from organic acid treatments. For example, acetic acid concentrations in mucilage are reported to be higher when turning is performed (Camu et al. 2008a). The impact of such fermentation practices on the flavour should be taken into account, as excessive acetic acid has been related to less pronounced flavour in the final product (Camu et al. 2008b).

4. CONCLUSION

The mobility of Cd in cacao nibs increases during fermentation, as indicated by enhanced water extractions and enhanced diffusible Cd identified by LA-ICP-MS imaging. Water-extractable nib Cd was negatively correlated to nib pH and increased through artificial acidification, indicating nib pH as the driving force behind nib Cd mobilisation. Migration of Cd from the nibs to the testa during fermentation is likely related to increased Cd mobility in the nibs due to acidification. Organic acid amendments before or during fermentation were ineffective in reducing the nib pH due to their negative impact on fermentation (lower fermentation temperatures and excessively high mucilage pH). Application of acetic or lactic acid as a post-fermentation treatment lowered the nib pH compared to blank and water treatments, but only when applying large doses. Although no significant reductions in nib Cd concentrations could be observed with acid treatment, the reduced nib pH did increase testa Cd concentrations and nib Cd mobility. Micro-fermentations with single pod-derived beans inside a commercial fermentation box indicated a factor 1.25 reduction in nib Cd. Optimisation of fermentation parameters for acetic acid production can likely offer similar nib acidification compared to acid treatments, without the added cost. However, a trade-off needs to be made between nib Cd reduction and flavour quality as excessive nib acidity is considered an off-flavour.

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