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1 **The impact of fermentation on the distribution of cadmium in cacao beans**

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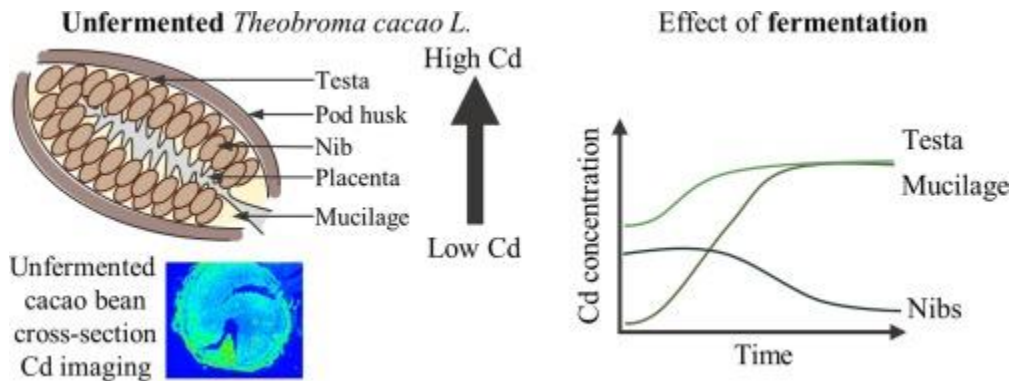
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17 HIGHLIGHTS

- 18 • Cadmium concentrations are highest in cacao testa followed by nibs and mucilage
- 19 • LA-ICP-MS was used successfully to visualize the distribution of Cd in cacao
- 20 • X-ray absorption spectroscopy showed Cd bound to O/N-ligands in cacao nib and testa
- 21 • Extensive fermentation can reduce the Cd concentration in the final product
- 22 • Nib pH controls Cd migration to outer tissues during cacao fermentation

23

24 **Graphical abstract**



25

26

27 ABSTRACT

28 A large fraction of the South-American cacao production is affected by new cadmium (Cd)  
29 regulations in cacao. This work was set up to characterize the distribution and speciation of Cd  
30 within the cacao fruit and to monitor potential Cd redistribution during cacao fermentation. In  
31 cacao fruits from four locations, Cd concentrations decreased with testa > nib ~placenta ~pod husk  
32 > mucilage. The distribution of Cd within cacao beans was successfully visualized using laser  
33 ablation inductively coupled plasma spectrometry (LA-ICP-MS) and confirmed higher Cd  
34 concentrations in the testa than in the nib. Speciation analysis by X-ray absorption spectroscopy  
35 (XANES) of unfermented cacao beans revealed that Cd was bound to O/N-ligands in both nib and  
36 testa. Fermentation induced an outward Cd migration from the nibs to the testa, i.e. against the  
37 total concentration gradient. This migration occurred only if the fermentation was sufficiently  
38 extensive to decrease the pH in the nib to <5.0, likely as a result of increased Cd mobility due to  
39 organic acid penetration into the nibs. The change in dry weight based nib Cd concentrations  
40 during fermentation was, on average, a factor 1.3 decrease. We propose that nib Cd can be reduced  
41 if the nib pH is sufficiently acidified during fermentation. However, a balance must be found  
42 between flavor development and Cd removal since extreme acidity is detrimental for cacao flavor.

43

## 44 1. INTRODUCTION

45 Although cacao-derived products are generally consumed in small quantities compared to staple  
46 foods, they can be an important source of dietary Cd because of potentially high Cd concentrations.  
47 The European Food Safety Authority estimated that cacao-derived products account for 4.3% of  
48 the total dietary Cd exposure in the European population (EFSA, 2012). Therefore, the European  
49 Commission approved threshold limits for Cd in cacao-derived products, which were enforced in  
50 January 2019 (European Commission, 2014). Similar limits were adopted by the Codex  
51 Alimentarius (Codex Alimentarius Commission, 2018). This new regulation on Cd in cacao will  
52 impact South-American cacao farmers, as Cd concentrations in South-American cacao are  
53 generally higher than those in cacao from other origins. Bertoldi et al. (2016) reported Cd  
54 concentrations in South-American cacao beans more than tenfold larger than those in West-  
55 African cacao. These findings have prompted researchers to explore potential techniques to lower  
56 Cd in the final product, focusing mostly on the relation between cacao bean Cd and soil Cd. For  
57 example, Argüello et al (2019) has mapped the concentrations of Cd in cacao beans and  
58 corresponding soils in Ecuador and was able to identify soil parameters to predict cacao bean Cd.  
59 Ramtahal et al. (2019) investigated potential soil amendments and showed that both biochar and  
60 lime application may reduce Cd accumulation in cacao.

61 A recent survey by Vanderschueren et al. (2019) showed that the Cd concentration in cacao-  
62 derived products is correlated to the cacao content of the product, indicating that Cd originates  
63 from the raw material (cacao nibs) rather than other ingredients or contamination during  
64 processing. Similar results have also been reported by Abt et al. (2018), Villa et al. (2014) and  
65 Yanus et al. (2014). The cacao fruit (*Theobroma cacao* L.) consists of an outer pod husk containing  
66 20–50 cacao beans (seeds), surrounded by a sugary mucilage and attached to a central tissue (cacao

67 placenta). Each cacao bean comprises of a seed coat or testa and two cotyledons known as the nib  
68 (Beckett, 2008). Chocolate production requires extensive post-harvest processing, starting with  
69 fermentation. The pod husk and placenta are discarded, and the cacao beans with surrounding  
70 mucilage are fermented for two to ten days depending on the cultivar and local practices.  
71 Fermentation is anaerobic during the first one to two days and the microbial population mostly  
72 consists of yeasts. Pectinolytic enzymes produced by the yeasts liquefy the mucilage and cause  
73 fermentation sweatings, which are drained through holes at the bottom of the fermentation boxes.  
74 This results in an increase in oxygen levels, allowing growth of lactic acid and acetic acid bacteria.  
75 The concentrations of lactic acid and acetic acid in the mucilage start to increase after two days of  
76 fermentation (De Vuyst & Weckx, 2016). The fermentation process results in a temperature  
77 increase from ambient temperature to 45–50 °C; an increase in mucilage pH from 3–4 to 4.5–5  
78 due to conversion of citric acid to ethanol; and a decrease in nib pH from 6.5–7 to 4.5–5 due to  
79 penetration of lactic and acetic acid (De Vuyst & Weckx, 2016; Thompson et al., 2007). After  
80 fermentation, cacao beans are dried to reach a moisture content of maximum 6–8% (Afoakwa,  
81 2010). During the second more industrial stage of the chocolate manufacturing process, the cacao  
82 beans are roasted and the testa is removed. The roasted nibs are then ground to cacao liquor and  
83 further processed to obtain consumer products (i.e. chocolate and cacao powder).

84 Post-harvest strategies that lower the Cd concentration in the cacao nib may offer viable options  
85 to lower the Cd concentration in the final product because the nib is the only part of the cacao fruit  
86 retained during processing. Developing such strategies requires better understanding regarding the  
87 distribution and speciation of Cd in the different cacao tissues, as well as the influence of  
88 conventional post-harvest processes on this distribution. Most previous work reports on the cacao  
89 nib, testa and pod husk with little attention to the mucilage and placenta. The Cd concentration in

90 the testa is generally reported higher than in the nibs. For example; Lewis et al. (2018) found more  
91 than twofold higher Cd concentrations in the testa compared to the nib, and Ramtahal et al. (2016)  
92 reported higher Cd concentrations in the testa compared to the nib, in cacao from Trinidad and  
93 Tobago. The reverse has also been observed. Chavez et al. (2015) measured the Cd concentrations  
94 in cacao from 19 Ecuadorian farms and found generally higher Cd concentrations in the nib  
95 compared to the testa. Sample treatment often differs, cacao samples were provided by chocolate  
96 manufacturers with little information regarding sample processing (Lee & Low, 1985), samples  
97 were either or not washed with water which can affect Cd concentrations in the outer tissues  
98 (Gramlich et al., 2018; Lewis et al., 2018; Ramtahal et al., 2016), or samples were washed with  
99 chelating agent solutions (Chavez et al., 2015). To the best of our knowledge, the influence of  
100 fermentation on the Cd distribution in cacao has not been reported to date. Thyssen et al. (2018)  
101 mapped the 2D distribution of Cd in sections of fermented cacao beans using laser ablation  
102 inductively coupled plasma mass spectrometry (LA-ICP-MS) and found elevated signals for Cd,  
103 Cu, K, Mg, Na, Pb and Zn in the testa compared to the nib, however they did not investigate  
104 unfermented samples. Fermentation has been reported as a possible technique for reducing Cd in  
105 rice (Zhai et al., 2019; Zhang et al., 2017). Zhai et al. (2019) reported Cd removal efficiencies  
106 >90% for rice fermented with lactic acid bacteria and related this to a combination of the Cd  
107 binding potential of the bacteria, and the effects of the organic acid production mobilizing Cd. This  
108 phenomenon may also occur during cacao fermentation due to the lactic and acetic acid production,  
109 but has not been studied to date.

110 The objectives of this study were (i) to determine the distribution and speciation of Cd between  
111 and within the different cacao fruit tissues, i.e. pod husk, placenta, mucilage, testa and nibs; and  
112 (ii) to investigate the influence of fermentation on the distribution of Cd between these different

113 cacao bean tissues. Better understanding of the distribution and speciation of Cd in cacao and the  
114 influence of post-harvest processing may shed light on opportunities to lower Cd concentrations  
115 in the final product.

## 116 2. MATERIALS AND METHODS

### 117 **2.1. Cacao material**

118 Ripe cacao fruits were collected at four fields in the provinces El Oro (batch A, CCN-51 cultivar),  
119 Guayas (batches B and C, Nacional cultivar) and Sucumbíos (batch D, Nacional cultivar) in  
120 Ecuador (Table 1). Unfermented cacao beans for XANES spectroscopy were collected at different  
121 fields in the provinces Esmeraldas (Nacional cultivar), Guayas (CCN-51 cultivar) and Sucumbíos  
122 (Nacional cultivar).

### 123 **2.2. Sampling and sample preparation**

124 A minimum of three intact cacao fruits was collected for each batch, and each fruit was considered  
125 as an independent replicate. The intact fruits were manually separated to obtain pod husk, placenta,  
126 mucilage and cacao beans. Residual mucilage was removed from the cacao beans using paper  
127 towels and all cacao tissues were oven dried for 72 hours at 65 °C. After drying, subsamples of  
128 intact cacao beans of batches C and D were collected for LA-ICP-MS imaging. The remainder of  
129 the cacao beans was manually separated in nibs and testa, and all dried fractions (pod husk,  
130 placenta, mucilage, testa and nib) were ground using a coffee grinder before chemical analyses.

### 131 **2.3. Fermentation**

132 Fermentation experiments were conducted to assess the effect of fermentation on the distribution  
133 of Cd within cacao tissues, and comprised two cultivars (CCN-51 in batch A and Nacional in  
134 batches B, C and C<sub>bis</sub>) and three common fermentation methods (cascade fermentation in batches  
135 A and B, single box fermentation in batch C and single box fermentation with pre-drying in batch



136 C<sub>bis</sub>). No fermentation experiments were set up for the cacao of batch D. Batch C was split in two  
137 fermentation batches, C and C<sub>bis</sub>, which comprised of the same cacao (same variety and plantation)  
138 but were subjected to different fermentation conditions (Table 1). Fermentation experiments were  
139 conducted in Ecuador following local practices using wooden boxes with perforated floors to allow  
140 drainage of the fermentation sweatings. The boxes were covered with jute bags to retain heat. For  
141 each batch, the total mass of cacao needed to fill two fermentation boxes (about 580–1180 kg,  
142 Table 1) was thoroughly mixed and divided in duplicate fermentation boxes. Different subsamples  
143 of 1 kg cacao mass were taken and placed in mesh bags (Figure S1) to facilitate bean sampling  
144 during fermentation. All mesh bag subsamples (3–7 per box, depending on the batch) were placed  
145 in the center of the fermentation boxes at the start of fermentation and relocated in the same  
146 position after mixing. Daily sampling was performed by taking out one of the mesh bag subsamples  
147 from each fermentation box. Each mesh bag subsample was considered an independent replicate  
148 for that fermentation day and the two fermentation boxes were considered as the duplicates for the  
149 batches or fermentation experiments. Fermentations A and B were performed in cascades of three  
150 wooden boxes measuring 60×60×60 cm (width×depth×height) and the fermenting masses were  
151 mixed every two days by depositing them in the next box of the cascade (Figure S2). Batches C  
152 and C<sub>bis</sub> were fermented in single 100×100×60 cm wooden boxes. This cacao was mixed manually  
153 after one day (C<sub>bis</sub>) or two days (C) and remained in the initial box throughout the fermentation  
154 period. Cacao beans of batch C<sub>bis</sub> were pre-dried over night before fermentation, mimicking a  
155 common practice in some fermentation facilities. In this pre-drying method, fresh cacao was spread  
156 out on a concrete floor and left to dry overnight. This method, also referred to as bean spreading,  
157 is a common practice to prevent excessive acidity of cacao beans during fermentation (Biehl et al.,  
158 1990; Meyer et al., 1989; Schwan & Wheals, 2004). The total fermentation time for each batch

159 was determined by local practices (Table 1). The endpoint of each fermentation was based on  
160 quality assessment by local farmers. Beans were sampled daily by removing one mesh bag  
161 subsample (1 kg cacao mass) from the center of each box. The cacao beans in the mesh bags were  
162 then manually separated in mucilage and beans and oven dried at 65 °C for 72 hours, with beans  
163 split in nibs and testa and further ground as described above.

#### 164 **2.4. Temperature and pH**

165 The mucilage pH was measured immediately after sampling or after opening of the cacao fruits.  
166 Cacao beans with mucilage attached were vigorously shaken for 2 min in a 1:10 solid to deionized  
167 water ratio and the pH of the suspension was measured. To determine nib and testa pH, dried and  
168 ground material was treated likewise in a 5:10 solid sample to deionized water ratio and filtered  
169 (F2040 filter paper, retention 7–9 µm, CHMLAB GROUP, Barcelona, Spain) to obtain a clear  
170 supernatant for pH measurement. The temperature in the fermentation experiments was measured  
171 daily in the center of the fermentation boxes using a digital thermometer (VWR International,  
172 Darmstadt, Germany).

#### 173 **2.5. Determination of the elemental composition of cacao beans**

174 Duplicates of 100 mg dry material were digested in 3 mL concentrated Suprapur® nitric acid  
175 (HNO<sub>3</sub>, 65% w/w; Merck, Darmstadt, Germany) in an open digestion block for 8 hours at a  
176 maximum temperature of 130 °C. Digests were diluted five times with Milli-Q water (18.2 MΩ  
177 cm<sup>-1</sup>) and the Cd concentration was determined by inductively coupled plasma mass spectrometry  
178 (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, USA). The ICP-MS analysis was  
179 performed in helium (He) collision cell mode monitoring the <sup>111</sup>Cd isotope, using <sup>103</sup>Rh as an on-  
180 line internal standard. The limit of quantification (LOQ) for the ICP-MS analysis was 0.02 µg Cd  
181 L<sup>-1</sup> which corresponded to a solid sample LOQ of 0.006 mg Cd kg<sup>-1</sup> dry matter. Blank samples (in

182 quadruplicate) and certified reference material NIST 2384 baking chocolate (in triplicate) were  
183 included in all digestions and treated the same way as the cacao tissue samples. Recoveries of the  
184 certified reference material ranged 98–108% and the coefficient of variation (CV) for the duplicate  
185 digestions ranged 0.1–23% (average CV 5%). The concentrations of several other elements were  
186 also determined, i.e.  $^{27}\text{Al}$ ,  $^{75}\text{As}$ ,  $^{44}\text{Ca}$ ,  $^{59}\text{Co}$ ,  $^{52}\text{Cr}$ ,  $^{63}\text{Cu}$ ,  $^{39}\text{K}$ ,  $^{24}\text{Mg}$ ,  $^{55}\text{Mn}$ ,  $^{95}\text{Mo}$ ,  $^{60}\text{Ni}$ ,  $^{31}\text{P}$ ,  $^{208}\text{Pb}$  and  
187  $^{66}\text{Zn}$ .

## 188 **2.6. Visualization of the elemental distribution by LA-ICP-MS in unfermented cacao beans**

189 Unfermented cacao beans for LA-ICP-MS imaging were sampled from batches C and D. For each  
190 LA-ICP-MS sample (which was a single bean), the Cd concentration was determined from other  
191 cacao beans obtained from the same fruit using the digest method and ICP-MS analysis as  
192 described previously. Transverse cacao bean cross-sections with a thickness of 65  $\mu\text{m}$  were made  
193 following the method described by Lombi et al. (2009) for rice grains. The cacao beans were sliced  
194 with a vibrating microtome (Microm HM 650 Vibratome, Thermo Scientific, Walldorf, Germany)  
195 using diamond blades (GFD Gesellschaft für Diamantprodukte, Ulm, Germany). The cross-  
196 sections were made at approximately 80% of the length of the cacao bean, measured from the  
197 cacao radicle side. Once a flat surface was obtained, the surface of the cacao bean was defatted  
198 using hexane (HiPerSolv Chromanorm 97%, VWR, Leuven, Belgium) to enable sticking of the  
199 tape on the cacao surface. Then, a piece of Kapton polyimide tape was pressed on the surface and  
200 the diamond blade cut underneath, leaving a cacao bean cross-section glued on the Kapton tape.  
201 For elemental detection, a quadrupole 7700cs ICP-MS (Agilent Technologies, Santa Clara, USA)  
202 was used, mounted with platinum (Pt) cones. The sensitivity and operational conditions (stability,  
203 background and mass calibration) were checked using a 1  $\mu\text{g L}^{-1}$  Y, Tl, Li, Ba and Ce tuning  
204 solution. The ICP-MS was then coupled to a 213-nm laser ablation system equipped with a TV2

205 cell (NWR213, ESI, Fremont, CA) and coupling was optimized using a NIST 612 glass by  
206 monitoring  $^{238}\text{U}$  and  $^{232}\text{Th}$  for maximum sensitivity and a U to Th ratio as close as possible to the  
207 unit. Imaging of cacao cross-sections was performed by subsequent line scans with a 20 Hz laser  
208 shot repetition rate, a fluency maintained between 5–5.3 J cm<sup>-2</sup>, a laser beam of 20 μm<sup>2</sup> and 50  
209 μm<sup>2</sup>, a scan speed of 23 and 50 μm s<sup>-1</sup>, and a distance between each line of 40 and 100 μm for high  
210 and low resolution images, respectively. The ablated cacao particles were transported with 800 mL  
211 min<sup>-1</sup> He and mixed with Ar gas from ICP-MS before the ICP torch inlet. The ICP-MS was used  
212 in He mode, allowing monitoring of  $^{111}\text{Cd}$  (0.2 s),  $^{114}\text{Cd}$  (0.2 s),  $^{31}\text{P}$  (0.1 s),  $^{39}\text{K}$  (0.005 s),  $^{44}\text{Ca}$   
213 (0.15 s),  $^{60}\text{Ni}$  (0.1 s) and  $^{64}\text{Zn}$  (0.1 s as integration time). The acquisition time was set according to  
214 the ablation time needed for one line. One data file recording the intensity of each element versus  
215 time was acquired for each line, and a homemade program under Python was used to generate 2D  
216 images of element intensities per pixel with a colour code.

## 217 **2.7. X-ray absorption near-edge structure spectroscopy**

218 The speciation of Cd in unfermented cacao was investigated using X-ray absorption near-edge  
219 structure spectroscopy (XANES). The samples comprised of cacao beans from CCN-51 and  
220 Nacional cultivars. The cacao testa and nib were separated manually and both materials were dried,  
221 ground and pressed into pellets for XANES analysis. The XANES spectra were collected at the X-  
222 ray absorption spectroscopy beamline (Australian synchrotron, ANSTO). The Cd K-edge (26711  
223 eV) was scanned rather than the Cd L<sub>III</sub>-edge (3538 eV) to avoid interference with the K K-edge  
224 (3608 eV), as K is expected to be abundant in cacao tissues. Sample spectra were measured in  
225 fluorescence mode with a 100-elements solid-state Ge detector, at 10 °K to prevent beam damage.  
226 One spectrum represents the average of 2–26 scans, depending on the concentration of Cd. Each  
227 scan was measured on a different spot on the pellet to limit beam damage. Reference X-ray spectra

228 for Cd metal foil were collected simultaneously with the sample spectra and were used for both  
229 energy calibration and spectral alignment. Several Cd reference compounds were also measured:  
230 Cd-chloride, Cd-phosphate, Cd-sulfate, Cd-oxide, Cd-acetate, Cd-lactate, Cd-citrate, Cd-histidine,  
231 Cd-phytate, Cd-malate, Cd-cysteine and Cd-glutathione (details about reference compound  
232 preparation are given in the supplementary information). Data extraction was performed using  
233 Sakura (<https://sakura.readthedocs.org>) while background subtraction, normalization and linear  
234 combination fitting (LCF) were done using Athena software (Ravel & Newville, 2005). For each  
235 sample spectrum, LCF was performed by fitting regions between -25 and 70 eV using the library  
236 of Cd reference compounds. Satisfactory fits were obtained with a combination of two reference  
237 compounds. Three compound LCFs were not retained as the residual factor (R-factor) used to  
238 assess the goodness of fit was not significantly smaller compared to the R-factor of the two  
239 compound LCFs. Linear combination results were normalized to 100% to compare the relative  
240 speciation between samples.

## 241 **2.8. Statistical analysis**

242 All statistical analysis was executed using JMP<sup>®</sup> Pro version 14.0.0 (SAS Institute 2018). The  
243 differences in Cd concentration between the different cacao tissues were tested using Tukey's  
244 Honestly Significant Difference test ( $P\text{-value} \leq 0.05$ ) using the mean data of sampling replicates  
245 (e.g. the two fermentation boxes), as the independent replicates. The effect of fermentation time  
246 on the elemental composition of the different tissues was tested using Pearson's correlation test  
247 ( $P\text{-value} \leq 0.05$ ).

## 248 **3. RESULTS AND DISCUSSION**

### 249 **3.1. Distribution of Cd in unfermented cacao beans**

250 Cacao beans from batch D showed the highest Cd concentrations in nib and testa, followed by  
251 batch C, and finally batches A and B (Table 2). The coefficients of variation (CV) of nib Cd  
252 between cacao fruits of the same batch ranged between 20 and 37%, indicating variation in cacao  
253 bean Cd between fruits from the same plantation. In a large survey of 159 Ecuadorian fields,  
254 Argüello et al. (2019) observed that the average CV in bean Cd concentration among fruits of  
255 different trees within the same field was 39%. They related this variation in bean Cd to the large  
256 spatial variation in soil Cd. The Cd concentrations were overall highest in the testa, followed by  
257 the nibs, placenta and pod husk (all similar in Cd content) and finally the mucilage. No information  
258 was found in literature regarding the Cd concentration in the placenta or the mucilage. Gramlich  
259 et al. (2018) measured the Cd concentration in cacao from 55 farms in Honduras and did not find  
260 a significant difference between the Cd concentration in the pod husks ( $1.1 \pm 0.2 \text{ mg kg}^{-1}$ ) and that  
261 in the nibs ( $1.1 \pm 0.1 \text{ mg kg}^{-1}$ ). Conversely, Ramtahal et al. (2016) reported higher Cd  
262 concentrations in the pod husks ( $0.53\text{--}4.49 \text{ mg Cd kg}^{-1}$ ) compared to the nibs ( $0.35\text{--}3.82 \text{ mg Cd}$   
263  $\text{kg}^{-1}$ ) for cacao from 45 farms in Trinidad and Tobago.

264 Testa Cd concentrations were higher than nib Cd concentrations in all batches (ratio testa Cd to  
265 nib Cd 1.8 for A, 1.7 for B, 1.5 for C and 1.7 for D). Considering the average weight fractions for  
266 nib (0.93) and testa (0.07), 91% of the total bean Cd was located in the nib and 9% in the testa in  
267 unfermented cacao beans. In accordance to the present work, Ramtahal et al. (2016) reported  
268 significantly higher Cd concentrations in testa ( $0.44\text{--}4.41 \text{ mg Cd kg}^{-1}$ ) compared to nibs ( $0.35\text{--}$   
269  $3.82 \text{ mg Cd kg}^{-1}$ ) for unfermented cacao beans from Trinidad and Tobago. Lewis et al. (2018)  
270 reported more than two-fold higher Cd concentrations in the testa (average  $1.83 \text{ mg kg}^{-1}$ ) compared  
271 to those in the nibs (average  $0.88 \text{ mg kg}^{-1}$ ) for unfermented cacao beans from the same genetic  
272 group and grown in a common garden. Similarly, Lee & Low (1985) determined the Cd

273 concentrations in raw cacao beans from two different sources and reported higher Cd  
274 concentrations in the testa ( $1.32 \pm 0.06$  and  $2.05 \pm 0.01$  mg Cd kg<sup>-1</sup>) compared to Cd concentrations  
275 in the nibs ( $0.76 \pm 0.02$  and  $1.01 \pm 0.01$  mg Cd kg<sup>-1</sup>) for the two sources, respectively. Conversely,  
276 Chavez et al. (2015) analyzed the Cd concentration in unfermented cacao nibs and testa from 19  
277 different small-scale farms in the south of Ecuador and consistently found higher Cd  
278 concentrations in the cacao nibs compared to the testa. However, the cacao beans in that study  
279 were washed with a hypochlorite solution before peeling which may have removed some of the  
280 Cd in the outer testa.

### 281 **3.2. Imaging of elemental distribution in unfermented cacao beans with LA-ICP-MS**

282 The elemental imaging maps obtained by LA-ICP-MS for <sup>111</sup>Cd and <sup>114</sup>Cd isotopes showed close  
283 agreement, which indicates that there were no interferences affecting the Cd measurement (Figure  
284 1, and Figures S3 and S4). There were no zones found with consistently elevated intensities for  
285 the measured isotopes (<sup>44</sup>Ca, <sup>111</sup>Cd, <sup>114</sup>Cd, <sup>39</sup>K, <sup>60</sup>Ni, <sup>31</sup>P and <sup>64</sup>Zn, Figures S3 and S4) in the  
286 obtained elemental maps, indicating that the samples were sufficiently planar for reliable image  
287 interpretation and comparison. Regions with consistently lower signal intensity corresponded to  
288 inherent cracks in the cacao bean samples as visible on the sample pictures (Figure 1). The overall  
289 Cd concentrations in the nib and testa from batch D were nearly seven times higher than the Cd  
290 concentrations in the tissues of batch C. When LA-ICP-MS imaging is performed at a higher  
291 resolution, a smaller surface area of the sample is ablated and thus less sample material is  
292 transported to the ICP-MS detector. Therefore, higher resolution imaging requires samples with  
293 larger elemental concentrations (or very long measurement times). Because of this, the batch D  
294 sample could be scanned at higher resolution (20 μm<sup>2</sup> laser beam size) than the cross-section of  
295 batch C (laser beam size 50 μm<sup>2</sup>). The ICP-MS integration time was equal in both scans to limit

296 the measurement duration. Therefore, the signal intensity (expressed as counts per second, cps) of  
297 the batch D sample was lower than that of the batch C sample even though the overall Cd  
298 concentrations in D were larger than those in C.

299 The testa layer was clearly distinguishable from the cacao nib and showed elevated Cd intensities  
300 for both samples (Figure 1), which is in line with the measured Cd concentrations in these tissues  
301 (Table 2). Apart from Cd, only Ca displayed clearly elevated intensities in the testa (Figures S3  
302 and S4). The distribution of K was approximately uniform between nib and testa; and P, Ni and  
303 Zn were more abundant in the nib compared to the testa (Figures S3 and S4). Cadmium and zinc  
304 were not co-located within the cacao bean tissues even though they are considered similar in  
305 chemical properties and are often transported in plants through similar mechanisms (Smolders &  
306 Mertens, 2013). Dissimilar Cd and Zn distribution patterns have also been visualized in rice  
307 (Meharg et al., 2008). This may indicate a difference in transport mechanisms for Cd and Zn into  
308 the cacao seed, possibly related to a defense mechanism of the plant as Zn is an important  
309 micronutrient while Cd has no known function in plant growth. The distribution of Cd within the  
310 cacao nib was not homogeneous throughout the cross-section. Further identification of nib zones  
311 with higher Cd intensities may shed light on the way Cd is translocated into the cacao bean during  
312 plant growth. Thyssen et al. (2018) created elemental maps of longitudinal cross-sections of  
313 fermented cacao beans using LA-ICP-MS and observed elevated signal intensities for Cd in the  
314 testa. However, they reported accumulation of P, K and Zn in the testa, which was not observed in  
315 this study. These differences compared to the present work may be explained by the influence of  
316 cacao fermentation or by possible differences in cultivars and overall different elemental  
317 concentrations between these samples.

### 318 **3.3. Speciation of Cd in unfermented cacao beans**



319 The LCF procedure identified two reference compounds to describe the speciation of Cd in all  
320 samples (Figure 2 and Figure S5). Optimal fits were obtained with a combination of Cd-histidine,  
321 where Cd is bound to amino and carboxyl groups, and Cd-citrate, where Cd is bound to alcohol  
322 and carboxyl groups. The proportions of both ligands were similar in most samples (39–58% Cd-  
323 histidine and 42–61% Cd-citrate), except for nib 3 (84% Cd-histidine and 16% Cd-citrate) and  
324 testa 1 (22% Cd-histidine and 78% Cd-citrate). These results indicate that Cd in the cacao nib and  
325 the testa is bound to O/N-ligands. In hyperaccumulator plants, Cd was found with both S-ligands  
326 and O-ligands, and the association with O-ligands was reported as a detoxification strategy in  
327 contrast to non-hyperaccumulating plants where S-ligands were predominant (Huguet et al. 2012,  
328 Isaure et al. 2006 & 2015, Vogel-Mikuš et al. 2007). In the present work, no evidence was found  
329 for complexation with S-ligands (thiols) in the cacao nib or testa.

#### 330 **3.4. Changes in pH and temperature during fermentation**

331 The nib pH in batches A and B decreased with fermentation from 6.2 to about 4.5, the mucilage  
332 pH increased from about 3.7 to 4.5, and the testa pH increased from 4.3 to 5.0 (Figure 3). Changes  
333 in pH were less pronounced in batches C and C<sub>bis</sub> (final nib pH 5.2 and 6.0, mucilage pH 3.8 and  
334 4.0, and testa pH 4.8 and 4.4 for the two batches, respectively). The fermentation times for C and  
335 C<sub>bis</sub> were shorter than in A and B (3–4 versus 5–7 days, Table 1), suggesting a lower extent of  
336 fermentation. The temperature profile was similar in all batches and increased from the start of  
337 fermentation reaching 45 °C after three to four days of fermentation. The pH and temperature  
338 values are in line with values reported in literature, which state that the temperature of the  
339 fermenting cacao bean mass increases from ambient temperature to about 45–50 °C and nib pH  
340 decreases from 6.3–7.0 to 4.0–5.5 during fermentation (Belitz et al., 2009; De Vuyst & Weckx,  
341 2016; Papalexandratou et al., 2011; Schwan & Wheals, 2004; Thompson et al., 2007).

### 342 **3.5. The influence of fermentation on the distribution of Cd in cacao beans**

343 One replicate sample of mucilage (fermentation day 3, batch B) showed an extreme Cd  
344 concentration (6.6 mg Cd kg<sup>-1</sup>) and was excluded from analysis. The concentration of Cd within  
345 the different cacao bean tissues before the start of fermentation (day zero, Figure 4) was in line  
346 with the values observed in intact fruits (testa > nib > mucilage, Table 2). The nib Cd concentration  
347 in batches A and B decreased with fermentation time by a factor 1.3 (Figure 4). The final nib Cd  
348 concentration in B was lower than 0.60 mg kg<sup>-1</sup>, which is commonly considered the maximum  
349 allowed Cd concentration in cacao beans destined for export to the EU. The mucilage and testa Cd  
350 concentrations in A increased with fermentation time (factor 2.1 in testa and 7.8 in mucilage)  
351 reaching a similar plateau concentration after four days of fermentation. The same was true for the  
352 mucilage Cd in B (increased by factor 2.5) but no significant trend in testa Cd was observed. The  
353 decreasing concentrations in the nibs during fermentation are unlikely related to a change in the  
354 ability to remove the high Cd testa from the low Cd nib. Budget analysis using concentration and  
355 weight fractions of the tissues showed that the nib Cd content (expressed in mg nib Cd kg<sup>-1</sup> total  
356 cacao bean) decreased and testa Cd content (mg testa Cd kg<sup>-1</sup> total cacao bean) increased for A  
357 and B (Figure S6). This suggests that Cd migrates from the nib to the testa and the mucilage during  
358 fermentation, resulting in lower Cd concentrations in the final cacao based product because the  
359 outer tissues (testa and mucilage) are removed at later stages of the post-harvest process. At the  
360 end of fermentations A and B, approximately 80% of the total cacao bean Cd was located in the  
361 nibs whereas 20 % was found in the testa.

362 As stated previously, batches C and C<sub>bis</sub> were fermented less extensively than batches A and B.  
363 This may explain why no change in nib Cd was observed by the end of fermentation in these  
364 batches. The mucilage Cd concentration in C increased significantly with fermentation time (factor

365 6.2) in a similar pattern as observed for batches A and B. But in contrast to A and B, the testa Cd  
366 concentration in C decreased with time. Fermentation of batch C<sub>bis</sub> had no significant influence on  
367 the Cd concentrations in the testa. The mucilage Cd concentration did increase by factor 1.7 but  
368 this change was not of the same magnitude as observed in the other batches. The overall Cd  
369 concentrations in C and C<sub>bis</sub> were higher than those in A and B. However, results from a pilot scale  
370 fermentation (5 kg) using the same high Cd cacao, showed that Cd concentrations in the nibs of  
371 this cacao did decrease with fermentation time if the cacao was fermented more extensively, i.e.  
372 four days with a decrease in nib pH from 6.1 to 4.8 (data not shown). This demonstrates that the  
373 pH, rather than the total Cd concentration, explains Cd migration. Mass balance calculations of  
374 bean Cd showed that the total bean Cd concentrations reduced with 15% by the end of fermentation  
375 in batches A, B and C (Figure S7). This may be related to the loss of mucilage through fermentation  
376 sweatings. No Cd loss was observed over the course of fermentation for batch C<sub>bis</sub> which had been  
377 air-dried prior to fermentation. Farmers estimate that the cacao loses approximately 25% of its  
378 fresh weight during pre-drying as the mucilage liquid runs off and evaporates. As a result,  
379 sweatings during the fermentation may be much smaller in such fermentation practices and the  
380 total Cd mass may remain constant over the course of fermentation.

381 The nib Cd concentration was strongly correlated to nib pH throughout fermentation in batches A  
382 and B, but not in C and C<sub>bis</sub> (Figure 5). The nib pH in C and C<sub>bis</sub> decreased by over one unit during  
383 fermentation but remained >5, while the pH in batches A and B dropped to 4.5. This may indicate  
384 the importance of fermenting long enough to reach nib pH values <5, in order to generate Cd  
385 migration from the nib outwards. XANES speciation analysis showed that Cd was mostly bound  
386 to O/N-ligands such as histidine and citrate. The pKa value for the dissociation of the second  
387 carboxylic group in citrate is 4.77, which can explain the increase in Cd mobility when the tissue

388 pH drops below that value. Conversely, the pKa values for histidine are 1.82 (carboxylic group),  
389 6.00 (N in the imidazole ring) and 9.17 (amine group). Zhai et al. (2019) stated that rice  
390 fermentation may decrease Cd concentrations and that this Cd removal capacity is related to the  
391 acid producing abilities of lactic acid bacteria present during fermentation. They reported a pH  
392 decrease from 6 (initial pH) to <4.5 by the end of fermentation, depending on the strain of lactic  
393 acid bacteria used. Testa Cd concentrations were approximately a factor two larger than nib Cd  
394 concentrations in unfermented cacao (Table 2). The migration of Cd during fermentation thus  
395 occurred against the total Cd concentration gradient. However, the cacao testa has been identified  
396 as a heavy metal adsorbent with potential applications in the treatment of industrial effluents  
397 (Meunier et al., 2003). Because of the Cd sorption capacity of the testa and the differences in pH  
398 between the nib and the testa, the concentration gradient of mobile Cd in fermented cacao beans  
399 may be the inverse of the total concentration gradient, a key speculation that requires further  
400 validation.

### 401 **3.6. The influence of fermentation on other elements in cacao**

402 Apart from Cd, several other elements were analyzed by ICP-MS (Al, As, Ca, Co, Cr, Cu, K, Mg,  
403 Mn, Mo, Ni, P, Pb and Zn) and the concentrations of most of these elements in each tissue (nib,  
404 testa, mucilage) were correlated with fermentation time (Table S1 and Figures S8 I–X).  
405 Concentrations of Al, As, Cr and Pb were <LOQ in relevant fractions of the nib samples (Al 98%,  
406 As 80%, Cr 72% and Pb 93%), the testa samples (Pb 22%) or the mucilage samples (As 37%) and  
407 were not further discussed. Fermentation had no significant effect on Mo in any of the cacao  
408 tissues. Elemental concentrations in the nibs generally decreased while testa and mucilage  
409 concentrations increased. The nib concentrations decreased in batches A and B for Cu (factor A  
410 1.4, B 1.2), K (A 1.6, B 1.4), Mg (A 1.4, B 1.3), Mn (A and B 1.1), Ni (A 1.7, B 1.6) and P (A

411 1.04, B 1.4). No significant changes were observed in the nib elemental composition for batches  
412 C and C<sub>bis</sub> with the exception of a significant increase in nib Ni concentration in C<sub>bis</sub> (factor 1.1).  
413 The testa concentrations increased with fermentation in batches A and B for Cu (A 3.2, B 1.8), K  
414 (A 2.8, B 2.6), Mg (A 4.4, B 2.9), Mn (A 3.1, B 3.0), Ni (A 3.9, B 3.4) and P (A 9.8, B). Calcium  
415 was the only element that displayed a reverse change in concentration, nib Ca increased while testa  
416 Ca decreased with fermentation time. The elemental concentrations in the mucilage generally  
417 increased, which might be caused by microbial deterioration of the outer layers of the testa. If  
418 present, this deterioration was not strong enough to cause a significant decrease in the testa weight  
419 fraction with fermentation time. The testa weight fraction remained in the range 0.05–0.10  
420 throughout fermentation in all batches. However, regardless of the minimal change in testa weight  
421 fraction with fermentation, changes in the morphology of nib and testa may still be possible. To  
422 confirm migration of the elements rather than changes in the morphology of the tissues during the  
423 fermentation process, the elemental concentrations in each tissue (nib and testa) were multiplied  
424 by the weight fraction of that tissue. The weight fraction corrected concentrations corroborated the  
425 migration of aforementioned elements (Cu, K, Mg, Mn, Ni and P) from the nib to the testa in  
426 batches A and B. The observed migration pattern of Ni might be of importance in the future  
427 because the European Commission mentioned cacao based products among important food sources  
428 of Ni in the European population (EFSA, 2015). Based on the similar behavior of Ni and Cd  
429 observed in this work, post-harvest strategies to lower Cd concentrations in cacao during  
430 fermentation will likely also be effective for Ni.

#### 431 4. CONCLUSION

432 In unfermented cacao fruits, Cd concentrations are highest in the testa, followed by nibs, placenta  
433 and pod husks which all contain similar Cd concentrations, and finally the mucilage. This study is

434 probably first to report the fate of Cd and its distribution in cacao tissues during fermentation.  
435 Migration of Cd from the nibs to the testa was only observed if the nib pH dropped below 5. This  
436 acidic pH resulted from longer fermentation times. More extensive fermentation can thus result in  
437 lower Cd concentrations in the final product as the testa and mucilage are removed later in the  
438 post-harvest process. After fermentation, nib Cd concentrations decreased by a factor 1.3,  
439 indicating that fermentation may be useful to comply to the new Cd requirements ( $0.60 \text{ mg Cd kg}^{-1}$   
440 <sup>1</sup>) in cacao beans with initial unfermented nib Cd concentrations up to  $0.78 \text{ mg Cd kg}^{-1}$ . Further  
441 work is required to assess the full potential of Cd migration from the nib to the testa during  
442 fermentation. Nevertheless, it is often recommended to avoid very low nib pH as this can cause an  
443 unpleasant acidic taste in the final product (De Vuyst & Weckx, 2015; Schwan & Wheals, 2004).  
444 A balance must thus be found between flavor quality and Cd concentration. This acidic flavor is  
445 the main reason for pre-drying practices and results confirmed that the nib pH in pre-dried cacao  
446 decreased less extensively compared to the other fermentation experiments. Our results indicate  
447 that pre-drying and short fermentation times may reduce the extent of outward Cd migration.

448

449 SUPPLEMENTARY INFORMATION

450 The supplementary information includes a description of the preparation of XANES Cd reference  
451 compounds, pictures of the mesh sample bags (Figure S1) and the cascade set-up (Figure S2) used  
452 in the fermentation experiments, LA-ICP-MS imaging of both cacao bean cross-sections for  $^{44}\text{Ca}$ ,  
453  $^{114}\text{Cd}$ ,  $^{39}\text{K}$ ,  $^{31}\text{P}$ ,  $^{60}\text{Ni}$  and  $^{64}\text{Zn}$  (Figures S3 and S4), XANES spectra for cacao tissue samples and  
454 Cd reference compounds (Figure S5), weight fraction corrected Cd concentrations in nib and testa  
455 throughout fermentation for all fermentation batches (Figure S6), Cd mass balances of the  
456 fermentation experiments (Figure S7), Pearson correlation coefficients indicating the effect of  
457 fermentation time on the elemental composition (Ca, Cd, Co, Cu, K, Mg, Mn, Mo, Ni, P and Zn)  
458 of each cacao tissue (nib, testa and mucilage) (Table S1), and the effect of fermentation on the  
459 elemental composition (Ca, Co, Cu, K, Mg, Mn, Mo, Ni, P and Zn) (Figures S8 I–X).

460

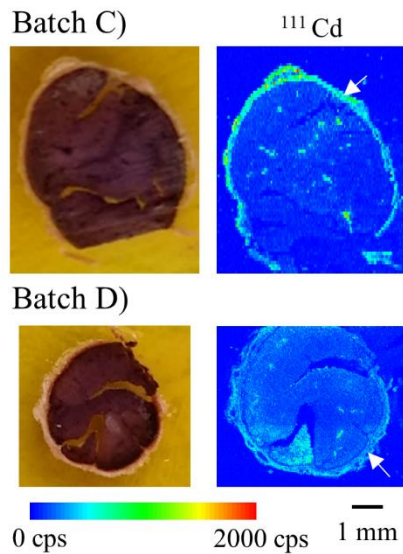
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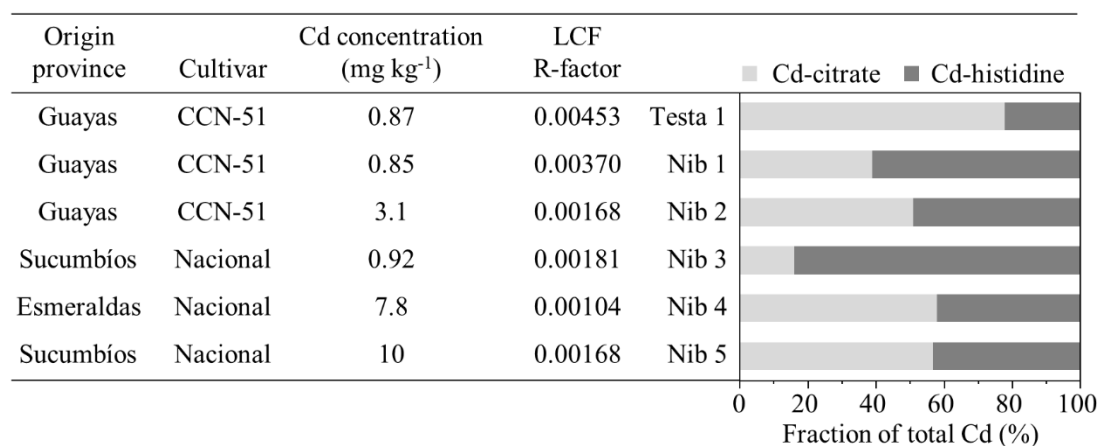


472 Figure 1 Relative distribution of  $^{111}\text{Cd}$  in two unfermented cacao bean transversal cross sections  
473 (batch C and batch D) using LA-ICP-MS, and photography images of the samples before LA-ICP-  
474 MS analysis. White arrows indicate cacao testa. The mean Cd concentrations in acid digested cacao  
475 beans from the same fruit (as determined by ICP-MS) were  $1.7 \text{ mg kg}^{-1}$  in the nib and  $3.1 \text{ mg kg}^{-1}$   
476  $^1$  in the testa (batch C); and  $11 \text{ mg kg}^{-1}$  in the nib and  $22 \text{ mg kg}^{-1}$  in the testa (batch D). The cross  
477 section from batch C was analyzed with a laser beam size of  $50 \mu\text{m}^2$  and a scan speed of  $50 \mu\text{m s}^{-1}$   
478  $^1$  whereas the cross section from batch D was analyzed with a laser beam size of  $20 \mu\text{m}^2$  and a scan  
479 speed of  $23 \mu\text{m s}^{-1}$ . cps = counts per second



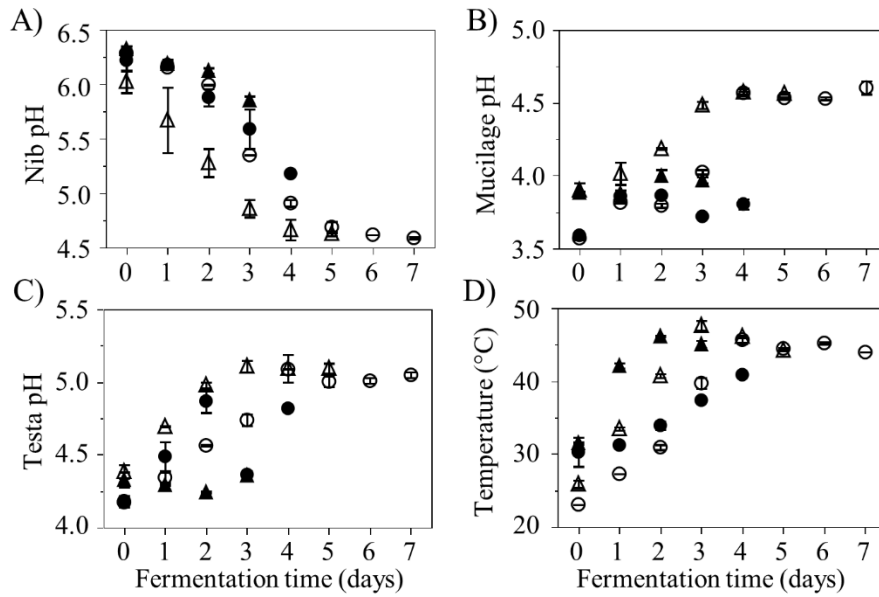
480

481 Figure 2 Fractions of Cd species in unfermented cacao nib and testa determined by XANES  
 482 followed by LCF analysis, and corresponding R-factors indicating the goodness of fit. Total Cd  
 483 concentrations in each tissue were determined by acid digestion and ICP-MS analysis. Detailed  
 484 XANES spectra and LCF fits for samples and reference compounds are given in the supplementary  
 485 information.



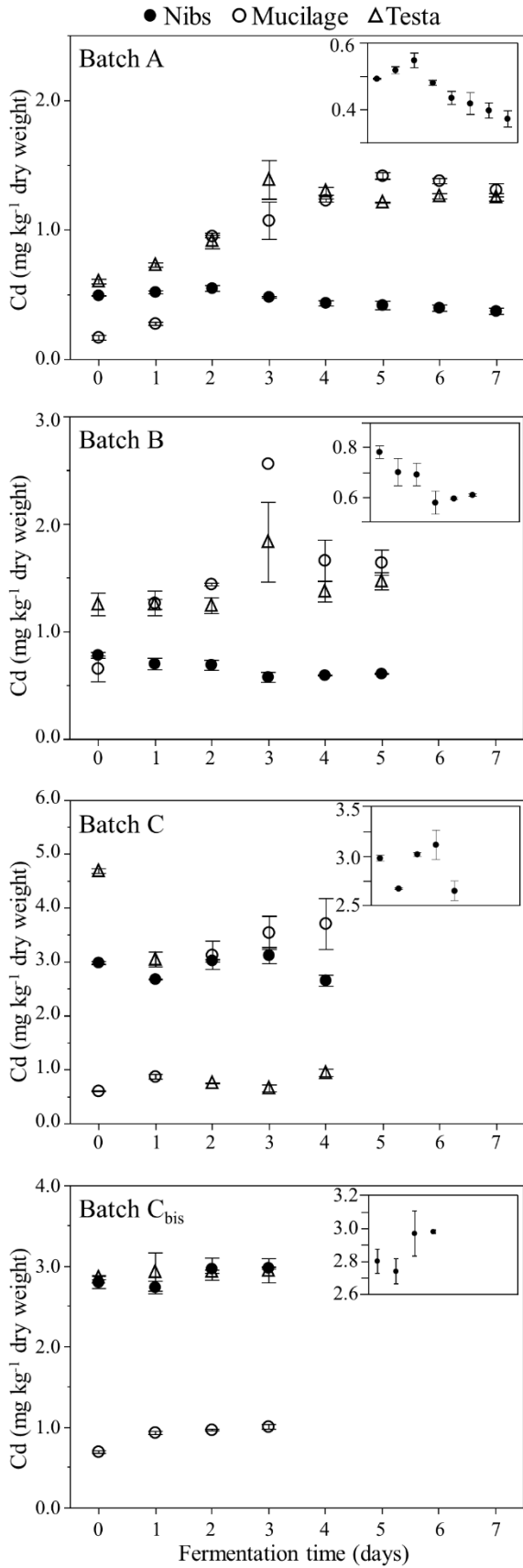
486

487 Figure 3 Change in nib pH (A), mucilage pH (B), testa pH (C) and temperature in the center of the  
488 fermenting mass (D), for three experimental set-ups: batch A (○), batch B (△), batch C (●) and  
489 batch C<sub>bis</sub> (▲). Each point represents the average of duplicate samples and the error bars are  
490 standard errors.

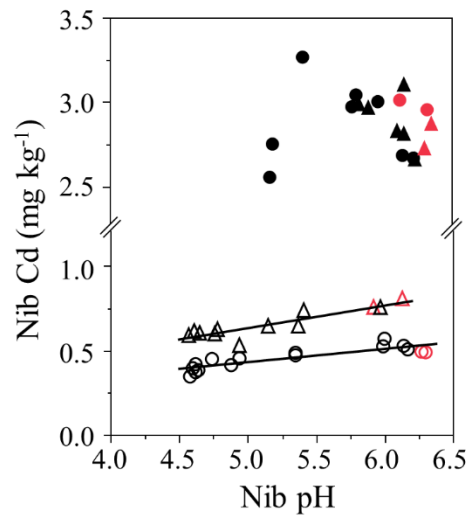


491

492 Figure 4 Concentrations of Cd in the different cacao bean tissues [nib (●), testa (▲) and mucilage  
493 (○)] measured at different days of fermentation. Each point represents the average of duplicate  
494 samples and error bars denote the standard error. Inset figures further zoom in on the changes in  
495 Cd concentration in the nibs to facilitate visualization.



497 Figure 5 Nib Cd concentration is significantly ( $P < 0.05$ ) correlated to nib pH during fermentation  
498 of batch A (○, Pearson correlation  $r = 0.87$ ) and batch B (△,  $r = 0.90$ ) but this correlation was  
499 neither significant in the fermentation of batch C (●) nor batch C<sub>bis</sub> (▲). The red color indicates the  
500 starting point of each independent fermentation replicate.



501

502 Table 1 Origin of the different cacao batches and set-up of the fermentation experiments. For batch D, no fermentation experiment was  
 503 executed.

	Origin	Cultivar	Box dimensions (w×d×h, cm)	Cacao per box (kg)	Box set-up	Times mixed in fermentation	Fermentation time (days)	Immediate fermentation
A	El Oro	CCN-51	60×60×60	290	Cascade	2	7	Yes
B	Guayas field 1	Nacional	60×60×60	290	Cascade	1	5	Yes
C	Guayas field 2	Nacional	100×100×60	590	Single	1	4	Yes
C <sub>bis</sub>	Guayas field 2	Nacional	100×100×60	590	Single	1	3	Pre-dried <sup>a</sup>
D	Sucumbíos	Nacional	/	/	/	/	/	

504 <sup>a</sup> Pre-dried overnight at ambient temperature before fermentation by spreading the cacao beans on concrete floor.

505 Table 2 Distribution of Cd among the different tissues of fresh cacao fruit (unfermented). Placenta  
 506 and pod husk materials were only collected for batches A and B. For batch D, no mucilage material  
 507 was collected. Concentrations are means ( $\pm$  standard deviation of sampling replicates), different  
 508 letters denote significant differences within each row (Tukey test,  $P < 0.05$ ).

Batch	N	Cd concentration (average $\pm$ standard deviation, mg kg <sup>-1</sup> )				
		Nib	Testa	Mucilage	Placenta	Pod husk
A	3	0.52 $\pm$ 0.11 <sup>bc</sup>	0.94 $\pm$ 0.22 <sup>a</sup>	0.08 $\pm$ 0.01 <sup>d</sup>	0.18 $\pm$ 0.008 <sup>cd</sup>	0.59 $\pm$ 0.21 <sup>ab</sup>
B	3	0.39 $\pm$ 0.07 <sup>ab</sup>	0.66 $\pm$ 0.26 <sup>a</sup>	0.09 $\pm$ 0.03 <sup>b</sup>	0.31 $\pm$ 0.12 <sup>ab</sup>	0.28 $\pm$ 0.01 <sup>b</sup>
C	6	2.4 $\pm$ 0.88 <sup>a</sup>	3.7 $\pm$ 1.6 <sup>a</sup>	0.48 $\pm$ 0.24 <sup>b</sup>	/	/
D	6	9.6 $\pm$ 2.3 <sup>b</sup>	16 $\pm$ 4.6 <sup>a</sup>	/	/	/

509

510



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