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# Effects of grain source and processing methods on the nutritional profile and digestibility of grain amaranth

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ABSTRACT

Amaranth grain is reputed to have a high nutritional value, and as a plant, be tolerant to adverse weather conditions. This suggests that grain amaranth could be useful in tackling malnutrition and the growing burden of cardiometabolic diseases. However, there is insufficient knowledge at present about how the nutrient composition and digestibility of amaranth grain varies with growing environment, crop genotype, and post-harvest processing. We investigated the effect of the source and processing of amaranth grains on the digestibility of protein and lipid present in the grains. There was variation in the composition and digestibility of raw grains from different sources, indicating a role of genotype and/or growing environment which warrants further investigation. The greatest differences in digestibility were measured between the different processing techniques. This indicates that efforts to increase the cultivation and consumption of grain amaranth need to be supported by education about effective processing and preparation techniques.

## 1. Introduction

Consumption of grain amaranth (*Amaranthus* sp.) has been associated with various positive health effects, in particular the reduction of cardiovascular disease risks such as blood cholesterol levels (Caselato-Sousa & Amaya-Farfan, 2012; Jimoh, Afolayan, & Lewu, 2018). Amaranth grain has an excellent nutritional composition as it is rich in macronutrients (~12–22% of proteins and ~6–13% of lipids), dietary fibre (~9 to 14%), vitamins, minerals, and other phytochemical compounds (polyphenols and phytosterols) (Venskutonis & Kraujalis, 2013; Karamac et al., 2019). Compared to traditional cereals, it has a superior protein quality with high lysine and methionine content (Segura-Nieto, Barba De La Rosa, & Paredes-López, 1994). The ability to improve nutritional quality of staple food products makes amaranth particularly suitable for people prone to food insecurity (e.g. urban consumers of low socioeconomic status, smallholder and subsistence farmers) (Mlakar, Turinek, Jakop, Bavec, & Bavec, 2010; Alemayehu, Bendevis, & Jacobsen, 2015).

The amaranth plant is reputed to be highly tolerant to pests, diseases and other environmental constraints such as drought stress (Myers, 1996). This pseudo-cereal has been seen as a useful food source for smallholder farmers facing challenging climatic conditions (Dinssa et al., 2016; Peiretti, 2018). However, it is unclear whether the nutritional benefits of the amaranth grain are retained in challenging climatological and agronomical conditions. Most attention has been dedicated to the study of growth, yield and nutritional composition of grain amaranth subjected to environmental stress or constraint (Aufhammer et al., 1995; Bielski & Szwejkowska, 2015; Dada, Imade, &

*Abbreviations:* ATR, attenuated total reflectance; AVRDC, The World Vegetable Center; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; FFA, free fatty acids; FTIR, Fourier transform-infrared; OPA, o-phthaldialdehyde; PBS, phosphate buffered saline; PCA, Principal component analysis; PSD, Particle size distribution; TAG, triacylglycerol

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Anifowose, 2017; Peiretti, Meineri, Longato, & Tassone, 2018). There are also some reports on the effect of genotype on grain yield and quality (Joshi et al., 2018). However, the studies looking at the impact of those factors, or post-harvest processing, on nutrient (especially lipid) bioaccessibility and digestibility of grain amaranth in the human gastrointestinal tract are scarce (Coelho, Silva, Martins, Pinheiro, & Vicente, 2018).

There is a paucity of research into how processing of grain amaranth changes its nutrient bioaccessibility and digestibility. Generally in plant-based foods, processing has been shown to affect nutrient bioaccessibility and digestibility (Grundy et al., 2016b; Aguilera, 2018). Indeed, the degree of disruption of the food structure has an impact on nutrient release and digestibility by notably modulating the integrity of the cell walls, promoting lipid coalescence and changes in protein structure and functionality (Foegeding & Davis, 2011; Edwards et al., 2015; Grundy, Lapsley, & Ellis, 2016; Aguilera, 2018; Capuano & Pellegrini, 2019). Processing is however not always beneficial and can result in the removal of food components (e.g. dietary fibres and polyphenols), formation of new networks (e.g. between proteins and lipids), and the release of antinutrients (e.g. phytates and oxalates) that can hinder macronutrient digestibility (Alzagtat & Alli, 2002; Oghbaei & Prakash, 2016; Cuadrado, Takacs, Szabó, & Pedrosa, 2019).

Despite the benefits listed above, amaranth is currently underutilized, particularly as a grain crop (with most production as a leafy vegetable), but it has great potential (Arendt & Zannini, 2013; Dinssa et al., 2016; Coelho et al., 2018). Grain amaranth originates from Mexico and central America, but nowadays it is also cultivated in Asia, the United States, Canada, Africa and Europe (the latter limited to ornamental varieties) (Arendt & Zannini, 2013; Coelho et al., 2018). In the current context, amaranth appears as a promising solution to tackle both climate change and malnutrition (Myers, 1996; Mlakar, Jakop, Bavec, & Bavec, 2008; Arendt & Zannini, 2013; Alemayehu et al., 2015). However, there is insufficient knowledge at present related to the effect of grain source (grains varying in genotype and grown under diverse climate and agronomic conditions) and processing on the nutritional value and the nutrients digestibility of grain amaranth.

The aims of this work were therefore to (i) screen a range of grain amaranth from different (geographic and genetic) sources to understand whether there is variation in nutritional composition and lipid digestibility, (ii) examine the effect of processing, and the role played by cell walls, on the bioaccessibility and digestibility of lipid and protein present in the amaranth grains.

## 2. Material and methods

### 2.1. Chemical and reagents

Pepsin from porcine gastric mucosa (#P6887, 2884 U/mg of solid), bovine bile extract (#B3883), pancreatin from porcine pancreas (#P7545, 65 U/mg of solid based on lipase activity), sodium chloride (99.8%), Pefabloc® SC (4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride), phosphate buffered saline (PBS) tablets, Nile Red, and Fast Green were purchased from Sigma (Poole, Dorset, UK). Sodium dihydrogen phosphate (99%), disodium hydrogen phosphate (99%), calcium chloride (99%), potassium chloride (99%), potassium dihydrogen phosphate (99%), sodium bicarbonate (99.5%), magnesium chloride hexahydrate (99%), ammonium carbonate (99.5%), and trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA, 98.5%) were purchased from Fisher Scientific (Loughborough, UK).

### 2.2. Amaranth grains and products

Amaranth grains (*Amaranthus sp.*; 9 seedlots) were collected from farmers in three environmentally diverse regions of Kenya (Bondo, Oyugis and Lugari) and from two commercial sources (retailer from the UK, origin India, and Annico Enterprises Ltd in Kenya). Table 1 presents the characteristics for each geographical area of collection in Kenya. No information about the species of amaranth provided by the farmers or retailers were available, however visual examination of plants grown from collected seeds in our labs suggested that they all were *A. cruentus*. Finally, 3 seedlots (Avam 1604, *A. hypochondriacus* from Tanzania; Avam 1607, *A. cruentus* from Madagascar; and V1064075, *A. hypochondriacus* from Kenya) were supplied by the World Vegetable Center (AVRDC) in Arusha, Tanzania. Before being digested under simulated duodenal conditions using the pH-stat method (see Section 2.5.1), these grains (14 seedlots in total) were ground to a flour at the University of Reading, UK, using a coffee grinder (CG618B, Shardor, Changsha, China). The grains were blended for 30 s with 3 periods of 10 s and a pause of 5 s between each period.

A second set of experiments were performed on amaranth grains that underwent different forms of processing. Five amaranth products were obtained from a small sized business (Annico Enterprises Ltd) near Nairobi, Kenya. Annico Enterprises Ltd collected raw grains (single source) and processed them into raw flour, toasted grains, toasted flour and puffed amaranth (Fig. 1). The raw grains were toasted and popped (puffed amaranth) using a hot pan (See Supplementary Material, Fig. S1a and b). The puffed amaranth was obtained by heating the raw grains for approximately 15 s until they popped; the grains were stirred with a wooden spatula whereas no stirring and more moderate heating was applied to obtain the toasted grains. The raw and toasted grains were milled using a hammer mill to generate the flour samples (See Supplementary Material, Fig. S2a and b). The hammer mill comprised a sieve of 2 mm aperture size and a milling speed of 75 m/s was used. Ten kilograms of grain were milled in approximately 5 min, the milled flour was then loaded again into the machine and milled for another 5 min.

### 2.3. Preparation of oil bodies and separated cells

Oil bodies were isolated from the amaranth grain (UK retailer variety) by soaking the grains overnight in an extraction medium (10 mM sodium phosphate buffer pH 7.5 containing 0.6 M sucrose at 1:5 w/v sample to medium ratio) followed by homogenisation (Kenwood BL315 blender; 2 min), filtration through 3 layers of cheese cloth and centrifugation (20,000 g at 4 °C for 20 min) of the slurry (Wilde, Garcia-Llatas, Lagarda, Haslam, & Grundy, 2019). Oil bodies were used as a control where lipids were fully bioaccessible and the cell wall did not encapsulate them and prevent their digestion. In order to gain some insight about the morphology of the amaranth cells, the latter were separated using a chelating agent (CDTA) as previously described (Grundy, Wilde, Butterworth, Gray, & Ellis, 2015).

### 2.4. Physical and chemical characterisation of the amaranth materials

The amaranth materials were analysed for protein (LECO, CHN628 Series Elemental Analyser; Dumas combustion analyser, with protein N factor of 5.85) (Segura-Nieto et al., 1994), lipid (Soxflo; hexane) (Brown & Mueller-Harvey, 1999), and total dietary fibre (Megazyme kit assay, Megazyme, Product Code: K-TDFR, AOAC procedure 32–05.01) content. Moisture (oven-dried at 105 °C to a constant weight) content was also determined. Each set of samples were analysed in duplicate.

The particle size distribution (PSD) of the oil bodies and flours was measured with a Beckman Coulter LS13320® laser analyser (Beckman Coulter Ltd., High Wycombe, UK). Water was used as a dispersant (refractive index of 1.330), the absorbance value was 0.001, and the refractive index was 1.473 for the oil bodies and 1.600 for the flours as measured using a refractometer (Rhino Brix90 Handheld Refractometer, Reichert, Inc., New York, USA). The particle size distribution, as average volume percentage, are presented as the means of at least three replicates.

The amaranth oils collected from the Soxflo were analysed with a Fourier transform-infrared (FTIR) spectrometer (Perkin-Elmer Spectrum 100 FTIR spectrometer, Shelton, US) equipped with a single

**Table 1**
Geographical and cultivar characteristics of the seeds collected by Kenyan farmers.

| | Altitude | Annual temperature | | Rainfall | | Annual rainfall (mm) | Soil characteristics | Agricultural practices | Seed cultivar |
|---|---|---|---|---|---|---|---|---|---|
| | (m) | Max | Min | Long rains | Short rains | | | | |
| **Bondo** | 1250 | 18 °C | 32 °C | Mar to Apr | Sep to Dec | 1300 | Moderate to well drained<br>Deep dark brown to very dark grey<br>Loamy sand to heavy clay texture | Monoculture | Cream |
| **Oyugis** | 1500 | 17 °C | 29 °C | Mar to Jun | Aug to Nov | 1600 | Well drained<br>Dark reddish grey to reddish brown<br>Clay loam texture with fine granular structure | Monoculture | Golden white |
| **Lugari** | 1600 | 9 °C | 26 °C | Mar to Jul | Dec to Feb | 1750 | Well drained<br>Dark brown<br>Loamy sand and red oxisols | Monoculture | Golden |

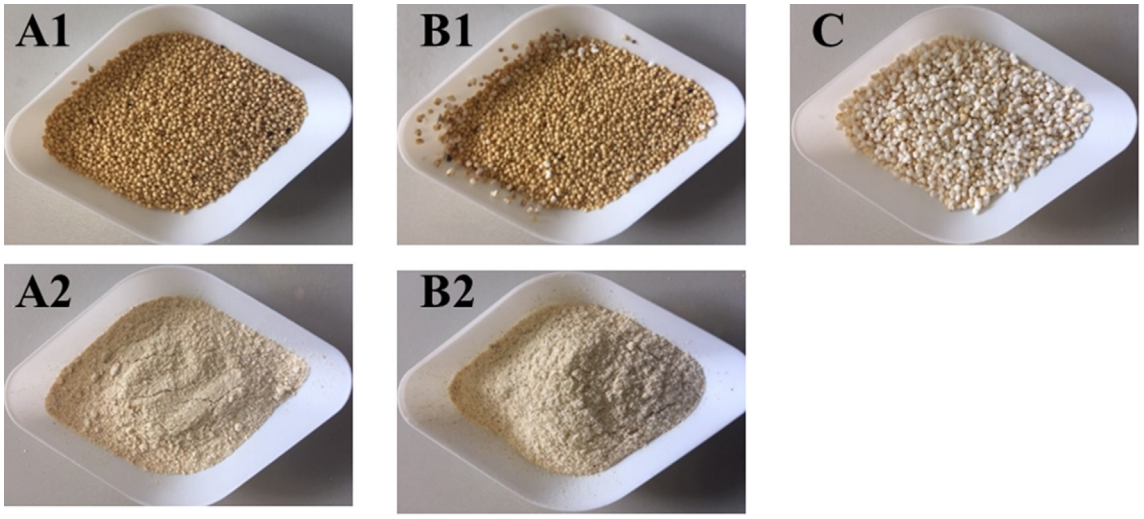


**Fig. 1.** Amaranth products provided by Annico Enterprises Ltd: raw grain (A1), raw flour (A2), toasted grain (B1), toasted flour (B2), and puffed amaranth (C).

bounce diamond/ZnSe attenuated total reflectance (ATR) crystal. The data were analysed with Spectrum™ software version 10.03.06 (Perkin Elmer, Shelton, US). Spectra were recorded in absorbance mode from 4000 to 600 $cm^{-1}$, using 64 scans and 4 $cm^{-1}$ resolution. Baseline was corrected and the spectra were normalized for presentation purpose.

### 2.5. In vitro digestion

#### 2.5.1. Duodenal digestion of lipid (pH-stat method)

The 14 grains ground into a flour and the amaranth products (5 forms of amaranth obtained from Annico Entreprise Ltd as well as the oil bodies) were subjected to simulated duodenal *in vitro* digestion using the pH-stat method (Grundy et al., 2015). Briefly, the samples (amount adjusted to obtain 300 mg of lipids) were mixed with simulated duodenal fluid (bile salts, NaCl and $CaCl_2$ dispersed in 10 mM phosphate buffer) and incubated in the pH-stat vessel at 37 °C for 1 h, pH 7. The final composition of the reaction system was 0.8 wt% lipid, 12.5 mM bile salts, 0.68 mg/mL of pancreatin, 150 mM NaCl and 10 mM $CaCl_2$. The rate and extent of free fatty acids (FFA) released during lipolysis of the intrinsic amaranth lipids were monitored by titration with 0.10 M NaOH. All experiments were carried out in triplicate.

#### 2.5.2. Gastrointestinal digestion of protein (Infogest standardised method)

Given that the amaranth grains provided by the farmers and AVRDC were available in a limited quantity (only about 50 g in total per sample), protein digestion following the Infogest protocol could not be performed on those grains.

The amaranth products were digested using the standardised *in vitro* static digestion protocol developed by the Infogest COST Action (Brodkorb et al., 2019) to follow protein digestion. After 2 min of incubation at 37 °C pH 7 (the amount of sample added was adjusted based on protein content − 0.7 g) to simulate the oral phase, the samples were subjected to gastric (120 min at pH 3) and duodenal (120 min at pH 7) digestions. The composition of the simulated fluids for each phase can be found elsewhere (Brodkorb et al., 2019). The protease reaction was stopped by adding 50 µL of 0.1 M Pefabloc prepared in deionised water at the end of the intestinal phase. Each digestion was performed in duplicate.

The extent of protein hydrolysis was determined by measuring both the products of proteolysis (supernatant) and the protein content recovered after digestion (pellet). The digested samples were centrifuged at 10,000 g for 10 min, and the supernatant was analysed using the standardised o-phthaldialdehyde (OPA) spectrophotometric assay in microplates (Mulet-Cabero, Rigby, Brodkorb, & Mackie, 2017). The recovered pellet from centrifugation was freeze-dried and the protein content measured using the LECO instrument. The amount of recovered protein measured from the pellet was then subtracted from the original protein content to obtain the percentage (%) of digested proteins.

### 2.6. Microstructural analysis

The amaranth grains from the 14 different sources and their separated cells were observed under confocal microscopy to identify any difference in the microstructure of the plant tissue/cells. A solution of Nile red (20 µg/mL), Fast Green (20 µg/mL), and Calcofluor White (40 µg/mL in water) in PBS (1 tablet dissolved in 200 mL of deionised

water, pH 7.4) was prepared. Nile red and Fast Green were used to stain the lipids and proteins in the amaranth grains respectively, while Calcofluor White was used to stain the cell wall. The amaranth samples (grains and separated cells preparations) were left to stain in 200 μL of the dye solution for at least 1 h. The samples were washed twice with PBS and the grains sectioned with a razor blade. The sectioned grains and cell preparations were then mounted on microscopy slides before being visualised with a confocal laser scanning microscope (Nikon, AR-1, confocal/resonant scanning microscope, Surrey, UK). Images were captured using 10x and 20x objective lenses. The samples were excited using an argon laser at 488 nm for Nile red, 638 nm for Fast Green, and 405 nm for Calcofluor White. The fluorescence emitted by the samples was detected at 510 to 600 nm (Nile red), 647 to 750 nm (Fast Green) and 410 to 480 nm (Calcofluor White).

### 2.7. Statistical analysis

The data obtained from the digestibility experiments (lipid and protein) and the particle size distribution were analysed using SPSS version 25.0 (IBM, Armonk, NY, USA). For all tests, the significance level was set at $P < 0.05$ (2 tailed). These data are expressed as means ± standard deviation of the replicate. Differences in lipid release, protein digestion and particle size between the amaranth products or the amaranth grains were analysed by Student's paired *t*-test. The correlation between nutritional composition, particle size distribution and nutrient digestibility were examined by Pearson correlation analysis. Principal component analysis (PCA) of the FTIR spectra from the amaranth oils were performed to reveal the spectral differences between samples using Orange software version 3.16 (Demšar et al., 2013). Linear baseline correction and vector normalization was applied to spectral region from 650 to 4000 $cm^{-1}$.

## 3. Results and discussions

### 3.1. Nutritional profiles of the amaranth grains

The compositions of the 14 grains (Table 2) and amaranth products (Table 3) were determined as described in Section 2.4. The highest amount of lipids was found in the Oyugis 3 and Annico samples (6.2%), and the lowest amount in the sample from the UK retailer (India with 4.2%). Avam 1607 and V1064075 presented higher protein content (16.2%) than the rest of the grains, Lugari 2 having the lowest quantity of protein (12.2%). Finally, the grains contained total dietary fibre values between 3.2 and 9.5% (Avam 1607 and Lugari 7, respectively). Overall, the composition of the grains used in the present study are within the range of values reported in the literature (Venskutonis & Kraujalis, 2013). Processing affected the crude lipid and protein composition of the grain amaranth as shown in Table 3. In particular, protein content showed the most variability, ranging from 13.3 to 15.5% (raw flour and puffed amaranth, respectively). These findings are in disagreement with those of a previous study that observed a reduction in protein levels upon popping, however the processing methods used differed from the ones of the current work (Gamel, Linssen, Alink, Mosallem, & Shekib, 2004).

**Table 3**
Proximate analysis of the amaranth products.

| | Raw grain | Toasted grain | Raw flour | Toasted flour | Puffed |
|---|---|---|---|---|---|
| Crude lipid (%)* | 6.2 | 6.3 | 6.2 | 7.0 | 6.1 |
| Protein (%)* | 15.2 | 14.0 | 13.3 | 13.8 | 15.5 |
| Moisture (%) | 10.4 | 9.4 | 9.2 | 10.1 | 3.4 |

* The data are expressed on a dry weight basis. Values are presented as means of duplicates.

### 3.2. Duodenal digestion of the amaranth flours

#### 3.2.1. Lipid digestion

Significant differences ($P < 0.05$) were observed in the lipolysis kinetics of the flours made from the 14 grains (Fig. 2). The majority of the flours demonstrated FFA release between 9.43 and 11.31 mmol/L following a 60 min digestion, while 4 seedlots showed marked disparities. Indeed, the flours made from the amaranth obtained from the UK retailer (India) and AVRDC (Avam 1607) had the highest extents of lipolysis (14.60 and 13.32 mmol/L, respectively) which was analogous to the oil bodies (13.80 mmol/L). On the other hand, the FFA release values were significantly lower for Avam 1604 and Oyugis 3 (7.61 and 7.38 mmol/L) than the corresponding values for the other flours ($P < 0.001$).

In order to shed light on the mechanisms that could explain the differences recorded in lipid digestion of the flours, their particle size was measured and their lipids analysed by FTIR.

#### 3.2.2. Characterisation of the flours and their extracted oils

The majority of the flours had similar particle size distributions (PSD) that were multimodal and broad, with an average size of 347 μm, ranging from 245 and 430 μm (Fig. 3). Two samples, Avam 1604 and Oyugis 3, had particles of larger sizes (average size of 832 and 744 μm, respectively) compared with the other flours ($P < 0.05$). The PSD of the flour milled by Annico using a hammer mill (Fig. S2a and b) was narrower and of smaller average size (177 μm). Therefore, it was expected that the lipids from this flour would have been digested to a greater extent compared with the other samples, which was not the

**Table 2**
Proximate analysis of the amaranth grains obtained from the World Vegetable Center (AVRDC), the farmers, and the retailers.

| | | Sample name | Moisture (%) | Crude lipid* (%) | Protein* (%) | Total dietary fibre* (%) |
|---|---|---|---|---|---|---|
| **AVRDC** | | *AVAM 1604* | 10.9 | 4.6 | 14.6 | 4.4 |
| | | *AVAM 1607* | 10.4 | 4.8 | 16.2 | 3.2 |
| | | *V1064075* | 10.8 | 5.1 | 16.2 | 4.6 |
| **Farmers** | **Bondo** | *Bondo 1* | 9.9 | 4.9 | 15.3 | 4.3 |
| | | *Bondo 2* | 10.2 | 5.4 | 15.8 | 4.1 |
| | | *Bondo 6* | 11.0 | 5.4 | 13.7 | 3.9 |
| | **Oyugis** | *Oyugis 1* | 10.6 | 5.5 | 13.5 | 5.2 |
| | | *Oyugis 2* | 10.0 | 4.9 | 14.8 | 3.7 |
| | | *Oyugis 3* | 10.4 | 6.2 | 13.4 | 4.2 |
| | **Lugari** | *Lugari 2* | 11.3 | 5.0 | 12.2 | 5.5 |
| | | *Lugari 7* | 10.5 | 5.9 | 13.3 | 9.5 |
| | | *Lugari 9* | 10.0 | 5.7 | 14.0 | 6.6 |
| **Retailers** | **Nairobi** | *Annico* | 10.4 | 6.2 | 15.2 | 4.2 |
| | **India** | *India* | 11.5 | 4.2 | 15.2 | 9.3 |

* The data are expressed on a dry weight basis. Values are presented as means of duplicates.

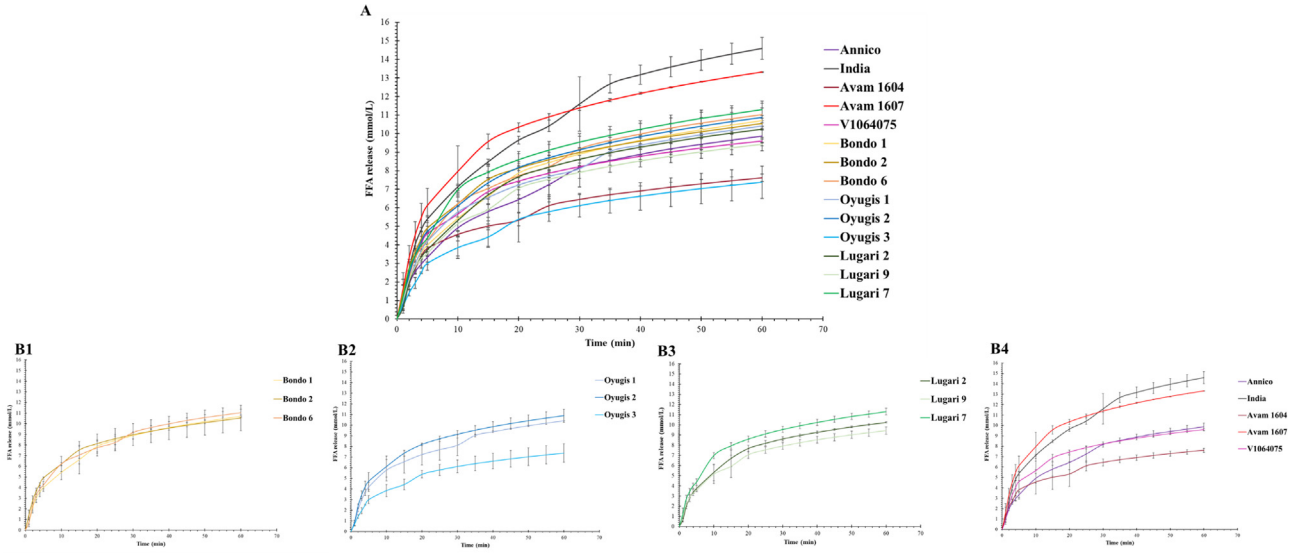


**Fig. 2.** Free fatty acids (FFA) released overtime during simulated duodenal digestion of the flours made from amaranth (A) collected in Bondo (B1), Oyugis (B2), Lugari (B3), and the World Vegetable Center (AVRDC) and the retailers (B4). The data are presented as mean concentrations in mmol/L ± SD (n = 3).

case. Indeed, particles of small size would have had a greater number of cells fractured and thereby nutrients released from the food matrix, such as that observed with almonds (Grundy et al., 2015). However, as expected, the two flours with the larger particles displayed the lowest amount of FFA release. The composition and organisation of the cell walls are known to affect the physico-chemical properties of grains (Waldron, Parker, & Smith, 2003) and the way they behave during mechanical processing (i.e. milling and grinding). Indeed, Table 2 shows variability in the dietary fibre (the main component of cell walls) content of the amaranth grains, ranging from 9.5 to 3.2%. Overall, no correlation existed between those values and the PSD or lipid digestibility.

Amaranth oils were analysed by FTIR to identify potential differences in lipid components between seedlots and the resulting spectra were analysed using PCA. Overall, the spectra displayed great similarity (Fig. 4A1 and A2) although analysis of the loadings plot from PC 1 highlighted significant differences (Fig. 4B1 and B2) for bands at 1745 (stretching of ester carbonyl group), 2850 (C–H symmetric stretching from aliphatic compounds) and 2920 (C–H asymmetric stretching from aliphatic compounds) $cm^{-1}$ (Roa, Santagapita, Buera, & Tolaba, 2014; Siwatch, Yadav, & Yadav, 2017). In particular, the Avam 1607 and Indian samples had shift in their peak positions compared with the other seedlots (Avam 1604 and Oyugis 2 are presented here as examples). The scores plots of the first two principal components (PC1 vs PC2) and their corresponding loadings plot are shown in Fig. S3 in the Supplementary Material section.

There were therefore differences in the digestibility as well as the composition of lipids in seeds from different sources. Variations in the types and quantities of lipids present in samples from the different sources could have been responsible for the recorded findings. For instance, certain lipid-soluble molecules, such as squalene, saponins, phytosterols and tocopherol, despite being present in minute amount could have affected the lipolysis due to their physico-chemical properties (Venskutonis & Kraujalis, 2013). Indeed, these compounds are surface active and can position themselves onto the interface, thereby interfering with lipase activity (Reis, Watzke, Leser, Holmberg, & Miller, 2010). While we cannot make controlled comparisons between different growing regions or plant genotypes, these factors may alter the composition of amaranth lipids (He, Cai, Sun, & Corke, 2002; Dada et al., 2017; El Gendy et al., 2017; Bozorov et al., 2018).

Analysis of the samples by FTIR revealed shift, for grains from certain sources, in the bands corresponding to the lipids. Differences in

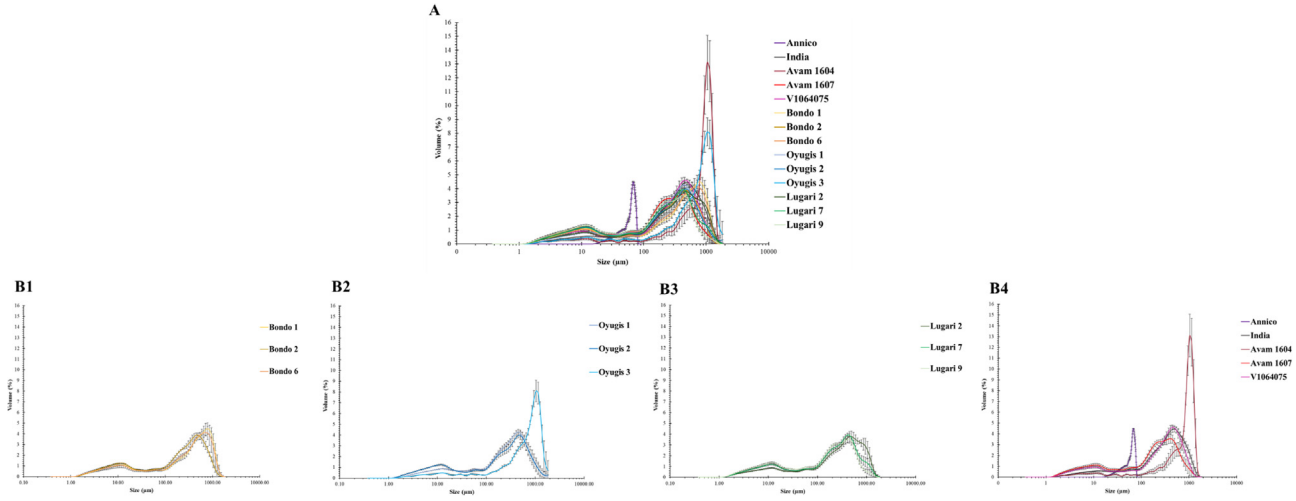


**Fig. 3.** Particle size distribution of flours made from amaranth (A) collected in Bondo (B1), Oyugis (B2), Lugari (B3), and the World Vegetable Center (AVRDC) and the retailers (B4) (n = 3).

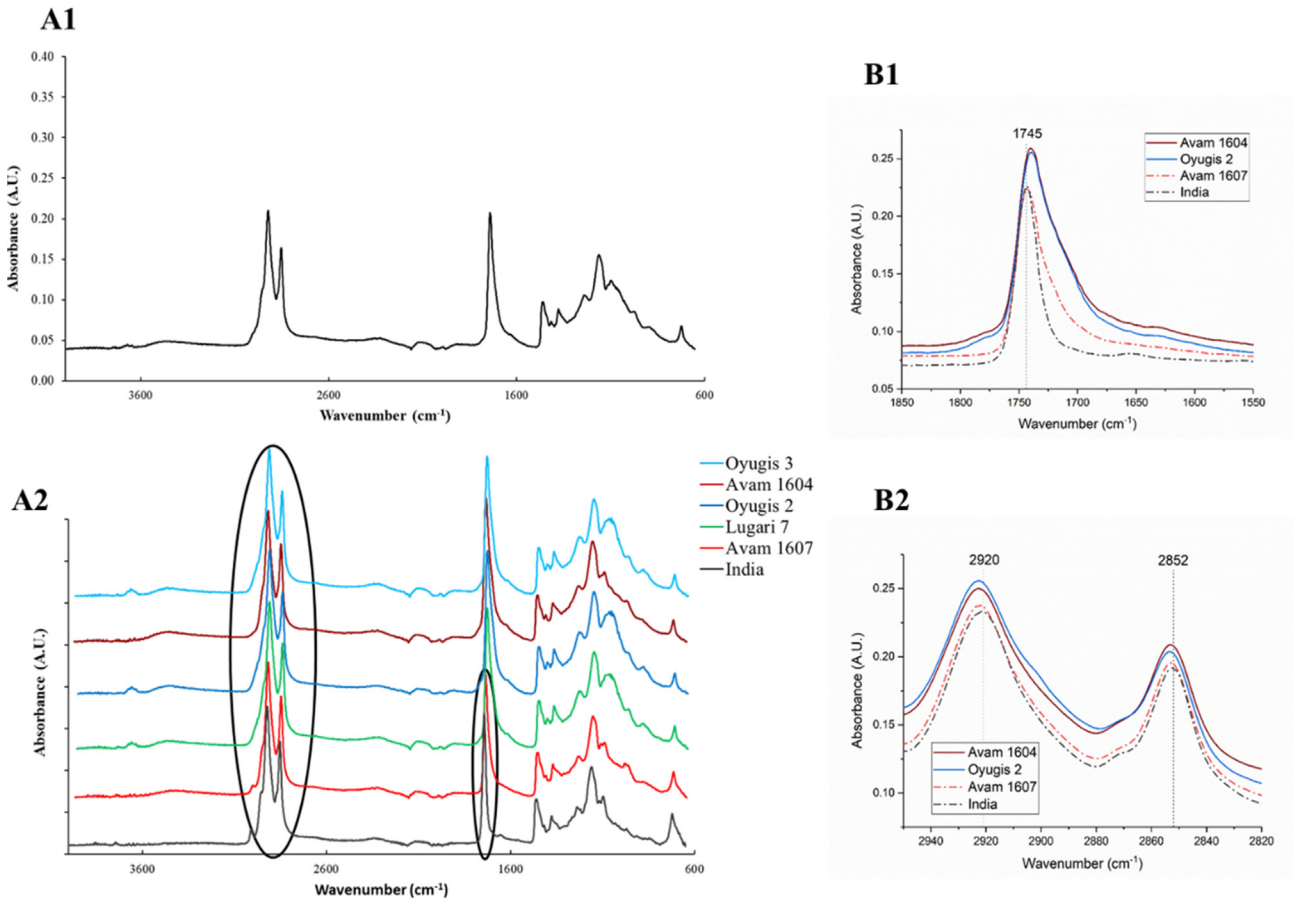


**Fig. 4.** FTIR spectra of oils recovered from the seedlots: average spectra of the 14 amaranth oils (A1) and spectra that were different from the others (A2). Areas of the peaks (B1 and B2) where significant differences were obtained as determined from the first principal component (PC1) of the PCA.

the particle size of the amaranth flours were also recorded. However, the relationship between those parameters (particle sizes and shift in FTIR spectra) and the digestibility of the flours is not clear. For instance, the flours with the lowest particle sizes were not the most digestible ones. Confocal microscopy observations were then performed in order to identify any potential disparities in the grains microstructure (e.g. cells shape, size and organisation within the plant tissue, or cell wall structure or thickness) that may explain the variation in lipolysis detected.

### 3.2.3. Microscopy observations of the amaranth grains

Typical images of the general morphology of the amaranth grain (A1 and A2), part of the endosperm (B and D), and the separated cells (C) are presented in Fig. 5. No structural differences could be observed between the different grains, including the two species clearly identified (*A. hypochondriacus and A, cruentus)*, and their corresponding separated cells. However, the images provided additional information about the general microstructure of the amaranth grain, in particular cell size, distribution of proteins and lipids within the plant tissues, and cell wall thickness. The perisperm cells located in the centre of the grain were stained in red indicating that they contained proteins and no or very little lipids. On the other hand, the endosperm cells appeared to be rich in lipids and tightly packed together (Fig. 5A2, B and D), which is in agreement with previous work (Irving, Betschart, & Saunders, 1981). A thin layer of lipid could also be distinguished underneath the seed coat (Fig. 5A2). The cell walls delimitating the perisperm (Fig. 5A1) and endosperm (Fig. 5B) cells can be clearly seen in blue, the latter having thicker cell walls than the former (Fig. 5D). In contrast to the proteins, the cell walls could not be detected in separated perisperm cells, even though the cells maintained their shape (Fig. 5C). It has been hypothesised that the starch granules adhered to the cell wall which could have preserved the cell shape (Irving et al., 1981).

The cells from the endosperm could not be separated (Fig. 5D) which suggests that, if intact, their content would have greater resistance to digestion. However, the lipolysis data (Figs. 2 and 6) revealed that a significant amount of the lipids in the flours were hydrolysed. It is therefore possible that these cells were fractured during grounding/milling thus making the cell content available to digestive agents. Moreover, the lipids located underneath the seed coat could have been released during processing and thus were readily available for lipase hydrolysis. The enzymes are unlikely to be able to diffuse through the amaranth cell wall given that the whole grains (intact cells), either raw or toasted, showed negligible extent of nutrient digestion (Fig. 6), although the seed coat may have formed an additional barrier that encapsulated the nutrients. Currently, there is no information about the permeability of amaranth cell walls (perisperm or endosperm), while the porosity of the cells of various nuts, grains and legumes have been investigated (Baron-Epel, Gharyal, & Schindler, 1988; Grundy et al., 2016a; Pallares Pallares et al., 2018; Li, Gidley, & Dhital, 2019). These studies confirmed that unless the cell wall is fractured, swollen or softened (via heat treatment), the digestive enzymes are unable to penetrate inside the plant cells and hydrolyse their substrates (starch, protein or lipid).

Unfortunately, at this stage, it was not possible to identify whether genotype, environment or a combination of both were responsible for the variabilities in particle size, oil composition and the subsequent lipid digestibility recorded between the grains. Environmental differences, such as weather conditions (heat stress), water availability, and

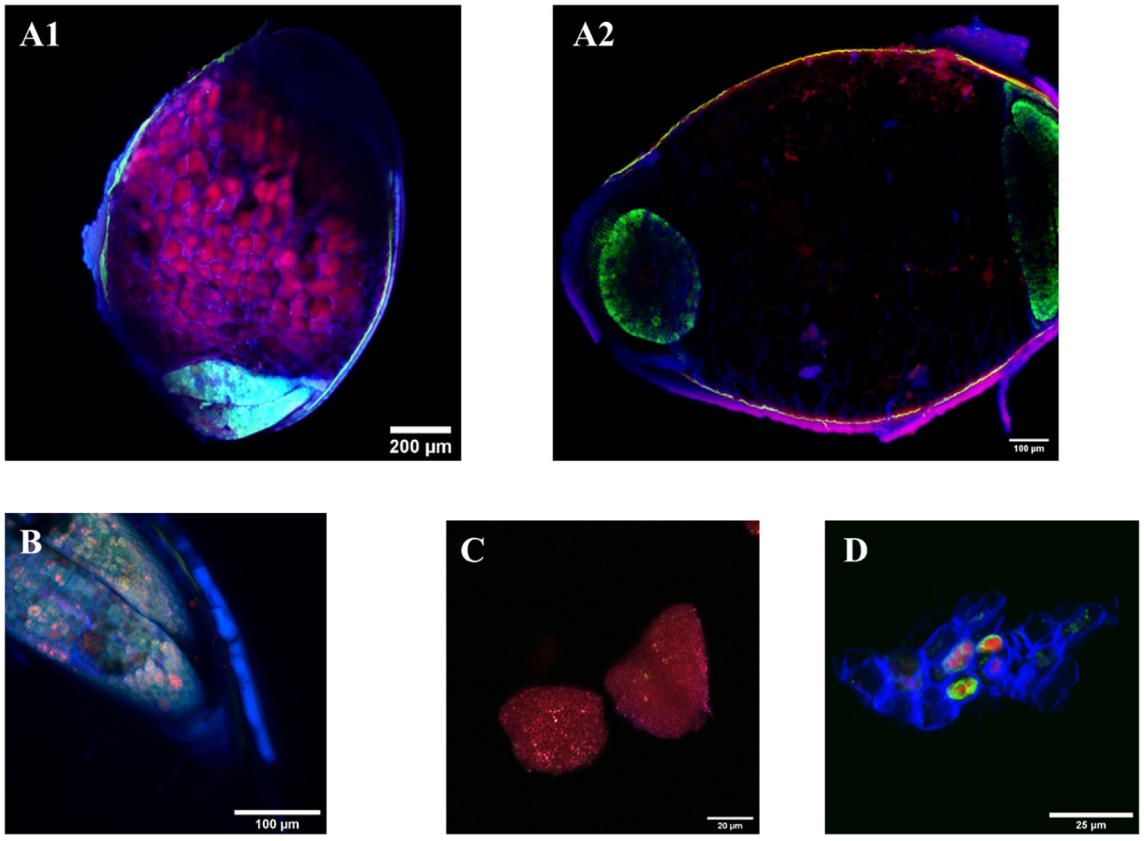


**Fig. 5.** Confocal laser scanning microscopy images of a cross-section of a typical amaranth grain (A1 and A2), part of the endosperm (B and D), and separated cells (C). Proteins are stained in red with Fast Green, lipids in green with Nile red, and cell walls in blue with Calcofluor White. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

soil quality, may have affected the cell walls (composition and organisation) which will have had consequences for its physico-chemical properties (including porosity) and subsequent behaviour during simulated digestion (Pogorelko, Lionetti, Bellincampi, & Zabotina, 2013; Rakszegi et al., 2014; Dada et al., 2017). Genetic variation is also likely to play a significant role (Sarker, Islam, Rabbani, & Oba, 2018). Further work, including glasshouse trials, performed in both Kenya and the UK, and the subsequent analyses of the 2nd generation of grains should permit to clarify the role played by each parameter.

### 3.3. Digestibility of the amaranth products

#### 3.3.1. Lipid digestion

The rate and extent of lipid hydrolysis were different among the amaranth products (Fig. 6). Amaranth grains, either raw or toasted, were the least digestible (0.22 and 0.80 mmol/L FFA released respectively). This is to be expected since processing of plant-based foods, leading to the disruption of the plant tissue, rupture and/or swelling of the cell walls, is necessary for gastrointestinal enzymes to access their substrates and for the digestion of macronutrients to occur (Heaton, Marcus, Emmett, & Bolton, 1988; Melito & Tovar, 1995; Noah et al., 1998; Edwards et al., 2015; Grundy et al., 2015). The greatest amount

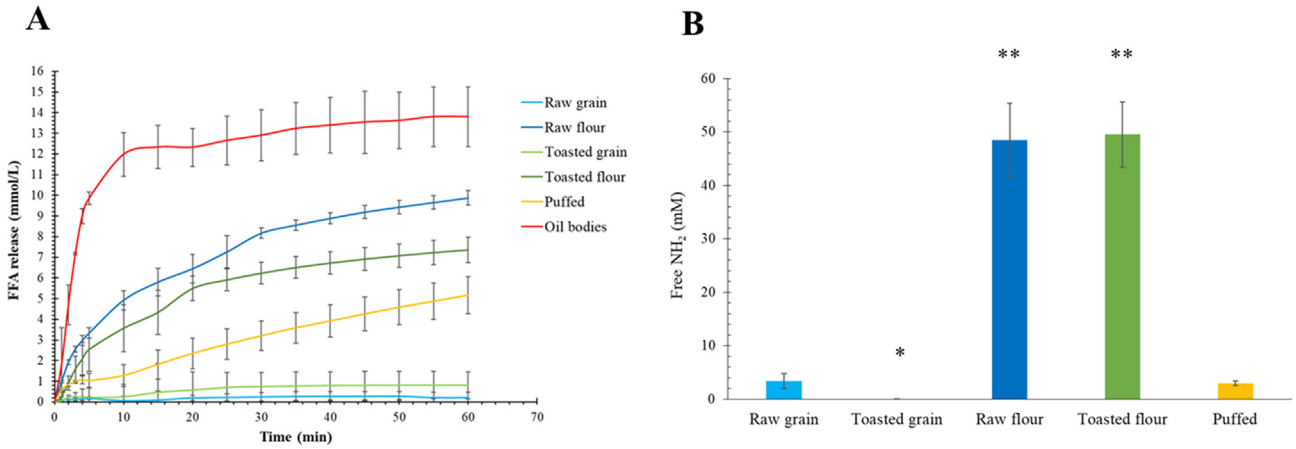


**Fig. 6.** Free fatty acids (FFA) released (A) and amount of protein hydrolysed (B) during simulated digestion of the oil bodies (A only) and the five amaranth products. Statistical differences between the processing methods and the raw grain were determined using Student's paired *t*-test (*$P < 0.05$ and **$P < 0.01$, n = 3).

of lipid digested was from the separated oil bodies, (FFA released 13.80 mmol/L), likely due to their small diameter (0.4 μm) and resultant large surface area available for lipase adsorption, in addition to their lack of a physical barrier (i.e. no cell wall so the lipids were fully bioaccessible). The lipids of the raw and toasted flours were also digested to a significant extent (9.88 and 7.36 mmol/L, respectively). Surprisingly, despite the change in structure through processing, the lipid digestion of the puffed amaranth was limited (5.16 mmol/L). A previous study reported high starch and protein digestibility in puffed amaranth (Lara & Ruales, 2002). Study-specific popping conditions (e.g. temperature, moisture content and heating time) can influence the physico-chemical properties of puffed amaranth and may have caused these differences in digestibility (Inoue et al., 2009). More importantly, the popped amaranth from Lara & Ruales, 2002 was milled before undergoing digestion, resulting in ruptured the cell walls thus allowing the nutrients to become highly bioaccessible. However, puffed amaranth is typically consumed without additional milling. The amaranth genotype and growing conditions, and resulting differences in the composition and organisation of the cell walls, are also likely to have an impact on the popping process. Some amaranth species are known to enter dormancy in response to stressful climate conditions, which may impact the seed coat and processability (Assad, Reshi, Jan, & Rashid, 2017). The method employed for obtaining the puffed amaranth used in this study may have led to partial grain popping, thus some of the cell walls may have still be intact and the macronutrients encapsulated within the cells.

#### 3.3.2. Protein digestion

The two methods (OPA for the supernatant and LECO for the recovered pellet) used to estimate the amount of digested proteins gave consistent results (Figs. 6 and S4 in Supplementary Material). Regardless of the level of processing, a large quantity of original amaranth product was recovered at the end of the simulated gastrointestinal digestion. Similarly to the lipids, the flours had the highest amount of protein digested (~53.0% based on the LECO analysis). Some proteins were hydrolysed in the raw grain sample (~2.5%) but no digestion was detected in the toasted grains. Thermal treatment, such as toasting, is likely to have led to the denaturation and/or loss in solubility of the proteins, thereby hindering their digestibility (Foegeding & Davis, 2011). The protein digestibility of the puffed amaranth was also low (~4.5%), which is in disagreement with other works (Lara & Ruales, 2002; Gamel et al., 2004; Muyonga, Andabati, & Ssepuuya, 2014). As mentioned above, the cell wall disruption of the puffed amaranth used in the present work may have been incomplete contrary to the flours used in these previous studies (milled to a particle size of 500 μm or less). Muyonga et al. (2014) incubated their samples in intestinal fluids for 24 h, which is not representative of physiological processes and may explain the discrepancy with our results. Protein digestibility of the raw and roasted amaranth grains were also surprisingly high in their study, which again could be due to the extensive intestinal digestion phase and the milling process.

As in many other plant-based food products, applying heat treatment to the amaranth grains seems to provide an overall improvement in digestibility of the nutrients they contain (Kauffman & Weber, 1990; Tovar, Valdivia, & Brito, 1994). However, not all heat treatments have the same effect on the food microstructure and macronutrients structure and organisation. In particular, proteins can unfold (denaturation), be hydrolysed, or form aggregates/complexes as a result of processing and thereby have their functional properties and digestibility altered (Foegeding & Davis, 2011; Capuano, Oliviero, Fogliano, & Pellegrini, 2018; Coelho et al., 2018). It is therefore possible that the proteins were entrapped into the gelatinised starch granules (viscous network) formed during the popping process and build-up of internal moisture. Particle size reduction (e.g. milling) also plays a critical role (Edwards et al., 2015; Grundy et al., 2015), which was the determinant processing parameter in this part of our study since the flours were more digested than the whole grains or the puffed amaranth.

Other processing techniques such as germination and fermentation have been employed to improve the nutritional value and protein digestibility of amaranth grain (Najdi Hejazi, Orsat, Azadi, & Kubow, 2016; Castro-Alba et al., 2019). Degradation of the antinutrients, especially phytates and oxalate, was demonstrated as the main mechanism behind the enhancement in protein digestibility. Cooking in excess water, but not popping, also reduced the antinutrient contents (Gamel, Linssen, Mesallam, Damir, & Shekib, 2006; Burgos, Binaghi, de Ferrer, & Armada, 2018).

## 4. Conclusions

The present study provides evidence that processing has a marked effect on the digestibility of macronutrients, namely lipids and proteins; ground amaranth being the most digestible form. These results suggest that the cell wall of amaranth acts as a physical barrier that prevents the diffusion of enzymes and other digestive agents. The rate and extent of lipolysis in 14 amaranth grains (in flour form) from different sources were also investigated. These experiments showed marked differences in lipid digestion between those grains, though the exact mechanism behind these effects is still unclear. It was hypothesised that depending on their source (genotype and/or the growing conditions), the amaranth grains acquire cell walls with specific physico-chemical properties that affect their behaviour during processing and digestion in the human gastrointestinal tract.

The amaranths were studied in forms that are currently commercialised in Kenya. This work provides further evidence that amaranth flour is rich in macronutrients with a favourable digestion profile and can be used to tackle malnutrition in particularly children. Additionally, a wider range of food products made from amaranth could be designed and promoted for populations in Western countries. For instance, because of their low digestibility, popped amaranth could be included in diets aiming at reducing the risks of cardiometabolic diseases.

## CRediT authorship contribution statement

**Myriam M.L. Grundy:** Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft, Writing - review & editing, Funding acquisition, Supervision, Project administration. **Dorah K. Momanyi:** Investigation, Data curation, Formal analysis. **Claire Holland:** Investigation, Data curation, Formal analysis. **Fanuel Kawaka:** Funding acquisition, Writing - review & editing, Methodology, Project administration. **Serene Tan:** Investigation, Data curation, Formal analysis. **Malinda Salim:** Formal analysis, Software, Writing - review & editing. **Ben J. Boyd:** Formal analysis, Software, Writing - review & editing. **Balazs Bajka:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Ana-Isabel Mulet-Cabero:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Jacob Bishop:** Conceptualization, Writing - review & editing, Funding acquisition, Methodology, Project administration. **Willis O. Owino:** Conceptualization, Project administration, Writing - review & editing, Methodology, Resources, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Ethics statements

The research did not include any human subjects and animal experiments.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104065.

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