Role of cell walls in the bioaccessibility of lipids in almond seeds

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ABSTRACT

Background: Certain nutrients and phytochemicals in almonds may confer protection against cardiovascular disease, but little is known about factors that influence their bioavailability. A crucial and relevant aspect is the amount of these dietary components available for absorption in the intestine, which is a concept referred to as bioaccessibility.

Objective: We investigated the role played by cell walls in influencing the bioaccessibility of intracellular lipid from almond seeds.

Design: Quantitative analyses of nonstarch polysaccharides (NSPs) and phenolic compounds of cell walls were performed by gas-liquid chromatography and HPLC, respectively. In a series of experiments, the effects of mechanical disruption, chewing, and digestion on almond seed microstructure and intracellular lipid release were determined. In the digestibility study, fecal samples were collected from healthy subjects who had consumed diets with or without almonds. Almond seeds and fecal samples were examined by microscopy to identify cell walls and intracellular lipid.

Results: Cell walls were found to be rich in NSPs, particularly arabinose-rich polysaccharides, with a high concentration of phenolic compounds detected in the seed coat cell wall. During disruption of almond tissue by mechanical methods or chewing, only the first layer of cells at the fractured surface was ruptured and able to release lipid. In fecal samples collected from subjects consuming the almond diet, we observed intact cotyledonary cells, in which the cell walls encapsulated intracellular lipid. This lipid appeared susceptible to colonic fermentation once the cotyledonary cell walls were breached by bacterial degradation.


KEY WORDS Almond seeds, plant cell wall, nonstarch polysaccharides, dietary fiber, phenolics, lipid digestion, bioavailability, microstructure, microscopy, chewing, fecal fat

INTRODUCTION

Studies show that diets rich in tree nuts, including almonds, have beneficial effects on risk factors for coronary artery disease (CAD) (1–7). Various nutrients and phytochemicals found in almonds and other tree nuts, including unsaturated fatty acids, plant sterols, α-tocopherol, dietary fiber, and phenolic components, could be responsible for modifying CAD risk factors (2, 3, 7). However, a major obstacle in evaluating the role of individual food components in modifying disease risk is the paucity of information on factors that influence their bioavailability (8).

One critical aspect of this information is the proportion of nutrients “released” from a complex food matrix and, therefore, potentially available for absorption in the gastrointestinal (GI) tract, a concept that was recently referred to as bioaccessibility (8). To study factors that affect bioaccessibility it is crucial to have a basic understanding of the physical and chemical changes in the food matrix during the digestive process (9–13).

In plant food tissues, the physicochemical structure and properties of cell walls (CWs) in the GI lumen are critical factors involved in influencing bioaccessibility (14, 15). Thus, for example, intact CWs of starch-rich leguminous seeds can act as a physical barrier to the action of α-amylase, thereby hindering the rate and extent of starch digestion (16, 17). In almond seeds, however, lipid is the main storage component, which comprises ~50% of the total weight of the seed and is located as intracellular oil bodies of diameter size ranging from ~1 to 5 μm (18). An important question is, therefore, do the CWs of almond tissue act as a physical barrier to the action of lipase after ingestion? Such an effect is likely to reduce the rate and extent of lipolysis in the proximal gut. Furthermore, if undigested lipid from almond tissue is transported to more distal sites of the GI tract, this could lead to increased excretion of fecal energy, which has implications for the regulation of body weight. An early, but somewhat limited, study showed that human subjects who had consumed a peanut-rich diet for 6 d excreted a significant amount of fecal lipid (19). More recent studies indicated that significant amounts of lipid were excreted in subjects on diets rich in either almonds (20) or pecans (21). However, none of these studies included an investigation of the factors that are responsible for this apparent “malabsorption,” but a decrease in lipid digestion caused by an intact CW barrier is one possible mechanism.

The physiologic effects of the almond seed CW and how intracellular lipid and other nutrients are released will depend on the physical state of the almond tissue in the gut lumen. Thus, a critical factor will be whether the CWs are disrupted during processing and chewing and in transit along the GI tract (14, 15).
In the present study, we investigated the role played by CWs in influencing lipid release during laboratory processing, chewing, and digestion of almond seeds (kernels). Quantitative chemical analyses of CWs were performed to provide some understanding of CW properties in vitro and in vivo. Also, a detailed examination of almond seed microstructure was carried out, focusing mainly on the topologic relation between the CW and the intracellular lipid. One important objective of this work was to identify undigested almond tissue in fecal material recovered from human subjects who consumed an almond-rich diet.

SUBJECTS AND METHODS

Test products

Almond seeds or kernels (Amygdalus communis L; variety, Nonpareil) selected for this study were kindly provided by the Almond Board of California. Roasted almond kernels of the same variety were also used in this investigation. All the samples were stored at a refrigerated temperature of 3–5 °C. The nutrient content (percentage by weight of edible portion) of the raw almonds was as follows: moisture 5.5%, protein 21.2%, lipid 55.1%, available carbohydrate (mainly sugars) 5.5%, dietary fiber 11.8% (derived from the CW), and ash (minerals) 3.1%. Values for moisture and lipid were obtained by proximate analysis (described in Chemical analysis) and for other nutrients from the US Department of Agriculture nutrient database (22).

Subjects

Seven human volunteers (5 men and 2 women) with a mean age (± SE) of 37 ± 5.9 y (range: 23–64 y) and an average body mass index of 24.7 ± 1.9 kg/m² (range: 17.4–32.5 kg/m²) participated in the chewing study. For the digestibility study, 3 healthy human subjects (3 men) with mean (± SE) age of 31 ± 5.4 y (range: 22–40 y) and body mass index of 24.3 ± 0.6 kg/m² participated.

Protocol of human studies

Chewing study

To assess the effects of physical disruption by chewing, a method mainly based on a technique developed by Granfeldt et al (9) was used. Each volunteer was asked not to eat for 2 h before the experiment. The subjects were instructed to rinse their mouths with drinking water (bottled still) and then chew a 2-g sample of almond seeds (both raw and roasted types chewed on separate occasions) 30 times for ≈30 s. In an earlier experiment, this technique was found to provide the conditions required to masticate the seeds to a physical consistency that allows swallowing to take place. The subjects were then asked to expectorate the contents of their mouths into a petri dish, samples of which were then immediately chemically fixed and later stained and examined by microscopy, as described in the section “Microstructural analysis.” Chewed samples from each of the 7 volunteers were examined separately. The subjects had previously given their informed consent to participate in the study, which was approved by the Research Ethics Committee of King’s College London (Reference: 01/02–26).

Digestibility study

Three human subjects consumed increasing amounts of raw almonds over a 3-d period (100, 150, and 200 g/d, which is equivalent to ≈55, 83, and 110 g/d lipid and 11.8, 17.7, and 23.5 g/d dietary fiber, respectively). Subjects were requested to limit intakes of other plant foods, to facilitate the location of the almond tissue in the fecal samples. On the day immediately after the feeding period (ie, day 4), a fecal sample was collected from each subject in a plastic bag with use of an under-seat frame and then weighed. Core biopsies of these fecal samples were immediately taken and then placed in a primary fixative within 10 min; the rest of the samples were frozen (−20 °C) and stored for future chemical analysis. In a similar manner, a fecal sample (negative control) from the same human subjects was also collected after a 3-d period, during which no almonds or other nuts were consumed. All fecal samples were coded, so that the investigators were not aware of which were the control and test samples. The fixed fecal samples from each of the subjects were examined with use of a full range of microscopy techniques, and other samples were quantitatively analyzed for macronutrients, as described in “Chemical analysis.” The subjects had previously given their informed consent to participate in the study, which was approved by the University of Toronto Research Ethics Board.

Laboratory processing of almond seeds

In the initial experiments, laboratory methods that simulate the chewing process were used to provide physically disrupted almond seed. Microscopic examination of this material provided useful information about the effects of trituration on the almond tissue under controlled conditions of physical processing. To simulate a cutting action during chewing, the whole almond seeds (raw and roasted types) were mechanically processed with use of a food processor (type 643; Moulinex, Alençon, France) for 30 s with the cutting blades set at the highest speed setting. To simulate a pulverizing action during chewing, a hand-grinding process of the almond samples was performed with use of a mortar and pestle, grinding continuously for 10 min.

To facilitate characterization of almond seeds macerated by the 2 above-mentioned methods it was decided to separate the particulate material into 4 fractions according to particle size distribution. This separation would facilitate the characterization of the almond tissue. Also, it was anticipated that more lipid would be released from the smaller particles, given the greater surface area-to-volume ratio of these particles, relative to the larger ones. These 4 fractions were separated by a mechanical sieving process (23) according to the following particle size ranges: between 3.35 and 2.00 mm, 1.99 and 1.00 mm, 0.99 and 0.5 mm, and <0.5 mm.

Microstructural analysis

The laboratory-processed, chewed, and fecal samples were examined by light, scanning, and transmission electron microscopy to assess the morphology of the CWs and the morphology and distribution of intracellular lipids released from any ruptured cells.

Laboratory-processed and chewed samples were rapidly fixed (5-min duration) in 1% (wt:vol) osmium tetroxide and then transferred to 2.5% (by vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7.2) and left overnight. Fecal samples were fixed in 2.5% (wt:vol) paraformaldehyde and 0.5% (by vol) glutaraldehyde. Subsequently, all the samples were washed twice, each time for 30 min, in 0.1 M sodium cacodylate or phosphate buffer and then postfixed in 1% (wt:vol) osmium...
tetroxide for 2 h. Samples were dehydrated in graded acetone or ethanol serial dilutions (ie, 50%, 70%, and 90%, by vol, acetone or ethanol and distilled water) for 30 min for each solution and then finally in 100% acetone or ethanol for 30 min (3 times).

For the transmission electron microscopy (TEM) and light microscopy, the almond samples were infiltrated with Spurr resin and embedded in molds and polymerized at 60 °C. Sections (1 μm for light microscopy) were cut on a Reichert Ultracut ultramicrotome (Leica Microsystems Ltd, Milton Keynes, United Kingdom), mounted on glass slides, and stained in 1% (wt:vol) Toluidine Blue, Sudan black B, or periodic acid–Schiff reagent. Sections of seed testa (skin) were also prepared and stained for lignin with use of phloroglucinol–HCl. Ultrathin sections (≈70 nm) were cut for TEM and viewed in a JEOl 100CX Mk.II transmission electron microscope (JEOL Ltd, Welwyn Garden City, United Kingdom).

For examination by scanning electron microscopy (SEM) the samples were critically point dried in a Polaron E3000 CP Drier (Quorum Technologies, Newhaven, United Kingdom), mounted on stubs, and sputter coated with gold in a Polaron E5101 sputter coating unit, and viewed in a JEOL 25SM and a Philips 501 scanning electron microscope (FEI Company, Cambridge, United Kingdom).

Chemical analysis

Cell wall components from cotyledon and seed coat of almond seeds

Cell wall material (CWM) was prepared from almond seeds (raw and roasted types) with use of methods adapted from previous work (24). Almond seeds were weighed (≈25 g of each type) and blended in 1.5% (wt:vol) sodium dodecyl sulfate (SDS) with use of a Ystral homogenizer (Ystral GmbH, Dottingen, Germany) for 5 min. A few drops of octanol were added to reduce foaming. The homogenate was filtered through a 75-μm nylon mesh. The residue was ball-milled at 0 °C in 0.5% (wt:vol) SDS at 60 rpm for 12 h. Microscopy was carried out to ensure that the CWM was free of any intracellular material. The CW residue was extracted with hot-ethanol (80 °C) and homogenized. This procedure was repeated twice. Further homogenization was carried out with residue resuspended in 200 mL deionized water. After filtering through 75-μm nylon mesh the residue was suspended in 100 mL ethanol and homogenized. This procedure was repeated twice. The residue was extracted with hot-ethanol (80 °C) and filtered through a 3-mm Whatman filter (Whatman plc, Brentford, Middlesex, United Kingdom) overlaid with a nylon mesh (75 μm). After several washes with acetone the CWM was allowed to dry overnight.

Analysis of CW sugar composition was carried out by acid hydrolysis of polysaccharides followed by reduction, acetylation, and quantitation of aldito acetates by gas-liquid chromatography, using methods adapted from Blakeney et al (25). Uronic acids were measured by the method of Blumenkranz and Asboe-Hansen (26).

Ester-linked phenolic components of CWM were analyzed by the method of Waldron et al (27). CWM (≈30 mg) prepared from almond seed was extracted with 0.1 M NaOH for 1 h at room temperature under N₂. The suspension was filtered through a Whatman (GFA) filter paper overlaid with a nylon mesh (75 μm). The residue was retained for further extraction. trans-Cinnamic acid was added (200 μL, 1.67 mg/50 mL methanol) to the filtrate as an internal standard. The filtrate was then acidified with concentrated HCl and extracted 3 times with ethyl acetate. The extracts were combined and rotary evaporated to dryness then reconstituted in 200 μL methanol and analyzed by HPLC. Previously retained residue was sequentially extracted further in 0.1 M NaOH (24 h), 1 M NaOH (24 h), and 2 M NaOH (24 h) with use of the same procedure. Extracted phenolic compounds were analyzed by HPLC (27).

Macronutrient analysis of raw almond seeds and human fecal samples collected during digestibility study

Total moisture (by oven drying) and lipid ( Soxhlet; light petroleum, bp 40–60 °C, diethyl ether) contents of the original almond seeds (on flour samples prepared with use of the Moulinex food processor, to pass through a sieve size 500 μm) were determined with use of standard methods of the Association of Official Analytic Chemists (AOAC) (28).

Frozen fecal samples were partially thawed, homogenized in a blender (Osterizer model; Sunbeam Corporation, Mississauga, Canada), freeze dried (freeze dry–shell freeze system; Labconco Corporation, Kansas City, KS), and then analyzed for moisture content and total lipids with use of methods of AOAC (28). The total moisture content of feces at the time of collection was determined by an AOAC method (28) and performed on samples that were partially thawed and homogenized only.

Statistical analysis

Paired Student’s t tests (2-tailed) were performed on the quantitative digestibility data (ie, fecal weight and fecal moisture and lipid contents). Statistical differences between the control and test (almond-containing) samples were accepted at P < 0.05.

RESULTS

Release of lipids by laboratory processing and chewing

Many of the cotyledonary cells of the almond seed tissue remained physically intact after mechanical trituration. Thus, the micrographs seen in Figure 1, A and B, were prepared from almond seed, which was physically disrupted by hand-grinding (pulverizing), and show structurally intact areas but not the fractured surfaces. Carbohydrates were located with use of Schiff’s reagent in the CW and intracellular inclusions (Figure 1A). In Figure 1B, the intracellular lipid bodies, stained with Sudan black, appear to fill much of the intracellular space. In all of the physically disrupted particles of almond tissue, the CW on the fractured surface (ie, the first layer of cells) were ruptured, so that much of the lipid was released from this location, as indicated in Figure 1, C and D. In these sections Toluidine Blue was used for staining the CW and intracellular components, including lipid, which is quantitatively the major nutrient of the seed. The lighter blue stain surrounding the seed particle section indicates lipid that was released from the ruptured cells during processing (Figure 1, C and D).

The SEM micrograph of almond samples disrupted by the cutting method revealed a surface mainly covered in lipid droplets (Figure 1E). These droplets were significantly larger in diameter size (≈10–40 μm) than the intact intracellular lipid bodies of ≈1–5 μm (TEM micrograph in Figure 2), indicating that these bodies had coalesced on the fractured surfaces of the cut
tissue. The presence of lipid on the particle surfaces (Figure 1E) indicates that the CW of the first layer was ruptured, which was seen more clearly when most of the lipid was removed by gentle washing with petroleum ether for 3–4 min (Figure 1F).

Examining the almond tissue over a range of particle sizes provided no evidence of cell separation, which is consistent with the view that cotyledonary cells are ruptured at the fractured surface, releasing intracellular lipids. The only explanation of this is that the cell-cell adhesion of the almond tissue is very strong, so that the cells can only be disrupted by breakage of the CW.

Similar results to those seen with use of laboratory processing were obtained under the physiologic conditions of chewing. Thus, in the samples of almond tissue that was masticated by healthy human subjects, the micrographs clearly showed cell breakage at the surface of the particles, rather than cell separation of whole cells (Figure 2). The micrograph in Figure 2A shows that the first layer of cells was disrupted by chewing, releasing lipid material from cells with ruptured CW, but many of the underlying cells remained physically intact with no evidence of structural changes to the CW or the intracellular contents. This lack of structural change can be seen more clearly in Figure 2B where the intact cells were examined at higher magnification. However, between these intact cells and the fractured layer are cells that appear to be damaged or distorted by chewing, with some showing evidence of ruptured CWs and perhaps even signs of lipid release (Figure 2A). The SEM micrographs (Figure 2, C and D) show the fractured surface of the almond cotyledon tissue with many of the individual cells filled with lipid. However, in some cells the intracellular contents were partly or wholly removed, presumably as a result of the chewing process. The micrograph in Figure 2D also indicates the distinctive epidermis of the testa or seed coat. The lipid droplets and CW can also be seen in these micrographs. The TEM micrographs (Figure 2, E and F) provide more structural details of the intracellular lipid bodies. The micrograph in Figure 2E shows the fractured surface of one of the almond particles, with its CW being ruptured by mastication and lipid bodies released from the cells, some of which have coalesced to form larger oil droplets. However, the micrograph in Figure 2F shows that the cells of tissue underlying the fractured surface are intact, as are the lipid bodies, with little evidence of lipid release. Also, it is important to note the presence...
of a thickened middle lamella at the cell junctions, indicative of a high concentration of pectic material (Table 1), which is likely to play an important role in maintaining cell-cell adhesion.

In all the microscopic sections examined, no differences in microstructure were observed between raw and roasted almond seed samples, whether in an intact or a physically disrupted state.

**Digestibility of almond seeds in healthy human subjects**

In fecal samples obtained from the human volunteers, the presence of almond seed tissue was positively identified (Figure 3, A–F). Indeed, in all the samples we observed an abundance of almond tissue, largely comprising structurally intact CWs enclosing intracellular components, including lipids. The CWs are composed mainly of nonstarch polysaccharides (ie, major constituents of dietary fiber), which by definition are not digested by endogenous enzymes of the upper GI tract. In this experiment an unknown quantity of CW tissue and intracellular substances also escaped fermentation in the large intestine. The micrographs of almond seed tissue stained with Toluidine Blue (Figure 3, A and B) show clear evidence of physically intact cells, which have...
TABLE 1
Concentration of sugar (monosaccharide) residues (dry weight) in the hydrolyzed cell wall material of raw and roasted almond seeds and almond seed testa (skin)

<table>
<thead>
<tr>
<th></th>
<th>Rhamnose</th>
<th>Fucose</th>
<th>Arabinose</th>
<th>Xylose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Uronic acids</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw almond</td>
<td>23.6</td>
<td>10.2</td>
<td>259.2</td>
<td>46.2</td>
<td>7.0</td>
<td>30.1</td>
<td>147.6</td>
<td>201.4</td>
<td>725.3</td>
</tr>
<tr>
<td>Roasted almond</td>
<td>31.6</td>
<td>13.9</td>
<td>335.1</td>
<td>64.0</td>
<td>9.4</td>
<td>40.2</td>
<td>174.6</td>
<td>205.7</td>
<td>874.5</td>
</tr>
<tr>
<td>Raw almond skin</td>
<td>22.8</td>
<td>8.0</td>
<td>143.1</td>
<td>25.0</td>
<td>10.3</td>
<td>26.0</td>
<td>157.7</td>
<td>199.7</td>
<td>592.6</td>
</tr>
<tr>
<td>Roasted almond skin</td>
<td>19.5</td>
<td>4.3</td>
<td>137.7</td>
<td>23.7</td>
<td>9.3</td>
<td>25.0</td>
<td>159.8</td>
<td>215.5</td>
<td>594.8</td>
</tr>
</tbody>
</table>

*Values for the total and each sugar concentration are the means of 4 replicates; the CV of the replicates was <3%.

Concentrations of fecal lipids in human subjects on control and almond-rich diets

All results in this section are presented as mean (± SE) values. In the digestibility study, the total wet weight of fecal samples collected from human subjects on the control and almond-rich (test) diets was 70 ± 20 and 196 ± 126 g, respectively (NS). The moisture content of fecal samples from subjects on the control and almond diets was 75.2 ± 3.1 and 72.7 ± 2.4%, respectively (NS). Analysis of the fecal samples showed a marked increase in lipid concentration in subjects on the almond-rich diet. Thus, the total lipid concentrations of fecal samples (wet weight basis) from subjects on the control and almond-rich diets were 3.5 ± 0.9% and 9.9 ± 1.1%, respectively (P < 0.015). On the basis of the total fecal weight values, the actual weight of lipid in the control feces was 2.8 ± 1.5 g, and in the test feces it was 21.4 ± 14.4 g.

Nonstarch polysaccharides (dietary fiber) and phenolic components of almond seed cell wall material

The concentrations of monosaccharide residues of the hydrolyzed CWM of seed cotyledon and testa were determined with use of gas-liquid chromatography. The predominant sugars found in the raw and roasted almond cotyledon were arabinose, uronic acids, glucose, xylose, galactose, rhamnose, fucose, and mannose, and in the skin they were uronic acid, arabinose, glucose, xylose, galactose, rhamnose, mannose, and fucose (concentrations shown in Table 1). The arabinose, rhamnose, and uronic acid contents represent more than 60% of the total sugar content, indicating the presence of one or more arabinose-rich polysaccharides, including pectic material. The presence of the latter is consistent with the relatively thick middle lamella at CW junctions, as seen in Figures 2F and 3F. The relatively high concentration of glucose found in the CWM suggests the presence of either cellulose or perhaps even a mixed linkage β-glucan. Another possibility is that the glucose could also form the cellulose backbone of a xylglucan, because xylose and galactose residues were also identified in the CWM preparations. The ratio of glucose, xylose, and galactose (3.19:1.00:0.65) is similar to ratios reported for the xylglucan group of CW storage polysaccharides (29, 30). The sugar concentrations found in the hydrolyzed CWM extracted from the roasted and raw almond samples were similar.

Several phenolic compounds, mainly protocatechuic acid, p-hydroxybenzoic acid, and vanillic acid, were identified in the CWM of almond skin, but not in the cotyledonal tissue in which only trace amounts were recorded. The mean concentrations (duplicate values) of the main phenolic components protocatechuic acid, p-hydroxybenzoic acid, and vanillic acid were 0.64, 0.46, and 0.10 µg/mg, respectively. A positive stain with use of phloroglucinol-HCl was observed for lignin in the same skin samples.

DISCUSSION

This paper provides an explanation of why in one study significant amounts of lipid were excreted in feces obtained from healthy human subjects who consumed an almond-rich diet (20). Microstructural examination of almond tissue showed that the CWs remained intact, except for the fractured surface and some of the underlying cells, even after laboratory processing and survived degradation by digestive secretions and microbial enzymes. The stain also picked up the presence of intestinal bacteria, which appear to be using the CWs and also the intracellular nutrients, including lipid. Interestingly, the slightly darker blue and more uniform staining on the periphery of the almond section reveals cotyledonal cells that are less packed together. Indeed, there is evidence of cell separation, caused possibly by bacterial degradation of the middle lamella, which is likely to consist mainly of pectic material [described in Results section under Nonstarch polysaccharides (dietary fiber) and phenolic components of almond seed cell wall material]. Further evidence of loss of the middle lamella and cell separation can be seen in the TEM micrographs (Figure 3, E and F).

More detailed pictures of microbial action on specific structures of the almond seed can be seen in the SEM micrographs. In Figure 3C, the relatively smooth surface of the seed coat or testa, which contains pigments and phenolic compounds, shows little evidence of bacterial degradation. Thus, no obvious structural changes were observed compared with structural changes seen in micrographs of predigested testa (Figure 2D). Also, there was little evidence of bacteria growing on the surface, because the number of bacteria observed in this location appeared to be very low. In contrast, large numbers of bacteria were detected on the CW surfaces of cotyledonal tissue. It appears that these bacteria could well be active, given that they co-locate with partially digested (fermented) CW polysaccharides (Figure 3D). In this case, microbial degradation created numerous holes in the CW, thus allowing microflora to enter contiguous cells and then use other potential substrates, notably the intracellular lipid. The TEM micrographs (Figure 3, E and F) can provide some insight into the behavior of these bacteria, in that in cells in which the CWs were largely but not completely intact, much of the intracellular contents appeared to be used by bacteria. In some cells, all the cellular contents had virtually disappeared with only the CWs surrounding the bacteria.
digestion in the GI tract. Thus, in the digestibility study, intact almond seed cotyledon and testa (seed coat) were identified in fecal samples obtained from subjects on the almond-rich diet. Moreover, intracellular lipid bodies that were observed in the original almonds were also positively identified in the same fecal samples. These results substantiate our original hypothesis that an increase in fecal fat excretion in subjects on a diet containing almonds is attributed to intact CWs hindering the digestion of intracellular lipid.

The lipid fraction of almonds constitutes the largest proportion of available energy in the seed. In people consuming a diet containing almonds, any fat that escapes digestion in the small intestine could represent an appreciable loss of energy. However, a proportion of this energy is likely to be salvaged in the large

FIGURE 3. Sections of almond tissues found in feces collected from healthy subjects. Panels A and B are light microscopy (LM) micrographs, which were stained with toluidine blue. (A) Shows intact almond tissue containing intracellular lipid and other nutrients and almond tissue surrounded by fecal bacteria. (B) Similar LM section to that seen in panel A, except showing the almond tissue at higher magnification and some evidence of cell separation of the outside layers of the tissue. In some parts of the tissue, it appears that the cell walls are ruptured, releasing lipid, and bacteria are located inside the cells. (C) Scanning electron microscopy (SEM) micrograph showing the epidermis of the seed coat, largely undamaged by endogenous secretions of the gut and by bacterial fermentation; few bacteria appear to be growing on the epidermal surface. (D) Similar SEM section to that seen in panel C, but showing the surface of the parenchyma cells, with bacterial fermentation appearing to erode the cell walls of fractured and contiguous cells. Numerous bacteria are seen to be growing on the cell wall surface. (E) TEM section showing fecal bacteria that have digested the cotyledonary cell walls and gained access to the nutrients inside the cell. (F) Similar TEM section to that seen in panel E, except that in one cotyledonary cell it appears that the bacteria have used all the intracellular nutrients, including the lipids. It also appears that bacterial enzymes have eroded the middle lamella, which has contributed to cell separation. Scale bars for micrographs A–F = 20 μm. BT, bacteria; CW, cell wall; SC, seed coat (testa).
intestine, where lipid that is more accessible after cell rupture could be used by resident microflora. Nevertheless, in the present study we observed a marked increase in fecal fat excretion in healthy subjects eating almonds relative to the control diet. This result is consistent with data from Zemaitis and Sabaté (20) and Haddard and Sabaté (21), who reported increases in fecal fat excretion in healthy subjects who consumed diets enriched with almonds or pecans. Because almonds and other tree nuts are considered to be high-fat foods and concern was expressed that high-fat diets could cause weight gain (31, 32), these results are of particular interest to health professionals concerned with obesity management. The importance of the health implications is underlined by the results of a recent study in which overweight and obese subjects, consuming a formula-based low-energy diet, showed greater weight loss on a diet enriched with almonds rather than complex carbohydrates (33).

After physical disruption by in vitro methods or by chewing, the cotyledonary cells of the almond seeds were seen to rupture, rather than separate, as seen in many of the micrographs. The process by which almond tissue fractures, like all plant tissues, depends on CW strength and cell-cell adhesion (15). Thus, in fruits and vegetables that are crisp or crunchy the process of tissue fracture occurs by cell rupture. However, during cooking and ripening of these plant foods a process of cell separation can occur, which leads to tissue softening, and is attributed mainly to the weakening of pectic substances involved in cell-cell adhesion (15, 34).

In the present study, the rupturing of CWs was manifest predominantly in the outer layer of almond seed particles, reflecting the fracture planes that were created during disruption. This rupturing resulted in the exposure and release of intracellular lipid bodies, which coalesced to form larger oil droplets (size range, \( \approx 10-40 \mu m \)). We presume that lipid from ruptured cells is much more accessible for emulsification and subsequent digestion by pancreatic lipase in the small intestine (35). The potential bioaccessibility of this lipid was demonstrated by the ease with which lipid could be solubilized and removed when the fractured surface of the almond tissue was gently washed with petroleum ether. This ease of removal of lipid from the fractured layer contrasted with the retention of intracellular lipid in intact cotyledonary cells after solvent washing. Moreover, in the digestibility study, the fractured surfaces of almond tissue examined in human feces were relatively free of coalesced lipid droplets. This finding suggests that lipid from ruptured cells was bioaccessible and, therefore, digested and absorbed, perhaps partly facilitated by the action of microbial enzymes. In contrast, however, there was no evidence that intracellular lipid retained by the CW barrier was degraded by lipases. In future studies, it will be necessary to quantify the extent and rate of release of lipids from the almond tissue and other lipid-bearing plant foods. The kinetics of lipid digestion and absorption is considered to be of importance in influencing postprandial lipemia, which can itself influence the atherosclerotic process of CAD (36, 37).

The encapsulation of intracellular lipids and other nutrients by CWs is not only likely to prevent their digestion in the upper GI tract, but also, from the results of our digestibility study in humans, it seems that a significant proportion of CWs and cell contents survived the fermentation process in the large intestine. However, the fecal samples provided indirect, but likely, evidence of bacterial fermentation of the almond tissue, including intracellular contents. This evidence is based on the presence of bacteria on the surface of CWs that appear to be actively growing and severe erosion of the walls, exposing the inside of the cells (micrograph in Figure 3D). Furthermore, the micrographs produced by TEM clearly show the presence of bacteria inside the cells, apparently “using” the intracellular contents.

These results raise interesting questions about the types of bacteria that use substrates from the almond seed, the extent to which these substrates are fermented, and the types of products that arise from this process (eg, short-chain fatty acids). Because the cells are particularly rich in lipid, it would be surprising if bacteria did not exploit this carbon source for metabolic purposes. Moreover, it was known for some time that polyunsaturated fatty acids can influence the growth and mucus adhesion of intestinal microflora and probiotic supplements (38–40). Although the triacylglycerols of almond lipids contain some polyunsaturated fatty acids, mainly linoleic acid, the predominant fatty acid is oleic acid, a monounsaturated variety comprising >65% of the total oil fraction (41). As far as we are aware the effects of almond seed lipids on bacterial growth and properties have not been investigated.

Evidence of microbial degradation of the almond seed CW is consistent with the plethora of data showing that CW constituents (ie, nonstarch polysaccharides) are fermented to a variable degree in the large intestine (42). This evidence raises a further interesting question about which polysaccharides are most susceptible to fermentation. The partial susceptibility of pectic substances to degradation by the gut microflora is borne out by the erosion of the middle lamella seen in the fecal samples, which can partly explain the cell separation seen in the outer layers of the particles of almond tissue. However, the effects of endogenous secretions of the gut and physical mixing as a result of peristalsis are also likely to play a role in any cell separation process. Other CW polysaccharides could also be susceptible to microbial degradation. However, further studies are required to establish the structures and relative proportions of polysaccharides in the almond seed CW, which can then be more closely linked to their susceptibility to microbial fermentation.

The degradation of the CW can also be significantly influenced by the presence of noncarbohydrate compounds, such as phenolic components (43), which are known also to have strong antioxidant properties (44, 45). In the current study only trace amounts of phenolic compounds were found in the cotyledonary tissue. This finding indicates that the considerable quantity of arabinose-containing polysaccharides lack significant phenolic substitution. However, significant concentrations of protocatechuic acid, \( p \)-hydroxybenzoic acid, and vanillic acid were found in the CW of the seed coat (skin). Also, (Klason) lignin was positively identified in the same skin samples. Some of these compounds are likely to play an important structural role and, therefore, modify the mechanical properties of the CW (15, 34), which could, in turn, confer greater resistance to bacterial fermentation and lipid release. Some evidence of this resistance comes from the microstructural examination of the seed coat, which appears to be largely intact in the fecal samples provided by the human subjects. Furthermore, the number of bacteria observed on the testa surface in the fecal samples appeared to be relatively low, certainly compared with that found on the surface of cotyledonary CWs. At this stage we are not able to quantify these observations.

In conclusion, we have successfully identified almond seed tissue in fecal material collected from healthy subjects on an
almond-rich diet. The main structures of almond tissue were found to be preserved after laboratory processing or even after chewing and digestion. In particular, the CWs were intact and hindered the release of intracellular lipid. Thus, an increase in fecal fat excretion after consumption of an almond-rich diet is primarily attributed to impairment of lipid bioaccessibility, as a result of an intact CW barrier.

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All the authors participated in the experimental design, the collection and interpretation of data, and the writing of the manuscript. Specifically, PRE and CWCK were involved in the design and data interpretation of the study overall and took the main responsibility for writing the manuscript. YR performed the laboratory processing and chewing experiments. DIAJ and CWCK were responsible for the digestibility part of the study. CP and KWW were responsible for the cell wall analysis. JFP was responsible for development of techniques and preparation of samples for microscopy analysis. None of the authors had any financial or personal conflict of interest.

REFERENCES