Supplementary Information (SI) for Food & Function.
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SUPPLEMENTARY MATERIAL

Almond Oil Extraction Residues as Functional Ingredients: Nutritional Composition, Polyphenol *In Vitro* Bioaccessibility, and Biological Activities

Rafael Mascoloti Spréa, Daniele B. Rodrigues, Manuel Román, Miguel A. Prieto, Joana S. Amaral, Lillian Barros

S1. Methodological details concerning the experimental procedures and analytical techniques employed in this study

1. Chemicals and reagents

All solvents and reagents used in the analyses, including those of analytical and LC/MS grade, as well as standards of phenolic compounds, soluble sugars, organic acids, tocopherols, and fatty acid methyl esters (FAME mix, Supelco FAME-MIX, 47885-U), were acquired from Sigma-Aldrich (St. Louis, USA), unless otherwise specified. Enzymes, namely α-amylase (cat# A1031, experimental activity 756 U/mg solid), pepsin (cat# P6887, experimental activity 2,014 U/mg solid), and pancreatin (cat# P7545, experimental activity 10.1 U/mg solid), along with bile salts (cat# B8631, 100 mmol/g), were also purchased from Sigma-Aldrich. Their experimental activities were determined immediately before use, following the protocol described by Brodkorb et al. (2019)[1], to ensure reproducibility and adherence to standardized digestion procedures. The commercial standard tocol was procured from Matreya (Pleasant Gap, USA).

2.3. Chemical parameters

2.3.1. Proximate composition and energetic value

The moisture content was measured using a moisture analyser (PBM, Adam Equipment, Oxford, USA). Ash, crude protein and total fat were determined according to AOAC methods [2]. Specifically, ash content was determined by gravimetry after sample incineration in a muffle furnace (550 °C, Lenton ECF 12/22, Hope Valley, UK), crude protein was calculated using a nitrogen-to-protein conversion factor of 6.25 after total nitrogen determination in a Kjeldahl distiller (Pro-Nitro-A, JP Selecta, Barcelona), and total fat was quantified by gravimetry after extraction in a Soxhlet apparatus (Behr Labor Technik, Dusseldorf, Germany).
FAMEs were identified based on the relative retention times of sample peaks with those of a standard mixture containing 37 FAMEs (47885-U, Sigma, St. Louis, USA). Results were expressed in relative percentage of each fatty acid.

2.3.6. In vitro digestion

A sample of approximately 2.5 g of each almond oil by-product or 2.5 g of distilled water as blank was combined with 2.5 mL of simulated salivary fluid and homogenized to achieve a paste-like consistency, serving as the initial material for the digestion process. Briefly, enzyme and bile solutions were freshly prepared prior to use to ensure accurate concentrations. The oral digestion phase was performed at 37 °C for 2 min on an orbital shaker set to 180 rpm. The pH of the mixture, consisting of the homogenized sample in water (1:2, w/w), was adjusted to a range of 5.6–6.9 using 0.1 M NaOH to optimize enzymatic activity. A volume of 600 µL of α-amylase solution (100 U/mL) was added to simulate salivary digestion. The gastric phase began by adjusting the pH to 3.0, followed by the addition of 50 µL of pepsin solution (25 mg/mL). The samples were then incubated under the same conditions as the oral phase (37°C, 180 rpm) for 60 min. Subsequently, the intestinal phase was initiated by raising the pH to 6.0 using 1 M $NaHCO_3$. Pancreatin (2 g/L; 5 mL) and bile salts (12 g/L; 2.5 mL) were added at a final concentration of 250 µL/mL to replicate pancreatic enzyme and bile activity in the small intestine. The mixture was incubated at 37 °C on an orbital

shaker at 180 rpm for 120 min. To stop enzymatic activity, reaction tubes were immediately placed in an ice bath after each digestion phase.

2.3.7. Prebiotic activity

For the assay, cultures were incubated for at least 3 days, followed by subculturing into fresh medium 12 h before the assay to ensure growth in the logarithmic phase. Then, the inoculum was adjusted to 0.5 McFarland turbidity (1.5×10^8 CFU/mL) in a densitometer (BEN-1B, Biosan, Riga, Latvia) and added to the sample or control solutions to achieve a final probiotic concentration of 5×10^5 CFU/mL. The mixtures were dispensed into U-bottom 96-well plates (200 µL/well), with 50 µL of sterile liquid paraffin added to each well to maintain anaerobic conditions. Additional controls included MRS broth containing 20 g/L glucose (positive control) and sample/control solutions without inoculum (blank controls). Prior to the assay, the strains were activated under anaerobic conditions in the same medium, with 0.05% L-cysteine added for B. animalis Bb12. Incubation was carried out at 37 °C under anaerobic conditions, with absorbance measurements at 620 nm registered every hour. After 48 hours, the data were analysed (Spectrostar nano, Ortenberg, Germany), and growth curves were generated, as previously described by Spréa et al. (2024) [6].

Table S1. Maximum growth rates (μmax , h^{-1}) of probiotic strains in prebiotic activity assays.

Probiotic strain	Positive control						Samples											
	GLU		FOS		Inulin		APC				APC 20				APC 24			
							Non-digested		Digested		Non-digested		Digested		Non-digested		Digested	
	Time (h)	OD	Time (h)	OD	Time (h)	OD	Time (h)	OD	Time (h)	OD	Time (h)	OD	Time (h)	OD	Time (h)	OD	Time (h)	OD
Bifidobacterium animalis ssp. lactis Bb12	20	2.87	42	1.44	42	2.33	38	2.26	48	2.10	40	2.24	30	1.86	42	2.11	42	2.29
Lactobacillus plantarum	21	3.00	40	1.72	33	2.39	33	2.15	40	1.72	21	2.11	31	2.15	40	2.22	31	1.55
Lactobacillus casei	21	3.19	26	1.65	33	2.35	36	1.67	33	1.66	30	2.03	30	2.10	37	1.83	38	1.58
Lactobacillus acidophilus LA-5	32	3.06	42	1.59	23	2.84	25	2.59	42	2.00	42	2.24	31	1.82	31	2.19	40	2.80

Maximum optical densities (OD, recorded at 620 nm) at the corresponding incubation times (in hours) of prebiotic activity assays. Probiotic strains were grown in medium containing either GLU (glucose) or FOS (fructooligosaccharides) and Inulin as positive controls, or samples (APC, APC20 and APC24 digested and non-digested) at 20 mg/mL (2% w/v) during 48h.

Table S2. Polyphenol concentration in bioaccessible fractions of almond by-products

Peak	Rt (min)	Tentative identification	Quantification (μg/g sample)		
			APC	**APC20**	**APC24**
1	4.77	Protocatechuic acid	15.83±0.89	16.88±0.34	19.42±2.22
2	5.17	Chlorogenic acid	4.62±0.27	5.06±0.54	4.65±0.37
3a	6.26	Amygdalin	31.26±1.81	32.07±1.74	31.42±2.01
3b		3-Hydroxy-1-(4-hydroxyphenyl)propan-1-one			
3c		Coumaric acid isomer			
3d		Vanillic acid-*O*-glucoside			
4a	6.53	Dihydroxybenzaldehyde	2.75±0.09	2.92±0.15	2.96±0.25
4b		Benzyl-*O*-hexosyl-pentoside			
4c		Lariciresinol hexoside			
5a	7.23	Vanillic acid	3.93±0.26	4.14±0.16	3.99±0.28
5b		4-Hydroxy acetophenone			
6a	7.69	Caffeic acid	3.07±0.22	3.34±0.29	3.33±0.29
6b		Vanillin			
7a	11.71	Secoisolariciresinol hexoside	111.33±6.61	110.79±12.24	104.38±10.26
7b		Procyanidin tetramer			
8a	12.40	*p*-Coumaric acid	6.98±0.67	7.04±0.58	6.93±0.44
8b		Shikimic acid			
8c		Isovanillin			
8d		Syringaldehyde derivative			
9a	13.17	Phenyllactic acid	6.14±0.18	5.77±0.37	5.67±0.57
9b		Syringaldehyde			
10a	14.53	Quercetin-3-*O*-glucoside	7.61±0.88	8.04±0.52	9.11±0.36
10b		ni			
11a	14.79	Taxifolin	24.58±0.57	20.98±1	21.36±1.33
11b		7-Hydroxysecoisolariciresinol			
12	16.9	Isorhamnetin-*O*-rutinoside	1.61±0.02	1.21±0.1	1.17±0.06
13a	17.65	7-Hydroxymatairesinol	2.37±0.12	2.41±0.09	2.57±0.26
13b		ni lignan			
14a	18.33	Isorhamnetin-*O*-hexoside	4.33±0.14	4.57±0.34	2.32±0.19
14b		ni lignan			
		Total phenolic content	226.4±5.27	225.21±16.27	219.29±18.28

APC: almond press-cake; APC20: almond press-cake submitted to SFE extraction at 20MPa; APC24: almond press-cake submitted to SFE extraction at 24MPa. Data are presented as mean ± SD (μg of phenolic compounds in

bioaccessible fractions per g of sample (starting materials, APC, APC20 and APC24, which were subjected to digestion). Peaks numbered according to the Table 2.

References

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