PAPER

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Screening of the effect of new food prototypes based on fermented lentil and quinoa flours on the colonic microbiota of older adults through a static *in vitro* colonic fermentation model†

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In view of the increasing demand for plant-based protein alternatives, along with the increased protein requirements of older adults, the formulation of new food concepts based on protein-rich ingredients from legumes or pseudo-cereals seems a promising approach. Previous studies have found that solid-state fermentation can improve the nutritional value and digestibility of plant-based commodities; however, scarce evidence exists regarding the effect on prebiotic potential. This study aimed to compare the effect of fermented and unfermented quinoa and lentil flours on the colonic microbiota, as well as that of new food prototypes (gels and breads) made with the flours. After simulating static colonic fermentation of the substrates by using a pool faecal inoculum obtained from four older adults, the microbiota composition (16S rRNA gene sequencing) and short-chain fatty acids (SCFA, gas chromatography GC-FID) were determined. The results showed statistically significant changes in the microbiota at the genus taxonomic level in the range of −5.8% to +17.6% relative abundance. Common findings in all experiments were increased *Bacteroides*, *Acidaminococcus* and *Parabacteroides* and decreased *Asteroleplasma*, Oscillospiraceae UCG-002 group and *Alistipes*, as well as increased SCFA production. Overall, the food matrix (flour, gel or bread) was the variable that most affected the changes in the microbiota composition and diversity, while the impact of fermentation of the lentils or quinoa was not statistically significant for most cases. Concretely, the gel-like prototypes produced the most beneficial changes in microbiota composition, while the bread-like formulations were more favourable for increased SCFA and decreased bSCFA production. In conclusion, new food prototypes based on lentil and quinoa flour could have beneficial prebiotic potential. However, few additional advantages were observed from previous solid-state fermentation for obtaining the fermented lentils and quinoa flour.

1. Introduction

Malnutrition represents a global challenge that affects various sectors of the population, including children and the elderly, which are among the most vulnerable groups, although the reasons for this are different depending on the geographical region.[1] In developed countries, the risk of malnutrition is present mainly in the oldest population due to several factors, including physiological aspects, such as loss of oral processing capacity and reduced efficacy in the digestion process, which lead to decreased nutrient uptake, and psycho-social aspects such as loneliness and depression, which trigger reduced food intake or inadequate dietary choices.[2–4] In this context, various strategies to supply older people with adequate foods that can meet their specific nutritional needs, such as higher protein content, have been proposed. Identifying food ingredients with a lower environmental impact to replace the traditional animal sources of protein (*e.g.*, meat, fish, and eggs) is also a current research target.[5] The conjunction of these two premises points to plant-based ingredients that are rich in protein, have a lower carbon footprint, and contain dietary fibre. The presence of dietary fibre in plant-based ingredients and foods makes them of special relevance in the context of the Western diets of developed countries, in which low intake

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of this nutrient is a common trend. According to the World Health Organization, adults should have a fibre intake of 25 grams per day.[6] Dietary fibre is found naturally in fruits, vegetables, legumes, nuts, and wholegrain cereals. These food matrices present textural properties that make them difficult for older adults to masticate and swallow. This is a possible reason why the dietary fibre intake does not meet the recommendation in this population group.[3] Therefore, developing new foods from plant-based ingredients would also help to tackle the challenge of meeting fibre intake recommendations. In turn, if such plant-based ingredients are derived from sources rich in protein, such as legumes or pseudo-cereals, the recommendation for increased protein intake would be also met.

For these reasons, in recent years, several studies have been conducted in the field of new food concepts made of plant-based ingredients. Examples include bars made of pumpkin seeds or yogurt-like prototypes made with hemp flour.[7,8]

Despite the progress that has been achieved, for these protein-rich plant-foods to be suitable for older adults, the digestibility of their proteins should be enhanced, as the impaired digestive systems of older people hinder protein bioavailability.[9] One strategy that has been explored to overcome this issue relates to the application of the fermentation of plant-based ingredients prior to the formulation of the food, which has been proven to increase digestibility.[10] One research line utilising the solid-state fermentation of legumes with *Pleurotus ostreatus* has demonstrated increased protein digestibility, along with decreased glycemic index. The enzymatic activity of the fungus on the substrate leads to the breakdown or partial hydrolysis of proteins, facilitating greater digestibility during gastrointestinal digestion. In addition, the fungus biomass also increases the protein content of the final product, and it is presumed that this type of protein is also easier to hydrolyse during digestion.[11–14]

However, a research gap related to the prebiotic potential of these foods remains. While it is well-known that the fibre in legumes and pseudo-cereals can lead to improved microbiota ecosystems and metabolic activity, it is currently unknown if their prebiotic potential changes after applying solid-state fermentation.

Therefore, the aim of the present study was to assess the prebiotic potential of fermented lentil and quinoa flours and some new food concepts containing these flours on the colonic microbiota of older adults in comparison to their non-fermented counterparts by using a static *in vitro* colonic fermentation model.

2. Materials and methods

2.1. Flours and food prototypes

Fermented lentil (*Lens culinaris*, Castellana variety) and quinoa (*Chenopodium quinoa Wild*, white variety) flours were obtained *via* solid-state fermentation (SSF) with the edible fungus *Pleurotus ostreatus* as previously reported.[11] After the incu-

bation period, the substrates were subjected to air-drying at 70 °C and milled using a food processor (Vorwerk, Thermomix® TM6-1, Wuppertal, Germany) at 10 000 rpm at 15 s intervals for 1 min. These conditions were selected based on previous studies.[12,13] The control flours were obtained by milling the commercial unfermented grains under the same conditions. The compositions of lentil and quinoa flours are reported in Sánchez-García *et al.*[12,13]

The gel-like prototypes were formulated as follows: a Mambo 8090 Multifunction food processor was used to mix all the ingredients (flour, water, extra virgin olive oil (EVOO), vinegar, bicarbonate) with water for 15 min at 90 °C and speed 4. This dough was then allowed to cool while stirring at speed 4 for 6 min. After this, the contents of the containers were poured into molds and gelled for 24 hours at 4 °C. The bread-like prototypes were prepared by mixing all the ingredients with a kitchen blender (Mambo 9090, Cecotec Innovaciones S. L., Valencia, Spain) for 1 min at 1100 rpm, adding the ingredients in the following order: flours, salt, sugar, yeast, water, and EVOO, making sure that the water was poured over the yeast to activate it. The water was previously heated with another blender (Thermomix, Vorwek Spain, M.S.L., Madrid, Spain) to a constant temperature of 40 °C before mixing with the other ingredients. After mixing, the batters were transferred into plastic bags, and 80 g of each was piped into aluminium cups. These were kept at 30 °C for a 1 h fermentation and subsequently baked at 160 °C for 16 min in an oven (Smeg Model ALFA144GH1, Smeg S.p.A., Guastalla, Italy), which was preheated to 160 °C for 30 min in humidity conditions. The breads were left at room temperature for 1 hour and then kept in resealable plastic bags for 24 h for the next-day analyses.

The formulations (in percentage of ingredients) of six gel-like and four bread-like prototypes are listed in Table 1, while their proximal compositions in terms of water, protein, fat, fibre and NaCl (g per 100 g) are shown in Table 2. As noted, the compositions of the prototypes elaborated with fermented flours differed from those of their unfermented counterparts due to the biotransformation of the components induced by solid-state fermentation with *P. ostreatus*, which mainly led to increased protein content.

In addition, the fungus plays a crucial role in the breakdown of lignocellulosic materials, which explains the reduction in insoluble fibre in lentil flour after fermentation. However, in quinoa flour, an increase in total fibre occurs at the expense of the formation of resistant starch.[12,13,15]

2.2. Static *in vitro* colonic fermentation

Prior to simulating colonic fermentation, the flours and prototypes were subjected to simulated gastrointestinal digestion using a static *in vitro* model specific to older adults.[16] After this process, the digest was centrifuged to obtain the liquid fraction containing the bioaccessible compounds (supernatant) and a solid pellet representing the undigested fraction. Then, *in vitro* colonic fermentation was carried out following the protocol described by Pérez-Burillo *et al.*[17] For this purpose, 0.5 g of the solid undigested fraction *in vitro* with

Table 1 Ingredient composition of the gel-like and bread-like prototypes (values expressed as weight %)

	Prototypes	Water	Quinoa flour	Lentil flour	Wheat flour	EVOO	Vinegar	Baking soda	Sugar	Instant yeast	Salt
Gels (G)	Fermented/unfermented quinoa gel (FQ-G/UQ-G)	67.3%	25%	—	—	4%	3%	—	—	—	0.7%
	Fermented/unfermented lentil gel (FL-G/UL-G)	68.8%	—	25%	—	4%	—	1.5%	—	—	0.7%
	Fermented/unfermented quinoa and lentil gel (FQL-G/UQL-G)	70.3%	12.5%	12.5%	—	4%	—	—	—	—	0.7%
Breads (B)	Unfermented lentil bread (UL-B)	38.37%	—	25.83%	25.83%	5.9%	—	—	1.85%	1.04%	1.18%
	Fermented lentil bread (FL-B)	44%	—	23.47%	23.47%	5.36%	—	—	1.67%	1.04%	1.08%
	Unfermented quinoa and lentil (UQL-B)	38.37%	12.92%	12.92%	25.83%	5.9%	—	—	1.85%	1.04%	1.18%
	Fermented quinoa and lentil bread (FQL-B)	38.37%	11.73%	11.73%	23.47%	5.9%	—	—	1.85%	1.04%	1.18%

Table 2 Proximal composition of the six gel-like and four bread-like prototypes made with unfermented and fermented lentil and/or quinoa flours (composition is expressed as g per 100 g of product)

	Prototypes	Water	Protein	Fat	CHO	Fibre	Ashes
Gels (G)	Unfermented quinoa gel (UQ-G)	72.3	3.2	5.6	17.7	1.7	0.7
	Fermented quinoa gel (FQ-G)	71.6	4.3	4.6	18.9	3.6	0.7
	Unfermented lentil gel (UL-G)	70.9	6.0	4.3	15.8	4.0	0.7
	Fermented lentil gel (FL-G)	70.5	7.2	4.5	15.7	3.6	0.7
	Unfermented quinoa/lentil gel (UQL-G)	72.4	4.6	5.0	16.7	2.8	0.7
	Fermented quinoa/lentil gel (FQL-G)	71.8	5.7	4.5	17.3	3.6	0.7
Breads (B)	Unfermented lentil bread (UL-B)	37.9	9.9	7.5	42.4	5.4	1.3
	Fermented lentil bread (FL-B)	42.4	10.4	7.1	38.9	4.6	1.2
	Unfermented quinoa/lentil bread (UQL-B)	37.9	8.3	8.3	36.7	4.1	1.3
	Fermented quinoa/lentil bread (FQL-B)	42.3	8.8	7.2	35.0	4.6	1.2

10% of the supernatant was used as a substrate, which was added to fermentation vials. The fermentation vials also contained 2 mL of faecal inoculum from a pooled faecal sample and 7.5 mL of a fermentation medium composed of peptone (14 g L^{-1}), cysteine (6.24 mg mL^{-1}), sodium sulfide hydrate (6.24 mg mL^{-1}), and resazurin (1 mg mL^{-1}) (Sigma-Aldrich®, Missouri, USA). The fermentation medium had a pH of 6.8–7.0 and was previously sterilized at 121 °C for 15 min. A negative control consisting of the fermentation medium and the faecal inoculum with water instead of a food sample was prepared as well. The vials were placed in a fermentation chamber from which oxygen was removed using Oxoid™ AnaeroGen™ (ThermoFisher). The experiment was allowed to proceed for 20 h at 37 °C under orbital agitation. After the simulation, the samples were stored at −80 °C until further analysis.

The faecal inoculum was prepared from fresh faecal samples from four healthy Spanish volunteers (mean age 64 ± 1 years old; body mass index [BMI] = 24.15 ± 2.65 kg m^{-2}). The faeces were mixed to obtain a pooled sample. Previous research has shown that pooling faecal samples from different donors to generate an inoculum for in vitro assays is a suitable approach. Among the advantages, pooled samples do not lead to the emergence of anomalous microbiota or metabolic activity compared to that of individual donors, as the effect of individual variation in faecal microbiota is minimised.[18] The inoculum from the pooled samples was prepared by adding phosphatase buffer 0.1 M to the pool in a proportion of 32% (w/v).

The participants who donated the faecal samples followed a normo-caloric diet (ESI Table 1†), did not suffer from digestive diseases, and had not consumed antibiotics, prebiotics or probiotics in the last three months. These participants signed an informed consent form after obtaining the approval of the Ethics Committee of the Universitat Politècnica de València (P02_22_03_2023).

Microbiota composition (expressed as relative abundance at the phylum and genus taxonomic levels) was determined at the end of in vitro colonic fermentation of the different flours and prototypes and in the pooled inoculum and the negative control sample as well. Changes in relative abundance were expressed as the percentage variation with respect to the negative control: positive values indicate an increase and negative values indicate a decrease.

2.3. Microbiota composition by 16s rRNA gene sequence analysis

DNA was extracted from colonic fermentation samples using a Stool DNA Isolation Kit (Norgen Biotek Corp® in Thorold, ON, Canada). The extraction process was conducted following the manufacturer's guidelines. The quantity of extracted DNA was determined using a Qubit fluorometer (Invitrogen Co., Carlsbad, CA, USA). Microbiological analysis involved amplifi-

cation with specific primers targeting the V3–V4 regions of the 16S rRNA using Illumina technology. The primer selection was based on the publication by Klindworth *et al.*[19]

A Bioanalyzer DNA 1000 chip was used to check the amplicons, and the libraries were sequenced using a 2 × 300 bp paired-end run (MiSeq Reagent kit v3) on a MiSeq-Illumina platform at the FISABIO sequencing service (Valencia, Spain). Two processing approaches were followed. The first was performed to obtain the relative abundances. Using the DADA2 package (1.28.0)[20] in the software package R (R version 4.3.3, released on 29 February 2024) for filtering, only reverse sequences were truncated at position 250. Those reads with an expected error (EE) of greater than 2 were removed, where EE = sum($10^{(-Q/10)}$). The DADA2 denoising algorithm was independently applied to the forward and reverse reads, and then the ASV were merged. Upon chimera removal, the minimum fold parent over abundance parameter was reset from 2 to 4. The SILVA database (version 138.1) was used in the taxonomy assignment up to the genus category depth. The R packages phyloseq (1.44.0) and microbiome (1.25.0) were used to work with the bacterial community data.

The other processing approach was performed only to estimate the alpha diversity (Shannon and Chao1 indexes) of samples. Filtering and quality assessment of raw sequences was performed using the fastp program,[21] which involved removing low-quality nucleotides. Illumina forward and reverse sequences were joined using the FLASH program.[22] Reads containing undetermined nucleotides were removed with the DADA2 R package. A denoising algorithm was employed to infer exact ASVs, and chimeras were removed. Taxonomy was assigned to ASVs up to the species level using the SILVA database (version 138.1). The R package phyloseq was used to estimate alpha diversity values.

2.4. Metabolic activity of microbiota: short-chain fatty acids (SCFA), ammonium and lactate

Short-chain fatty acids (SCFA) were analysed from the aliquots taken *in vitro* after colonic fermentation using gas chromatography (GC-FID), according to the protocol adopted by Adorno *et al.*[23] Analytical calibration curves were obtained to quantify the target volatile acids, including the linear-chain SCFA acetic acid (AA), propanoic acid (PA), butyric acid (BA), caproic acid (CA), and the branched-chain SCFA (bSCFA) isovaleric acid (IVA), isobutyric acid (IBA) and isocaproic acid (ICA), over a concentration range from 0 to 30 mM. Samples (2 mL) were blended with 5 mL of H_2SO_4 (9.2 M), and a small amount of NaCl was added using the tip of a spoon to eliminate any residual water in the extract. Subsequently, 0.4 mL of the internal standard solution (52.9 mM 2-methylhexanoic acid) and 2 mL of diethyl ether were added and vortexed for 1 min. The samples were centrifuged at 3000*g*-force for 3 min. The resulting supernatant was transferred to chromatography vials and subsequently injected into an Agilent GC7890B-5977B GC-FID instrument (Agilent, Santa Clara, CA, USA) equipped with a multipurpose sampler with a SUPELCOWAX™ 10 Capillary GC Column (30 m × 0.25 mm × 0.25 μm, Merck,

Rahway, NJ, USA). The temperature program of the oven was set at 90 °C for 1 min, followed by a ramp to 190 °C at a rate of 5 °C min⁻¹, and a final hold at 250 °C for 30 min. Helium served as the carrier gas with a 1 mL min⁻¹ flow rate, and the inlet temperature was maintained at 250 °C. The injection volume was 2 μL. The results were expressed in millimolar concentration (mM).

The ammonium concentration was determined by using a deproteinizing sample preparation kit (Sigma-Aldrich). The protein present in the samples was first precipitated with perchloric acid (PCA) and then neutralized. The ammonia concentration was determined using the R-Biopharm Enzytec™ Liquid Ammonia (Darmstadt, Germany) and assessed using a spectrophotometer (UV/vis, Beckman Coulter, Brea, CA, USA) following the manufacturer's provided instructions. The results were expressed in mM. The lactate concentration was determined using a Lactate Assay kit (Sigma Aldrich®, St Louis, MO, USA). Measurements were performed using a Multiskan FC microplate reader (ThermoScientific 51119100, Waltham, MA, USA) following the instructions provided by the manufacturer. The results were expressed in μM.

2.5. Statistical analysis

Data were summarised as the mean and standard deviation of at least three replicates. Statgraphics Centurion was used for the statistical analysis. ANOVA was applied to study the statistical significance of the unfermented *vs.* fermented flours and prototypes on the colonic microbiota (relative abundance of phylum and genus and metabolites (SCFA), ammonium and lactate). The analyses were conducted with at least a significance of 95% (p-value < 0.05). Moreover, a multifactor analysis of variance (multivariate ANOVA) was also performed with a confidence interval of 99% ($p < 0.001$) and 95% ($p < 0.05$) to establish the effect of the factors (matrix and fermentation) on the response variables (relative abundance of bacteria at genus taxonomic level, metabolites and Shannon and Chao1 indexes) based on the F-ratio. The F-ratio value is directly related to the statistical effect of each factor on the response variables. The higher the F-ratio, the higher the impact. Pearson correlations were used to assess the possible relationships between the microbial genera and the production of metabolites.

3. Results and discussion

3.1. Baseline gut microbiota

The microbiota compositions at both the phylum and genus levels in the faeces of the four donors and in the faecal pooled inoculum are included in ESI Table 2.† In all the individual samples, the most dominant phyla were Bacteroidota and Firmicutes, representing more than 95% of the total microbiota, followed by Proteobacteria. The distribution pattern was aligned with that typically found in the gut microbiota of older adults,[24] confirming the representativity of the sample in terms of genera. *Bacteroides* was the most abundant genus, followed by *Alistipes* and *Faecalibacterium*. The results did not

show a significant variation among the individual faecal samples, with the proportion of *Bacteroides* ranging from 34.27 to 46.76%. The proportions of *Alistipes* and *Faecalibacterium* were found in the ranges of 6.99% to 16.36% and 3.09% to 9.15%, respectively. Of note, one donor showed a high proportion (20.97%) of an atypical genus (*Asteroleplasma*). Although its biological role in human physiology remains unclear, previous studies have associated *Asteroplasma* with non-communicable diseases.[25] This finding could be explained by the extreme variability that can be present in the gut microbiota of the elderly.[24] The high abundance of this genus was maintained in the faecal pool. The alpha-diversity, expressed as the Shannon Index, in the individual samples was found to range between 4.86 and 5.39. It is important to note that the faecal donors in this study followed a high-fibre diet (ESI Table 1†), with some even exceeding the minimum recommended daily intake, a rare occurrence in the elderly population.[26] As a result, the effects of the studied prototypes may differ when applied to other dietary patterns, particularly those with more typical low fibre content. Other studies have shown that the impact of the same substrates on the microbiota and its metabolites can vary depending on the type of diet.[27]

When the samples were mixed to obtain the pooled inoculum, the resulting microbiota was characterized by enriched bacterial communities (Shannon index 5.78) compared to the individual samples. The Bacteroidota phylum represented 47.72%; while the relative abundance of the Firmicutes phylum increased to 48.43%, and that of Proteobacteria increased to 3.02%. Verrucomicrobiota in the pooled inoculum exhibited in the same relative abundance as in the individual sample with the highest value. At the genus level, *Bacteroides* was the most abundant, with a relative abundance of 21.89%, followed by *Alistipes* (15.3%), *Oscillospiraceae UCG-002* (9.9%), *Faecalibacterium* (4.8%), *Eubacterium* (3.8%), *Odoribacter* (2.9%) *Asteroleplasma* (2.9%), and *Parasuterella* (2.1%). In terms of richness and diversity, this pooled basal microbiota had Shannon and Chao-1 indexes of 5.78 and 1412. Overall, these results suggest a higher bacterial diversity than in the individual faecal samples, providing a microbiota that is a more representative of the whole population. This supports the decision to work with a pooled inoculum rather than individual faecal inoculums in the context of the present study.[18]

3.2. Changes in faecal microbiota composition

After simulating the colonic fermentation of the undigested fraction of the different samples, changes in the basal pooled inoculum in terms of colonic composition and metabolic activity were observed to different extents and patterns depending on the type of matrix (flour, gel-like prototype, or bread-like prototype), with some of them being considered as potentially prebiotic.[28] Of note, the composition of the undigested fraction was not specifically characterised, but can be assumed to differ from the composition of the food prototype. Throughout gastrointestinal digestion, macronutrients (*i.e.*, carbohydrates, proteins, and lipids) are broken down into smaller bioavailable units such as monosaccharides, amino acids, and fatty acids that can be absorbed, while resistant starch and dietary fibre remain unaltered and are not absorbed.[29] Therefore, considering that up to 97% of macronutrients are normally digested and absorbed,[30] the composition of the undigested fraction after simulated gastrointestinal digestion is expected to be mainly fibre (containing the potentially prebiotic compounds) and small fractions of undigested macronutrients, these being the actual substrates available for the pooled inoculum during simulated colonic fermentation.

After simulated colonic fermentation, the largest changes in the microbiota at the phylum taxonomic level ranged between −11% and +3.8% of relative abundance, mainly in Bacteroidota, Firmicutes, and Proteobacteria (ESI Table 3†). In detail, the quinoa-based flours (UQ-F and FQ-F) induced a significant decrease in Bacteroidota (−6% and −11%, respectively) at the expense of increased Firmicutes, especially FQ-F (+9%). Similar results were found by Li *et al.*,[31] who analysed the relative abundance levels of Bacteroidota in adults after consuming quinoa-enriched bread. The opposite pattern was obtained after colonic fermentation with the lentil-based flours, as Bacteroidota increased by 1.1% and 1.98% and the phylum Firmicutes decreased by −1.63% and −3.64% for UL-F and FL-F, respectively. A study by Siva *et al.*[32] in which rats were fed a lentil-based diet showed the same results in terms of increased Bacteroidota and decreased Firmicutes, indicating the nutritional potential of lentils to improve both the body weight of rats and to suppress intestinal colonisation by pathogens, which generally belong to the phylum Firmicutes.

The results for the gel-like prototypes formulated both with and lentil flour depicted a similar trend. The phylum Bacteroidota increased significantly in both prototypes, except in the cases of UQL-B, in which it showed a decrease of −2.54%, and UL-G, in which it showed no significant changes. In contrast, the phylum Firmicutes decreased significantly in all the prototypes or showed no difference in relative abundance (UQL-B). Another study revealed that mung bean proteins also stimulate the growth of taxa belonging to Bacteroidota, and consequently reduce the Firmicutes/Bacteroidota (F/B) ratio in the gut microbiota.[33] Of note, all the prototypes (except for FL-G) showed a significant decrease in the Proteobacteria phylum, with this difference being more pronounced in the case of FQL-B, with a decrease of 3.4%. An increase in the phylum Proteobacteria is often considered a symptom of dysbiosis;[34] thus, the Proteobacteria-reducing effect provided by the prototypes could be potentially beneficial for gut health.

Focusing on the results at genus level, larger changes in relative abundance were found than in the phylum taxonomy, with changes in the range between −5.1% and +17.6% (Fig. 1). After colonic fermentation, a common observation was an increase in the genera *Bacteroides*, *Acidaminococcus* and *Parabacteroides* and a decrease in *Asteroleplasma*, *Oscillospiraceae UCG-002 group*, and *Alistipes*. The specific effects of the fermentation and food matrix structure are commented on later, while this section focuses on the most rele-
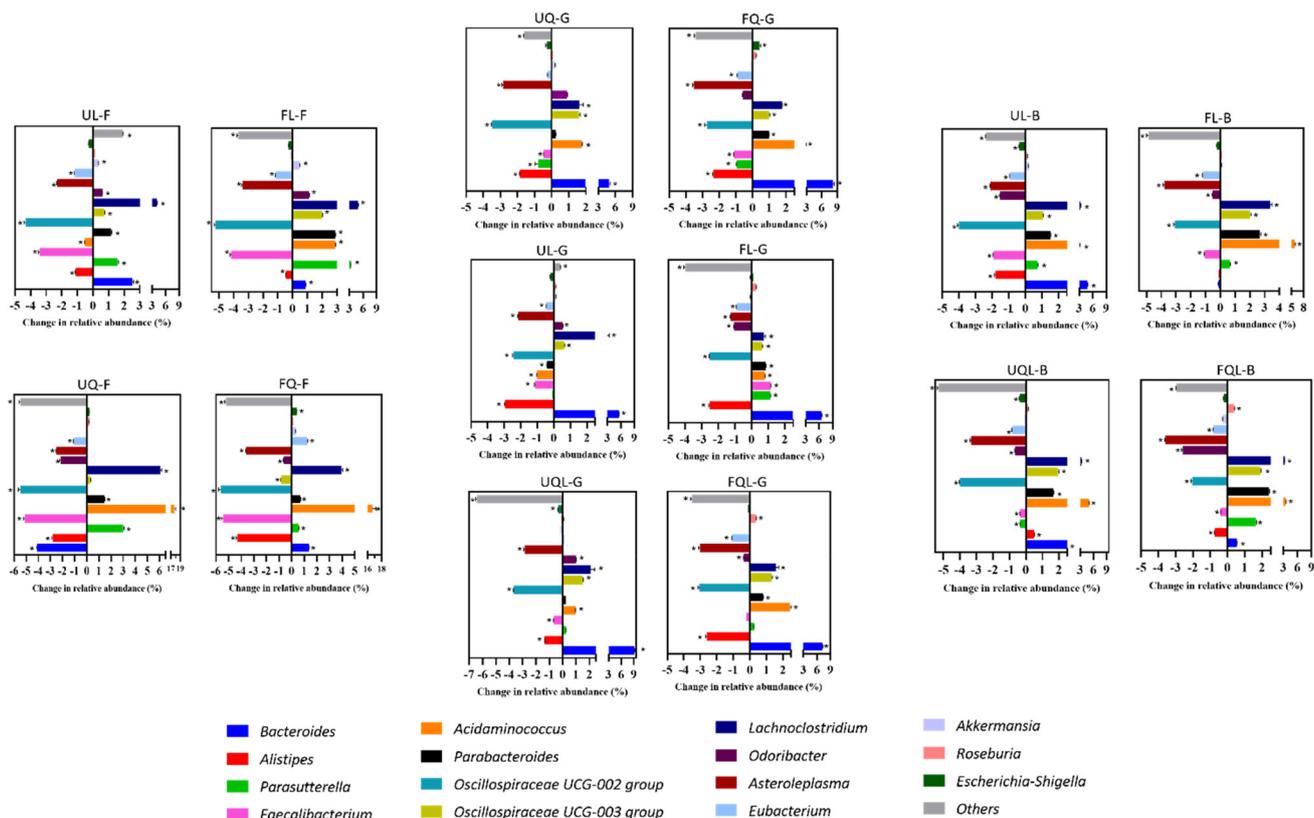
Fig. 1 Changes in bacterial relative abundance with respect to the basal control microbiota composition after colonic fermentation of flour substrates and gel-like and bread-like prototypes. Unfermented lentil flour (UL-F), fermented lentil flour (FL-F), unfermented quinoa flour (UQ-F), fermented quinoa flour (FQ-F), unfermented quinoa gel (UQ-G), fermented quinoa gel (FQ-G), unfermented lentil gel (UL-G), fermented lentil gel (FL-G), unfermented quinoa and lentil (UQL-G), fermented quinoa and lentil gel (FQL-G), unfermented lentil bread (UL-B), fermented lentil bread (FL-B), unfermented quinoa and lentil bread (UQL-B), and fermented quinoa and lentil bread (FQL-B).

vant changes in bacterial populations. For the flours, greater variations occurred for quinoa (fermented and unfermented), with *Acidaminococcus* showing increases of +17.1% for UQ-F and +17.6% for FQ-F. This is considered a negative result, as some species within *Acidaminococcus* have shown associations with gut inflammation and other detrimental health-related outcomes.[35] Another impact that was not desirable was the increase in *Lachnoclostridium*, as this genus has been associated with the risk of cardiovascular disorders, as well as with high-fat diets.[36] Flour substrates significantly increased the production of *Lachnoclostridium* after colonic fermentation by up to +5.9% (FQ-F). However, the structural matrix of the prototypes seemed to affect its growth, especially in the case of gels.

In contrast, a positive finding was the significant reduction in *Asteroleplasma* induced by all the flours and prototypes. Another relevant result was the increase in *Bacteroides*, as it tends to decline with age, is associated with the prevention of diabetes and obesity, and contributes to an increase in the production of beneficial metabolites, such as lactate, acetate, and propionate.[37,38] For example, it increased 4.9% in UQ-G and 9% in UQL-G. Of note, the increase induced by the bread-like prototypes and flours was not as pronounced as that induced

the gels. This finding can be supported by previous research showing a considerable increase in *Bacteroides* due to lentil starch or chickpea ingestion in obese mice and elderly mice, respectively.[39,40]

Interestingly, most of the study substrates led to increased *Parabacteroides*, with the fermented flour-based products showing the highest increases. This finding is of special relevance, as this genus is linked to gut health through alleviating inflammation, regulating immunity and producing SCFA. Moreover, its decline has been linked to diseases such as obesity and inflammatory bowel disease.[41] Additionally, the significant reduction in the *Alistipes* genus for all of the substrates (except UQL-B) was a positive result, as it has negative interactions with the immune system, particularly in older adults.[42]

Finally, all the fermented prototypes, regardless of the flour source, caused a significant decrease in the relative abundance of *Odoribacter*. For instance, the impact of solid-state fermentation of the quinoa and lentil bread recipes showed an important decrease (−0.67% for UQL-B *vs.* −2.54% for FQL-B) in the *Odoribacter* genus. Previous studies reported negative associations between *Odoribacter* and the quality of dietary carbohydrates, which could provide added value to foods formulated

with flours obtained through solid-state fermentation by *Pleurotus ostreatus.*[43]

3.3. Metabolic activity: SCFA, lactate and ammonium

The flours and prototypes also induced changes in the basal metabolism of the microbiota in terms of SCFA, bSCFA, ammonia and lactate production (Fig. 2 and 3). In general terms, all the substrates (flours and prototypes) led to increased total SCFA and reduced bSCFA (with some exceptions), with unclear patterns in terms of food matrix or flour processing (fermented/unfermented). Focusing first on the most abundant metabolites, *i.e.*, the SCFA, all the study samples led to significantly increased values (25–35 mM) compared to the control (20 mM). This result suggests that the incorporation of the study substrates into the diets of older people, either as flours or structured matrices and with either fermented or unfermented flours, could help mitigate the reduced SCFA production in the gut of this population group, because of alterations in microbiota composition.[44] The increase in total SCFA production is in accordance with previous studies conducted with cereals and legumes, and demonstrates a potential prebiotic effect as an added value in addition to nutritional quality.[45,46] The different extents to which SCFA were increased in the different substrates could depend on the processing (*e.g.*, drying or fermentation). In particular, solid-state fermentation is a biotechnological process that can lead to modified physical structure and macronutrient composition.[12,13] Previous studies have shown that *Pleurotus ostreatus* is a lignocellulosic fungus that hydrolyses cellulose, lignin and hemicellulose for its own growth due to the

secretion of enzymes such as endoglucanase, xylanases, laccases or glucosidases, among others.[47] Therefore, fermentation with this fungus can alter the composition of fibre,[48] making it more accessible as a substrate for microbiota utilisation. As shown in Fig. 2, the fermented flours showed increased production of total SCFA, which is the final product of fibre utilisation, supporting this explanation.

Focusing on the specific SCFA, acetic acid (AA), propionic acid (PA) and butyric acid (BA) were identified and present in concentrations up to 10 times higher than the branched-chain series (bSCFA) (2.5–4.5 mM): isovaleric (IVA), isobutyric (IBA) and isocaproic acid (ICA).

BA was the most abundant metabolite after colonic fermentation, particularly in the prototypes made with lentil flours. Similar results were found in a previous study[49] showing that fermentation of lentil substrates improved the BA production. For the prototypes, significant differences in the SCFA production after *in vitro* colonic fermentation were found. These differences might be explained by the different macronutrient composition: breads presented higher fibre than gels, resulting in a higher concentration of SCFA production.

While increased production of SCFA is considered to be beneficial, as these metabolites have proven to have several positive effects on different health aspects,[50] those with branched-chain structures should be considered separately. The bSCFA are produced by colonic fermentation of branched-chain amino acids. The production of bSCFA increases with aging due to a physiological decline in the gut microbiota as well as to the lower dietary fibre intake typical in the older adult population.[51] The results showed that bSCFA decreased
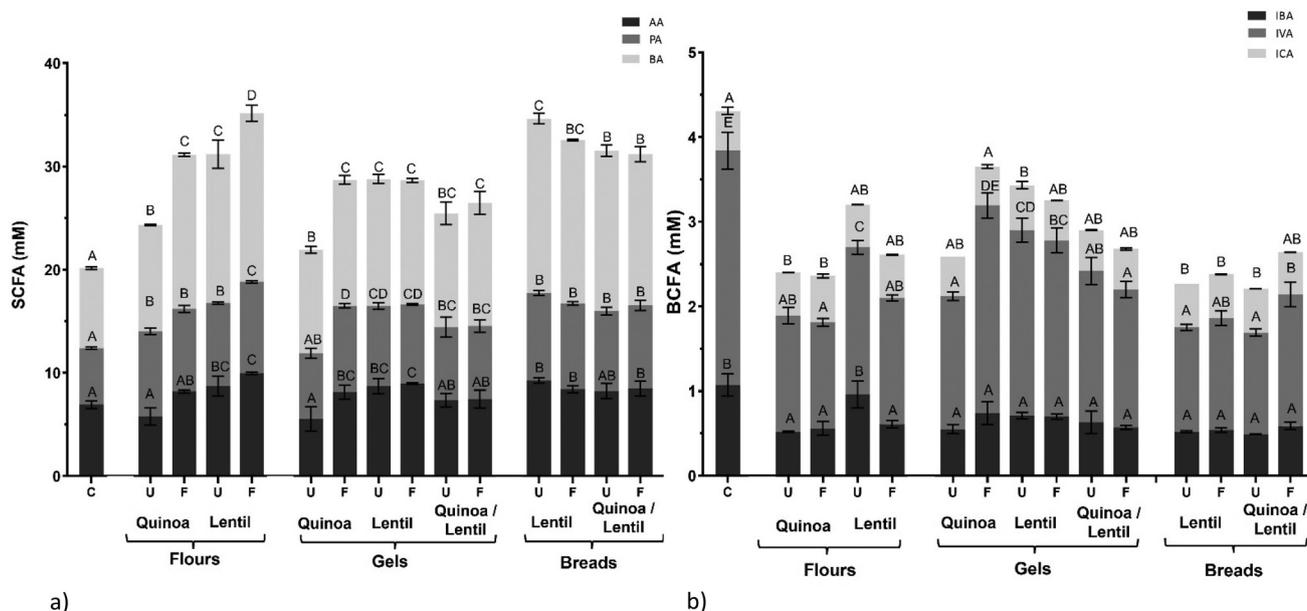

Fig. 2 Metabolite production after static *in vitro* simulation of colonic fermentation of flour samples and gel-like and bread-like prototypes. SCFA concentration (a) including acetic acid (AA), propionic acid (PA), butyric acid (BA), and BCFA concentration (b) including isobutyric acid, (IBA), isovaleric acid, (IVA) and isocaproic acid (ICA). Different capital letters (A–C) indicate significant differences between each study sample and the control. Simple ANOVA was conducted for each study sample. The data shown are mean values from independent triplicates and the standard deviation.
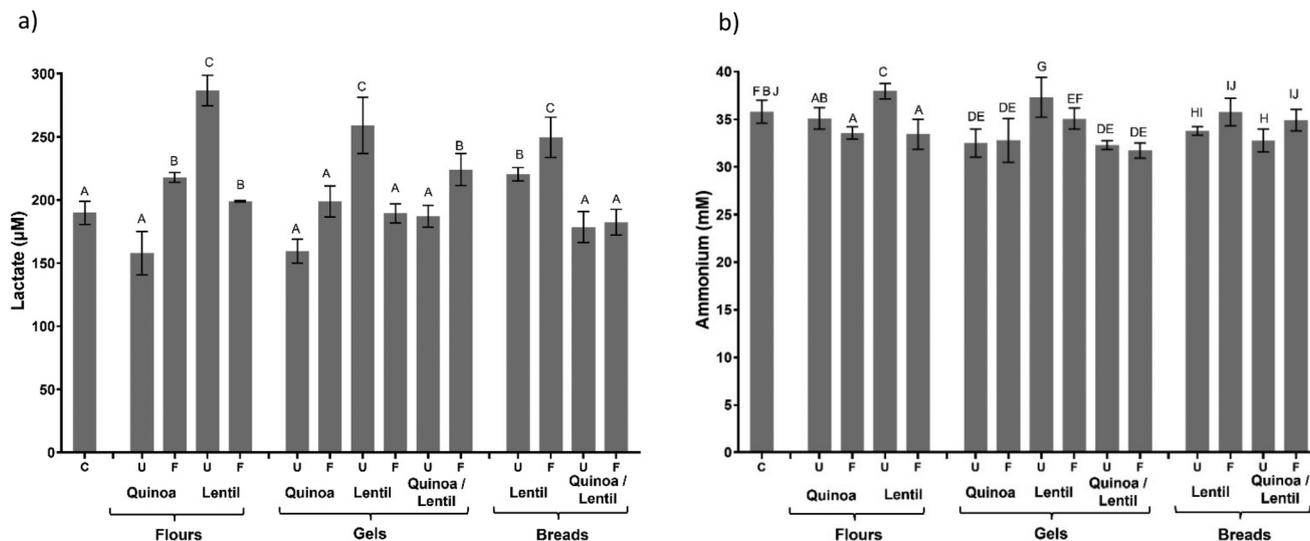
Fig. 3 Metabolite production after static *in vitro* simulation of colonic fermentation of flour samples and gel-like and bread-like prototypes. Lactate concentration (mM) (a); simple ANOVA was conducted for each study sample. Different capital letters (A–C) indicate significant differences. Ammonium concentration (mM); (b) simple ANOVA was conducted for each study sample. Different capital letters (A–C) indicate significant differences between flour study simples and the control, (D–G) indicate significant differences between gel prototypes and the control, and (H–J) indicate significant differences between bread prototypes. The data shown are mean values from independent triplicates and the standard deviation.

after *in vitro* colonic fermentation in all the studied matrices (Fig. 2). This fact could be explained by the fact that both quinoa and lentil substrates are rich in starch, a polysaccharide that has been reported to have a negative correlation with bSCFA production.[52] Regarding the impact of fermentation, only fermented lentil flour (FL-F) induced a significant reduction in the bSCFA concentration compared to non-fermented flour.

Moving on to the production of other metabolites, lactate and ammonium were determined (Fig. 3). Lactate is a metabolite responsible for maintaining eubiosis in the large intestine and cross-feeding of the intestinal ecosystem. According to the literature, the production of lactate is reduced with aging due to the reduction in lactate-producing genera such as *Bifidobacterium*.[53] In this study, the colonic fermentation of FQ-F, UL-F, FL-F, UL-G, FQL-G, UL-B and FL-B led to increased lactate production.

Some studies have shown that very high levels of protein and peptides in the colon may lead to increased production of harmful metabolites, such as sulphur, ammonia, and other nitrogenous metabolites that can lead to colon cancer.[54] As shown in Fig. 3, fermented flours from either lentils or quinoa led to a greater reduction of ammonia levels compared to unfermented flours. However, the benefits attributed to solid-state fermentation in the reduction of this metabolite were not found in the prototypes. In the gel prototypes, quinoa flour, regardless of fermentation, promoted a greater reduction in ammonia whether alone or in combination with lentil flour, with the highest reductions for UQ-G, FQ-G, UQL-G, and FQL-G (12.48, 8.36, 9.88, and 11.49%, respectively). For bread-like prototypes, formulations with unfermented flours

achieved even lower ammonia concentrations than their unfermented flour-counterparts.

3.4. Statistical significance of the studied variables (solid-state fermentation and food matrix) and the response variables (microbiota composition and metabolite production)

The previous sections presented and discussed the results in the colonic microbiota for each specific studied product (flours and prototypes) compared to the microbiota. However, an additional analysis was conducted to establish statistical significance of the two study variables, *i.e.*, (1) the processing to which the samples were subjected (solid-state fermentation with *Pleurotus ostreatus vs.* unfermented); and (2) the structure of the food matrix (flour *vs.* bread prototype *vs.* gel prototype) on the response variables. Thus, multifactorial ANOVA was applied to show the magnitude of the effect (*F*-ratio) that the two study variables had on the different response variables (Table 3). The analysis demonstrated that overall, the "food matrix" significantly affected most of the results, including the microbiota composition at genus level, diversity and metabolic activity, while the "Fermentation process" did not have significant effects on diversity or metabolite production, but did have a significant impact on some bacterial genera.

In particular, the food matrix was shown to significantly affect the most abundant bacterial genera, including *Bacteroides*, *Alistipes*, *Parasutterella*, *Faecalibacterium*, *Acidaminococcus*, *Parabacteroides*, *Oscillospiraceae UCG-003*, *Lachnoclostridium*, *Odoribacter*, *Akkermansia*, *Roseburia* and *Escherichia-Shigella*.

These results are consistent with the current knowledge. The incorporation of fermented flours in the formulation of

Table 3 *F*-ratios obtained from factorial ANOVA analysis for the different genera, metabolites, and Chao1 and Shannon values. Factors included in the analysis were food matrix and fermentation

		Principal effects			
Response variable		Food matrix		Fermentation	
Microbiota composition (genus level)	*Bacteroides*	34.37	**	5.28	*
	Alistipes	7.39	*	0.24	ns
	Parasutterella	20.61	**	12.90	*
	Faecalibacterium	73.21	**	1.5	ns
	Acidaminococcus	6.63	*	0.64	ns
	Parabacteroides	48.77	**	49.58	**
	Oscillospiraceae UCG-002	0.27	ns	0.57	ns
	Oscillospiraceae UCG-003	6.58	*	2.06	ns
	Lachnoclostridium	44.42	**	0.04	ns
	Odoribacter	5.80	*	7.17	*
	Asteroleplasma	1.76	ns	1.11	ns
	Eubacterium	1.34	ns	11.09	*
	Akkermansia	5.24	*	4.46	*
	Roseburia	11.75	*	35.10	**
	Escherichia-Shigella	4.01	*	4.34	*
	Others	0.33	ns	2.40	ns
Alpha diversity	Chao1	4.35	*	0.36	ns
	Shannon index	11.28	*	1.56	ns
Metabolites	SCFA	1.47	ns	0.00	ns
	bSCFA	9.25	*	0.18	ns
	Ammonia	0.78	ns	1.23	ns
	Lactate	2.32	ns	0.07	ns

ns: non-statistical differences ($p > 0.05$). $p < 0.05$ *: $p < 0.001$ **.

the prototypes could lead to an expected improvement of the composition of the microbiota, as fermented foods are a vehicle for beneficial microorganisms reaching the gut.[55,56] However, the processing of the fermented flours in the present study included a subsequent step of heat drying, therefore inactivating the viability of the fungus. Thus, the possible positive effect that fermenting lentils and quinoa could have on microbiota was based on changes in the structure and composition of dietary fibres as a consequence of the fungal metabolism, as previously reported for other plant-based ingredients.[57] Indeed, as shown in Fig. 2, the fermented quinoa and lentil flours allowed for increased total SCFA production compared with the unfermented ones, but when included as ingredients in the formulation of the food prototypes, the increased prebiotic effect seems to be reduced to the matrix effect.

Contrarily, there are several studies proving that the food matrix structure can modify the fate of food components[58] even in foods with the same composition and different structures.[59,60] Despite the smaller amount of evidence available on the effect of the food matrix on colonic microbiota, mechanisms related to how food structure shapes disintegration, nutrient release, and absorption could support the different effects observed in the colonic microbiota in the present study. In this context, a study by Bui *et al.* (2021) evidenced that in flour-based food formulations, the food matrix form had a greater impact than the amount of carbohydrates on microbiota composition and metabolic activity.[61] Therefore, when formulating new food concepts with plant-based ingredients with a focus on improving gut microbiota,

the structure should be considered as a relevant factor for the prebiotic potential.

3.5. Correlation between gut microbiota and metabolic activity

A correlation analysis between the abundance of the bacterial genera and metabolite concentrations was applied to estimate the metabolic role of the different components of the microbiota (Fig. 4). The results showed three genera were mainly
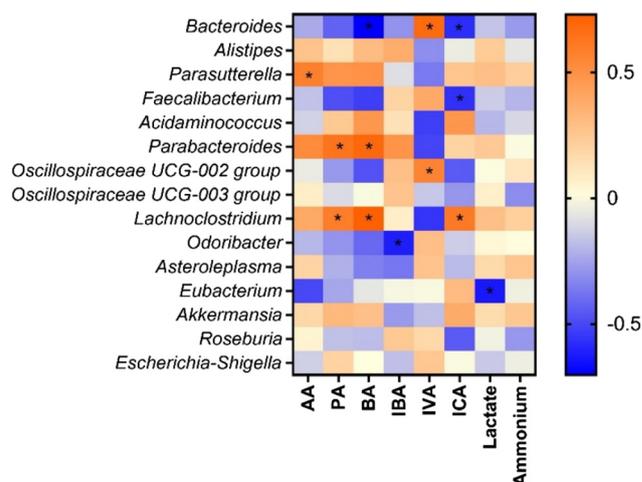

Fig. 4 Pearson correlation between bacterial genera and metabolites. Acetic acid (AA), propionic acid (PA), butyric acid (BA), isobutyric acid, (IBA), isovaleric acid, (IVA) and isocaproic acid (ICA), lactate and ammonium.

responsible for producing the SCFA (BA, PA, and AA): *Parabacteroides*, *Parasuterella*, *and Lachnoclostridium*. Of note, the bSCFA IBA and the genus *Odoribacter* were negatively correlated ($r = -0.620$; $p = 0.003$). The genera *Bacteroides* and *Faecalibacterium* had positive correlations with another bSCFA (IVA) ($r = 0.0703$, $p = 0.04$; $r = 0.570$, $p = 0.033$, respectively). The metabolites ammonium and lactate were not significantly correlated with any genus, except for a negative correlation between *Eubacterium* and lactate ($r = -0.635$; $p < 0.05$; $p = 0.015$).

4. Conclusion

This study aimed at elucidating the potential of new food prototypes elaborated with fermented plant-based ingredients to improve gut microbiota and metabolic activity in the older adult population by using an *in vitro* model for simulating colonic fermentation. The results showed that the food matrix structure was the factor that affected the study outcomes the most, in the sense that bread prototypes presented the most relevant changes in metabolic activity and gel formulations in the microbiota composition (increased *Bacteroides and Roseburia*). In contrast, applying solid-state fermentation with *Pleurotus ostreatus* does not seem to provide additional benefits compared to the non-fermentation approach, other than increasing *Parabacteroides*, *Eubacterium* and *Roseburia*, suggesting that this biotechnological process could impart beneficial changes in the colonic microbiota, albeit with a lower impact than the food structure.

In conclusion, new food prototypes made with plant-based ingredients such as quinoa or lentils could provide a potential probiotic effect for modulating the gut microbiota and metabolite production in the older adult population. Depending on the raw materials and the food matrix structure used, such new food prototypes could have different impacts on shaping the microbiota, so these variables should be considered in future studies focused on developing new plant-based foods with prebiotic potential.

Author contributions

Elena Gomez-Gomez: writing – review & editing, writing – original draft, visualization, supervision, methodology, investigation, formal analysis, conceptualization. Andrea Asensio-Grau: writing – review & editing, visualization, formal analysis, conceptualization. Jorge García-Hernández: writing – review & editing, supervision, conceptualization. Ana Heredia: writing – review & editing, supervision, resources, project administration, methodology, funding acquisition, conceptualization. Joaquim Calvo-Lerma: writing – review & editing, visualization, formal analysis, conceptualization. Ana Andrés: writing – review & editing, supervision, resources, project administration, methodology, funding acquisition, conceptualization.

Data availability

The data supporting this article have been included as part of the references section.

Data will be made available upon reasonable request.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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