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Enhancing nutritional profile and digestibility of lentil flour by solid state fermentation with *Pleurotus ostreatus*

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Lentils (*Lens culinaris*) present with an excellent nutrient profile. However, the increasing displacement of legumes from the diet and the possible negative effects of food matrix and antinutrient factors encourage the application of new strategies to improve nutrient digestibility and to produce food concepts that contribute to increase legume consumption. This study approached the solid-state fermentation of lentils with an edible fungus (*Pleurotus ostreatus)* in order to produce improved lentil flour. Fermentation contributed to increase protein (23%), resistant starch (9.8%), and polyphenols (from 2.1 to 3.2 mg gallic acid equivalent/g dry matter). After simulating *in vitro* digestion, fermented flours presented a higher fraction of digested protein (17%) along with lower starch hydrolysis (34 *vs.* 24%), while polyphenols content increased from 3.1 to 7.73 mg gallic acid equivalent/g dry matter. Thus, this study supports the application of solid-state fermentation with this edible fungus to obtain lentil flours with enhanced digestibility profile as compared to non-fermented counterparts. It could be used as novel raw material in the formulation of new food concepts with enhanced nutritional profile.

Introduction

Despite of a drop in their consumption in the last decades, legumes are an essential component of the human diet, especially in the Mediterranean Sea surrounding territories. Environmentally, they promote sustainable agriculture and contribute to climate change mitigation, and its ability to fix nitrogen can improve soil fertility and reduce the carbon footprint. [1]

Legumes offer an excellent nutrient profile, including high protein content (around 21-31% of dry weight)[2]. Legumes are also rich sources of starch, fibre, antioxidant compounds, vitamins and minerals[3]. For these reasons, consumption of legumes is associated with cardiovascular and gastrointestinal health, prevention of obesity and diabetes among others benefits [4]. The role of legumes in a healthy diet is such that the World Health Organisation recommends legume intake weekly[5]. Their high protein content becomes of particular relevance in population groups with increased protein needs such as athletes, elderly or post-surgery patients, and in the scope of the growing tendency of veganism. Overall, facilitating strategies to promote legume consumption seems a worthwhile purpose.

Among legumes, lentils (*Lens culinaris*) are one of the most consumed varieties, taking the fourth place in the worldwide production volume [6]. Despite the potential of lentils' nutrient profile, the digestion process could impair bioaccessibility, because of food matrix structure and existing interactions of antinutritional factors with digestive enzymes [7]. Among antinutritional factors, proteinaceous compounds (such as protease inhibitors) are responsible of decreasing trypsin and chymotrypsin activity, and non-protein factors (including phytic acid and phenolic compounds) can bind to minerals and protein, resulting in lower protein solubility and proteolysis inhibition [8]. To prevent these possible negative effects, processing strategies can be targeted, including heating (such as boiling), mechanical forces such as milling, sprouting or fermentation. Amongst them, a biotechnological process such as fermentation raises as a promising option, as it reduces the amount of anti-nutrients by biotransformation, and allows for enriching protein content and functional components [9].

Solid-state fermentation is carried out in the absence of water but with enough moisture in the substrate to enable microorganism growth [11]. It presents numerous advantages versus the liquid-state modality, such as being rapider, less expensive, and approaching the most to the natural environment of several microorganisms [12]. It has been applied in by-products valorisation, and more recently, some attempts have been performed in beans [13], lentils [14] or peas [15]. While bacteria and yeasts have been used to perform fermentation in solid state, less research relates to fungus, although some literature report positive findings with the common edible fungus *Pleurotus ostreatus* [16].

This study raises the production of solid-state fermented lentil flour with *Pleurotus ostreatus* and aims at analysing the impact of fermentation on nutrient and antioxidant profile of fermented lentil flour before and after *in vitro* digestion, by assessing digestibility and bioaccessibility. The ultimate goal is to evidence the potential of fermented lentil flour as an ingredient to use in the formulation of new food products.

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Materials and methods

Materials

Lentils (*Lens culinaris*) from "Castellana" variety were purchased at a local supermarket (Valencia, Spain). The strain of *Pleurotus ostreatus* was acquired at the Spanish Culture Type Collection (CECT) (20311/ Batch 18-10-2016) (Universitat de València, València, Spain). To formulate the broth, yeast, malt, glucose and agar extracts were supplied by Scharlab (Barcelona, Spain). To prepare the simulated digestive fluids, the following products were used: pepsin from porcine gastric mucosa (2500 U/g protein), bovine bile extract, pancreatin from porcine pancreas (8 x USP) , KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$ and $CaCl_2$ (Sigma-Aldrich; St Louis, MO, USA), and NaOH and HCl (AppliChem Panreac; USA). The analytical determinations required the following reagents: CH_3OH, CH_3CH_2OH, bovine serum albumin, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), $FeCl_3{\cdot}6H_2O$, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), Acetic acid, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS), $K_2S_2O_8$, trichloroacetic acid (TCA), dinitrosalicylic acid (DNS), glucose, N-K tartrate, invertase (Sigma-Aldrich; Barcelona, Spain), EDTA and crystalline urea for analysis (ACS), (AppliChem Panreac; USA), and amylogucosidase (Megazyme; Ireland).

Fungal solid-state fermentation

Starter culture preparation. For the *Pleurotus ostreatus* strain recovery, isolated colonies from the agar plate culture, were taken, and cultured in Petri plates with a broth made of 0.4% yeast extract, 0.1% malt extract, 0.4% glucose and 1.5% agar [12], and then incubated for 14 days at 28 °C (Selecta J.P.200207, Germany). The obtained mycelium was inoculated with an inoculating loop in a flask containing the broth and then incubated again at 28 °C to reach a final amount of 264 mg dry biomass in 100 broth mL [16]. Dry biomass was determined by top funnelled paper filtration to separate the mycelium from the liquid broth; the obtained filter paper with the mycelium was placed in an empty Petri plate and vacuum-dried at 60°C until constant weight.

Fermentation process. Lentils were subjected to solid state fungal fermentation (in triplicate) according to Espinosa-Páez et al. (2017) with slight modifications [12]. Lentils (70 g) were autoclaved in glass jars (1 L) at 121 °C during 20 min, in a lentil:distilled water proportion of 1:0.74 (w/v). Once sterilised, inoculation with 8 mL of vortexed liquid broth was performed (Heidolph, Reax top), and then glass jars were incubated at 28 °C during 14 days (**Figure 1**). For control samples (in triplicate), lentils were autoclaved at these same conditions but without inoculation.

Flour preparation. After fermentation time, fermented lentils were heat-dried (Selecta J.P. 200207, Germany) during 48 h at 60 °C. Once dehydrated, milling was applied (Ø80 mm grinding dial at 1980 rpm), resulting in fermented lentil flour (FLF). In order to make a control lentil flour, control lentils were also subjected to the same heat-drying and milling processes as for FLF, resulting in lentil flour (LF).

In vitro digestion process

LF and FLF were subjected to static *in vitro* digestion according to Asensio-Grau et al., (2018) [17]. The salivary fluid (SF) was obtained from a healthy volunteer and alpha-amylase activity was measured according to Brodkorb et al., (2019) [18]. The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared daily from stock solutions according to Minekus et al. (2014) [19]. For the oral stage, SF (pH 7) was added to a 50 mL falcon tube containing 5 g of flour sample and homogenized during 1 min. The gastric stage followed by incorporating the SGF (pH 3) containing gastric pepsin (2000 U/mL) to the oral bolus in proportion 1:1 (v/v) and tubes were head-over-heels rotated at 55 rpm for 2 hours at 37°C in an incubator chamber (JP Selecta SA, Barcelona). Finally, the intestinal stage was simulated by adding to the gastric chime

Fig. 1 Flow chart illustrating the fungal solid-state fermentation process conducted with *Pleurotus ostreatus*

the SIF (pH 7) that has a mixture of a pancreatin (100 Trypsin U/mL) and bile salts solution (bovine bile, 10 mM) in a proportion 1:1 (v/v) and tubes were agitated during 2 h at 37°C as in the gastric stage. At the end of the intestinal stage, the undigested fraction was separated by centrifugation (4000 g-force, 15 min) and aliquots from the supernatant (bioaccessible fraction) were used for analytical determinations.

Analytical determinations

Macronutrient characterisation. Lipid, protein, ashes and water content were determined by the AOAC methodologies in LF and FLF (AOAC, 2000) [20]. Carbohydrates were estimated considering total solids and subtracting lipid, protein and ashes contents.

Colour. Instrumental measurements of colour were conducted at room temperature in a Minolta spectrophotometer (model CM-3600d), placing the sample in a 20 mm thick, transparent plastic cell, and using a black plate as the background to standardize the measurements. Visible absorption spectra were recorded between 380 and 770 nm by reflectance to obtain tristimulus values of CIELa*b*, using illuminant D65 and standard observer (10° visual field) as references. Saturation (C*) and tone (h*) were calculated from a* and b* coordinates. Colour differences (ΔE) were calculated according to Eq. (1).

$$\Delta E = \sqrt{\Delta a^{*2} + \Delta b^{*2} + \Delta L^{*2}} \quad (1)$$

Particle size. Particle size distribution of LF and FLF were measured using a laser light scattering instrument (Mastersizer 2000, Malvern, UK). The results were reported as the average of the mean particle ($d_{3,2}$) and the particle size distribution [17].

Proteolysis. Proteolysis was determined as the amount of soluble protein in trichloroacetic acid (TCA) according to Lamothe, et al., 2014 [22]. Digested samples (1 mL) were mixed with TCA solution (50 %) and remained reacting during 20 minutes. Then, samples were centrifugated at 7168 g-force during 15 minutes (Eppendorf MiniSpin Plus). Supernatant was diluted with a buffer (50 mM EDTA and 8M UREA, pH 10) and the absorbance was measured by ultraviolet spectrophotometry (Helios Zeta UV/Vis, Thermo Scientific) at 280 nm. A calibration line was determined using bovine serum albumin as a standard. Proteolysis extent was expressed as grams of TCA soluble protein in 100 grams of total protein concentration initially present in each tube.

Glycolysis. Sugars release during digestion was determined as monosaccharides with the dinitrosalycilic acid (DNS) colorimetric method, after sample hydrolysis with invertase + amilogycosidase [23]. During both gastric and intestinal digestion, 1 mL digesta aliquots were taken and mixed with 4 mL of ethanol during 30 min. Then, samples were centrifugated (100 g-force, 10 min, 20 °C), and 50 µL of supernatant were mixed with 250 µL of an enzymatic solution (1% amiloglycosidase and 1% invertase) and 750 µL of a DNS solution (1% glucose, 1% NaOH 1M and 5% DNS) in glass tubes. To facilitate the colorimetric reaction, tubes were heated at 100 °C during 15 min and then diluted in 5 mL of distilled water. Spectrophotometry measurements were taken at 530 nm (Helios Zeta UV/Vis, Thermo Scientific, City, Country). Glycolysis extent was expressed as grams of free glucose in 100 grams of total glucose concentration initially present in each tube.

Rapid Digested Starch (RDS) was determined at 20 minutes of intestinal digestion time, while Slowly Digested Starch (SDS) was considered as those released after 120 min. Resistant Starch (RS) was not hydrolysed into glucose after digestion, and it was estimated as the difference between total starch and released glucose during digestion. For this determination, total starch in lentil and fermented lentil flours was quantified by means of enzymatic hydrolysis [24].

Total polyphenols. Total polyphenols were quantified before and after digestion. An extraction with methanol (80%) during 2 h in agitation (55 rpm, 25 °C; Intelli-Mixer RM-2) was performed in order to recover phenolic compounds from the samples. In the case of undigested samples, methanol was added in proportion 1:10 (w/v)[16]. After 120 min of intestinal digestion, methanol was added in proportion 1:20 (w/v) to 1 mL of the liquid phase (or bioaccessible fraction). After 2 h in agitation, samples were centrifuged (20 min, 14 g-force, 20 °C). Supernatant was used to quantify polyphenols with the Folin-Ciocalteu method, according to Chang et al. (2006) using a spectrophotometer (Helios Zeta UV/Vis, Thermo Scientific) [25]. Gallic acid calibration curve (0 —0.5 g/L) was used to quantify total polyphenols. Results were expressed as milligrams of gallic acid per gram of dry matter.

Antioxidant activity. Antioxidant activity was determined in flours and in the bioaccessible fraction of the *in vitro* digested samples. Extraction with methanol 80% was performed as for the polyphenols determination. Three different spectrophotometric methods (DPPH, FRAP and ABTS) were conducted according to Thaipong et al., (2006) using a spectrophotometer (Helios Zeta UV/Vis, Thermo Scientific) [26]. In all the methods, a trolox calibration curve (0 —0.5 g/L) was used to quantify antioxidant activity. Results were expressed as milligrams of trolox per gram of dry matter.

Statistical analysis

Simple factorial variance analyses (ANOVA) were performed with a confidence interval of 95% ($p < 0.05$) to assess possible significant differences between LF and FLF samples (Statgraphics Centurion-XV). The Duncan's multiple range test was then applied. Pearson correlation analysis was conducted to detect associations between the antioxidant activity obtained through different methods and total polyphenols content. Fermentation was performed in triplicate and the in vitro digestion process of each flour sample in duplicate. All the analyses were determined in all replications (n=6) per flour.

Results and discussion

Impact of fermentation on physical characteristics and nutrient profile.

The physical and nutritional parameters were assessed in lentil flours with (FLF) and without fermentation (LF). Fermentation led to a slight change in the particle size distribution spectrum (**Figure 2**). Taking the mean size ($d_{3,2}$) into account, smaller particles were obtained in fermented flour ($d_{3,2}$ fermented flour (56.15±1.05) < $d_{3,2}$ control flour (59.77±1.15)). This change could be possibly related to the degradation or partial consumption of the substrate (lentil) by the fungus. Some of the components that the fungus takes as substrates are present in the plant cell walls [25], so its degradation could facilitate eventual structural breakdown in the milling process as compared to unfermented substrates. The reduction of the particle size of a food substrate has been repeatedly associated with increased digestibility and bioaccessibility of nutrients in nuts and seeds [28,29]. This observation is explained by the fact that the higher the matrix disruption, the easier the nutrients release to the digestion medium, leading to higher enzyme-substrate accessibility.

Fig. 2 Particle size distribution in lentil flour (LF, control flour) and fermented lentil flour (FLF)

Fermentation also affected optical properties. As shown in **Table 1**, significant changes in luminosity (L*), red colour (+a*) and yellow colour (+b*) were detected, leading to an overall difference of colour of 8.2 ± 0.9. Fermented flour was darker, with a browner tone (h*) and with more colour intensity (C*). Fungi are vulnerable to thermal and mechanical damages. In this sense, both heat drying and milling are processes that impart changes in the physical properties of the samples. Colour changes were possibly related to the mycelia colour alterations as a heat-drying effect, causing darkening of the fermented flour [30].

Apart from physicochemical above-commented changes, fermentation implied compositional changes as a consequence of the substrate consumption by the microorganism for its growth (**Table 2**). Significant differences were observed in protein and carbohydrate content, with a net increment of 18.5% in the case of protein and a decrease of 6% for carbohydrate, while no changes were evidenced in lipid content. Carbohydrates are the main carbon

source for fungus growing, this fact explaining the decrease of carbohydrates in the fermented flour samples [31]. The increase in protein content was also attributed to the bioconversion of some carbohydrates into protein [32]. In addition, it is suggested that the acidophil nature of Pleurotus species and its ability to reduce pH by releasing organic acid, prevents ammoniac volatilisation driven nitrogen losses [33]. On the other hand, the increment of protein can be also attributed to *Pleurotus ostreatus* growth, i.e. to an increase of unicellular protein biomass [34]. Additionally, the extracellular fungal enzymes produced during growing and bioconversion, could explain the overall increase in protein content in fermented flours [35].

Polyphenols content and antioxidant activity in flours did also experiment changes as a consequence of fermentation of lentils (**Table 3**). The net increment of polyphenols content in the fermented flour was even higher (53%) than in protein. *Pleurotus ostreatus* is known to contain high amounts of phenolic compounds, so its growth and proliferation explain the increase of these compounds in the fermented lentil flours [24]. Additionally, according to Espinosa-Páez et al. (2017), *Pleurotus ostreatus* is a basyliomycetes fungus, and secretes different lacases (phenol-oxidases), which are utilized to degrade lignin and obtain nutrients as substrate [12]. Lacases catalyze oxidation reactions and can polymerize, depolymerize or transform large amounts of phenolic compounds. According to these authors, other possible reasons for the reported increment in polyphenols could be the hydrolysis of bounded phenols and the diamination of aromatic amino acids and phenylalanine and tyrosine precursors. Complementarily, Hur et al., (2014) reported that microbial enzymes (glucosidase, amylase, cellulase, tannase, esterase, invertase or lipase), which are generated during fermentation, are capable of hydrolysing glucosides and breaking the cell matrix of seeds and starch, enabling the release of phenolic compounds [36].

The increase in phenolic compounds led to enhanced antioxidant activity (**Table 3**). As three different methods were used to measure antioxidant activity (DPPH, FRAP and ABTS), Pearson correlations, were applied to study the associations between antioxidant activity obtained by each method and polyphenols content. It was the FRAP that showed the highest value (0.8446), so it should be the most suitable method to study antioxidant activity in fermented lentils flour. According to previous studies the ferric reducing potential is associated to the presence of polyphenols. This result indicates a relationship between the polyphenolic content and the ferric reducing capacity, in which the presence of phenolics compounds contributes to increase the antioxidant activity measured by FRAP assay in the flours [37].

Table 1. Colour coordinates in lentil flour (LF) and fermented lentil flour (FLF)

	LF	FLF
L*	60.2 ± 1.5 [a]	52.5 ± 0.9 [b]
a*	5.4 ± 0.4 [b]	7.05 ± 0.15 [a]
b*	14.9 ± 0.8 [b]	16.7 ± 0.3 [a]
C*	15.8 ± 0.9[b]	18.1 ± 0.3[a]
h*	1.2 ± 0.2[a]	1.17 ± 0.05[a]
ΔE	8.2 ± 0.9	

Values sharing a same letter in the same column are not significantly different ($p < 0.05$)

Table 2. Proximal composition of lentil flour (LF) and fermented lentil flour (FLF)

	LF	FLF
Moisture (g/ g dry matter)	0.018 ± 0.004[b]	0.033 ± 0.008[a]
Protein (g/ g dry matter)	0.233 ± 0.004[b]	0.276 ± 0.007[a]
Lipid (g/ g dry matter)	0.025 ± 0.001[a]	0.023 ± 0.001[a]
Carbohydrate (g/ g dry matter)	0.740 ± 0.006[a]	0.697 ± 0.011[b]
Ashes (g/ g dry matter)	0.003-± 0.001[a]	0.003 ± 0.001[a]
Energy (kcal/ g dry matter) *	6.5	7.5

Values sharing a same letter in the same row are not significantly different ($p < 0.05$).* Calculated as the sum of estimated energy provided per macronutrient: 4 kcal/g for protein and carbohydrates, 9 kcal/g for lipid

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Table 3. Polyphenols content and antioxidant activity in lentil flour (LF) and (FLF)

	LF	FLF
Polyphenols (mg gallic acid/ g dry matter)	2.09 ± 0.02[b]	3.2 ± 0.06[a]
DPPH (mg trolox/ g dry matter)	4.6 ± 0.2[a]	4.45 ± 0.18[a]
FRAP (mg trolox/ g dry matter)	1.53 ± 0.14[b]	1.82 ± 0.07[a]
ABTS (mg trolox/ g dry matter)	2.504 ± 0.008[a]	2.6 ± 0.3[a]

Values sharing a same letter in the same row are not significantly different ($p < 0.05$)

Effect of fermentation on digestibility, bioaccessibility and antioxidant activity in the bioaccessible fraction

Proteolysis is a well-defined phenomenon occurring throughout gastrointestinal digestion, in which proteins are hydrolyzed mainly by pepsin in the stomach and subsequently by pancreatic proteases in the small intestine, resulting in small peptides and free aminoacids that are eventually absorbed [38]. In the present study, fermentation did not only lead to increased protein content, but also it enhanced the hydrolyzed protein fraction (**Figure 3**). This result could be explained by the fact that in fermented flour, protein was already partially hydrolysed by fungal lytic mechanisms during fermentation, this representing one of the main advantages of applying this pretreatment. When focusing on total proteolysis extents, gastric digestion accounted for 20 and 28% (g of TCA soluble protein/100g protein) in LF and FLF respectively, which represented half of total proteolysis achieved after intestinal digestion, i.e. 40 and 57% respectively. This is in accordance to previous studies of fermented legume flours, in which protein digestibility ranged between 30 and 70% [12]. The relatively low total proteolysis obtained could be attributed to the presence of antinutritional factors in lentils, which exert an inhibitory role on proteases [39]. Despite of this fact, fermentation process suggests to have had a reduction effect on these antinutrient factors, probably as a consequence of the

elimination of phytic acid and tannins [40]. Further studies should assess changes in antinutrient components to confirm this result.

Another factor contributing to the incomplete proteolysis in both samples could be pancreatic proteases having lower affinity for plant proteins than for animal protein, according to Alpers (1987) [38]. In fact, previous studies conducted with similar methodologies than in the present study, showed that animal protein reach proteolysis extents close to 100% [41].

The other majoritarian macronutrient in lentils, apart from protein, are carbohydrates, which require complex transformations

Fig. 3 Proteolysis extent after an *in vitro* gastrointestinal digestion of lentil flour (LF, control flour) and fermented lentil flour (FLF). *Different low case letters indicate statistically significant differences (p<0.05) between digestion times. Different capital letters indicate statistically significant differences (p<0.05) between LF and FLF at each digestion time. Data are presented as mean and standard deviation over six replications (n=6) per type of flour

during digestion in order to become absorbable. Starch hydrolysis starts in the oral stage by the action of alpha-amylase, which gets rapidly inactivated by the action of the acid pH in the stomach. The hydrolytic reaction is resumed in the duodenum, where pancreatic alpha-amylase accounts for the largest carbohydrate digestion by hydrolyzing alpha 1-4 glyosidic bounds. Resulting monosaccharides can therefore be absorbed. Resistant starch escapes enzymatic action and reaches the colon, in which they are fermented by the microbiota, resulting in absorbable short chain fatty acids [38]. Considering that starch digestion can be expressed as g of glucose released/100 g of initial starch content, **Figure 4** represents starch digestibility in both flours along the different stages of digestion. In both samples, a gradual increase in free glucose as digestion progressed was observed, which reached maximum extents of 34%

Fig. 4 Glycolysis extent throughout *in vitro* simulated gastrointestinal digestion of lentil flour (LF, control flour) and fermented lentil flour (FLF). *Different low case letters indicate statistically significant differences (p<0.05) between digestion stages. Different capital letters indicate statistically significant differences (p<0.05) between LF and FLF at each digestion stage. Data are presented as mean and standard deviation over six replications (n=6) per type of flour

in the LF at 120 min of intestinal digestion, and 24% in the fermented counterpart, evidencing lower amylolysis due to fermentation. The overall low carbohydrates digestibility extent in this study had been previously identified by Sandhu & Lim (2008), who attributed similarly low values (hydrolysis indexes varying from 8 to 20% in a range of legumes) to starch granule diameter and molecular weight of amylose and amylopectin [42]. Other types of starch that are present in cereals, such as in rice, have exhibited a complete hydrolysis of starch under *in vitro* digestion studies [43], suggesting that legumes seem to be more hypoglycemic than cereals.

Taking into account the classification of starch according to the digestion time when it becomes hydrolysed, fast digestion starch (FDS), slow digestion starch (LDS) and resistant starch (RS), which is the proportion that remains undigested after 120 min, can be differentiated. A slower starch digestion and a higher proportion of resistant starch are associated with a lower glycemic index [44]. Foods with low glycemic index are recommended, as the progressive increase of glucose concentration in plasma prevents from abrupt secretion of insulin, in contrast to foods with easily digestible starch or simpler carbohydrates. Phased secretion of insulin is associated with reduced risk of type II diabetes, obesity and cardiovascular disease [45]. In addition, resistant starch contains the fraction of carbon sources that can be eventually utilized by the colon microbiota, promoting the growth of the species associated with health beneficial outcomes [46]. In this study, results of the different starch fractions reveal a significant increase of resistant starch fraction in FLF (66.5% $\pm$ 0.8 vs. 76.2% $\pm$ 0.8; p<0.05) along with a significant reduction of the fast-digestible starch in the FLF (29% $\pm$ 2 vs. 21.8% $\pm$ 0.5; p<0.05), probably due to its consumption as substrate for growing the fungus during fermentation.

As previously discussed, an increment in polyphenols and antioxidant activity were observed in fermented lentil flours. However, this increase could be considered as beneficial only if bioaccessibility of these compounds guarantees satisfactory extents. In **Table 4**, polyphenols content and antioxidant activity in lentil flours after *in vitro* digestion process are presented. If these results were compared to the initial amount in the undigested samples, a substantial increase in polyphenols in the bioaccessible fraction of digesta was observed, and an increase of the antioxidant activity was also detected. These results highlight the role of the structural matrix in which these compounds are contained in terms of extractability, and also a protective effect against degradat

ion during digestion. The majority of polyphenols are bounded to high molecular weight compounds in the food matrix, such as protein or carbohydrate, this fact hindering or impeding that polyphenols can be extracted unless subjected to digestion. Hence, hydrolysis of protein and starch, as well as the acidic conditions of the medium at some digestion stages, can promote the release and solubilization of polyphenols [47]. On the other hand, the obtained results show that the difference in polyphenols content between digested and undigested samples is lower than those attributed to the fermentation process in the undigested samples. In addition, despite polyphenols content of fermented flour digesta was higher than in the control one, antioxidant activity was lower or equivalent (depending on the method), which suggests a higher lability to digestion of those de novo generated polyphenolic compounds during fermentation.

Table 4. Effect of gastrointestinal digestion on polyphenols and antioxidant activity in the bioaccessible fraction of lentil flour (LF) and fermented lentil flour (FLF)

	LF	FLF
Polyphenols (mg GA eq./ g dry matter)	7.11 ± 0.07^{b}	7.73 ± 0.16^{a}
DPPH (mg TE/g dry matter)	6 ± 0.3^{a}	4.7 ± 0.4^{b}
FRAP (mg TE/g dry matter)	3.63 ± 0.05^{a}	2.35 ± 0.07^{b}
ABTS (mg TE/g dry matter)	5.61 ± 0.54^{a}	5.6 ± 0.3^{a}

Values sharing a same letter in the same row are not significantly different (p < 0.05)

Conclusions

Solid-state fermentation of lentils with *Pleurotus ostreatus* prior flour production accounts for increased protein and polyphenols content,

along with increased proteolysis and antioxidant activity. In addition, increased resistant starch to digestion is achieved by means of solid-state fermentation process with this fungus, what could be translated into reduced glycaemic index in the *in vivo* scenario. The present work supports the application of solid-state fermentation with this edible fungus to obtain legume flours with enhanced nutrient content and digestibility profile as compared to non-fermented counterparts. Developed new food products on the basis of these flours could be considered as functional foods, and used as substitutes of other raw materials of similar characteristics and costs, or as alternative to animal-origin protein. Solid-state fermentation has shown promising results, and could be therefore easily incorporated as a new technology in the food industry to deliver legume-based flours with enhanced nutritional profile. Further studies should confirm the present results in the *in vivo* setting.

Conflicts of interest

"There are no conflicts to declare".

References

1 F. Stagnari, A. Maggio, A. Galieni, and M. Pisante, Multiple benefits of legumes for agriculture sustainability: an overview, *Chem. Biol. Tech. Agricult*., 2017, **4**, 2.

2 R.N. Tharanathan, S. Mahadevamma, Grain legumes-A boon to human nutrition, 2003, *Trends Food Sci Technol*, **14**, 507-518.

3 M. A. Martín-Cabrejas, Legumes: An overview, 2019, *Food Chem. Funct. Anal*, **8**, 1-18.

4 M.W. Vasconcelos, and A.M. Gomes, The legume grains: when tradition goes hand in hand with nutrition. In Traditional Foods, 2016, (pp. 189-208). Springer, Boston, MA.

5 World Health Organization. (2018). A healthy diet sustainably produced: information sheet (No. WHO/NMH/NHD/18.12). World Health Organization.

6 F. Longobardi, V. Innamorato, A. Di Gioia, A. Ventrella, and A. Agostiano, Geographical origin discrimination of lentils (Lens culinaris Medik.) using 1H NMR finger printing and multivariate statistical analyses, *Food Chem.*, 2017, **237**, 743-748.

7 C. Quesada, B. Bartolomé, O. Nieto, C. Gómez-Cordovés, T. Hernández, and I. Estrella, Phenolic inhibitors of α-amylase and trypsin enzymes by extracts from pears, lentils, and cocoa, *J. Food Protect*., 1996, 59, **185** -192.

8 S.M. Bessada, J.C. Barreira, M. B. P. Oliveira, Pulses and food security: Dietary protein, digestibility, bioactive and functional properties, 2019, *Trends Food Sci Tech*, 93, 53-68.

9 E. Zannini, E. Pontonio, D.M. Waters, and E.K. Arendt, Application of microbial fermentations for production of gluten-free products and perspectives, *App. Microbiol.*, 2012, **93**, 473-485.

10 A. Pandey, Solid state fermentation, *Biochem. Eng. J*., 2003, **13**, 81–84.

11 S. Rodríguez, and M. Sanromán, Application of solid-state fermentation to food industry - A review, *J. Food Eng.,* 2006, **73**, 291-302.

12 E. Espinosa-Páez, M.G. Alanis-Guzmán, C.E. Hernández-Luna, J.G. Báez-González, G.A. Amaya-Guerra and A. Andrés-Grau, Increasing Antioxidant Activity and Protein Digestibility in Phaseolus vulgaris and Avenasativa by Fermentation with the Pleurotus ostreatus Fungus, *Molecules*, 2017, **22**, 2275.

13 M.I. Torino, R.I. Limón, C. Martínez-Villaluenga, S. Mäkinen, A. Pihlanto, and C. Vidal-Valverde, Antioxidant and antihypertensive properties of liquid and solid state fermented lentils, *Food Chem*., 2013, **136**, 1030–1037.

14 Z. Ma, J. Boye, and X. Hu, Nutritional quality and techno-functional changes in raw, germinated and fermented yellow field pea (Pisumsativum L.) upon pasteurization, *LWT*, 2018, **92**, 147-154.

15 S. Kulkarni, S. Nene, and K. Joshi, A comparative study of production of hydrophobin like proteins (HYD-LPs) in submerged liquid and solid state fermentation from white rot fungus *Pleurotus ostreatus, Biocat. Agricult. Biotechnol*., 2020, **23**, 101-440.

16 J. Hu, and Z. Duvnjak, Z, The production of a laccase and the decrease of the phenolic content in canola meal during the growth of the fungus Pleurotus. ostreatus in solid state fermentation processes. *Engl. Life Sci*., 2004, **4**, 50–55.

17 A. Asensio-Grau, I. Peinado, A. Heredia, and A. Andrés, Effect of cooking methods and intestinal conditions on lipolysis, proteolysis and xanthophylls bioaccessibility of eggs, *J Funct Foods*, 2018, **46**, 579-586.

18 A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assunção, and S. Balance, INFOGEST static in vitro simulation of gastrointestinal food digestion, *Nat. Protoc.*, 2019, **14**, 991-1014.

19 M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carriere, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D. J. McClements, O. Menard, I. Recio, C. N. Santos, R. P. Singh, G. E. Vegarud, M. S. Wickham, W. Weitschies and A. Brodkorb, A standardised static in vitro digestion method suitable for food - an international consensus, *Food Funct*., 2014, **5**(6), 1113–1124.

20 AOAC, 2000. Official methods of analysis. Association of Official Analytical Chemists. 15th Edition.

21 O. Tatirat and S. Charoenrein, Physicochemical properties of konjac glucomannan extracted from konjac flour by a simple centrifugation process, *LWT*, 2011, **44**, 2059-2063.

22 S. Lamothe, N. Azimy, L. Bazinet, C. Couillard, and M. Britten, Interaction of green tea polyphenols with dairy matrices in a simulated gastrointestinal environment, *Food Funct.*, 2014, **5**, 2621-2631.

23 R. Armellini, I. Peinado, A. Asensio-Grau, P. Pittia, M. Scampicchio, A. Heredia, and A. Andrés, In vitro starch digestibility and fate of crocins in pasta enriched with saffron extract, *Food Chem*., 2019, **283**, 155-163.

24 S. Mishra, J. Monro, and D. Hedderley, Effect of processing on slowly digestible starch and resistant starch in potato *Starch-Stärke*, 2008, **60**, 500-507.

25 C.H., Chang, H.Y. Lin, C.Y. Chang, and Y.C. Liu, Comparisons on the antioxidant properties of fresh, freeze-dried and hot-air-dried tomatoes. *J. Food Eng.*, 2006, **77**(3), 478-485

26 K. Thaipong, U. Boonprakob, K. Crosby, L. Cisneros-Zevallos, L. and D.H. Byrne, Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts, *J. Food Comp. Anal*., 2006, **19**, 669-675.

27 C. Sotomayor, J. Frías, J. Fornal, J. Sadowska, G. Urbano, and C. Vidalvalverde, Lentil starch content and its microscopical structure as influenced by natural fermentation. *Starch-Stärke*, 1999, **51**, 152–156.

28 C. Paz-Yépez, I. Peinado, A. Heredia, and A. Andrés, Influence of Particle size and intestinal conditions on in vitro lipid and protein digestibility of walnuts and peanuts, *Food Res. Int*., 2019, **119**, 951-959.

29 J. Calvo-Lerma, C. Paz-Yépez, A. Asensio-Grau, A. Heredia, and A. Andrés, Impact of Processing and Intestinal Conditions on in Vitro Digestion of Chia (Salvia hispanica) Seeds and Derivatives, *Foods*, 2020, **9,** 290.

30 P.I. Angulo-Bejarano, N.M. Verdugo-Montoya, E.O. Cuevas-Rodríguez, J. Milán-Carrillo, R. Mora-Escobedo, J.A. Lopez-Valenzuela, Tempeh flour from chickpea (Cicer arietinum L.) nutritional and physicochemical properties, *Food Chem.*, 2008, **106**, 106-112.

31 Y. Zhao, D. Sun-Waterhouse, M. Zhao, Q. Zhao, C. Qiu, and G. Su, Effects of solid-state fermentation and proteolytic hydrolysis on defatted soybean meal, *LWT*, 2018, **97**, 496-502.

32 M. Adamovic, G. Grubic, I. Milenkovic, R. Jovanovic, R. Protic, L. Sretenovic, and L.J. Stoicevic, The biodegradation of wheat straw by Pleurotus ostreatus mushrooms and its use in cattle feeding, *Anim. Feed Sci. Tech*, 1998, **71**, 357–362.

33 R. Cohen, L. Persky, and Y. Hadar, Biotechnological applications and potential of wood-degrading mushrooms of the genus *Pleurotus*, *App. Microbiol*, 2002, **58**, 582–94.

34 K. Deepalakshmi, and S. Mirunalini, S, Pleurotus ostreatus: An Oyster Mushroom with Nutritional and Medicinal Properties. *J. Biochem. Technol*., 2014, **5**, 718-726.

35 M. Kadari, Changes in intracellular and extracellular enzyme activities of *Lentinus subnudus* during sporophore development, *Biosci. Res. Coummun*., 1999, **11**, 127-130.

36 S.J. Hur, S. Lee, Y.C. Kim, I. Choi, and G.B. Kim, Effect of Fermentation on the Antioxidant Activity in Plant-Based Foods, *Food Chem*., 2014, **160**, 346–356.

37 S. Dudonne, X. Vitrac, P. Coutiere, M. Woillez, and J.M. Mérillon, Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays, *J. Agr. Food Chem*., 2009, **57**, 1768-1774.

38 D.H. Alpers, Digestion and absorption of carbohydrates and proteins, In Physiology of the Gastrointestinal Tract, 1987, 1469-1487.

39 M.A. Osman, Changes in sorghum enzyme inhibitors, phytic acid, tannins and in vitro protein digestibility occurring during Khamir (local bread) fermentation, *Food Chem*., 2004, **88**, 129–134.

40 G. Di Lena, E. Patroni, and G.B. Quaglia, Improving the nutritional value of wheat bran by a white-rot fungus, *Int. J. Food Sci. Tech.,* 1997, **32**, 513–519.

41 A. Asensio-Grau, I. Peinado, A. Heredia, and A. Andrés, In vitro study of cheese digestion: Effect of type of cheese and intestinal conditions on macronutrients digestibility, *LWT*, 2019, **113**, 108278.

42 K.S. Sandhu, and S.T. Lim, Digestibility of legume starches as influenced by their physical and structural properties. *Carbohydr. Polym*., 2008, **71**, 245-252.

43 M. Frei, P. Siddhuraju, and K. Becker, Studies on the in vitro starch digestibility and the glycemic index of six different indigenous rice cultivars from the Philippines, *Food chem.,* **83**, 2003, 395-402.

44 N.G. Asp, J.M.N. Van Amelsvoort, and J.G.A. Hautvast, Nutritional implications of resistant starch, *Nutr. Res. Rev*., 1996, **9**, 1–31.

45 D.S. Ludwig, The glycemic index: physiological mechanisms relating to obesity, diabetes, and cardiovascular disease, *JAMA*, 2002, **287**, 2414-2423.

46 D. Haenen, J. Zhang, C. Souza da Silva, G. Bosch, I.M. van der Meer, J. Van Arkel, A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine, *J. Nutr*., 2013, **143**, 274-283.

47 J. Pérez-Jiménez, and F. Saura-Calixto, Literature data may underestimate the actual antioxidant capacity of cereals, *J. Agr. Food Chem*., 2005, **53**, 5036-5040.