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ORIGINAL RESEARCH

Comparing the Effectiveness of Three Different Biorefinery Processes at Recovering Bioactive Products from Hemp (*Cannabis sativa* L.) Byproduct

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Abstract

Hemp (*Cannabis sativa* L.) seeds are considered a nutritional powerhouse, rich in proteins and unsaturated fatty acids. The market for hemp seed food products is growing, due to the loosening of constraints in industrial cultivation. During the food processing chain, the external part of the seed is discarded, although it contains a significant amount of proteins. Converting this material into value-added products with a biorefinery approach could meet the ever-increasing need for sustainable protein sources while reducing food waste. In this study, creating value from hemp byproducts was pursued with three different approaches: (i) chemical extraction followed by enzymatic digestion, (ii) liquid fermentation by strains of *Lactobacillus* spp., and (iii) solid-state fermentation by *Pleurotus ostreatus*. The resulting products exhibited a range of in vitro antioxidant and antihypertensive activity, depending on the proteases used for enzymatic digestion, the bacterial strain, and the length of time of the two fermentation processes. These byproducts could be exploited as functional ingredients in the food, pharmaceutical, and cosmetic industries; the suggested biorefinery processes thus represent potential solutions for the development of other protein-containing byproducts or wastes.

Keywords Protein hydrolysates · *Lactobacillus* spp. · *Pleurotus ostreatus* · Solid-state fermentation · ACE-inhibitory activity · Antioxidant activity

Abbreviations

6BHI	L. *fermentum* BHI6
AA	Ascorbic acid
AAeq	Ascorbic acid equivalents
ABTS	2,2,-Azino-bis (3-ethylbenz-thiazoline-6-sulfonic) acid
ACE	Angiotensin-converting enzyme
Alc	Alcalase
BSA	Bovine serum albumin
C1112	L. *rhamnosus* C112
C1272	L. *acidophilus* C1272
Chy	Chymotrypsin
DPPH	1,1-Diphenyl-2-picrylhydrazyl
Fla	Flavourzyme
HPM	Hemp meal
HPB	Hemp bran

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HPBPI	Hemp bran protein isolate
L.	*Lactobacillus*
L98b	*L. plantarum* 98b
LB325	*L. plantarum* 325
LB82	*L. plantarum* LB82
LC	*L. paracasei* LC
LCD1	*L. casei* LCD1
MR13	*L. fermentum* MR13
MRS	de Man-Rogosa-Sharpe
MW	Molecular weight
Neu	Neutrase
P. ostreatus	*Pleurotus ostreatus*
Pan	Pancreatin
Pep	Pepsin
PLH4	4 h *P. ostreatus* fermented sample
PLH9	9 h *P. ostreatus* fermented sample
PLH13	13 h *P. ostreatus* fermented sample
PLH17	17 h *P. ostreatus* fermented sample
PRLF	*L. fermentum* PRLF
Pro	Protamex
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
spp.	Species
SSF	Solid-state fermentation
Try	Trypsin

Introduction

Agro-food industrial chains generate a large number of byproducts and waste, with negative environmental, economic, and social impacts (Torres-León et al. 2018). These materials often contain significant quantities of secondary chemical building blocks (such as proteins, polysaccharides, lipids, micronutrients, phytochemicals, and dietary fiber) which could be recovered by biorefinery processes. The ever-increasing need for proteins of the food sector based on the continuous growth of the world population requires new sustainable protein sources due to depletion of renewable feedstocks, reduction in arable land, climate change, and environmental pollution.

Protein extraction from byproducts can be achieved using different techniques, like chemical extraction, liquid fermentation by microorganisms, and solid-state fermentation (SSF) by fungi (Baiano 2014). Chemical extraction is the most useful technique, taking advantage of the solubility of proteins at alkaline pH. Further hydrolysis of protein extracts with enzymes, while keeping the nutritional value of protein isolates, can improve their functional properties and allow the acquisition of specific bioactivities related to the release of bioactive peptides. To date, protein hydrolysates with antioxidant, antihypertensive, anticancer, antimicrobial, hypocholesterolemic, immunostimulating, and other properties have been produced from a number of sources (Martínez-Alvarez et al. 2015; Rizzello et al. 2016). Similar results can be achieved by liquid fermentation using *Lactobacillus* spp., thanks to their safety, efficient proteolytic system, and ability to adapt to different environments and matrices (Rizzello et al. 2016). For example, lactobacilli fermentation can improve the procyanidins and antiradical activity of cocoa bean (Di Mattia et al. 2013), or can improve the prebiotic activity of fibers present in plant-based sources (Sánchez-Zapata et al. 2013; Garcia-Amezquita et al. 2018; Nissen et al. 2020a). During fermentation, the biological activity of lactobacilli or the release of proteolytic enzymes into the media can change the nutritional and bioactive properties of the raw materials, producing peptides with various biological activities (Hafeez et al. 2014; Raveschot et al. 2018). Another biotransformation process is SSF by fungi; for example, mushrooms such as white-rot fungi are produced worldwide, both for their edible value and for their bio-compounds, which have medicinal properties. Recent studies have shown that it is possible to improve the quality (i.e., nutritional value and biological properties such as antioxidant) of cereals by applying SSF with mushrooms (Subramaniam et al. 2014; Zhai et al. 2015). Fungi through SSF are able to transform different wastes from industrial food products (Murthy and Naidu 2012; Nath et al. 2016). This biotechnology can be applied to the production of enzymes from agro-industrial wastes such as wheat bran, rice straw, banana waste, tea waste, and kinnow pulp (Oberoi et al. 2010). For example, xylanase can be produced by SSF on coffee byproducts (Murthy and Naidu 2012), or lipase can be produced from wheat industry byproducts (Treichel et al. 2010). Besides, other compounds than enzymes can be produced with SSF by fungi, likely optically pure L-lactic acid that can be obtained from whey byproduct of dairy industry (Taskin et al. 2012; Wu et al. 2011). SSF by fungi of whey, such as *Aspergillus niger* have received popularity for industrial glutamic acid production, while filamentous fungi for the production of lactobionic acid (Nath et al. 2016). SSF by fungi of apple pomace can be used to improve the mobilization of phenolic compounds, obtaining a product with superior antioxidant activity (Ajila et al. 2012). Bao et al. (2013) reported that the amount of crude proteins, crude fats, polysaccharides, reducing sugars, polyphenols, total amino acid, and adenosine significantly increased when rice was fermented with *Pleurotus eryngii*. White-rot fungi are known to efficiently attack the lignocellulosic matrix by secreting specific extracellular oxidative and hydrolase enzymes (Abdel-Hamid et al. 2013), allowing the extraction of proteins present in the vegetable cell wall. In particular, *Pleurotus ostreatus* is able to produce specific enzymes through SSF processing of different solid agro-food wastes (Zilly et al. 2012; Fernández-Fueyo et al. 2014; Akpinar and Urek 2012).

In the present work, we compared the effectiveness of these three bioprocesses at extracting and transforming the protein fraction of a byproduct generated in the hemp (*Cannabis sativa* L.) food processing chain. In the initial processing phase, hemp seeds are mechanically pressed, producing hemp meal (HPM) in the form of small cylindrical bars (along with hemp oil). These bars, after being ground, undergo a series of sieving steps to separate the small particles, the flour, from the large particles. This latter fraction, the hemp bran (HPB), is normally discarded or used for animal feed, despite its important protein content (Pojić et al. 2014). Recently a technological study has compared different mechanical pretreatments to improve ultrasonic extraction of polyphenols from hemp seed cake (Teh et al. 2014), but no literature is found over biotechnological approaches.

The three processes (alkaline extraction followed by enzymatic hydrolysis, liquid fermentation by *Lactobacillus* spp., and SSF by *P. ostreatus*) were optimized to recover HPB's protein fraction. The soluble proteins, antioxidant properties, and ACE-inhibitory activity in the resulting biorefinery products were evaluated and compared, in order to foresee their exploitation by the food, cosmetic, and pharmaceutical industries.

Material and Methods

Material

HPB, a byproduct remaining after mechanical pressing of hemp seeds and subsequent grinding and sieving, was supplied by a local company (Hemp Positive World, Cesena, Italy). Original hemp variety was Futura 75. General reagents and enzymes for enzymatic hydrolysis were analytical grade from Merck KGaA (Darmstadt, Germany). The o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe(NO2)-Pro) and o-aminobenzoylglycine (Abz-Gly) were from Bachem Holding (Bubendorf, Switzerland). MRS broth (de Man-Rogosa-Sharpe) (dextrose 20%; peptone 10%; beef extract 8%; sodium acetate 5%; yeast extract 4%; ammonium citrate 2%; dipotassium phosphate 2%; polysorbate80 1%; magnesium sulfate 0.2%; manganese sulfate 0.05%) was from Oxoid (Thermo Fisher Scientific, Waltham, MA, USA). SuperFi Platinum Taq was from Invitrogen (Thermo Fisher Scientific, Carlsbad, CA, USA). Pre-cast gels, the MW marker for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), bovine serum albumin (BSA), and related reagents were from Bio-Rad (Hercules, CA, USA).

HPB Protein Extraction and Enzymatic Hydrolysis

Protein Extraction and Evaluation of Total Protein Content

Protein extraction from HPB was made following the procedure described by Tang et al. (2009). The milled hemp bran was mixed with distilled water in the ratio of 1:20 (w/v) at room temperature, and the mixture was adjusted to pH 10.0 with 2 N NaOH. After 1 h stirring, samples were centrifuged at 8000 × *g* for 30 min at 4 °C. The pellet was discarded, and the supernatant was collected and adjusted to pH 5.0 with 2 N HCl. Then, the isoelectric precipitate was collected by centrifugation (8000 × *g* for 10 min), resuspended in deionized water, and homogenized. The suspension was adjusted to pH 7.0 with 2 N NaOH and lyophilized in a Heto PowerDry LL300 freeze dryer (Thermo Fisher Corporation) to produce hemp bran protein isolate (HBPI).

In preliminary experiments for evaluating the efficiency of different extraction protocols, the effects of HPB defatting, alkali extraction time, and ultrasonic treatment of HPB were also evaluated. Briefly, HPB was defatted adding hexane to the bran in a ratio of 1:3, stirring at 250 rpm for 30 min, and centrifuging at 5000 × *g* for 10 min at room temperature. The procedure was repeated thrice and the defatted HPB was dried overnight, under the hood. In ultrasonic assisted method, HPB and distilled water were mixed in a ratio of 1:20 in an ice bath and pH was adjusted to 10. Ultrasonication was performed using Hielscher UP400S (Hielscher Ultrasonic GmbH, Teltow, Germany), at 100 W for 5, 10, 20, and 30 min. Removal of insoluble material and acid precipitation were made following the method of Tang et al. (2009), previously described.

The total protein content of HBPI was determined by the Kjeldahl method (N = 6.25), according to AOAC standard methods (Latimer 2016).

Enzymatic Hydrolysis

The HBPI was dissolved in deionized water (1:8, w/v) and hydrolyzed with 8 different enzymes in separate aliquots. The reactions with pepsin, trypsin, and pancreatin (enzyme concentration 1%, v/v) were performed at 37 °C and pH 2.0, 7.0, and 7.5, respectively. Chymotrypsin (1%, v/v) and Flavourzyme (0.2%, v/v) treatments were made at 40 °C and pH 8.5 and 6.0, respectively. Hydrolysis with Alcalase (2%, v/v), Protamex (1%, v/v), and Neutrase (3%, v/v) were performed at 50 °C and pH 8.0, 7.0, and 7.0, respectively. After 2-h incubation, enzymes were inactivated by heating at 85 °C for 15 min. After cooling down to room temperature, solutions were centrifuged at 14000 × *g* for 10 min, and supernatants were collected and stored at − 80 °C for further analysis. The codes of the samples obtained by enzymatic treatments are reported in Online Resource 1.

Bacterial Fermentation of HPB

For bacterial hydrolysis, ten different strains of *Lactobacillus* spp. were selected in a first fermentation (screening fermentation), including *L. plantarum* LB82, 98b, and 325, *L. casei* LCD1, *L. paracasei* LC, *L. acidophilus* C1272, *L. fermentum* PRLF, MR13, and BHI6, and *L. rhamnosus* C1112. All these bacterial strains belong to the microbial collection of the Department of Agricultural and Food Sciences of the University of Bologna (Italy), and have been previously isolated from sourdough and extensively studied (Babini et al. 2017; Taneyo-Saa et al. 2018; Taneyo-Saa et al. 2019; Nissen et al. 2019; Nissen et al. 2020a; Babini et al. 2020). Bacteria were revived from − 80 °C glycerol stock by two successive growths in MRS broth (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for at least 24 h. Afterwards, for each bacterial strain, an inoculum of Log_{10} 7 CFU/mL was used to hydrolyze independently two sterilized (121 °C and 1 bar for 15 min) HPB/water suspensions (1:6 and 1:12 w/v). Incubations were made in 50-mL volumes at 37 °C for 24 h.

The best performer strains of *Lactobacillus* spp. on the best sterile HPB/water suspension from the screening fermentation (*L. plantarum* 98b, *L. fermentum* 325, and *L. rhamnosus* C1112) were applied in a second fermentation (process fermentation), on 1:6 (w/v) sterile HPB/water suspension. These three strains and a pool of them were employed at a concentration of Log_{10} 7 CFU/mL to ferment the HPB/water suspension at 37 °C up to 72 h.

Prior, over, and after both the screening and the process fermentations, pH values and bacterial growths were recorded. Not-inoculated sterile HPB/water suspensions were used as controls. Two biological replicates and two independent experiments were performed.

A complete list of bacterial strains used for the first and the second fermentation and their codes are reported in Online Resource 1.

pH Values of Bacterial Fermented HPB

The pH levels of bacterial fermented HPB/water suspensions were determined with a pH meter (Crison, Alella, Spain) at 20 °C, appropriately calibrated with three standard buffer solutions at pH 9.21, 4.00, and 2.00. The pH values were measured in duplicate at different times, prior, over, and after the process.

Bacterial Cell Measurement

Bacterial quantification was obtained by both culture-dependent and culture-independent protocols. The first consisted in plating on selective MRS agar supplemented with 0.05 g/L L-cysteine serial dilutions (NaCl 0.9%, w/v) of the samples, then incubated for 24 h at 37 °C. Values were expressed as Log_{10} CFU/mL. Culture-independent quantifications were obtained by qPCR with the SYBR Green I chemistry applying genus-specific primers as Lac1 for *Lactobacillus* spp. (forward: 5′-GCAGCAGTAGGGAA TCTTCCA-3′ and reverse: 5′-GCATTYCACCGCTA CACATG-3′) (Castillo et al. 2006). Genetic standards were prepared from relative PCR amplicons from DNA of pure cultures of the target bacteria using a Pro-Flex PCR apparatus (Applied Biosystem, Foster City, CA, USA) as described previously (Nissen et al. 2019, 2020a, b). Bacterial DNA extraction and qPCR reactions were performed according to previous protocols employing a RotorGene 6000 (Qiagen, Hilden, Germany) (Nissen et al. 2019, 2020a, b). Quantification results are reported as the means of the values of the two techniques employed and expressed as Log_{10} cells/mL (Nissen et al. 2019, 2020a, b). Parameters of successful reactions and appropriate quantifications of qPCR are reported in Online Resource 5.

P. ostreatus SSF on HPB

SFF Process

Fifty grams of hemp bran was placed in a 500-mL Pyrex bottle with a cotton cap, wetted with 15 mL of potassium phosphate buffer 0.1 M pH 5.8 and sterilized by autoclaving at 120 °C for 20 min. This substrate was inoculated with 8.5 g of *P. ostreatus* (commercial strain purchased from Azienda Agricola Funghi Mara, BO) grown on malt extract agar (Masutti et al. 2015). The fermentation was made at 27 °C, in the dark for a period of 17 days. The fermentation process was monitored sampling after 4, 9, 13, and 17 days. The samples were recovered under sterile conditions by adding in the fermenter a specific quantity of buffer, necessary to collect the enzymes produced during the SSF process. The "waste water," the portion of solution not adsorbed onto the substrate and containing the enzymes, was withdrawn by a sterile pipette. Each sample was centrifuged for 20 min at 4255 × *g* to remove the solid fraction and lyophilized in a Coolsafe 55-4 freeze dryer (Scanvac, Lillerod, DK). Samples collected after 4, 9, 13, and 17 days of SSF were named, respectively, PLH4, PLH9, PLH13, and PLH17 (Online Resource 1).

Determination of Cellulase, Xylanase, Pectinase, and Amylase Activities

The cellulase, xylanase, and pectinase activities were determined as reducing sugars following the method described by Bailey et al. (1992), using cellulose filter paper (ca. 50 mg), xylose from beechwood, pectin from apple, and starch from potato as respective substrates. The reaction for cellulase activity was carried out at 50 °C for 1 h, in citrate buffer 0.05 M

at pH 4.8. Reactions for xylanase, pectinase, and amylase activities were carried out at 30 °C for 3 min at variable pH (3-6) in citrate buffer 0.05 M. The glucose, xylose, and galacturonic acids released were detected by a UV-Vis spectrophotometer (UVIKON 923, Biotech Kontron, Augsburg, Germany) at 550 nm. The total activities were expressed as μmol of monomer released per minute. Each test was repeated three times for each sample. Concerning the assays conducted at variable pH, only the activities at optimum pH were reported: pH = 6 for xylanase, pH = 4 for pectinase, and pH = 5 for amylase (Table 2).

Determination of Peroxidase and Laccase Activities

The peroxidase activity was determined following the method described by Setti et al. (1998). Briefly, it was employed an oxidative coupling reaction of MBTH (3-methyl 2-benzothiazolinone hydrazone) in the presence of hydrogen peroxide and methoxyphenols at 30 °C, in 25 mM sodium phosphate buffer at pH 6.5. The reaction produces a red-colored azo-dye compound, then detected by a UV-Vis spectrophotometer (UVIKON 923, Biotech Kontron), at 502 nm.

The laccase activity was determined following the method described for the peroxidase activity, but without hydrogen peroxide (Setti et al. 1999). The total activities were expressed as μmol/min of MTBH. Each test was repeated three times for each sample.

Determination of Arylesterase Activity

The arylesterase activity was determined according to the method of Giuliani et al. (2001) with slight modifications. In particular, 0.9 mL of 100 mM sodium phosphate buffer pH 6.0, 0.1 mL of methyl ferulate or methyl caffeate, and 0.05 mL of the extract were mixed in a quartz cuvette. Immediately after adding the extract, the absorbance decrement at 335 nm was measured in a spectrophotometer (UVIKON 923, Biotech Kontron) for a time needed to detect the disappearance of methyl ferulate or methyl caffeate. These measurements were conducted three times on each sample. The activities were expressed in μmol/min of methyl ferulate and methyl caffeate hydrolyzed.

Determination of Protease Activity

The protease activity was estimated by the method described by Kunitz (1947) with slight modification. A total of 1 mL of enzyme solution was added to 1 mL of casein 2% (w/v) in 0.1 M sodium phosphate buffer at variable pH (6-9); each mixture was incubated at 37 °C for 1 h and the reaction was stopped by adding 2 mL of TCA 10% (w/v). The samples were centrifuged for 10 min at 8000 × *g* and for each supernatant, the tyrosine equivalents released were detected by a UV-Vis spectrophotometer (UVIKON 923, Biotech Kontron) at 280 nm.

The activity was expressed in μmol tyrosine/min and the one at optimum pH 9 was reported (Table 2). Each measurement was conducted in triplicate on the same sample.

Evaluation of Soluble Protein Content

Soluble protein concentration was evaluated by Bradford assay using the Quick Start Bradford Protein Assay kit from Bio-Rad (Hercules, CA, USA). Absorbance of samples after addition of the dye was measured after 5-10 min, at 595 nm in a microplate reader SPARK 10M (TECAM, Switzerland). The standard curve was obtained with bovine serum albumin (BSA) from 0.5 to 10 μg/mL. Results were expressed in μg/mL, as mean ± SEM (standard error of mean) of three replicates.

Protein Pattern Analysis by SDS-PAGE

Protein pattern was analyzed on 4% stacking and 14% (v/v) resolution Tris SDS-polyacrylamide gels using Mini-PROTEAN® equipment from Bio-Rad (Hercules, CA, USA). Protein ladder of 10-200 kDa was used as molecular weight (MW) marker.

Antioxidant Activity Assays

Antioxidant activity was analyzed by ABTS (2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay, ferrous ion-chelating ability assay, and ferric reducing antioxidant power (FRAP) assay. All analyses were performed on a microplate scale, and absorbance was measured in a microplate reader SPARK 10M (TECAM, Switzerland). Results were expressed as mean values of three replicates. The ABTS (2,20-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) scavenging activity was determined according to the procedure of Re et al. (1999). Briefly, an ABTS stock solution (7 mM in 2.45 mM $K_2S_2O_8$) was diluted with sodium acetate 20 mM pH 4.5, to reach an absorbance of 0.70 ± 0.02 at 734 nm. This solution (198 μL) and the sample (2 μL) were mixed and incubated at room temperature in the dark for 30 min. The absorbance was measured at 734 nm. The results were expressed as mg AA eq/1 L of sample solution by means of a dose-response calibration curve of ascorbic acid (AA) at concentration from 0 to 5 mg/L. Ferrous ion-chelating activity was measured according to the method reported by Tang et al. (2009). Sample aliquots (25 μL) at different concentrations were mixed with 100 μL of 50 μM ferrous sulfate ($FeSO_4$) and 100 μL of 300 μM ferrozine. After incubation at room temperature for 10 min, the absorbance of solutions was measured at 562 nm. The results were expressed as μg EDTA eq/mL of sample solution by means of a dose-response calibration curve of EDTA (from 0 to 10 μg/L). The FRAP assay was performed following the method of Benzie and Strain (1996), based on the reduction of a ferric-tripyridyltriazine complex to

its ferrous, colored form in the presence of antioxidants. Briefly, the FRAP reagent was prepared freshly mixing 1 mL of a 10-mM TPTZ (2,4,6- tripyridy-*s*-triazine) solution in 40 mM HCl, 1 mL of 20 mM $FeCl_3$, and 10 mL of 300 mM acetate buffer, pH 3.6. Aliquots of 10 μL of sample were mixed with 300 μL FRAP reagent and the absorbance of reaction mixture was measured at 593 nm after incubation at room temperature for 10 min. AA at different concentrations in the range from 0 to10 μM was used as standard solution. Results were expressed as the volume of antioxidants having a ferric reducing ability equivalent to that of AA.

ACE-Inhibitory Activity Assay

Inhibition of angiotensin I-converting enzyme was determined through a fluorimetric assay using o-aminobenzoylglycyl-*P*-nitro-*L*-phenylalanyl-*L*-proline (Abz-Gly-Phe(NO2)-Pro) as substrate (Sentandreu and Toldra 2006). The assay was performed under the following conditions: 50 μL of sample were mixed with 50 μL ACE solution 7.5 μg/mL and pre-incubated at 37 °C for 10 min. The reaction was started by addition of 200 μL of 0.45 mM Abz-Gly-Phe(NO2)-Pro dissolved in 150 mM Tris buffer, pH 8.3, containing 1.125 M NaCl. The reaction mixture was incubated at 37 °C for 30 min and the fluorescence, generated by the release of the Abz-Gly group, was measured in a fluorometer microplate reader SPARK 10M (TECAN, Switzerland), using excitation and emission wavelengths of 355 and 405 nm, respectively. Different concentrations of o-aminobenzoylglycine (Abz-Gly) in the range of 5-30 μM were used to obtain the reference curve. All analyses were performed in triplicate. The IC_{50} values were determined using nonlinear regression analysis, fitting the spectrophotometric data with the log (inhibitor) vs. response model generated by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Statistical Analysis

Statistical tests were performed using the SPSS software (IBM Corp, Armonk, NY, USA). Statistical significance of differences among several means was determined using one-way analysis of variance ANOVA with Duncan test, with a significant level of $P < 0.05$. Post hoc Tukey's HSD test was performed with Statistica 8.0 (Tibco Inc., Palo Alto, CA, USA).

Results and Discussion

The byproduct (HPB), used in the present work, was coming from the refining process of HPM through a 0.35-μm sieve, to obtain type 2 semi-wholemeal flour. This material represents about the 10% (w/w) of the seed, which means that about 10% of hemp seed is normally discarded during the food processing chain. The protein content of HPB was 20.63 ± 0.91% (w/w) in dry matter, much higher compared to the amount measured by Pojić et al. (2014) in an equivalent fraction of HPM (10.6 ± 0.91%, w/w, in dry matter). This difference can be related to the variability of protein content in the whole seed (20-25% w/w, in dry matter) and in the HPM (30-50%, w/w in dry matter), depending on the variety of hemp and the oil extraction procedure (cold pressing or solvent) and efficiency (Malomo et al. 2014).

Protein Extraction and Enzymatic Hydrolysis of HPBPI

Protein extraction was obtained by the aqueous alkaline extraction method followed by isoelectric precipitation. In order to optimize the process, different extraction times were evaluated as well as preliminary defatting of the substrate, and the addition of an ultrasonication step for different times, after alkaline extraction (Online Resource 4). HPBPI from non-defatted HPB, obtained with alkali exposure time of 1 h, contained the highest amount of soluble proteins (27.45 g/L). This sample was therefore used for hydrolysis reactions with 8 different enzymes, which were performed in specific pH and temperature conditions. Reaction time was 2 h as the maximum hydrolytic activity is usually reached after 2-h incubation, as reported for a hemp protein isolate digested by six different proteases (Tang et al. 2009). The efficacy of the hydrolytic processes was checked analyzing the amount of soluble proteins released from the matrix and the protein/peptide profile of hydrolysates on SDS-PAGE (the "Protein Solubility and Profile of Biorefinery Products" section).

Screening Fermentation of HPB by Ten Strains of *Lactobacillus* Spp.

Preliminary Screening of *Lactobacillus* Spp. Strains

Screening fermentation was made to select the best combinations among ten different *Lactobacillus* spp. strains and two HPB/water suspensions (w/v). pH results (Online Resource 2) showed that the two HPB suspensions were similarly subjected to significant acidification already after 6 h, especially when C1112 and 98b were used ($P < 0.05$). Besides, this trend was kept up to 24 h and the C1112 was the best performer (pH 4.12 ± 0.07). However, the pH decrease was less than expected for a *Lactobacillus* spp. fermentation on a plant-based substrate, probably due to a buffering effect exerted by HPB. For example, sauerkrauts are acidified by lactic acid bacteria from an initial pH around 6.8 down to pH values minor than 4 after 12 h (Wiander and Korhonen 2011), or similar results were obtained by Fonteles et al. (2012) that after 24 h of fermentation of cantaloupe juice with *L. casei*, NRRL B-442 reached pH lower than 3.5.

qPCR reactions averagely resulted of $R = 0.9968$ and of $R^2 = 0.9936$, with an efficiency of 69%, and the same melt temperature specificity at 87 °C (Online Resource 5). Quantifications showed no significant differences between 1:12 and 1:6 (w/v) HPBI suspensions ($P > 0.05$). In detail, the best output was reached on 1:6 (w/v) suspension by LB325 and MR13 that grew up to Log_{10} 12.25 ± 0.09 and Log_{10} 12.71 ± 0.26 cells/mL, respectively (Online Resource 2).

Process Fermentation of HPB by Selected *Lactobacillus* Spp. Strains

Process fermentation was carried out inoculating a 1:6 (w/v) sterile HPB/water suspension with the three most competitive *Lactobacillus* spp. strains resulted from the screening fermentation. Two were selected for their top growth values (LB325, MR13), while a third for its top acidification performance (C1112). In addition, a pool of these three strains was assayed as the fourth inoculum. In this second part, the ratio HPB/water suspension of 1:6 (w/v) was chosen. The starting inocula and the incubation conditions were the same of the screening part, but fermentation was extended up to 72 h. Results for pH measurements (Table 1) indicated that each inoculum was able to lower the pH of more than 1 unit at the earl time point, and the top acidification was recorded after 48 h by C1112 with pH value of 4.21 ± 0.02. Considering the overall fermentation time, MR13 hit the larger delta of 2.26 pH unit (Fig. 1a). Results from *Lactobacillus* spp. quantification (Table 1) indicated that the maximum growth was reached by the bacterial pool at the endpoint, attaining at Log_{10} 12.64 ± 0.28 cells/mL with a delta value of Log_{10} 6.66 cells/mL (Fig. 1b). The growth of C1112 and MR13 strains recorded maximum levels after 48 h, but declined before the endpoint. However, each single inoculum showed a very intensive growth between 6 and 48 h and reached a growth plateau from 48 h to the end of incubation (Table 1). The high growth of these strains could be due even to a possible buffering capacity of the matrix. In fact, even at the endpoint, the pH had a limit value of 4.31 ± 0.04, as that recorded by MRI3. To our knowledge, there are no reports in literature about any *Lactobacillus* spp. growing more than Log_{10} 12.71 cells/mL for more than 48 h. For example, in Fonteles et al. (2012), after 20 h of cantaloupe juice fermentation by *L. casei*, NRRL B-442 reached just Log_{10} 8.39 CFU/mL. Nwamaioha and Ibrahim (2017) have demonstrated that *L. reuteri* 20016 and *L. rhamnosus* ATCC 53103 can grow up to Log_{10} 9 CFU/g on MRS in anaerobic conditions and at 42 °C for 72 h. Other authors, in the attempt to optimize the components of MRS to achieve massive growth of *L. plantarum* JNU 2116, reached Log_{10} 9.15 CFU/g in aerobic conditions at 37 °C for 48 h (Yoo et al. 2018). These results demonstrated the potential of HMB as a good substrate to deliver high amounts of metabolites (including peptides) without inhibition of *Lactobacillus* spp. growth.

SSF on HPB with *P. ostreatus*

SSF on HPB with *P. ostreatus* was carried out to stimulate the overexpression of specific enzymatic activities capable of doing the demolition of hemp cell wall, delignifying the hemp bran and hydrolyzing its carbohydrate polymers to encourage the protein extraction. As Masutti et al. (2012) showed that increasing SSF enzyme collection frequency brought to a greater production of enzymes, we decided to collect samples of enzymes produced with a frequency of 4 days of SSF and the total enzymatic activities were determined as concentration per mL of waste water collected at different pH (Table 2). After 4 days of fermentation, a relevant pectinase activity appeared as a consequence of the chemical structure characterizing the wall cell plant of the dicotyledons such as hemp plant. In these wall cell plants, the pectic chains surround the hemicellulose and cellulose network and the hydrolysis of these chains can make the fibers located below, more susceptible toward a further hydrolase action (Hatfield 1993). In comparison, Zilly et al. (2012) reached a higher pectinase activity (620 U/L) after 14 days of *P. ostreatus* SFF on passion fruit waste that more than fibers have a superior content of sugars than hemp. The production of significant amylasic and xylanasic activities was observed after 9 days of fermentation evidencing the presence of starch as well as arabinoxylane in the hemp bran. Our results about amylase and xylanase are

Table 1 pH and Log_{10} cells/mL[1] of cultures of *Lactobacillus* spp. (LB325, C1112, MR13, and their pool) on hemp bran/water suspension 1:6 (w/v), during process fermentation. Means with different letters are statistically significant by post hoc Tukey's HSD test ($P < 0.05$). Means with different symbols are statistically significant by post hoc Tukey's HSD test ($P < 0.05$)

Samples		0 h	6 h	24 h	48 h	72 h
LB325	pH	$6.59 \pm 0.05^{*}$	$5.45 \pm 0.10^{**}$	$4.43 \pm 0.08^{\dagger}$	$4.39 \pm 0.02^{\dagger}$	$4.56 \pm 0.04^{\dagger}$
	Log cells/mL	5.95 ± 0.08^{a}	7.88 ± 0.09^{b}	10.41 ± 0.31^{c}	11.78 ± 0.35^{d}	12.13 ± 0.19^{e}
C1112	pH	$6.50 \pm 0.03^{*}$	$5.30 \pm 0.09^{**}$	$4.12 \pm 0.07^{\$}$	$4.21 \pm 0.04^{\$}$	$4.51 \pm 0.04^{\dagger}$
	Log cells/mL	5.97 ± 0.05^{a}	7.94 ± 0.16^{b}	10.42 ± 0.11^{c}	11.82 ± 0.29^{d}	11.72 ± 0.14^{d}
MR13	pH	$6.62 \pm 0.01^{*}$	$5.24 \pm 0.09^{\dagger}$	$4.43 \pm 0.08^{\dagger}$	$4.35 \pm 0.04^{\dagger}$	$4.36 \pm 0.04^{\dagger}$
	Log cells/mL	5.94 ± 0.08^{a}	9.33 ± 0.14^{c}	10.81 ± 0.32^{d}	12.37 ± 0.23^{e}	12.29 ± 0.05^{e}
Pool	pH	$6.49 \pm 0.02^{*}$	$5.82 \pm 0.08^{**}$	$4.59 \pm 0.04^{\dagger}$	$4.39 \pm 0.05^{\dagger}$	$4.63 \pm 0.04^{\dagger}$
	Log cells/mL	5.98 ± 0.04^{a}	8.35 ± 0.25^{b}	10.85 ± 0.32^{d}	11.73 ± 0.35^{d}	12.64 ± 0.28^{e}

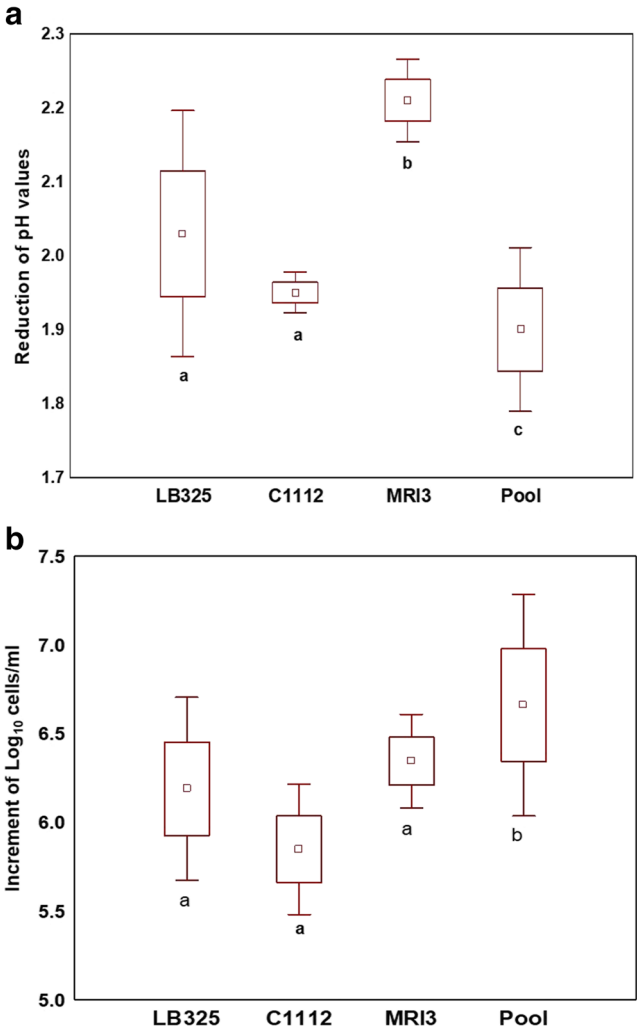

Fig. 1 Plots of mean values of **a** delta pH and **b** delta Log_{10} cells/mL of *Lactobacillus* spp. strains on hemp bran/water suspension 1:6 (w/v) during process fermentation. Marker: mean value; box: mean ± standard deviation; whiskers: mean ± 1.96* standard deviation. $^{a-c}$Means with different letters are statistically significant by post hoc Tukey's HSD test ($P < 0.05$)

better than what obtained by Zilly et al. (2012) after 14 days of *P. ostreatus* SSF on wheat bran, accounting for 67 and 500 U/L, respectively. Peroxidase activity appeared, then followed by laccase activity after 9 days which was a symptom of the fungal attack of lignin (Dashtban et al. 2010), since it is the more external structure of seed hull. Considering peroxidase and laccase, the outputs that we obtained showed for the former a minor activity in comparison to SSF by *P. ostreatus* on both wheat bran (63 U/L) and passion fruit wastes (150 U/L) almost at the same time point, but a larger activity for the latter already after 9 days (Zilly et al. 2012). Caffeoyl and feruloyl arylesterase activities were slightly produced remaining quite constant during all the period of the SSF even if the presence of caffeic and ferulic acid derivatives is common and in high quantity in monocotyledon spp. seed bran (Tetlow and Emes 2017; Peanparkdee et al. 2017). Arylesterase broke down the bounds between lignin and hemicellulose (Ralph and Helm 1993) in order to favor the next attack of the xylanasic activity and the release of reducing sugars. The cellulasic activity was quite negligible of the enzymes produced: It should be due to the "selective delignification" carried out by some kind of fungal attack that leaves the cellulose fibers almost intact (Narayanaswamy et al. 2013).

The scarce protease activity resulted surprising due to the high amount of proteins after the hydrolysis of the main polysaccharide structures.

Overall, these results on enzymatic hydrolysis revealed some information about the chemical and structural composition of hemp bran: Highly lignified, it is made of pectin that surrounds the hemicellulose-cellulose network, as in every dicotyledon plant. It also reveals the presence of starch, common in monocotyledon spp. bran and suggests the presence of protein moieties linked to cellulose fibers, the only ones which have not undergone hydrolysis.

Table 2 Enzymatic activities of the samples collected after 4, 9, 13, and 17 days of *Pleurotus ostreatus* SSF on hemp bran, expressed as μmol/min of converted substrate. Within each enzymatical activity, means followed by the same letter did not differ significantly by *t* test ($P > 0.05$). n.d. = not detected

Enzyme	Activity (μmol/min)			
	4 days	9 days	13 days	17 days
Peroxidase	27.58 ± 2.08^{a}	51.33 ± 1.73^{b}	31.82 ± 3.11^{b}	53.50 ± 3.70^{b}
Laccase	3.12 ± 0.28^{a}	99.01 ± 8.49^{b}	74.21 ± 7.87^{b}	105.27 ± 7.66^{b}
Cafferoil-esterase	2.49 ± 0.19^{a}	3.55 ± 0.78^{b}	3.74 ± 0.38^{b}	3.06 ± 0.32^{b}
Feruroil-esterase	3.72 ± 0.59^{a}	8.02 ± 0.83^{b}	5.03 ± 0.71^{b}	6.12 ± 0.85^{b}
Xylanase	65.77 ± 7.63^{a}	456.58 ± 14.99^{b}	318.38 ± 26.07^{b}	501.09 ± 21.99^{b}
Pectinase	111.08 ± 7.68^{a}	258.60 ± 6.08^{b}	186.70 ± 19.43^{b}	325.37 ± 16.38^{b}
Amylase	66.88 ± 8.89^{a}	296.38 ± 20.00^{b}	270.62 ± 14.12^{b}	355.83 ± 28.82^{b}
Cellulase	1.63 ± 0.04^{a}	2.26 ± 0.33^{b}	2.62 ± 0.35^{b}	2.41 ± 0.28^{b}
Protease	n.d.	5.74 ± 1.17^{b}	3.24 ± 0.42^{a}	5.80 ± 0.38^{b}

Protein Solubility and Profile of Biorefinery Products

The yield of products obtained by the three processes was variable, depending on the process and experimental conditions (Online Resource 3). In general, *Lactobacillus* spp. fermentation produced the highest amount of material (around 20 g/100 g HPB for the 6-, 24-, and 72-h MR13 samples, 6- and 24-h LB32520 samples, and 6- and 24-h samples fermented by the pool of bacteria), followed by the chemical extraction procedure (7 g/100 g HPB) and the *P. ostreatus* SSF process (average value of the four samples, about 1.7 g/100 g HPB).

The amount of soluble proteins is reported in Fig. 2. To compare the three processes, data are expressed as mg of soluble protein/g total proteins in HPB sample. The amount obtained after protein extraction (15.7 mg/100 g HPB) was significantly increased ($P < 0.05$) by the subsequent enzymatic treatments, with the only exception of the Flavourzyme digested sample (Fig. 2a). These results indicate that enzymes were able to solubilize proteins from the extracts. Protein solubility is a function of hydrophilicity and electrostatic repulsions which are generally increased after cleavage of peptide bonds, due to the higher number of ionizable amino and carboxyl groups (Lamsal et al. 2007). Pepsin treatment was particularly efficient, leading to the solubilization of about 10% (107.64 mg/g HPB protein) of the initial HPB proteins. The release of soluble proteins by microbial fermentation was dependent on *Lactobacillus* spp. strain and incubation time. The maximum amount was observed after 6/24 h for C1112 (18.36 and 19.06 mg/g HPB protein), after 6 h for MR13 (35.24 mg/g HPB protein), and after 24 h for strain LB325 (38.48 mg/g HPB protein), with the solubilization of 1.83, 3.52, and 3.84% of the initial HPB proteins, respectively. As the substrate was sterilized before incubation, the increase in soluble proteins can be assigned to the hydrolysis of substrate proteins by bacterial proteases. Similarly, fermentation of sorghum, green gram, and their composite meal for 24 h increased the soluble protein from 5.1 to 6.2% while prolonged fermentation beyond 24 h did not cause any further increase in soluble proteins (Chavan et al. 1988). In a previous research, pea seeds were fermented by *L. plantarum* 299v in monoculture under different time (3 h, 3, and 7 days) and temperature (22, 30, and 37 °C) conditions. Results showed an initial slight increase in soluble proteins in the first 3 h, a decrease in the subsequent 3 days, and again an increase that led to the highest content after 7 days fermentation at 30 °C, suggesting that peptide hydrolysis occurred mainly through secondary fermentation (Jakubczyk et al. 2013). SSF of HBP by *P. ostreatus* was effective in releasing proteins starting from 9 days of incubation, when about 2% of HBP proteins were solubilized (18.63 mg/g HPB protein). The effect of SSF by *P. ostreatus* was previously evaluated on kidney beans and oats, leading to an increase of soluble proteins of 13% and 6%, respectively (Espinosa-Paez et al. 2017). Other reports showed the effect of SSF by *Aspergillus oryzae* and *Rhizopus* spp. on defatted rice bran and wheat bran. *Rhizopus* spp. significantly increased protein solubility after 48 h of fermentation, while the effect of *A. oryzae* fermentation was observed only after 72 h (Moreira da Silveira and Badiale-Furlong 2009).

The profile of soluble proteins was achieved through SDS-PAGE (Fig. 3). The hydrolysis pattern was observably different. Degradation of HPBPI proteins (ranging from around 50 to 10 kDa) to smaller peptides was observed after any enzyme treatment. Pepsin, Flavourzyme, Alcalase, and Protamex showed the highest degree of hydrolysis with only a smeared band of MW around 10 kDa. Trypsin and chymotrypsin hydrolysates showed bands between 10 and 15 kDa, while those of pancreatin and Neutrase showed higher MW proteins in the range of 20-50 kDa, indicating a lower proteolytic activity. *Lactobacillus* spp. fermented samples showed the presence of a main smeared band at around 10 kDa, and two minor bands at 20 and 25 kDa. The intensity of the minor band decreased after 24 and 72 h, with strains C1112 and MR13,

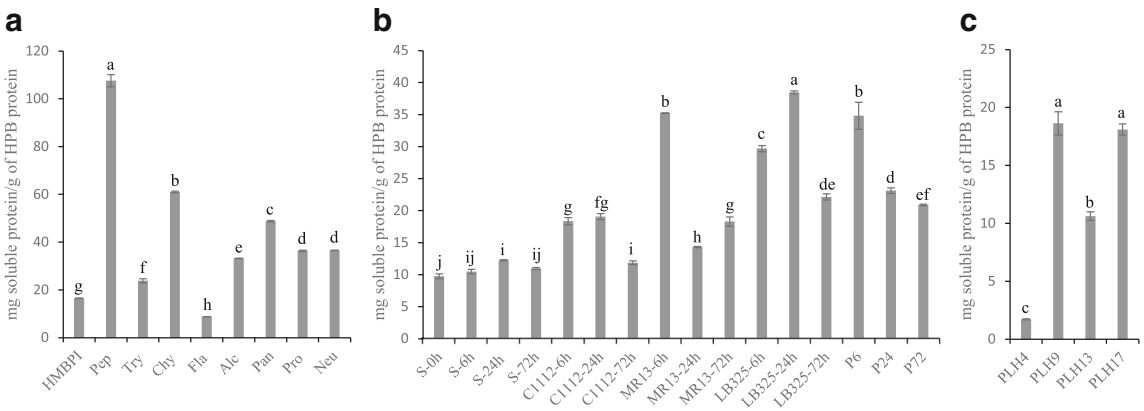

Fig. 2 Soluble protein content (mg soluble protein/g of HPB total proteins) of products obtained after biorefinery processes of HPB. **a** Enzymatic hydrolysis. **b** Fermentation by *Lactobacillus* spp. (LB325, C1112, MR13, and their pool, at different incubation times). **c** SSF by *Pleurotus ostreatus* (samples PLH after 4, 9, 13, and 17 days of incubation). [a-j]Means followed by the same letter in each plot did not differ significantly (Tukey test, $P < 0.05$)

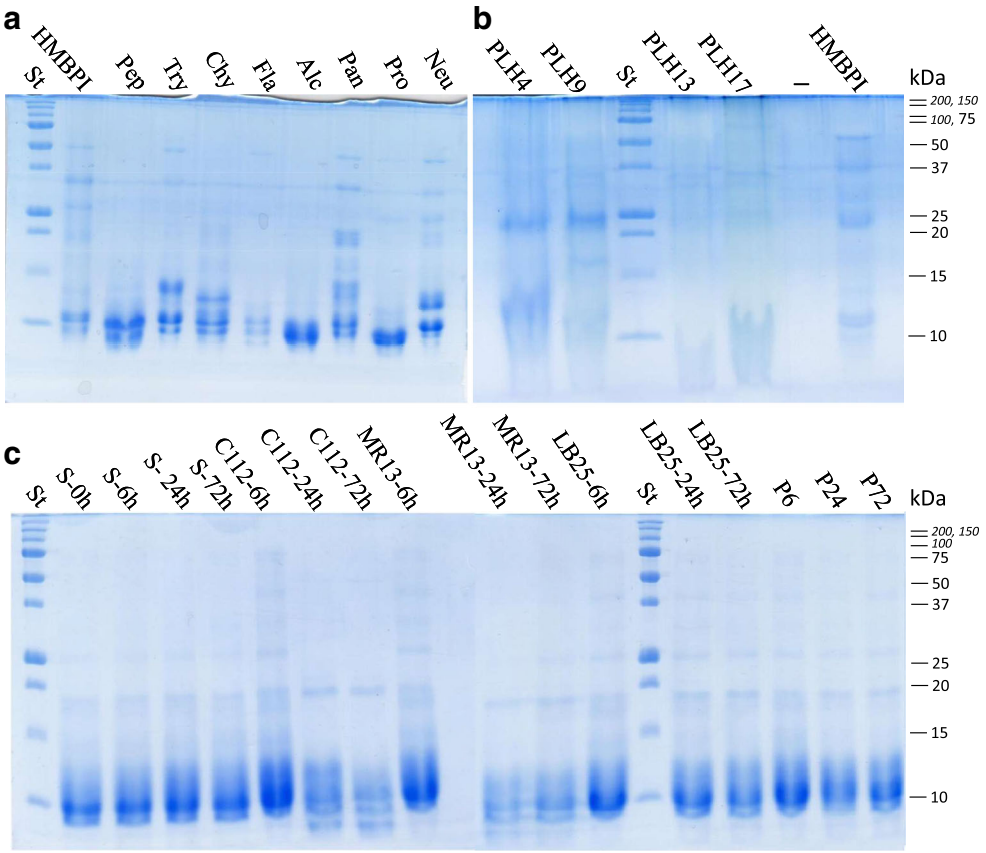

Fig. 3 SDS-PAGE of products obtained after biorefinery processes of HPB. **a** Enzymatic hydrolysis. **b** Fermentation by *Lactobacillus* spp. (LB325, C1112, MR13, and their pool, P, at different incubation times). **c** SSF by *Pleurotus ostreatus* (sample PLH after 4, 9, 13, and 17 days of incubation)

suggesting a degradation to smaller peptides, not detectable by this technique. Finally, the 4 days SSF sample showed the presence of one main band of MW between 20 and 25 kDa and a smear between 10 and 15 kDa. The latter after 9 days started to decrease to lower MW and the 20-25 kDa band completely disappeared after 13-day incubation. These results are in line with the protease activity measured in SSF samples, which started after 4 days of incubation.

The above results indicated that the three processes were able to partially solubilize HPB proteins and hydrolyze them to proteins/peptides with MW around and below 15 kDa. The more effective treatment was pepsin hydrolysis of HPBPI (yield of soluble proteins of about 10%), followed by the 24 h fermentation by LB325 (about 4%) and the *P. ostreatus* SSF after 9 days of treatment (2%).

Antioxidant Capacity of Biorefinery Products

The in vitro antioxidant capacities of biorefinery products were evaluated by ABTS, Fe^{2+} chelating, and FRAP assays (Fig. 4). The first method exploits the scavenging capacity of hydrogen-donating antioxidants toward the free radical $ABTS^{+}$. The second one measures the ability of antioxidants to chelate transition metal ions like Fe^{2+}, while the third is used to evaluate the ability of natural antioxidants to donate electrons, reducing reactive radical species. To make a comparison between the products obtained by the different processes, results are referred to the amount of lyophilized powder obtained by each of them.

Protein hydrolysates of HPB (Fig. 4a) had similar ABTS and FRAP profiles, showing a general increase of activity after enzymatic treatment, with respect to the initial extract. For both assays, chymotrypsin hydrolysate had the best activity, respectively about 7 times and 10 times higher than HPBPI. Fe^{2+} chelating assay showed a completely different trend, with only a slight increase of activity observed, again, after chymotrypsin treatment. *Lactobacillus* spp. hydrolyzed samples (Fig. 4b) showed only slight increase of ABTS and FRAP values after fermentation, with the highest activity (about 1.3 times than control) measured after 72-h incubation with the pool of *Lactobacillus* spp. strains. More relevant variations were observed for Fe^{2+} chelating activity which increased of about 1.8 times after 6-h incubation with LB325 and with the pool of bacteria. The antioxidant activity of SSF products (Fig. 4c) changed depending on the treatment time. ABTS and FRAP trends were similar with the highest activity after 9 days of treatment, while the highest Fe^{2+} chelating activity was observed after 13 days.

Comparing the activities of products obtained by different processes, it is possible to note that chymotrypsin hydrolysate had ABTS activity value similar to the sample derived from 9 days of SSF by *P. ostreatus* (respectively 7.0 and 8.0 mg AA eq/g), while bacterial fermented products had in general lower activity (with a maximum of 3.8 mg AA eq/g, observed for the

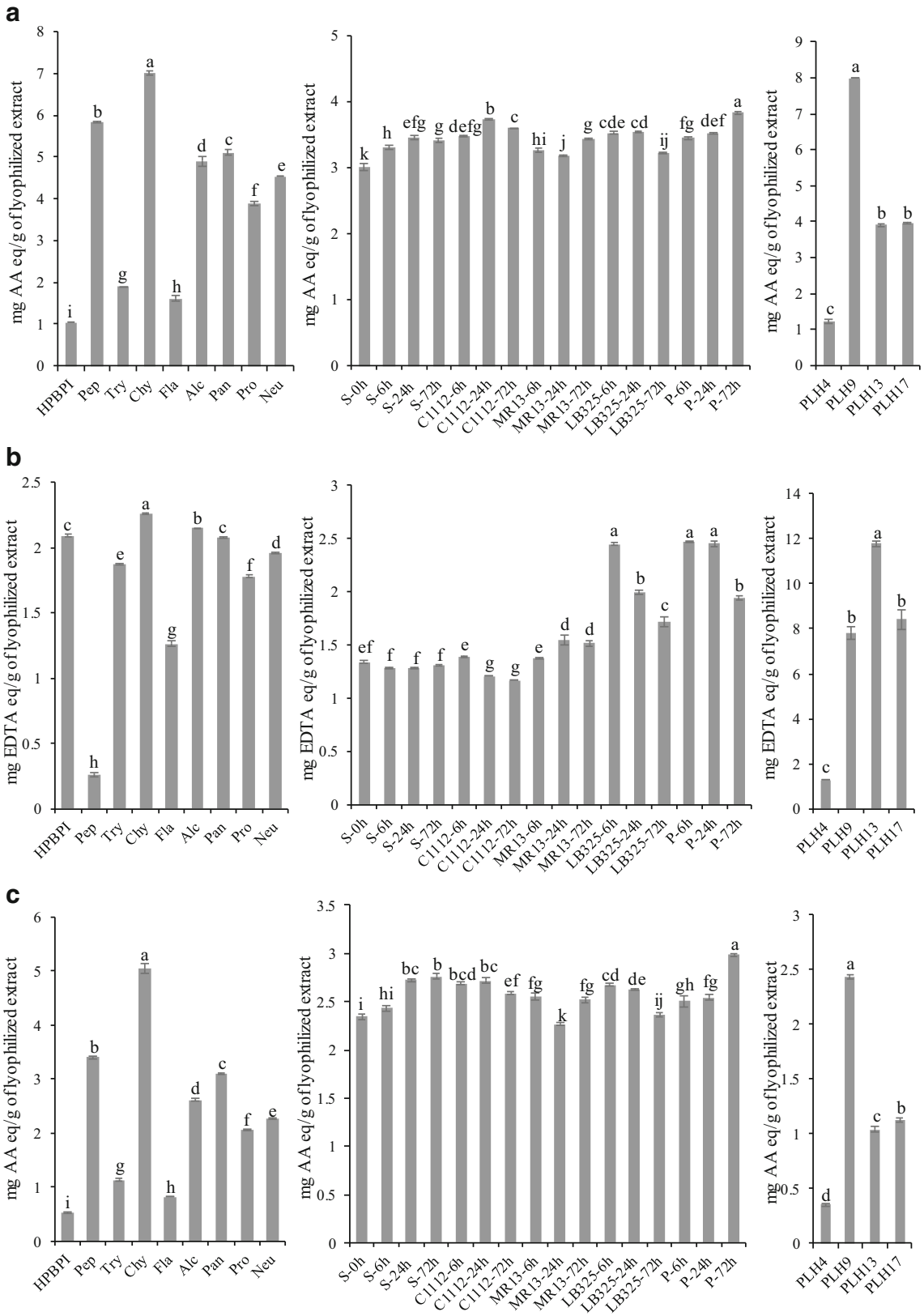

Fig. 4 Antioxidant activity of products obtained after biorefinery processes of HPB by enzymatic hydrolysis, fermentation by *Lactobacillus* spp. (LB325, C1112, MR13, and their pool, at different incubation times), and SSF by *Pleurotus ostreatus* (sample PLH after 4, 9, 13, and 17 days of incubation), evaluated by different assays. **a** ABTS (mg AA eq/g of lyophilized extract). **b** Fe^{2+} chelating activity (mg EDTA eq/g of lyophilized extract). **c** FRAP (mg AA eq/g of lyophilized extract). $^{a-k}$Means followed by the same letter in each plot did not differ significantly (Tukey test, $P < 0.05$)

72-h sample fermented by the pool of bacteria). Fe^{2+} chelating activity was particularly high in the 13 days *P. ostreatus* fermented sample (11.8 mg EDTA eq/g), about 6 times the best activities measured in enzymatic and microbial samples (2.2 mg EDTA eq/g for chymotrypsin hydrolysate and 2.5 mg EDTA eq/g for 6-h samples fermented with LB325 and the pool of bacteria). Finally, the highest FRAP values were obtained for chymotrypsin hydrolysate (5.0 mg EDTA eq/g) with respect to *Lactobacillus* spp. strains and *P. ostreatus* samples (highest values of 3.0 and 2.4 mg EDTA eq/g for the 72-h bacterial pool fermented sample and the 9 days *P. ostreatus* treated sample, respectively). The above results indicated that all the three processes were able to convert HPB into products with antioxidant properties, though the mechanisms responsible of the observed activities cannot be defined at this stage of research. Antioxidant properties have been previously reported for hemp seed protein hydrolysates obtained by enzymatic treatment of protein extracts (Tang et al. 2009; Girgih et al. 2013; Wang and Xiong 2019). On a similar experiment conducted on rapeseed protein hydrolysates, the EC50 was 0.71 mg/mL, similar to what we found in HPB prior microbial hydrolysis (Pan et al. 2011). Despite a comparison of data cannot be made, due to differences in experimental conditions, a variability in the antioxidant activity dependent on the type of protease, as observed in the present work, was reported for hemp seed protein isolate treated by six different proteases (Tang et al. 2009). A simulated gastrointestinal digest of hemp seed protein isolate improved some of its antioxidant activities (DPPH and hydroxyl and superoxide radical scavenging), while the crude protein extract and some peptide fractions showed significantly stronger metal reducing and chelating activities (Girgih et al. 2013). Products with antioxidant properties obtained by bacterial or fungal fermentation of hemp seeds have not been previously reported, but many examples are reported in the literature for other substrates. Flours from different cereals, pseudo-cereals, and legumes showed antioxidant properties after fermentation with lactic acid bacteria, and activity was dependent on peptides sharing compositional features typical of other antioxidant peptides (Rizzello et al. 2016). SSF by *Rhizopus oryzae* RCK2012 was an efficient method for the improvement of antioxidant potential (DPPH and ABTS radical scavenging) of cereals, caused by the release of soluble bioactive compounds. Among them are phenolic compounds and some other water-soluble compounds such as bioactive small peptides and oligosaccharides (Bhanja Dei and Kuhad 2014). Recently, the enhancement of antioxidant activity of fermented okara was obtained by SSF with *Rhizopus oligosporus* and *Aspergillus oryzae*. The release of isoflavone-derived compounds and small molecular weight peptides and amino acids, produced during the fermentation process, was considered to play role for the enhanced activities obtained using both fungi (Sitanggang et al. 2019).

ACE-Inhibitory Activity of Biorefinery Products

The antihypertensive properties of biorefinery products were measured by an in vitro assay testing the ability to inhibit angiotensin-converting enzymes. Results are expressed as IC_{50} values, i.e. the concentration of samples (in mg of lyophilized powder/L), able to induce a 50% inhibition of the enzyme (Table 3).

A strong variability in the activity was observed among the samples. Enzymatic hydrolysis with different proteolytic enzymes generally released ACE-inhibitory peptides from HPBPI. Indeed, the IC_{50} value of the latter (about 4268 mg/L) decreased over 35 times in pepsin hydrolysate (about 120 mg/L), over 25 times in Neutrase and Alcalase hydrolysates (respectively 155 and 176 mg/L) and, to a lesser extent, in all the other hydrolysates. Many other studies have demonstrated that antihypertensive peptides with varying potencies can be generated simply by treating protein substrates with different enzymes and enzyme: substrate ratios (Rizzello et al. 2016; Malomo et al. 2015). The differences in the activities are dependent on the different peptide patterns generated by enzymes. It is well known that ACE interaction with substrate or inhibitors is related to the presence of hydrophobic amino acids, to the size of the peptides and to the specific location of amino acids in peptide chain (Mundi and Aluko 2014). *Lactobacillus* spp. fermentation also led to a decrease of IC_{50} values with respect to the initial sample (S-0 h, 4298 mg/L). The lowest values, indicating highest antihypertensive activity, were measured after 24 and 72 h of fermentation with C1112 strain (837 and 720 mg/L, respectively). For the other *Lactobacillus* spp. fermented samples (with exception of LB325 after 72 h), IC_{50} ranged from around 1000 to 1300 mg/L. According to previous research on wholemeal wheat and pea, *Lactobacillus* spp. were able to release peptides with antihypertensive effects depending on the strain and incubation time (Rizzello et al. 2016). All the samples obtained by SSF with *P. ostreatus* had ACE-inhibitory activity. The IC_{50} values decreased from 1160 mg/L after 4 days of incubation to 738, 877, and 601 mg/L after 9, 13, and 17 days, respectively. As a protease activity was starting to be detected in the 9 days sample, it is possible that the antihypertensive activity, which significantly increased after that time, could be related to the release of bioactive peptides from substrate proteins. The results of ACE-inhibitory assay indicated that the three biorefinery processes were able to convert HPB into antihypertensive products. The highest activities measured in samples that underwent the *Lactobacillus* spp. and the *P. ostreatus* processes were similar, and 6 and 5 times lower than the best activity measured in enzymatic-treated samples. These values are in the range of previous results obtained on hemp seed protein hydrolysates. In particular, a pepsin-pancreatin digest of hemp seed protein isolate produced hydrolysates with ACE-inhibitory activity and IC_{50} value of 670 mg/L (Girgih

Table 3 ACE-inhibitory activity expressed as IC_{50} values (mg/L), of products obtained after biorefinery processes of HPB by (A) enzymatic hydrolysis; (B) fermentation by *Lactobacillus* spp. (LB325, C1112, MR13, and their pool); and (C) SSF by *Pleurotus ostreatus*. Means with different letters are statistically significant by post hoc Tukey test ($P < 0.05$)

Process and samples	ACE-inhibitory (IC_{50} mg/L)
Enzymatic hydrolysis	
HMBPI	4268.00 ± 1.02^{a}
Pepsin	120.30 ± 1.85^{i}
Trypsin	2145.00 ± 1.03^{b}
Chymotrypsin	290.80 ± 1.08^{f}
Flavourzyme	1440.00 ± 1.04^{c}
Alcalase	176.00 ± 1.06^{g}
Pancreatin	849.70 ± 1.05^{d}
Protamex	329.60 ± 1.02^{e}
Neutrase	155.80 ± 1.05^{h}
Bacterial fermentation	
Control after 0 h	4298.00 ± 1.01^{b}
Control after 6 h	4384.00 ± 1.06^{a}
Control after 24 h	2079.00 ± 1.04^{d}
Control after 72 h	2211.00 ± 1.09^{c}
C1112 after 6 h	1287.00 ± 1.01^{f}
C1112 after 24 h	836.70 ± 1.05^{o}
C1112 after 72 h	719.50 ± 1.08^{p}
MR13 after 6 h	1233.00 ± 1.02^{h}
MR13 after 24 h	1101.00 ± 1.05^{m}
MR13 after 72 h	1010.00 ± 1.05^{n}
LB325 after 6 h	1184.00 ± 1.03^{k}
LB325 after 24 h	1202.00 ± 1.03^{j}
LB325 after 72 h	1836.00 ± 1.06^{e}
Pool after 6 h	1249.00 ± 1.01^{g}
Pool after 24 h	1224.00 ± 1.02^{i}
Pool after 72 h	1147.00 ± 1.00^{l}
Solid-state fermentation	
PLH after 4 days	1160.00 ± 1.02^{a}
PLH after 9 days	737.50 ± 1.03^{c}
PLH after 13 days	877.20 ± 1.04^{b}
PLH after 17 days	601.10 ± 1.04^{d}

et al. 2011). Besides, IC_{50} values from 16 to 228 mg/L were measured in hemp seed proteins treated by different enzymes (Malomo et al. 2015). Thus, HPB can be a source of antihypertensive compounds with comparable activity to that obtained from the entire hemp seed. Interestingly, fractionation of hemp hydrolysates did not improve activity and isolated peptides responsible for antihypertensive activity had similar IC_{50} values (17 mg/L for WVYY and 269 mg/L for WYT, Girgih et al. 2014b). These data suggest the potential direct use of these hydrolysates, without further fractionation or purification steps, which are normally required to enhance potency, but increase the cost of the process. Another work has shown that when hemp seed and hemp meal hydrolysates obtained by simulated gastrointestinal digestion were administered in the diet of spontaneously hypertensive rats, it had strong hypotensive effects (Girgih et al. 2014a). Despite the antihypertensive activity in vivo of the biorefinery products from HPB has still to be verified, the preliminary results of the in vitro assays are encouraging starting points for an industrial exploitation as ingredients for functional foods, nutraceuticals, or pharmaceutical formulation for the prevention and treatment of hypertension.

Conclusions

The development of sustainable solutions to recover potentially valuable food byproducts and waste is one of the main challenges of our society. In the present work, three different biorefinery processes for recovering bioactive compounds from protein-containing HPB were assessed and compared. The chemical procedure consisted of an alkaline protein extraction followed by hydrolysis by one of the eight different proteolytic enzymes. The microbial liquid fermentation was performed using different *Lactobacillus* spp., while *P. ostreatus* was assessed in a solid-state biorefinery system. The resulting products exhibited different levels of antioxidant (including ABTS radical scavenging, Fe^{2+} chelating capacity, and ferric reducing power) and antihypertensive activity (evaluated by in vitro assays). The antihypertensive activity was particularly noteworthy, as the IC_{50} values of ACE inhibition were comparable to values in protein hydrolysates from the whole seed. These results indicate that bioactivity is not impaired by industrial processing, and further, these compounds remain in the generated byproducts. Pepsin treatment was the most promising biorefinery process in terms of ACE inhibition. A weaker effect, but still significant, was observed for samples from *Lactobacillus* spp. fermentation (*L. rhamnosus* C1112 was the most active) and SSF by *P. ostreatus* (albeit after 9 days of incubation). These results suggest that HPB could be transformed into value-added products that would increase the hemp seed market in the food, cosmetic, and pharmaceutical sectors. Product yield and bioactivity could be further increased by process combination and scale-up optimization.

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Authors' Contributions Leonardo Setti: conceptualization, data curation, funding acquisition, methodology, project administration, resources, supervision, validation, visualization, writing—original draft, and writing—review and editing.

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Irene Maggiore: data curation, formal analysis, investigation, visualization, writing—original draft, and writing—review and editing.

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