LWT - Food Science and Technology 138 (2021) 110685

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Fermentation of spent coffee grounds by *Bacillus clausii* induces release of potentially bioactive peptides

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ARTICLE INFO

Keywords:
Spent coffee grounds
B. clausii
Bioactive peptides
Biological activity
Fermentation

ABSTRACT

Spent coffee grounds (SCG) are residues generated during coffee beverage preparation that contain 12–17% protein and are a rich source of peptides. Bacteria can generate peptides with potential bioactivity through protein hydrolysis in a fermentation process. This study aimed to obtain digested protein hydrolysates with potential bioactivity from *Bacillus clausii*-fermented SCG. The fermentation was performed with 1.5×10^8 colony-forming units/mL of bacteria at 37 °C for 39 h. Total and soluble proteins and protein hydrolysates were quantified using spectrophotometric techniques. Pepsin/pancreatin protein hydrolysates were characterized using ultra-performance liquid chromatography-mass spectrometry. The physicochemical properties and potential bioactivity of peptides were evaluated using *in silico* analysis. The fermentation process increased the amounts of total proteins, soluble proteins, and protein hydrolysates by 2.7, 2.2, and 1.2-fold, respectively, compared to non-fermented SCG. Fermented SCG samples, increased the abundance of seven peptides that displayed potentially antioxidant capacity, angiotensin-converting enzyme activity, and dipeptidyl peptidase-IV-inhibitor activity. The YGF and GMCC peptide sequences presented the highest bioactivity scores (0.97 each), followed by the YWRYDCQ (0.65) and RMYRY (0.60) peptides. In summary, fermented SCG had increased abundance of peptides with high bioactive potential that may be exploited in managing oxidative stress, hypertension, and diabetes.

1. Introduction

The research on biologically active compounds obtained from natural sources is continually growing. Peptides are among the candidate bioactive compounds because of their utility, including their benefit to human health (Dullius, Fassina, Giroldi, Goettert, & Volken de Souza, 2020). Peptides can be obtained from different food sources of animal and plant origin; currently, they are obtained from residues in the food industry (Kehinde & Sharma, 2020).

Spent coffee grounds (SCG) are residues obtained during the process of instant coffee preparation. These residues are considered waste and have occasionally been used as fertilizers (Murthy & Madhava Naidu, 2012). The use of SCG has been diversified to obtain biodiesel, food color, antioxidant dietary fiber, and dietary matrix for the extraction of phenolic compounds using different technologies (Nguyen, Nguyen, Wang, Juan, & Su, 2020; Passos et al., 2017; Vázquez-Sánchez et al., 2018; Zuorro & Lavecchia, 2013). SCG protein hydrolysates with antioxidant and angiotensin-converting enzyme (ACE)-inhibitory activity have been obtained by enzymatic action (Valdés, Castro-Puyana, & Marina, 2020).

SCG have lipid, carbohydrate, and protein contents of ~24, 13, and 11 g/100 g, respectively; these are favorable characteristics for processing as a fermentable substrate. After a simulated digestion-fermentation process with SCG, a high chemoprotective activity against oxidative stress was observed in HepG2 cells, and anti-inflammatory properties were demonstrated in lipopolysaccharide (LPS)-stimulated RAW 64.7 macrophages; these activities were mainly related to hydrolyzed lignin and short fatty acids (López-Barrera, Vázquez-Sánchez, Loarca-Piña, & Campos-Vega, 2016; Martinez-Saez et al., 2017; Panzella et al., 2017).

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https://doi.org/10.1016/j.lwt.2020.110685
Received 19 August 2020; Received in revised form 29 November 2020; Accepted 30 November 2020
Available online 2 December 2020

Fermentation is a natural, alternative process for protein hydrolysate production. The activity of microbial proteolytic systems is used for hydrolyzing food proteins to release the peptides and amino acids used as a nitrogen source by microorganisms for growth (Montesano, Gallo, Blasi, & Cossignani, 2020). This protein hydrolysate production method is especially relevant in the dairy sector for harnessing the antioxidant, antimicrobial, antimutagenic, and antihypertensive activities of proteins (García-Burgos, Moreno-Fernández, Alférez, Díaz-Castro, & López-Aliaga, 2020; Halavach, 2020; Rochín-Medina, Ramírez-Medina, Rangel-Peraza, Pineda-Hidalgo, & Iribe-Arellano, 2018). However, in recent years, this method has been used for the preparation of food-plant protein hydrolysates, which act as inhibitors of an enzyme involved in the pathogenesis of metabolic syndromes and provide other health benefits (Montesano et al., 2020).

Bacillus clausii is a Gram-positive spore-forming bacteria with the capacity to release phenolic compounds from different food matrices (Ramírez, Quintero-Soto, & Rochín-Medina, 2020; Rochín-Medina, Ramírez, Rangel-Peraza, & Bustos-Terrones, 2018). Additionally, this microorganism produces alkaline proteases (Kazan, Denizci, Öner, & Erarslan, 2005) with the potential to yield protein hydrolysates through its antimicrobial activity during fermentation (Rochín-Medina, Ramírez-Medina et al., 2018). However, there are no reports on bioactive peptides in fermented SCG. The objective of this study was to induce the release of peptides from *B. clausii*-fermented SCG digested proteins, identify them, and assess their bioactivity potential by *in silico* analysis of their peptide sequences as an alternative for obtaining potentially valuable compounds in the prevention of chronic diseases.

2. Materials and methods

2.1. SCG fermentation

SCG were obtained from commercial Arabica coffee beans (World Table®, Mexico) roasted at a medium level. The SCG were sterilized and inoculated with a suspension of 1.5×10^8 CFU/mL of *B. clausii* (ATCC 700160), grown in trypticase soy broth (MCD Lab, Mexico). The inoculated samples were fermented at 37 °C for 39 h (Rochín-Medina, Ramírez, et al., 2018).

2.2. Protein and peptide characterization

2.2.1. Total protein

Fermented (FSCG) and non-fermented (NFSCG) SCG total proteins were determined using a micro-Kjeldahl system (Tecator, Sweden), according to a previously established method (Martinez-Saez et al., 2017). Total protein concentrations are expressed as g/100 g of sample (db).

2.2.2. Protein extraction and separation

FSCG and NFSCG were dried at 50 °C for 3 d and defatted with 10 mL hexane by steeping; the mixture was stirred (Vari-Mix, ThermoFisher, USA) at 20 °C for 16 h. Samples were centrifuged at 15 000×*g* for 15 min, and the pellets were washed by centrifugation with 20 mL of trichloroacetic acid in acetone (10%, w/v), followed by 0.2 mol/L ammonium acetate in methanol (80%, v/v), and then with acetone (80%, v/v) under the same centrifugation conditions. This procedure was repeated until the supernatant was colorless. For protein extraction, the pellet (1 ± 0.1 g) was resuspended in 20 mL of extraction buffer I (0.01 mol/L $CaCl_2$, 0.01 mol/L $MgCl_2$, and 0.001 mol/L PMSF), stirred (Vari-Mix, ThermoFisher, USA) at 20 °C for 12 h, and centrifuged at 15 000×*g* at 4 °C for 30 min. The supernatant was recovered, and the pellet was mixed with 10 mL of extraction buffer II [0.1 mol/L Tris-HCl (pH = 8.0), 0.17 mol/L $NaCl_2$, 0.001 mol/L PMSF, and 0.01 mol/L EDTA] for 8 h. The supernatants were recovered by centrifugation and then mixed. The proteins were precipitated from the supernatants by using two volumes of 0.1 mol/L ammonium acetate in methanol (−20 °C, 8 h) and then recovered by centrifugation (3000×*g* at 4 °C for 10 min). The pellet was washed with pure methanol followed by acetone (80%, v/v) and then dried to remove any solvents. The protein pellet was resuspended in 1 mL of deionized water and dialyzed against distilled H_2O at 4 °C for 3 d. Soluble protein content was determined using bovine serum albumin (Merck, Germany) as the standard (Bradford, 1976). Protein extracts equivalent to 30 mg of sample were separated by SDS-PAGE using 0.125 mg/mL polyacrylamide gel (acrylamide:bisacrylamide = 29:1, w/w) and stained with Coomassie blue R-250.

2.2.3. Protein hydrolysates

Defatted and depigmented FSCG and NFSCG proteins were hydrolyzed by sequential enzymatic *in vitro* digestion using pepsin-pancreatin (Merck, Germany) (Megías et al., 2004). One gram of each sample was incubated at 37 °C for 3 h with a pepsin solution [20 mL 0.1 mol/L HCl, 15 mg pepsin (pH = 2.0)]; then, the reaction was neutralized with 10 mL 0.2 N NaOH. For the second incubation, samples were treated with pancreatin [7.5 mL phosphate buffer, 40 mg pancreatin (pH = 8.0)] at 37 °C for 3 h. The enzymatic reaction was stopped by heating the samples at 80 °C for 20 min. The supernatant was separated by centrifugation (5000×*g* at 4 °C for 10 min) and filtered with PVDF (0.45 μm; PALL. USA). The hydrolysates were separated by centrifugation with a membrane filter of 10 kDa, vacuum dried, and resuspended in 500 μL of HPLC grade water. Protein hydrolysate content was determined as described before for soluble proteins.

2.2.4. Protein hydrolysate profiling by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) for hydrolysate peptide identification

A 15 μL aliquot of protein hydrolysates was injected into a UPLC-diode array detection (DAD) system (ACCELA, Thermo Scientific, USA). The separation was performed in a C18-Luna column (150 × 4.6 mm) (Phenomenex, Inc, USA) using 1% (v/v) formic acid (A) and acetonitrile (B) with a linear gradient of 0.5–30% B for 25 min at a rate of 0.2 mL/min. The detection was performed at 254 and 280 nm. The UPLC-DAD system was connected to a mass spectrometer with an electrospray ionization (ESI) source (LTQ XL, Thermo Scientific, USA) operating in positive mode (35 V, 300 °C). Data were analyzed using the Xcalibur 2.2 software (Thermo Scientific, USA), and full scan spectra were acquired in the *m/z* range of 100–2000. Selected ions for MS^n experiments were fragmented by collision-induced dissociation by applying 10–45 V. Helium and nitrogen were used for collision and drying, respectively.

2.2.5. In silico physicochemical properties and biological activities of FSCG and NFSCG peptides

The physicochemical properties of FSCG- and NFSCG-identified peptides were evaluated by *in silico* analysis (Zhang et al., 2020). The hydrophobicity, net charge, and pI (isoelectric point) of peptides were calculated and predicted using the PepDraw tool (http://pepdraw.com/) (Zhang et al., 2020). Potential biological activities were obtained using the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) (Minkiewicz, Iwaniak, & Darewicz, 2019; Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). The PeptideRanker tool (http://distilldeep.ucd.ie/PeptideRanker/) was used to classify peptides as bioactive based on the predicted probability (Ding, Liang, Yang, Sun, & Lin, 2020). Values close to 1 (on a scale of 0–1) indicate a greater probability that the amino acid sequence exhibits bioactivity.

2.3. Statistical design

A one-way factorial design was used. The comparison of the means ± SD among groups was performed using Tukey's test for multiple comparisons. Each experiment was replicated three times. Differences with $p < 0.05$ were considered significant at 95% confidence intervals. Statistical analysis was performed using Minitab 16.0 (State College, PA).

3. Results and discussion

The total protein content of NFSCG was 15 g/100 g (Table 1), a value within the reported range of 12.8–16.9% (Cruz et al., 2012; Jiménez-Zamora, Pastoriza, & Rufián-Henares, 2015). Fermentation of SCG significantly ($p < 0.05$) increased protein content by 2.8-fold relative to that of NFSCG. This increase in total protein content by fermentation has been observed in coffee pulp fermented with *Streptomyces* strains at 28–45 °C for 10 d (a 1.5-fold increase) (Orozco et al., 2008) and in *Lactobacillus plantarum*-FSCG incubated at 37 °C for 48 h (a 1.2-fold increase) (Choi, Rim, Na, & Lee, 2018). Soluble protein in FSCG (Table 1) increased significantly ($p < 0.05$) by 2.2-fold compared to that in NFSCG. There are few reports of the soluble protein content in coffee; a variation of 4.9–9.1 g/100 g of soluble proteins has been reported in several *C. arabica cultivars* (Baú, Mazzafera, & Santoro, 2001). The increase in protein upon fermentation can be attributed in part to the type of bacteria, fermentation conditions, increase in biomass, loss of dry matter due to the action of microorganism(s), and microbial degradation of complex proteins releasing peptides and amino acids (Pranoto, Anggrahini, & Efendi, 2013).

The significant increase ($p < 0.05$) in protein hydrolysates (Table 1) in FSCG samples (1.2-fold) can be attributed to the generation of small peptides after pepsin-pancreatin hydrolysis and of proteases produced by the fermentation process (Chauhan & Kanwar, 2020).

FSCG and NFSCG proteins were separated by SDS-PAGE, and their expression profiles are shown in Fig. 1. The two low-molecular-weight bands found in both samples correspond to the subunits of legumin-like proteins from 11S globulins in coffee (Coelho et al., 2010). The protein 11S-legumin is a 55 kDa polypeptide that, under denaturing conditions, is cleaved into two legumin subunits: a basic form (α, 32 kDa) and an acidic form (β, 22 kDa) (Rogers et al., 1999). A 76.3 kDa putative heat shock protein (HSP) was observed in FSCG and NFSCG (Fig. 1); this result is associated with a proteomic study of coffee that identified a 76.3 kDa HSP (HSP70) (K. G. do Livramento, Borém, Jose, et al., 2017; K. Livramento, Borém, Torres, et al., 2017) related to the stress response of coffee seed. The HSP could be synthesized as a response to high temperatures during the brewing process.

Fig. 2 shows the chromatographic separation of protein hydrolysates from FSCG and NFSCG. The highest peak intensity for FSCG (a 1.7-fold increase relative to that for NFSCG) was observed between 0 and 10 min, followed by the second-highest peak intensity (1.5-fold relative to that for NFSCG) in the retention time interval between 20 and 30 min and a 0.9-fold increase between 11 and 20 min. We detected seven peptide sequences in both FSCG and NFSCG (0–30 min); each peptide contained between two and seven amino acids and was 247.3–1033.3 Da in size (Table 2). This indicates that the fermentation increased the amounts of these peptides in FSCG.

The predicted physicochemical properties of the identified peptides in protein hydrolysates from FSCG and NFSCG are shown in Table 2. Hydrophobicity, charge, peptide sequence, and low molecular size play an important role in some bioactivity properties in peptides from food matrices (Sun, Acquah, Aluko, & Udenigwe, 2020). In our study, the hydrophobicity of the identified peptides ranged from +6.63 to +10.59 kcal/mol. In addition, for all the peptides, the pI ranged from 4.84 to 10.13. Only two peptides [YSR (Tyr-Ser-Arg) and RMYRY (Arg-Met--Tyr-Arg-Tyr)] were positively charged in the neutral solvent with net charges of +1 and + 2, respectively, which is related to the type of transport across the gastrointestinal epithelium into blood circulation and to their bioactive functions (Sun et al., 2020).

The bioactive potential of identified peptides from FSCG and NFSCG (Table 3) was determined by an *in silico* analysis using the BIOPEP database. Seven peptides with potential biological activity were found in both samples and were identified to potentially exhibit antioxidant, ACE, and dipeptidyl peptidase-IV (DPP-IV) inhibitor activities. The probability of bioactivity of each peptide was predicted by PeptideRanker. The YGF (Tyr-Gly-Phe) and GMCC (Gly-Met-Cys-Cys) peptide sequences presented a higher score (0.97), followed by the YWRYDCQ

Table 1
Total protein, soluble protein, and protein hydrolysates in fermented (FSCG) and non-fermented (NFSCG) spent coffee grounds samples.

Sample	Total protein g/100 g of sample (db)	Soluble protein (%)[a]	Protein hydrolysates (%)[b]
FSCG	41.67 ± 5.35[a]	38.92 ± 4.40[a]	81.97 ± 8.90[a]
NFSCG	15.00 ± 1.00[b]	17.41 ± 3.51[b]	69.85 ± 7.02[b]

Data are the mean ± SD of three independent experiment. Columns with different letters indicate statistical differences (*t*-test; $p < 0.05$).
[a] Percentage based on total protein content (db).
[b] Percentage based on soluble protein content (db).

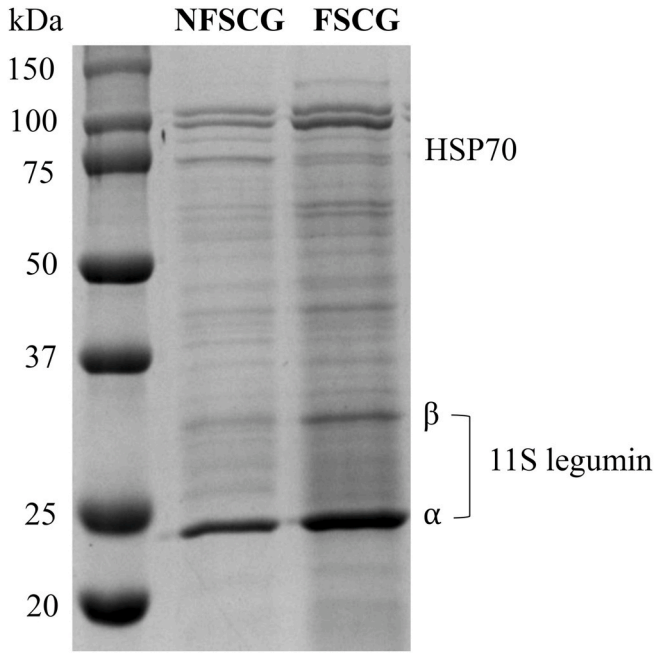
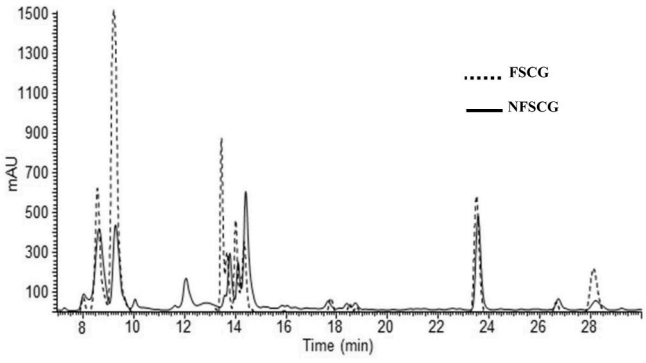


Fig. 1. Separation of fermented (FSCG) and non-fermented (NFSCG) spent coffee grounds soluble proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Molecular weight standards are shown on the left. HSP, heat shock proteins.



Retention time (min)	Area		Fold-increase
	FSCG	NFSCG	
0-11	3.8×10^7	2.2×10^7	1.7
11-20	2.4×10^7	2.6×10^7	0.9
20-30	1.6×10^7	1.0×10^7	1.5

Fig. 2. Chromatogram of protein hydrolysates from fermented (FSCG) and non-fermented (NFSCG) spent coffee grounds.

Table 2
Physicochemical properties of identified peptides from fermented (FSCG) and non-fermented (NFSCG) spent coffee grounds.

Retention time (min)	Sequence	MH^+ (Da)	Error (ppm)	Molecular mass (Da)	Hydrophobicity[a] (Kcal/mol)	Isoelectric point[a]	Net charge[a]
7.92	YWRYDCQ	1033	8.61	1033.3	+10.59	6.06	0
8.64	YGF	365	0.78	385.5	+6.63	5.38	0
8.89	QT	247	2.02	247.3	+8.92	5.29	0
9.12	RMYRY	885	9.89	787.4	+9.43	10.13	+2
9.38	GMCC	415	3.87	309.5	+8.34	4.84	0
14.08	YSR	422	8.23	424.2	+9.46	9.64	+1
23.48	YQH	445	2.01	446.2	+10.29	7.60	0

[a] The hydrophobicity, net charge and pI were calculated by the PepDraw tool (http://pepdraw.com).

Table 3
Bioactive potential of peptides identified after digestion with pepsin/pancreatin of fermented (FSCG) and non-fermented (NFSCG) spent coffee grounds proteins, using mass coupled ultra-performance liquid chromatography.

Retention time (min)	Peptide sequence[a]	Score[b]	Bioactive sequence[c]	Activity[c]
7.92	YWRYDCQ	0.65	RY, YW WRY, WR, YD, YW	Antioxidant DPP-IV inhibitor
8.64	YGF	0.97	YG, GF YG, GF	ACE-Inhibitor DPP-IV inhibitor
8.89	QT	0.05	QT	DPP-IV inhibitor
9.12	RMYRY	0.60	RY, MY YRY, MY MY, RM, YR	ACE-Inhibitor Antioxidant DPP-IV inhibitor
9.38	GMCC	0.97	GM	ACE-Inhibitor
14.08	YSR	0.32	YS	DPP-IV inhibitor
23.48	YQH	0.21	YQ, QH	DPP-IV inhibitor

[a] Aminoacids: Y: Tyr; W: Trp; R: Arg; D: Asp; C: Cys; Q: Gln; G: Gly; F: Phe; T: Thr; M: Met; S: Ser; H: His.
[b] Peptide score by PeptideRanker for predicting the bioactivity potential (http://distilldeep.ucd.ie/PeptideRanker/).
[c] The biological activity was predicted by the BIOPEP database (http://www.uwm.edu.pl/biochemia/). ACE, angiotensin-converting enzyme; DPP-IV, dipeptidyl peptidase-IV.

(Tyr-Trp-Arg-Tyr-Asp-Cys-Gln) and RMYRY peptides, with scores of 0.65 and 0.60, respectively. The peptide sequences with the highest scores (Table 3) were found within the first 10 min in the chromatogram, where peptides from FSCG exhibited peak intensities that were increased by 1.7-fold (Fig. 2). The structures and the MS/MS spectra of the four major peptides from FSCG and NFSCG are shown in Fig. 3. Some reports have shown that predicted peptides with a PeptideRanker score above 0.5 are more likely to be bioactive (Ding et al., 2020; Shang et al., 2018). Protein hydrolysates from globulins are a pool of peptides with antioxidant activity (Ma, Zhang, Bao, & Fu, 2020; Torres-Fuentes, Contreras, Recio, Alaiz, & Vioque, 2015) and hypoglycemic potential (Mojica, Gonzalez de Mejia, Granados-Silvestre, & Menjivar, 2017) or more combined bioactivities such as antioxidants and ACE-inhibitors (Valdés et al., 2020; Vecchi & Añón, 2009; Zou, Wang, Wang, Aluko, & He, 2020); antioxidants and anti-inflammatory potential (Mojica et al., 2017; Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014); antioxidants and DPP-IV inhibitors (Nongonierma, Le Maux, Dubrulle, Barre, & FitzGerald, 2015); and antioxidants, ACE and DPP-IV inhibitors (Zaharuddin et al., 2020).

There is no published information regarding the bioactivity of peptides presented in FSCG protein hydrolysates, and only scarce information for NFSCG peptides; however, some peptide fragments that present the hydrophobic amino acid Tyr (Y) on the C-terminal side are similar to the peptides obtained after enzymatic hydrolysis (thermolysis with alcalase) of Arabica-SCG (Valdés et al., 2020) and gastrointestinal digestion (with pepsin and pancreatin) of coffee silverskin (Pérez-Míguez, Marina, & Castro-Puyana, 2019). In the present study, five of the seven identified peptides contained Tyr (Y) in their sequences (Table 3, Fig. 3), which probably contributed to their bioactivity. Dipeptides containing Tyr (Y), Trp (W), Cys (C), or Met (M) and protein hydrolysates rich in these dipeptides are potential components of functional foods that enhance human health (Zheng, Zhao, Dong, Su, & Zhao, 2016). Other studies indicated that most antioxidant, ACE-inhibitory, and DPP-IV inhibitory bioactive peptides contain hydrophobic [Leu (L), Ileu (I), Val (V), and Met (M)] and aromatic [Phe (F) and Tyr (Y)] acid residues, as well as the imidazole-ring-containing His (H) (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007; Ma et al., 2020; Mojica, Chen, & de Mejía, 2015).

The proposed mechanism of action for the antioxidant activity of peptides reported at the cellular level include the regulation of antioxidant enzymes, the thiol and hydroxyl groups available in peptide sequences, the balance of intracellular levels of reactive oxygen species, and the reduction of lipid oxidation (Esfandi, Walters, & Tsopmo, 2019). The activity of some peptides to inhibit ACE and DPP-IV enzymes have been related to conformational changes due to hydrophobic and electrical interactions of the peptide sequences with the enzymes (Liu, Cheng, & Wu, 2019; Yan et al., 2020).

This exploratory study has potential limitations. The composition of protein hydrolysates described in our work could be dependent on the coffee variety, the growing conditions, and the grain storage; factors directly related to the peptide sequence obtained from SCG (Figueroa Campos, Sagu, Saravia Celis, & Rawel, 2020; Rendón, de Jesus Garcia Salva, & Bragagnolo, 2014). Our results demonstrated that the fermentation and enzymatic hydrolysis of FSCG effectively produced an increase in soluble and hydrolysate proteins that may generate bioactive peptides. An advantage of FSCG is the use of an agro-industrial waste to obtain and increase through fermentation protein hydrolysates with a pool of low molecular weight peptides (<1 kDa) reported as sizes of peptide sequences with high bioactivity (Zou et al., 2020) that could be used as ingredients to formulate bioactive foods or food supplements.

4. Conclusions

This investigation may contribute relevant, novel information to the literature regarding the production of protein hydrolysates from agro-industrial waste with added value. The fermentation process increased the abundance of peptides; SCG fermentation with *B. clausii* improved the production of bioactive peptides with the potential to alleviate the effects of oxidative stress, hypertension, and diabetes. The peptide sequences with a bioactivity probability value greater than 0.6 (YGF, GMCC, YWRYDCQ, and RMYRY) will be considered for further analysis.

Funding

This work was supported by the Tecnologico Nacional de Mexico (TecNM) under Grant number 5433.19-P.

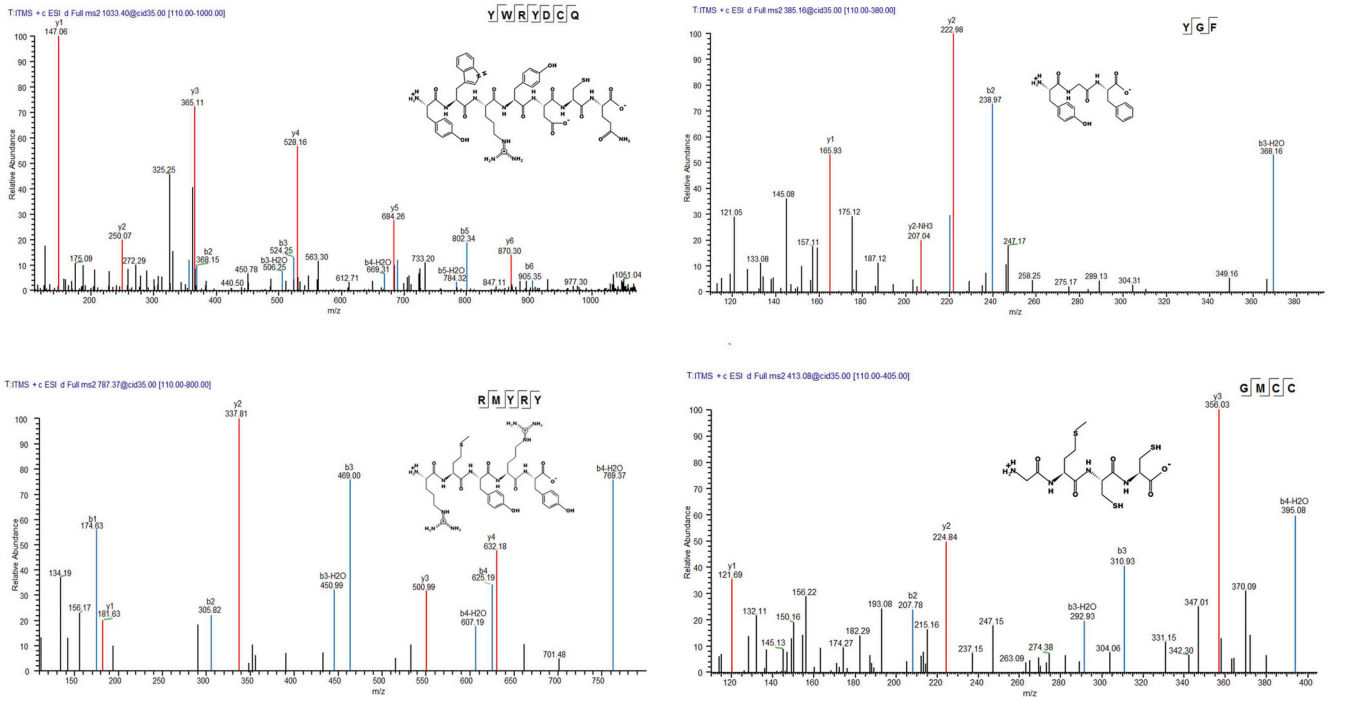


Fig. 3. Mass spectra of the four major peptides from both fermented (FSCG) and non-fermented (NFSCG) spent coffee grounds.

CRediT authorship contribution statement

Karina Ramírez: Conceptualization, Formal analysis, Investigation, Writing - original draft. **Karen V. Pineda-Hidalgo:** Supervision, Resources, Investigation, Methodology, Writing - review & editing. **Jesús J. Rochín-Medina:** Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Project administration, Writing - original draft.

Declaration of competing interest

The authors reported no potential conflict of interest.

Acknowledgments

We thank Cristina Madahí Serrano Payán for her technical assistance.

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