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The health-promoting potential of peptides from brewing by-products: An up-to-date review

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ABSTRACT

Background: Substantial amounts of by-products are generated annually by the brewing industry, being currently undervalued. Nevertheless, the two major by-products (brewer's spent grain – BSG – and yeast – BSY) have potential to be converted into profitable and marketable products. Indeed, they are sources of proteins that can be hydrolysed to obtain biologically active peptides with promising application as food ingredients, supplements, or nutraceuticals due to their health-promoting properties. Moreover, the recovery of such peptides follows a sustainable and environmentally friendly principle allied with economic and social benefits.

Scope and approach: This review is an up-to-date summary of the current knowledge regarding the biological properties exerted by BSG and BSY peptides and the methodologies used to obtain those peptides.

Key findings and conclusions: BSG and BSY hydrolysates or isolated peptides have been recognized to possess antioxidant, immunomodulatory, antihyperglycemic, antihypertensive, antithrombotic, lipid-lowering, antimicrobial, antiulcer, and antiproliferative activities, some of them proving to be multifunctional. Although more research has been conducted on BSG peptides, *in vivo* confirmation of bioactivities was carried out mostly for BSY, thus being more advanced for a possible translation to humans. Nevertheless, both could be commercialized as functional food ingredients, supplements, or nutraceuticals, presenting general beneficial functions, diseases risk reduction, or ameliorate pathological conditions such as non-communicable diseases, primarily cardiovascular diseases. However, further validation using animal models followed by human clinical trials needs to be carried out to translate the current findings into commercial applications, but the results collected so far indicate that such studies are justified and very promising.

1. Introduction

Beer is one of the most popular alcoholic beverages, making the brewing industry a huge global business. Particularly, in the European Union, beer production and consumption have increased over the last decade (The Brewers of Europe, 2019), which also represents an increase of millions of tons of brewing by-products and their disposal represents an ecological and economical problem. Most breweries managed to reuse their by-products mainly into animal feed (Buffington, 2014; Ferreira, Pinho, Vieira, & Tavarela, 2010). However, they present valuable nutrients that urge innovative applications to obtain more profitable and marketable added-value food products.

Barley, the main raw material used for beer production, is malted in a controlled germination process to solubilize starch inside grains prior to brewing. Malted barley is dried, milled, and submitted to enzymatic degradation (mashing) resulting in the release of fermentable carbohydrates and proteins/peptides/amino acids (Mussatto, Dragone, & Roberto, 2006). The sweet liquid produced is called wort and the residual solid fraction (the insoluble part of the barley grain comprising mainly the grain coverings) is separated (lautering) and discarded, being commonly called brewer's spent grain (BSG). Then, the wort is boiled with the addition of hops, which impart bitterness and flavour. At the end, the liquid extract is separated from the brewer's spent hops (BSH) to be fermented. Yeasts are responsible for alcoholic fermentation, converting sugars to alcohol and carbon dioxide (Dashko, Zhou, Compagno, & Piškur, 2014). The most used brewing yeasts belong to the genus *Saccharomyces* and can be traditionally divided into two groups: ale and lager yeasts, also known as top- and bottom-fermenting yeasts,

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respectively. Ale yeasts are mainly *Saccharomyces cerevisiae* species, while lager yeasts are *Saccharomyces pastorium*, the latter being responsible for more than 90% of the beer produced worldwide (Capece, Romaniello, Siesto, & Romano, 2018). At the end of the fermentation stage, most yeasts are collected and generally reused several times (4–6 times), taken from one fermentation to another, and finally discarded as brewer's spent yeast (BSY) (Ferreira et al., 2010).

During beer production, three main agro-industrial by-products can be collected: BSG, BSH, and BSY (Fig. 1). BSG is the major by-product, representing around 85% of the total by-products obtained (UNICER, 2013). Approximately 20 kg of fresh BSG is produced per 100 L of brewed beer (Reinold, 1997) and, in the European Union, the BSG average annual production is estimated to be about 3.4 million tons (Stojceska, 2019). BSY is the second major brewing by-product, representing approximately 15% of the total by-products (UNICER, 2013). During the fermentation process, yeasts rise numerous times, which results in a marked increase of yeast biomass (3–6 fold) (Ferreira et al., 2010). At the end, 2–4 kg of BSY is generated per 100 L of beer (Europe, 2002). BSH represents, by far, the smallest amount produced, and, as such, it is the least exploited brewing by-product. For this reason, it will not be addressed in the present review.

The chemical composition of BSG and BSY is variable according to the brewing conditions and raw materials used that differ among breweries (Bokulich & Bamforth, 2013; Lynch, Steffen, & Arendt, 2016). Both by-products are valuable sources of proteins, namely 19–30% (Lynch et al., 2016) and 35–60% (Thiago, dos, Pedro, & Eliana, 2014) dry weight, respectively, being rich in essential amino acids (Fărcaş et al., 2017; Podpora, Swiderski, Sadowska, Piotrowska, & Rakowska, 2015; Vieira, Carvalho, et al., 2016). These proteins, besides their nutritional properties, also provide physiologically active peptides, called bioactive peptides, which can provide additional health benefits, namely antihypertensive, antioxidant and immunomodulatory activities, among others (Tables 1 and 2). Protein hydrolysates contain a mixture of peptides with diversified lengths and amino acid composition that explain the wide range of functional activities reported in literature. Isolated peptides with a particular molecular weight can be fractionated to separate the bioactive ones from the mixture.

Research studies concerning the valorisation of brewing by-products as raw materials to produce bioactive peptides emerged at the beginning of the 21st century, however, only recently has gained major relevance due the interest to reduce the environmental and economic costs, while providing a sustainable and effective way of handling waste, which is highly encouraged in The Sustainable Development Goals of United Nations. Furthermore, the health-beneficial properties exerted by bioactive peptides from BSG and BSY, either protein hydrolysates or isolated peptides, make them worthy candidates for their application as functional food ingredients, supplements, or nutraceuticals with therapeutic purposes, for instance, in metabolic syndrome disorders as hypertension, abdominal adiposity, dyslipidaemia, hyperinsulinemia and glucose intolerance that are predictive of cardiovascular disease and type 2 diabetes mellitus (Cornier et al., 2008). Nevertheless, to translate these biopotential into commercial applications, manufacturers face major challenges to get consumers' acceptance regarding sensory and taste qualities as well as to meet the regulatory requirements. In addition, appropriate, efficient, and cost-effective scalable production methods that ensure stability during the manufacturing process and storage are required (Chakrabarti, Guha, & Majumder, 2018; Chalamaiah, Keskin Ulug, Hong, & Wu, 2019).

To the best of our knowledge, there is no literature reviewing the bioactivities of both BSG and BSY peptides. In this way, the present review aims to gather and summarize the current knowledge regarding the biological properties exerted by peptides obtained from the two main brewing by-products: BSG and BSY. Additionally, the methodology used to produce such peptides will also be addressed and is summarized in tables to provide a relevant basis for food and food supplement industries planning future investments and research.

2. Bioactive peptides from BSY

BSY is generally recognized as a safe (GRAS) raw material (Vieira, Carvalho, et al., 2016) and a potentially rich source of bioactive peptides encrypted within its protein sequences. However, yeasts' cell wall needs to be disrupted to release these cytosolic and membrane proteins (Marson, de Castro, Belleville, & Hubinger, 2020). Additionally, cell lysis also releases yeast's numerous vacuole proteases (Hecht, O'Donnell, & Brodsky, 2014) which can promote protein degradation (autolysis) through a sustainable process and, thus, generate bioactive peptides.

The first study ascribing the beneficial biological properties of BSY peptides is from 2005 by Kanauchi, Igarashi, Ogata, Mitsuyama, &

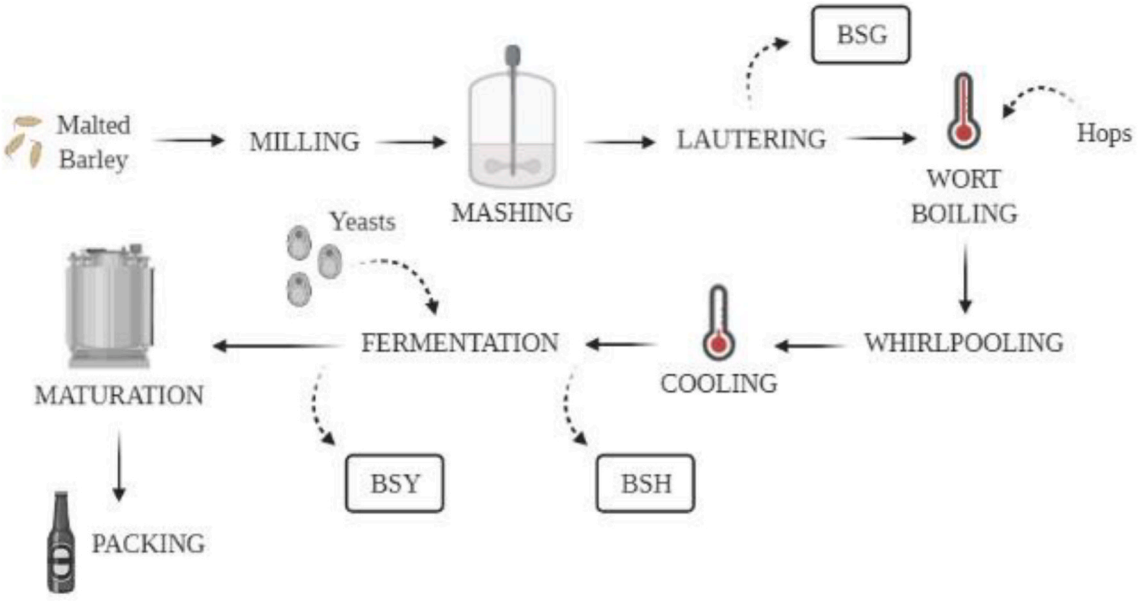

Fig. 1. Schematic representation of the brewing process highlighting the removal of the by-products brewer's spent grain (BSG), hops (BSH), and yeast (BSY). Created with BioRender.com.

Table 1
Peptides/protein hydrolysates obtained from brewer's spent yeast (BSY) and their bioactivities.

Bioactive peptides' production			Most promising peptide(s)	Bioactivity				References
Pre-hydrolysis	Protein hydrolysis	Post-hydrolysis		*in vitro*		*in vivo*		
					assay and results	*(animal model)*	*doses tested*	
–	Alcalase (12 h at 50 °C)	–	AF and GF peptides	ACE-inhibitory	IC_{50}: 3.0 and 3.4 μmol/L, respectively [a]	Antihypertensive (SHR)	BSG peptides: 3% in diet (HED = 10 g [b]), 5 days; AF or GF peptides: 8 mg/1mL/rat, single dose	Kanauchi et al. (2005)
–	Flavouzyme, Neutrase, Alcalase, Protamex or Ficin (E/S 1/100, 48 h at 50 °C)	Filtration (10 kDa)	Flavouzyme (E/S 1/100, 48 h at 50 °C)	Antioxidant High CHP content	DPPH: 59.9% at 2.5 mg/mL; ABTS: 66.5% at 1.5 mg/mL 674.0 μg/g	Glucose tolerance (diabetes type 1 – ICR mice treated with streptozotocin – and type 2 – C57BL/KsJ-leprdb/leprdb mice)	100 mg/kg (HED = 8.1 mg/kg [c])	Jung et al. (2011)
Autolysis (4 h at 70 °C) + Filtration (10 kDa)	*Cynara cardunculus* proteases (E/S 4% [v/v], 4 h, 55 °C)	Filtration (3 kDa)	<3 kDa	Antiproliferative (leukaemia cells)	>50% inhibition at 2.5 μg/mL	Antiulcer (Wistar rats treated with ethanol – gastric mucosa)	100, 300, and 1000 mg/kg (HED = 16.2, 48.6 and 162.2 mg/kg, respectively [c]), orally gavaged	Amorim et al. (2016)
Mechanical disruption	Autolysis (6 h at 36 °C)	Filtration (5 kDa) + SGID	Digests	Antioxidant ACE-inhibitory	FRAP: 405 ± 9 μM TE/mL; ROS production in Caco-2 cells (0.5, 1.0 and 2.0 mg of digested peptides/mL, 24h) under normal and oxidative stress conditions IC_{50}: 345 ± 10 μg peptide/mL	–	–	Vieira, Neves, et al. (2016)
Mechanical disruption	Autolysis (6 h at 36 °C)	–	NI	Antioxidant ACE-inhibitory	FRAP: 374 μM TE/mL; TPC: 385 μM GAE/mg IC_{50}: 379 μg peptide/mL	–	–	Vieira, Melo, and Ferreira (2017)
Autolysis (5 h at 70 °C)	*Cynara cardunculus* proteases (E/S 4% [v/v], 4.5 h, 55 °C)	Filtration (3 kDa) + SGID	<3 kDa digests	ACE-inhibitory	IC_{50}: 84.2 μg protein/mL	–	–	Amorim, Pinheiro, and Pintado (2019)
Autolysis (5 h at 70 °C) + Filtration (>10 kDa)	*Cynara cardunculus* proteases (E/S 4% [v/v], 4.5 h at 55 °C)	Filtration (3 kDa)	<3 kDa: SPQW, PWW and RYW peptides	Antioxidant ACE-inhibitory	ORAC: 7.25 μM TE/mg of sample IC_{50}: 84.2 ± 10.8 μg/mL	Antihypertensive (SHR)	<3 and >3 kDa from the 10 kDa retentate: 300 mg/kg (HED = 48.6 mg/kg [c]) in 1 mL of water, single-dose	Amorim, Marques, et al. (2019)
Autolysis (24 h at 50 °C) or Mechanical disruption or Enzymatic hydrolysis (Brauzyn)	Brauzyn + Flavourzyme, Brauzyn + Protamex or Brauzyn + Alcalase (E/S 0.5, 10 or 5.23%, 60, 70 or 80 °C, variable time)	–	Enzymatic hydrolysis + Brauzyn + Alcalase (10% E/S at 60 °C)	Antioxidant	DPPH: 35.2 ± 0.6 μmol TE/g; FRAP: 12.6 ± 0.7 μmol TE/g	–		Marson et al. (2019)
Heat (70 °C)	Alcalase + Brauzyn + Protamex, Alcalase + Brauzyn, Alcalase + Protamex or Brauzyn + Protamex (2000 U/$g_{protein}$, 2h at 50 °C)	–	Differ among antioxidant assays	Antioxidant	FRAP, ORAC and DPPH	–		Marson, de Castro, Machado, et al. (2020)

[a] Order of the most promising peptide(s).
[b] According to the authors.

[c] Calculation according to Reagan-Shaw et al., (2008); ABTS – 2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); ACE – angiotensin-converting enzyme; CHP – cyclo-His-Pro; DPPH – 2,2-diphenyl-1-picrylhydrazyl; E/S – enzyme/substrate ratio; FRAP – Ferric ion Reducing Antioxidant Power; GAE – Gallic Acid Equivalent; HED – Human Equivalent Dose; IC_{50} – half maximum inhibitory concentration; NI – Not identified; ORAC – Oxygen Radical Absorbance Capacity; ROS – Reactive Oxygen Species; SGID – simulates gastrointestinal digestion; SHR – Spontaneously Hypertensive Rats; TE – Trolox Equivalent; TPC – Total Phenolic Content.

Andoh. At the time, it was a surprisingly innovative work involving *in vivo* demonstration of the antihypertensive effect of BSY protein hydrolysates and isolated peptides. The succeeding publication only occurred in 2011, by Jung et al. and presents *in vitro* and *in vivo* studies. Nevertheless, in the last decade animal welfare awareness and legislation has increased globally, and the approval of ethic committees to undertake animal studies became more strict, which may explain why recent research on the biological properties of BSY peptides has been done mainly *in vitro*. The recent tendency of industry by-products reuse has prompted new investigation towards antioxidant, antihypertensive, antihyperglycemic, antiulcer, and antiproliferative properties of BSY peptides. The methodology used to produce such peptides and the most relevant results are summarized in Table 1.

2.1. Antihypertensive potential

Hypertensive patients are characterized by hyperactivation of the renin-angiotensin-aldosterone system (Catt et al., 1971), an important system physiologically involved in the regulation of blood pressure, mainly *via* angiotensin II (Muñoz-Durango et al., 2016) generated by angiotensin-converting enzyme (ACE). Angiotensin II increases arterial pressure through vasoconstriction and regulation of renal ion and water handling. When ACE activity is increased, the levels of angiotensin II are augmented which causes increased vessel contraction, decreased lumen diameter and excessive blood volume, contributing to the development and progression of hypertension (Forrester et al., 2018). Therefore, bioactive peptides that present ACE-inhibitory activities are of major interest to control increased blood pressure. Furthermore, high blood pressure is also associated with oxidative stress and dysfunctional endogenous antioxidant mechanisms (Lassègue & Griendling, 2004) that can be reduced by antioxidant peptides.

As prementioned, Kanauchi, Igarashi, Ogata, Mitsuyama, and Andoh (2005) examined the antihypertensive effect of BSY peptides *in vivo*. Two BSY dipeptides (AF and GF) were obtained by enzymatic treatment (Table 1) and isolated due to their promising ACE-inhibitory *in vitro* activity. Spontaneously hypertensive rats (SHR) were fed with diets containing these peptides, either mixed or isolated, showing a significant decrease in systolic blood pressure (measured by the tail-cuff method) compared to the control group. The bioactive peptides demonstrated ~60% of the potency of captopril, a drug commonly used to inhibit ACE as part of hypertension management.

On the other hand, based on *in vitro* studies, Vieira, Melo, and Ferreira (2017) obtained a BSY (*Saccharomyces pastorium*) protein autolysate with maximized ACE-inhibitory and antioxidant activities (Table 1). Bearing in mind the commercialization of such bioactive peptides as functional food ingredients, supplements or nutraceuticals, the oral route would be the elected administration *via*. Thus, the efficacy of orally ingested bioactive peptides to exert systemic effects depends on their absorption capacity and stability to retain function to reach the blood circulation and, ultimately, their respective target(s) in an active form/concentration to induce physiological effects. Thus, *in vitro* simulated gastrointestinal digestion (SGID) is a common approach to assess the stability of peptides to gastrointestinal enzymes and, therefore, assess the bioavailability of active peptides by oral administration (Chakrabarti et al., 2018). Peptides' stability and transport across epithelial cells (from the gastrointestinal tract) are usually estimated using differentiated Caco-2 cell monolayers or co-cultures of Caco-2/HT29-MTX that mimic essential aspects of the intestinal epithelial barrier, and allow the evaluation of the cytotoxicity of bioactive compounds (Lea, 2015). These *in vitro* approaches elucidate, to a certain extent, the oral and absorption dynamic, serving as a basis for *in vivo* research, reducing the number of experiences with animals, applying the 3R's principles. In this way, Vieira, Neves, et al. (2016) studied and estimated the impact of gastrointestinal digestion and intestinal absorption on a particular fraction (<5 kDa) of BSY autolysates. The authors concluded that SGID enhances the antioxidant and ACE-inhibitory activities, and the *in vitro* permeability assays performed suggest that these BSY peptides are well absorbed at the gastro-intestinal tract (considering the criteria defined by Yee, 1997). In addition, no cytotoxic effects were observed on Caco-2 cells confirming the safety of these compounds. Also, a cellular protective effect exerted by the digested BSY autolysate against oxidative stress was demonstrated. Therefore, the BSY bioactive peptides obtained here by autolysis seem to be well absorbed and maintain their *in vitro* bioactivity, supporting its potential as functional ingredients or nutraceuticals with antioxidant and antihypertensive abilities. Nevertheless, confirmatory *in vivo* studies were not performed.

Other BSY (*Saccharomyces pastorium*) protein hydrolysates presenting ACE-inhibitory activity were recently obtained by Amorim, Pinheiro, and Pintado (2019). The authors performed autolysis for disruption of the cell wall, followed by protein hydrolysis using an extract of proteases from *Cynara cardunculus*, a flower of the wild thistle (Table 1). Peptides lower than 3 kDa exhibited higher ACE-inhibitory activity, which was preserved after *in vitro* SGID. Later, the same research group (Amorim, Marques, et al., 2019b), following a similar procedure prepared BSY peptides tested *in vitro* to evaluate the antioxidant potential and ACE-inhibitory activity, and the most promising fractions (<3 and >3 kDa from the 10 kDa retentate) were selected for *in vivo* studies with SHR (Table 1). The <3 kDa fraction, which contains the identified peptides SPQW, PWW, and RYW, caused the most noticeable decrease in systolic, diastolic, and mean blood pressure (telemetric measurement) of SHR compared to control ($p < 0.05$), wherein the systolic blood pressure decrease was similar ($p > 0.05$) to captopril. The success of these fraction, according to the authors, might be due to the low molecular weight peptides present, a feature that has been associated with ACE-inhibitory effect, and particularly the hydrophobic amino acids content as C-terminal. These results corroborate those of Vieira et al. that BSY bioactive peptides are absorbed and reach their target in an active form or, alternatively, are degraded into active metabolites that exert the observed effects.

2.2. Antioxidant activity

The antioxidant potential of BSY hydrolysates was firstly observed by Jung et al. (2011). Hydrolysis was performed with the peptidase preparation Flavourzyme®, followed by ultrafiltration (Table 1) resulting peptides presenting antioxidant activity *in vitro*. More recently, Marson, Machado, de Castro, and Hubinger (2019) also studied the *in vitro* antioxidant potential of BSY (*Saccharomyces pastorium*) hydrolysates. They compared three methods for yeast cell wall rupture: autolysis, mechanical disruption, and varied enzymatic hydrolysis (Table 1), and concluded that the inner yeast cell compounds were more effectively released after enzymatic hydrolysis, particularly after sequential hydrolysis using Brauzyn® and Alcalase®, exhibiting higher protein recovery when compared to the other methods (83% in relation to non-treated yeast). Furthermore, the sequential hydrolysis was the only method that improved the antioxidant properties over 60% compared to non-treated yeast. Later, after these conclusions on the pre-hydrolysis treatments, the same group (Marson et al., 2020) tested the protein hydrolysis' conditions to maximize the antioxidant activity evaluated by multiple *in vitro* assays that assess different antioxidant mechanisms. The maximum effect in each assay varied according to the

Table 2
Peptides/protein hydrolysates obtained from brewer's spent grain (BSG) and their bioactivities.

Bioactive peptides' production			Most promising peptide(s)	Bioactivity				References
Pre-hydrolysis	Protein hydrolysis	Post-hydrolysis		*in vitro*		*in vivo*		
					assay and results	*(animal model)*	*doses tested*	
Heat (121 °C)	*Bacillus cereus* peptidases (0, 2, 8 h or 24 h at 32 °C)	–	24 h hydrolysates	Antimicrobial	agar well diffusion assay for Gram-negative bacteria (*Escherichia coli*)	–	–	Kotlar et al. (2013)
Protein alkaline extraction	Alcalase, Flavourzyme, Corolase PP, Protex 6L, Protamex, Corolase L10, Promod 144 MG, Promod 24P, Prolyve or Trypsin 250 (E/S 1 or 2.5% at 50 or 60 °C, pH 7 or 9)	Filtration (3 and 5 kDa)	Prolyve and Flavourzyme <5 kDa (1% at 50 °C)	Antioxidant	U937 cells (0.5% v/v protein, 24 h) under oxidative stress conditions: SOD activity (Prolyve): 140.47 ± 9.29% of control; DNA damage (Flavourzyme)	–	–	McCarthy et al. (2013a)
			Alcalase (2.5% at 60 °C) > 5 kDa and Protamex (1% at 50 °C)	Anti-inflammatory (↓ IFN-γ)	Concanavalin-A-stimulated Jurkat T cells (0.5% v/v BSG protein/hydrolysates, 24 h): 58.32 ± 13.46 and 53.28 ± 17.17% reduction, respectively [a]			
Protein alkaline extraction	Corolase PP, Flavourzyme or Alcalase (E/S 1 or 2.5% [w/w] or [v/w], 4 h at 50 or 60 °C, pH 7 or 9)	–	Alcalase (E/S 1% [w/w] at 60 °C)	(low) Antioxidant	DNA damage in U937 cells (0.5% v/v protein, 24 h) under oxidative stress conditions	–	–	McCarthy et al. (2013b)
			Corolase PP (E/S 2.5% [w/w] at 50 °C)	Anti-inflammatory (↓ IFN-γ)	Concanavalin-A-stimulated Jurkat T cells (0.5% v/v BSG protein/hydrolysates, 24 h): >20% reduction of control	–	–	
Protein alkaline extraction	Alcalase, Flavourzyme, Prolyve 1000, Protex 6L, Protamex, Corolase PP, Corolase L10, Promod 144 MG, Promod 439, Promod 24P or Trypsin (E/S 1 or 2.5% [w/w or v/w], 4 h at 50 or 60 °C, pH 7 or 9)	–	Prolyve 1000 (E/S 1 [w/w], at 50 °C)	ACE-inhibitory	89.25 ± 2.46% at 1 mg/mL [b]	–	–	Connolly et al. (2014)
			Trypsin (E/S 1 [w/w] at 50 °C)	α-glucosidase inhibitory	66.81 ± 4.896% at 7.5 mg/mL			
			Corolase PP (E/S 1 [w/w] at 50 °C)	DPP-IV inhibitory	75.00 ± 3.06% at 3.5 mg/mL			
Protein alkaline extraction	Alcalase	Filtration (3 and 5 kDa) + Incorporation in milk (1.125% [v/v]) + SGID	NI	Anti-inflammatory (↓ IL-6)	Concanavalin-A-stimulated Jurkat T cells (2.5% [v/v] digests, 24 h): 79.4–85.1% reduction of control	–	–	Crowley et al. (2015)
Protein alkaline extraction	Alcalase, Corolase PP, Flavourzyme or Promod (E/S 1% [w/w or v/w] at 50 °C, pH 7, variable time)	SGID + Filtration (3 and 5 kDa)	Alcalase (4 h): IVY and ILDL peptides	ACE-inhibitory	IC_{50}: 80.4 ± 11.9 and 96.4 ± 8.36 μM, respectively [a]	–	–	Connolly et al. (2015)
			Undigested Corolase PP < 5 kDa and digested Alcalse peptide ILLPGAQDGL	DPP-IV inhibitory	IC_{50}: 2.01 ± 0.09 mg/mL and 145.5 ± 10.7 μM, respectively [a]	–	–	Connolly et al. (2017)
Protein alkaline extraction	BSY-extracted proteases (0.29/1 U/mg, 6 h at 50 °C)	–	NI	Antioxidant	FRAP: 1.88 mg TE/mL; TPC: 1.65 mg GAE/mL	–	–	Vieira, Teixeira, and Ferreira (2016)
Protein alkaline extraction	Neutrase or Alcalase or BSY-extracted proteases (E/S 10:100 [v/v], 4h at 50 °C)	Filtration (3 and 10 kDa)	<10 kDa	Antioxidant	FRAP (Neutrase): 0.157 mg TE/mg dwb; TPC (Neutrase): 0.082 mg GAE/mg dwb; ROS production (BSY proteases) in Caco-2 and HepG2 cells (1.0 mg/mL, 24 h) under normal and oxidative stress conditions	–	–	Vieira, Dias, Carmo, and Ferreira (2017)
–	Alkaline protease + Flavourzyme or neutral protease + Flavourzyme	SGID + Filtration (1 kDa)	Digests <1 kDa	Antithrombotic	thrombin inhibitory activity, delayed thrombin and activated partial thromboplastin time respect to the control at 2 g/L protein	–	–	Cian et al. (2018)
				Antioxidant		–	–	

(*continued on next page*)

Table 2 (*continued*)

Bioactive peptides' production			Most promising peptide(s)	Bioactivity				References
Pre-hydrolysis	Protein hydrolysis	Post-hydrolysis		*in vitro*		*in vivo*		
					assay and results	*(animal model)*	*doses tested*	
Protein alkaline extraction or Carbohydrases	Prolyve 1000+Protease P or Alcalase + Protease P or Alcalase + Flavourzyme or Corolase PP + Flavourzyme (E/S 2% [w/w] 2 h + 1% [w/w] 2 h at 50 °C)	Filtration (30 and 10 kDa) + SGID	Alkaline extraction + undigested Prolyve 1000+Protease P		ORAC: 1383.91 ± 34.42 μmol TE/g protein; FRAP: 295.76 ± 14.92 μmol TE/g protein; ABTS: 72.62 ± 4.18%			Connolly et al. (2019)
			Alkaline extraction + undigested Prolyve 1000+Protease P and Carbohydrases + Alcalase + Flavourzyme (<10 kDa)	Anti-inflammatory (↓ IL-6)	Lipopolysaccharide-stimulated RAW 264.7 (0.01% [w/v] hydrolysates, 24 h): 58.30 ± 13.75 and 58.06 ± 5.83% with respect to the untreated control cells, respectively [a]			
				Alkaline extraction and Carbohydrases digests	DPP-IV inhibitory			
similar values (87.01–90.26% at 2 mg/mL)								
Carbohydrases	Alcalase + Flavourzyme (E/S 2%/g $BSG_{protein}$, 2 h + 1%/g $BSG_{protein}$, 2 h at 50 °C)	SGID	IPY and LPY peptides	Antioxidant	ORAC IPY: 0.37 ± 0.02 μmol/μM TE; LPY: 0.34 ± 0.02 μmol/μM TE	Antihypertensive (SHR)	hole hydrolysate: 50 mg (HED = 8.1 mg/kg [c]) spray-dried sample/kg body weight, 1 mL, orally gavaged	Cermeño et al. (2019)
			IPVP peptide	DPP-IV inhibitory	$IC_{50:}$ 38.67 ± 5.94 μM			
		–	IPLQP and LPLQP peptides	ACE-inhibitory	$IC_{50:}$ 3.10 ± .60 and 3.17 ± 0.60 μM, respectively [a]			
Protein alkaline extraction	Neutral protease-Purazyme + Flavourzyme	–	WNIHMEHQDLTTME (↓cholesterol esterase) and DFGIASF and LAAVEALSTNG (↓pancreatic lipase)	Lipid-lowering	cholesterol esterase inhibition: 15.5 ± 1.5% at 0.2 mg protein/mL; pancreatic lipase inhibition: 51.9 ± 2.3% at 0.06 mg protein/mL	–	–	Garzón et al. (2020)
Xylanase (5 h at 50 °C)	*Lactiplantibacillus plantarum* PU1 (60:40 ratio BSG extract)	SGID + Filtration (50, 30, 10 and 3 kDa)	LFGFTYLR, LVLANAIYFK, VGYVANFCK, IFLENVIR and EVQMDFVR	Antioxidant	DPPH	–	–	Verni 2020
Phenolic extraction + Carbohydrases	Alkaline protease-Protex (2 h at 55 °C)+Flavourzyme (2 h at 50 °C) or Purazyme (2 h at 50 °C)+Flavourzyme (2 h at 50 °C) (E/S 55 g/kg)	SGID	Undigested Purazyme + Flavourzyme and Alkaline protease-Protex + Flavourzyme	Anti-inflammatory (↓TNF and IFN-γ + ↑IL-10)	Spleen cells, macrophages and T lymphocytes from Wistar rats or mice (C_{57}BL/6J, TLR_4KO, and TLR_2KO) stimulated with Concanavalin-A or lipopolysaccharide (0.1 or 0.01 g/L of protein, 24 or 48 h)	–	–	Cian et al. (2020)
Protein alkaline extraction + Ultrasonic waves [40 ot 50 kHz] or Heat [50 or 100 °C]	Alcalase (0.024 U/$g_{protein}$, 1, 2, 3 or 4 h at 60 °C)	–	Ultrasonic waves 50 kHz +4 h hydrolysis	Antioxidant	DPPH: 63.11%; ORAC: 127 μmol/L TE; MCA: 22%; SRSA: 48.84%; HRSA: 55.45%	–	–	Ikram et al. (2020)

[a] Order of the most promising peptide(s).
[b] Compared to the BSG protein-enriched isolate.
[c] Calculation according to Reagan-Shaw et al., (2008); ABTS – 2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); ACE – angiotensin-converting enzyme; BSY – brewer's spent yeast; DPPH – 2,2-diphenyl-1-picryl-hydrazyl; DPP-IV – Dipeptidyl peptidase IV; dw – dry weight E/S – enzyme/substrate ratio; FRAP – Ferric ion Reducing Antioxidant Power; GAE – Gallic Acid Equivalent; HED – Human Equivalent Dose; HepG2 – human hepatocarcinoma; HRSA – hydroxyl radical scavenging activity; IC_{50} – half maximum inhibitory concentration; IFN-γ – interferon-γ; IL – interleukin; MCA – metal chelating activity; NI – Not identified; SGID – simulates gastrointestinal digestion; ORAC – Oxygen Radical Absorbance Capacity; ROS – Reactive Oxygen Species; SHR – Spontaneously Hypertensive Rats; SRSA – superoxide radical scavenging activity; TE – Trolox Equivalent; TNF – tumour necrosis factor; TPC – Total Phenolic Content; U937 cells – human monocytic blood cell line.

combination of enzymes used, as expected, since different enzyme combinations lead to the formation of distinct peptides that can exert their effects by different mechanisms.

2.3. Antihyperglycemic potential

Jung et al. (2011) explored the antihyperglycemic potential of BSY peptides, particularly, the content of cyclo-His-Pro (CHP), a cyclic dipeptide that in humans is suggested to be related with glycaemic control in diabetes. Thus, they tested BSY hydrolysates obtained with several commercially available enzymes (Table 1) and verified that the highest CHP content (674.0 μg/g) was obtained using Flavouzyme. Therefore, they went further to *in vivo* studies with Flavouzyme hydrolysates. The oral glucose tolerance test was performed in an animal model of type 1 (ICR mice treated with streptozotocin) and type 2 (C57BL/KsJ-leprdb/leprdb mice) diabetes by measuring plasma glucose after a 2.5 g glucose/kg load in a group of animals that initially received the BSY hydrolysates (Table 1). The blood glucose levels were more than 100 mg/dl lower in the yeast hydrolysate group compared to control for type 1 diabetes. Smaller, but still significant ($P < 0.05$), differences were also reported between the two groups for type 2 diabetes. Thus, in both models, glucose tolerance effect was observed, suggesting the potential of BSY hydrolysates as an antidiabetic material for the preparation of functional foods or supplements.

2.4. Antiulcer activity

Amorim et al. (2016) investigated the potential protective role of BSY peptides on gastric mucosa against ulcerative lesions. After BSY autolysis, ultrafiltration, hydrolysis by *Cynara cardunculu* proteases and nanofiltration (Table 1), the <3 and >3 kDa fractions were administered to Wistar rats, followed by absolute ethanol treatment as an ulcerogenic agent. The peptide fraction <3 kDa was able to significantly ($p < 0.001$) reduce gastric injuries, in a dose-dependent manner, up to 63.4%. This cytoprotective effect appears to depend, at least in part, on a prostaglandin-mediated mechanism since the inhibition of the prostaglandin synthesis' pathway (using indomethacin) resulted in the inability of peptides to protect against gastric lesions.

2.5. Antiproliferative activity

Amorim et al. (2016) also studied the antiproliferative activity of <3 and >3 kDa peptide fractions (0.25–250 μg/mL) (Table 1) on several human tumour cell lines (glioma, melanoma, human breast adenocarcinoma, breast expressing phenotype multiple drug resistance, kidney, lung, colon adenocarcinoma, and leukaemia). Results exhibited a promising antiproliferative activity (cell growth inhibition of more than 50%) of both fractions (specially <3 kDa that fully inhibited the cell growth) against the leukaemia cells, suggesting the potential use of these BSY peptides in the prevention and treatment of this disease.

3. Bioactive peptides from BSG

Barley is used since ancient times in human nutrition, however, the reuse of BSG as a source of proteins that can be hydrolysed to produce bioactive peptides with diverse health-promoting benefits in humans is recent. First studies were published less than a decade ago, in 2013 and, since then, several bioactive properties, including antioxidant, immunomodulatory, antihyperglycemic, antihypertensive, antithrombotic, lipid-lowering, and antimicrobial have been associated to BSG protein hydrolysates and peptides (Table 2). Although only one *in vivo* study was reported so far, numerous *in vitro* studies encourage the use of BSG proteins to obtain bioactive peptides that should be incorporated in functional foods or as nutraceuticals to enhance health. The following subsections provide more details regarding the bioactivities of BSG peptides. The methodology used to produce such peptides and the most relevant results are summarized in Table 2.

3.1. Antihypertensive potential

The *in vitro* ACE-inhibitory activity of BSG hydrolysates was demonstrated by Connolly, Piggott, and FitzGerald (2014). The authors used the alkaline extraction method to obtain a BSG protein-rich fraction, and tested 11 commercial enzymes for protein hydrolysis (Table 2). Results demonstrated that BSG hydrolysates significantly increased ACE-inhibition in a dose-dependent manner, and the hydrolysate obtained with Prolyve® 1000 displayed the highest ACE inhibition (Table 2), only slightly superior to the Alcalase hydrolysate. Later, Connolly, O'Keeffe, Piggott, Nongonierma, and Fitzgerald (2015) evaluated the impact of SGID and ultrafiltration on ACE-inhibitory activity. In this study, and contrary to the previous one, the Alcalase hydrolysate displayed the most potent effect *in vitro*, which increased after SGID by 84.6 ± 2.89%, which can be explained by the different enzymatic conditions tested, namely the pH used for Alcalase. For this reason, it was fractionated and the peptides containing higher abundance of hydrophobic amino acids and/or proline were chosen for further studies. Ultimately, two bioactive peptides, IVY and ILDL, were identified as holding the most potent ACE-inhibitory effect (Table 2).

Further *in vivo* studies by Cermeño et al. (2019) assessed the blood pressure-reducing properties of a BSG hydrolysate using the SHR model. The extraction of proteins from BSG is challenging due to their entrapment within a complex carbohydrate structure, thus, the authors performed a BSG pre-treatment with carbohydrases. Proteins were hydrolysed with the commercial enzyme Alcalase followed by Flavourzyme and the resulting peptides were administered to rats (Table 2), and blood pressure and heart rate recorded by telemetry. Systolic, diastolic, and mean blood pressure significantly decreased (37.8 ± 0.50, 24.14 ± 7.86 and 31.93 ± 3.12 mmHg, respectively), reaching minimum levels 6 h after hydrolysate ingestion. Due to the satisfactory hypotensive effects, hydrolysates were fractionated, and four peptides identified: IPLQP and LPLQP demonstrated the highest ACE-inhibitory activity, while IPY and LPY presented the highest antioxidant activity (Table 2). Thus, we might conclude that the antihypertensive effects of BSG hydrolysate might have the contribution of ACE-inhibitory as well as antioxidant peptides.

3.2. Antioxidant activity

Vieira, Dias, Carmo, and Ferreira (2017) demonstrated and compared the antioxidant capacity of BSG protein hydrolysates obtained by different enzymatic approaches. BSG alkaline-extracted proteins were hydrolysed using equal amounts of commercial enzymes or BSY-extracted proteases (Table 2). The use of BSY proteolytic enzymes is advisable from both economic and environmental standpoints due to the reuse of the two major brewing by-products. After ultrafiltration, chemically based antioxidant activities were assessed, which revealed the enhanced antioxidant potential of the <10 kDa fraction, highest for the hydrolysate prepared with the protease Neutrase (Table 2). Subsequently, the cytotoxic effects and the protective ability of <10 kDa fractions against oxidative stress on cell lines were evaluated. All <10 kDa hydrolysates (1 mg/mL) exerted a protective effect against oxidative stress in the Caco-2 cell line, inhibiting intracellular reactive oxygen species (ROS) generation. However, in the HepG2 cell line, only when BSY proteases were applied the peptides where able to decrease ROS levels (over 30%) under stress conditions. This study highlights the importance of advanced *in vitro* approaches (cell-based) before moving forward to animal models. Indeed, commercial enzymes at first seemed to be the best choice to produce antioxidant peptides; however, when tested in intestinal and hepatic cell line models, the use of BSY proteases demonstrated to be the most promising method. BSG protein hydrolysis using BSY proteases (Table 2) was optimized by Vieira, Teixeira, and Ferreira (2016) to obtain maximum antioxidant properties (Table 2).

Thus, BSG peptides with improved antioxidant properties can be obtained using a yeast extract from BSY, reusing and valuing both agro-industrial by-products.

Recently, the influence of pre-treatment (ultrasonic waves or heat) on the antioxidant potential of alkaline-extracted protein hydrolysates obtained using the protease Alcalase (Table 2) was scrutinized by Ikram, Zhang, Ahmed, and Wang (2020). The release of peptides with the strongest antioxidant potential was observed with ultrasound pre-treatment at 50 kHz and the enzymatic hydrolysis after 4 h (Table 2).

Connolly et al. (2019) compared two pre-hydrolysis methods for BSG proteins' extraction: alkaline and carbohydrases. Additionally, several enzymatic combinations were tested (Table 2) and the impact of SGID and fractionation on some carbohydrases hydrolysates evaluated. The results demonstrated that the antioxidant activities were not significantly different between the alkaline and carbohydrases hydrolysates, though the Prolyve 1000+Protease P alkaline hydrolysate displayed the highest potential (Table 2). In addition, SGID appears to have a negative impact on the antioxidant properties of carbohydrases hydrolysates and ultrafiltration resulted in fractions with varied bioactivities. Nevertheless, the authors believe these results demonstrate that carbohydrases pre-treatment compared to alkaline protein-extraction is a feasible approach to produce bioactive peptides, which is of greater interest because when the latest method is applied, the final extract contains large amounts of salts that are not desirable in a bioactive ingredient.

The cellular antioxidant protection of BSG hydrolysates was studied by McCarthy et al. (2013a). Alkaline-extracted proteins hydrolysed using different commercially available enzymes, and filtered (Table 2) were added to a human monocytic blood cell line culture to evaluate the superoxide dismutase (SOD) activity (an endogenous antioxidant enzyme) and the cellular DNA damage under oxidative stress conditions. Flavourzyme fractions <3 and >5 kDa and Alcalase protein hydrolysate presented increased SOD activity, but the highest value was observed for Prolyve hydrolysate (Table 2). Only the Flavourzyme fraction <5 kDa demonstrated protection against DNA damage. Hence, in this study, Flavourzyme hydrolysis revealed the best BSG antioxidant peptides. In another study of McCarthy et al. (2013b), the SOD activity and DNA damage were evaluated again, but none of the samples revealed positive results, except one prepared with Alcalase that protected against oxidant-induced DNA damage (Table 2). The authors in both reports investigate multiple conditions, namely enzyme/substrate ratio, temperature, and pH, and these apparently slight differences are responsible for such discrepant results.

Recently, Verni et al. (2020) developed a biotechnological protocol that applies a BSG pre-treatment with commercial enzymes (xylanase) followed by fermentation with *Lactiplantibacillus plantarum* (lactic acid bacteria) to release active BSG antioxidant peptides. Although data was not reported, the authors claim that BSG peptides demonstrated antioxidant activity, which increased after SGID and remained with fractionation. Thus, they hypothesized that the <3 kDa fraction would contain the active peptides, and was further purified revealing 5 peptides (LFGFTYLR, LVLANAIYFK, VGYVANFCK, IFLENVIR and EVQMDFVR) holding the strongest antioxidant activity, possibly through a synergic effect. These peptides are rich in hydrophobic amino acids and present at least one aromatic amino acid, often included in antioxidant peptides' structure.

3.3. Antihyperglycemic potential

Delaying digestion of carbohydrates to absorbable glucose by inhibition of α-amylase and intestinal α-glucosidase is a therapeutic approach to control postprandial hyperglycaemia in pre-diabetes and diabetes (Mcdougall & Stewart, 2005). The α-amylase and α-glucosidase inhibitory potential of BSG hydrolysates was firstly investigated by Connolly et al. (2014). Hydrolysates, obtained as referred in subsection 3.1, were tested for *in vitro* bioactivity, and significantly increased α-glucosidase inhibition in a dose-dependent manner (Table 2). However, no significant change in α-amylase inhibition was observed.

Dipeptidyl peptidase IV (DPP-IV) inhibition is another strategy for the management of type 2 diabetes since this protease is responsible for the degradation of incretin, a hormone that enhances insulin secretion in the presence of glucose (Drucker, 2006; Nongonierma & FitzGerald, 2013). Connolly et al. (2014) also investigated the DPP-IV-inhibitory potential of BSG hydrolysates *in vitro,* and noticed a significantly increased DPP-IV inhibition in a dose-dependent manner, wherein digestion with the commercial enzyme Corolase® PP resulted in the highest inhibition (Table 2). In another report, Connolly, O'Keeffe, Nongonierma, Piggott, and FitzGerald (2017) tested the impact of SGID and filtration on the DPP-IV inhibitory potential of BSG hydrolysates (obtained as previously described in subsection 3.1). In agreement with the previous results, the highest mean DPP-IV-inhibition was observed for the Corolase PP hydrolysate. While ultrafiltration favoured Corolase PP hydrolysates, particularly the <5 kDa fraction (Table 2), SGID increased the inhibition of Flavourzyme and Alcalase hydrolysates, but not Corolase PP. Further fractionation of Alcalase hydrolysates (which had shown better antihypertensive activity) allowed the identification of the peptide ILLPGAQDGL displaying the highest DPP-IV inhibitory activity (Table 2). Interestingly, this peptide had already shown to inhibit the ACE (Connolly et al., 2015), and, thus, presenting a dual promising activity.

Connolly et al. (2019), beside the antioxidant capability (subsection 3.2), also determined the DPP-IV-inhibitory potential of alkaline and carbohydrases hydrolysates (Table 2). The alkaline hydrolysates showed a higher extent of DPP-IV inhibition than carbohydrases hydrolysates for all enzymatic combinations although not always statistically significant. SGID of carbohydrases hydrolysates highly increased the bioactivity, equalling the inhibitory potential of the peptides obtained after alkaline proteins' extraction (Table 2).

Finally, in the original article of Cermeño et al. (2019), the authors also analysed the *in vitro* DPP-IV inhibitory activity of BSG hydrolysate (Table 2). The investigators persued peptides with high content of proline residues due to the link with good DPP-IV inhibitory activity and identified the peptide IPVP holding the highest inhibitory potential (Table 2). Further studies exploring BSG peptides with anti-hyperglycemic potential *in vivo* are required to prove its effects and hereafter develop food or supplements with benefits against diabetes.

3.4. Immunomodulatory effect

Immunomodulation refers to modifications in the body's immune system triggered by agents that activate or supress its function. The immune system can initiate the inflammatory process as a protective response to harmful stimuli. However, sometimes it is necessary to control such effect, either by enhancing or reducing it to obtain the desired outcome. In particular, the ability of a compound to reduce the production of pro-inflammatory cytokines, may be beneficial in acute and/or chronic inflammation resulted from events such as tissue injury, infections, or autoimmune diseases, when uncontrolled inflammation becomes damaging.

McCarthy et al. (2013b and 2013a), beside the antioxidant activity, also focused on the potential immunomodulatory effects of BSG proteins and hydrolysates (Table 2). Both studies were conducted on Concanavalin-A-stimulated Jurkat T cells, a cell model for immunomodulatory experiments. McCarthy et al. (2013b) analysed several cytokines' production and verified that both BSG proteins and several hydrolysates only significantly reduced interferon-γ (IFN-γ) production by a minimum of 20%, thus exhibiting selective immunomodulatory effects that may be of benefit in the control of inflammatory diseases. McCarthy et al. (2013a) dedicated solely to IFN-γ production, and conclude that Alcalase (>5 kDa fraction) and Protamex hydrolysates display the greatest reduction of IFN-γ production (Table 2).

Following a similar approach, Crowley et al. (2015) investigated the

anti-inflammatory potential of BSG protein hydrolysates incorporated in low-fat milk and subjected to SGID (Table 2). In this work, stimulated (Concanavalin-A) Jurkat T cells and (lipopolysaccharide) RAW 264.7 murine macrophages were exposed to digests and several cytokines production was evaluated, namely IFN-γ, IL-6, IL-2, IL-1β, and tumour necrosis factor (TNF)-α. The results were not fully satisfactory since only a significant reduction in IL-6 production on Jurkat T cells was verified (Table 2). This study highlights the influence of the food matrix on BSG peptides' bioactivity, which should be considered when seeking to incorporate peptides into a food product or consume them with other foods.

The effect of BSG hydrolysates (produced as prementioned in subsection 3.2) on IL-6 production in lipopolysaccharide-stimulated RAW 264.7 was evaluated by Connolly et al. (2019). A significant reduction in IL-6 production was found with the alkaline hydrolysates Prolyve 1000+Protease P and also with the <10 kDa fraction of the carbohydrases hydrolysate Alcalase + Flavourzyme (Table 2). However, after SGID, no effects on IL-6 production were observed.

More recently, Cian et al. (2020) studied the immunoregulatory effects of BSG hydrolysates and explored the signalling pathways involved. After phenolic extraction and carbohydrate hydrolysis, BSG protein hydrolysates were obtained using commercial enzymes (Table 2). Spleen cells, macrophages and T lymphocytes were cultivated in the presence of hydrolysates or *in vitro* SGID products and stimulated (Concanavalin-A or lipopolysaccharide) for a later evaluation of the IL-10, TNF, and IFN-γ content. All rat cells presented an anti-inflammatory profile with inhibition of the pro-inflammatory mediators, TNF and IFN-γ, and induction of the anti-inflammatory IL-10. Undigested Purazyme + Flavourzyme and Alkaline protease-Protex + Flavourzyme presented the most promising results. The study of signal transduction pathways indicated the major involvement of factor nuclear-kB and the contribution of mitogen-activated protein kinases p38, c-Jun N-terminal kinases, and extracellular signal-regulated kinases 1/2, known as crucial pathways in the regulation of immunomodulatory effects, demonstrating that BSG hydrolysates regulate the immune response, an effect partly maintained after SGID.

3.5. Antithrombotic activity

Antithrombotic agents reduce the formation of blood clots and are used therapeutically to prevent thrombosis. Indeed, when a blood vessel is injured, a blood clot is formed to prevent blood losses. However, this process can lead to the obstruction of the blood flow, which can culminate in death. Thus, the use of compounds that have antithrombotic activity can prevent such situation and be beneficial to the vascular health.

The antithrombotic activity of BSG hydrolysates was only evaluated by Cian, Garzón, Martínez-Augustin, Botto, and Drago (2018). Hydrolysates were produced using sequential enzymatic systems followed by SGID and ultrafiltration (Table 2). All samples showed thrombin inhibitory activity and peptides <1 kDa delayed thrombin and activated partial thromboplastin time respect to the control, an effect that was more pronounced after SGID. Thus, BSG peptides are capable to delay clotting time and new applications for this by-product based on this novel bioactivity should be exploited.

3.6. Lipid-lowering activity

Lipid-lowering agents are used to prevent hyperlipidaemia, an abnormal elevation of lipids in the blood, mainly triglycerides and cholesterol. This type of agents can manage hyperlipidaemia, an important risk factor in cardiovascular diseases and thus, are associated with beneficial health in the cardiovascular system.

Garzón, Cian, Aquino, and Drago (2020) isolated and identified BSG peptides with good lipid-lowering activity evaluated through the inhibition of lipid metabolism-related enzymes, namely cholesterol esterase and pancreatic lipase, which measure the ability of peptides to reduce the absorption of dietary cholesterol and triglycerides, respectively. Alkaline-extracted proteins were hydrolysed sequentially by Neutral protease-Purazyme + Flavourzyme (Table 2), subjected to a fractionation and purification process and each fraction was evaluated for bioactivity. Ultimately, three relevant peptides were identified. The peptide WNIHMEHQDLTTME presented the highest cholesterol esterase inhibition (Table 2), which could be explained by the high presence of hydrophobic and basic residues, characteristics observed in inhibitors of this enzyme. On the other hand, the peptides DFGIASF and LAAVEALSTNG demonstrated the highest pancreatic lipase inhibitory capacity (Table 2). These last peptides have a molecular size within the range reported for pancreatic lipase inhibitors (700–1500 Da). *In silico* studies showed that these peptides were susceptible to gastrointestinal digestion and, thus, could be hydrolysed by digestive proteases to new peptides with greater, equal, or lesser activity, which should be exploited. This novel lipid-lowering BSG peptides have potential to ameliorate hyperlipidaemia.

3.7. Antimicrobial activity

The antimicrobial potential of BSG hydrolysates was only evaluated by Kotlar, Ponce, and Roura (2013). BSG was hydrolysed using *Bacillus cereus* extracellular peptidases (Table 2) and the antimicrobial activity was assessed using an agar well diffusion assay. A Gram-positive (*Listeria monocytogenes*) and Gram-negative (*Escherichia coli* O157:H7) indicators were subjected to BSG hydrolysates for 24 h at 30 °C. The Gram-positive bacteria was found to be resistant against all tested hydrolysates. On the other hand, the diameters of inhibitory zones for the Gram-negative bacteria increased with the increase in the proteolytic digests time, suggesting strong antimicrobial activity against *Escherichia coli*. Since BSG biopeptides seem to be effective in inhibiting the growth and survival of *Escherichia coli*, thus might be used as an ingredient to improve food safety.

4. Discussion and conclusion

The present up-to-date review provides a relevant basis for food and supplement industries planning future investments and investigation, by summarizing the bioactivities of peptides obtained from the two major brewing by-products, BSG and BSY, as well as mentioning the methodologies used to discover and evaluate such potentialities.

Although the interest of exploring bioactive peptides started from BSY proteins back in 2005, the latter (2013) awareness on BSG peptides' potential turned out to be superior, currently finding in the literature almost twice more published investigations. On the other hand, when refering to the impact and translation of the research, only one *in vivo* study was performed for BSG bioactive peptides, while almost half of the publications dedicated to BSY bioactive peptides confirmed its bioactivities using animal models. Nevertheless, increasing evidence has been supporting the presence of peptides within BSG and BSY proteins holding several bioactivities, including antioxidant, immunomodulatory, antihyperglycemic, antihypertensive, antithrombotic, lipid-lowering, antimicrobial, antiulcer, and antiproliferative.

The composition of BSG and BSY is variable according to the brewing conditions and raw materials used that differ among breweries, resulting in variations on the protein content and the peptides obtained and, unfortunately, this information is often omitted in the reports. Hydrolysates can be obtained by means of varied approaches such as commercially available enzymes or proteases from yeasts, bacteria, or plants, but the enzymatic treatment with commercial enzymes is the common choice for BSG and BSY hydrolysates production.

Beside the plausible differences in BSG/BSY starting proteins, the various methodologies used, and even the varied conditions and pretreatments applied within the same protease, lead to the obtainment of extremely varied peptides, which explain the different bioactivities.

In addition, manifold *in vitro* assays were performed to evaluate the same bioactivity, exploring different mechanisms that result in the same ultimate effect. Nevertheless, all these variations make it very difficult to compare or validate the results or even to draw major conclusions regarding the best methodology or the peptides with superior effects.

The composition, sequence, hydrophobicity, steric and electronic properties of the peptides' amino acid residues impact their bioactivities. For example, peptides containing hydrophobic amino acids seem to be more effective ACE inhibitors, particularly those with positively charged amino acids in the N-terminus and proline in the C-terminus (Mada, Ugwu, & Abarshi, 2020). Notwithstanding, although peptide amino acid sequence has been shown to be a good predictor of bioactive potential, several distinct structural features have been associated with the same functional effect (Ganguly, Sharma, & Majumder, 2019). Furthermore, there are several pathways that lead to the same ultimate *in vivo* effect, making it difficult to predict the outcome by analysing the peptide' composition.

The short number of *in vivo* studies with BSG/BSY bioactive peptides can be explained by the recent animal welfare awareness and legislation (e.g. European Union directive 2010/63/EU) that correctly demand strong *in vitro* assessment to provide the necessary evidence to justify moving forward to animal studies. Nonetheless, it is also important to debate the concentrations used in the *in vivo* studies mentioned in the present review. When protein hydrolysates are tested, ≤1000 mg/kg rat body weight could be considered low doses (Aluko, 2018) that, based on animal-to-human dose translation, are equivalent to ≤162.2 mg/kg human body weight (Reagan-Shaw et al, 2008). However, when a small fraction of peptides or isolated peptides are administered to animals, the effect is potentiated, and considerably lower doses are tested.

In conclusion, BSG and BSY bioactive peptides might be valuable food ingredients, supplements, or nutraceuticals not only for promoting and enhancing health but also to reduce the risk, ameliorate or treat several pathological conditions, namely non-communicable diseases such as cardiometabolic diseases. Attractively, some of those peptides/hydrolysates demonstrated multifunctional activities, which is an advantage as a therapeutic purpose. Nonetheless, to translate the health-promoting potential of BSG and BSY peptides into commercial applications, additional validation needs to be carried out using animal models followed by human clinical trials. Furthermore, most bioactive constituents of BSG and BSY protein hydrolysates are still unknown, which needs further identification and characterization. Thus, additional studies are required to support the effective usefulness of BSG and BSY hydrolysates in the development of foods or nutraceuticals, but the results collected so far indicate that such studies are justified and promising.

Declarations of interest

None.

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