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Review – Potentials and limitations of utilising brewer's spent grain as a protein source in aquaculture feeds

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ABSTRACT

On a worldwide basis, aquaculture is among the most important food-producing industries. The steadily growing population has led to an increased demand for aquaculture products, which in turns has intensified the farming of aquatic species along with aquafeed production. Fishmeal and soybean meal represent two of the most widely used protein sources in aquafeeds. Increasing demands and costs have fostered a need for supplementing fishmeal and soybean meal with sustainable alternatives in the coming years. Brewer's spent grain (BSG) is the major by-product generated in beer brewing. With a relatively high protein content, low market price and stable annual availability BSG represents a potential protein source for aquaculture feeds. However, in its untreated form BSG contains high levels of anti-nutritional factors (ANFs) such as lignin and fibre which are known to reduce digestive performance in fish. Furthermore, BSG appears to be perishable under inappropriate storage conditions due to its high moisture content. Therefore, the main objective of this review was to compile an overview of different methods that may help facilitate the implementation of BSG in aquafeed. Before BSG may be utilised as a protein source, it must undergo refinement to remove lignocellulosic material. Here, we focus on two general approaches commonly used: a fractionation-and a conversion-based method. The fractionation-based approach relies on chemical extractions to separate BSG into its constituents, thereby removing the ANFs from the protein component. The conversion-based approach aims to transform ANFs into digestible substances by applying a combination of chemical, biological and enzymatic treatments. Application of appropriate preservation methods can help prevent microbial colonisation of BSG. Three different preservation methods were compared including freeze-drying, oven-drying and lactic acid bacteria (LAB) treatment. Of these, LAB treatment appears to be the most favourable with respect to energy-costs and potential health benefits. At present, BSG has not found any practical use in aquafeed production. However, the collection of methods presented in this review may provide a basis for incorporating BSG in aquafeeds and highlight possible future directions for realising this aim.

IPisoelectric precipitation.
SSsuperheat steam drying.

1. Introduction

Globally, aquaculture constitutes one of the fastest-growing food-producing sectors (OECD/FAO, 2016). Due to a steadily growing population, the demand for food has increased resulting in the intensification of aquaculture production (FAO, 2020). Moreover, the recent COVID-19 pandemic will inevitably influence the global food industries including aquaculture; though, it is still not fully understood how and to which extend food production will be affected. It is expected that the pandemic will lead to increasing demand for healthy food, such as fish products and a need for adopting new practices in food production (Galanakis, 2020). Fishmeal and plant-based meals are, at present, the most commonly applied protein sources in commercial fish feed formulations (Jannathulla et al., 2019). However, these ingredients are associated with environmental and economic concerns which have necessitated the search for alternative protein sources (Boyd, 2015). Brewer's spent grain (BSG) represents ~85% of the total by-products generated during the beer manufacturing process (Rachwał et al., 2020). From 1998 to 2020, the global beer production increased from 1.30 to 1.82 billion tonnes (Conway, 2021). In 2020, the production of BSG was estimated at ~36 million tonnes assuming that about 20 kg BSG is generated per hL of brewed beer. Further, BSG is high in proteins by comparison with other abundant agro-industrial by-products (Sousa et al., 2018). Owing to its immense production volumes, stable annual availability and comparatively high protein content, BSG constitutes a

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Abbreviations

AA	amino acid
ADL	acid detergent lignin
ADF	acid detergent fibre
AF	aflatoxin
ANF	anti-nutritional factor
AX	arabinoxylan
BSG	brewer's spent grain
CAA	crystalline amino acid
CP	crude protein
DAA	dispensable amino acid
DES	deep eutectic solvent
DM	dry matter content
EAA	essential amino acid
HMF	hydroxymethylfurfural
IA	indispensable amino acid
IL	ionic liquid
LAB	lactic acid bacteria
MRP	Maillard reaction product
NDF	neutral detergent fibre
NEAA	non-essential amino acid
NSP	non-starch polysaccharide

potential, unexploited protein source for aquaculture feed. Even though the biotechnological potential of BSG has been extensively investigated in the literature, a limited number of studies have focused on its utilisation in aquafeed production. Therefore, the main objective of this review was to address novel approaches that may be suitable for transforming unrefined BSG into a high-quality protein source for aquafeed applications with focus on fish feed.

2. Aquaculture feeds

Protein is the main and most expensive ingredient in fish feeds. Currently, fishmeal and soybean meal are the most widely used protein sources in aquafeeds. Yet, these ingredients are not regarded as economically sustainable due to their excessive prices which result from stagnating production volumes and competitive demands (Salin et al., 2018). Thus, the continued growth of aquaculture production relies on the implementation of alternative protein sources in aquafeeds. This section provides an overview of the general composition of aquaculture feeds with emphasis on the protein source while comparing the advantages and drawbacks of utilising fishmeal, soybean meal, or BSG as protein sources in aquafeeds. Formulation of fish diets requires knowledge of the nutritional requirements of target species. The nutritional content of a feed is essentially divided into two major categories: the micro-and macronutrients (Murai, 1991). The micronutrients are comprised of minerals and vitamins while macronutrients provide an energy source for ATP production and building blocks for growth. The macronutrients are categorised into three distinct nutrient classes: carbohydrates, lipids and proteins which yield different amounts of energy when fully oxidised by the organism. The requirement and utilisation efficiency of these three macronutrients differ considerably between fish species which is reflected in their natural feeding behaviour. Carnivorous fish species are the least efficient in digesting and utilising carbohydrates, whereas herbivorous and omnivorous fish species exhibit high efficiency in carbohydrate exploitation (Wilson, 1994). Furthermore, the protein requirement appears to be positively correlated with the trophic level of the fish with carnivorous species having the highest protein requirement (Teles et al., 2020). It is also well-established in the literature that the protein requirement of fish varies with size, life stage and abiotic factors such as water temperature and salinity (Wilson, 1986). Despite the lower metabolism of ectothermic organisms compared to endotherms such as birds and mammals, fish have a higher dietary protein requirement, ranging from 25 to 70% (Teles et al., 2020). This may appear counterintuitive, because fish, unlike endotherms, do not spend energy on maintaining a high body temperature. Furthermore, fish primarily excrete nitrogen as ammonia which is less costly to produce in terms of energy in comparison to urea and uric acid that are the primary nitrogenous compounds excreted in the urine of terrestrial animals (Pandian and Vivekanandan, 1985). However, fish ingest less feed (in % of body mass) and have a faster growth trajectory than endotherms and therefore require more protein in their diets. In the following section, we compare different dietary protein sources based on their nutritional quality.

2.1. Protein sources

Fish, like other monogastric animals, do not have a true protein requirement. Instead, fish require an appropriate mixture of essential (EAAs) and non-essential amino acids (NEAAs) that proteins are composed of (Teles et al., 2020). While NEAAs are synthesised *de novo* from intermediates of the glycolytic pathway or the citric acid cycle, EAAs cannot be produced by the organism and must therefore be supplied in the diet (Litwack, 2018). In most organisms, NEAAs are used as building blocks in a multitude of essential metabolic functions (Hou et al., 2015). NEAAs production may sometimes be insufficient and therefore dietary supplementation is needed to meet the requirements for maximal growth and health (Peres and Oliva-Teles, 2006). The EAAs and NEAAs for fish, which are also referred to as indispensable (IAAs) and dispensable amino acids (DAAs), respectively, are presented in Table 1 (Murai, 1991).

A high-quality protein source for fish feed must fulfill multiple criteria, namely: high palatability and attractability, a balanced AA profile and low quantities of toxins and anti-nutritional factors (ANFs). Antinutritional factors are defined as biologically active compounds that inhibit nutrient absorption and utilisation when present in feed (Glencross et al., 2020). Employing protein sources of high palatability and attractability is crucial to enhance feed consumption as fish are more willing to ingest feeds that appear attractive and palatable to them (Al-Souti et al., 2019). The level of macronutrients, toxins and ANFs, as well as the presence of stimulants and attractants, largely influence feed palatability and attractability. These chemical factors determine the physical characteristics of the feed including taste, smell and visibility which indirectly affect feed palatability and attractability (Tantikitti, 2014). Feeding fish with palatable and attractive diets is of vital importance to increase feed consumption thus reducing the accumulation of organic waste derived from undigested feed (Al-Souti et al., 2019). High levels of ANFs in feed can be problematic for the health and

Table 1
The two major categories of amino acids (AA) for fish (Murai, 1991).

Essential/indispensable amino acids	Non-essential/dispensable amino acids
Arginine	Aspartate
Histidine	Glutamate
Lysine	Alanine
Leucine	Tyrosine
Isoleucine	Serine
Methionine	Glutamine
Valine	Asparagine
Phenylalanine	Cysteine
Tryptophan	Proline
Threonine	Glycine

nutrition of fish. Impairment of health and nutritional status can be ascribed to reduced protein digestibility and availability resulting from high ANF concentrations (Gilani et al., 2012). To maximise feed utilisation and ensure profitable aquaculture production, the ANF content of the protein sources used in aquaculture feed must be kept at a minimum. Finally, a well-balanced AA profile of the protein source is required for optimal growth, feed utilisation and nitrogen retention of the cultured fish. Several previous studies have demonstrated that the AA composition, as well as the proportion of EAAs and NEAAs, have a significant impact on nitrogen retention and excretion. A study performed by Green et al. (2002a) showed that maximal N retention was achieved when the AA profile of the feed met the AA requirement of the target fish species. By contrast, N excretion increased as levels of dietary AA exceeded the AA requirement of target fish species (Green et al., 2002a). Several studies have investigated how the EAA/NEAA ratio of dietary protein influences protein utilisation efficiency in different fish species including Nile tilapia (*Oreochromis niloticus*) (Mambrini and Kaushik, 1994), rainbow trout (*Oncorhynchus mykiss*) (Green et al., 2002b) and European sea bass (*Dicentrarchus labrax*) (Peres and Oliva-Teles, 2006). Based on the results from these studies, the following generalisations can be made about the EAA/NEAA ratio:

1) N excretion decreases with increasing EAA/NEAA ratio.
2) N retention increases with increasing EAA/NEAA ratio until reaching a plateau value (>50:50) with maximum N retention and minimum N excretion.
3) Feed intake decreases when the EAA/NEAA ratio exceeds 50:50.

An EAA/NEAA ratio of 50:50 appears preferable for aquafeeds as feed intake and growth are maximised at this level. An EAA/NEAA ratio beyond 50:50 is often associated with higher N retention but a lower feed intake (Peres and Oliva-Teles, 2006). Thus, formulating feed with an EAA/NEAA ratio of 50:50 establishes an optimal balance between N retention and feed intake ensuring that N retention is maximised without compromising feed intake.

2.1.1. Fishmeal, plant-based meals and BSG

Current protein sources used in commercial aquafeeds can be divided into two main categories: 1) marine-based meals and 2) plant-based meals. Fishmeal, which is mainly obtained from fisheries production of small pelagic fish species unfavorable for human consumption, constitutes the most common marine-based protein source in aquafeed (Péron et al., 2010).

In 1994, the global production of fishmeal from non-targeted captures peaked at ~7.3 million tonnes (FAO, 2020). Since then, fishmeal production from wild fish stocks had followed a declining trend with minor annual fluctuations reaching a level of ~5.0 million tonnes in 2020 (EUMOFA, 2021). Historically, aquafeed production has relied on fishmeal as the sole protein source because of its excellent nutritional properties including high protein content, high palatability, a well-balanced AA profile and lack of ANFs; the latter two resulting in high protein digestibility (Oliva-Teles, 2012). Beyond that, fishmeal consumption entails low excretion of ammonia and undigested protein in faeces which can be explained by the favourable AA composition and the absence of ANFs, respectively. However, reliance on fishmeal as the sole protein source in aquafeed is not feasible for long-term aquaculture production as increasing demand for aquaculture commodities may cause overexploitation of the wild fish species used to produce fishmeal (Olsen and Hasan, 2012). During the last decades, the rise in fishmeal prices, due to declining catches and increased market demand, has strengthened efforts to transition to plant-based meals in aquafeeds (Jannathulla et al., 2019). Plant-based meals are derived from a variety of different vegetables, predominantly soybean but also rapeseed, cottonseed, groundnut and sunflower (Tacon et al., 2011). Soy protein is available as either meal or protein concentrate, with soybean meal being the most widespread soy product in aquafeed production. Meal and concentrate are distinct in their content of protein, ANFs and AA profile. Soy protein concentrates are rich in proteins (>75%) with negligible amounts of ANFs, while soybean meals have a lower protein content (40–50%) and contain high levels of certain ANFs (Hardy, 2010). Furthermore, soy protein concentrates have an AA profile that resembles fishmeal (Dersjant-li, 2002) whereas soybean meals are deficient in specific AAs, particularly methionine and lysine (Table 2) (Alashi et al., 2013). As a result, soybean meals have lower nutritional value as compared to soy protein concentrates which have restricted their use as fishmeal replacement at high inclusion levels. Besides these nutritional constraints, the incorporation of soybean meal, as well as other plant-based protein sources, in aquafeed may harm the environment mainly due to intensified use of arable land for crop production (Naylor et al., 2021). Pelletier et al. (2018) studied the environmental impact of the production of soybean meal and concentrates for aquafeed in two different geographical regions (US and Brazil). This study found that the production of these soy products was generally associated with great land use and high greenhouse gas emission resulting from deforestation (Pelletier et al., 2018). In addition, the high phytic acid concentration of soybean poses nutritional and environmental constraints for its use in fish feed as phytic acid has been shown to reduce mineral utilisation and

Table 2
Content of crude protein (CP), essential amino acids (EAA) and non-essential amino acids (NEAA) in fishmeal, soybean meal and brewer's spent grain (BSG).

	Fishmeal[a]	Soybean meal[a]	BSG[b]
CP (% DM)	50–70	40–50	20–30
EAA (% DM)			
Tryptophan	1.16	1.30	0.03
Phenylalanine	4.33	5.00	1.03
Valine	5.41	4.80	1.02
Isoleucine	4.53	4.00	0.73
Leucine	7.79	7.80	1.35
Methionine	2.80	1.30	ND
Threonine	4.51	4.00	0.16
Lysine	8.60	6.40	3.17
Arginine	6.43	7.20	1.00
Histidine	3.49	2.60	5.81
NEAA (% DM)			
γ-amino butyric acid	ND	ND	0.06
Alanine	6.52	4.30	0.91
Asparagine	ND	ND	0.33
Aspartate	9.50	11.70	1.06
Glutamate	12.65	18.70	3.67
Glycine	6.89	4.20	0.39
Serine	3.68	5.10	0.83
Tyrosine	3.65	3.20	0.57
Cysteine	0.91	1.60	ND
Glutamine	ND	ND	0.02
Hydroxyproline	1.30	ND	ND
Proline	4.70	5.10	ND
Taurine	0.77	ND	ND
Phenylalanine	4.33	ND	ND
Hydroxylysine	0.29	ND	ND
Ornithine	0.11	ND	ND
EAA/NEAA	0.97	0.82	1.84

DM: dry matter content.
ND: not determined.
[a] EAA and NEAA contents are reported as percentages of fishmeal and soybean meal in the original work (Alashi et al., 2013; Gamboa-Delgado et al., 2017). The contents of individual amino acids in fishmeal were retrieved from Gamboa-Delgado et al. (2017), while the amino acid contents in soybean meal were extracted from Alashi et al. (2013).
[b] EAA and NEAA contents are reported as percentages of total protein content in the original work (Waters et al., 2012). To render values comparable to those reported for fishmeal and soybean meal, the amino acid contents were converted into a percentage of BSG using the following formula: $\frac{X_{AA} * X_{protein}}{100\%}$. X_{AA} is the amino acid content measured as a percentage of the total protein content ($X_{protein}$) which was estimated at 22% by Waters et al. (2012).

increase faecal phosphor excretion (Dalsgaard et al., 2009). The challenges imposed by the utilisation of fishmeal and soybean meal necessitate a search for novel, alternative protein sources with low environmental impact and high nutritional value. From a nutritional point of view, BSG and soybean meal have similar drawbacks: low palatability, high ANF content and deficiency in certain AAs (Table 2). Nonetheless, when comparing their AA profiles it can be argued that BSG provides a better protein source than soybean meal. This can be explained by the higher EAA/NEAA ratio for BSG (1.84) (Waters et al., 2012) compared to soybean meal (0.82) (Alashi et al., 2013) which may enhance N retention in fish and diminish N excretion to the surrounding aquatic environment. When considering the crude protein (CP) content, unprocessed BSG represents a less favourable protein source compared to soybean meal and fishmeal, because it has a significantly lower CP content (Lynch et al., 2016). In a sustainability context, BSG is expected to be superior to both soybean meal and fishmeal owing to its unfluctuating annual availability, vast production volumes and low market price (Mussatto et al., 2006). In terms of circular economy, BSG can be defined as a side stream which is a by-product generated unintentionally from an industrial process. Hence, BSG production does not require any additional energy or resource use other than that needed for beer production. To date, only a few studies have addressed the use of raw and partially refined BSG as a second-generation feedstuff in aquafeeds. Jayant et al. (2018) demonstrated that soybean meal could be substituted with up to 50% of unprocessed BSG without affecting growth and nutrient utilisation in striped catfish (*Pangasianodon hypophthalmus*). In a similar study, Nazzaro et al. (2021) evaluated the influence of partial fishmeal replacement with raw BSG on protein digestibility in rainbow trout and gilthead seabream (*Sparus aurata*). This study revealed that inclusion of 20% BSG did not adversely affect the protein digestibility in these two carnivorous fish species. He et al. (2020) investigated the effect of substituting fishmeal with a BSG-derived product on the feed utilisation and growth performance of Pacific white shrimp (*Litopenaeus vannamei)*. This product was produced by a protease-aided fractionation process as described in He et al. (2019) and had a high content of crude protein (46%). The most interesting finding of this study was that the BSG product could replace up to 50% of fishmeal without affecting the feed utilisation, although higher inclusion levels had a negative influence on the growth performance. Taken together, these studies suggest that the use of unprocessed BSG in aquafeed is feasible at low inclusion levels (20–50%). Due to the low nutritional value, unrefined BSG cannot be employed at high inclusion levels in aquafeed without impairing the growth and N-metabolism of the fish. Thus, BSG must undergo intensive valorisation in order to increase its nutritional value to a level that allows for a higher inclusion (60–90%) in aquafeeds.

2.1.2. Current pros and cons of utilising BSG in aquafeeds

As outlined above, the use of BSG as a protein source in aquafeeds has potential due to a relatively high protein content, a favourable amino acid profile, low market price and stable availability. Despite this, BSG has yet to be implemented in aquafeed formulation, because it has a high content of ANFs and indigestible components which reduces the nutritional value. Before BSG can be incorporated as a protein source in aquafeeds, it must undergo refinement which is an energy and time-consuming process. Several approaches have been considered to utilise BSG in its raw or semi-refined form, including biogas production, enrichment of construction materials such as bricks, substrate for microbial growth and enzyme production and a fibre source in food products (Jackowski et al., 2020). Furthermore, Assandri et al. (2021) reported that unprocessed BSG can be converted into an efficient composting agent by means of a simple procedure in which the composting properties of BSG are improved by addition of manure and a lignocellulosic bulking agent. From a holistic perspective, recycling BSG as a composting agent may lead to a more sustainable cultivation of crops that could potentially be employed in aquafeeds. While this certainly promotes the environmental sustainability in the production of plant-based ingredients for aquafeeds, utilising BSG as a composting agent will not overcome the nutritional challenges associated with plant-based protein sources, nor will it reduce the demand for arable land. The alternative applications of BSG discussed here represent more climate-smart and economic strategies for valorising BSG compared to the extensive refinement needed to enable high inclusion levels of BSG-derived protein in aquafeeds. The implementation of BSG and other protein-rich agro-industrial residues, may help reduce the consumption of fishmeal and soybean meal thereby contributing to a more sustainable aquaculture production which justifies efforts to valorise and implement BSG in aquafeed production.

3. Brewer's spent grain (BSG)

3.1. Production of BSG

Barley (*Hordeum vulgare*) is the core ingredient in the production of beer. Barley grain is composed of three main constituents: the germ (embryo), the endosperm comprising a matrix of starch and protein enclosed by a layer of aleurone cells, and the grain covering (Fig. 1a) (Willows et al., 2017). The grain covering is further divided into three distinct layers constituting the inner seed coat (testa), the middle pericarp and the outer protective husk layer which primarily consists of dead cells with a high content of lignocellulosic material (Fig. 1b) (Mosher and Trantham, 2017).

Beer brewing encompasses three key steps: malting, mashing and fermentation (Fig. 2). During malting, barley grains undergo germination resulting in a chemically modified endosperm with a high concentration of hydrolytic enzymes. The germinated barley (malt) is milled to separate the grain covering from the internal grain parts and to increase the accessibility of the endosperm macromolecules (Lewis and Young, 2001). The milled malt then undergoes mashing; a process in which endosperm starch and proteins are converted to fermentable sugars and AAs, respectively, by the action of enzymes produced during malting. This process generates a sweet liquid known as the wort which constitutes the fermentation medium for beer production. After supplementation with sugars and hops, the wort is filtered through a bed comprising the insoluble and non-degraded parts of malted barley which is deposited at the bottom of the mash tun. The remaining solid fraction obtained after filtration is BSG (Xiros and Christakopoulos, 2012).

3.2. Chemical composition of BSG

Brewer's spent grain is chiefly composed of the grain covering from the original barley seed in addition to a minor portion of endosperm fragments (Lynch et al., 2016). Contents of the main constituents in BSG are presented in Fig. 3.

3.2.1. Lignocellulose

BSG is a heterogeneous, lignocellulosic material comprising high amounts of lignin (~12–28% of dry matter) and non-starch polysaccharides (NSP) (~30–50% of dry matter) derived from the barley husk cell walls. In addition, traces of starch and β-glucans (~1%) derived from the endosperm are also present in BSG (Steiner et al., 2015). Lignin is a complex phenolic polymer synthesised by radical-induced polymerisation of three methoxylated benzene derivatives referred to as phenylpropanoid alcohols (p-coumaryl, coniferyl and sinapyl alcohol). During lignin synthesis, these alcohol monomers are coupled via various types of C–C and ether linkages leading to the formation of a diverse and recalcitrant aromatic network (Zhu et al., 2013). The NSP component consists of cellulose and hemicelluloses in approximately equal amounts, with arabinoxylans (AX) being the most abundant hemicellulose. Arabinoxylans are amorphous, non-cellulosic polysaccharides that function as reservoirs of phenolic acids, primarily ferulic acid, which has antifungal properties (Asiegbu et al., 1996).

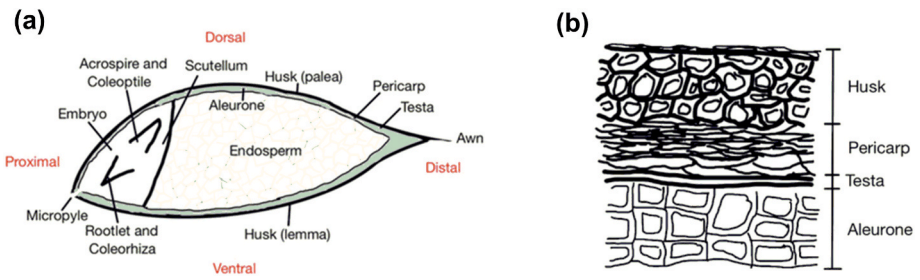

Fig. 1. (a) Longitudinal cross-section of a barley grain showing its primary components (germ, endosperm and grain covering) and (b) structure of the grain covering. Figures are adapted from (Mosher and Trantham, 2017).

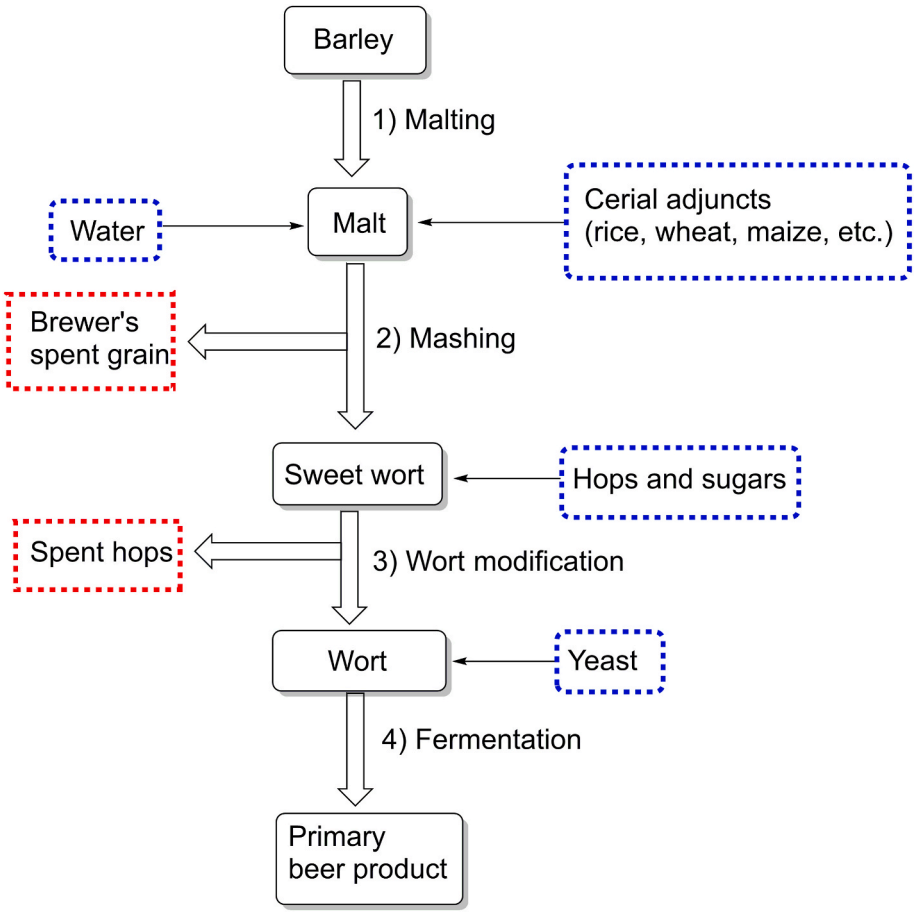

Fig. 2. Schematic representation of key steps in the beer-brewing process. 1) Malting: production of hydrolytic enzymes. 2) Mashing: production of the sweet wort by hydrolysis of endosperm starch and proteins. 3) Wort modification: boiling and clarifying of the sweet wort after addition of hops and sugars. 4) Fermentation: the transformation of modified wort into a primary beer product that requires further processing to obtain the final beer product (not shown). Generated by-products and supplementary ingredients are highlighted in red and blue, respectively.

Structurally, AX consists of a xylose backbone mainly substituted with arabinose at O-2, O-3, or both (Mendis and Simsek, 2014). Some of the arabinose residues may undergo esterification with ferulic acid at O-5 of arabinose which may lead to the formation of diferulic acid cross-linkages between adjacent AX strands under oxidative conditions (Fig. 4) (Mathew and Abraham, 2004). Besides arabinose, the xylose backbone can also be linked to other substituents including uronic acid, methylated uronic acid and acetyl groups (Coelho et al., 2016). The three lignocellulosic components are arranged in such a manner that cellulose forms hydrogen bonds with hemicellulose which in turn is attached to non-core lignin through ether and ester linkages as illustrated in Fig. 4 (Mandalari et al., 2005).

3.2.2. *Protein*

In addition to lignocellulose, protein is the second most predominant constituent of BSG accounting for up to 30% of the dry matter content (Lynch et al., 2016). Cereal proteins, such as those found in BSG, have been divided into four categories based on their solubility in different solvents: 1) albumins (water-soluble), 2) globulins (salt-soluble), 3) prolamins (alcohol-soluble) and 4) glutelins (acid/alkaline soluble) (Byers et al., 1983). The majority of proteins found in BSG are hordeins which are barley prolamins (>50% of total protein) (Vieira et al., 2014). Glutelins represent the second most abundant protein type in BSG while albumins and globulins constitute a smaller fraction of the total amount of proteins in BSG (~2%) (Celus et al., 2006). The proteins of BSG are classified as high-quality proteins because they contain high levels of EAAs. The 10 AAs which are essential to fish account for ~65% of the total protein content of BSG (Table 2). Lysine, which is derived from barley hordeins, constitutes the most abundant EAA followed by leucine, phenylalanine and isoleucine. Other minor constituents present in BSG comprise lipids (~7–10%), minerals (~1%), simple sugars including glucose, fructose and sucrose (<15%), and traces of vitamins (Waters

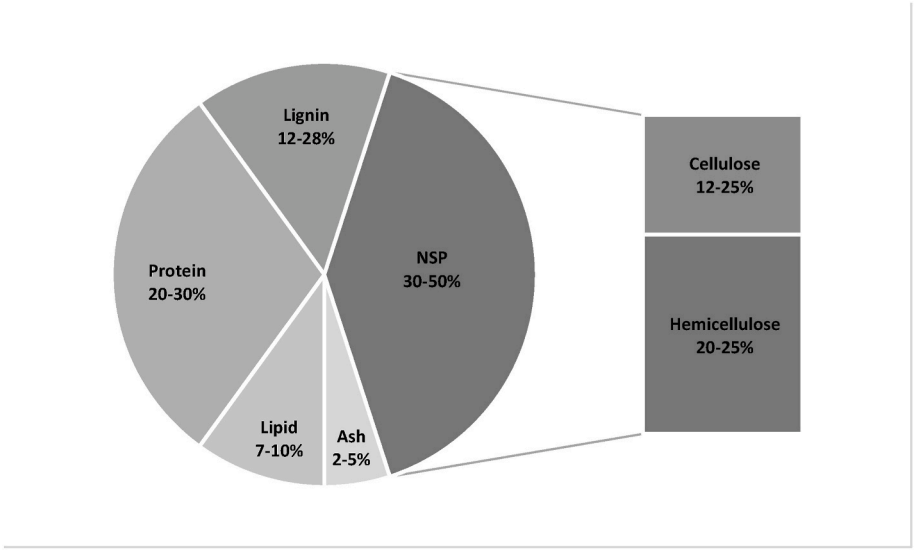

Fig. 3. *Chemical composition of brewer's spent grain (BSG). NSP: non-starch polysaccharides. Percentages of all constituents are reported as ranges because the composition of BSG is known to vary greatly with barley variety, brewing regime and time of harvesting among others as shown by* Santos et al. (2003). *Figures are adapted from* (Lynch et al., 2016).

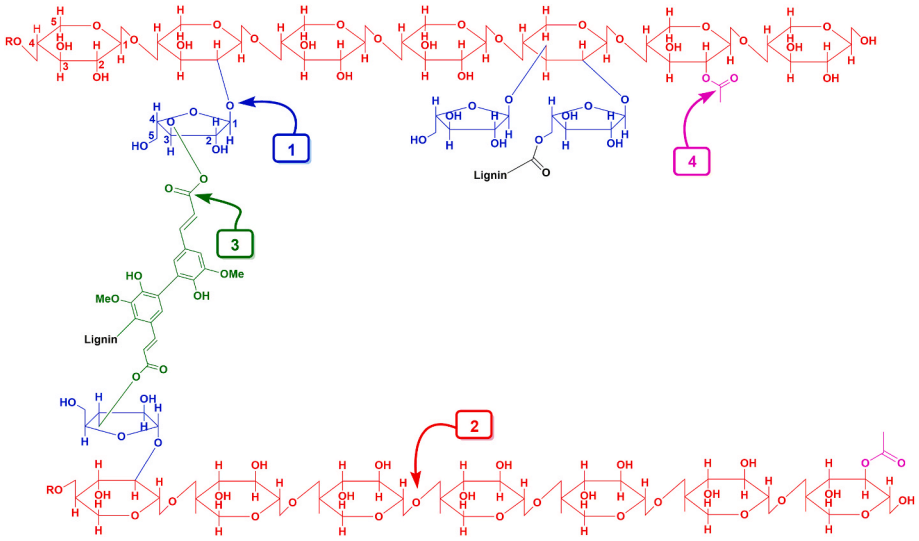

Fig. 4. Generalised structure of arabinoxylans (AX) showing two cross-linked AX strands and two possible sites of lignin coupling. Arabinose (blue) and acetyl groups (pink) are linked to the xylose backbone (red) through ether and ester bonds, respectively. AX strands are cross-linked by C–C coupling between two ferulic acid moieties (green). Enzymatic activities involved in AX hydrolysis are indicated with arrows and numbered as follows: 1; α-L-arabinofuranosidase, 2; endo-1,4-xylanase, 3; ferulic acid esterase and 4; acetyl xylan esterase.

et al., 2012).

4. Prospects of BSG utilisation in aquaculture feed

4.1. Benefits of applying BSG in aquaculture feed

The use of BSG as a protein source in aquaculture feed is expected to be beneficial in several respects. The relatively high content of protein and EAA makes BSG a potential alternative for the conventional meals used in aquafeed formulation which may reduce the reliance on fishmeal and increase the availability of arable land (Salin et al., 2018). As a result, the implementation of BSG as a protein source represents a more environmentally sustainable alternative to current protein sources. Owing to its high annual abundance and low prices, the application of BSG is also expected to be more economically feasible in comparison with conventional protein sources (Mussatto, 2014). Currently, BSG is sold cheaply as feed supplements for livestock, discarded as waste, or incinerated for energy production; the latter two constituting an environmental burden (Johnson et al., 2010). However, employing BSG in animal feed is believed to alleviate the environmental impact resulting from BSG incineration and disposal presuming that appropriate methods are chosen for the storage and processing of BSG (Petit et al., 2020).

4.2. Limitations of applying BSG in aquaculture feed

At present, there are various challenges associated with the utilisation of unrefined BSG in aquafeeds including: 1) a high content of ANFs, 2) a low protein concentration and a deficiency in certain AAs and 3) the choice of adequate preservation methods (Johnson et al., 2010). In the following section, key aspects of these complicating factors will be described in detail. Additionally, we will discuss the potential for using different refinement methods for increasing the nutritional value of BSG to a level that allows for its implementation in aquaculture feed. The practicability of a specific refinement method is highly dependent on its economic performance and environmental implications. At present, the economic costs and environmental impact of the various refinement methods considered in the next chapter are not fully understood and will need further investigation in future research employing

techno-economic analysis and life-cycle assessment. What we know about the economic aspects of BSG valorisation is chiefly based on the work of He et al. (2021) who performed a techno-economic analysis of the production of BSG protein concentrates through wet fractionation. The study found that the production cost of BSG protein concentrates (1043 USD/t) was lower than the average fishmeal price (1449 USD/t) which implies that BSG-derived products may be economically superior to fishmeal in aquafeeds provided that low-cost and time-efficient methods are used to produce them.

4.2.1. Anti-nutritional factors

Anti-nutritional factors encompass a variety of different compounds including lectins, phytic acid, alkaloids, protease inhibitors, tannins and structural components of plant cell walls (NSP and lignin) (Glencross et al., 2020). In unrefined BSG, lignin, cellulose and hemicellulose comprise the main ANFs while tannins and phytic acid account for a minor portion of the ANF content. Comparison of the ANF content in soybean meal and BSG indicates that BSG has higher concentrations of lignin and fibre, whereas the tannin content is considerably higher in soybean meal (Table 3) (Martins et al., 2017). Additionally, BSG and soybean meal contain similar amounts of phytic acid and therefore they may have similar drawbacks concerning the excretion of phosphor (Çantaş and Yildirim, 2020). This review, however, will solely focus on problems related to the anti-nutritional effects of lignocellulose in BSG and how these can be addressed by appropriate refinement strategies.

Previous studies have established that ANFs reduce protein digestibility and bioavailability via different mechanisms depending on the type of ANF in question. For most ANFs, protein digestibility is reduced either by inhibition of digestive enzymes or by chelation with dietary proteins (Gilani et al., 2012). Furthermore, some ANFs may also act as feed deterrents due to their bitter taste resulting in rejection of feed items (Glencross et al., 2020). In a comparative study, Waters et al. (2012) estimated the quantity of phenolic compounds in BSG which include ferulic acid and hydroxycinnamic acids derived from AX and lignin, respectively (Guido and Moreira, 2017). They concluded that BSG contains higher levels of phenolic compounds (131 mg/L) compared to malt (112 mg/L) and barley (90 mg/L) which implies a gradual build-up of lignin and AX during the brewing process. Most literature descriptions have emphasised the discrete effects of various lignocellulosic constituents on protein digestibility of diets fed to different fish species with rainbow trout being the most studied. From these, it can be inferred that the distinct components of lignocellulose have varying impacts on protein digestibility and that their magnitude of impact is commensurate with inclusion levels (Glencross et al., 2012). Cellulose had only a marginal impact on protein digestibility whilst lignin adversely affected this parameter in several species including rainbow trout (Glencross et al., 2012), Nile tilapia (Amirkolaie et al., 2005) and barramundi (*Lates calcarifer*) (Irvin et al., 2016). Conversely, Hansen and Storebakken (2007) found that cellulose inclusion reduced energy and dry matter digestibility although it improved pellet texture in terms of increased durability and hardness. For these reasons, cellulose may prove useful as a filling agent in feed production if applied in quantities that do not negatively affect protein digestion. Furthermore, interactive effects of different lignocellulosic components on protein digestibility have been demonstrated revealing negative synergistic interactions between lignin and certain polysaccharides included in the matrix of lignocellulose (Irvin et al., 2016). From a nutritional point of view, the high content of lignocellulosic material in BSG precludes its utilisation in aquafeed in an unrefined form. As a result, BSG must undergo considerable refinement before it can be considered a suitable protein source for fish. The following section outlines different methods for valorising BSG.

Table 3
Content of anti-nutritional factors (ANFs) in unrefined brewer's spent grain (BSG) and soybean meal.

ANF	Soybean meal	BSG	References
Lignin[a] (% DM)	0.4	5.4[d]	(Heuzé et al., 2017; Heuzé et al., 2020)
Cellulose[b] (% DM)	5.3	16.5[d]	(Heuzé et al., 2017; Heuzé et al., 2020)
Hemicellulose[c] (% DM)	4.8	34.4[d]	(Heuzé et al., 2017; Heuzé et al., 2020)
Phytic acid (% DM)	1.00	1.01[e]	(Ktenioudaki et al., 2015; Martins et al., 2017)
Tannin (mg/g DM)	8.22	1.4[d]	(Heuzé et al., 2017; Martins et al., 2017)

DM: dry matter content.
[a] Lignin content is measured as acid detergent lignin (ADL) and therefore it does not include acid-soluble lignin.
[b] Cellulose content is calculated from the acid detergent fibre (ADF) content, which is the sum of ADL and hemicellulose, using the formula: cellulose = ADF – ADL.
[c] Hemicellulose content is calculated from the neutral detergent fibre (NDF) and the ADF content by the formula: hemicellulose = NDF – ADF, where NDF includes ADL, cellulose and hemicellulose.
[d] The reported values are based on dehydrated, unground BSG.
[e] The phytic acid content is based on milled BSG flour.

4.2.1.1. Refinement of BSG. An extensive body of literature has described different approaches for addressing the nutritional issues associated with high levels of fibre and lignin in plant-derived protein sources such as those found in BSG. These approaches can be clustered into two main refinement categories: 1) conversion of lignocellulose to digestible components using biological, chemical and enzymatic treatments (Fig. 5), and 2) chemical fractionation (Fig. 6). The overarching goal of both strategies is to produce a protein-rich concentrate containing negligible amounts of fibre and lignin.

4.2.1.1.1. Conversion of lignocellulose

4.2.1.1.1.1. Hydrolysis of cellulose and hemicellulose

Prior studies have mainly focused on the depolymerisation of cellulose and hemicellulose into their respective monosaccharides which consist of glucose, xylose and arabinose using chemical, hydrothermal and enzymatic pretreatment of BSG. As described by D'Este et al. (2018), monosaccharides can serve as precursors for fermentative AA production in the presence of an amino-group donor such as glutamate. Therefore, the application of native or engineered microorganisms, specialised in producing specific AAs by fermentation, can be considered a useful method for increasing certain AAs to a level that matches the nutritional requirement of the fish species concerned. Chemical pretreatment of BSG fibre involves strong alkaline or acidic hydrolysis of cellulose and hemicellulose. Such harsh conditions are likely to induce the production of assorted sugar decomposition products such as furfural, 5-hydroxymethylfurfural (HMF) and acetic acid (Cardenas--Toro et al., 2015). These by-products have proven to be deleterious for most fermenting bacteria and fungi used in the downstream processing of pretreated lignocellulosic material and their production must therefore be minimised. For instance, a study performed by Martín and Jönsson (2003) concluded that the ethanol production of *Saccharomyces* and *Zygosaccharomyces* was inhibited by furfural and HMF. In the same way, Dessie et al. (2019) showed that high concentrations of furfural and HMF had an inhibitory effect on the production of succinic acid production by *Actinobacillus succinogenes*. In addition to the formation of inhibitory by-products, utilising strong alkaline or acidic solution to depolymerise the cellulose and hemicellulose content in BSG may also hydrolyse proteins and other constituents leading to complete deterioration of the material. Some studies have suggested milder hydrolysis conditions for minimising the production of inhibitory by-products. Carvalheiro et al. (2004) investigated the depolymerisation of the NSP component present in BSG and found that the formation of sugar decomposition products could be reduced by application of

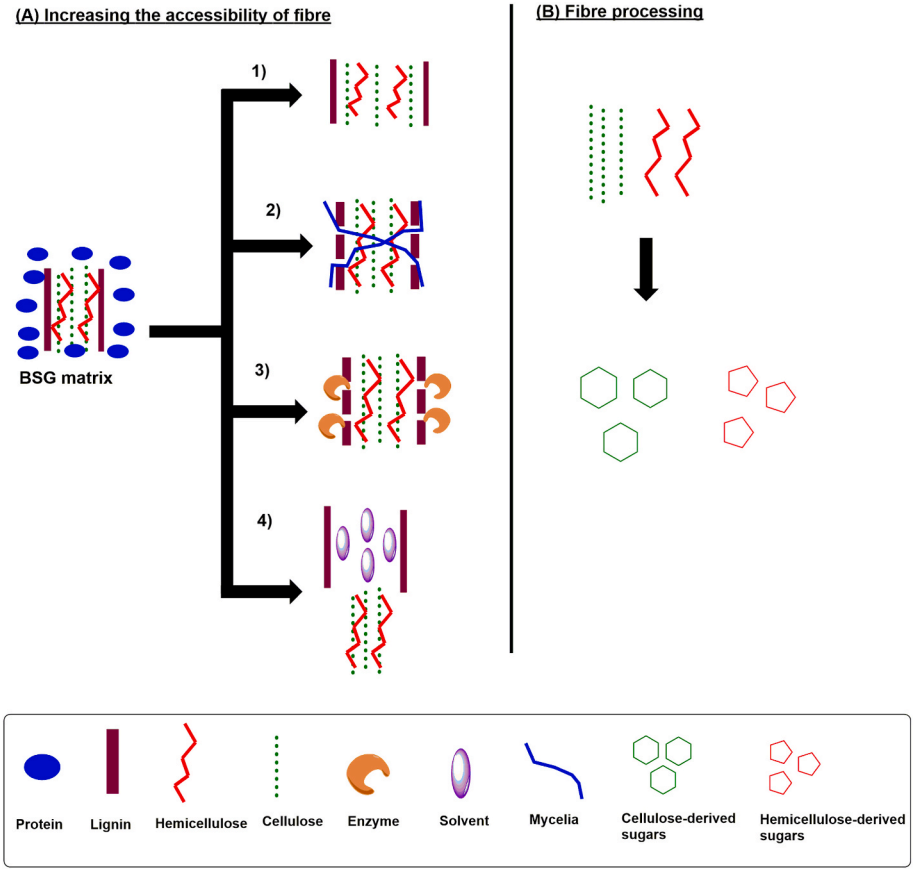

Fig. 5. Conversion of lignocellulose in two steps. (A) Increasing fibre accessibility by 1) disruption of intermolecular forces between cellulose, hemicellulose and lignin, 2) fungal lignin degradation, 3) enzymatic lignin degradation using enzymes excreted during the cultivation of lignin-degrading fungi and 4) lignin extraction with a suitable solvent system. (B) Fibre processing by: 1) production of fermentable monosaccharides by hydrolysis of cellulose and hemicellulose and 2) production of specific amino acids by fermentation of monosaccharides. For the sake of simplicity, proteins are not shown in the downstream processing of lignocellulose and all chemical structures are displayed as pictograms.

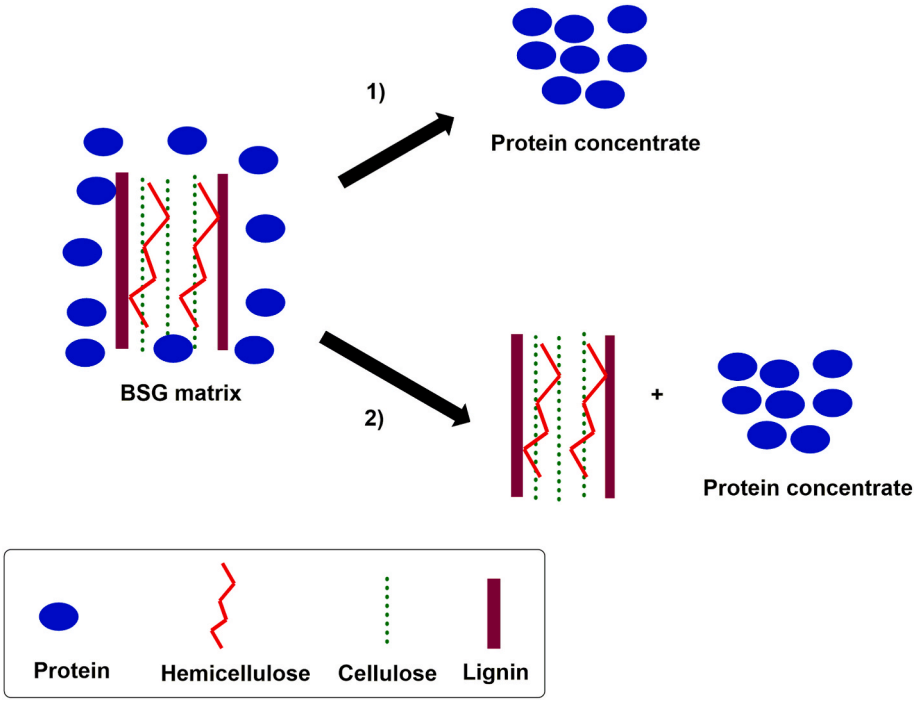

Fig. 6. Chemical fractionation. Protein concentrates can be obtained from brewer's spent grain (BSG) by two different approaches: 1) direct protein extraction using a repertoire of different solvent systems and 2) extraction of fibre and lignin from BSG.

autohydrolysis; a hydrothermal pretreatment in which water is used as the only reagent under elevated temperatures. They found that monosaccharide production was enhanced with prolonged reaction time and increased temperature. At the same time, optimum conditions for monosaccharide production increased the relative content of lignin and by-products. For instance, the lignin content increased from ~29 g/100

g solid to ~58 g/100 g solid after 60 min of incubation at 170 °C. Correspondingly, the content of acetic acid, formic acid, furfural and HMF was increased by 250%, 480%, 490% and 480%, respectively (Carvalheiro et al., 2004). As a result, the findings of this study imply that autohydrolysis is not a feasible method for the complete hydrolysis of cellulose and hemicellulose. The issue of by-product formation encountered by chemical and, to some extent, hydrothermal pretreatment, may be avoided by using an enzymatic approach. In a study performed by Forssell et al. (2008), the solubilisation of BSG fibre was examined with the use of different commercial enzyme mixtures of carbohydrate degrading enzymes. One interesting finding of this study was the incomplete hydrolysis of AX which can be ascribed to the lack of key enzymes involved in the cleavage of the various bonds present in AX. Due to the high structural complexity of AX, complete hydrolysis requires the simultaneous action of several enzymes. On large-scale production, applying multiple enzymes for a single process is infeasible from both an economic and a practical perspective. This issue can be addressed by employing fungal species as multi-enzyme factories. Such systems have been extensively studied using *Fusarium oxysporum* and *Neurospora crassa* as model organisms (Xiros et al., 2008a, 2008b). Application of the crude enzyme extract produced by these species has proven successful in hydrolysing AX from BSG completely. Finally, and perhaps one of the most interesting results from the study conducted by Forssell et al. (2008) was the low hydrolysis efficiency observed for all tested enzyme mixtures. This may be attributable to the restricted enzyme accessibility of cellulose and hemicellulose stemming from the closely interlinked network of the three lignocellulosic components as described by Mussatto et al. (2008). To render the fibre more accessible for enzymatic action and thus facilitate complete depolymerisation, lignin must be removed before fibre hydrolysis. A description of the different delignification strategies will be given in the following section.

4.2.1.1.1.2. Delignification

Generally, two different strategies can be applied to delignify lignocellulosic biomasses—a biological and a chemical approach. The overriding advantage of the biological approach is that it diminishes the formation of by-products. Moreover, biological delignification is believed to be more sustainable in terms of energy consumption and environmental impact because it occurs under milder conditions compared to chemical delignification (Christopher et al., 2014). A compilation of literature has explored various solvent systems suitable for lignin extraction from lignocellulosic biomasses including ionic liquids (ILs), alkaline solutions, ethanol, sodium chlorite and an acetone/water mixture (Denilson, 2010; Melro et al., 2020; Pereira et al., 2016; Prado et al., 2013; Siqueira et al., 2013). However, the majority of presently used extraction solvents are neither environmentally friendly nor safe to handle due to their corrosive and volatile properties. Further, application of these solvent systems is often associated with co-extraction and disintegration of proteins, hemicellulose and cellulose thus preventing further processing of these macromolecules after lignin removal (Prado et al., 2013). Ionic liquids are defined as ionic compounds, composed of organic cations and inorganic/organic counter anions, which exist as dissociated ions at temperatures below the boiling point of water (Singh, 2019). These solvents present novel systems for extracting biodegradable polymers from biomass as they possess several favourable characteristics including low volatility, high thermal stability, safe handling and low environmental impact. Furthermore, ILs have proven to be excellent solvents for lignin extraction because they preferentially dissolve the lignin fraction of lignocellulose. This was validated in a study by Prado et al. (2013) who made a comparative analysis of ethanol, alkaline and IL extraction of lignin from apple tree prunings. Subsequent analysis of the three resulting lignin extracts showed that the greatest purity was achieved when employing IL extraction. In contrast, the lignin extracts obtained from ethanol and alkaline extraction contained high amounts of impurities stemming from co-extraction of other macromolecules in the material (Prado et al., 2013). Studies on the mechanistic aspects of lignin dissolution in ILs suggest that lignin is dissolved due to hydrogen bonding between the hydroxyl groups of lignin and the anion of ILs, which disrupts the network of hydrogen bonds between lignin molecules (Ji et al., 2012). In addition, the study undertaken by Liu et al. (2017) revealed that treatment with deep eutectic solvents (DES), which are a class of ILs comprised of Brøndsted acids (proton donors) and bases (proton acceptors), promotes separation of lignin and hemicellulose by disrupting the hydrogen bonds between the two. Despite the evident benefits of using ILs for lignin removal, these solvents are currently not applicable on an industrial scale due to their high production costs. Alternatively, fibre accessibility can be increased by using chemical methods that break the intermolecular forces between the three lignocellulosic components thus separating them from each other. A study by Mussatto et al. (2008) demonstrated that cellulose of BSG could be disentangled from hemicellulose and lignin by a sequence of dilute acid and alkali treatments. This approach, however, does not remove lignin from the BSG matrix and therefore it cannot be considered a suitable refinement method in the context of feed production from plant-based materials. Biological delignification relies on the use of lignin-degrading microbes that possess inducible enzymatic machinery adapted for complete or partial lignin deconstruction of biomasses. Lignin decomposers are classified into two groups comprising fungi and bacteria with fungi generally being more efficient in lignin degradation than bacteria (Janusz et al., 2017). White-rot fungi are believed to be the most efficient lignin degraders as they are capable of oxidising lignin completely to CO_2 and water (Sahadevan et al., 2016). The white-rot fungi *Phanerochaete chrysosporium* constitutes the most well-studied lignin-degrader. Previous studies have unraveled the effect of culture conditions on the growth and enzymatic activity of the ligninolytic enzymes produced by this fungal species. Based on the results from these studies, it has been established that the ligninolytic activity is maximised under high oxygen tension and nitrogen-limited conditions (Kirk et al., 1978). In addition, *P. chrysosporium* grows optimally at a broad range of pH values and at temperatures as high as 40 °C (Richard et al., 1987). In the context of valorising the BSG fibre, *P. chrysosporium*, as well as other white-rot fungi, represent potential candidates for BSG delignification because they selectively degrade lignin and leave the fibre untouched (Wong, 2009). Although white-rot fungi hold great potential for delignification of BSG, their use may be obstructed by the occurrence of antifungal agents such as ferulic acid bound to AX of BSG (Fig. 4). Asiegbu et al. (1996) examined the effect of ferulic acid on the biomass-degrading capacity of several white-rot fungi. An interesting finding from this study was that the growth of *P. chrysosporium* was significantly depressed by ferulic acid, even at low concentrations (0.5 g ferulic acid/L growth medium). Another study performed by Huang et al. (1993) revealed that *Saccharomyces cerevisiae* efficiently transformed ferulic acid into 4-hydroxy-3-methoxystyrene via a decarboxylation pathway. Using yeast-induced transformation of ferulic acid may thus be a useful strategy for eliminating the antifungal effect of ferulic acid ultimately enhancing the efficacy of fungal delignification of BSG. However, to the best of our knowledge, no previous studies have examined how the conversion of ferulic acid affects fungal delignification efficiency. Therefore, further research is required to evaluate the viability of using ferulic acid transformation as a pretreatment method before BSG delignification with white-rot fungi. Another major disadvantage of utilising white-rot fungi in the delignification of BSG is the accumulation of chitin which is the main structural component of fungal cell walls. The presence of fungal-derived chitin in BSG may be an impediment to its application in aquafeeds since high chitin levels have been shown to be correlated with reduced protein digestibility in some fish species (Karlsen et al., 2017). Accumulation of chitin could be prevented by adopting an enzymatic approach for degrading the lignin in BSG. A number of studies have investigated the enzymatic delignification of different lignocellulosic biomass including BSG. For instance, Fan et al. (2019) identified the optimum conditions for small-scale delignification of corn stover catalysed by lignin peroxidase produced by the fungus *Aspergillus oryzae*. Likewise, Giacobbe

et al. (2019) showed that laccase-pretreatment caused efficient delignification of BSG. Although these results are promising, more research is needed in order to develop enzymatic systems that are economically viable for large-scale production.

4.2.1.1.2. Chemical fractionation. The term chemical fractionation describes the process in which a heterogeneous mixture is separated into its different components with the aid of chemical extractions. In general, a fractionation process is characterised by three distinct stages including 1) pretreatment, 2) extraction and 3) purification. The pretreatment involves mechanical processing of the material and serves to increase the accessibility of target compounds, such as proteins, to extraction solvents. During extraction, the target compound is separated from other matrix components by solubilisation in an extraction solvent. For heterogeneous biomasses, such as BSG, simultaneous extraction of other matrix compounds is often unavoidable as these compounds may be soluble in the applied extraction solvent. As a result, the crude extract may contain a mixture of the target compound and various co-extracted compounds. To isolate the target compound from the crude extract, a purification step is required. The most common purification modes used for the isolation of target compounds are precipitation and filtration (Galanakis, 2015). The protein fraction of BSG may be obtained by two distinct fractionation procedures: 1) extraction of lignin and fibre or 2) direct extraction of protein (Fig. 6). Lignin extraction can be accomplished by deploying the solvent systems mentioned in the section "4.2.1.1.1.2 Delignification". Likewise, a variety of procedures for carbohydrate extraction of BSG have been described including sequential alkaline extraction, pressurised hot water extraction, microwave-assisted extraction and hydrothermal treatment (Mandalari et al., 2005). However, the majority of these methods are used to selectively extract AX and are therefore less suitable for extracting other carbohydrates present in the biomass.

4.2.1.1.2.1. Protein extraction

Over the last decades, protein extraction has been extensively investigated for miscellaneous plant-derived biomasses inclusive of BSG. Several methods have been deployed for extracting BSG proteins where alkaline extraction, sequential (Osborne) extraction and ultrafiltration have received the greatest attention (Wen et al., 2019). Table 4 summarises the various protein extraction methods discussed in this section and provides an overview of solvents, protein yields and the extend of ANFs co-extraction for each extraction method. Alkaline extraction, which represents the most common method for protein extraction of plant-based biomass, is based on the high solubility of plant proteins in alkaline solution. Following extraction, solubilised proteins are precipitated by isoelectric precipitation (IP) in which the pH value is lowered to the isoelectric point of the proteins. This process takes advantage of the fact that most plant proteins are highly insoluble at their isoelectric point (Lam et al., 2018). A potential pitfall in alkaline protein extraction of lignocellulosic biomasses is the occurrence of co-extraction of lignin because lignin is partially soluble at high alkalinities (Melro et al., 2020). Nevertheless, deploying IP for protein purification may help prevent lignin contamination as simultaneously extracted lignins and proteins are separated in this process. The efficacy of alkaline protein extraction is influenced by multiple factors including pH value of the aqueous solution, temperature, time, solid to liquid ratio (S:L) and the lipid content of the biomass being extracted (Vei de Vilg and Undeland, 2017). Alkaline protein extraction has several benefits in the form of excellent extraction efficiency, low economic costs and ease of operation (Wen et al., 2019). Notwithstanding, alkaline protein extraction has its limitations. Firstly, the formation of non-endogenous ANFs is promoted by the harsh conditions used in alkaline protein extraction. Some of these ANFs include oxidised sulfur-containing AAs which result from oxidation of thiol (-SH) groups. Under alkaline conditions, extracted L-amino acids or L-peptides may be converted into their D-isomers by racemisation. In the literature, D-amino acids have been defined as non-endogenous ANFs although this definition is problematic because most organisms can absorb and utilise D-amino in absence of L-amino

Table 4
Summary of protein extractions from brewer's spent grain (BSG) reported in the literature.

Extraction method	Conditions	Protein yield	ANFs content[a]	Reference
Extraction using protease treatment	Alcalase: 7.415 U/g BSG, $(NH_4)_2CO_3$ buffer (pH 8.0), S:L = 4.0% w/v, 24 h, 60 °C.	~52%	Not reported	Qin et al. (2018)
Protease treatment of carbohydrolase-pretreated BSG	Shearzyme and Ultraflo: 75 μL/g BSG, demineralised water (pH 5.0), S:L = 1:10, 4 h, 50 °C. Alcalase and Flavourzyme, demineralised water, 4 h, 50 °C.	~63%	Not reported	Connolly et al. (2019)
Water-alkaline-acid sequential extraction	*Water extraction* MiliQ water, S:L = 1:20, 25 °C, 1.5 h. *Alkaline extraction* 110 mM NaOH, S:L = 1:20 w/v, 50 °C, 1 h. Acid extraction 1 M H_2SO_4, S:L = 1:20, 25 °C, 1 h	*Water extraction* 43% *Alkaline extraction* 37% *Acid extraction* 14% Total 94%	Total lignin: ~65% Total cellulose: ~65% Total hemicellulose: ~65%	Qin et al. (2018)
Acidic extraction	40 mg H_2SO_4/g solution, S:L = 1:285, 25 °C, 1 h.	90%	Lignin: ~18% Cellulose + hemicellulose: ~68%	Qin et al. (2018)
Hydrothermal extraction	MiliQ water, S:L = 4.0%, 60 °C, 24 h.	66%	Lignin: ~16–17% Cellulose + hemicellulose: ~20%	Qin et al. (2018)
Extraction with acid detergent (AD) solution	2 g BSG/100 mL AD solution, refluxing, 1 h.	90%	Not reported	Crowe et al. (1985)
Alkaline extraction of cellulase-pretreated BSG	2 g BSG/50 mL citrate buffer (pH 4.9), 3 mg cellulase, 37 °C, 24 h. ~2 g BSG/100 mL NaOH solution (pH 10.5), rt, 2 h.	41%	Not reported	Crowe et al. (1985)
Modified Osborne extraction	25 g BSG/100 mL solvent, 10 min, 25 °C. Solvents: 1) distilled water, 2) NaCl solution, 70% ethanol solution, 3) acetic acid solution and 4) NaOH solution.	12%	Not reported	Crowe et al. (1985)
Ultrasound assisted extraction and protein isolation by ultrafiltration	Ultrasound: Na_2CO_3 buffer (pH 10), S:L = 1:10, for 1 h. Ultrafiltration: membranes: 5 and 30 kDa, 25 °C.	20%	Not reported	Tang et al. (2009)

[a] Anti-nutritional factors (ANFs) include lignin, cellulose and hemicellulose. The ANFs content is calculated as the percentage of extracted ANFs relative to the total ANF amount in untreated BSG and used to measure the purity of protein concentrates. The solid to liquid ratio is denoted as S:L.

acids (Gilani et al., 2012). Yet, the metabolic conversion of D-amino acids may sometimes be too slow to meet the nutritional requirements of the organism and in this case D-amino acids can be classified as non-endogenous ANFs (Friedman and Levin, 2012). Maillard reaction products (MRPs), which are formed in a reaction between a reducing sugar and an AA at high temperatures, comprise another group of non-endogenous ANFs (Gilani et al., 2012). Secondly, alkaline protein extraction has been reported to induce structural and functional changes to isolated proteins thereby affecting their nutritive value. Connolly et al. (2013) found that extraction temperature had a significant impact on AA composition as well as the protein yield. The loss of specific AAs at high temperatures may be explained by their consumption in the Maillard reaction. Furthermore, a positive correlation between extraction temperature and protein yield was reported by Connolly et al. (2013). These observations were consistent with the findings from a study by Ervin et al. (1989) which implied a sizeable loss of glutamate and proline when the extraction temperature was increased from 75 to 100 °C. Notably, this study also revealed an enrichment of several EAA including lysine, leucine and valine, among others, resulting from the temperature increase (Ervin et al., 1989). However, the use of high extraction temperatures is energy-intensive and may therefore not be considered a viable method for large-scale extraction of proteins with a specific AA composition. In addition to this, alkaline extraction is not considered an eco-friendly method as it requires large volumes of solvent. Sequential protein extraction, also referred to as Osborne extraction, is a well-established method for extracting cereal proteins. This extraction method relies on differences in solubility between the four different protein classes found in cereal grains. These protein classes are obtained in separate fractions using a series of different solvent systems (Byers et al., 1983). Celus et al. (2006) deployed a modified Osborne extraction method to identify the different protein types in BSG and to demonstrate that alcohol-soluble hordeins are the most abundant protein class. A study undertaken by Byers et al. (1983) examined the effect of several parameters on the AA composition of protein fractions obtained from milled wheat by Osborne extraction. Results from this study indicated that the AA profile was markedly influenced by the applied extraction conditions including temperature, extraction solvent and the presence of a reducing agent such as mercaptoethanol. These findings suggest that deploying appropriate extraction conditions may help facilitate isolation of proteins with a specific AA composition (Byers et al., 1983). This may serve as a useful strategy for the selective production of protein concentrates with AA profiles that reflect the AA requirements of fish. Similar to alkaline extraction, Osborne extraction is energy consuming and requires large volumes of solvent. Protein recovery by ultrafiltration, which is based on membrane separation processes, offers several advantages over the above-mentioned extraction methods including reduced amounts of solvent, low energy consumption and high purity of protein concentrates (Tang et al., 2009). Tang et al. (2009) examined the applicability of ultrafiltration for obtaining proteins and observed protein retentions greater than 90%. Furthermore, they concluded that ultrafiltration had high efficiency in removing salts originating from the buffer solutions used to solubilise proteins from the BSG matrix. This method is not without drawbacks; the most critical being fouling of membrane surfaces caused by a build-up of a concentrated polarisation layer (Marshall et al., 1993). Recent advances in membrane technology have led to better fouling control and thus increased the efficiency of membrane processes. A systematic review by Mohammad et al. (2012) evaluated the applicability of various abatement strategies for membrane fouling. These include the use of chemically modified membranes, manipulation of operating parameters, such as the transmembrane pressure and temperature, application of ultrasound and vibrating membrane systems for disrupting the fouling layers, backwashing and chemical cleaning of membranes (Mohammad et al., 2012). Chemical modification of membranes is mainly achieved by incorporation of hydrophilic groups as increased hydrophilicity has proven to enhance the antifouling properties of membranes (Asatekin et al., 2007). Employing a combination of these mitigation approaches could potentially increase the economic feasibility of using ultrafiltration as a method for recovering proteins from BSG. It is generally known from the literature that isolated BSG proteins suffer from poor solubility in aqueous media which may impede their use in animal feed applications (Celus et al., 2007). The low water solubility of BSG proteins may be due to the formation of insoluble protein aggregates during mashing as indicated in the work of Celus et al. (2006). Yet, recent research has established that enzymatic hydrolysis of isolated BSG proteins can improve their solubility over a broad range of pH values. This can be explained by the reduction in molecular weight (MW) and the protein unfolding which result in increased exposure of hydrophilic AA residues to the aqueous medium (Celus et al., 2007). Most previous research has reported a relatively poor extractability of BSG proteins. A possible reason for this might be that proteins are tightly encapsulated in the lignocellulosic matrix of BSG. In an attempt to overcome this problem, previous studies have examined the benefits of various pretreatments on protein yield. Most of the previous research has focused on identifying the effect of carbohydrate solubilisation on protein extraction efficiency. For instance, Qin et al. (2018) sought to assess how protein extraction of BSG was effected by different pretreatments including alkaline, acidic, enzymatic, hydrothermal and a combination of those. This study demonstrated a positive relation between carbohydrate solubilisation by the distinct pretreatments and protein extraction yield. Strikingly, the hydrothermal pretreatment appeared to be the most promising in terms of high selectivity for proteins during extraction as the resulting protein concentrates were less contaminated with lignin and carbohydrate residues (Qin et al., 2018). The findings of this study are in agreement with the results from the study of Crowe et al. (1985) who found that protein yield was increased significantly by enzymatic hydrolysis of cellulose prior to extraction of BSG. Moreover, a study by Connolly et al. (2019) concluded that the protein yield increased when BSG proteins were converted into shorter peptides by protease pretreatment. Interestingly, this study found that extraction of the generated peptides did not require a strong alkaline solution and that the resulting peptide-containing isolates exhibited enhanced water solubility in comparison to isolates comprised of intact proteins.

4.2.2. *Quality of BSG proteins*

High protein quality is crucial for the successful implementation of agro-industrial residues in aquafeed production (Nunes et al., 2014). As stated previously, the AA content along with the EAA/NEAA ratio are major determinants used to evaluate the applicability of novel protein sources in animal feed. Ideally, the AA profile and the CP content of the feed must meet the protein and AA requirements of the target species to avoid excretion of excess nutrients and malnutrition. Former studies have documented a deficiency in specific EAA in plant-based products with lysine and methionine generally being the first limiting AAs (Nunes et al., 2014). Within the field of feed formulation, two different methodologies are used to fortify deficient AAs of the diet: 1) ingredient-based formulation and 2) nutrient-based formulation. In ingredient-based formulation, deficient AAs are raised to desirable levels by increasing the inclusion of selected raw materials. In nutrient-based feed formulation, fortification of deficient AAs is achieved by supplementation of crystalline amino acids (CAAs). Despite being more environmentally sound, this method also entails some shortcomings concerning the utilisation efficacy of supplemented CAAs. The nutritional consequences of CAAs supplementation in previous studies appear to be inconsistent (Nunes et al., 2014). Some studies have determined that protein synthesis and growth are depressed when protein-bound AAs are substituted with their CAAs counterparts (Choo et al., 1991; Zarate et al., 1999). A reasonable explanation of this could be that CAAs are readily absorbed from the gastrointestinal tract into the bloodstream from where they are transported quickly to different organ systems of the fish. This theory was supported by Rolland et al. (2015) who found that postprandial plasma concentrations of CAAs in rainbow

trouts peaked transiently after a single meal with a CAA-supplemented diet. Thus, the cellular uptake of protein-bound AAs will be delayed relative to that of CAAs resulting in excess CAAs in the cells; a problem that can be overcome by continuous feeding. In the absence of AAs derived from proteins, CAAs tend to be used as an energy source, which may impede normal protein synthesis in the cells (Zarate et al., 1999). Other studies, however, have found similar or even better utilisation efficiencies of CAAs when compared to protein-bound AAs (Peres and Oliva-Teles, 2005; Rolland et al., 2015, 2016; Williams et al., 2001). Since these studies were conducted for different fish species, it can be argued that the observed inconsistencies in CAA utilisation efficiency originate from inter-species variability in absorption and assimilation rates of CAAs. The significance of different CAA conversion routes was illustrated in a study by Rolland et al. (2016) who found that increasing the dietary levels of crystalline methionine increased AA oxidation in rainbow trout. Surprisingly, the enhanced methionine catabolism did not appear to be correlated with higher ammonia excretion which is possibly due to the conversion of glutamate to glutamine by fixation of free ammonia (Rolland et al., 2016). Thus, for some aquatic species, CAA supplementation provides an adequate and inexpensive method of increasing the dietary content of specific AAs. For unprocessed BSG, lysine and histidine are reported to be the most abundant AAs whereas the sulfur-containing AAs, including methionine and cysteine, appear to be deficient (Waters et al., 2012). However, subjecting BSG to the various chemical and biological refinements outlined in the previous section may induce substantial alterations in the AA composition which are currently unknown. As emphasised previously, unrefined BSG is not a suitable protein source and therefore assessment of protein quality must be undertaken for the BSG-derived protein concentrates rather than for the untreated BSG. When alkaline extraction is employed for achieving protein isolates from BSG, some AAs are enriched whereas others are diminished relative to the original AA composition of raw BSG. This was confirmed by Connolly et al. (2013) who examined the dependence of AA profile on varying extraction temperatures. The most intriguing observation of this study was that concentrations of histidine and lysine decreased during extraction. This finding was supported by He et al. (2019) who also observed a marked decline in the content of histidine and lysine when BSG was subjected to alkaline extraction. Moreover, the content of individual AAs appeared to be more balanced after extraction except for glutamate which was still abundant in the concentrates. This partial equalisation of the various AAs during extraction may prove useful for feed formulation in that it can help abate the need to fortify specific AAs. In addition to the protein extraction approach, fungal pretreatment of BSG may also provide a useful tool for adjusting the AA content as desired. The work of Watson (1976) illustrated how the AA composition of *Saccharomyces cerevisiae* was influenced by the nitrogen source used in the culture medium. Remarkably, the results from this study revealed that the AAs supplied in the culture medium were accumulated proportionally in fungal tissue during the growth period. This trend was observed for all AAs with exception of glutamate that was found in high concentrations irrespective of the AA source (Watson, 1976). For BSG, a more balanced AA profile may be attained by growing *S. cerevisiae* on a BSG medium supplied with one or more CAAs. Furthermore, using fungi with high AA-accumulating capacity in the biological removal of ANFs constitutes a more efficacious and economic refinement strategy for BSG. This can be exemplified by an approach that applies an AA-accumulating fungus to degrade lignin from BSG thereby merging AA optimisation and delignification into a single step. To our current knowledge, yeast are the only fungi reported to accumulate AAs from the culture medium. Therefore, future studies should focus on identifying other fungi with this ability, especially species relevant for the bioconversion of lignocellulosic residues such as BSG.

4.2.3. Preservation of BSG

Owing to the high level of nutrients and moisture, BSG is susceptible to microbial colonisation. Previous studies have established that BSG is microbiologically stable at the point of production; though, under inappropriate storage conditions, it undergoes spoilage (Robertson et al., 2010a, 2010b, 2010a). Microbial proliferation will eventually lead to the deterioration of BSG, which may hinder its use in industrial applications such as aquaculture feed. Therefore, storage and preservation methods are crucial in preventing chemical alteration of BSG (Bartolomeé et al., 2002). The most commonly applied preservation methods for BSG are freezing and drying of which the latter can be subdivided into different types including freeze-drying, oven-drying and superheat steam (SS) drying. Previous studies have focused on describing the chemical alterations that occur when BSG is preserved by freezing, freeze-drying and oven-drying. Bartolomeé et al. (2002) showed that the AX content was considerably reduced when freezing was employed while it was unaffected by the other preservation methods. This is probably due to the enhanced activity of freeze-resistant microbes during the thawing of BSG resulting in depolymerisation of the AX component. In a similar study, Santos et al. (2003) observed a slight decrease in the protein and lipid content when oven-and freeze-drying was applied. This can be explained by the fact that proteins and lipids are partly degraded to form volatile compounds under harsh conditions. These studies suggest that all three methods are less suitable for the preservation of BSG as they give rise to compositional changes of the material. Furthermore, due to the high energy consumption and demand for bulk storage, drying and freezing are not economically feasible on an industrial scale (Johnson et al., 2010). Superheat steam drying provides a means by which these challenges can be circumvented since protein and NSP content remains unaltered (Tang et al., 2005). However, one major disadvantage of this technique is that it requires highly specialised equipment compared to the conventional drying methods.

4.2.3.1. Aflatoxins. Besides compositional deterioration, the production of aflatoxins may also complicate the use of BSG in aquafeeds from a food safety perspective. Aflatoxins comprise a group of 20 structurally related mycotoxins of which only 4 have been described in detail concerning their health effects and toxicity. Based on differences in physical and chemical properties, these aflatoxins are designated AFB_1, AFB_2, AFG_1 and AFG_2 with AFB_1 being the most toxigenic and well-studied (Santacroce et al., 2008). These four aflatoxins have a general structure consisting of a bifuran moiety linked to a cyclopentenone-containing coumarin core. As a consequence of their structural features, aflatoxins are lipophilic compounds that are readily absorbed and metabolised to form highly reactive and toxic intermediates such as aflatoxin-exo-8,9-epoxide. Owing to its strong affinity for various biologically important macromolecules, this intermediate facilitates adduct formation with DNA, RNA and proteins, which may ultimately lead to the onset of organ damage or cancer (Santacroce et al., 2008). Previous studies have confirmed the occurrence of inherent aflatoxin-producing fungi from BSG including several species of *Aspergillus*, *Cladosporium* and *Penicillium* (Gerbaldo et al., 2011). Furthermore, Sodhi et al. (1985) found a positive correlation between fungal proliferation and AFB_1 production under inadequate storage conditions for longer periods. Application of contaminated fish feed in aquaculture may lead to the accumulation of AFB_1 or its more reactive metabolites in fish tissue, particularly in fish species with low aflatoxin tolerance such as rainbow trout (Mwihia et al., 2018). Furthermore, the consumption of fish products containing traces of aflatoxin residues is believed to have severe consequences for human health including aflatoxicosis, nutrient malabsorption, immunosuppression and cancer development (Bbosa et al., 2013). Although a large body of literature has established that the health effects on fish caused by aflatoxin exposure are similar to those observed for humans, there appears to be great interspecific variation in aflatoxin susceptibility. For instance, feeding trials with contaminated feeds have revealed that

rainbow trout is one of the most sensitive species, while Nile tilapia exhibits greater tolerance towards aflatoxin exposure (Mwihia et al., 2018). On the other hand, silver catfish (*Rhamdia quelen*) are highly resistant to high aflatoxins concentrations (Anater et al., 2020). Existing literature suggests that BSG contamination can be minimised largely by proper storage conditions or the removal of aflatoxins from the raw material. Recent studies have focused on AFB_1 elimination through fermentation carried out by lactic acid bacteria (LAB). It has been suggested that LAB can detoxify AFB_1 by reversible binding, thereby abating ingestion of the toxin (Hernandez-Mendoza et al., 2009). The work of Asurmendi et al. (2014) corroborated that improperly stored BSG generally contained low levels of AFB_1 which can be attributed to the activity of native LAB species present in BSG. In addition, Mokoena et al. (2006) have shown that the addition of supplementary LAB cultures to maize-based meals enhanced AFB_1 removal considerably under prolonged storage. These findings may provide a pathway to eliminate AFB_1 and facilitate the implementation of BSG in feed for a broader range of fish species. From a sustainability perspective, preservation of BSG by LAB treatment is preferred over the conventional drying techniques (Petit et al., 2020). However, exposing a feed ingredient to microbial treatments, necessitates thorough evaluation of any pathogenic, or other side-effects that microbial residues may have on fish health. Since there is a general acceptance of the beneficial, probiotic effects of LAB for fish, these bacteria are expected to play a crucial role in protecting fish against harmful pathogens when added to the feed ingredients. Overall, the collection of literature on LAB treatment discussed here, implies that treating BSG with LAB cultures has several advantages including improved preservation, efficient aflatoxin removal and enhanced probiotic effect of the resulting feed.

5. Conclusion

The increasing demand for aquaculture commodities has led to a compelling need for alternative protein sources. Brewer's spent grain (BSG) can be considered a potential protein source in fish feeds because it has a high content of crude protein and essential amino acids (EAA) as compared to other agro-industrial by-products. However, the direct implementation of unrefined BSG in aquafeeds is hindered by high levels of lignin and non-starch polysaccharides (NSP) which constitute anti-nutritional factors (ANFs) for fish and by poor storability due to susceptibility to microbial growth. In this review, we have discussed how these limiting factors can be addressed to facilitate the implementation of BSG in aquafeeds. Different strategies were considered for eliminating ANFs from BSG with emphasis on two major approaches. The first approach aims at removing the ANFs, which may be achieved by chemical extraction of lignin and NSP or by extracting proteins directly from BSG. The second approach, however, relies on the chemical conversion of BSG whereby ANFs are degraded to smaller molecules that can either be digested by the fish or used as starting materials in the synthesis of other substances such as amino acids (AA). Due to a high water content, BSG is susceptible to microbial colonisation. This may cause biomass deterioration and production of aflatoxins which are known to have adverse health effects in several fish species as well as in humans consuming aflatoxin-contaminated fish products. As a result, it is critical to deploy proper storage conditions and preservation of BSG. The most widespread preservation methods include drying and freezing, which are associated with high energy consumption and demand for large storage areas, respectively. Some studies have demonstrated the positive effects of lactic acid bacteria (LAB) including their probiotic properties and efficient aflatoxin removal. The protein quality of refined BSG products, which is determined by their respective AA profiles, must also be taken into consideration. Some studies have shown that the AA profile of BSG is altered considerably by protein extraction while other studies have indicated that certain yeast species are able to change the composition of protein-bound AAs by accumulating crystalline AAs present in the culture medium. These approaches may be applicable for adjusting the AA profile of refined BSG products in such a manner that it satisfies the AA requirement of the target fish species. Presently, none of the discussed methods have been applied to convert BSG into high-quality, protein-rich raw material for aquafeeds. In our opinion, biological refinement of BSG appears to be the most eco-friendly in terms of resource use and energy consumption. From an economic point of view, this approach may be less favourable because most biological conversions are time-consuming and ineffective for short-term production. Therefore, future research might focus on optimising the efficiency of biological systems to facilitate their implementation in the valorisation of BSG as well as other relevant agro-industrial residues. Specifically, enzymatic conversion of lignin, cellulose and hemicellulose should be the primary focus of future studies rather than fungal and bacterial degradation which is often time-consuming and involves a risk of accumulating other ANFs, such as chitin, during the cultivation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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