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Review

RuBisCO as a protein source for potential food applications: A review

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ABSTRACT

RuBisCO is a complete protein, widely abundant and recognized as ideal for human consumption. Further, its biochemical composition, organoleptic and physical features mean RuBisCO has potential as a nutritionally beneficial food additive. Nonetheless, despite growing plant-based market trends, there is a lack of information about the applications of this protein. Here, we explored the biochemical features of RuBisCO as a potential food additive and compared it with other plant protein sources currently available. We describe potential advantages, including nutritional content, digestibility, non-allergenicity and, potential bioactivities. Despite the lack of industrial procedures for RuBisCO purification, a growing number of novel methods are emerging, justifying discussion of their feasibilities. Overall, this information can help both researchers and industry to review the use RuBisCO as a sustainable source of protein for plant-based food products or formulation of novel functional foods.

1. Introduction

The world is current witnessing an unprecedented demand for plant-based protein sources. The global plant protein market was valued at $29.4 billion in 2020, and is projected to reach $162 billion by 2030, according to Bloomberg Intelligence (New York magazine, August 2020). Moreover, plant-based diets have gained popularity in recent decades due to their potential health advantages and environmental and ethical considerations (Aimutis, 2022). This growing interest has contributed to the surge in demand for plant-based proteins. Consumers worldwide are encouraged to consume more protein, with a preference for plant-based sources and a potential shift away from animal protein. This is motivated by several factors, including the belief that plant-based diets may improve health, concerns about the negative effects of animal protein on human health, the environmental impact and ethical concerns surrounding animal protein production, and the popularity of high protein diets like the ketogenic and Atkins diets (Hertzler et al., 2020). Accordingly, the food industry swiftly adjusted to the growing demand for plant protein by introducing a whole new line of food products. However, these commercially available plant-based proteins have several drawbacks. One of the major challenges is their inconsistent functional characteristics, as the nutritional, molecular, and physicochemical properties of plant proteins are significantly distinct from those of animal proteins. Compared to animal-derived food products, plant-derived products typically have a lower percentage (w/w) of protein. From a nutritional point of view, plant-based products may contain naturally occurring substances that can impact nutrient absorption and digestion (Popova & Mihaylova, 2019). In addition, plant-based proteins are frequently deficient in essential amino acids, such as lysine in cereal seeds, cysteine and methionine in legume seeds, and tryptophan in general (McGuire & Beerman, 2013). Another major problem related to both animal- and plant-based proteins may pose a significant problem in terms of potential allergenicity. It is estimated that around 220 to 250 million individuals suffer from food allergies, with developed countries reporting an incidence of 5 to 8% in children and 1 to 2% in adults (Dorsan & Chaudaty, 2013; Jones, 2014). Food allergies have a significant impact on people's quality of life and can also affect the national and global economy due to the costs associated with diagnosis and treatment, as well as the production of allergen-free foods (Mills et al., 2007; Dorsan & Chaudaty, 2013). One of the most consumed plant-based protein derives from soy seeds, which is among the eight major food allergens according to the Food and Agriculture Organization of the United Nations (FAO). As a result, it is crucial to identify suitable botanical sources and extraction methods to produce consistent and functional plant proteins.

The food industry currently lacks a plant-based protein that can offer

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all the benefits of animal protein without the previously mentioned drawbacks. The enzyme ribulose bisphosphate carboxylase/oxygenase (EC.4.1.1.39; RuBisCO), a protein that plays a vital role in photosynthesis, could be an excellent option as a plant protein source, as it overcomes most of the serious constraints referred before. RuBisCO's potential for human consumption relates to its high nutritional value, as it is highly digestible and contains a balanced proportion of essential amino acids that align with the recommendations of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), as noted by Pouvreau et al. (2013). In addition to its natural abundance, RuBisCO offers various nutritional benefits compared to other plant-based proteins. It is regarded as a non-allergenic protein (Jeurink & Savelkoul, 2005; Cirkovic Veličković & Gavrovic-Jankulović, 2014) and possesses numerous organoleptic, functional, and physical properties that are highly promising for the food industry. However, there is currently a lack of efficient methods for large-scale RuBisCO purification and subsequent utilization in the food sector.

In this review, we examine the potential of RuBisCO as a safe and efficient plant protein source for human consumption by highlighting its nutritional and biochemical value, functional properties compared to other commercially available plant-based proteins, and bioactivities for human health. Additionally, we discuss its major industrial applications and evaluate current methods for large-scale purification, weighing the advantages and disadvantages of each. To the best of our knowledge, this is the first review about RuBisCO's features as a food additive. Given the current trend towards innovation in plant-based proteins and the growing number of methods reported for isolating RuBisCO for food purposes, this review can make a significant contribution to both the food industry and researchers.

2. Biochemical features of RuBisCO

RuBisCO is responsible for one crucial step in photosynthesis that makes most life as we know possible. Its primary function is to catalyze various chemical reactions, with the most significant being the first reaction of the Calvin cycle. This sequence of reactions begins with the absorption of carbon dioxide and ends with the synthesis of carbohydrates, the carbon skeletons upon which virtually all life on Earth depends. As a result, RuBisCO is responsible for fixating more than 90% of the inorganic carbon which is converted into biomass. However, RuBisCO can also react with oxygen instead of carbon dioxide, leading to a chain of reactions called photorespiration. This process consumes energy and oxygen while releasing carbon dioxide and ammonia, making RuBisCO an inefficient catalyst: in the presence of oxygen, RuBisCO inevitably spends a minimum of 25% of the time reacting with oxygen. In addition, RuBisCO is a sluggish catalyst, catalyzing its reactions at a rate that is ca. 1 million times lower than other enzymes such as catalase (Erb & Zarzycki, 2018). To overcome these inefficiencies and to grow at appropriate rates, plants must accumulate large amounts of RuBisCO in their leaves, making it the most abundant protein in nature (Spreitzer & Salvucci, 2002). In a plant leaf, there exist more than 10,000 diverse proteins, out of which RuBisCO constitutes a significant portion, ranging from 30 to 65% of the total protein content. It is also one of the most ubiquitous proteins in nature, being found in the photosynthetic tissues of all "green" plants, algae, cyanobacteria, a variety of photosynthetic bacteria and some non-photosynthetic bacteria as well (i.e., chemolitotrophic bacteria; Raven, 2013). It has been estimated that for every person on earth there is 5 kg of RubisCO, most of which synthesized yearly (Erb & Zarzycki, 2018). Altogether, this makes RuBisCO the most abundant protein in the global carbon cycle that literally feeds life on earth.

RuBisCO has a molecular weight of ca. 550 kDa, consisting of eight large subunits (LSU – "Large SubUnit") of approximately 51 to 58 kDa each and eight small subunits (SSU – "Small SubUnit") of molecular weight between 12 and 18 kDa (Jensen & Bahr, 1977), as described in Fig. 1.

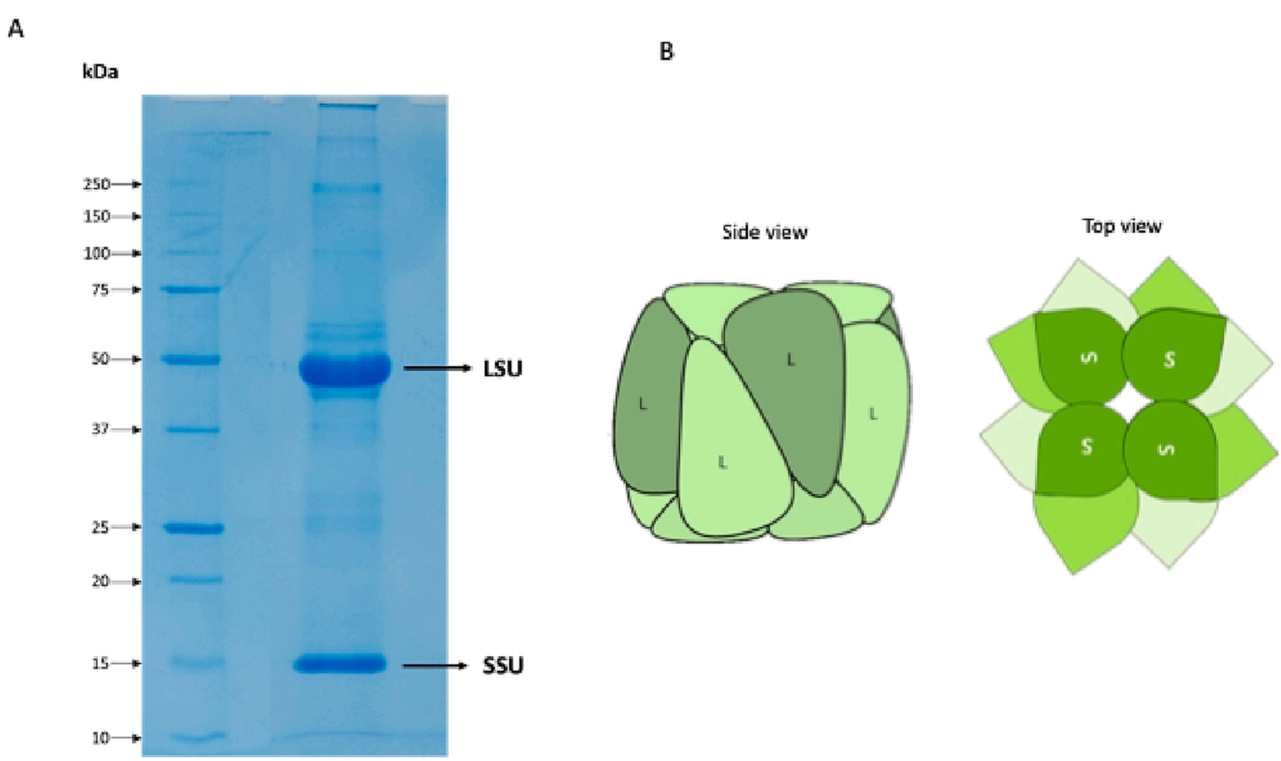

Fig. 1. RubisCO from photosynthetic organisms consists of a set of eight large subunits (L or LSU) of 51 to 58 kDa each, and eight small subunits (S or SSU) of 12 to 18 kDa each, forming an L_8S_8 quaternary structure. A: SDS-PAGE separation of RuBisCo subunits from spinach leaves. The estimated molecular masses of standards are indicated on the left and are expressed in kDa. B: Schematic structure of RubisCO showing the spatial arrangement of the 16 subunits; left: side view; right: top view.

Although the amino acid sequence of the large subunit remains relatively consistent among higher plant species, exhibiting over 80% homology, the small subunit's amino acid sequence displays significant differences, with around 70% homology (Udenigwe et al., 2017). Consequently, various crops may exhibit slight differences in the amino acid profiles of RuBisCO and, in turn, experience differences in certain biochemical characteristics, including isoelectric point, molecular weight, bioactivities, and others. The large subunit is the product of a single gene, which is encoded in the chloroplast genome (and therefore inherited from the female progenitor), whereas the small subunit is the product of a gene family and is encoded in the nucleus (meaning that it is inherited from both progenitors). These characteristics significantly impact both the amino acid composition and overall yield of extracted RuBisCO from plant leaves. Therefore, when aiming to extract this protein on a large scale for food purposes, they must be taken into consideration.

RuBisCO is present in the soluble fraction of chloroplasts and constitutes over 50% of the foliar protein in C3 plants (Kawashima & Wildman, 1970). In C4 plants, however, RuBisCO accounts for a lower percentage, ranging from 8% to 23% (Ku et al., 1979). The effective concentration of RuBisCO in foliage depends on various factors, including the availability of nitrogen, the plant species, its age, and the intensity of light (Barbeau & Kinsella, 1988).

3. Advantages of RuBisCO as a protein source for human consumption

3.1. Animal proteins vs RuBisCO

The most prominent animal protein sources that are currently in use by the food industry are creatine, milk (caseins), whey and egg (Table 1). Creatine, a non-protein amino acid, has several advantages in terms of muscle metabolism and sports performance (Balsom, Söderlund, and Ekblom, 1994). According to Greenhaff et al. (1993), supplementing with creatine increases the energy reserve in the form of adenosine triphosphate (ATP). However, it is worth noting that creatine is also associated with weight gain and several side effects, including muscle cramps and gastrointestinal disorders (Stone et al., 1999; Schilling et al., 2000). Caseins are milk proteins that are often used as a positive control model in food allergy studies. Additionally, the two most potent milk allergens, α-lactalbumin and β-lactoglobulin, are present in cheese whey, a by-product of cheese production that is rich in proteins, minerals, and lactose. Cheese whey can be used directly or after fermentation, in the preparation of many products, incorporated as ingredient or additive, and as a sports supplement. The presence of lactose is associated with intolerances, whereas α-lactalbumin and β-lactoglobulin, also present in cheese whey, are associated with allergies. Whey protein concentrates (WPC) find application in a wide range of food products such as confectionery items, nutrition bars, cereals, processed cheeses, baked goods, sports beverages, and muscle gain formulations. Egg protein is considered as a model protein because it is quite complete in essential amino acids and displays several useful functional properties. Despite being a good source of proteins, it has a high allergenic potential, having an incidence in children from 1.6% to 2.6% (Croegaert et al., 2013). The egg contains 24 major proteins, of which ovomucoid, ovalbumin, conalbumin and lysozyme are the ones with the highest allergenic potential (Tosca et al., 2014).

Apart from its plant origin, RuBisCO has two significant advantages, as presented in Table 1: a) it is a complete protein with a balanced proportion of all the essential amino acids, and b) it is a non-allergenic protein that is commonly used as a negative control in allergenic assays (Astwood et al., 1996; Thomas et al., 2004; Jeurink & Savelkoul, 2005; Hoff et al., 2007; Jongh et al., 2013; Cirkovic Veličković & Gavrovic-Jankulović, 2014). These nutritional features make RuBisCO a viable substitute for animal proteins commercially available in the current market.

3.2. Plant-based proteins vs RuBisCO

People's awareness about healthy foods continues to increase, with the recognition that many serious human diseases (e.g., cancer and neurodegenerative disorders) may be delayed, minimized or prevented by the selection of appropriate diets. Furthermore, the current demand to reduce the environmental impact of agrifood systems and the improvement of food production capacity are problems that need to be solved (Godfray et al., 2010). Plant-based proteins could be a promising alternative. However, the plant-based proteins currently available in the market are insufficient to solve the aforementioned problems. Most plant-based proteins are allergenic, have unbalanced compositions of essential amino acids, and/or possess unpleasant tastes. The large-scale extraction and purification of RuBisCO, which is a non-allergenic, complete and readily digestible protein found in very high abundance in all photosynthetic tissues, could potentially address several global issues, such as protein malnutrition, which is a widely recognized public health concern (Müller & Krawinkel, 2005; Stefano et al., 2018).

Given that RuBisCO appears to address all the constraints mentioned earlier and possesses the ideal characteristics required for a protein intended for both animal and human consumption, a critical question

Table 1
Advantages and disadvantages of animal proteins commercially available vs RuBisCO.

Animal	Commercially available	Advantages	Disadvantages
Proteins	Creatine	Improves muscle metabolism and sports performance	A non-protein amino acid that may cause: - Weight gain - Muscle cramps - Gastrointestinal disorders
	Caseins	Balanced amino acid profiles containing all the essential amino acids in their composition	Allergenic proteins. They are present in milk and are associated with lactose intolerances
	Cheese whey proteins	Balanced amino acid profiles containing all the essential amino acids in their composition	Allergenic proteins. They contain lactose in their composition, which is associated with lactose intolerances
	Whey protein concentrates (WPC)	Balanced amino acid profiles containing all the essential amino acids in their composition	Allergenic proteins
	Albumin	Balanced amino acid profile containing all the essential amino acids in its composition	Allergenic protein
RuBisCO		Balanced amino acid profile containing all the essential amino acids in its composition. It is a non-allergenic protein. It is a plant-derived protein, present in all photosynthetic tissues, where it occurs in very high abundance. Good digestibility	None

arises: Why has it not yet become commercially available in the market?

3.2.1. RuBisCO nutritional properties

RuBisCO appears to have the most potential among all proteins for commercial applications and fits the "ideal" protein profile for both human and animal nutrition. The composition of RuBisCO is well-balanced in essential and non-essential amino acids, being the closest (when compared to soy protein and whey protein) to the egg protein, but it is not over-rich in essential amino acids (Table 2) – It is considered as the model protein in nutritional terms. Lysine, the sulphur-containing amino acids, and tryptophan, regarded as critical in low-meat diets, are present at high concentrations in RuBisCO (Pouvreau et al., 2013). In comparison to other plant proteins, spinach RuBisCO exhibits higher quality according to the protein quality model established by FAO/WHO in 2007, with a higher proportion of essential amino acids. The quality of a protein is measured using the chemical index, which is the ratio of the amount of an essential amino acid in a food protein to the amount of the same amino acid in a reference protein. The reference protein used by FAO/WHO (2007) is the pattern of essential amino acids in egg protein. A higher chemical index indicates a better protein quality. The chemical index is defined by:

Chemical index = (mg of limiting amino acid per g of the protein under analysis)/(mg of amino acid per g of the reference protein).

It is common for plant-based proteins available in the market, mostly derived from pulses and cereals, to be deficient in some essential amino acids. When compared to animal-based proteins, plant-based proteins present a lower percentage of essential amino acid content (Gorissen et al., 2018). All cereal seeds, like rice and wheat contain much less protein than animal protein sources or legume seeds and are often incomplete, mainly in lysine (an essential amino acid; McGuire & Beerman, 2013), a problem that demands genetically modified plants and consequently discussions about transgenic cultures (Alink et al., 2008; Nguyen et al., 2012). The supplementation of poultry feedstock with lysine is a well-known example of addressing amino acid deficiencies in animal feed. On the other hand, legume seeds such as soy, pea, and lupin are deficient in the sulfur-containing amino acids, namely cysteine and methionine, while both legume and cereal seeds generally lack sufficient tryptophan (McGuire & Beerman, 2013).

Table 2
Comparison between the content in essential amino acids of RuBisCO and those of other proteins with the chemical index and nutritional recommendation of FAO/WHO ().

Essential amino acids	'Ideal' protein according to FAO/ WHO ($mg.g^{-1}$ protein)	Egg protein ($mg.g^{-1}$ protein)	Cheese whey protein ($mg.g^{-1}$ protein)	Soy protein ($mg.g^{-1}$ protein)	Spinach RuBisCO ($mg.g^{-1}$ protein)
Histidine	15	22	13	26	26
Isoleucine	30	54	50	49	43
Leucine	59	86	94	82	82
Lysine	45	70	68	63	71
Methionine + Cysteine	22	57	34	26	40
Phenylalanine + Tyrosine	38	93	128	90	96
Threonine	23	47	68	67	52
Tryptophan	6	17	27	14	17
Valine	39	66	67	50	60
Chemical index [a]		100	87	100	100
Chemical index [b]		100	60	46	70

[a] Calculated in comparison to the 'ideal' protein as recommended by FAO/WHO (2007).
[b] Calculated in comparison to the egg protein.
adapted from Pouvreau et al., 2013

3.3. Allergenicity and digestibility

Another major advantage of RuBisCO as a food protein source of great potential is that it is considered a non-allergenic protein (Jeurink & Savelkoul, 2005; Cirkovic Veličković and Gavrovic-Jankulović, 2014). Surprisingly, RuBisCO has even been utilized as a negative control in several allergy trials and tests (Astwood et al., 1996; Thomas et al., 2004; Hoff et al., 2007; Jongh et al., 2013). As far as we are aware, there is a single case study in which a 23-year-old woman is reported to have developed an allergic reaction after ingestion of spinach leaves. Based on proteomic analysis, the authors stated that RuBisCO was responsible for the sensation of asphyxia and angioedema, both in the lips and in the tongue (Foti et al., 2012).

It is important to note that all proteins commercially available for human and animal consumption are integral members of the lists of major allergens, both in Europe and the US. In soy protein alone, 15 allergenic proteins were found, which implies a very significant nutritional restriction on the use of this well-known protein source by people suffering from allergies (Ogawa et al., 1991). In addition, most of the soy produced worldwide is transgenic, an issue that has been the subject of many scientific and ethical discussions about its environmental, economic and social effects on present and future societies (Damato, 2009). Also, soy protein contains a high concentration of isoflavones that exhibit estrogenic bioactivity. So, soy consumption in large amounts is harmful, especially to men and children (Setchell et al., 2002). Erectile dysfunction and male infertility are some of the side effects that may arise as a result of excessive soy consumption (Mollakhalili-Meybodi et al., 2021). The Kunitz and Bowman-Birk inhibitors present in soybeans can interfere with the digestion of protein and the absorption of nutrients. However, heat treatment, such as cooking or roasting, can denature these inhibitors and improve the digestibility of soy protein.

As for the other pulses, lupin for instance is included in the allergen lists in the EU and Australia (Jappe & Vieths, 2010) and according to Food and Drug Administration (FDA), people who suffer from peanut allergies are more susceptible to suffer from lupin allergies as well, and the reactions could be severe and life-threatening. Rice and peas are generally considered to be hypoallergenic foods, which means that they are less likely to cause allergic reactions than other foods. Ca. 80% of patients suffering from food allergies have immunoglobulins E (IgE) to rice protein (Goliáš et al., 2013). Some rice proteins, including α-globulin (26 kDa), glyoxalase (33 kDa), and a glycoprotein (56 kDa), have been identified as allergenic, with the α-amylase/trypsin inhibitors family (with molecular weights between 14 and 16 kDa) having the highest incidence of allergenicity (Krishnan & Chen, 2013). Even though it is not common, cases of people who have allergy to peanut reacting to pea protein have been described in the literature (Wensing et al., 2003; Lavine & Ben-Shoshan, 2019).

Wheat grains, which contain over 100 main proteins, have been identified as allergenic, largely due to the presence of gluten (Tatham & Shewry, 2008). Gluten intolerance is a well-known issue associated with wheat consumption (Pasha et al., 2016). Celiac disease is another important consideration, which consists of chronic inflammation that affects the gastrointestinal tract, resulting in an atrophy of intestinal villi and consequent malabsorption of nutrients (Gasbarrini & Mangiola, 2014). On this topic, several studies show that the novel gluten-free foods, which have been arising in the market, often contain a low percentage of protein and a higher percentage of carbohydrates and lipids compared to gluten-equivalent products (Berti et al., 2004; Caponio et al., 2008; Segura & Rosell, 2011).

In terms of digestibility, RuBisCO is easily and readily digested by pepsin (digestive protease present in the gastric juice responsible for the hydrolysis of dietary proteins into peptides; Thomas et al., 2004; Jong & Nieuwland, 2011). RuBisCO has been used as a positive control in digestibility assays due to its high susceptibility to proteolytic attack (pronase, trypsin, proteinase K, α-chymotrypsin or subtilisin; see for example Fig. 3 in reference Monteiro et al., 2015). This is not the case for many plant proteins, especially those that are allergenic and may not be fully hydrolyzed by digestive enzymes before reaching the intestines, reducing their digestibility. Soy protein, for example, is deficient in methionine, which can further lower its digestibility (Friedman & Brandon, 2001). Protein digestibility can also decrease up to 50% due to the high concentration of trypsin inhibitors (which are proteins themselves) present in this legume seed (Friedman & Brandon, 2001; Gilani et al., 2012). Wheat protein extraction involves an alkaline treatment, decreasing its digestibility (up to 40%) and causing a drastic reduction (up to 100%) in protein quality. This extraction has a low yield in protein production, meaning that it takes a large amount of this cereal to obtain a small amount of protein (Gilani et al., 2012). Pea, lupin, and rice proteins are easily digested (House et al. 2010; Zuber et al. 2018). However, a study showed that animal protein digestion is quicker than rice protein isolate (Jäger et al., 2013). The process of cooking was also demonstrated to decrease significantly the gastric and gastrointestinal protein digestibility of rice (Liu et al., 2019).

Among the hundreds of millions of proteins known to exist, only a few % (which still comprise a considerable number) have such a well-balanced amino acid composition as RuBisCO. If we now consider other protein properties, such as complete absence of toxicity, including allergenicity (something that cannot be said for the egg protein, soybean protein and whey protein), high digestibility and good functional properties, very few proteins (if any) besides RuBisCO will remain. In addition, for commercial viability, the protein must occur naturally in high concentrations and be suitable for extraction and purification at an industrial scale. Therefore, it is apparent that RuBisCO stands out among all the proteins in nature in terms of its potential to be used as a protein source for human and animal consumption.

The discussion and information provided in Sections 4 and 5 reinforce the question raised in the last paragraph of Section 3.2: despite its exceptional properties, why hasn't RuBisCO become commercially available yet?

4. Functional and organoleptic properties of RuBisCO

Proteins are incorporated in food products for different reasons, with the most significant being their nutritional value and functional properties. These properties are closely related to solubility, water and fat retention, emulsion, gelatinization, foaming capacity, flavor, and durability. As mentioned earlier, the plant-based food industry faces a significant challenge in creating products with desirable appearance, texture, flavor, mouthfeel, nutrition, and functionality. Therefore, the ability of plant proteins to thicken, gel, emulsify, foam, and retain fluids are some of the most important features for their use in plant-based foods (McClements & Grossmann, 2021). In this respect, RuBisCO presents several physical and chemical properties, as described below and in Table 3.

4.1. Solubility

Protein solubility is one the most important factors because it dictates the other protein functionalities. RuBisCO's high solubility is defined by multiple factors, such as pH, ionic strength, protein concentration, temperature, the presence of divalent cations and the species from which the protein was purified (Sheen, 1991). When treated with magnesium, RuBisCO extracted from tobacco leaves tends to precipitate at pH values lower than 7.5, suggesting the influence of divalent cations and pH value (Prevot-D'Alvise *et al.*, 2004). However, when the same conditions were applied to RuBisCO purified from spinach leaves, it did not exhibit the same behavior, indicating the influence of the species of origin. RuBisCO from alfalfa leaves was soluble when the pH was closer to its isoelectric point (pH 5.0 to 6.5), but almost insoluble under acidic solutions (only 20% w/v soluble). When the solution was alkaline (pH 7 to 12) the solubility increased from 10% to 90% (Prévot-D'Alvise et al., 2004). According to Van del Velde (2011), RuBisCO purified from

Table 3
Advantages and disadvantages of plant-based proteins commercially available vs RuBisCO.

Plant-based proteins	Commercially available	Advantages	Disadvantages
	Rice	Considered an hypoallergenic food Easily digested High water/fat retention Good gelling capacity	Low percentage of protein Incomplete protein, mainly in lysine Low solubility Poor emulsifying capacity Low foaming capacity Off-flavors
	Wheat	Great foaming capacity No flavor	Low percentage of protein Incomplete protein, mainly in lysine and tryptophan Allergenic protein Low protein digestibility Low solubility Low water/fat retention Poor emulsifying capacity
	Soy	High percentage of protein High water/fat retention Good gelling capacity	Incomplete protein, mainly in cysteine, methionine and tryptophan Allergenic protein Low protein digestibility Low solubility Poor emulsifying capacity Low foaming capacity Off-flavors
	Pea	Considered an hypoallergenic food Easily digested	Incomplete protein, mainly in cysteine, methionine and tryptophan Low solubility Low water/fat retention Poor emulsifying capacity Low gelling capacity Low foaming capacity Off-flavors
	Lupin	High percentage of protein Easily digested High water/fat retention Good foaming capacity	Incomplete protein, mainly in cysteine, methionine and tryptophan Allergenic protein Low solubility Poor emulsifying capacity Low gelling capacity Off-flavors
RuBisCO		Well-balanced in essential and non-essential amino acids Non-allergenic protein Easily and readily digested by pepsin High solubility High water/fat retention Great emulsifying capacity Good gelling capacity Great foaming capacity No flavor	None

spinach leaves is highly soluble at pH values commonly found in food, ranging from pH 6 to 8. Concerning other proteins commonly used for human consumption, the protein extracted from different pea seed genotypes revealed to be almost insoluble at pH 5.0. However, its solubility increased below and above this pH value and the proteins exhibited excellent solubility at pH 7 and 8 (Barac et al., 2010). Based on these findings, it can be inferred that pea proteins are suitable for use in food products with a neutral or basic pH. Despite being a commonly used plant-based protein, soy protein is known to have a significant drawback of low solubility (Malhotra & Coupland, 2004). Only 10% of soy proteins are soluble at pH values close to their isoelectric points (4.6; Freitas et al., 2017). Lupin protein, being a legume, shares similarities with soy protein. Meanwhile, rice protein, despite being considered a hypoallergenic alternative, has low solubility at acidic environments, with a solubility of less than 2% (w/v), similar to soy protein, which can affect its other functional properties as well (Wang et al., 2018).

4.2. Water and fat retention capacities

The water binding capacity of a protein can greatly influence its potential for use in food applications, as it ultimately affects the texture of the final product. A protein with low water binding capacity can result in a dry product or may lose water during storage. On the other hand, fat retention is linked to flavor in food products. RuBisCO's water and fat retention capacities have been tested in a few works. Results showed that RuBisCO had a capacity of water retention of 428% by volume, while soy protein, which was already partially denatured, had a capacity of 612% by volume (Douillard & Mathan, 1994). Regarding the fat retention capacity, RuBisCO from alfalfa showed a 208% increase in volume whereas soy seed protein showed an increase of 159% (Douillard & Mathan, 1994). Pea protein water/fat retention is low. Recently, in an attempt to increment pea protein water/fat retention, an assay was performed where pea protein was modified via sequential deaminidation and conjugation to enhance these functionalities (Shen et al., 2022). In the case of rice proteins, the water retention capacity ranged from 387% (w/w) to 560% (w/w), and the fat retention from 374% (w/w) to 918% (w/w), values that are comparable to the well-known casein (Chandi & Sogi, 2007). In what concerns total seed preparations, Raikos and colleagues (2014) demonstrated that lupin flour had a great water holding capacity due to its high protein content, and the results were identical to other studies reported (Khalid & Elharadallou, 2013). On the other hand, wheat flour did not show good water holding retention capacity due to its low protein content (Raikos et al., 2014).

4.3. Emulsifying capacity

Emulsion is the functional property which dictates the protein ability to emulsify oil (Kaushal et al., 2012). RuBisCO from spinach leaves shows a great emulsifying capacity after pH precipitation followed by lyophilization. However, heat coagulation has a negative effect on this property (Rault et al., 1993). RuBisCO showed emulsion stability comparable to the popular egg white protein (Lamsal et al., 2007). Rice proteins have a poor emulsifying capacity due to their low water solubility and high molecular weight (Agboola et al., 2005). As mentioned above (in the solubility section), pea protein is almost insoluble at pH 5.0, which poorly influences its emulsifying capacity at this pH value. However, the emulsion ability increases significantly below and above this pH value (Barac et al., 2010). The emulsifying ability of wheat protein is also low, which negatively affects it's used in the food industry, so to improve this function a conjugation of gluten with fructose via Maillard reaction was performed (Song et al., 2018). The emulsifying capacity of soy protein is limited at pH 5.0 (pH value where protein solubility is lower), something which compromises its utilization in food matrixes (Deng, 2021). Soy protein emulsion capacity can be improved by modifying certain physico-chemical properties (Tang, 2017). Lupin protein has a similar behavior as soy and pea proteins. To improve this function, high pressure treatments have been applied to lupin protein (Chapleau & Lamballerie-Anton, 2003).

4.4. Gelation

Protein denaturation followed by network formation is the process that triggers gelation, which is a functional property that can have a significant impact on the texture of food products (Abaee et al., 2017). The gel formation capacity of RuBisCO has also been examined, with the most favorable outcomes observed for alfalfa-derived RuBisCO after being subjected to heat treatment at 80 °C and pH 12 for 30 min (Barbeau & Kinsella, 1988). The gel produced by RuBisCO derived from spinach and tobacco leaves has also demonstrated favorable outcomes, and similarly to its emulsifying capacity, these gels exhibit greater resistance when compared with those formed by soy protein (Van del Velde et al., 2011). Soy seed protein has been found to own a strong gelling capacity, with as little as 10% (w/w) of soy protein showing excellent rheological properties (Batista et al., 2005). Pea protein gelling properties showed some limitations and were weaker and less elastic when compared with soy protein (Shand et al., 2007). Another constraint of pea protein is its high gelation temperature, which requires temperatures of approximately 95 °C to induce gelation. As typical cooking temperatures reach only around 75 °C, this makes it challenging to utilize pea protein as a gelling agent (Health and Human Services, 2017). Lupin protein exhibits a behavior similar to pea protein: the gels formed with lupin protein were weaker and more deformable when compared to the ones made with soy protein, resulting in a poor gelling ingredient for the food industry (Berghout et al., 2015). Agboola et al. (2005) demonstrated that isolated Australian rice proteins had good gelling properties (See Table 4).

4.5. Foaming capacity

Proteins exhibit great foaming capacities because they contain both hydrophilic and hydrophobic moieties. When comparing the foaming capacity of RuBisCO to the well-known protein found in egg whites, it was observed that both proteins produced similar volumes of foam. However, the foam created by RuBisCO was 33% more stable (Douillard & Mathan, 1994). Recent trials have demonstrated that RuBisCO exhibits a more desirable foaming capacity than soy and whey proteins at pH values of 4.5 and 7.0 (Van del Velde et al., 2011). Foaming capacity of soy protein isolates in their native form was low, which can be explained by their tertiary and quaternary structures (Martínez et al., 2009; Martínez et al., 2011). Lupin protein's noteworthy foaming ability in acidic solutions sets it apart from soy protein. This characteristic makes lupin protein more appropriate for use in food products such as yoghurts or salad dressings (Jayasena et al., 2010). Rice proteins foaming capacity was improved when pressure was applied to these proteins (Zhu et al., 2016). At pH 5.0, pea protein had the lowest foaming capacity, but this property increased significantly when the pH was increased to 7.0 and 8.0 (Barac et al., 2010). Gluten (wheat storage proteins) is composed by glutenin and gliadin, and the last mentioned is responsible for the foaming properties of gluten (Keller et al., 1997; Banc et al., 2007). This protein has a great foaming capacity at neutral and alkaline pH values, but low foaming capacity at pH 2, which can be improved by adding NaCl (Thewissen, Celus, Brijs, and Delcour, 2011).

4.6. Flavor

A significant obstacle in substituting animal proteins for plant-based proteins is the occurrence of undesirable flavors, known as off-flavors. Aldehydes, ketones, and alcohols are the main compounds responsible for the "green" and "grassy" flavor in pea proteins (Heng, 2005). Off-flavors can arise during the harvesting, storage, and subsequent processing of plant-based proteins due to the oxidation and enzymatic degradation of unsaturated fatty acids (Azarnia et al., 2011; Roland

Table 4
Current industrial methods for RuBisCO isolation as a food additive and corresponding advantages and disadvantages.

		Plant source	Method	Advantages	Disadvantages
Company	France Lucerne	Alfalfa	Not disclosed	Capacity to purify large amounts of RuBisCO at a low price	Low degree of purification. Potential allergenic character
	TNO - Innovation For Life	Sugar beet leaves	The process involves pressing, centrifuging and ultrafiltration	Capacity to produce 10 kg protein/h	Low degree of purification. Potential allergenic character
	NIZO	Spinach	The process involves protein extraction, with decantation and pressing, followed by purification by aggregation, precipitation and affinity separation, with a final concentration step by filtration and evaporation	The final product contains 90% RuBisCO. Low % of chlorophylls in the final product	Complex procedure, not suitable to the food industry
	Green Project	*Lemna* spp. (aquatic flowering plants)	Not disclosed	Capacity to purify large amounts of RuBisCO at a low price	The final product has green colour
Project	GreenProtein	Sugar beet leaves	The process involves heat precipitation, decantation, micro-filtration and ultra-filtration, with a final concentration step by spray-drying	The final product is a white powder. Capacity to purify large amounts of RuBisCO at a low price	The use of organic solvents to remove pigments. Low degree of purification. Potential allergenic character

et al., 2017; Lan et al., 2019). Lupin protein, as the majority of legume seed proteins, has a unique flavor profile that has been described as moldy, beetroot-like, grassy, metallic, cooked potato-like and pea-like (Schlegel et al., 2019). The taste perceptions pattern was identified as bitter, salty and astringent (Schlegel et al., 2019). Despite being one of the most commonly utilized plant-based proteins for human consumption, soy protein has significant flavor-related limitations. The presence of isoflavones, saponins, phenolic acids, etc. is responsible for soy protein bitter and astringent tastes (Damodaran & Arora, 2013). Also, residual amounts of phospholipids, which contain polyunsaturated fatty acids (PUFAs), can suffer oxidation and create volatile compounds that are accountable for the beany, grassy, or green odors (Damodaran & Arora, 2013). Rice bran contains a high quantity of lipids, which can trigger the activity of lipases and ultimately lead to the development of a rancid flavor in rice (Champagne, 2004). Degermed wheat and corn flours (endosperm products) have little or no flavor (Rackis et al., 1979). In contrast, RuBisCO has already been highly purified as a tasteless, odorless and colorless powder (Van del Velde et al., 2011).

5. Bioactivities of RuBisCO

Aside from all the advantages mentioned above, RuBisCO has been acknowledged for its functional properties related to human health, especially those that arise from the peptides produced during its proteolytic breakdown, making it a potentially valuable food additive (Udenigwe et al., 2017). Indeed, some authors have postulated that the ability of RuBisCO to produce bioactive peptides upon digestion is one of the features that makes this protein a suitable source for food applications, particularly to produce functional foods (Udenigwe et al., 2017) with important health benefits. Arguably, bioactive peptides have been defined as specific protein fragments that have beneficial influences on the body function and on health in general (Kitts and Weiler, 2003). A comprehensive review of the bioactive peptides generated from RuBisCO has been documented elsewhere (Udenigwe et al., 2017). The list encompasses a wide array of activities, including antihypertensive properties, opioid and anxiolytic activities, antibacterial and antioxidant activities, among others. For instance, a specific bioactive peptide derived from RuBisCO has demonstrated antihypertensive properties by regulating blood pressure (Kapel et al., 2006; Udenigwe & Aluko, 2012). This particular peptide has been shown to inhibit angiotensin I-converting enzyme (ACE) under *in vitro* conditions. ACE plays a vital role in the renin-angiotensin system (RAS), which regulates blood pressure. Additionally, this bioactive peptide has been found to decrease blood pressure in hypertensive animals (Kapel et al., 2006). Systolic blood pressure of hypertensive rats was reduced after oral administration of RuBisCO's hydrolysates (500 mg/kg body weight; Kapel et al., 2006). The dipeptide responsible for this bioactivity was identified as *N*-valyl-tryptophan (VW) and found to be located within RuBisCO's large subunit at different positions (f67-68, f382-383 and f459-460), as well as in the small subunit (at f60-61; Kapel et al., 2006). The bioactive peptides produced from RuBisCO have also been found to possess opioid functionality (Teschemacher, 2003; Perlikowska & Janecka, 2017). Rubiscolin-6, a peptide derived from RuBisCO extracted from spinach leaves, has demonstrated anxiolytic activity (Yang et al., 2001; Hirata et al., 2007). Another interesting effect of rubiscolin-6, detected in mice, was the ability to boost memory consolidation (Yang et al., 2003). Additional studies have indicated that selected RuBisCO-derived peptides possess antimicrobial properties. The hydrolysis of RuBisCO's small subunit has been found to produce bacteriostatic activity against pathogenic Gram-negative bacteria such as *Salmonella arizonae* and *Shigella sonnei* (Trovaslet et al., 2007). Bioactive peptides obtained after hydrolysis of RuBisCO extracted from alfalfa green juice also exhibited antimicrobial activity against Gram negative (*Escherichia coli*) and Gram positive (*Micrococcus luteus*, *Listeria innocus* and *Bacillus subtilis*) bacteria (Kobbi et al., 2015). RuBisCO's derived peptides have also been studied for their potential antioxidative capacity. An *in vitro* assay showed a potent antioxidant activity of RuBisCO's bioactive peptides (Kobbi et al., 2017). The activity was dose-dependent and the mechanism behind it was the inhibition of linoleic acid oxidation, which led to the reduction of ferric ion and scavenging of a radical cation (Kobbi et al., 2017). *Solieria chordalis,* a red seaweed, has been identified as a source of antioxidative peptides in previous research (Bondu et al., 2015). More recently, it has been reported that 60% of the identified antioxidative peptides in *Solieria chordalis* were derived from RuBisCO (Bondu et al., 2015).

6. Technologies for RuBisCO extraction and food applications

The available literature describes many methods for obtaining highly pure RuBisCO, but these methods are either economically unfeasible for industrial-scale production or involve the use of reagents that are harmful to human and animal health.

Despite its natural abundance and ubiquity among photosynthetic cells and organisms, its potential use in food products has been limited due to the difficulty of efficiently purifying it on a large scale in a cost-effective form that is safe for human consumption, similar to other typical proteins. In the last decade however, several patented methods and projects have yielded several procedures, more or less expensive, to produce industrial amounts of RuBisCO, either as a white, tasteless powder or as a green protein additive (Table 3). The first company that produced RuBisCO on a large scale was France Luzerne, a French company participating in Fralupro, a European project whose objective was to discover a possible use for the leaves from 32 million ha of alfalfa each year. In 1998, this company started to produce 1,200 tons of alfalfa RuBisCO per year, at the same cost of producing soy protein. However, the project failed to achieve a significantly higher degree of RuBisCO purification beyond that already present in the total soluble protein extract. It is worth recalling that RuBisCO is already ca. 50% pure (relative to the total leaf protein) in the leaves of C3 plants. This extract contains thousands of different proteins, many potentially allergenic and/or otherwise undesirable, in addition to tannins and other phenolic compounds, which can negatively contribute to the nutritional quality of the protein concentrate.

TNO – Innovation For Life is a Dutch company that also produces RuBisCO from sugar beet leaves on a large scale. The process involves pressing, centrifugation and ultrafiltration, and has a production capacity of 10 kg protein/h, with a low degree of purification, around 35 to 40%. Therefore, RuBisCO is not obtained in a purified state. Another Dutch company, NIZO, filed a patent application for its RuBisCO purification process in 2010. The end-product resulting from the purification process described in the patent application by NIZO contains at least 90% of the leaf RuBisCO in a soluble form and less than 0.1% (w/v) chlorophylls. However, the process is complex and has not yet been able to be scaled-up to an industrial level. RuBisCO Foods, another Dutch company that produces protein gels and powders for the purpose of their application in food and feed, has developed and patented a unique technology that allows the extraction and purification of soluble proteins from *Lemna* spp. (aquatic flowering plants), including RuBisCO. However, the protein extracts still contain chlorophylls in their composition.

GreenProteins is a European project that presents, as its main objective, the production of high-quality proteins for food applications. Proteins are extracted from sub-products produced by the food industry as a way to promote sustainability. This project, which began in 2016 and ended in 2021, was based on a new methodology used for purifying RuBisCO from sugar beet leaves, as described in patent US20150335043A1. Although the final product achieved is a white powder, which indicates complete removal of chlorophylls, the method described uses organic solvents for the removal of the pigments, which is neither compatible with healthy food nor feasible for further applications in the food industry. It is also important to note that although the disclosure to the public states that RuBisCO is purified, the end-product actually consists of the total extract of soluble proteins found in sugar beet leaves.

The large number of patents and industry-level projects focused on RuBisCO extraction and purification indicates a significant level of interest and demand for this protein. Nonetheless, whilst there are several methods described in the industry, most of these are based on fractionation that yield highly impure RuBisCO, whereas more complex methods typically hinder RuBisCO production at a reasonable price. Therefore, some do not produce the protein in a purified form and those that do are not cost-effective. Some of these methods produce RuBisCO in a form that is insoluble and/or green. These observations may well explain the absence of commercially available RuBisCO in the food markets. Nevertheless, there has been a rising number in novel patents submitted with new methods that claim to purify RuBisCO, which are scalable and suitable for the food industry. Hopefully, with the emerging novel methods, RuBisCO may fulfill its potential as the future of commercialized plant-based proteins.

As a food additive, RuBisCO has already been tested in several of the above-mentioned projects, mostly as a protein additive in foods, using several food matrixes. Noteworthy, the isolated RuBisCO produced by GreenProteins has been successfully used as a protein additive in wheat dough, with prominent published results. Ducrocq, Boire, Anton, Micard, & Morel (2020) supplemented wheat doughs with RuBisCO, gluten and pea protein and the results were quite promising. Whilst gluten and pea protein decreased dough stiffening during heat treatment, RuBisCO showed the ability to bind (weakly and covalently) to the dough and remarkably, the dough treated with RuBisCO had a higher concentration of large polymers after being exposed to heat, hence enhancing its functional properties (Ducrocq et al., 2020).

7. Advantages to the food industry

Overall, RuBisCO seems to present all the advantages to render it the perfect plant-based protein for today's market, including its sustainability, nutritional composition, digestibility, organoleptic and physical properties and even bioactivities for human health, in the absence of allergenicity. Fig. 2 summarizes all the major advantages of RuBisCO as a food additive.

Whilst the production of other proteins available in the market (especially those of animal origin) have typically negative environmental consequences, RuBisCO is a very good candidate from a circular economy and sustainability point of view. Due to its capacity to react with O_2 and catalytic inefficiency, this protein is present in very large amounts in all photosynthetic cells and green tissues, thus allowing its isolation from green food waste produced by the industry. Although its isolation has been mostly reported from alfalfa green juice and sugar beet leaves (Tenorio et al., 2016; Kobbi et al., 2017, respectively), RuBisCO purification can be achieved from a wide variety of crops, a good proportion of which generate common industrial sub-products, like carrot or tomato leaves, hence becoming a very sustainable source of plant-based protein. Other photosynthetic options exist as potential sources of RuBisCO, including macro and microalgae. These algae can also serve as an excellent protein source due to their fast growth and ability to produce high masses in short periods of time.

8. Conclusion

Overall, RuBisCO possesses unique characteristics, a favorable nutritional profile, beneficial bioactivities, and desirable physical attributes, making it a promising candidate for use in food applications. Based on all its well-studied advantages, it seems clear that RuBisCO may be regarded as the ideal protein for both human and animal consumption. The potential applications of this protein for the food industry are vast. To date, what is missing is a good and reliable procedure for RuBisCO purification, i.e., cost-effective, suitable to be scaled-up to an industrial level and relatively easy to perform. Novel methods to purify RuBisCO with all the characteristics described above have been arising and could help to place this protein on a higher position in the plant-based food market. Given the current trends in the plant-based market and RuBisCO's unique features, ubiquity and quantity, it seems plausible that new and improved methods for its large-scale purification will continue to emerge in the coming years. These developments will likely be accompanied by further research uncovering new bioactivities and functions associated with RuBisCO.

Most probably, in the near future, RuBisCO will become a common protein additive used to enhance the nutritional value of various food products such as protein bars, bread, beverages, yogurts, and puddings. Furthermore, since RuBisCO is also known for its bioactive peptides, it could not only enhance the nutritional value of different food products but also add health-promoting value and functional food features to the final product.

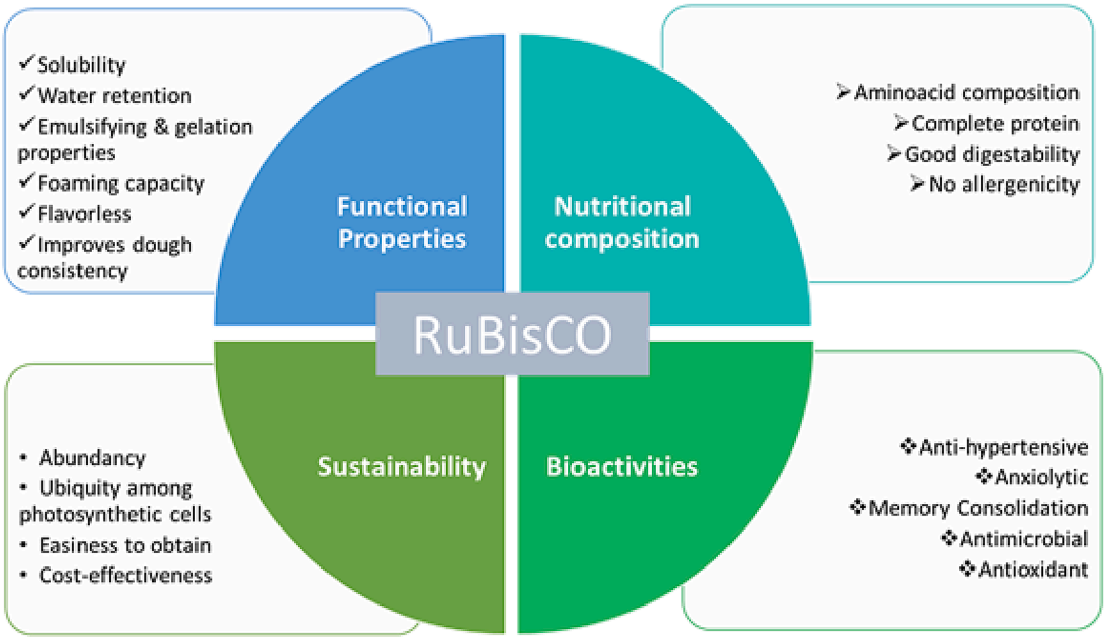

Fig. 2. Advantages of RuBisCO as a food additive.

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.

Data availability

No data was used for the research described in the article.

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