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Artisanal cocoa bean fermentation: From cocoa bean proteins to bioactive peptides with potential health benefits

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ABSTRACT

Post-harvest processing of cocoa beans, such as fermentation, not only determines the formation of aroma and flavor compounds, but also contributes to the formation of bioactive compounds with health benefits. Nevertheless, most studies associate these beneficial effects to the polyphenols present and little is known about the potential health effects of other cocoa components such as bioactive peptides. Thus, in the present review we focus on the role of cocoa bean protein hydrolysis for the release of bioactive peptides. Also, the potential health benefits of cocoa bean hydrolysates and peptides, specifically, antioxidant, antihypertensive, antidiabetic, anti-Alzheimer, antiobesogenic and antitumor activities are presented. However, more research is needed that considers bioaccesibility and bioavailability studies, as well as *in vivo* studies and clinical trials, for testing cocoa bean bioactive peptides. This knowledge may allow the development of nutraceuticals and food products with specific biological activities as well as good aroma and flavor.

1. Introduction

Bioactive peptides are usually composed of two to 20 amino acid residues and have a molecular mass of less than 6 kDa. Peptides may be released from the native protein by the action of proteases from microorganisms, by the addition of proteolytic enzymes during food processing, or the action of gastrointestinal enzymes once ingested (Hernández-Ledesma et al., 2004; Udenigwe & Aluko, 2012). Moreover, peptides can be active in foodstuffs or can be released during the digestion process in the gastrointestinal tract (Daliri et al., 2017). Their bioactivity is based on their amino acid composition and sequence (Maestri et al., 2016; Rawel et al., 2019). Also, dipeptides with the proline-proline sequence in their C-terminal, are the least prone to degradation by proteolytic enzymes in humans (Maestri et al., 2016). In fact, peptides produced in different food matrices were reported to be involved in a variety of biological activities including antihypertensive, antioxidant, opioid and antidiabetic (Doyen et al., 2014; Beltran-Barrientos et al., 2018; Haroon et al., 2018). Although milk and dairy products, particularly fermented dairy products, have been extensively studied as a source of bioactive peptides (Capriotti et al., 2016), many bioactive peptides are also found in other animal (Udenigwe & Aluko, 2012) and plant sources (Singh et al., 2014; Tovar-Pérez et al., 2019). By-products and agro-industrial wastes also represent a relatively inexpensive source of bioactive peptides, in particular, for the production of antioxidant and ACE-inhibitory peptides either by enzyme treatment or by fermentation (Piovesana et al., 2018).

Functional molecules such as bioactive peptides derived from different foods have been widely pursued for restoring health against metabolic disorders such as hypertension, hyperglycemia, hyperlipidemia and obesity (Udenigwe, 2014). The effects of bioactive peptides mainly aim at the protein level mostly by inhibiting metabolic enzymes perhaps due to protein–protein interactions, expression of gene regulation responsible for abnormal signalling pathways and by physical interaction and direct removal of metabolites, therefore maintaining physiological homeostasis (Udenigwe, 2014). Recently, there are emerging advancements in the strategies for food bioactive peptide discovery triggered by their multiple food and health implications and the fact that the classical approach to food peptide production (involving protein hydrolysis and conducting bioactivity assays on the hydrolysates) is laborious, time-consuming and not cost-effective (Agyei et al., 2018). In order to circumvent the challenges that the classical approach presents, the use of bioinformatic tools ("*in silico*") has emerged as an approach for the prediction of novel peptide sequences from food proteins. Thus, once the prediction of bioactive peptides is

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achieved by *in silico* tools, actual laboratory production of the peptides is undertaken followed by their identification through analysis with high-throughput peptidomics that relies on liquid chromatography-mass spectrometry techniques (Agyei et al., 2018).

Most recently, there is growing interest of the scientific community for the identification of bioactive peptides derived from plant food proteins due to the public opinion toward plant based foods, with respect to their higher sustainability than animal foods (Rizzello et al., 2016; Piovesana et al., 2018). In fact, several studies have shown the potential beneficial health effects related to cocoa and chocolate consumption, attributed to different bioactivities such as antioxidant, anti-inflammatory, improvement of endothelial function and antiplatelet activity (Grassi et al., 2010; Latif, 2013).

From a functional point of view, cocoa is considered a rich source of different bioactive compounds, particularly, the group of polyphenols, to which some mechanisms have been proposed that may be responsible for their beneficial effects (Quiñones et al., 2011; Lee et al., 2003; Gültekin-Özgüven et al., 2016). While cocoa polyphenols and their antioxidant capacity have been widely studied, reports on the potential health benefits related to other compounds such as peptides are currently considered a topic of interest (Daliri et al., 2017). Furthermore, cocoa shells which are a by-product with high-value bioactive components derived from the chocolate industry (Panak et al., 2018; Pavlovic et al., 2020) have been underexplored for the production of bioactive peptides.

On the other hand, post-harvest processing of cocoa beans, such as fermentation, not only determines the formation of aroma and flavor compounds, but also contributes to the formation of bioactive compounds with health benefits (Marseglia et al., 2019). Nevertheless, most studies associate these beneficial effects to the polyphenols present (Mayorga-Gross & Esquivel, 2019) and little is known about the potential health effects of other cocoa components such as bioactive peptides (Marseglia et al., 2019). In fact, it has been reported that peptides and amino acids produced during artisanal cocoa fermentation are known to be the most important precursors for the development of cocoa aroma (Marseglia et al., 2014); nevertheless, scarce studies are available on the evaluation of their potential bioactivity and contribution to health. Moreover, fermented foods are having a resurgence due to consumerś growing interest in foods that are wholesome, natural and health promoting (Terefe & Augustin, 2019). Thus, cocoa bean fermentation may not only be studied for the production of palatable food and beverage products but also for the production of healthy functional products.

Thus, in the present review we focus on the role of cocoa bean protein hydrolysis for the release of oligopeptides as precursors of bioactive compounds. Most studies presented in this review used the classic approach for bioactive peptide studies, nevertheless, the use of bioinformatics and peptidomic approaches are emerging strategies for the discovery and analysis of cocoa bean bioactive peptides.

2. Cocoa bean valorization

The origin of cacao is controversial, however, cacao seeds were brought to Europe by the Spaniard conqueror Cortez, who came across cocoa beans when he arrived in Mexico. Nevertheless, it was until 1828, with the invention of the cocoa press, that allowed the separation of cocoa powder from cocoa butter, that the making of modern chocolate started (Ozturk & Young, 2017).

Cocoa beans are the main ingredient for the production of chocolate, and are responsible for its characteristic flavor (Owusu, et al., 2013; Voigt et al., 2018). Principal botanical varieties for the production of cocoa beans from *Theobroma cacao L.* are Forastero, Criollo and Trinitario. Forastero varieties constitute almost 95% of the total worldwide production and the Trinitario and the Criollo varieties, which are regarded as the fine varieties for flavor production, account for less than 5% of the total world production (Rawel et al., 2019). According to the International Cocoa Organization (ICCO), 4731 thousands of tons of cocoa beans were produced worldwide in 2016–2017, from which America contributed 16%, with a total of 758 thousand tons between 2016 and 2017 (ICCO, 2019). In Mexico, the state of Tabasco and Chiapas are the major cocoa bean producers with 18.2 and 9.81 tons during 2018 (SIAP, 2018).

It is worthwhile to acknowledge that although the main worldwide known use for cocoa bean is in the manufacture of chocolate; in Mexico, cocoa beans may be used as one of the main ingredients in traditional maize-cocoa beverages that have been consumed since pre-hispanic times (Soleri et al., 2013). In the family of cacao beverages made with maize, there is a great variety of them that may differ in the manner in which the grain is processed and this may vary depending on the region in southern Mexico. Interestingly, some of these drinks may start with a solid state fermentation of maize and cacao (Castillo-Morales et al., 2005). These beverages have been scarcely studied, although there are few reports on their chemical and nutritional composition (González-Amaro et al., 2015) and biochemical and microbiological changes occurring during fermentation (Castillo-Morales et al., 2005). However, the possible presence of bioactive peptides derived from either cacao bean or maize protein fermentation or both has not been addressed.

3. Cocoa bean composition and fermentation

Cocoa beans are composed of an embryo, two cotyledons (86–90% of seed dry weight) and the mucilaginous pulp that surrounds the seeds (which constitutes of about 10–14% of the dry weight), and may vary depending on their origin (Rawel et al., 2019; Santander et al., 2019). Cocoa pulp consists of sugars (glucose, fructose and sucrose), citric acid and pectin that serve as substrates for the microorganisms involved during fermentation. Additionally, cocoa beans have a high content of polyphenols (15–20% in fat-free fresh seeds), which provide an astringent, bitter and unpleasant flavor that decreases to 5% during fermentation (Afoakwa et al., 2008). Thus, cocoa beans must undergo post-harvest processing that involves fermentation, drying and roasting for the development of flavor precursors that will provide desirable aroma and taste profiles, as well as bioactive peptides (Rawel et al., 2019).

It is noteworthy to mention that the manufacture of chocolate with cocoa beans, is one of the few artisanal fermented foods where microorganisms come naturally and drive the fermentation process, rather than being manipulated with a starter culture. Thus, cocoa bean fermentation is one of the few remaining artisanal microbial processes that occur through traditional practices and may be performed in heaps, boxes, baskets, or trays or on direct platforms (Lefeber et al., 2010); this being the result of the unique history/evolution of cocoa bean production and its transformation to chocolate (Ozturk & Young, 2017). Additionally, a lack of success in replacing this traditional natural cocoa bean fermentation with starter cultures like those used in other fermented foods, hindered its transition at the farm level (Ozturk & Young, 2017). Thus, the first stage of the chocolate-making process involves both pectinolysis and fermentation of cocoa beans (Buzzini et al., 2017).

The most common types of fermentation during food processing are alcoholic, lactic and acetic acid based fermentations (Steinkraus, 2002), and in some cases, as it is the case of cocoa bean fermentation for chocolate production, these occur almost simultaneously (Camu et al., 2007; Lagunes et al., 2007; Lefeber et al., 2012; Sandhya et al., 2016) making the process of studying these microbial consortia extremely complex. Particularly, fermentation is an essential step since it allows the development of aroma and flavor precursors by the action of the microorganisms involved, that include yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Fig. 1) (Janek et al., 2016, Voigt et al., 2018) and takes place for approximately four to six days. However, after 5 days of fermentation, some off-flavors may be formed by some fungal species (Sârbu & Csutak, 2019).

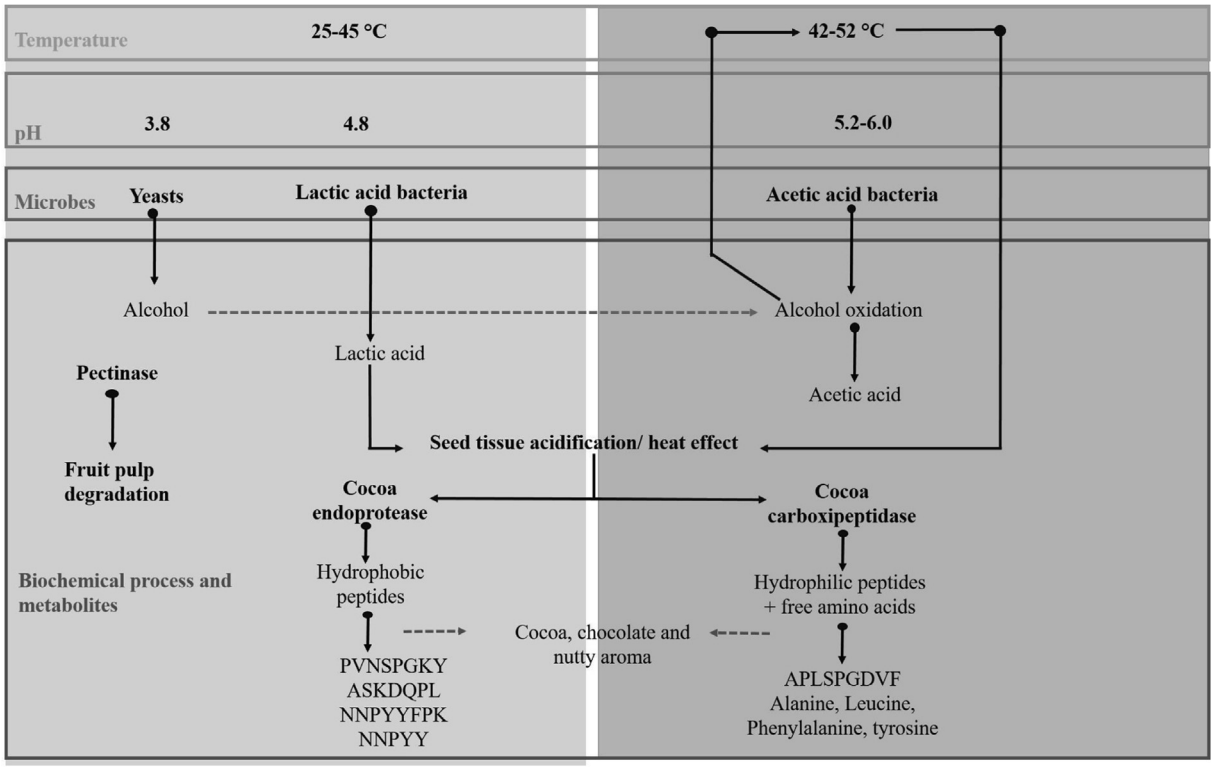

Fig. 1. Microbial development and biochemical processes during artisanal cocoa fermentation (Adapted from Kadow et al., 2015 and Voigt et al., 2018).

During this three stage process that involves yeasts, LAB and AAB, metabolites such as ethanol, lactic and acetic acids are produced, pH decreases from 7 to 4–4.5, and temperature increases up to 45 °C (Santander et al., 2019). This process triggers the activation of endogenous hydrolytic enzymes (Lagunes et al., 2007; Nielsen et al., 2007; Sandhya et al., 2016). It has been previously reported that endogenous proteolytic enzymes are responsible for the production of several precursor aroma compounds such as peptides and amino acids derived from protein hydrolysis (John et al., 2019; Salger et al., 2019); although, these enzymes are active during a short period of time, they are further inactivated by other enzymes (Sârbu & Csutak, 2019). These pH and temperature changes cause a series of processes on cocoa beans, that include pulp degradation and cotyledon death (Sârbu & Csutak, 2019), as well as the production of aroma compounds such as alcohols, carboxylic acids, aldehydes, ketones, esters and pyrazines (Aprotosoaie et al., 2016; Janek et al., 2016, Voigt et al., 2018) and bioactive peptides (Marseglia et al., 2019).

The microbial strains involved during spontaneous fermentation may be originated from the direct manipulation of cocoa beans, such as workers' hands, tools used during the post-harvest process, insects, banana leaves, heaps, boxes, baskets, trays or direct platforms, or fruits surfaces. However, not all microorganisms will participate during cocoa bean fermentation (Camu et al., 2007; Sârbu & Csutak, 2019). Nevertheless, the order of the microbial communities involved during the spontaneous fermentation of cocoa beans are the same worldwide (Sârbu & Csutak, 2019).

4. Cocoa bean proteins and oligopeptide formation during fermentation

Proteomic techniques with different protein ionization methods, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS), have enabled the identification of food proteins (Rawel et al., 2019). In this context, the whole cocoa bean proteome was characterized by nano-UHPLC-ESI MS/MS analysis using tryptic digests of cocoa bean protein extracts leading to a total of more than 1000 proteins. Nevertheless, vicilin and albumin, classified as storage proteins, showed the highest abundance among all detected proteins, with relative amounts of 3.9% and 11.5%, respectively. Proteins were also classified according to their main biological function, with most of these related to metabolism and energy, protein synthesis and processing and response to different stress (Scollo et al., 2018).

During fermentation, microorganisms and enzymes extensively modify proteins to release peptides and amino acids that are flavour precursors (Marseglia et al., 2014). In this sense, it has been widely reported that cocoa proteins undergoing fermentation are cleaved into hydrophilic and hydrophobic peptides as well as amino acids by two endogenous enzymes, aspartic endoprotease and carboxypeptidase. In fact, the sequential proteolytic degradation of the storage proteins was followed, forming longer peptides in the early stages of fermentation and an increasing number of shorter peptides at the latter stages of fermentation, thereby establishing the peptide profile of a bean as a reliable indicator of its degree of fermentation (DSouza et al., 2018). In this work, more than 800 fermentation peptides were identified and it was concluded that these findings could be a key in cocoa metabolomics which could open several avenues of novel research, as well as reinforcing the knowledge on cocoa flavor chemistry (DSouza et al., 2018).

Proteomic and peptidomic analysis allowed a comprehensive study of storage protein degradation in cocoa beans during fermentation. In fact, main differences in protein content of non-fermented cocoa beans were attributed to their geographical origin (Kongor et al., 2016). On the contrary, peptides could not be correlated to the geographic origin but rather to the degree of fermentation associated to the fermentation method applied in the country of origin. In fact, well-fermented beans could be distinguished from partially fermented and under-fermented ones by the number of oligopeptides (Kumari et al., 2018).

Studies have reported that metabolites produced by yeasts and bacteria during spontaneous fermentation are ideal to stimulate the

Table 1
Potential beneficial effects of cocoa bean protein hydrolysates.

	Sample	Model	Bioactivity	Reference
Antioxidant	Unfermented cocoa bean hydrolysate	*In vitro*	FRAP and beta-carotene-linoleic bleaching inhibition assay	Samardi et al., 2011
	Enzymatic hydrolysate from extracted cocoa bean proteins	*In vitro*	DPPH, ABTS and ORAC	Tovar-Pérez et al., 2017
	Fermented cacao beans	*In vitro*	ABTS and ORAC	Preza et al., 2010
Antihypertensive	Hydrolysate of unfermented cocoa bean	*In vitro*	ACEI	Samardi et al., 2011
	Fermented cocoa beans	*In vitro* simulated gastrointestinal digestion	ACEI	Marseglia et al., 2019
Antidiabetic	Hydrolysate of unfermented cocoa bean	*In vitro*	Inhibition of α-amylase and α-glucosidase	Samardi et al. 2012
	Hydrolysate of unfermented cocoa bean	BRIN-BD 11 cell line	Insulin secretion activity	Samardi et al., 2012
	Fermented cocoa beans	*In vitro*	Inhibition of dipeptidyl peptidase-IV (DPP-IV)	Ryan et al., 2017
	Hydrolysate of unfermented cocoa bean	*In vivo*Diabetic rats	Hypoglycemic effect	Samardi et al., 2012
Anti-Alzheimer	Enzymatic hydrolysate of cocoa by-product	*In vitro*	Highest prolyl endopeptidase inhibition	Martorell et al., 2013
	Enzymatic hydrolysate of cocoa by-product	*In vivoCaenorhabditis elegans* CL4176	Amelioration of body paralysis progression, protection oxidative stress	Martorell et al., 2013
Antiobesogenic	Extracted cocoa proteins	*In vivo*Rats induced with obesity	Reduction in body weight, proinflammatory factors, serum lipids, insulin, leptin	Coronado-Cáceres et al., 2019
Anti-tumor	Semi-fermented cacao beans protein fractions	Murine lymphoma L5178Y model	Reduction in ascetic fluid and packed cell volume, inhibition of cell growth	Preza et al., 2010

Angiotensin converting enzyme: ACEI

activity of these enzymes (Camu et al., 2007; Marseglia et al., 2014; Mota-Gutierrez et al., 2018). Interestingly, during the first 24 h of fermentation the cocoa bean protein degradation begins; and after 72 h, protein degradation slowed down and reached its maximum after 120 h. In fact, specific identified peptides at 24 and 120 h (APLSPGDVF and PVNSPGKY) were proposed as markers for a good fermentation process in addition to being important aroma precursors (Kumari et al., 2016). Changes occurring to vicilin from the non-fermented stage up to the dried cocoa bean, showed an initial increase and subsequent decrease in the diversity of peptides with an increasing degree of fermentation (Kumari et al., 2016). The proteolytic degradation of vicilin started within the first 24 h of fermentation, with the formation of 65 different vicilin oligopeptides, that continued to increase until 72 h of fermentation, after which the number of peptides decreased, with no free peptides at 144 and 168 h of fermentation, as well as in the dried samples (Kumari et al., 2016).

Another study reported 44 identified oligopeptides released during cocoa bean fermentation from different geographical origins. The formation of these peptides was dependent on the cleavage specificity of proteases and the protein structure. Authors suggested that not only endogenous proteases may be involved during proteolysis, but also proteases from microbial origin may play a role, even though the penetration of microbial enzymes into beans has not yet been reported (Marseglia et al., 2014).

In general, during the first 72 h of fermentation, when pH is around 3.5, endopeptidases are activated, liberating hydrophobic peptides. As fermentation continues, pH slightly increases to 5.5–6.0, then, carboxypeptidases release more hydrophilic peptides and hydrophobic amino acids (Voigt et al., 2018; John et al., 2016). Although, cocoa carboxypeptidase could be substituted by a commercial carboxypeptidase, endogenous cocoa bean protease could not be substituted by any other commercial protease, due to its particular specificity cleavage, which is essential for the generation of the precursors of the typical cocoa aroma compounds (Janek et al., 2016; Scalone et al., 2019). Other studies have reported that during the first 24 h of fermentation, the degradation of proteins begins and after 72 h, the degradation reaches out its maximum, and by the end of fermentation, proteolysis was undetected (Kumari et al., 2016). Nevertheless, other authors reported the presence of peptides having a length of 7–23 amino acids after 96 h of fermentation, and even after seven days, there was an increase in the diversity of amino acids, di and tri-peptides (ÓSouza et al., 2018).

In fact, studies have reported that the presence of two common peptides, APLSPGDVF and PVNSPGKY may be markers of a good fermentation (Kumari et al., 2016; ÓSouza et al., 2018; Voigt et al., 2018; John et al., 2019). Furthermore, other peptides such as ASKDQPL, NNPYYFPK, NNPYY, APLSPGDVF and SPGDF were reported as cocoa aroma precursors (Fig. 1) (Marseglia et al., 2014; Kumari et al., 2016; Voigt et al., 2016). In addition to being aroma precursors, the formation of peptides through proteolysis during cocoa bean fermentation, may provide bioactive compounds either in a native state or as a hydrolyzed product after consumption (Rawel et al., 2019).

5. Potential bioactive peptides

Cocoa beans represent an interesting source of peptides that are formed during cocoa bean fermentation (Marseglia et al., 2014; Voigt et al., 2018; ÓSouza et al., 2018; John et al., 2019) from two major storage protein fractions, albumin and vicilin-like (Marseglia et al., 2019). Authors have studied oligopeptides not only from the point of view of generating chocolate flavor, but also from the potential biological effect that oligopeptides from cocoa beans may present. Exploring these avenues, several studies reported that within the cocoa bean protein are peptide sequences that may exert different potential beneficial health effects when released (Preza et al., 2010; Sarmadi et al., 2011; 2012; Martorell et al., 2013; Tovar-Pérez et al., 2017; Coronado-

Cáceres et al., 2019). Studies have been focused on bioactive peptides released by three pathways: 1) after inducing cocoa bean autolysis (Sarmadi et al., 2011; 20122), 2) hydrolysis with exogenous commercial enzymes (Tovar-Pérez et al.,2017; Martorell et al., 2013) and 3) after cocoa bean fermentation (Preza et al., 2010; Marseglia et al., 2014, 2019; Ryan et al., 2017) (Table 1).

5.1. Antioxidant

Reactive oxygen species (ROS) play an important role during physiological processes of living organisms, including cell growth and metabolism, as signaling compounds in gene expression systems and intracellular signaling transduction pathways (Wang et al., 2018; Nwachukwu & Aluko, 2018). During these processes, ROS such as hydroxyl radicals, singlet oxygen, superoxide anions and hydrogen peroxide may be neutralized and eliminated by living organisms through their endogenous antioxidant defense mechanisms. These mechanisms are enzymatic antioxidants that include catalase, glutathione peroxidase, and superoxide dismutase and non-enzymatic antioxidants which include ascorbic acid, glutathione, tocopherol, and melatonin (Wu et al., 2017; Nwachukwu & Aluko, 2018). Nevertheless, an imbalance between antioxidants and ROS, may result in oxidative damage to proteins, lipids, and nucleic acids (Nwachukwu & Aluko, 2018). Additionally, this oxidative damage may lead to the development of chronic diseases such as cancer, diabetes, cardiovascular diseases and inflammatory disorders (Wu et al., 2017; Manzanares et al., 2019).

Therefore, to help diminish and prevent oxidative damage, consumption of different fruits, vegetables, beverages, cereals and other food products may be a source of exogenous antioxidants. These exogenous antioxidants include ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids and polyphenols (Möller & Loft, 2006; Lourenço et al., 2019). Moreover, other antioxidant sources such as peptides have been a growing interest. In this sense, several antioxidant peptides from different food proteins have been identified, and their antioxidant capacity has been studied. This antioxidant capacity has been explored in terms on the ability of peptides to scavenge free radicals, to inactivate ROS and protect cells against oxidative stress, to chelate oxidative metals, as well as promote to activate intracellular antioxidant enzymes (Bamdad et al., 2015; Manzanares et al., 2019).

The release of hydrolysates and peptides from different plant protein sources with antioxidant capacity has been widely studied (Nwachukwu & Aluko, 2018). Antioxidant activity of cocoa beans have been attributed to phytochemicals such as polyphenols (Oracz & Nebesny, 2016). However, recent studies have determined the potential antioxidant activity of hydrolysates and peptides from cocoa beans (Preza et al., 2010; Samardi et al., 2011; Tovar-Pérez et al., 2017).

In this regard, Samardi et al. (2011) evaluated the antioxidant activity of autolysates after inducing autolysis with endogenous enzymes by subjecting unfermented cocoa beans to optimum pH (3.5 or 5.2) and temperature. After antioxidant evaluation with the ferric reducing/antioxidant power (FRAP) test, autolysates presented the ability to donate electrons, and this activity was directly proportional to sample concentration. Furthermore, a beta-carotene-linoleic bleaching inhibition assay, a model used for membrane based lipid peroxidation was also evaluated. Results demonstrated that autolysates had the ability to scavenge linoleate derived free radicals in a dose-dependent manner.

On the other hand, enzymatic hydrolysis with commercial enzymes have been employed to obtain protein hydrolysates and peptide fractions presenting bioactivity. Tovar-Pérez et al. (2017) extracted different protein (albumin, globulin, prolamin and glutelin) fractions from cocoa beans. Glutelin represented 80% of total protein; thus, this protein fraction was hydrolyzed with alcalase to obtain the hydrolysates, and then purified to obtain peptide fractions. Then, the antioxidant activity of protein fractions, hydrolysates and peptide fractions was determined by DPPH, ABTS and ORAC. Furthermore, the highest antioxidant activity was reported for two peptide fractions (1–3 kDa), and was similar to that from glutathione (a peptide based endogenous antioxidant). Thus, authors concluded that with this enzymatic treatment applied to cocoa beans, potentially antioxidant peptides may be released (Tovar-Pérez et al., 2017).

5.2. Antihypertensive

Hypertension is an important risk factor for the development of other cardiovascular diseases (WHO, 2014). Therefore, to decrease this risk factor, blood pressure pharmacological therapy may be recommended. Among these drugs, angiotensin converting enzyme (ACE) inhibitors have been commonly used. ACE is crucial in blood pressure regulation, since it converts angiotensin I to angiotensin II, a potent vasoconstrictor; and inactivates bradykinin, a vasodilator (Iwaniak et al., 2014). Hence, its inhibition helps regulate blood pressure. Though, side effects after long term treatment of ACE inhibitors have been reported; such as dry cough, skin rashes, and taste disturbances (Gu & Wu, 2013). Thus, peptides from plant extracts with ACE inhibitory activity have been studied (Daskaya-Dikmen et al., 2017).

In this subject, Samardi et al. (2011) reported that autolysates from cocoa beans presented the ability to inhibit ACE (30–80%), and had an IC_{50} of 3–9.7 mg/mL. Moreover, autolysates produced at pH 3.5 presented more ACE inhibition than those produced at pH 5.2; and were dependent on protein concentration. Authors concluded that high ACE inhibition of autolysates was due to high contents of hydrophobic and aromatic amino acid residues. On the other hand, low ACE inhibition from autolysates that were produced at pH 5.2, may be due to the presence of hydrophilic amino acid residue fractions (Samardi et al., 2011).

It has been widely reported that not only the identification of peptides during food processing with a potential biological effect is important, but also the evaluation of their resistance through the gastrointestinal digestion, and bioavailability should be considered. In fact, during gastrointestinal digestion new peptides may be formed, and the biological effect may be affected. To the best of our knowledge, only one study has identified cocoa peptides released through *in vitro* gastrointestinal digestion and their potential ACE inhibition activity (Marseglia et al., 2019). Moreover, authors also determined the potential molecular mechanisms of cocoa peptides with ACE inhibition with a computational *in silico* approach. In this respect, well fermented cocoa beans, cocoa paste and dark chocolate subjected to a simulated gastrointestinal digestion model were studied. Interestingly, results indicated that after digestion, the amount of peptides present in fermented cocoa beans was reduced. Contrary to this, cocoa paste and dark chocolate presented a higher amount of peptides released after gastrointestinal digestion, suggesting that peptides were resistant to protease activities (Marseglia et al., 2019). Moreover, cocoa digests showed high ACEI; however, from 65 identified peptides, only 20 were predicted active by an *in silico* approach. Also, two of the potentially active synthesized peptides presented very weak activity, thus suggesting that the inhibitory capacity of cocoa digestates was not attributed to a single peptide, but rather to a synergistic effect of all cocoa peptides (Marseglia et al., 2019).

5.3. Antidiabetic

Diabetes mellitus (DM) is a chronic degenerative disease that is characterized by prolonged hyperglycemia that occurs when insulin is not produced sufficiently by the pancreas or when the body does not effectively use the produced insulin. Long-term uncontrolled hyperglycemia may lead to serious damage of other body systems, principally nerve and cardiovascular systems (WHO, 2018). Simple lifestyle modifications such as maintaining healthy body weight, exercising, eating healthy diet (avoiding sugar and saturated fats), and avoiding smoking, may help prevent or delay the onset development of DM (WHO, 2018). However, it has been reported that food-derived

bioactive compounds from animal and plant sources may help control glycemic functions, such as increase insulin secretion, insulin action or may inhibit glucose absorption (Samardi et al., 2012).

In fact, the potential hypoglycemic effect of autolysates obtained at different pH (3.5 and 5.2) from fresh unfermented cocoa beans was determined by exploring the inhibition of two enzymes (α-glucosidase and α-amylase) involved in glucose and carbohydrate digestion (Samardi et al., 2012). Results demonstrated that cocoa autolysates did not present inhibition of α-glucosidase. Contrary to this, autolysates presented an inhibition of greater than 50% on alpha amylase activity and was directly proportional to their protein content. Moreover, the ability to stimulate insulin secretion from BRIN-BD 11 cell lines was determined with different protein concentrations of cocoa autolysates. Protein concentrations from 0.3 to 2.5 mg/mL of cocoa autolysates obtained at pH 3.5 and 5.2, presented higher insulin secretion than the control. Additionally, since the *in vitro* evaluation is not always linked to an *in vivo* effect, the antidiabetic effect was also evaluated with diabetic rats induced with streptozotocin. Different doses of these autolysates were administered to diabetic rats, and after a single oral dose, 600 mg/kg presented the highest antihyperglycemic effect. After amino acid composition determination, the hypoglycemic effect was attributed to the presence of hydrophobic amino acids in specific peptide sequences (Samardi et al., 2012).

On the other hand, Ryan et al. (2017) reported that the fermentation process of cocoa beans and products, enhanced the dipeptidyl peptidase-IV (DPP-IV) inhibitory activity, the enzyme that inactivates incretin hormones such as the glucagon-like peptide-1 and the gastric inhibitory peptide. Although the compounds responsible for this activity remain to be elucidated, authors concluded that polyphenols and flavonols do not participate in the inhibitory activity of DPP-IV (Ryan et al., 2017). In fact, authors highlighted the fact that although cocoa bioactivity has been focused on flavonols or total polyphenols as the principal bioactive substances in previous works, their study suggested that the exploration of other potentially bioactive compounds in cocoa, may play an important role in cocoa bean bioactivity (Ryan et al., 2017). Finally, these authors concluded that the mechanisms or compounds responsible for DPP-IV inhibition are not straightforward and remains to be elucidated. Although this study focused on DPP-IV inhibition, these results may extend to other biological activities and further research is necessary to identify other bioactive compounds present in cocoa (Ryan et al, 2017).

5.4. Anti-Alzheimer

Neurological diseases are any disorder that involves the central and peripheral nervous system (WHO, 2020a). Among neurological diseases, dementia is a clinical syndrome characterized by progressive decline of cognitive function; it affects memory, orientation, comprehension, language and judgement (Crous-Bou et al., 2017; WHO, 2019). Alzheimer disease is the most common cause of dementia, which contributes approximately 60–70% of cases (WHO, 2020a). Several characteristic changes in brain tissue have been reported in patients with Alzheimer diseases. Thus, several anti-Alzheimer therapies have been studied, including antioxidant and anti-inflamatory peptides (Ribaric, 2018).

To this end, a cocoa by-product (Barquillo) obtained from cocoa processing, was hydrolyzed with two proteases to evaluate their potential beneficial effects in an *in vitro* and *in vivo* Alzheimer model (Martorell et al., 2013). First, results indicated that hydrolyzed samples presented the highest prolyl endopeptidase (PEP) inhibition, which is a brain enzyme that participates in several aspects of the central nervous system (CNS), including learning, memory and mood (Hsieh et al., 2016). PEP activity has been found to be significantly higher in the brains of Alzheimer's patients than in normal individuals. Thus, PEP inhibitors are used as therapeutic agents for memory deficits and cognitive dysfunction related to aging and neurodegenerative diseases of the central nervous system (Hsieh et al., 2016). Thus, the hydrolyzed cocoa protein fraction with the most PEP inhibition activity was selected for the *in vivo* study. The transgenic strain CL4176 of the nematode *Caenorhabditis elegans* with induced oxidative stress was used as an Alzheimer model. Results showed that hydrolyzed samples presented greater amelioration of body paralysis progression than non-enzymatic treated samples (Martorell et al., 2013). In the hydrolyzed sample, a 13 residue peptide sequence (DNYDNSAGKWWVT) provided the most protection against oxidative stress, producing a significant delay in nematode paralysis. Also, a reduction of Aβ deposit was observed. Furthermore, transcriptomic analysis of nematodes treated with this peptide revealed modulation of the proteasome and synaptic functions as the main targets of this peptide. Therefore, authors concluded that this peptide released from cocoa by-products with commercial proteases may be used as a therapeutic agent for the prevention of age-related diseases (Martorell et al., 2013).

5.5. Antiobesogenic

Obesity is a disease characterized as abnormal or excessive fat accumulation, which has increased worldwide. This disease is a risk factor for the development of other diseases, such as hypertension, coronary artery disease, fatty liver, stroke, type-2 diabetes mellitus, several cancers (kidney, breast, liver, colon and endometrial), among other diseases (WHO, 2020b). Different factors such as sedentary lifestyle, poor diet, environmental, or hereditary factors may help develop obesity (Hruby & Hu, 2015); however, different lifestyle changes such as a balanced diet and physical activity may help decrease and prevent obesity (WHO, 2020b). Although several pharmacological treatments are available to diminish this disease, such as Orlistat, phentermine/topiramate, lorcaserin, bupropion/naltrexone, among others, they may present undesirable side effects; thus, limiting the availability as safe anti-obesogenic drugs (Kumar & Aronne, 2017). Recently, a rising interest in food-derived compounds for obesity management has been taken. Different bioactive food compounds have been reported for the prevention of obesity and its related diseases (Torres-Fuentes et al., 2013).

The anti-obesogenic effect of cocoa proteins (CP) has also been reported (Coronado-Cáceres et al., 2019). After the administration during 8-weeks of 150 mg/kg/day of CP to diet-induced obesity of male Wistar rats fed with a high fat diet (HF), results showed that total weight gain and relative weight on white adipose tissue (WAT) were significantly lower than rats administered with HF diet. Moreover, serum pro-inflammatory biomarkers, such as TNF-α and MCP-1, were also decreased after the administration of CP with HF diet, compared to rats that received HF diet. Additionally, serum profile was also improved in rats administered with CP and HF, since triglycerides, non-essential fatty acids, insulin and leptin were significantly reduced and HDL levels were increased. After rats were administered with CP and HF diet, several gene expressions were determined. In this regard, AMPK, PPAR-γ, PPAR-α, SIRT1, Plin1, and PGC-1α were activated. Contrary to this, TNF-α, SREBP-1c, leptin and ACC of the mRNA of transcription factors and proteins related to WAT dysfunction were repressed. Overall, these results suggested that the administration of cocoa proteins may help decrease the development of inflammatory diseases related to obesity (Coronado-Cáceres et al., 2019). Although authors attributed the anti-obesogenic effect and the potential mechanistic pathway on the administration of cocoa proteins, it should not be overlooked that bioactive peptides are released through gastrointestinal digestion and these may be having the effect. Thus, further studies are needed to identify and determine which peptides may be playing a role in the anti-obesogenic effect.

5.6. Antitumor

Cancer is a large group of diseases characterized by abnormal

uncontrollably cell growth in any organ or tissue of the body, that may spread to other organs. In 2018, cancer was the second leading cause of death worldwide (WHO, 2020c). Several different treatments such as chemotherapy, surgery and radiation, have been implemented to these patients; nevertheless, their applications may be expensive and some adverse effects may present. Thus, bioactive natural components such as peptides, are being studied as an innovative strategy for the prevention or treatment of cancer. In this sense, several antitumor and anticancer peptides from natural sources have been reported with the ability to reduce tumor progression through multiple mechanisms including antioxidant, apoptotic, anti-proliferative, anti-angiogenic and immunomodulatory activities (Hernández-Ledesma & Hsieh, 2015). Therefore, it is important to develop *in vitro* studies that may be used as screening methods to identify food compounds that may be used for the prevention of cancer (Sausville et al., 2001). In this sense, the determination of antioxidant activity may be a useful tool to predict and compare if a food derived compound of interest may have oxidation/reduction potential (Preza et al., 2010).

In this regard, the inhibitory effects of plant-derived peptides on proliferation and cytotoxicity on different tumor cell lines, such as murine lung, liver and mammary gland cancer and ovarian neoplasm, leukemia, rat osteoblast-like sarcoma, human nasopharyngeal carcinoma, among others have been reported (Ma et al., 2014). In particular, the antitumor effect (using a murine lymphoma L5178Y model) of protein fractions from unfermented and semi-fermented (24 h of fermentation) cocoa bean proteins was determined (Preza et al., 2010). Preliminary assays demonstrated that this effect was not dose-dependent. Moreover, semi-fermented cacao albumin fraction presented the highest cell growth inhibition (59.98%), and this effect was attributed to the amino acid profile, which is rich in cysteine, leucine, arginine and lysine. Similarly, a higher antioxidant activity with ORAC and ABTS methods was observed with semi-fermented cocoa bean proteins. In fact, glutelin fractions from semi-fermented cocoa bean presented the highest antioxidant activity and were 7.1-fold higher than reduced glutathione (a non-enzymatic antioxidant). However, authors reported there was not a direct correlation between the antitumor effect and antioxidant activity (Preza et al., 2010).

6. Future trends

There is growing interest in cocoa bean peptides and their potential health benefits; thus more studies are needed to evaluate the production of peptides during cocoa bean fermentation, their bioactivities and sequence identification (Chakrabarti et al., 2018). In particular, *in silico* studies would contribute to decrease the experimental work required for the identification of cocoa peptides formed or resistant to gastrointestinal digestion and associated to a specific bioactivity, thus allowing to select specific peptides that may be synthesized and subjected to *in vitro* assays.

Also, the evaluation of bioaccessibility and bioavailability of these compounds is crucial, since their stability after gastrointestinal digestion needs to be addressed (Puangkam et al., 2017). Bioaccessibility being defined as the fraction which is released from the food matrix in the gastrointestinal tract that becomes available for absorption, which is usually evaluated by *in vitro* digestion models, sometimes followed by Caco-2 cells uptake (Carbonell-Capella et al., 2014).

On the other hand, the term bioavailability can be defined as the fraction of an ingested nutrient or compound that reaches the systemic circulation and is utilized (Carbonell-Capella et al., 2014). Thus, several *in vitro* simulated gastrointestinal digestion models have been proposed to evaluate the resistance of these compounds after oral intake; and then, the potential bioactivity should be determined. In fact, after an *in vitro* gastrointestinal digestion study, an *in vivo* effect may be most predictable (Puangkam et al., 2017). Indeed, potential beneficial health effects of cocoa bean protein hydrolysates and peptides need to be evaluated by *in vitro*, *in vivo* animal models and clinical trials. In addition to the study of bioactive peptides produced during the fermentation of cacao beans *per se*, it would be relevant to explore the potential health benefits that may offer traditional maize-cacao beverages associated with peptides released during fermentation of maize and cacao proteins. This knowledge may help revalue these traditional Mexican beverages that are hardly known beyond their regions.

On the other hand, over the past decade there is growing interest in the valorization of agro-industrial byproducts, since it not only has economic benefits, but it may also decrease environmental contamination due to their disposal. Therefore, food industry and researchers have been focused on the exploration of food byproducts as natural sources of bioactive compounds (Simitzis, 2018; Nieto et al., 2020). In this regard, cocoa shells, which are produced as a waste product during chocolate manufacturing, are a rich source of dietary fiber and protein, and have been reported to have several industrial applications due to its nutritional value and high-value in bioactive compounds such as methylxanthines and phenolics (Panak et al., 2018; Pavlovic et al., 2020). However, cocoa shells may also be a good source of bioactive peptides that needs to be explored.

Up to now, there is no evidence that suggests that microbial proteases may participate in cocoa bean protein hydrolysis besides cocoa bean endogenous enzymes (Marseglia et al., 2014, 2019; Kumari et al., 2016). Nevertheless, it has been well established that in different food fermentations with microorganisms, they do not only enhance the organoleptic profile of the final products, but also release bioactive peptides through their proteolytic system. Proteolytic enzymes from microorganisms first degrade protein through their cell envelope proteinase, then it transports them via peptide transport systems into the cell, where intracellular peptidases continue to hydrolyze them (Hajfathalian et al., 2017). Thus, it would be of interest to evaluate if microbial proteases participate in the release of bioactive peptides during cocoa bean fermentation.

Furthermore, cocoa bean microbiome composition and diversity was studied by metagenomic analysis using high-throughput DNA sequencing aiming at understanding their contribution towards the enhancement and tailoring of the cocoa fermentation process for flavor modulation (Serra et al., 2019; Agyirifo et al., 2019) and possibly for bioactive peptide production. Also, it may be possible to establish a standardized fermentation process with consistent quality (Romanens et al., 2018; Figueroa-Hernández et al., 2019) and to reduce fermentation time (Magalhães et al., 2017). This knowledge and understanding may provide the means for customizing the different stages of fermentation with the aim of improving aroma generation and bioactive peptide production.

7. Conclusions

As it was evidenced in this review, cocoa oligopeptides are not only important as precursors for chocolate aroma and flavor generation, but are also important for bioactive peptide release. To date, few studies have evaluated the potential biological effect of protein hydrolysates generated by cocoa bean fermentation. Nevertheless, it is important to highlight that cocoa bean protein fractions should be evaluated after subjecting them to gastrointestinal digestion and only those fractions resulting, may be subjected to bioactivity and bioavailability studies. Also, there is a need to study bioactive peptides released during the different stages of cocoa bean fermentation so that nutraceutical or food products may be tailor-made with desired bioactivities as well as good aroma and flavor.

Since the cocoa bean fermentation process is influenced not only by the microbial diversity, but also by the environment and agriculture practices, it is important that each cocoa producing region characterizes its own microbial diversity in order to be able to customize the different stages of fermentation for the production of fermented cocoa bean of consistent customized quality.

8. Ethics statement

The present review did not include and human subjects or animal experiments.

CRediT authorship contribution statement

Leydy A. Domínguez-Pérez: Investigation, Writing - original draft. **Lilia M. Beltrán-Barrientos:** Investigation, Writing - review & editing. **Aarón F. González-Córdova:** Supervision, Visualization. **Adrián Hernández-Mendoza:** Supervision, Visualization. **Belinda Vallejo-Cordoba:** Conceptualization, Investigation, Writing - review & editing, Project administration.

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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