

# The effects of the phytochemistry of cocoa on the food chemistry of chocolate(s) and how disease resistance in cocoa can be improved using CRISPR/Cas9 technology

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Epicatechin (PubChem CID: 1203)  
Anthocyanin (PubChem CID: 11979368)  
Proanthocyanidin (PubChem CID: 108065)  
Theobromine (PubChem CID: 5429)  
Caffeine (PubChem CID: 2519)  
Theophylline (PubChem CID: 2153)

## ABSTRACT

Consumers' rating of "chocolate brands" are majorly based on texture and taste rather than packaging. The texture/taste of a chocolate bar is largely influenced by the cocoa variety used for its production, whereas, its bioactive constituent is directly affected by the seed processing/chocolate manufacturing technique(s) adopted, and the additives used. Cacao is the key ingredient for chocolate production; therefore, the choicest varieties must be used to protect consumers' interest. Currently, the availability of the African variety is the only reason why it is globally sought-after for chocolate production rather than its taste. Therefore, a transfer of genetic materials from quality cocoa breeds into the high-yielding and resilient African variety or vice versa, would inferably increase the availability of quality cocoa beans all-year-round, and also increase the chances of obtaining sumptuous and palatable chocolates anywhere in the world.

## 1. Introduction

The cocoa (cacao) plant is one of the most important crops in the world with high economic value, and a complete repertoire of bioactive substances that are useful in medicine and physical therapy; cocoa also have a partial proclivity to religion and folk medicine (Vooru et al., 2019). In the past, it was used as food, drink (beverage) and a "sacred" item for performing rituals, a tradition that is still practiced in some

parts of Africa. The generic name "*Theobroma*" is a combination of two (2) Greek words (*Theo*- "God" and *broma*- "Food"). Currently, cocoa is fast becoming more important to humanity than in ancient times i.e., it is now mostly used by food manufacturing industries, pharmaceutical companies, tobacco, wine industries etc., amongst others. The term "cocoa beans" simply refer to the seeds found in the fruit of the *Theobroma cacao* tree (Cocoa "English" or Cacao "Spanish") covered by a layer of sweet spongy pulp (Vooru et al., 2019). Presently, the economic

**Abbreviations:** ORAC, Oxygen Radical Absorbance Capacity; RDA, Recommended Dietary Allowance; eNOS, Endothelium-derived Nitric Oxide Synthase; LDL, Low-Density Lipoprotein; ROS, Reactive Oxygen Species; HDL, High-Density Lipoprotein; TNF- $\alpha$ , Tumor Necrosis Factor  $\alpha$ ; IL-1 to IL-10, Interleukin 1 to 10; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas9, CRISPR-associated protein 9; sgRNA, Single Guide Ribonucleic Acid; crRNA, CRISPR RNA; tracrRNA, Trans-Activating CRISPR RNA; PAM, Protospacer-Adjacent Motif; psgR-Cas9-At, Phosphorylated Single Guide RNA-Cas9-At; BbsI, Biobricks Standard Idempotency (Restriction Endonuclease); PCR, Polymerase Chain Reaction; HindIII, Site-specific Deoxyribonuclease Enzyme from *Haemophilus Influenzae*; EcoRI, Restriction Endonuclease Enzyme isolated from *E. coli*; CTAB, Cetyl trimethylammonium bromide; RFLP, Restriction Fragment Length Polymorphism; dCAPs, Derived Cleaved Amplified Polymorphic Sequences; AFLP, Amplification Fragment Length Polymorphism; T-DNA, Transfer Deoxyribonucleic Acid; T generation, Transgenic generation; cGMP, Cyclic Guanosine Monophosphate.

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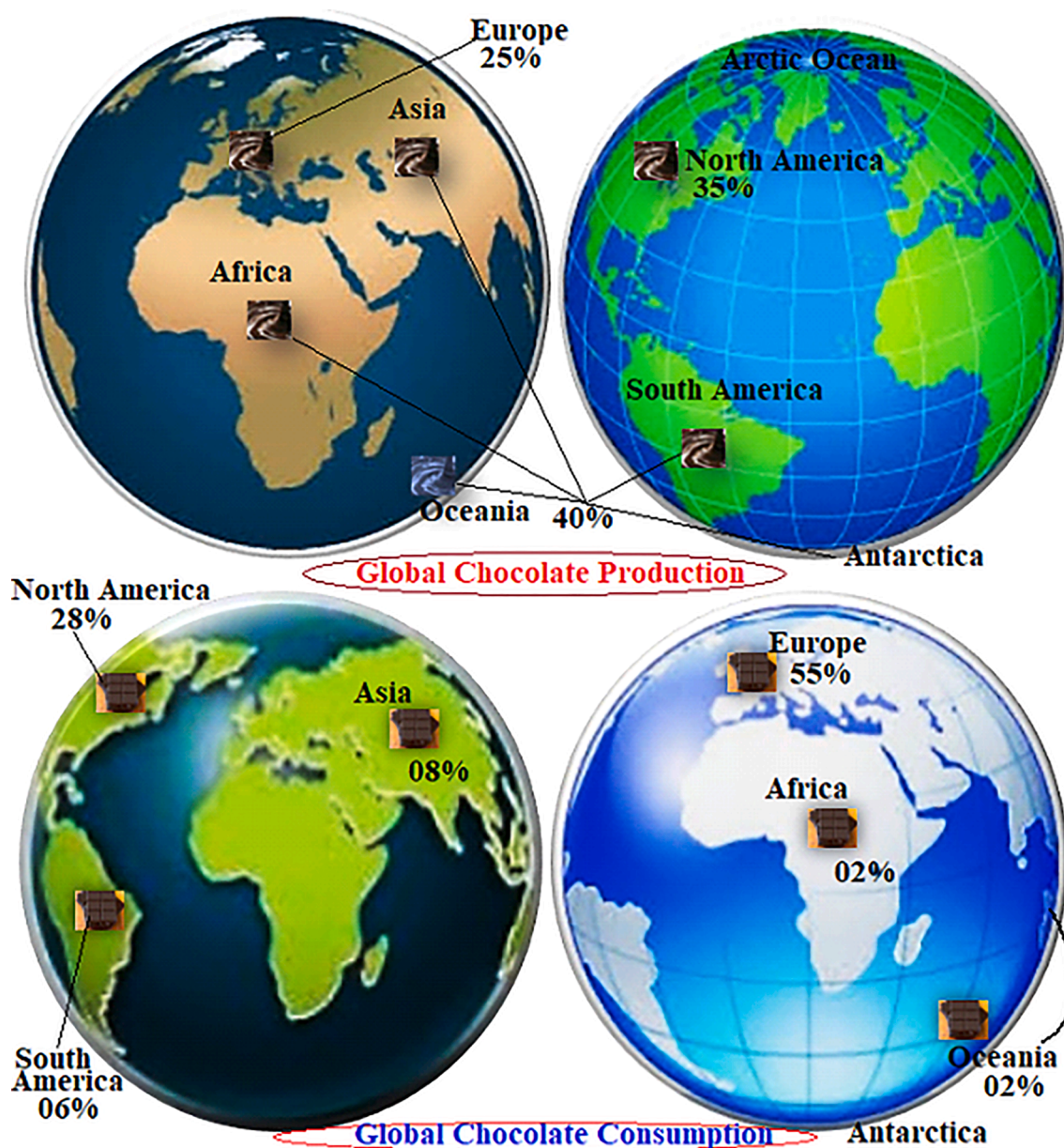
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value of cocoa can only be rivaled by few cash crops (Etaware, 2019; Etaware & Adedeji, 2019; Etaware, Adedeji, Osowole, & Odebode, 2020)

A major consumer of cocoa beans available in the global market was and still is the “chocolate industry”, with net value of over 43% per annum (Eghbal, 2018; Market Watch, 2019). In 2016, the global chocolate market was dominated by North America (35%) and Europe (25%), who were leading producers of chocolate in the world (Poelmans and Swinnen, 2019). The rate of global chocolate consumption was on the increase too from Asia (0.12 kg/person/year) through America (5.18 kg/person/year) to Europe (11.85 kg/person/year), with Europe top on the global consumption chart (55%), trailed by North America (28%), Asia (8%), Latin America (6%) and lastly, Africa and Australia with a share of 2% each (Fig. 1). Chocolates are made from cocoa liquor and sweeteners (e.g. sugar, saccharine or natural honey). The “cocoa liquor” is regarded as the paste obtained from pulverized and processed cocoa beans (nibs), and it contains about 55% cocoa butter (Theobroma

oil) and 45% cacao cake (Wolters Kluwer Health, 2020). The proportion of cocoa liquor in the final product determines how dark the chocolate will become e.g. 70–85% in dark chocolate, 35% in semisweet or bittersweet chocolate, 20% in white chocolate and 10–12% in milk chocolate (Callebaut, 2017), Cocoa powder made by partial de-fattening of cocoa liquor contains only 22% cocoa butter.

Sadly, cacao beans are only produced almost exclusively within 20°N and 20°S of the equator (Voora et al., 2019), strictly based on climate, edaphic and other environmental factors. The beans are majorly produced in Africa, with over 70% of the world’s cocoa beans supplied by West Africa alone (Shahbandeh, 2020), ironically, Europe and America jointly account for consumption of over 60% of cacao beans produced worldwide (Fig. 2). unfortunately, the yield per hectare of land in West and Central Africa is very low (200–700 kg per ha), as a result over 40% of cocoa farmers within that region are running at a loss, and the only available option is to totally abandon their cocoa farmlands or sell it to other investors (Etaware, 2019; Etaware & Adedeji, 2019; Etaware et al.,



Source: Poelmans and Swinnen (2019)

Fig. 1. Global Statistics on Chocolate production and consumption (2016).



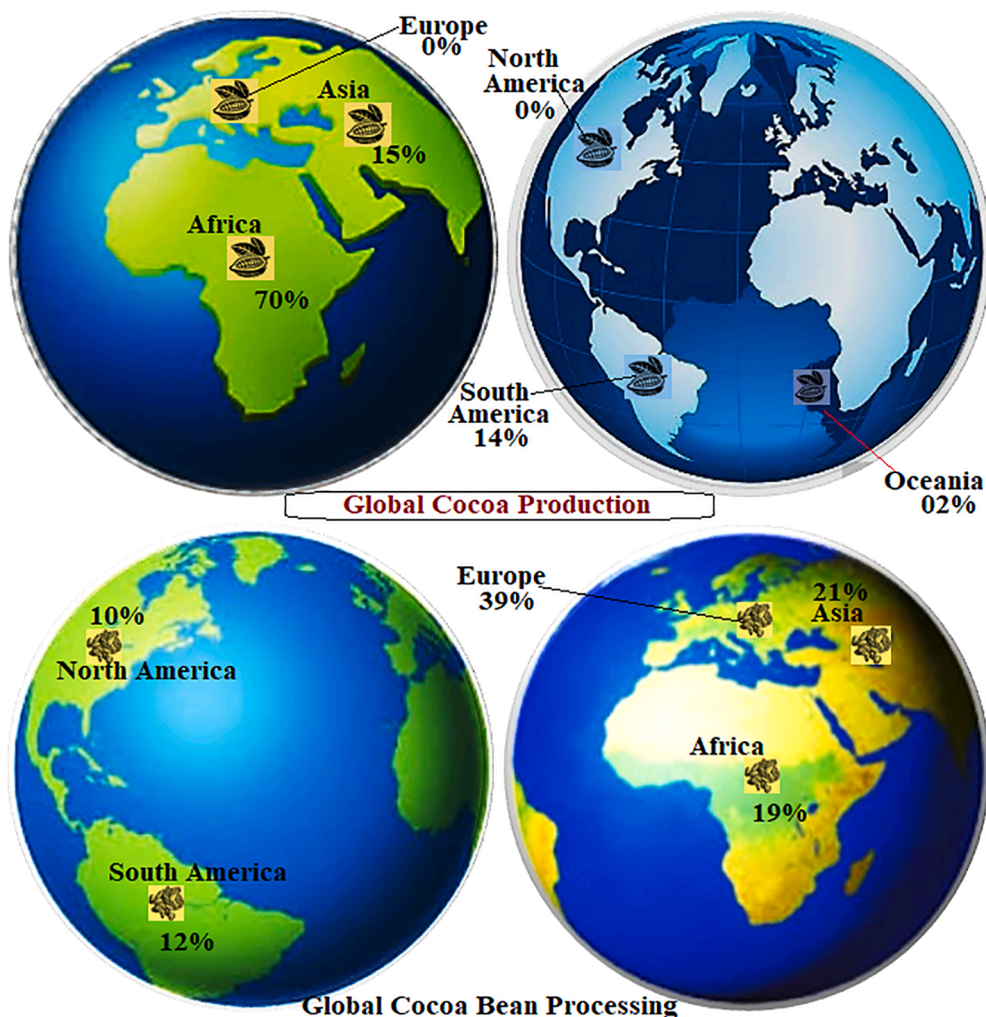


Fig. 2. Statistics on world cocoa production and processing (2018/2019 and 2019/2020).

2020). To turn this situation around, most cocoa farmers had to intensify their efforts in cocoa cultivation by increasing their farm size to further offset the Production-Loss ratio. This was only a temporary solution, because at the long run, the percentage loss was almost more than the profits realized after sales.

A more effective solution is to develop genetically modified cocoa species with greater resilience to diseases and pests, high yielding, drought tolerant, durable with good flavour and high bean quality etc. which will help increase the yield per hectare of cocoa produced in West and Central Africa, as well as repurpose Africa towards sustainable cocoa production in the nearest future. For this aim to be achieved, rigorous genome editing should be conducted on the existing cocoa species within Africa, in order to achieve a genetically stable (Transgenic or Mutagenic) variety with greater quality, flavour and improved phytochemistry, that can easily adapt to the climatic and edaphic factors of the African terrain, and also positively improve the food quality of chocolates and other products manufactured from cacao beans, since their quality mostly depends on the quality of the cocoa cultivar. The good news is that some researchers perturbed by the sharp decline in cocoa production are currently adopting the latest cutting-edge technology in gene editing “CRISPR/Cas9” to produce quality cocoa varieties that are both resilient to common diseases limiting cocoa production in the world e.g., black pod disease of cocoa, frosty pod rot etc., and also, better flavoured due to the genetic modification of genes responsible for the production of bioactive substances in cocoa (Pokou et al., 2019; Fister et al., 2018a; Fister et al., 2018b; Gallego et al., 2018;

Fister et al., 2016a; Fister et al., 2016b; Fister et al., 2016c; Fister et al., 2015), but how well these genetically modified cocoa varieties will fit into the global cocoa market is yet to be ascertained.

### 1.1. Types of cocoa

There are three (2) broad varieties and one (1) natural mutagen of cocoa cultivated around the world, other cultivars found in the global market are either mutagens or transgenic progeny obtained from genetic manipulation or crossbreeding of these varieties (Waters, 2017). They are:

#### 1.1.1. Criollo

The Criollo cacao variety is mostly cultivated in South (Latin) America. The cocoa pods are mostly red, white, green or yellow with rough or warty skin and pointed tips. The beans are light purple to white in colour, and they have the finest flavour amongst all the existing cocoa varieties (Lindt & Sprungli, 2021). Criollo varieties possess the finest and best flavoured beans which is highly priced in the global market, but its production is very minimal due to its low level of resilience to diseases and pests.

#### 1.1.2. Forastero

It is popularly grown in Africa, in countries like Cote D’Ivoire, Ghana, Nigeria, Cameroun, Sao Tome and Principe etc. It accounts for the highest population of cocoa beans in circulation around the world

(70 – 75% of global production). The colour of the pod ranges from red to yellow. The tree is much hardy, durable, and a bit resilient to disease than other varieties and it produces beans with poor flavour, astringent taste and low quality compared to other varieties. The thick-skinned smooth pods yield flat, violet coloured beans (Lindt & Sprungli, 2021).

### 1.1.3. Trinitario

The Trinitario cocoa cultivar is an unintentional cross breed between the very subtle Criollo cocoa variety and the hardy Forastero cultivar which took place in Trinidad and Tobago around 1730. It was first planted as a separate cultivar in Ceylon between 1834 and 1880, and later introduced to other countries like Madagascar, Fiji, Singapore, Samoa and Tanzania etc. Due to its fine flavour, it is mostly used as substitute for Criollo variety in making chocolates. The pods are soft, mostly red, white, green or yellow with coarse skin.

## 1.2. The cocoa bean phytochemistry and its health benefits to humans

Cacao beans contain beneficial phytochemicals that are anti-cholesterolemic, antidiabetic, hypotensive, promote fat combustion in the body, enhance bowel contraction, prevent tumor formation (anti-cancerous), prevent bronchial asthma, improve proper brain functioning and prevent memory loss (amnesia or alzheimer). It also contains beneficial amino acids like glutamine, arginine and leucine (Fig. 3) that can facilitate healing of cuts or wounds in humans. Regular consumption of cocoa is good for the heart and skin, it can reduce immense muscular fatigue and can eradicate moodiness due to the presence of stimulants “Theobromine” and “Caffeine” in minute quantities etc. Some of the benefits of consumption of cacao beans are further explained below:

### 1.2.1. Induce hypotension in humans

The flavonoids present in the cacao beans are very useful in lowering blood pressure level in humans. It has been reported that cacao flavonoids (Fig. 4) can help improve the elasticity of blood vessels, thereby, increasing the functionality of the human circulatory system (Heiss et al., 2015; Sansone et al., 2015).

### 1.2.2. Reduce the risk of amnesia and alzheimer

Regular consumption of cocoa can help improve the functionality of the brain by increasing the flow of blood to the brain. This can help minimize the risk of loss or impairment of semantic memory in children and adults (Amnesia and Alzheimer) and can also help cure vascular disorder in humans (Socci et al., 2017).

### 1.2.3. Antihypercholesterolemia

Cocoa beans contain stearic acid that can reduce hypercholesterolemia because of its hypoglycemic effects on cholesterol and glucose in the blood stream (Heiss et al., 2015; Sansone et al., 2015).

### 1.2.4. Antidiabetics

Frequent consumption of cocoa can effectively increase the functioning of the “Islets of Langerhans” by improving the sensitivity of the insulin produced, which will further facilitate glucose metabolism within the system (Mellor et al., 2018). Proanthocyanidins also prevents the formation of cataracts which is promoted by diabetic situations (Health Benefits, 2020).

### 1.2.5. Antiasthma

Cacao beans contain “Theophylline” and “Xanthine” (Fig. 5) that are very effective in the relaxation of bronchial spasms with rapid dilation of constricted bronchial tubes. It is a more effective remedy that promotes the flow of air easily within the lungs and it can also serve as a cure for allergies, shortness of breath and other asthmatic conditions (Health Benefits, 2020).

### 1.2.6. Antiobesity remedy

Obesity is a situation caused by high intake of fatty substances. Regular consumption of cocoa can help increase the metabolism of fats and lipids in the human body, and also reduce the level of production and storage of fat within the adipose tissues. It can also help regulate body temperature by promoting the production of body heat from the combustion of fats stored in the liver and adipose tissues (Health Benefits, 2020).

### 1.2.7. Dermatological effects in humans

Cocoa butter is an important component of skin moisturizing cream and lotions, and its beneficial effects on skin may extend beyond topical application. Research conducted on cocoa butter application to the skin showed that cocoa flavanols are essential in protecting skin from damage caused by exposure to UV light. Cocoa butter also contains phytochemicals that can improve blood flow to the skin, thereby providing adequate nourishment to the skin and slowing down the rate at which the skin ages. Furthermore, regular topical application of cocoa butter on the skin can help rejuvenate the skin, remove scars, wrinkles and stretchmarks or other skin blemishes from the skin (Wilson and Watson, 2017).

### 1.2.8. Minerals, amino acids and vitamins present in the cocoa bean

Cacao beans contain more than 300 volatile compounds, majority of which are responsible for its distinctive aroma and flavour. Cocoa flavour is characterized by aliphatic esters, polyphenols, aromatic carbonyls, and Theobromine (Wolters Kluwer Health, 2020), which are majorly responsible for prevention of rancidity of cocoa butter. A review of the composition of 1 g of pulverized cacao bean showed that it contains 3% mineral elements (i.e. Copper [0.04 mg], Iron [0.14 mg], Manganese [0.04 mg], Magnesium [4.99 mg], Phosphorus [7.34 mg], Zinc [0.07 mg], Potassium [15.24 mg], Calcium [1.28 mg] and Selenium [0.14 µg], respectively), 5% amino acids (i.e. Valine [11.77 mg], Isoleucine [7.60 mg], Tryptophan [2.93 mg], Threonine [7.76 mg], Leucine [11.90 mg], Lysine [9.83 mg] and Histidine [3.40 mg], respectively) as shown in Fig 3, 92% nutrient (i.e. Carbohydrate [0.28 g], Total dietary fiber [0.37 g], Protein [0.20 g], and Total lipid fat [0.14 g], respectively), and < 1% vitamins (i.e. Vitamin B2 [0.002 mg] and Niacin [0.02 mg], respectively) (Health benefits, 2020).

### 1.2.9. The health benefits of cocoa polyphenols, stimulants and other phytochemicals

Cocoa is also a dietary source of resveratrol which is a better remedy for hypercholesterolemia in humans, it can also increase the level of insulin sensitivity, it is anti-cancerous, neuro- and cardio-protective, antiaging and anti-inflammatory etc. The pharmacologically active ingredients of cocoa seeds include amines (Fig. 3), alkaloids, polyphenols (Fig. 4), mineral elements, phenylethylamine, N-acyl ethanolamine (Fig. 5) and fatty acids (Fig. 6). Cocoa also contain Theobromine (0.5–2.7%), Caffeine (≥0.25%), theophylline, Phenylethylamine, and Trigonelline. The distinctive bitter taste of cocoa is produced by the reaction of Diketopiperazine with Theobromine during roasting (Wolters Kluwer Health, 2020).

### 1.2.10. Antioxidant activities of the cocoa bean

Cocoa beans contain flavonoids, which is one of the most beneficial subunits of polyphenols. Flavonoids are cardio-protective based on their antioxidant and antiplatelet activities, immune-regulatory effects, and their effects on endothelial cells (Sansone et al., 2015; Socci et al., 2017; Zyzelewicz et al., 2018). Cocoa intake increases serum antioxidant capacity, protecting the endothelium from oxidative stress and endogenous ROS. The epicatechin content of cocoa is primarily responsible for its favorable impact on vascular endothelium, regulating both acute and chronic upregulation of nitric oxide production. Cocoa procyanidins have also been shown to affect signaling pathways of polymorphonuclear cells, white blood cells involved in inflammation and injury.



Kenny and his compatriots demonstrated that flavanol fractions can enhance secretion of the cytokines TNF- $\alpha$ , IL-1, IL-6, and IL-10 from stimulated human peripheral blood mononuclear cells. Cocoa procyanidins decrease IL-2 at the transcriptional level. Ramiro *et al.* demonstrated that IL-4 release can be enhanced in the presence of cocoa flavonoids, thus downregulating T-lymphocyte activation and the acquired immune response.

### 1.3. The food chemistry of chocolate and its health benefits to humans

Chocolate contain part or all of the classes of phytochemicals present in cocoa bean and also, those added during production either through additives, nutrient fortifiers and other ingredients. The quality and quantity of the phytochemicals passed on from the cacao bean to chocolates depends on several factors, such as:

1. The type or variety of cocoa beans used in the chocolate production
2. The processing techniques applied during the preparation of the cocoa beans e.g. the period of fermentation, the type of drying process employed, the duration of drying of the cocoa beans etc.
3. The storage conditions of the processed beans and the percentage of moisture present in the stored beans.
4. The cocoa bean quality i.e. healthy or diseased or badly processed beans.
5. The production or manufacturing techniques adopted for chocolate production and the chemical nature of additives or other ingredients used in chocolate production.

These are some of the factors that can increase or reduce the benefits obtained from the consumption of chocolates. Described below are various health benefits associated with the consumption of chocolates.

#### 1.3.1. Hypocholesteremic effects induced by chocolate consumption

In two (2) independent experimental trials conducted by Mursu and his colleagues, milk chocolate and white chocolate inhibited LDL oxidation. In the latter study, a marker of lipid peroxidation decreased by 11.9% after consumption of white chocolate, dark chocolate, or dark

chocolate enriched with polyphenols. These results indicate that the fatty acids in chocolate may play an important role in LDL oxidation and hypercholesterolemia development (Wolters Kluwer Health, 2020). Though, the lipid content of chocolate is comparatively high, analysis of the lipid composition of cocoa butter, which is a major constituent of chocolate, showed that stearic acid (18:0) was one of the major lipid component found therein. According to the research carried out by Katz and his colleagues, it was discovered that stearic acid is non-atherogenic (Anti-atherosclerosis), non-hepatotoxic, hypocholesterolemic, non-nephrotoxic, cardio-defensive and neuro-protective. Stearic acid is unusual in that it does not elevate serum lipid levels to the same degree that other saturated fatty acids do. Clinical trials conducted by Grassi and his companion using patients with hypertension showed that daily consumption of 100 g flavonoid-rich chocolate well over two (2) weeks, significantly reduced the total level of cholesterol found in the patients' blood serum by 7% and LDL by 12%. Consumption of cocoa and dark chocolate increased HDL by 4% in another clinical trial carried out by Wan and his colleagues. Mellor *et al.* detected substantial increase in HDL levels in subjects consuming a high-polyphenol chocolate, in contrast to those consuming low-polyphenol chocolates over the course of 16 weeks. However, in another clinical trial conducted by Kristherton and his colleagues, HDL increased by 11.4% and 13.7% when subjects consumed dark chocolates and polyphenol-enriched dark chocolates, respectively, but patients who consumed white chocolate showed no appreciable increase in HDL levels (McCulloch, 2018).

#### 1.3.2. The importance of dietary fibre in chocolates

The consumption of 1 kcal of energy producing chocolate, containing 70–85% cocoa liquor (dark chocolate), can provide the body with 170 mg of fibre, while those made from 35% (bittersweet or semisweet chocolate) and 10–12% (milk chocolate) cocoa liquor can provide the body with 120 and 60 mg of total dietary fibre, respectively. Though, soluble fibre is important in the reduction of serum cholesterol and other low-density lipoprotein formed within the blood stream, total dietary fiber is recognized as important for weight maintenance, and insoluble fiber has been associated with reduced risk of type-2 diabetes (Heiss *et al.*, 2015; Sansone *et al.*, 2015).

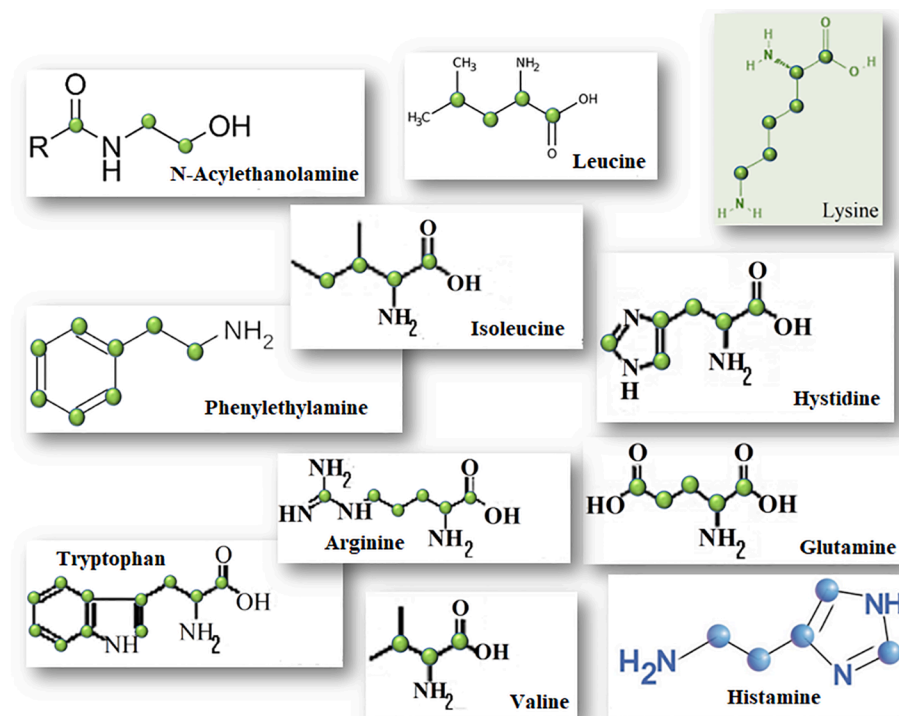


Fig. 3. Amino acids found in cocoa beans.

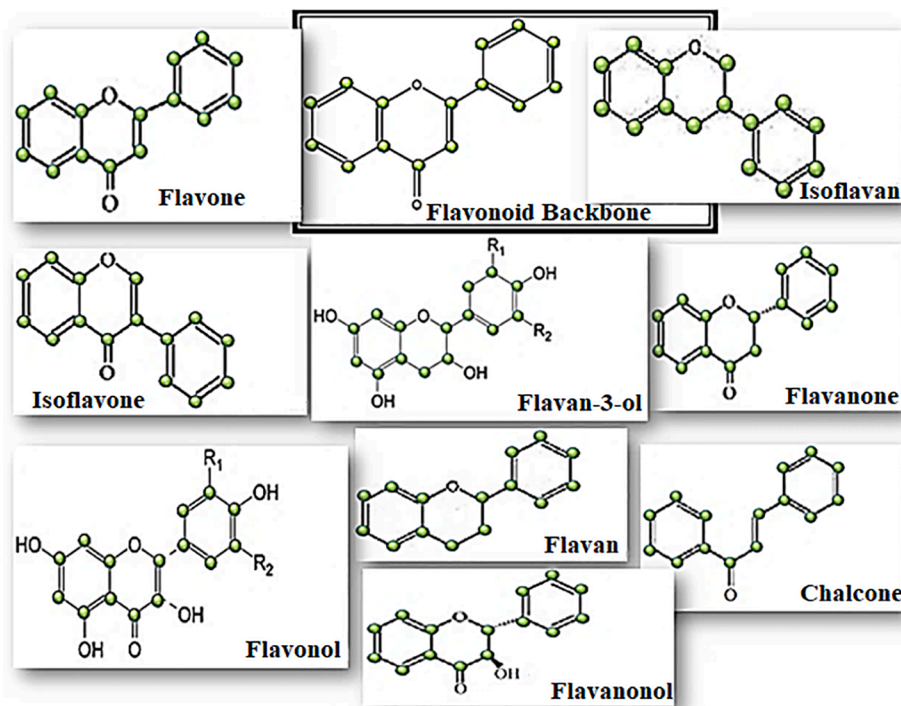


Fig. 4. Classes of flavonoids present in the cocoa beans.

### 1.3.3. The health benefits of minerals found in chocolate

A portion of dark chocolate that can provide the body with 100 kcal of energy, contains 36 mg of magnesium, which is 9% of the USA recommended dietary allowance (RDA) for men between 45 and 65 years old, one-third of which can be supplied by the same amount of milk chocolate (11 mg). Milk chocolate provides 10% of the USA RDA for copper per 100 kcal energy portion, whereas dark chocolate provides

31% and cocoa powder 23% per tablespoon of each product. Dark chocolate contains 114 mg potassium (2% RDA) in every 100 kcal energy portion and milk chocolate contains 67 mg (1% RDA). Iron (Fe) deficiency is one of the most important nutritional problems in the world. Milk chocolate contains 5% of the RDA for Iron for adult men and postmenopausal women (0.42 mg) per 100 kcal energy portion consumed, whereas, dark chocolate provides 25% of the RDA (1.90 mg).

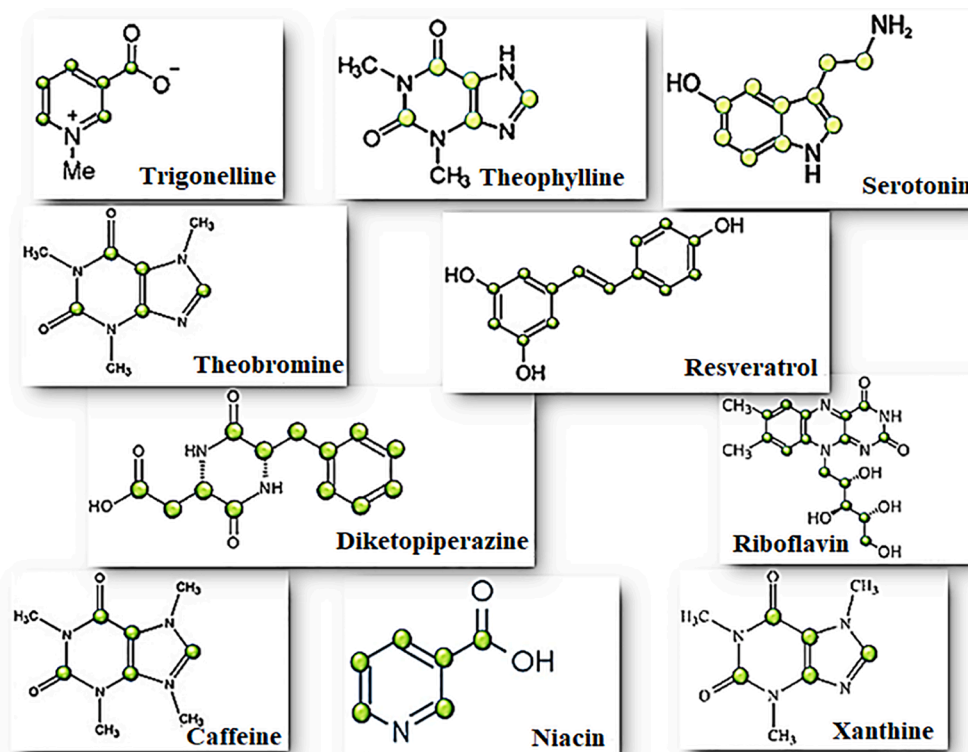


Fig. 5. Stimulants, anti-tumor, anti-hypercholesteremic, cardio-protective compounds and some essential vitamins found in cocoa.

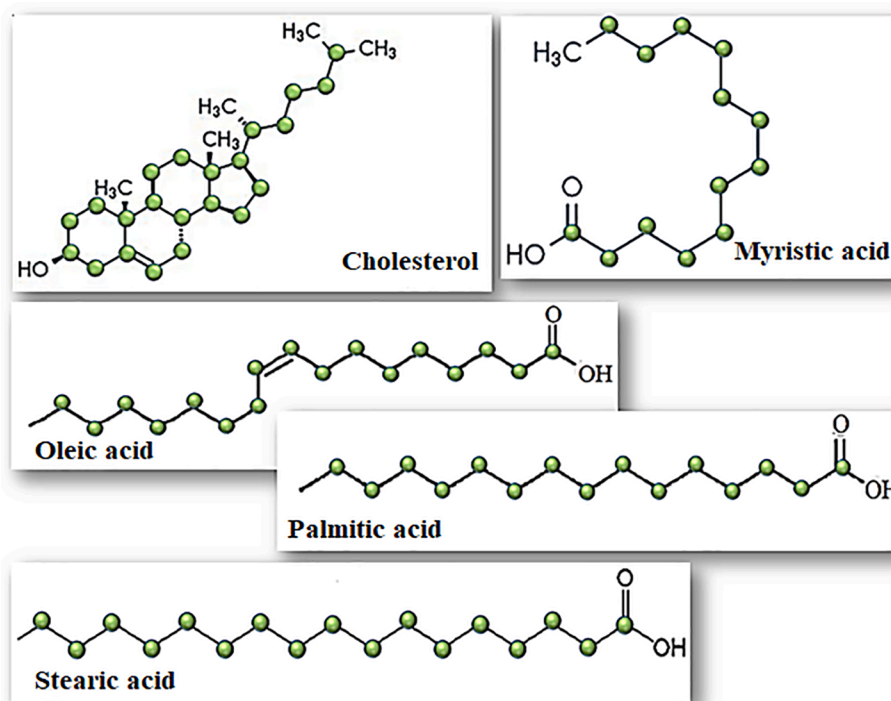


Fig. 6. Triglyceride fatty acids found in cocoa, cholesterol and myristic acid structure.

Magnesium is a pertinent component in the synthesis of protein. It is an important element that aids muscle relaxation, and energy production. Normal amount of Magnesium in the diet can help fight against arrhythmia and hypertension. Copper is highly relevant in the formation of a series of cellular enzymes and is required for several metabolic processes including iron transport, glucose metabolism, infant growth, and brain development. The deficiency in the required proportion of dietary copper in humans can result in anaemia, pancytopenia, hypertension, inflammation and myocardial hypertrophy. However, an increase in the amount of copper in the blood stream may be harmful to human health. High serum copper concentration has been known to increase the risk of death from heart failure and cancer. Fortunately, it is very unlikely that chocolate consumption would elevate serum copper concentrations to harmful levels. Dietary potassium may protect against hypertension caused by excess sodium intake. Low potassium intake has been associated with increased risk of cardiovascular mortality.

#### 1.3.4. Antioxidant activities of chocolates

Analysis of the antioxidant properties of chocolate showed that a typical chocolate candy contains a combined “catechin-epicatechin” (C-E) content of 460 to 610 mg/kg. Epicatechins are responsible for the improvement of vascular functions, reduction of blood pressure, improvement of insulin sensitivity, and reduction of platelet activity (Sansone et al., 2015). Hurst and his team of researchers in 2009 investigated various commercially manufactured chocolates, they found out that milk chocolate bars maintain oxygen radical absorbance capacity (ORAC), total polyphenols, and flavan-3-ol monomers for at least 50 weeks in commercial preparations. It is believed that flavonoids most likely represent the active ingredients responsible for immunomodulatory effects.

#### 1.3.5. Cognitive mood improvement through chocolate consumption

The consumption of chocolate can elevate mood and elicit joy to a greater extent in adults and children more than an apple, but these effects are mostly pronounced few minutes after consumption. This suggests that the sensory effects experienced by most individuals while eating chocolate was indeed responsible for mood improvement

(Kowalska et al., 2019), rather than the previous notion held by scientist on the chemical induction of the brain cells by stimulants from the chocolate, which would be expected to be manifested much later after consumption and the effects would seemingly last longer. Research show that chocolate consumption can give a short-term improvement of “negative mood”, but that the improvement is likely attributable to chocolate’s palatability rather than neuro-stimulation (Socci et al., 2017).

#### 1.4. Cocoa genome engineering

The International Cocoa Genome Sequencing Consortium (ICGS), coordinated by CIRAD presented a detailed analysis of the genomic sequence of the Criollo cocoa variety. The genome sequence identified 28,798 protein-coding genes, and about 20% of the cacao genome consists of transposable elements. Several genes were identified as coding for flavonoids, aromatic terpenes, theobromine and many other metabolites involved in cocoa flavour and quality traits, among which a relatively high proportion of genes were identified as codons for polyphenols, which constitute up to 8% of cacao pod’s dry weight. The information retrieved from the [Cacao Genome Database \(2018\)](#) showed that:

1	Cocoa Variety:	Criollo
2	Chromosome type:	Diploid
3	NCBI genome ID:	572
4	Genome size:	345.99–430 Mbp
5	Number of chromosomes:	10 pairs (2n = 20)
6	Year of Determination:	2010
7	Source:	Cocoa Genome Database (2018)

The genome sequence is a welcome approach that will facilitate cacao molecular biology and breeding for elite varieties through marker-assisted selection, with preference to breeding for improved flavour and high cocoa bean quality, genetic resistance to fungal (oomycetes), viral and other related diseases responsible for huge yield losses annually. Furthermore, numerous researches are ongoing in Africa and around the world to produce transgenic and mutagenic cocoa varieties



that are resilient to diseases and pests, high yielding, drought tolerant with good bean quality and high survival rate. One of such research is that initiated by the commercial chocolate company “Mars, Incorporated” and the University of California, Berkeley in the use of the novel CRISPR/Cas9 technology to edit cocoa DNA to improve the hardness, flavour and productivity of cocoa varieties around the world. The aforementioned approach is in line with the aim of this article.

The current article is focused on the deletion of the cacao Non-Expressor of Pathogenesis-Related 3 (TcNPR3) gene, identified by Fister and his colleagues as the gene that suppresses defense response in cocoa. The use of CRISPR/Cas9 to knock out this gene in cocoa (using *Agrobacterium tumefaciens* as the conveyor vehicle) would enhance disease resistance in cocoa i.e. the genetically modified cocoa plants will be resilient to black pod disease caused by *Phytophthora* sp. worldwide (Fister et al., 2018b). black pod disease is a major limiting factor to global cocoa production (Etaware, 2019; Etaware & Adedeji, 2019; Etaware et al., 2020; Obiakara et al., 2020). The CRISPR/Cas9 method of genome editing, proposed in this article as a potential tool for production of viable transgenic cocoa varieties in Africa is described below:

## 2. Note: The procedure adapted for genome editing of cocoa cultivars in this report was an excerpt from Liu et al. (2015).

### 2.1. Synthesis of sgRNA fragment and recognition of target loci

The sgRNA is the principal driver for gene insertion or deletion. It consists primarily of a customized crRNA sequence attached to the backbone structure known as tracrRNA sequence. The sgRNA fragment can be designed manually or synthesized *in vivo* or *in vitro*. In principle, the specificity of the Cas9 nuclease is determined by the 20-nt guide sequence within the sgRNA. A potential target sequence must instantaneously precede the PAM (5'-NGG-3') to be recognized by the first 20-nt of sgRNA via Watson-Crick base pairing. The recognition between sgRNA and its target will lead to Cas9 cleavage at 3 bp upstream from the PAM.

**Note:** “N” represents specific nucleotides like Adenine, Thymine, Cytosine, Guanine etc.

### 2.2. Synthesis of oligonucleotides used as guide

Once a 20-nt target site is identified, a pair of DNA oligonucleotides (oligos) can be synthesized as follows,

Forward oligo: 5'-gattGNNNNNNNNNNNNNNNNNN NGG-3'

Reverse oligo: 3'-ctaaCNNNNNNNNNNNNNNNNNN NCC-5'

### 2.3. Vector construction

#### 2.3.1. Single strand (1 ×) sgRNA-Cas9 plasmid

To construct a single strand (1 ×) sgRNA-Cas9 plasmid

1	Phosphorylate and anneal each pair of oligos.
2	Digest 0.5 µg of psgR-Cas9-At with BbsI nuclease for at least 2hrs at 37 °C.
3	Ligate the BbsI-digested vector with the oligo duplex.
4	Transform <i>E. coli</i> with the ligation product.
5	Identify positive clones of <i>E. coli</i> by colony PCR.

#### 2.3.2. Double strand (2 ×) sgRNA-Cas9 plasmid

To construct a double strand (2 ×) sgRNA-Cas9 plasmid

1	Construct two separate single strands of sgRNA-Cas9 plasmid
2	Amplify the double strand sgRNA module from the single strands sgRNA-Cas9 plasmids
3	Digest the PCR product of the single and double strands sgRNA plasmids and module
4	Ligate the digested products to obtain a double strand sgRNA-Cas9 plasmid.
5	Transcribe the ligation product into <i>E. coli</i> .
6	Identify positive clones of <i>E. coli</i> by colony PCR.

#### 2.3.3. Sub-clone the sgRNA-Cas9 cassette into the plant expression vector

1	Digest the pCambia1300 vector and 1 × or 2 × sgRNA-Cas9 plasmid with HindIII and EcoRI
2	Run the reactions on a 1% agarose gel,
3	Set up the ligation reaction and incubate at 16 °C for 2hrs.
4	Transcribe the ligation product into <i>E. coli</i> .
5	Identify positive clones of <i>E. coli</i> by colony PCR.
6	Purify plasmid from the culture of the positive clone
7	Transcribe the plasmid into <i>Agrobacterium tumefaciens</i>

### 2.4. Deletion of TcNPR3 in the desired cocoa variety

A detailed procedure for introduction of the transformed *Agrobacterium tumefaciens* into the desired cocoa variety, in order to complete the knockout of the TcNPR3 gene, was described by Fister et al. (2018b).

### 2.5. Examination of sgRNA-Cas9-mediated gene modifications

The examination of the target genes to ascertain if they were modified during the process was aided by the following methods:

#### 2.5.1. Detection of targeted gene modification in T1 generation

Since the leaves of plants in the T1 generation mostly contain chimeric DNAs, detection of targeted gene modifications can be performed with a single piece of leaf. Genomic DNAs of the leaf samples should be extracted using CTAB method (Springer 2010). At least 24 representative samples should be analyzed for each T1 population.

#### 2.5.2. Detection of targeted gene modifications by RFLP

A prerequisite for the use of this assay is that the selected cleavage sites for CRISPR/Cas9 (3 bp upstream of PAM) should be found within the restriction endonuclease site, so that PCR amplicons from gene-modified samples would be resistant to restriction enzyme digestion.

- Create DNA amplicons from the genetically modified samples using PCR
- Digest the PCR products
- Digested products should be analyzed by electrophoresis in a 1.5% agarose gel.

**Note:** PCR amplicons should be resistant to restriction enzyme digestion by showing uncleaved bands.

#### 2.5.3. Detection of targeted gene modifications by dCAPs assay

For targeted gene sites that contain no restriction enzyme sites for RFLP assay, dCAPs assay can be used as an alternative choice for mutation detection (Liu et al., 2015).

#### 2.5.4. Detection of targeted gene modifications by Surveyor assay

Surveyor nuclease is a mismatch-specific DNA endonuclease that cleaves with high specificity at the 3' side of any mismatch site in both DNA strands, including base substitutions, insertions or deletions (Qiu et al., 2004).

- Design the relevant primers needed for Surveyor assay
- Amplify the DNA fragments using PCR
- Analyze 5.0 µL of the PCR product by electrophoresis on a 1.5% agarose gel

#### 2.5.5. Detection of targeted gene deletion by AFLP

In a situation whereby two adjacent genomic sites (within a range of

100-bp to 1-kb) are targeted, long fragment deletions between the two targeted sites occur frequently, which facilitate the detection of gene deletion by AFLP. Primers used for AFLP assay are designed to amplify DNA fragment containing both target sites.

#### 2.5.6. Detection of targeted gene modification by DNA sequencing

PCR primers are designed to amplify a specific DNA fragment surrounding the genomic target site. PCR products are purified and sequenced using either primer. A typical chromatogram for chimera shows unique peaks before the sgRNA target site but, immediately after the target site, multiple peaks start to appear in each nucleotide position.

#### 2.6. Selection of stable genes in T2 generation inherited from the T1 generation

T2 seedlings from those T1 lines are then transplanted to soil and used for further screening for the desired gene modifications. The selected lines are kept for further cultivation to produce seeds. In cases of low efficiency in detection of the target genes or in a situation where multiplex gene modifications are expected, the population size used for mutation analysis should be increased.

#### 2.7. Selection of stably inherited gene modifications in T3 generation

A good number of T3 seeds should be planted for each of the selected T2 lines for segregation and identification of T-DNA free plants with stable inherited genes. PCR analyses for T-DNA sequence should be performed using the appropriate primers for the Cas9 genes.

**Examples:** Cas9-3451F: 5'-CCCAAGAGGAACAGCGATAAG-3'

Cas9-4115R: 5'-GGTCGATGGTGGTCAAAG-3'

### 3. Summary

It is indeed obvious that the food chemistry of chocolate is primarily determined by the cultivar or variety of cocoa used for chocolate production i.e. the quality and quantity of phytochemicals present in chocolate(s) is determined by the number and quality of the phytochemicals present in the primary source (cocoa beans). Also, the processing techniques used in the preparation of the cacao beans and the procedure adopted in the manufacturing of chocolate(s) can help sustain or deplete these beneficial phytochemicals. The problem of short-supply of cocoa beans in the global cocoa market can be averted by the implementation of genetically modified disease resistant varieties through point deletion of specific genes. The alteration of this gene "TcNPR3" will only affect the defense mechanism of the cocoa plant positively, other beneficial factors will remain unaltered. Finally, whether or not this genetically engineered cocoa varieties will be accepted in full by manufacturers and consumers of cocoa products, is still under debate.

### 4. Conclusion

It is indeed obvious that a highly flavoured cacao bean will increase the palatability of chocolates produced from it. The satisfaction obtained from the consumption of chocolate is largely attributed to its food components, directly obtained from cocoa beans. Therefore, the current article elucidated the link between the phytochemical component of the cacao bean and that of the food chemistry of chocolates, their mechanism(s) of action and the role(s) they play or function(s) they perform in the human body. Currently, the availability of the African cocoa variety (Forastero) is the only reason why it is globally sought-after for chocolate production, NOT because of its flavour, phytochemical or nutrient composition. Therefore, a transfer of genetic materials from foreign cocoa breeds into the high-yielding and resilient African varieties or vice versa, would inferably increase the availability of quality cocoa beans in

the global market.

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### 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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