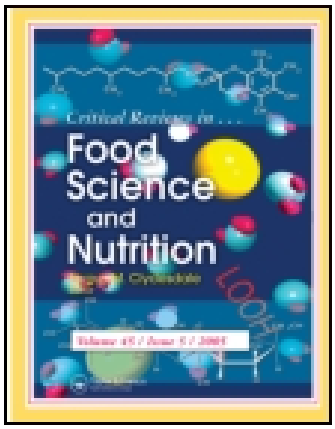

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Cocoa Agronomy, Quality, Nutritional, and Health Aspects

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Cocoa Agronomy, Quality, Nutritional, and Health Aspects

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The history of cocoa and chocolate including the birth and the expansion of the chocolate industry was described. Recent developments in the industry and cocoa economy were briefly depicted. An overview of the classification of cacao as well as studies on phenotypic and genetic diversity was presented. Cocoa agronomic practices including traditional and modern propagation techniques were reviewed. Nutrition-related health benefits derived from cocoa consumption were listed and widely reviewed. The specific action of cocoa antioxidants was compared to those of teas and wines. Effects of adding milk to chocolate and chocolate drinks versus bioavailability of cocoa polyphenols were discussed. Finally, flavor, sensory, microbiological, and toxicological aspects of cocoa consumption were presented.

Keywords Cocoa, genetic diversity, health benefits, antioxidants, contamination, flavor

1. INTRODUCTION

1.1. The History of Cocoa and Chocolate

Cacao, designated *Theobroma cacao* by the 18th Century botanist, Carolus Linnaeus, is an important Neotropical, perennial crop, on which the thriving global chocolate industry is based. Cacao's putative centre of genetic diversity is at the headwaters of the Amazon River, South America (Cheesman, 1944) and it is indigenous to the Amazon and Orinoco rainforests. "Cocoa" is the dried and fermented fatty seed of the cacao tree from which chocolate is made. The term "cocoa" is also used to refer to cocoa powder, the dry powder made by grinding cocoa seeds and removing the cocoa butter from the cocoa solids. "Cacao" was the Aztec word for chocolate and *Theobroma* means "Food of the Gods", in keeping with the Aztecs' regard for the drink they made from cacao seeds. According to Aztec mythology, the cacao tree was brought to earth by the god, Quetzalcoatl, and planted in Southern Mexico and the Yucatan peninsula (Young, 1994).

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In pre-Columbian times, the fruity pulp surrounding cacao seeds was used in South America as "a refreshing source of liquid or was fermented to produce *chicha* (an alcoholic beverage often made from maize or manioc/cassava)" (Henderson et al., 2007). It is believed that cacao was transported to Central America by Amerindians from South America. Henderson et al. (2007) have provided evidence demonstrating that cacao beverages were consumed in northern Honduras from as early as 1100 B.C. Circa 1000 B.C., cacao's earliest known name, "kakawa," came into use among the Olmec Indians, inhabitants of the Mexican Gulf Coast, who built the first of the great Mesoamerican civilizations. The Olmecs are believed to be the first to grow cacao as a domestic crop (Coe and Coe, 1996). By 400 B.C. to A.D. 100, the Mayan Indians of northern Guatemala adopted the word "cacao" from the Olmecs. The Mayans later established the earliest known cocoa plantations in the Yucatan. By the time the Mayan civilization collapsed circa 900 A.D. and the Toltec state emerged, cocoa beans were a major Mesoamerican commodity. Circa 1500 A.D., the Aztec empire, founded in the late 14th century in the area that is now Mexico City, annexed the richest cocoa region in Mesoamerica: Xoconochco (later Soconosco) along the Pacific coast of modern Chiapas, Mexico and Guatemala (Bergmann, 1969).

Subsequently, cultivation of cacao and chocolate processing were expanded in Central America, and fermented cacao *chichas* were also produced there by the time the Spanish conquistadors, under Hernán Cortés, arrived in Mexico in 1519 (Thomas, 1994). The preparation by the Aztecs involved fermenting, drying, optionally toasting, and grinding cocoa seeds then mixing the resulting mass with water, red pepper (chilies), and vanilla, among other ingredients such as honey (Presilla, 2001) in a thick, frothed, bitter suspension to produce "xocolatl", the Aztec translation of "bitter water". The Aztec Emperor, Montezuma, drank large quantities of this highly-regarded beverage reputed to have aphrodisiac and health properties. The drink was reserved for the male elite, nobility/royalty and religious ceremonies. However, when the Spanish were offered this drink by Montezuma, they found it unpalatable.

The conquistadors also discovered that cocoa beans were used throughout Mesoamerica as currency, a practice that probably existed for many centuries. In 1502, Columbus and his crew encountered off the coast of present-day Honduras some Amerindians, who were very protective of the "almonds" (cocoa seeds/beans) they were carrying in their canoe (Presilla, 2001).

1.2. The Birth and Expansion of the Chocolate Industry

Cortéz took cocoa beans back to the Spanish court of King Charles V in 1528 along with the equipment necessary for brewing the drink. The Spaniards altered the Aztec recipe and added sugar and other spices (Presilla, 2001) while deleting chili to make a sweet drink that was more to their liking. Monks in Spanish monasteries were directed to produce the chocolate drink, but keep the recipe a secret, and this they did for nearly a century. The Spanish Empire embarked upon establishing a thriving cocoa industry, cultivating cocoa in its colonies in the Americas (Alden, 1976).

An Italian traveler discovered the secret of drinking chocolate in 1606 and spread its fame further afield. Chocolate eventually became popular in Europe in the 17th Century. Its consumption spread quickly to France, Italy, Germany, and then England where the first chocolate house was opened in 1657. In 1659, the first chocolate-confection maker was opened in Paris.

"Eating chocolate" was first introduced to the public in 1674 when the first chocolate *lozenge* appeared in England. In 1720, Italian chocolate-makers received prizes in recognition of the quality of their products. Then in 1765, North America *discovered* cocoa products (Coe and Coe, 1996). At first, eating chocolate was seen as a luxury and was only enjoyed by the "upper class." However, at the end of the 19th Century, chocolate became more popular and accessible to the wider population. In this way, chocolate consumption spread across Europe and around the world, and slowly the presentation of chocolate changed.

Cocoa powder was originally produced by the Dutch in 1828 with the development by van Houten of a hydraulic press to remove excess fat. The chocolate bar originated in Great Britain in 1830, and the Swiss successfully entered the chocolate market with milk chocolate in 1830, followed shortly thereafter with chocolate filled with hazelnuts. The Swiss chocolatier, Rodolphe Lindt, introduced the important process of conching into the chocolate manufacturing process in 1879 (Presilla, 2001). This increases the smoothness of the cocoa mass through agitating with cocoa butter for several hours to "round off" the edges of the sugar crystals.

Chocolate consumption expanded rapidly and continuously thereafter. Pharmacological uses for cocoa and cocoa by-products were increasingly investigated, not unexpectedly since the earliest consumers of cocoa attributed to it "strengthening, restorative and aphrodisiac" properties.

1.3. More Recent Developments in the Chocolate Industry

A relatively recent development is the introduction of a system of classifying chocolates according to the different origins and types of cocoa used in their manufacture – the application of "Appellation d'Origine Controllé" (Controlled Origin Name) label, similar to that used for good wines. The Italian chocolate company, ***Amedei***, now markets "Trinidad", "Venezuela," and "Madagascar" chocolates. The French chocolate company, ***Valrhona***, produces "Gran Couva" from beans purchased from San Juan Estate in Trinidad. This development coincides with an increasing demand for quality (dark) chocolate, recognized for distinct flavors.

Another fairly recent development in the cocoa market is the emergence of trade in cocoa certified as *Organic Cocoa* (ICCO, 2006) [8]. The organic chocolate market is experiencing a strong growth in demand, as evidenced by the success of *Green & Black's*, the United Kingdom's leading organic chocolate maker. This company's turnover increased by 69% to £22.4 million, in 2004, compared with a growth rate of 2% for the global chocolate industry (http://news.bbc.co.uk/2/hi/business/4543583.stm, accessed June 6, 2010). Its *Maya Gold* brand earned it the UK's first Fair-trade mark. However, despite the strong growth that occurred over the past five years, the share of organic cocoa remains small in the cocoa market. The organic cocoa market is estimated to represent less than 0.5% of total cocoa production. However, the demand for organic cocoa products is growing since consumers are increasingly concerned about food safety and environmental issues.

In order for cocoa beans to be certified as organic they must have been produced on land which has been free of prohibited substances for three years prior to harvest (FAO/WHO, 2001). Production methods are strictly regulated (fertilizers, soil conditioners, and pesticides). For chocolate products to be certified as organic, 95% of the ingredients (not counting added water and salt) must be organically produced, and the processor must be a certified organic handler. However, special provisions allow labeling to state that a product is "100% Organic," if the product contains 100% organically produced ingredients. Twenty countries, six in Africa (including Ghana) and 14 in the Americas

(including the Dominican Republic, Costa Rica, Colombia, Ecuador, and Venezuela), are already certified as producers of organic cocoa. Cameroon, Côte d'Ivoire, Guyana, Haiti, Honduras, Indonesia, and the Philippines were in the process of seeking certification in 2006 (ICCO, 2006).

1.4. The Cocoa Economy

The global production of cocoa beans for 2009 to 2010 was estimated as 3.596 million tonnes (ICCO website: http://www.icco.org/about/press2.aspx?Id=zwt12743, assessed June 1, 2010) [11]. The annual global consumption of cocoa beans, in various forms, is approximately 3.63 million tonnes, and is valued at in excess of USD 5 billion. The global chocolate market reached a value of USD 40.6 billion in 2001 with most of the consumption occurring in developed countries such as the United States of America, Germany, France, the United Kingdom, Switzerland, and Belgium. World grindings are expected to increase from almost 3.7 million tonnes in 2007/2008 to 4.1 million tonnes in 2010/2011, at an estimated average growth rate of nearly 2.7% per annum. According to Guiltinan (2007), cocoa and cocoa butter are the raw materials in an approximately US$70 billion chocolate manufacturing, cosmetic, and related products industry.

1.5 Summary

The emergence of cacao as a domesticated crop, from which diverse and sophisticated processed products are derived, has spanned many decades. The flavor attributes as well as the nutritional and health benefits of cocoa have been appreciated from ancient to modern times and this has precipitated the expansion of the cocoa industry and has made the chocolate industry very lucrative.

2. CHARACTERIZATION OF CACAO VARIETIES AND BEANS

2.1. Types of Cacao

Currently, the majority of the global cacao cultivation is based on traditional varieties collected prior to 1950 by collectors such as Dr. F.J. Pound (Lockwood & End, 1993). All of the existing wild, semi-wild, and cultivated cacaos form one interbreeding population. The genetic diversity of cacao existent in the wild is not properly represented in the areas of domestication (Anon, 1981). This may be attributed to the "Founder effect" (Mayr, 1954) since only a relatively small part of the wild genepool was taken into cultivation. Only one-third of the cacao cultivated involves hybrid and clonal cultivars developed by breeding programs in Trinidad, Côte d'Ivoire, Ghana, Brazil, Malaysia, and other producing countries (Eskes & Lanaud, 2001).

Cacao classification is based on botanical/morphological traits, particularly those of the fruit (pod) and seed (bean). Under domestication, three major categories or classes of cacao were recognized: Criollo ("native"), Forastero (Upper and Lower Amazon and Guianese), and Trinitario (Cheesman, 1944; Lachenaud, 1997; Lachenaud et al., 1997; Wood & Lass, 2001). Trinitario is a cross between Criollo and Forastero, and is the type of cacao traditionally grown in Trinidad. Hybridization that produced Trinitario cacao is believed to have first occurred in Trinidad hence the designation.

Amazon Forastero trees provide 95% of the world's cocoa (ICCO website—http://www.icco.org/) and comprise close to 70% of cacao cultivation globally (Eskes and Lanaud, 2001). The production of aromatic cocoas from Criollo and Trinitario cacao trees represents only 4 to 5% of the world market. Criollo and Trinitario beans are collectively known as "fine or flavor" cocoa that is used to manufacture fine chocolates throughout the world, and commands premium prices on the world market. Their flavor ranges from spicy, fruity to that of caramel, nuts, and vanilla.

Criollo fruits or pods are characteristically pointed, red or yellow in color with a warty surface and thin pod walls. The pods may also be smooth or five-angled "pentagona" with five ridges. The plump beans (cotyledons) are white to light purple due to the absence or low concentration of anthocyanin. Criollo cacao is grown throughout Central America, in Mexico and Venezuela. Popular Criollos include *Ocumare 61* and *Chuao* of Venezuela. The best known pure Criollo varieties are designated *Porcelana* and are from Venezuela. Currently, there is a paucity of authentic Criollo trees globally because Criollo varieties tend to be susceptible to diseases and are not vigorous.

In South America, the eastern Cordillera of the Andes with its north-eastern extension marked the boundary between the early distributions of Criollo and Forastero. The divergence of these two groups was most likely mediated by humans. According to Cheesman (1944), man was most probably the agent of dispersal, by selecting the pale-colored, plump beans (least astringent and more aromatic) of Criollo cacao on the eastern side of the Cordillera, crossing the ridge with them and transporting them north-west to Central America. The Isthmus of Panama probably acted as a secondary barrier permitting further differentiation of a distinct Central American Criollo (Cuatrecasas, 1964).

Soria (1970), Figueira et al. (1994), Motomayor et al. (2002; 2003), Sereno et al. (2005), Loor et al. (2009), Johnson et al. (2009), Zhang et al. (2009), and Motilal et al. (2010) present comprehensive information on the various cacao varieties cultivated in Tropical America. Motomayor et al. (2002) and Motilal et al. (2010) have reported on the low genetic diversity and heterozygosity found among the Criollo germplasm they studied.

Susilo et al. (2011) determined, using Simple Sequence Repeat (SSR) markers, that Java (Indonesia) fine/flavor cocoa has a heterogeneous genetic background that excludes direct parentage of pure Criollo cacao or a single cross as was previously believed. The DR cacao clones (1 and 38), which are the main clones that produce Java cocoa, have been found to have

Trinitario, Upper Amazon, and Lower Amazon Forastero cacao as candidate parents.

The term Forastero, meaning "exotic," was originally used in Venezuela to denote a type of cacao introduced from Trinidad in 1825. The Forastero cacaos of Ecuador, Brazil and West Africa are distinct from the original "Forastero" of Venezuela, and are best described as Amazon Forastero. The beans are relatively small and flat and the cotyledons are purple. They are strong in cocoa flavor and thus suited to the manufacture of milk chocolate. The beans have a higher fat content than Criollo cacao, which makes them more desirable in the cocoa trade since butter fat attracts a significant part of the income derived from the sale of cocoa. Amazon Forastero trees are hardy and robust, which explains why they currently provide 95% of the world's cocoa. Iwaro et al. (2003) observed that a relatively larger proportion of individuals from this group show resistance to disease compared to other groups.

The Forastero group contains a variety of pod types, from those with shapes similar to that of some Criollos (oblong, rugose with an attenuate or acute apex) to Amelonado, typical of Brazil, the Orinoco Basin, and the Guyanas, which is inconspicuously furrowed, with an almost smooth surface texture, and blunt or obtuse apex. The fruits are almost always green, ripening yellow. In some forms, there may be traces of red pigmentation in the unripe pods, as is evident in the Parinari accession group, collected in Peru and among the Guianese germplasm collected in French Guiana (Lachenaud and Motamayor, 2004). The fruit wall is relatively thick, with a hard, woody mesocarp.

The Trinitario group comprises a wide range of hybrids between Criollo and Amazon Forastero that originated in Trinidad (Cheesman, 1944), but are also found in various other Caribbean countries, and in Central America, Papua New Guinea and wherever there has been a mixture of Criollo and Amazon Forastero germplasm (Purseglove, 1988; Wood & Lass, 2001). This is thus a very heterogeneous group, displaying extreme phenotypic and genetic diversity.

Apart from the three aforementioned traditionally recognized classes of cacao, there is also Refractario cacao. This group originated in Ecuador and is well represented in the International Cocoa Genebank, Trinidad (ICG,T) (Iwaro et al., 2003). It has considerable potential value for germplasm enhancement, since it contains germplasm selected on farms in Ecuador in the 1920s and 1930s among cacao seedlings, which showed no symptoms of Witches' Broom disease despite high disease pressure (Pound, 1938 and Bartley, 2001). Zhang et al. (2008) reported on the distinct genetic grouping of the Refractarios among a diverse germplasm sample including Parinari accessions (Upper Amazon Forasteros) and Imperial College Selections (Trinitarios) as well as Nacional cacao. The latter might have been derived from a local wild population in the Pacific Coast region of Ecuador (Loor et al., 2009), was classified as Forastero by Cheesman (1944) and Soria (1970), and was found to be genetically close to Forastero (Lerceteau et al., 1997). Nacional cocoa beans are distinguished by their strong floral flavor, which is designated as *Arriba* and are thus in high demand in the cocoa market.

2.2. Cacao Phenotypic and Genetic Diversity

Bekele et al. (2006) found that the phenotic relationships among nearly 600 accessions from the ICG,T were congruent to their genetic relationships. There was a distinct separation between the Trinitario and Amazon Forastero genetic groups or classes based on phenotypic data. A similar finding for Criollo and Forastero clones was reported by Engels (1983; 1986), who also noted a greater affinity between Trinitario and Criollo varieties in terms of bean characteristics. Using some morphological descriptors, N'Goran et al. (1994) were also able to differentiate Criollo from Forastero genotypes.

Ronnig and Schnell (1994) found that Trinitarios differed genetically from Forasteros at four of six loci based on allozyme data for eight polymorphic loci encoding six enzymes. They also found that Caribbean and Central American groups were distinct from South American types. Laurent et al. (1993) distinguished amongst Criollo, Trinitario, and Amazon Forastero groups of cacao using ribosomal probes. N'Goran et al. (1994) clearly separated Lower Amazon Forasteros from Upper Amazon Forasteros and Criollos using RAPD markers. The Trinitarios they studied were spread between the Lower Amazon Forastero and Criollos. N'Goran et al. (2000) also differentiated Criollo from Amazon Forastero genotypes using RFLP analysis. Lerceteau et al. (1997) also showed a clear discrimination between Criollo and Forastero clones using RAPD and RFLP markers. Motomayor et al. (2002) further differentiated between "Modern" and "Ancient" Criollo.

The research reviewed above reinforced the conventional classification of cacao. High genetic diversity was observed among the Forastero group by several scientists including Laurent et al. (1994), N'Goran et al., 1994; 2000; Motomayor et al. (2003), Sounigo et al. (2005), and Zhang et al. (2006), among others.

Bekele et al. (2006) differentiated the Trinitarios and Forasteros from the Refractarios they studied, and Zhang et al. (2008) confirmed that the Refractario is a distinct genetic group. Motilal et al. (2010) found that the Refractario accessions they studied were classified between the Upper Amazon Forastero and Trinitario.

However, the research findings of Motamayor et al. (2008) were significant in that they revealed 10 genetic groups in cacao based on results of genotyping 1241 accessions from a broad geographic area with 106 microsatellite markers and subjecting the data to Bayesian statistics. A new classification of cacao was thus proposed by Motamayor et al. (2008) that was expected to enhance the management of cacao germplasm.

Further studies combining morphological and molecular data should facilitate a better understanding of the patterns of diversity within cacao genebanks, and allow us to determine whether the diversity conserved *ex situ* compares favorably with that in the centre of diversity of cacao. If not, further collection in the wild, and/or *in situ* conservation would be recommended especially since much of the diversity of cacao remains untapped and is threatened by deforestation (Anon, 1981; FAO, 1987).

Progress in genotyping cacao under field conditions should be facilitated in the future by the recent optimization of a Single Nucleotide Polymorphism (SNP) assay for such purposes by Livingstone III et al. (2011) [38]. The latter have replaced the need for a real-time PCR system for genetic fingerprinting with a fluorescence microplate reader and standard thermocycler that reduce the cost of SNP genotyping.

2.3 Summary

The recognized major classes of cacao, *viz.*, Criollo, Forastero, and Trinitario, can be identified in terms of observable traits including flavor as well as by using molecular techniques. The phenotypic and genetic diversity of cacao conserved in the international and national collection centers has been extensively studied and further work is ongoing. Progress is underway to fully characterize flavor/quality attributes and associated volatiles and chemicals of different varieties of cacao from specific geographic areas.

3. CACAO AGRONOMY: CONVENTIONAL AND MODERN APPROACHES

Cacao is cultivated in the Tropics primarily between latitudes 10 degrees N to 10 degrees S of the Equator, usually at altitudes below 294 m. Temperatures must be between 18 and 30°C, with precipitation fairly constant and minimally 1500 mm per annum (ICCO website: http://www.icco.org/about/growing.aspx, accessed on June 7, 2010). The soil should be fairly loose, and rich in potassium, nitrogen, and trace elements. Young cocoa trees are particularly delicate, vulnerable to direct sunlight and wind, and must develop initially in the protective shade of other trees. Mature cocoa trees grow best under moderate shade that may be provided by bananas, cassava, or other useful crops grown between the rows of cacao. Such shade crops supplement farmers' incomes. Permanent shade trees, such as leguminous types, *Inga* and *Gliricidia* spp. and the immortelle/coral tree (*Erythrina* spp.), are usually planted amongst the cacao (Wood and Lass, 2001) at a wider spacing. Recently, more consideration is being given to diversifying cacao production systems by introducing more timber and other useful trees to plantations. Previously, coconuts were used for inter-cropping in South-East Asia, but there were problems with common pests and diseases of both crops. Good models with economically valuable shade or companion trees are under development (Somarriba and Calvo, 1998).

Cacao comes into bearing within two to five years, depending on the variety. Its small flowers, and the fruits (pods) that develop from them after fertilization, over a period of about six months, are borne directly on the trunk and main branches. The interior of the fruit has five cells or loculi, in each of which is a row of from 5 to 12 seeds embedded in a soft, acid pulp. Each fruit contains from 20 to 60 or more seeds, which constitute the raw cocoa or "beans" of commerce. The tree grows to about five meters within three years, and may reach eight meters at about 10 years. A tree normally lives for 30 to 40 years. In most well-maintained plantations, new cocoa trees replace older ones at 25-year intervals.

Cocoa plantations generally have a density of 1000 to 1200 cocoa trees per hectare (ha^{-1}). A producing tree can deliver on average 0.5 to 2 kg of dried seeds per year. In the 1980s, an efficient, modern plantation was capable of producing approximately 1500kg of dried cocoa per hectare (Wood and Lass, 2001). More recently, it is possible to attain yields of up to 2000 to 3000 kg ha^{-1} (Butler, 2004; Maharaj et al., 2005; Pang, 2006). A newly established cocoa plantation can be expected to be profitable after approximately six years.

Apart from requiring specific growing (including soil) conditions, cacao is susceptible to diseases such as fungal infections, and infestation by insects, rodents, and parrots (Wood and Lass, 2001). Among fungal diseases is witches broom caused by *Moniliophthora perniciosa* (particularly in South America and the Caribbean), and black pod rot caused by *Phytophthora* spp. (particularly in Africa). Some insects can cause younger trees to wither or "dieback". In South-East Asia, the "cocoa pod borer" insect (larvae of the moth, *Conopomorpha cramerella* Snellen) causes considerable damage. Diseases and pests can destroy 20 to 30% of total cocoa production. This is why modern plantations devote so much attention to crop protection. The research on cacao has thus been focused on the development of high-yielding, resistant cultivars (Iwaro et al., 2003) that negate the need for heavy use of fungicides that are expensive, may pose risks to human health, and are often deleterious for the environment.

It is important to note that as a result of differences in soil, climate, and cultivation practices, cocoa beans possess different properties, of which flavor is of paramount importance.

3.1. Traditional and Modern Propagation Techniques

Rooted cuttings, grafting (top and side), and budding are traditional vegetative propagation techniques applied to cacao (Wood and Lass, 2001). In addition, a large proportion of plants has been and is still generated from seed. More recently, somatic embryogenesis has been investigated under laboratory and field conditions as a feasible *in vitro* clonal propagation method for commercial production of cacao plants (Maximova et al. 2005; 2008). Maximova et al. (2008) found that somatic embryo-derived plants demonstrated normal phenotypes and growth under field conditions when compared to plants propagated by traditional methods. The suitability and cost efficiency of this technique are under investigation and will determine the prospects for commercial application. Silva et al. (2009) refined the aforementioned transformation system through their evaluation of the effects of polyamines and antibiotics on somatic embryogenesis and the efficiency of *uidA* gene transfer by *Agrobacterium tumefaciens* in the genetic transformation in cacao.

3.2. Planting Cacao

Cacao should be planted in the most suitable pattern and density according to the varietal requirements to ensure high productivity and easy management of the farms. During the first three years, weeding is often required. Once the canopy has closed, reduction in light will prevent weed growth. Young trees need no pruning. However, low branches should be pruned to facilitate harvesting from older trees. Vertical growth is usually restricted to the first jorquette (fan of branches) (Wood and Lass, 2001)

3.3. Plant Densities

Maharaj et al. (2005), Souza et al. (2009), among others, found that it is possible to optimize the regional cocoa yields by implementing high planting densities (1736–2500 trees ha^{-1}). However, Souza et al. (2009) observed plant density by year interaction where increased yields were restricted to the first half of the crop period. In the second half, a lower planting density (1059 trees ha^{-1}) resulted in the best yield.

Mooleedhar and Lauckner (1990) demonstrated the need for determining the optimal variety × spacing design. Pang (2006) reported that the optimal planting density appears to be higher for many of the Upper Amazon types with high general combining ability for yield efficiency than it is for some of the Trinitarios. He recommended selection for adaptation to planting density as a higher priority than selection for yield efficiency.

4. MOLECULAR APPROACHES: APPLICATION OF GENOMIC TOOLS, GENE DISCOVERY AND GENETIC TRANSFORMATION

Significant success has been achieved in adopting modern techniques (such as tissue culture; somatic embryogenesis) to facilitate propagation in cacao. In addition, linkage mapping of the cacao genome to identify quantitative trait loci (QTL) and marker-assisted selection to accelerate progress in breeding for improved planting material are ongoing (Lanaud et al., 2004; Guiltinan, 2007; Brown et al., 2008; Kuhn et al., 2009).

The selection criteria employed in cocoa improvement programs are based on the characters of economic importance. Among traits, which superior varieties should have, are good bean weight, a high number of beans per pod, good yield potential, and resistance to diseases [preferably horizontal/nonspecific and durable resistance (Simmonds, 1994)]. Other selection criteria include vigor, uniform plant type, precocity (early flowering), drought tolerance, and bean quality (flavor and butterfat content). Characters that can be selected at an early stage of plant development are preferred. The identification of QTLs for the aforementioned characters would facilitate the development of superior genotypes for planting and evaluation under specific environmental conditions.

Guiltinan (2007) and Clement et al. (2004) described the value of the recently constructed cacao bacterial artificial chromosome (BAC) libraries for gene discovery in cacao. Marcano et al. (2009) reported on a genome-wide mapping study for yield factors and morphological traits in cacao. Lanaud et al. (2009) presented their findings on a meta–QTL analysis of resistance traits to *Phytophthora* spp. and other diseases in cacao. Kuhn et al. (2011) identified 19 COSII SNP markers that co-locate with existing QTLs in cacao, and that can be used for genotyping and off-typing in cacao breeding programs and for genetic mapping and studies to trace co-location of genes regulating important traits in cacao and other species. Feltus et al. (2011) used a BAC-based physical map to identify candidate genes for bean shape, pod weight, and Black Pod resistance contained within QTL intervals.

While research on genetic transformation in cacao has been useful in the analysis of gene function, public opposition precludes the use of this system in crop improvement (Guiltinan, 2007). Nevertheless, the future outlook for improved cacao planting material was deemed promising based on the ongoing research; conventional breeding and that involving genomic tools (Wilkinson, 2001; Figueira and Alemanno, 2005; Guiltinan, 2007; Guiltinan et al., 2008; Kuhn et al., 2011).

Progress in the application of genomic tools, gene discovery, and molecular approaches culminated in the release of a preliminary cacao genome map by two independent research teams in 2010. In September, 2010, Mars, IBM, USDA released the results of their cacao genome mapping project on the internet (Cacao Genome Database website: http://www.cacaogenomedb.org/). While the sequence provided is a preliminary release, it covers 92% of the genome with approximately 35,000 genes. The sequencing was done using the highly homozygous cultivar, Matina 1–6. The other research team genetically sequenced an ancient Mayan variety of cacao, a highly homozygous Criollo designated as B97–61/B2 that was domesticated about 3000 years ago in Belize. They identified the genes that influence the production of flavonoids in cacao, which are thought to offer cardiovascular benefits, and isolated the gene that determines the melting point of chocolate as well as of hundreds of genes potentially involved in pathogen resistance. Their genetic map includes approximately 87% of the assembled sequences on 10 *pseudochromosomes* (*Theobroma cacao L.* has only 10 pairs of chromosomes) and identified 28,798 genes that code for proteins. They assigned 88% or 23,529 of these protein-coding genes to one of the 10 chromosomes in the Criollo cacao tree studied. Additional details are available on the following website: http://cocoagendb.cirad.fr/gbrowse/cgi-bin/gbrowse/theobroma/ and in the publication by Argout et al. (2011), which was released online in December 2010.

4.1. Summary

Much progress has been made in the last decade in cacao genomics. The sequencing of the cacao genome has elucidated the

connection between genes and traits of interest and will facilitate scientists and breeders in more easily developing superior cacao plants in terms of disease and pest resistance, yield, flavor, flavanol (antioxidant) content, and other traits of economic interest or with potential health benefits. This augurs well for the future of cocoa farmers and the global cocoa industry. The emergence of cocoa as a commodity with health/nutraceutical benefits will have significant economic and social impacts. Research in this area is expected to expand rapidly in the future.

5. NUTRITIONAL AND HEALTH BENEFITS

The belief that cocoa and cocoa products may have some health benefits is not a new concept (Keen, 2001) as the Pre-Columbian societies were known to use chocolate as medicine, too (Lippi, 2009). The recognition of these products as significant sources of phytochemicals with healthful effects has been significant. Chocolate is no longer considered a guilty pleasure, and has positive health benefits when eaten in moderation as part of a balanced diet. The long-term effectiveness and appropriate dose range of cocoa consumption are not clear. Future research efforts should concentrate on higher-quality and more rigorous randomized trials with longer follow-ups to resolve the uncertainty regarding the clinical effectiveness. Then chocolate can really be eaten without feeling guilty!

5.1. Nutritional Components

Cocoa is the nonfat (or rather low-fat) component of cocoa liquor (finely ground cocoa beans) which is used in chocolate making or as cocoa powder (commonly 12% fat) for cooking and drinks (Cooper et al., 2008a). Using triacylglycerol profiling, the detection limit was 1% cocoa butter equivalents (CBE) in chocolate fat (0.3% CBE in milk chocolate, having a fat content of 30%). For quantification, the average error for prediction was 1.2% CBE in chocolate fat, corresponding to 0.4% in milk chocolate (30% fat content) (Buchgraber et al., 2007). Cocoa liquor contains about 55% cocoa butter which contributes to cocoa solids (Buchgraber et al., 2007). The term chocolate refers to the combination of cocoa, cocoa butter, sugar into a solid food product. Chocolate contains high concentrations of refined sugar and have a very saturated fat content derived from the cacao bean itself, added milk powder, or the addition of vegetable oils to chocolate products (McShea et al., 2008). Fat and sugar are major components of chocolate, and provide significant energy that needs to be taken into account when assessing possible risks and benefits of recommending chocolate consumption for health purposes (Cooper et al., 2008b). Although chocolate contains a high amount of saturated fats, the two major fatty acids are palmitic and stearic acids (Borchers et al., 2000) with about one–third of the lipid in cocoa butter being composed of stearic acid (Pearson, 1994; Kris-Etherton, 1997; Steinberg et al., 2003). The fat in chocolate comes from cocoa butter and consists of approximately equal amounts of oleic acid (a monounsaturated, heart-healthy fat) and stearic and palmitic acids (both saturated fats) (Alspach, 2007). Stearic acid seems to have fewer implications for the progression of coronary heart disease (Borchers et al., 2000), does not seem to augment the risk of coronary heart diseases and nutritionally linked cancer as other fatty acids (Weisburger, 2001) and exerts a neutral effect on cholesterol (Gu et al., 2002; Steinberg et al., 2003; Ding et al., 2006; Alspach, 2007) in neither raising nor lowering LDL-cholesterol levels (Alspach, 2007). The underlying reason appears to be one of limited absorption from the intestinal tract in contrast to most other lipids (Weisburger 2001). However, Connor (1999) suggested that stearic acid represented a risk for heart disease, as do other saturated fats. The health benefits of a chocolate bar are controversial due to the large amounts of saturated fats despite exhibiting strong antioxidant activity (Lee et al., 2003).

Free amino acids, oligopeptides, and reducing sugars are cocoa aroma precursors formed during the fermentation process. The content and distribution of free amino acids in raw cocoa from different origins varied greatly (5–25 mg g^{-1} fat free dry matter) (Rohsius et al., 2006). On average, the hydrophobic amino acids accounted for more than 60 mol% (15 mol% leucine, 15 mol% alanine, 10 mol% phenylalanine, 9 mol% valine, 5 mol% isoleucine, and 5 mol% tyrosine), while acidic free amino acids and the remainders accounted for 21 mol% per group (Rohsius et al., 2006). Up to 38 minutes of roasting, the total soluble protein and albumin concentration of the roasted nibs diminished slightly, all amino acids showed significant losses with the exception of methionine, phenylalanine, and histidine and the liquor had acceptable sensory characteristics (Abecia–Soria et al., 2005).

Cocoa fiber is rich in dietary fiber (mainly insoluble dietary fiber) and has significant amounts of soluble dietary fiber (Lecumberri et al., 2007a). The consumption of cocoa fiber with a hypercholesterolemic diet in rats was proven to improve the lipidemic profile and reduced lipid peroxidation, suggesting that it might have contributed to a reduction of cardiovascular risk (Lecumberri et al. 2007b). The effect of cocoa powder containing indigestible dextrin (a water-soluble dietary fiber) significantly increased the defecation frequency and fecal amount compared to those of placebo intake periods, nonintake period, and intermediate nonintake period on 40 male and female volunteers (Furukawa et al., 2004).

The nutritional quality of fiber-enriched baked products benefited from a decrease of both fat and digestible polysaccharide contents by using fibers as partial replacers of fat and/or starch without significant loss of dough functionality (Collar, 2007). Cocoa shell is a major by-product from the cocoa industry, and it is rich in dietary fiber (Moulay et al., 2006). Dietary fibers from cocoa shell which were added up to 6% resulted in sensory acceptable, and long-term stored innovative fiber-enriched wheat bread formulations (Collar et al., 2009).

A study of the mineral element composition of 30 powdered cocoa beverages (PCBs), made in Nigeria, revealed that

the products were rich sources of Ca and P (Shittu and Badmus, 2009). Fe, Cu, and Cr were found in trace amounts. Most PCBs samples had Pb content above the maximum permissible level of 1.0 mg/g. The calcium content correlated significantly with phosphorus content (Shittu and Badmus, 2009). In determining the bioavailability of Fe in cocoa powder using the Hb regeneration efficiency method suggested that cocoa was a significant source of moderately bioavailable Fe using a rodent model (Yokoi et al., 2009). Cocoa powders may be significant Fe sources for human subjects. Cocoa and cocoa products contained relatively higher amount of magnesium compared to black tea, red wine, and apples (USDA, 2011).

5.2. Glycemic Effects

Cocoa extract may possess potential hypoglycemic and hypocholesterolemic effects on serum glucose levels and lipid profiles (Ruzaidi et al., 2005). These same authors suggested that further work would be required to elucidate the exact mechanism by which polyphenols present in cocoa extract can lower the serum glucose levels and improve lipid profiles in diabetic rats (Ruzaidi et al., 2005). A further study to evaluate the protective effect of cocoa polyphenol-rich extract (CE) on glucose levels in streptozotocin-induced diabetic rats showed that in the 20 mg CE-pretreated group there was a 143% increase in plasma glucose levels, compared with a 226% increase in diabetic control rats. Hence, the pretreatment with CE from roasted cocoa beans could prevent the development of diabetes induced by STZ injection in rats (Ruzaidi et al., 2008).

The glycemic index of chocolate and chocolate-containing confectionery was shown to elicit relatively low levels of postprandial glycemia compared with equicarbohydrate amounts of starchy staples such as bread, rice and potatoes (Foster-Powell et al., 2002). Dark chocolate, for example, had a glycemic index (GI) of 50 compared with many varieties of bread, rice, and potato which have GI values > 70 (Foster-Powell et al., 2002). The low glycemic response can be attributed at least in part to the sugar content of chocolate confectionery (Brand-Miller et al., 2003).

In a study involving 15 healthy young adults with typical Italian diets, comparisons were made on the basis of supplementing daily with 100 g dark chocolate versus 90 g white chocolate, each of which provided 480 kcal. The dark chocolate supplement was associated with improved insulin resistance and sensitivity and decreased systolic blood pressure, whereas white chocolate had no effect (Grassi et al., 2004). The presence of cocoa powder in foods has been shown to lead to greater postprandial insulin secretion than alternate flavorings. In general, the chocolate product produced 28% greater insulinemia than the alternate flavor, ranging from 45% greater in the chocolate milk versus the strawberry milk, to 13% greater in dark versus white block chocolate (Brand-Miller et al., 2003).

5.3. Gastrointestinal Effects

Lactose intolerance is a common gastrointestinal disorder which is associated with incomplete digestion of lactose. A study of the tolerance to lactose in milk chocolate among symptomatic lactose maldigesters suggested that 12 g of lactose present in milk chocolate is well tolerated. Ingestion of milk chocolate produced only minor gastrointestinal complaints and did not differ significantly from those experienced by persons eating lactose-free chocolate (Järvinen et al., 2003).

The prebiotic potential of cocoa flavanols was assessed in a randomized, double-blind, crossover, controlled intervention study (Tzounis et al., 2011). The daily consumption of high-cocoa flavanol drink for four weeks significantly increased the bifidobacterial ($P < 0.01$) and lactobacilli ($P < 0.001$) count but significantly decreased clostridia counts ($P < 0.001$) when compared to low-cocoa flavanol. The study revealed that consumption of cocoa flavanols could significantly affect the growth of select gut microflora in humans, thus providing for potential prebiotic benefits associated with the dietary inclusion of flavanol-rich foods (Tzounis et al., 2011).

5.4. Cognitive and Mood Elevation

The findings of a report on the acute cognitive function associated with cocoa flavanol consumption suggest that the consumption of 520 mg cocoa flavanol and to a lesser extent 994 mg CF may be beneficial to performance and mood during highly effortful cognitive processing (Scholey et al., 2010). Chocolate contains tryptophan, which is a chemical the brain uses to produce serotonin, a proven anti-depressant. It also generates feelings of euphoria. In addition, the theobromine and phenylethylamine in cocoa have a stimulating effect. The suppression of mitogen-induced degradation of tryptophan due to an inhibition of activated indoleamine 2,3-dioxygenase by tested cacao extracts enhanced the availability of tryptophan for serotonin synthesis, which could have a mood elevating effect (Jenny et al., 2009).

5.5. Summary

Chocolate can be high in refined sugar and saturated fat content derived from the cocoa bean itself, added milk powder, or the addition of vegetable oils to chocolate products. Fat and sugar of chocolate provide significant energy. This high caloric content must be considered when assessing possible risks and benefits (strong antioxidant activity) of recommending chocolate consumption for health purposes. Unlike chocolate, cocoa is not as high in sugar and fat. The glycemic index of chocolate and chocolate-containing confectionery was shown to elicit relatively low levels of postprandial glycemia compared with equicarbohydrate amounts of starchy staples such as bread, rice, and potatoes. It is suggested that by balancing total calorie

intake, flavanols from cocoa products may provide some cardiovascular benefit if included as part of a healthy diet for patients with essential hypertension. Stearic acid seems to have fewer implications for the progression of coronary heart disease and does not seem to augment the risk of coronary heart diseases and nutritionally linked cancer as other fatty acids and exerts a neutral effect on cholesterol. The consumption of cocoa fiber with a hypercholesterolemic diet in rats was proven to improve the lipidemic profile and reduced lipid peroxidation, suggesting that it might have contributed to a reduction of cardiovascular risk. Chocolate also contains tryptophan, which is a chemical that brain uses to produce serotonin, a proven anti-depressant and also generates feelings of euphoria.

6. SELECTED COCOA COMPONENTS

6.1. Polyphenols & Flavonoids

Cocoa beans and cocoa-containing products are rich source of dietary polyphenols (Cooper et al., 2007; Rimbach et al., 2009; Rusconi and Conti, 2010) contributing to about 10% of the dry weight of the whole bean and its derivative chocolate, particularly dark chocolate (Rusconi and Conti, 2010). Cocoa beans from different countries of origins and the methods of preparation (primary and secondary) could also partially influence the antioxidant polyphenols of cocoa products (Jalil and Ismail, 2008).

Cocoa and chocolate products are rich in flavanol monomers, oligomers, and polymers [procyanidins (PC)] (Langer et al., 2011). Cocoa flavan-3-ols or catechins are a subclass of flavonoids which are, in turn, a subclass of polyphenols (Arts et al., 1999; Santos-Buelga and Scalbert et al., 2000; Wollgast and Anklam, 2000; Fraga et al., 2005; Lamuela-Raventós et al., 2005; Ramiro-Puig and Castell, 2009; Neilson and Ferruzzi 2011). PCs were found to be the most potent antioxidant species using a novel liquid chromatography-mass spectrometry (LC-MS) for the identification and quantitative analysis of antioxidants in methanolic extracts of cocoa powder (Calderón et al., 2009). The correlation coefficient between antioxidant capacity and PCs in chocolates was 0.92, which suggest that the PCs were the dominant antioxidants in cocoa and chocolates (Gu et al., 2006). PCs contain 2.3 or up to 10 of the catechin or epicatechin units linked, which is fairly distinctive (Natsume et al., 2000; Miller et al., 2008). Catechin and epicatechin were the most abundant antioxidants followed by their dimers and trimers in cocoa as screened by liquid chromatography-mass spectrometry (Calderón et al., 2009). Epicatechin was in part linked to the reported vascular effects observed after the consumption of flavanol-rich cocoa beverages (Schroeter et al., 2006).

Using rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols, epicatechin concentrations could be used to predict the content of other polyphenols, especially epicatechin-4-8-epicatechin and epicatechin-4-8-epicatechin-4-8-epicatechin and total polyphenols content (Cooper et al., 2007). The (−)-catechin content was not predictable from the epicatechin content, and it is concluded that this is the main form of polyphenol that varies according to manufacturing conditions and cocoa origin (Cooper et al., 2007). Theobromine and caffeine were major compounds detected in the resulting fractions (F1 and F2) from extracted cocoa crude extracts by LC-MS. Monomer, dimer, and trimer were identified in fraction 3 (F3) (Maleyki and Ismail, 2010). Catechin and epicatechin in F3 was stable when stored at 4°C and −20°C for five months. Methylxanthines (theobromine, caffeine, and theophylline) which were separated from cocoa powder showed low antioxidant capacity compared with other fractions. However, their presence could reduce antioxidant capacity of flavonoids in cocoa powder (Maleyki and Ismail, 2010).

Natural cocoa was found to contain the highest levels of antioxidant activities, total polyphenols, and PCs followed by baking chocolates, dark chocolates and baking chips, and finally milk chocolate and chocolate syrups (Miller et al., 2006; Miller et al., 2009). Natural cocoa powders (average 87% of nonfat cocoa solid) contained the high levels of total antioxidant capacity (826 ± 103 μmol of TE/g) and PCs (40.8 ± 8.3 mg/g) (Gu et al., 2006) and were reported to contain up to 70 mg polyphenols/g (expressed as catechin) (Vinson et al., 1999). Unsweetened chocolates or chocolate liquor (50% nonfat cocoa solids) contained 496 ± 40 μmol of TE/g of AOC and 22.3 ± 2.9 mg/g of procyanidins. Milk chocolates, which contain the least amount of nonfat cocoa solids (7.1%), had the lowest concentrations of AOC (80 ± 10 μmol of TE/g) and PCs (2.7 ± 0.5 mg/g) (Gu et al., 2006). The hydrophilic antioxidant capacity contributed >90% of total antioxidant capacity in all products.

High cocoa content dark chocolate was found to be the richest in polyphenols, although each chocolate was different in polyphenol content (Cooper et al., 2007). *Trans*-resveratrol and *trans*-piceid were identified in dark chocolate and cocoa liquor extracts. The exceptional antioxidant activity of chocolate could be related to its high PC content rather than to the presence of stilbenes (Counet et al., 2006). Flavanol levels of 12 commonly consumed brands of dark chocolate were quantified and correlated with% theobromine and% nonfat cocoa solids (Langer et al., 2011). Epicatechin comprised the largest fraction of total chocolate flavonoids. All dark chocolate contained higher levels of total flavanols (93.5–651.1 mg of epicatechin equiv/100 g of product) than a milk or a white chocolate (40.6 and 0.0 mg of epicatechin equiv/100 g, respectively).

One serving of cocoa (5 g) or chocolate (15 or 40 g, depending upon the type of chocolate) provided 2000 to 9100 μmol of Trolox equivalents (TE) per gram of antioxidant capacity and 45 to 517 mg of PCs (Gu et al., 2003), amounts that exceed a serving of the majority of foods consumed in America (Gu et al., 2006). Chocolate is more variable with some products containing no flavonoids (0.09 mg PC/g), whereas others are high in flavonoids (4 mg PC/g) (Kris-Etherton and Keen, 2002). Thus, approximate estimates of flavonoid-rich chocolate needed

to exert acute and chronic effects are 38 and 125 g, respectively (Kris-Etherton and Keen, 2002)

The PC content was related to the nonfat cocoa solid (NFCS) content. The natural cocoa powders (average 87% of nonfat cocoa) contained the highest levels of antioxidant capacity (AOC: 826 ± 103 μmol of TE/g) and PCs (40.8 ± 8.3 mg/g) (Gu et al., 2003). Unsweetened chocolates or chocolate liquor (50% nonfat cocoa solids) contained 496 ± 40 μmol of TE/g of AOC and 22.3 ± 2.9 mg/g of PCs. Milk chocolates, which contain the least amount of NFCS (7.1%), had the lowest concentrations of AOC (80 ± 10 μmol of TE /g) and PCs (2.7 ± 0.5 mg/g) Gu et al., 2003). In dark chocolates, the nonfat cocoa solids were linearly related to total polyphenols (Cooper et al., 2008a). For dark chocolates, a strong linear correlation was associated between nonfat cocoa solids and oxygen radical absorbance capacity ($R^2 = 0.9849$), total polyphenols ($R^2 = 0.9793$), and procyanidins ($R^2 = 0.946$), respectively (Miller et al., 2006). On the basis of principal component analysis, 81.4% of the dark chocolate sample was associated with nonfat cocoa solids, antioxidant activity, total polyphenols, and PCs (Miller et al., 2006). A study of some major brands of commercial cocoa products available in the Spanish market indicated that (−)-epicatechin was in the range of 116.02–730.26 μg/g, whereas (+)-catechin was in the range of 81.40–447.62 μg/g (Andres–Lacueva et al., 2008).

Cocoa-containing products tested range from cocoa powder with 227.34 ± 17.23 mg of PCs per serving to 25.75 ± 9.91 mg of PCs per serving for chocolate syrup (Miller et al., 2009). A rough estimate of the dosage consumed during nominal consumption of antioxidant-rich dark chocolate corresponded to a dose of up to 2.8 g of (−)-epicatechin per 40 g serving (or 40 mg/kg for an average human) (McShea et al., 2008).

Methylxanthines (theobromine, caffeine, and theophylline), which have been separated from cocoa powder, have shown low antioxidant capacity compared with other fractions (Maleyki and Ismail, 2010). Also, the presence of methylxanthines could reduce the antioxidant capacity of flavonoids (Maleyki and Ismail, 2010).

6.2. Processing Effects

Many of the confectionery manufacturing processes (i.e. fermentation, drying, roasting, and alkalizing) could destroy considerable amounts of flavonoids, so most cocoa mixes and chocolate products sold on contain little or no flavonoids (Ariefdjohan and Savaiano, 2005). Cocoa and chocolate, when processed appropriately, may contain relatively large amounts of flavonoids, particularly catechin and epicatechin (Ariefdjohan and Savaiano, 2005). The composition of the cocoa or chocolate must be carefully defined with regard to the proportions of polyphenols in the monomeric, oligomeric, and polymeric forms, as further research on milk chocolate to settle the bioavailability issue is required (Cooper et al., 2008a). The concentration of flavanols in any chocolate would depend on both the flavanol content of the cocoa bean and the processes used for transforming the cocoa into chocolate (Fraga et al., 2005). During processing, flavan-3-ols are lost and, as a consequence, are not necessarily the major components in many commercial cocoas and dark chocolates (Gu et al., 2006). Depending on the processing methods, cocoa and chocolate could contain significant amounts of antioxidants, mainly as the flavanol monomers of catechin and epicatechin (Hammerstone et al., 1999). Over 10% of the weight of cocoa powder in beverages was flavonoids (Hammerstone et al., 2000).

The flavor of chocolate was due to a significant concentration of polyphenols embedded in the triglyceride matrix of fermented, dried, and roasted cacao seeds (Stark et al., 2006; McShea et al., 2008). "Raw" chocolate which was underfermented and under-roasted with very high cacao solid percentages (90–100%) may contain higher levels of antioxidant polyphenols, but would likely to be exceedingly unpalatable (McShea et al., 2008). The more processed the chocolate, the more flavonoids (and all of their related health benefits) are lost. The flavanol content could vary in cocoa-based products due to the use of different processing techniques, for instance fresh cocoa beans of 9776 mg/100 g total flavanols (Gu et al., 2002), cocoa powder of 4784 mg/100 g total flavanols (Rein et al., 2000) and dark chocolate (highly alkalized) of 3.3 mg/serving (37g) (Schramm et al., 2001). It was reported that differences in cocoa bean blends and processing, with the possible exception of dutching, were minor factors in determining the level of antioxidants (Miller et al., 2006).

Quercetin-3-arabinoside and isoquercitrin were the major flavanols in the cocoa powder products ranging from 2.10 to 40.33 μg/g and from 3.97 to 42.74 μg/g, respectively, followed by quercetin-3-glucuronide (0.13–9.88 μg/g) and quercetin aglycone (0.28–3.25 μg/g) (Andres–Lacueva et al., 2008). The alkali treatment resulted in 60% loss in these cocoa products of the mean total flavonoid content. Among flavanols, (−)-epicatechin presented a larger decline than (+)-catechin (38%), probably because of its epimerization into (−)-catechin, a less bioavailable form of catechin (Andres–Lacueva et al., 2008). Also, a decline was also confirmed for di-, tri-, and tetrameric PCs (Andres–Lacueva et al., 2008). These changes due to the alkali treatment, could affect the antioxidant properties and the polyphenol bioavailability of cocoa powder products (Andres–Lacueva et al., 2008). Hannum et al. (2002) reported that when two commercially available cocoa products were analyzed, the gently alkalized cocoa contained 0.36 mg of flavanols per gram, while the highly alkalized cocoa contained only 0.07 mg. Alkalized cocoa (Dutched powders, average 80% nonfat cocoa solids) were found to have contain lower antioxidant capacity and contain less PCs than natural cocoa powders (Gu et al., 2003).

The antioxidant activity and flavan-3-ol levels of milk and dark chocolates were found to be stable over typical shelf lives of one year under controlled storage and over 2 years in ambient storage in the laboratory. The un-substituted flavan-3-ols (e.g., epicatechin, PC oligomers, and PCs polymers) were very

stable under representative commercial retail and room storage conditions (Hurst et al., 2008).

6.3. Comparison of Cocoa Polypenols and Antioxidative Properties to those of Teas and Wines

Chocolate was the second major source of proanthocyanidins in the American diet with apples being the major (32.0%) (Gu et al., 2004). In a Dutch population study, chocolate was reported to be a good source of dietary catechins, second only to black tea in (Arts et al., 1999). Also, one serving of cocoa had higher amount of polyphenols than that of green tea (Subhashini et al., 2010).

Cocoa flavonoids were considered powerful antioxidants having higher scavenging capacity than those in black tea, green tea, red wine, or apples (Lee et al., 2003; Lamuela-Raventós et al., 2005). When the phenolic and flavonoid contents and total antioxidant capacities of cocoa were compared to those of black tea, green tea, and red wine, it was found that cocoa contained much higher levels of total phenolics (611 mg of gallic acid equivalents, GAE) and flavonoids (564 mg of epicatechin equivalents, ECE) per serving than black tea (124 mg of GAE and 34 mg of ECE, respectively), green tea (165 mg of GAE and 47 mg of ECE), and red wine (340 mg of GAE and 163 mg of ECE) (Lee et al., 2003). A comparison of the flavonoid content between chocolate and red wine indicated that 49 g of dark chocolate had the equivalence of flavonoids as that of 196 ml of Tannat wine, which was the daily wine intake recommended to produce health benefits in an adult of 70 kg body weight (Pimentel et al., 2010). A 71% cocoa dark chocolate had flavonoids of 21.6 ± 2.4 μmol of catechin equivalents/g and polyphenols = 62.9 ± 0.1 μmol of catechin equivalents/g (Pimentel et al., 2010).

Overall, cocoa-containing and chocolate products rank second after red wines and grape juice in foods with the highest levels of total *trans*-resveratrol in the diet (Hurst et al., 2008). Among the 19 top selling commercially available cocoa-containing and chocolate products from the U.S. market, *trans*-resveratrol levels were highest in cocoa powders (1.85 ± 0.43 μg/g), followed by unsweetened baking chocolates (1.24 ± 0.22), semisweet chocolate baking chips (0.52 ± 0.14), dark chocolates (0.35 ± 0.08), milk chocolates (0.10 ± 0.05), and chocolate syrups (0.09 ± 0.02). These cocoa-containing and chocolate products have about three to five times more *trans*-piceid than *trans*-resveratrol. Levels of *trans*-piceid were highest in the cocoa powders (7.14 ± 0.80 μg/g), followed by unsweetened baking chocolates (4.04 ± 0.14), semisweet chocolate baking chips (2.01 ± 0.18), dark chocolates (1.82 ± 0.36), milk chocolates (0.44 ± 0.06), and chocolate syrups (0.35 ± 0.06) (Hurst et al., 2008). Dietary resveratrol (3,4′,5-trihydroxystilbene) has been implicated in the health benefits. Studies also have shown that consumption of resveratrol in wines increases vasodilation (Orallo et al., 2002) inhibits platelet aggregation and coagulation (Bertelli et al., 1995).

6.4. Added Milk Effects—Bioavailability of Cocoa Polyphenols

An understanding of how food matrices may influence flavan-3-ol absorption could provide a basis to design and develop functional products which positively influence flavan-3-ol absorption and, by extension, potential bioactivity (Neilson and Ferruzzi, 2011). The effect of milk on the bioavailability of cocoa flavonoids considering phase II metabolites of epicatechin has been the subject of considerable debate.

Cocoa powder dissolved in milk, as one of the most common modes of cocoa powder consumption, seems to have a negative effect on the absorption of polyphenols. In a study in which 21 volunteers received three interventions in a randomized crossover design with a 1-week interval [250 ml of whole milk (control), 40 g of cocoa powder dissolved in 250 ml of whole milk, and 40 g of cocoa powder dissolved with 250 ml of water] revealed that milk does not impair the bioavailability of polyphenols and thus their potential beneficial effect in chronic and degenerative disease prevention (Roura et al., 2007).

An investigation of the effects of milk on the bioavailability of cocoa flavan-3-ol metabolites detected two flavan-3-ol metabolites in plasma and four in urine revealed that milk had only minor effects on the plasma pharmacokinetics of an (epi)catechin-*O*-sulfate and had no effect on an *O*-methyl-(epi)catechin-*O*-sulfate (Mullen et al., 2009). The milk significantly lowered the excretion of four urinary flavan-3-ol metabolites from 18.3% to 10.5% of the ingested dose (Mullen et al., 2009). Metabolomics was proposed as a powerful tool to characterize both the intake and the effects on the metabolism of dietary components (Fiehn et al., 2006). Human urine metabolome modifications after single cocoa intake were investigated in a randomized, crossed, and controlled trial. After overnight fasting, 10 participants randomly consumed either a single dose of cocoa powder with milk or water, or milk without cocoa (Llorach et al., 2009). The better characterization of the urinary metabolome would be able to delve in the metabolism of phytochemistry and its relation with human health. Of the 27 metabolites related to cocoa-phytochemicals, including alkaloid derivatives, polyphenol metabolites, and processing-derived products such as diketopiperazines were identified as the main contributors to the urinary modifications after cocoa powder intake (Llorach et al., 2009).

The matrix in which polyphenols are consumed can affect their metabolism and excretion, and this may affect their biological activity (Roura et al., 2008). One (−)-epicatechin glucuronide and three (−)-epicatechin sulfates were detected in urine excreted after the intake of the two cocoa beverages (40 g cocoa powder dissolved in 250 mL whole milk and 250 mL whole milk) (Roura et al., 2008). Another study investigating the effect of milk on the urinary excretion of microbial phenolic acids after cocoa powder consumption in humans (Urpi-Sarda et al., 2010) indicated that of the 15 metabolites assessed, the excretion of nine phenolic acids was affected by the intake of milk. The urinary concentration of 3,4-dihydroxyphenylacetic,

protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric, caffeic, and ferulic acids was diminished after the intake of cocoa with milk, whereas urinary concentration of vanillic and phenylacetic acids was increased. Hence, the milk partially affected the formation of microbial phenolic acids derived from the colonic degradation of PCs and other compounds present in cocoa powder (Urpi-Sarda et al., 2010). Whether similar protective effects were associated with the consumption of many commercial chocolate and cocoa products containing substantially lower amounts of flavan-3-ols, especially when absorption at lower doses is obstructed by milk, needs to be determined.

Unlike for milk chocolates, it was shown that the consumption of plain, dark chocolate resulted in an increase in both total antioxidant capacity and the (−)-epicatechin content of blood plasma (Serafini et al., 2003), however, these effects were markedly reduced when the chocolate was consumed with milk or if milk was incorporated as milk chocolate (Serafini et al., 2003). These findings indicated that other dietary constituents such as milk may interfere with the absorption of antioxidants from chocolate *in vivo* and may therefore negate the potential health benefits that can be derived from eating moderate amounts of dark chocolate.

Twenty-three samples of chocolate were surveyed for (+) and (−)-catechin. At all concentrations studied, it was found that the intestinal absorption of (−)-catechin was lower ($p < 0.01$) than the intestinal absorption of (+)-catechin (Donavan et al., 2006). Also, plasma concentrations of (−)-catechin were significantly ($P < 0.05$) reduced compared to (+)-catechin ($p < 0.05$), which provide an explanation for the low bioavailability of (−)-catechin in chocolate and cocoa-containing products (Donavan et al., 2006).

The major factors to be considered in the biological activity of flavonoids in general, and flavanols in particular are: (i) bioavailability from foods; (ii) absorption and metabolism at the gastrointestinal tract; (iii) tissue and cellular distribution after absorption; and (iv) which are the chemical form(s) biologically available to the cell/tissue and their potential metabolism at cellular level (Galleano et al., 2009).

6.5. Summary

Cocoa flavonoids had higher scavenging capacity than those in black tea, green tea, red wine, or apples. Cocoa is one of the most concentrated sources of flavanols, a subgroup of the natural antioxidant compounds called flavonoids. Catechin and epicatechin were the most copious antioxidants followed by their dimers and trimers in cocoa as screened by liquid chromatography-mass spectrometry. PCs were most important antioxidant components in a methanolic extract of cocoa powder. Quercetin-3-arabinoside and isoquercitrin were the major flavanols in the cocoa powder products. On a gram weight basis, epicatechin, and catechin content of the products follow in decreasing order: cocoa powder > baking chocolate > dark chocolate = baking chips > milk chocolate > chocolate syrup. Cocoa-containing and chocolate products were ranked second after red wines and grape juice in foods with the highest levels of total *trans*-resveratrol in the diet. Methylxanthines in cocoa powder could reduce antioxidant capacity of flavonoids.

Processing such as alkali treatment resulted in the loss of flavanols and, thus they may not be the major components in many commercial cocoa products. The antioxidant activity and flavan-3-ol levels of milk and dark chocolates were found to be stable over typical shelf lives of one year under controlled storage and over 2 years in ambient storage in the laboratory.

There is conflicting evidence as to whether milk could adversely affect the bioavailability of flavan-3-ols. Unlike for milk chocolates, it was shown that the consumption of plain, dark chocolate increased both total antioxidant capacity and the (−)-epicatechin content of blood plasma, however, these effects were markedly reduced when the chocolate was consumed with milk or if milk was incorporated as milk chocolate. Human urine metabolome modifications have shown that consumption of plain, dark chocolate increased both the total antioxidant capacity and (−)-epicatechin content of blood plasma.

7. HEALTH ASPECTS OF COCOA POLYPHENOL/ FLAVANOLS

Dietary intake of flavanols has attracted increasing interest in epidemiological mechanistic and human intervention studies suggesting the potential beneficial health effects. Flavan-3-ol bioavailability depends on various factors, including digestive release, absorption, metabolism, and elimination. In addition to these, in vivo factors, such as the complexity of whole-food systems (physical form, flavan-3-ol form and dose, macronutrient and micronutrient profile, processing, etc.) influence the absorption efficiency and circulating profile of flavan-3-ols (Neilson and Ferruzzi, 2011). PCs were stable in the stomach environment, with a gastric transit lasted ≈50 to 60 minutes (Rios et al., 2002). Most ingested PCs reached the small intestine intact and were available for absorption or metabolism (Rios et al., 2002). The PC oligomer dimer B2, and the flavanol monomers epicatechin and catechin, can be absorbed into the circulation and can be detected in human plasma as early as 30 minutes after consumption of a flavanol-rich food such as cocoa (Holt et al., 2002). Table 1 indicates some studies which were published between 2005 and 2011, relating to the health benefits of cocoa and cocoa products containing polypenols/flavanols.

7.1. Effects and Low-Density Lipoprotein Oxidative Susceptibility and Hypocholesterolemic Aspects

Many studies support the interference that cocoa and cocoa products lower low-density lipoproteins (LDL) oxidizability. In a meta-analysis of eight randomized controlled trials (215 participants) of short-term effect of cocoa product consumption on lipid profile, cocoa consumption significantly reduced blood cholesterol, but the changes were dependent on the dose of

Table 1 Relating polyphenol/flavanol components of cocoa and cocoa products to health benefits- some studies published between 2005 and 2011

Polyphenol/flavanol components	Effects	Implications	References
Chocolate intake assessed by a semi-quantitative food frequency questionnaire, using used generalized estimating equations to estimate adjusted odds ratios	The literature on clinical trials assessing the effects dark chocolate on blood pressure, platelet function, and endothelial function suggested that consumption of small amounts of chocolate might provide additional benefits in reducing coronary heart disease risk	Consumption of chocolate was inversely related with prevalent coronary heart disease in a general United States population. Consumption of nonchocolate candy was associated with a 49% higher prevalence of coronary heart disease comparing 5/week vs. 0/week	Djoussé et al. (2011)
Chocolate consumption and risk of stroke in the population-based Swedish Mammography Cohort.	Examination of the association between chocolate consumption and risk of stroke in the population-based Swedish Mammography Cohort. Comprising of 39,227 women. Chocolate consumption was assessed using a self-administered food-frequency questionnaire. In a stratified analysis by hypertension, the multivariable relative risks of total stroke for 50 g per week increment of chocolate consumption were 0.89 (95% CI: 0.73 to 1.09) for women with a history of hypertension and 0.85 (95% CI: 0.74–0.97) for women without hypertension.	A high chocolate consumption was associated with a lower risk of stroke.	Larsson et al. (2011)
Wistar rats were fed with a 5 or 10% cocoa-enriched diet beginning two weeks before arthritis induction and until the end of the study	The effects of two cocoa-enriched diets on adjuvant arthritis in rats were assessed by taking into account not only clinical and biochemical inflammatory indices, but also antibody response and lymphocyte composition.	The cocoa-enriched diets during AA did not significantly decrease joint inflammation but modified T-helper cell proportions and prevented specific antibody synthesis	Ramos-Romero et al. (2011)
Consumption of cocoa flavanols, which improve endothelium-dependent flow-mediated dilatation, can modify blood pressure responsiveness	Twenty-one volunteers were randomized to consume single servings of either a high-flavanol (HF, 701 mg) or a low-flavanol (LF, 22 mg) cocoa beverage in a double-blind, cross-over design with three to seven day washout between treatments. Pre-exercise blood pressure was similar after taking LF and HF. The blood pressure response to exercise was attenuated by HF compared with LF. BP flow mediated dilation measurements were higher after taking HF than after taking	The consumption of cocoa flavanols may be able to enhance muscle blood flow to allow for improved nutrient delivery and removal to exercising muscles and attenuate the blood pressure responses to exercise, which could allow for safer and more efficient exercise performance in an at-risk population such as that included in the present study, thus placing less stress on the cardiovascular system during exercise	Berry et al. (2010)
Sucrose with cocoa added	Twenty-four male participants were exposed to CPTs while holding one of three tastants in their mouths: water, sucrose, or sucrose with cocoa bitterness. The addition of cocoa to sucrose produced a lower cold tolerance than did the sucrose solution	The results suggest that taste qualities, probably due to their values as a signal for nutrition, are responsible for mediating cold pain tolerance	Eggleston et al. (2010)
Administration of flavanol-rich cocoa powder and spirulina	The antioxidant impact of spirulina could be expected to amplify the bioactivity of the NO evoked by cocoa flavanols in inflamed endothelium	By optimizing cerebrovascular perfusion while quelling cerebral oxidant stress, cocoa powder and spirulina could collaborate in prevention of senile dementia	Mc Carty et al. (2010)
Consumption of varying servings of chocolate per month. No regular intake of chocolate, one to three servings of chocolate per month, three to six servings per week and _1 servings per day	Investigation of the association between chocolate intake and incidence of heart failure. A prospective cohort study of 31,823 women aged 48 to 83 years without baseline diabetes or a history of HF or myocardial infarction who were participants in the Swedish Mammography Cohort	Moderate habitual chocolate intake was associated with a lower rate of heart failure hospitalization or death, but the protective association was not observed with intake of $\leq$1 servings per day	Mostofsky et al. (2010)
Cocoa PCs and major antioxidant PC B2	Protected rat pheochromocytoma cells against 4-Hydroxynonenal HNE-induced apoptosis by blocking mitogen-activated protein kinase kinase activity and intracellular reactive oxygen species accumulation.	Implications for neurodegenerative diseases such as Alzheimer's disease	Cho et al. (2009)

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Table 1 Relating polyphenol/flavanol components of cocoa and cocoa products to health benefits- some studies published between 2005 and 2011 *(Continued)*

Polyphenol/flavanol components	Effects	Implications	References
Participants self-reported usual chocolate consumption over the preceding 12 months with a standardized questionnaire and then underwent a health examination three months after discharge	Assessment of the long-term effects of chocolate consumption amongst patients with established coronary heart disease. A population-based inception cohort study of 1169 nondiabetic patients hospitalized with a confirmed first acute myocardial infarction between 1992 and 1994 in Stockholm County, Sweden	Chocolate consumption was associated with lower cardiac mortality in a dose dependent manner in patients free of diabetes surviving their first acute myocardial infarction	Janszky et al. (2009)
Intake of cocoa polyphenols. Each subject received 40 g cocoa powder with 500 mL skim milk/day or only 500 mL skim milk per day for four weeks	Evaluation of the effects of chronic cocoa consumption on cellular and serum biomarkers related to atherosclerosis in 42 high-risk patients of cardiovascular disease included in a randomized crossover feeding trial	The intake of cocoa polyphenols may modulate inflammatory mediators in patients at high risk of cardiovascular disease. These antiinflammatory effects may contribute to the overall benefits of cocoa consumption against atherosclerosis	Monagas et al. (2009)
Consumed drinks containing 520 mg and 994 mg cocoa flavanol (CF) and a matched control, with a 3-day washout between drinks	A randomized, controlled, double-blinded, balanced, three period crossover trial 30 healthy adult. Assessments included the state anxiety inventory and repeated 10-min cycles of a Cognitive Demand Battery comprising of two serial subtraction tasks (Serial Threes and Serial Sevens), a Rapid Visual Information Processing (RVIP) task and a "mentalfatigue" scale, over the course of 1 hour	Increase in self-reported "mental fatigue" was significantly attenuated by the consumption of the 520 mg CF beverage only.	Scholey et al. 2009
Dark chocolate containing flavanols and added sterol esters	Reductions in serum total, LDL cholesterol and systolic blood pressure in normotensive population with elevated cholesterol	Regular consumption of products containing cocoa flavanols may offer an added cardiovascular benefit	Allen et al. (2008)
1-year administration of a cocoa polyphenolic extract (Acticoa powder), orally delivered at the dose of 24 mg/kg per d between 15 and 27 months in old Wistar-Unilever rats.	We examined whether one-year administration of a cocoa polyphenolic extract orally delivered at the dose of 24 mg/kg per day between 15 and 27 months of age, affects the onset of age-related cognitive deficits, urinary free dopamine levels and lifespan in old Wistar-Unilever rats	Acticoa powder may be beneficial in retarding age-related brain impairments, including cognitive deficits in normal ageing and perhaps neurodegenerative diseases	Bisson et al. (2008)
Dark chocolate and cocoa versus a placebo	Short-term dark chocolate and cocoa consumption (six weeks) by healthy volunteers failed to support the predicted beneficial effects on any of the neuropsychological or cardiovascular health-related variables	Short-term effects of dark chocolate was associated with higher pulse rates	Crews et al. (2008)
High-flavanol cocoa (HF, 902 mg flavanols) and exercise; low-flavanol cocoa (LF, 36 mg flavanols) and exercise	HF increased brachial artery flow-mediated dilation acutely (two hours post-dose) by 2.4% and chronically (over 12 weeks) by 1.6% and reduced insulin resistance by 0.31%, diastolic pressure by 1.6 mmHg and mean arterial blood pressure by 1.2 mmHg independent of exercise in a study involving 49 overweight and obese adults	Reducing cardiometabolic risk factors in the overweight and obese adults; HF consumption did not enhance the effects of exercise on body fat and fat metabolism in obese subjects	Davison et al. (2008)
Regular consumption of dark chocolate	1317 subjects of the Moli-sani ongoing cohort study, Italy were selected as having declared eaten any chocolate during the past year (mean age 53 $\pm$ 12 years; 51% men) and 824 subjects who ate chocolate regularly in the form of dark chocolate only (50 $\pm$ 10 years; 55% men) were selected. After adjustment for age, sex, social status, physical activity, systolic blood pressure, BMI, waist:hip ratio, food groups, and total energy intake, dark chocolate consumption was significantly inversely associated with C-reactive protein	Significant inverse association between dark chocolate and serum C-reactive protein adds new insight into the relationship between flavonoid-rich foods, inflammation, and cardiovascular protection. Consumers of up to one serving (20 g) of dark chocolate every three day had serum CRP concentrations that were significantly lower than nonconsumers	di Giuseppe et al. (2008)

Table 1 Relating polyphenol/flavanol components of cocoa and cocoa products to health benefits- some studies published between 2005 and 2011 *(Continued)*

Polyphenol/flavanol components	Effects	Implications	References
In phase 1, subjects were randomly assigned to consume a solid dark chocolate bar (containing 22 g cocoa powder) or a cocoa-free placebo bar (containing 0 g cocoa powder). In phase 2, subjects were randomly assigned to consume sugar-free cocoa (containing 22 g cocoa powder), sugared cocoa (containing 22 g cocoa powder), or a placebo (containing 0 g cocoa powder).	Randomized, placebo-controlled, single-blind crossover trial of 45 healthy adults. Blood pressure decreased after the ingestion of dark chocolate and sugar-free cocoa compared with placebo	Acute ingestion of both solid dark chocolate and liquid cocoa improved endothelial function and lowered blood pressure in overweight adults. Sugar content may attenuate these effects, and sugar-free preparations may augment them	Faridi et al. (2008)
Effect of cocoa extract containing polyphenols and methylxanthines prepared from cocoa powder; cocoa extract (600 mg/kg body weight/day) was given to obese – diabetic rats for 4 weeks.	Oral glucose tolerance test revealed that cocoa supplementation reduced plasma glucose at 60 and 90 minutes compared to in un-supplemented obese-diabetic rats	Cocoa supplementation could reduce circulating plasma free fatty acid and 8-isoprostane and may enhance the antioxidant defense system	Jalil and Ismail, 2008
Cocoa flavanol-rich beverage	Eight% increase in brain blood flow after one week and 10% increase after two weeks in a study with healthy, older adults ages 59 to 83	Long-term improvements in brain blood flow could impact cognitive behavior, offering future potential for debilitating cerebrovascular brain ischemic syndromes including dementia	Sorond et al. (2008)
Cocoa powder containing low-polyphenolic compounds (placebo-cocoa group) or three levels of cocoa powder containing high polyphenolic compounds (13, 19.5, and 26 g/day for low-, middle-, and high-cocoa groups, respectively)	Decreased the susceptibility of LDL to oxidation and increased HDL-cholesterol concentrations in plasma in normocholesterolemic and mildly hypercholesterolemic human subjects	Improvement in LDL and HDL cholesterol concentrations and inhibition of oxidized LDL may reduce the development of atherosclerotic lesions; decrease in the incidence of arteriosclerotic disease	Baba et al. (2001)
Dark and control chocolates were prepared by Nestlé, Switzerland)	Effect of flavonoid-rich dark chocolate compared with cocoa-free control chocolate on coronary vascular and platelet function in 22 heart transplant recipients in a double-blind, randomized study. Two hours after ingestion of flavonoid-rich dark chocolate, coronary artery diameter was increased significantly (whereas it remained unchanged after control chocolate. Endothelium-dependent coronary vasomotion improved significantly after dark chocolate in the placebo group ($p = 0.01$). Platelet adhesion decreased ($p = 0.04$) in the dark chocolate group but remained unchanged in the control group	Dark chocolate induced coronary vasodilation, improves coronary vascular function, and decreases platelet adhesion 2 hours after consumption. These immediate beneficial effects were paralleled by a significant reduction of serum oxidative stress and were positively correlated with changes in serum epicatechin concentration.	Flammer et al. (2007)
Randomly assignment to receive for 18 weeks either 6.3 g (30 kcal) per day of dark chocolate containing 30 mg of polyphenols or matching polyphenol-free white chocolate	Determination of the effects of low doses of polyphenol-rich dark chocolate on blood pressure. From baseline to 18 weeks, dark chocolate intake reduced mean systolic and diastolic blood pressure ($p < 0.001$) without changes in body weight, plasma levels of lipids, glucose, and 8-isoprostane. Hypertension prevalence declined from 86% to 68%. Blood pressure decrease was accompanied by a sustained increase of S-nitrosoglutathione ($p < 0.001$), and a dark chocolate dose resulted in the appearance of cocoa phenols in plasma. White chocolate intake caused no changes in blood pressure or plasma biomarkers.	The inclusion of small amounts of polyphenol-rich dark chocolates part of a usual diet efficiently reduced blood pressure and improved formation of vasodilative NO.	Taubert et al. (2007)

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Table 1 Relating polyphenol/flavanol components of cocoa and cocoa products to health benefits- some studies published between 2005 and 2011 *(Continued)*

Polyphenol/flavanol components	Effects	Implications	References
Cocoa-containing foods	The lower cardiovascular mortality risk related with cocoa intake is mediated by mechanisms other than lowering blood pressure; higher cocoa in the elderly was inversely associated with blood pressure.	Cocoa intake was positively associated with calorie intake; lower systolic and diastolic blood pressure in the highest cocoa intake compared to the lower cocoa intake.	Buijsse et al. (2006)
Administration of flavanol-rich cocoa to 15 young (<50 years) and 19 older (>50 years) healthy subjects for several days on blood pressure and peripheral arterial responses	Flow-mediated vasodilation, measured by tonometry in the finger, was enhanced with flavanol-rich cocoa in 15 young (<50 years) and 19 older (>50) healthy subjects but was significantly more so among the old group ($p = 0.01$). The nitric oxide synthase (NOS) inhibitor N-nitro-l-arginine-methyl-ester (L-NAME) induced significant pressor responses following cocoa administration only among the older subjects.	Flavanol-rich cocoa enhanced several measures of endothelial function to a greater degree among older than younger healthy subjects.	Fisher and Hollenberg (2006)
Ingestion of flavanol-rich cocoa.	An increase in the oxygenation level-dependent signal intensity in response to the cognitive task following ingestion of flavanol-rich cocoa (172 mg cocoa flavanols daily for five days in a study with 16 healthy young women (18–30 years). Altered neuronal activity or changed vascular responsiveness, or both; the net effect was dependent on which of the two effects was dominant.	The flavanol-rich cocoa increased the cerebral blood flow to brain gray matter, suggesting the potential of cocoa flavanols in the treatment of vascular impairment, including dementia and strokes, and in maintaining cardiovascular health.	Francis et al. (2006)
High flavanol (326 mg/day) or low flavanol (27 mg/day) cocoa powder dissolved in 100 mL water.	UV-induced erythema was significantly decreased in the high flavanol group; ingestion of high flavanol cocoa led to increases in blood flow of cutaneous and subcutaneous tissues, and to increases in skin density and skin hydration in this group of women	Regular consumption of a beverage rich in flavanols can confer substantial photoprotection as well as help maintain skin health by improving skin structure and function	Heinrich et al. (2006)
Consumption of a flavanol-rich cocoa snack food containing phytosterols.	Reduces plasma total and LDL cholesterol levels in a population with hypercholesterolemia.	Management of serum cholesterol levels-atherosclerosis risk factor.	Polagruto et al.(2006)
Dietary intake of flavanol-rich cocoa-high flavanol cocoa beverage	Brachial artery hyperemic blood flow increased by 76% after the six-week cocoa intake in the high cocoa flavanol group [446 mg total flavanols vs. 32% in the low CF (43 mg total flavanols)] group in 32 hypercholesterolemic postmenopausal women. The 2.4-fold increase in hyperemic blood flow with high cocoa flavanol was closely correlated (with decrease plasma levels of soluble vascular cell adhesion molecule.	Decreased risk for cardiovascular disease after menopause associated with improved endothelial function.	Wang-Polagruto et al. (2006)
Flavanol-rich dark chocolate	Decreases blood pressure, serum LDL cholesterol, improved flow mediated dilation and ameliorated insulin sensitivity in hypertensives.	May improve cardiovascular benefit.	Grassi et al.(2005)
Flavanol-containing milk chocolate	Decreases diastolic blood pressure, plasma cholesterol, LDL-cholesterol and increases in vitamin E/cholesterol.	Cardiovascular health and oxidant stress.	Fraga et al. (2005)
Flavanols in cocoa drink	Increased bioactive nitrogen oxide and endothelium-dependent vasodilation in smokers.	Supports the concept that dietary flavanols can reverse endothelial dysfunction.	Heiss et al (2005)
Cocoa polyphenols-high flavanol cocoa beverage consumption by 20 individuals at risk for cardiovascular diseases	Increase plasma concentrations of nitroso compounds and flow-mediated dilation of the brachial artery.	May reverse endothelial dysfunction through enhancement of NO bioactivity.	Sies et al. (2005)
Flavonoid-rich cocoa beverage that provided 0.25 g/kg body weight	Epicatechin, catechin, 3′-*O*-Me epicatechin and (−)-epicatechin-(4- > 8)-epicatechin (Dimer B2) were detected in the plasma within 1 hour after the consumption of the beverage. Also, the susceptibility of erythrocytes to hemolysis was reduced significantly following the consumption of the beverages.	Reduced susceptibility to free radical-induced hemolysis.	Zhu et al. (2005)

cocoa consumption and the healthy status of participants (Jia et al., 2010). Cocoa consumption significantly lowered LDL cholesterol by 5.87 mg/dL and marginally lowered total cholesterol by 5.82 mg/dL (Jia et al., 2010)

In a randomized, double-blind parallel arm study design, subjects were randomized to one of two dietary treatments of either a cocoa flavanol-enriched snack bar containing 1.5 g phytosterol or a control product containing no phytosterols (Table 1; Polagruto et al., 2006). The consumption of the phytosterol-enriched snack bars, but not the control bars for six weeks was associated with significant reductions in plasma total and LDL cholesterol and the ratio of total to high-density lipoprotein cholesterol. A significant reduction in lipid-adjusted serum β-carotene was observed in the phytosterol, but not the no-phytosterol-added group. The study supported the use of a novel flavanol-rich cocoa snack bar to effectively reduce plasma total and LDL cholesterol levels in a population with hypercholesterolemia (Polagruto et al., 2006).

Mathur et al. (2002) administered cocoa supplements (a 36.9 g dark chocolate bar and a 30.95 g cocoa powder beverage) providing approximately 651 mg of PCs per day) for six weeks to healthy human volunteers and found that LDL oxidation lag time was significantly higher after the cocoa supplements than at baseline. Overall, these data suggested that the rise in plasma epicatechin concentrations improves the ability of plasma to scavenge for free radicals, which indirectly works to inhibit lipid oxidation (Mathur et al., 2002).

Polyphenolic extracts from cocoa powder containing monomeric flavanols as well as PC dimers and trimers were found to protect human liver cells against oxidative susceptibility by modulating glutathione concentration, reactive oxygen species generation, malondialdehyde production, and antioxidant enzyme activity (Martin et al., 2008). Pretreatment of HepG2 cells with cocoa polyphenolic extract prevented apoptosis through the reduction of reactive oxygen species generation and the modulation of the apoptotic pathways activated by *tert*-butyl hydroperoxide (Martin et al., 2010). Also, cocoa polyphenolic treatment also activated survival signaling proteins, such as protein kinase B and extracellular regulated kinases and increased the activities of two antioxidant enzymes, glutathione peroxidase and glutathione reductase. Hence, cocoa polyphenolic treatment was an effective inductor of glutathione peroxidase and glutathione reductase activities via extracellular-regulated kinases activation and that this upregulation seems to be required to attenuate *tert*-butyl hydroperoxide induced injury.

An ex vivo study shows that epicatechin, a major polyphenol in chocolate and chocolate extracts, is a powerful inhibitor of plasma lipid oxidation due to polyphenols' ability to bind to LDLs (Vinson et al., 2006). Conversely, the fat from chocolate alone is a pro-oxidant in this model as demonstrated in an in vivo human study. After consumption of dark chocolate and cocoa powder, the LDLs isolated from plasma were protected from oxidation compared to the lipoproteins isolated after cocoa butter consumption, which were put under oxidative stress (Baba et al., 2007). The polyphenolic substances derived from the cocoa powder contributed to the elevation of HDL cholesterol, which could decrease the incidence of arteriosclerotic disease (Table 1; Baba et al., 2007).

The level of excretion of epicatechin in urine was significantly higher in the cocoa group (volunteers were given 36 g of cocoa powder—containing 2610 mg of polyphenols) than that in the control group thus suggesting that the antioxidants in cocoa powder might be absorbed and increase the resistance of human LDL to oxidation (Osakabe et al., 2001).

A three-week clinical supplementation trial of 45 nonsmoking, healthy volunteers who consumed 75 g daily of either white chocolate, dark chocolate, or dark chocolate enriched with cocoa polyphenols found that cocoa polyphenols may increase the concentration of HDL cholesterol, whereas chocolate fatty acids may modify the fatty acid composition of LDL and make it more resistant to oxidative damage (Mursu et al., 2004).

Cocoa supplementation with a cocoa extract containing polyphenols and methylxanthines may have protective effects against lipid peroxidation with a concomitant boost in the antioxidant defense system. Moreover, cocoa supplementation could reduce circulating plasma free fatty acid and 8-isoprostane and may enhance the antioxidant defense system (Table 1; Jalil and Ismail, 2008). The health-promoting properties of cocoa extracts could be attributed to polyphenol compounds as well as methylxanthines (caffeine and theobromine) and minerals.

A study which examined the effects of chocolate on lipid profiles, weight, and glycemic control of 12 individuals with Type 2 diabetes on stable medication revealed that high polyphenol chocolate was effective in improving the atherosclerotic cholesterol profile (Mellor et al., 2010). The participants consumed 45 g chocolate with or without a high polyphenol content for eight weeks and then crossed over after a four-week washout period. The HDL cholesterol increased and improved the cholesterol:HDL ratio without affecting weight, inflammatory markers, insulin resistance or glycemic control.

7.2. Endothelial Functions, Inflammatory Mediators, and Atherosclerosis

Endothelial dysfunction is an early stage of atherosclerosis, which is characterized by a decreased bioactivity of NO-enhanced formation of oxygen-derived free radicals and impaired flow-mediated vasodilation (Tsuchiya et al., 2002; Ambrose and Barua, 2004; Schroeter et al., 2006). Atherosclerosis is an inflammatory disease of the arterial vascular wall. Inflammation is a complex process which involves separate cellular actions such as cell migration as well as synthesis of extracellular matrix (ECM) leading to the atherosclerotic fibrous plaque. In later stages, ECM degradation results in plaque disruption responsible for the clinical atherothrombotic syndromes (Páramo, 2008). The expression and activation of matrix metalloproteinase (MMP)-2 takes part in the migration and invasion of human aortic vascular smooth muscle cells (VSMC) originating from normal human tissue, which is strongly linked

to atherosclerosis (Montero et al., 2006; Lee et al., 2008; Rodriguez et al., 2008). Cocoa PCs are potent inhibitors of MEK and MT1-MMP, and subsequently inhibit the expression and activation of pro-MMP-2, and also the invasion and migration of VSMC, which may in part explain the molecular action of antiatherosclerotic effects of cocoa.

Arterial stiffness and endothelial function are prognosticators of cardiovascular risk (Aznaouridis et al., 2004). Table 1 shows that among patients at high risk of cardiovascular disease, the intake of cocoa polyphenols may modulate inflammatory mediators in patients at high risk of cardiovascular disease which may contribute to the overall benefits of cocoa consumption against atherosclerosis (Monagas et al., 2009). In a randomized, single-blind, crossover fashion (eating 100 mg of dark chocolate 74% rich in cocoa and sham eating) with 10 healthy volunteers, wave reflection as an index of arterial stiffness and left ventricular after load were studied using a validated system that employs high-fidelity arterial tonometry and appropriate computer software for pulse wave analysis (Aznaouridis et al., 2004). Chocolate led to an increase in aortic systolic and diastolic pressures and a substantial decrease in augmentation index that denotes a decrease in wave reflection along the arterial tree. Resting brachial diameter and flow-mediated dilation increased by 0.17 mm denoting improvement in endothelial function (Aznaouridis et al. 2004)

The acute ingestion of both solid dark chocolate and liquid cocoa improved endothelial function and lowered blood pressure in overweight adults (Table 1, Faridi et al., 2008) Sugar content may attenuate these effects, and sugar-free preparations may augment them. Further investigation is clearly warranted to determine longer term effects of habitual solid and liquid cocoa ingestion, optimal dosing of chocolate for cardiovascular benefit, variation in beneficial effects among diverse populations, and, ultimately, the influence of dietary cocoa intake on cardiac events (Faridi et al., 2008). In healthy humans, flavanol-rich cocoa induced vasodilation via activation of the nitric oxide (NO) system. Four days of flavanol-rich cocoa induced consistent and striking peripheral vasodilation. On day five, pulse wave amplitude significantly exhibited a large additional acute response to cocoa (Fisher et al., 2003). The hypothesis that vascular responsiveness to flavanol-rich cocoa increases with advancing age was tested (Table 1; Fisher and Hollenberg, 2006). Flavanol-rich cocoa enhanced several measures of endothelial function to a greater degree among older than younger healthy subjects. The nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine-methyl-ester (L-NAME) induced significant pressor responses following cocoa administration only among the older subjects in 19 older (>50 years) healthy subjects than in 15 young (<50 years). Flow-mediated vasodilation, measured by tonometry in the finger, was enhanced with flavanol-rich cocoa in both groups, but significantly ($p = 0.01$) more so among the old (Fisher and Hollenberg, 2006). The NO-dependent vascular effects of flavanol-rich cocoa may be greater among older people, in whom endothelial function is more disturbed (Fisher and Hollenberg, 2006). The increase in circulating NO pool may contribute to beneficial vascular health effects. The antiinflammatory actions of certain cocoa flavanols and/or flavanol-rich cocoa have the ability to alter eicosanoid and cytokine production, inhibit platelet aggregation, and promote favorable levels of NO (Kaplan and Frishman, 2001; Pockley, 2002).

The ingestion of flavanol-rich cocoa in 16 healthy men (25–32 years) was coupled with acute elevation in the levels of circulating NO species, enhanced flow-mediated vasodilation response of conduit arteries, and augmented microcirculation (Schroeter et al., 2006). The oral administration of pure (−)-epicatechin to humans closely emulated acute vascular effects of flavanol-rich cocoa. The concept that chronic intake of high-flavanol diets is associated with prolonged, augmented NO biosynthesis was supported by data that indicate a correlation between the chronic consumption of cocoa flavanol-rich diet and augmented urinary excretion of NO metabolites. Thus, ingestion of the (−)-epicatechin flavanol can be effectively linked to reported vascular effects of consuming flavanol-rich cocoa (Schroeter et al., 2006).

Flavonoid-rich dark chocolate intake improved the endothelial function which was associated with an increase in plasma epicatechin concentrations in healthy adults (Engler et al., 2004). High-flavonoid chocolate consumption improved the endothelium-dependent flow-mediated dilation of the brachial artery (mean change = 1.3 ± 0.7%) as compared to low-flavonoid chocolate consumption (mean change = −0.96 ± 0.5%) ($p = 0.024$) (Engler et al., 2004).

The effects of chronic dietary intake of flavanol-rich cocoa on endothelial function and markers of cardiovascular health were studied in 32 hypercholesterolemic postmenopausal women. Thus, consumption of flavanol-rich cocoa has beneficial vascular effects in hypercholesterolemic postmenopausal women (Table 1; Wang-Polagruto et al., 2006). Brachial artery hyperemic blood flow increased by 76% after the six-week cocoa intake in the high cocoa flavanol (CF) group versus 32% in the low cocoa flavanol group (Wang-Polagruto et al., 2006). The 2.4-fold increase in hyperemic blood flow with high CF cocoa closely correlated ($r = 0.8$) with a decrease (11%) in blood plasma levels of sol. vascular cell adhesion mol^{-1}. The risk for cardiovascular disease after menopause was associated with improved endothelial function.

The ingestion of a high-flavanol cocoa drink but not the low-flavanol cocoa drink significantly increased plasma concentrations of nitroso-compounds and flow-mediated dilation of the brachial artery. Therefore, ingested flavonoids may reverse endothelial dysfunction through enhancement of NO bioactivity (Table 1; Sies et al., 2005). Cocoa polyphenols inhibited human 5-lipoxygenase, the key enzyme of leukotriene synthesis. (−)-Epicatechin and other cocoa flavan-3-ols proved to be inhibitory at the enzyme level (Sies et al., 2005). The NO-promoting effects of cocoa polyphenols may also contribute to the reported decreases in blood pressure among elderly individuals after intake of dark chocolate (Taubert et al., 2003).

In a study involving 12 smokers, the circulating pool of bioactive NO and endothelium-dependent vasodilation were acutely

increased following the oral ingestion of a flavanol-rich cocoa drink during abstinence (>12 hours) from smoking (Table 1; Heiss et al., 2005). It is suggested that this increase in circulating NO pool may contribute to beneficial vascular health effects of flavanol-rich food. Impaired release of NO results in endothelial dysfunction, in which vessels tend to constrict and impede flow in response to stimuli that should lead to dilation and flow augmentation (Rubanyi, and Vanhoutte, 1986; Mursu et al., 2004). The increase in the circulating NO pool may contribute to beneficial vascular health effects of flavanol-rich food (Rubanyi, and Vanhoutte, 1986; Mursu et al., 2004; Heiss et al., 2005). However, long-term studies are needed to investigate whether short-term promising effects transform, unambiguously, into long-term health benefits.

The ingestion of flavanol-rich cocoa powder was shown to provoke increased endothelial production of NO—an effect likely mediated by epicatechin—and thus may have considerable potential for promoting vascular health (Table 1; McCarty et al., 2010). Joint administration of flavanol-rich cocoa powder and spirulina may have particular merit, inasmuch as cocoa can mask the somewhat disagreeable flavor and odor of spirulina, whereas the antioxidant impact of spirulina could be expected to amplify the bioactivity of the NO evoked by cocoa flavanols in inflamed endothelium (McCarty et al., 2010). Moreover, there is reason to suspect that, by optimizing cerebrovascular perfusion while quelling cerebral oxidant stress, cocoa powder and spirulina could collaborate in prevention of senile dementia.

7.3. Inflammation

Table 1 shows that consumers of up to 1 serving (20 g) of dark chocolate every three days had serum C-reactive protein concentrations that were significantly lower than nonconsumers in a healthy Italian population which suggests that regular consumption of small doses of dark chocolate may reduce inflammation (di Giuseppe et al., 2008). Intake of flavonoid-rich chocolate in humans was reported to increase the plasma level of (−)-epicatechin and concurrently significantly decrease the plasma level of proinflammatory cysteinyl leukotrienes. The (−)-epicatechin and its low-molecular PCs inhibited both dioxygenase and 5,6-leukotriene A4 synthase activities of human 5-lipoxygenase which may contribute to a putative antiinflammatory effect of cocoa products (Schewe et al., 2002). Mexico City (MC) residents, who were exposed to fine particulate matter and endotoxin, exhibit inflammation of the olfactory bulb, substantia nigra, and vagus nerve. Mice were exposed to MC air, received no treatment or oral dark chocolate, and were compared to clean-air mice either untreated or treated intraperitoneally with endotoxin (Villarreal-Calderon et al., 2010). After 16 months of exposure, the dorsal vagal complex dexhibited significant inflammation in endotoxin-treated and MC mice. Sustained dorsal vagal complex inflammation in mice exposed to MC air is mitigated by chocolate administration (Villarreal-Calderon et al., 2010). Cocoa-enriched diets during adjuvant arthritis in Wistar rats were not able to significantly decrease joint inflammation but modified T helper-cell proportions and prevented specific antibody synthesis (Table 1; Ramos-Romero et al., 2011)

7.4. Cardiovascular Risk and Hypertension

In a systematic review of 136 publications, revealed that the body of short-term randomized feeding trials suggested cocoa and chocolate may exert beneficial effects on cardiovascular risk via effects on lowering blood pressure, antiinflammation, antiplatelet function, higher HDL, and decreased LDL oxidation (Ding et al., 2006; Kondo 2007).

There has been increased interest in the potential health-related benefits of antioxidant and phytochemical-rich dark chocolate and cocoa. Dark chocolate was seen to be more protective than milk or white chocolate (Fernández-Murga et al., 2011). Several studies have demonstrated the benefits of cocoa consumption including hypertension resistance, prevention of cardiovascular diseases, and decreasing platelet aggregation. Cocoa and particularly dark chocolate are rich in flavonoids and recent studies have demonstrated blood pressure lowering effects of dark chocolate (Fernández-Murga et al., 2011).

A systematic review and meta-analysis confirmed the blood-pressure lowering capacity of flavanol-rich cocoa products in a larger set of trials than previously reported (Desch et al., 2010). However, significant statistical heterogeneity across studies was found. The review of epidemiological and mechanistic studies supports the role of flavonoids, particularly cocoa and tea flavanols, in protecting the cardiovascular system against cardiovascular disease (Grassi et al., 2009). A limitation of the data was the few and very small studies, no crossover designed studies and a wide range of dose and type of flavonoids tested. Thus, although flavonoid rich foods and beverages are likely to protect cardiovascular system, further research is needed to characterize the mechanism of action on flavanol-rich foods. Long-term clinical trials are also needed to definitively clarify the benefits deriving from long-term consumption of flavanol-rich foods, particularly focusing on the lowest effective levels as well as synergism or antagonistic actions between different classes of flavonoids commonly found in foods.

The Zutphen Elderly Study is the continuation of the Zutphen Study, the Dutch contribution to the Seven Countries Study (Keys et al., 1966) on the association of cocoa and blood pressure. An observational study, which involved a cohort of 470 elderly men in the Zutphen Elderly Study and free of chronic diseases at baseline, found that habitual cocoa intake was inversely associated with blood pressure and 15-year cardiovascular and all-cause mortality. Table 1 shows that blood pressure was measured at baseline and 5 years later, and causes of death were ascertained during 15 years of follow-up (Buijsse et al., 2006).

A meta-analysis of 10 randomized controlled trials assessing the antihypertensive effects of flavanol-rich cocoa products confirmed the blood-pressure lowering capacity of flavanol-rich cocoa products (Desch et al., 2010). The primary outcome mea-

sure was the change in systolic and diastolic blood pressure between intervention and control groups. However, these authors indicated that questions on the most appropriate dose and the long-term side effect profile warrant further investigation before cocoa products could be recommended as a treatment option in hypertension.

In a study with 198 healthy subjects, the higher cocoa intake was an independent determinant of low arterial stiffness and wave reflection indexes and was also independently associated with significantly lower central (aortic) pulse pressure (Vlachopoulos et al., 2007). These authors suggested that chocolate consumption may exert a protective effect on the cardiovascular system (Vlachopoulos et al., 2005).

Five randomized controlled studies of cocoa administration, involving a total of 173 subjects with a median duration of two weeks, indicated that the pooled mean systolic and diastolic blood pressure were −4.7 mmHg [95% confidence interval (CI) −7.6 to −1.8 mmHg; $p = .002$] and −2.8 mmHg (95% CI, −4.8 to −0.8 mmHg; $p = 0.006$) lower, respectively, compared with the cocoa-free controls (Taubert, 2007). The intake of low habitual amounts of dark chocolate caused progressive reductions of systolic and diastolic blood pressure in older subjects (44 adults aged 56–73 years) with prehypertension or stage 1 hypertension without inducing weight gain or other adverse effects (Taubert et al., 2007). The decrease in systolic and diastolic blood pressure was associated with an increase in circulating levels of vasodilative *S*-nitrosoglutathione, which implies a causative role of *S*-nitrosoglutathione for blood pressure regulation. Short-term interventions of at most two weeks indicated that high doses of cocoa can improve endothelial function by formation of vasodilative NO and reduce blood pressure due to the action of the cocoa polyphenols, but the clinical effect of low habitual cocoa intake on blood pressure and the underlying blood pressure-lowering mechanisms are unclear (Taubert et al., 2007). High flavanol cocoa was also found to increase brachial-artery flow-mediated dilation, which could reduce cardiometabolic risk among those who are overweight or obese (Table 1; Davison et al., 2008).

A randomized study in which patients with essential hypertension received either 100 g dark chocolate per day (containing 88 mg flavanols) or 90 g per day flavanol-free white chocolate in an isocaloric manner for 15 days found that the ambulatory blood pressure decreased after dark chocolate intake, but not with white chocolate consumption (Table 1; Grassi et al., 2005). Also, this same study indicated that dark chocolate was associated with decreased serum LDL cholesterol, improved flow-mediated dilation, and ameliorated insulin sensitivity in hypertensives. In another study, the daily consumption of flavanol-containing dark chocolate was associated with a significant mean reduction of 5.8 mmHg in systolic blood pressure (Erdman et al., 2008). Together the results of these human dietary intervention trials provide scientific evidence of the vascular effects of cocoa *flavanols* and suggest that the regular consumption *of cocoa* products containing *flavanols* may reduce *risk of* cardiovascular diseases (Erdman et al., 2008).

In a randomized cross-over study, 28 healthy young (18–20 years) male soccer players consumed either 105 g of flavanol-containing milk chocolate (FCMC) (168 mg of flavanols) or cocoa butter chocolate (CBC) (<5 mg of flavanols) as part of their normal diet for 14 days (Table 1 Fraga et al., 2005). The consumption of FCMC was significantly associated with a decrease in diastolic blood pressure, mean blood pressure, plasma cholesterol, LDL-cholesterol, malondialdehyde, urate, and lactate dehydrogenase activity and an increase in vitamin E/cholesterol. No relevant changes in these variables were associated with CBC. FCMC consumption was associated with changes in several variables which are related to cardiovascular health and oxidant stress (Fraga et al., 2005).

A study involving 49 adults with serum total cholesterol concentrations of 5.20–7.28 mmol/L and blood pressure of ≤59/99 mmHg indicated that regular consumption of chocolate bars containing plant sterols and cocoa flavanols resulted in reductions of 2.0 and 5.3% in serum total and LDL cholesterol ($p < 0.05$), respectively. Consumption of cocoa flavanols also reduced systolic blood pressure after eight weeks (−5.8 mmHg; $p < 0.05$) (Table 1- Allen et al., 2008).

Table 1 shows that a short-term effect of flavonoid-rich dark chocolate in terms of inducing coronary vasodilation and improving coronary vasomotion and shear stress-dependent platelet adhesion resulted in the potential to beneficially affect atherothrombosis (Flammer et al., 2007).

A cross-sectional design study with 4970 participants of the National Heart, Lung, and Blood Institute Family Heart Study, USA assessed chocolate intake through a semi-quantitative food frequency questionnaire. The consumption of chocolate was inversely related with prevalent coronary heart disease in the United States (Table 1; Djoussé et al., 2011). Chocolate consumption was associated with lower cardiac mortality in a dose-dependent manner in patients free of diabetes in the Stockholm Heart Epidemiology Program, Sweden, surviving their first acute myocardial infarction (Table 1; Janszky et al., 2009)

A study which examined the association between chocolate consumption and risk of stroke in the population-based Swedish Mammography Cohort of 39,227 women revealed that only women in the highest quartile of chocolate consumption (median 66.5 g/week) had a significantly reduced risk of stroke, suggesting that higher intakes are necessary for a potential protective effect (Table 1; Larsson et al., 2011). Table 1 indicates that in a population of middle-aged and elderly Swedish women, moderate habitual chocolate intake was associated with a lower rate of heart failure hospitalization or death, but the protective association was not observed with intake of ≤1 servings per day (Mostofsky et al., 2010). Converse to the previous findings above, a double-blind, placebo-controlled, randomized trial failed to support the predicted beneficial effects of short-term (6 weeks) dark chocolate and cocoa consumption on any of the neuropsychological or cardiovascular health-related variables in healthy older adults (Table 1; Crews et al., 2008).

The facilitation of vasodilation and attenuating exercise-induced increases in blood pressure, cocoa flavanols may

decrease cardiovascular risk and enhance the cardiovascular benefits of moderate intensity exercise in at-risk individuals (Table 1; Berry et al., 2010).

In an investigation to determine whether certain flavan-3-ols and PCs can have a positive influence on cardiovascular health, it was found that homeostatic modulation of flavan-3-ols and PCs production offered an additional mechanism by which flavan-3-ols and PCs -rich foods can potentially benefit cardiovascular health. Cells from individuals with low baseline levels of cytokine transforming growth factor ($n = 7$) were stimulated by individual flavan-3-ols and PCs fractions (25 μg/ml). Cytokine transforming growth factor release was enhanced in the range of 15–66% over baseline [$p < 0.05$; monomer, dimer, and tetramer (Mao et al., 2003)].

7.5. Hemolysis and Antiplatelet Formation

A number of studies have demonstrated that the consumption of flavonoid-rich cocoa products could inhibit epinephrine-induced platelet activation and reduce the susceptibility to free-radical hemolysis, which could offer cardiovascular benefits. Table 1 shows that the susceptibility of erythrocytes to free radical-induced hemolysis was reduced significantly ($p < 0.05$) following the consumption of flavonoid-rich cocoa beverages. Epicatechin, catechin, 3′-*O*-Me epicatechin, and (−)-epicatechin-(4β>8)-epicatechin (Dimer B2) were detected in the plasma within one hour after the consumption of the beverage (Table 1; Zhu et al., 2005).

Cocoa consumption suppressed unstimulated and stimulated platelet activation in whole blood, which may explain the effects of dietary polyphenols. The consumption of a cocoa drink resulted in the inhibition of epinephrine-induced platelet activation. The platelet activation and reactivity to physiologic agonists were inhibited 2 and 6 hours after the ingestion of a cocoa beverage by healthy subjects without a history of cardiovascular disease. This was shown in a controlled clinical setting, using specific antibodies against the activated conformation of the fibrinogen-binding receptor GPIIb-IIIa GPIIb-IIIa and granular membrane protein P-selectin on the platelet surface (Rein et al., 2000). These authors suggest that additional research is required to determine the exact chocolate components that are responsible for the observed antiplatelet effects, the amounts needed to reach effective plasma levels, and the clinical significance to coronary vascular diseases and thrombosis.

Thirty-two healthy subjects were assigned to consume active (234 mg cocoa flavanols and PCs /day) or a placebo ($\leq$ 6 mg cocoa flavanols and PCs/day) tablets in a blinded parallel-designed study. It was revealed that regular consumption of flavanols and PCs from cocoa, in a range easily accommodated in a normal diet, increased plasma concentrations of epicatechin and catechin, but there were no effects on markers of oxidation. The cocoa flavanol and PC treatment modestly reduced platelet aggregation and activation (Murphy et al., 2003). The mean platelet count was significantly higher ($p = 0.0001$) and the mean platelet volume was significantly lower ($p = 0.004$) in the active group than in the placebo group on day 28. There were no significant differences in arachidonic acid-induced platelet aggregation between the active and placebo groups on day 28 or changes within each group from days 0 to 28.

Cocoa extract and individual cocoa flavanols and PC oligomers demonstrated a dose-dependent antioxidant activity against *in vitro* erythrocyte hemolysis. Epicatechin, catechin, Dimer B2, and Dimer B5 were detected in plasma within 30 minutes following the feeding of a cocoa extract. Concordant with this rise, an increase in plasma antioxidant capacity, and a parallel decrease in erythrocyte hemolysis were reported (Zhu et al., 2002).

Dark chocolate consumption on ex vivo platelet function acutely reduced collagen-stimulated platelet aggregation and reduced plasma NO production (Innes et al., 2003; Kennedy et al., 2003). A single-investigator, blind, randomized study carried out in Dundee examined the ex vivo effects of chocolate on platelet activity in healthy volunteers. White chocolate had no effect on ex vivo platelet function. Milk chocolate was associated with a trend toward reduced (not of statistical significance) platelet-rich plasma aggregation and had no significance on platelet NO production. Whole-blood platelet aggregation was reduced significantly ($p < 0.05$) for stimulation with 1 mcg/mL collagen. Dark chocolate was associated with a reduction in plasma NO and a significant ($p = 0.018$) reduction in ADP-stimulated aggregation (Innes et al., 2003; Kennedy et al., 2003).

7.6. Cerebral Blood Flow, Memory, and Alzheimer's disease (Neurodegenerative Diseases)

Neuroinflammatory processes are known to contribute to the flow of events culminating in the neuronal damage which underpins neurodegenerative disorders. Evidence suggests that flavonoids may express such ability through a multitude of physiological functions, including an ability to modulate the brains immune system. A review highlighted the evidence for their potential of flavanols to inhibit neuroinflammation through an attenuation of microglial activation and associated cytokine release, iNOS expression, NO production, and NADPH oxidase activity. Flavonoids represent important precursor molecules in the quest to develop of a new generation of drugs capable of counteracting neuroinflammation and neurodegenerative disease (Spencer et al., 2012).

Transcranial doppler ultrasound was used to measure mean blood flow velocity in the middle cerebral artery in 34 healthy, elderly volunteers (72 $\pm$ 6 years) in response to the regular intake of flavanol-rich cocoa (FRC) or flavanol-poor cocoa (FPC) (Table 1; Sorond et al., 2008). After two weeks of the dietary intake of FRC, a significant increase in cerebral blood flow velocity was observed. The data suggest a promising role for regular cocoa flavanol consumption in the treatment of cerebrovascular ischemic syndromes, including dementias and stroke (Table 1;

Sorond et al., 2008). This benefit may extend to the brain and have important implications for learning and memory (Sorond et al., 2008).

An increase in the blood oxygenation level-dependent signal intensity in response to the cognitive task following ingestion of flavanol-rich cocoa (172 mg cocoa flavanols daily for 5 days) was due to altered neuronal activity or changed vascular responsiveness, or both; the net effect was dependent on which of the two effects was dominant (Table 1; Francis et al., 2006). The flavanol-rich cocoa increased the cerebral blood flow to brain gray matter, suggesting the potential of cocoa flavanols in the treatment of vascular impairment, including dementia and strokes, and in maintaining cardiovascular health.

Neurodegenerative disorders such as Alzheimer's disease (AD) are associated with oxidative stress. It has been suggested that apoptosis is a crucial pathway in neuronal cell death in AD patients. 4-Hydroxynonenal (HNE), one of the aldehydic products of membrane lipid peroxidation, is reported to be elevated in the brains of AD patients and mediates the induction of neuronal apoptosis in the presence of oxidative stress. In patients, HNE-induced nuclear condensation and increased sub-G1 fraction, both markers of apoptotic cell death, were inhibited by the cocoa PC fraction and its major antioxidant PC B2 (Table 1; Cho et al., 2009). The antiapoptotic effects of cocoa PC fraction and PC B2 were mediated by blocking the activation of the mitogen-activated protein kinase 4 activity—c-Jun N-terminal protein kinase (MKK4–JNK) pathway and intracellular reactive oxygen species (ROS) accumulation, and the subsequent suppression of caspase-3 cleavage, poly(ADP-ribose) polymerase (PARP) cleavage, and downregulation of antiapoptotic protein.

The adult proliferation rate declines with aging and is altered in several neurodegenerative pathologies such as Alzheimer's disease. A natural diet rich in polyphenols and polyunsaturated fatty acids (LMN diet) can modulate neurogenesis and have a significant effect combating the cognitive function decline during both aging and neurodegenerative diseases such as Alzheimer's disease (Valente et al., 2009). The LMN diet rich in polyphenols, dry fruits and cocoa, was able to decrease behavioral deterioration caused by aging and Tg2576 genotype in 13-month-old mice and to delay the Aβ plaque formation. These results corroborate the increasing importance of polyphenols as human dietary supplements in amelioration of the cognitive impairment during aging and neurological disorders (Fernández-Fernández et al., 2011).

Table 1 shows that in a study which examined a one-year administration of a cocoa polyphenolic extract (Acticoa powder), orally delivered at the dose of 24 mg/kg per day between 15 and 27 months of age, affected the onset of age-related cognitive deficits, urinary free dopamine levels and lifespan in old Wistar-Unilever rats (Bisson et al., 2008). The lifespan of AP24-treated rats (Acticoa powder—cocoa polyphenolic extract) at the dose of 24 mg/kg per day was prolonged relative to placebo by approximately 11% over the 27-month test period.

7.7. Cancer

A review of the epidemiologic evidence for protective effects against cancer and overall mortality revealed a very small number of observational epidemiologic studies, which offered weak support for a reduction in mortality and little data related to cancer (Maskarinec, 2009). However, several intervention studies, despite their short duration, have reported some favorable changes in biomarkers assessing antioxidant status, but very few findings related to inflammatory markers (Maskarinec, 2009). A review of the chemopreventive effects of cocoa polyphenols on the major chronic diseases of the world indicated that few studies show that reactive oxygen species associated with the carcinogenic processes is also inhibited (Weisburger, 2001). There have not been many studies on a possible lower risk of various types of cancer either in humans or in animal models consuming cocoa butter or chocolates.

7.8. Immune Response

The innate immune system comprises one of the first lines of defense against pathogens. In addition to providing physical and chemical barriers, the innate immunity consists of several specific immune cells, including monocyte/macrophages, natural killer (NK) cells, and polymorphonuclear cells. The innate immunity in host protection and the initiation of cytokine networks are gaining increasing recognition. A study investigated the effect of select cocoa flavanols and PCs on innate responses in vitro. Peripheral blood mononuclear cells and purified monocytes and CD4 and CD8 T cells were isolated from healthy volunteers and cultured in the presence of cocoa flavanol fractions which differed in the degree of flavanol polymerization (Kenny et al., 2007). The chain length of flavanol fractions had a significant effect on cytokine release from both unstimulated and lipopolysaccharide-stimulated peripheral blood mononuclear cells. The flavanol oligomers were potent stimulators of both the innate immune system and early events in adaptive immunity (Kenny et al., 2007).

Cocoa was shown to have regulatory effects on the acquired immune response in both *in vitro* and *in vivo* experiments (Ramiro-Puig and Castell, 2009). In rats, high cocoa intake modulated intestinal and systemic immune cell functionality. It seems that the immune cell function is controlled by redox-sensitive pathways, flavonoids, which are potent antioxidant compounds and thus responsible for cocoa's beneficial effects. Also, there was evidence to show that certain cocoa flavonoids can directly interact with cell signaling and gene expression factors.

7.9. Thymus Maturation

Cocoa intake enhanced total antioxidant capacity in all tissues especially in the thymus of young rats (Ramiro-Puig et al.,

2009). Moreover, thymus superoxide dismutase and catalase activities were also dosedependently increased by cocoa. Cocoa diet enhanced the thymus antioxidant defenses and influenced thymocyte differentiation. In the thymus, the richest cocoa diet produced a strong increase in the activity of antioxidant enzymes and may also enhance thymic maturation in young rats (Ramiro-Puig et al., 2009).

7.10. Anticariogenic

Glucans comprise of an extracellular slime layer produced in the presence of sucrose that promotes adhesion and the formation of a dental plaque biofilm by oral *Streptococci*. This study evaluated the effect of a mouth-rinse of cocoa bean husk extract (CBHE) on plaque accumulation and *Streptococci mutans* count. A concentration of 1 mg/ml of CBHE was proven to be effective. When the CBHE treatment was compared with the placebo treatment, there was a significant ($p < 0.001$) 20.9% reduction in *Streptococci* counts and a 49.6% reduction in plaque score with the former (Srikanth et al., 2008). The rinse can be incorporated in chocolates, chewing gums, mouth rinses, and beverages to prevent dental caries.

7.11. Microcirculation in Human Skin

The external application of cocoa was reported to have a variety of benefits, including soothing burns, disinfecting skin wounds, and acting as a moisturizer for the skin (Dillinger et al., 2000). The ingestion of cocoa was associated with photoprotection against ultraviolet (UV)-induced erythema and improved dermal blood circulation and skin hydration (Table 1; Heinrich et al., 2006). The photoprotection offered by cocoa flavanols was within the range of that reported for dietary carotenoids, such as β-carotene or lycopene (Heinrich et al., 2006). Ten healthy women ingested a cocoa drink (100 mL) with high (329 mg) or low (27 mg) content of flavanols. The ingestion of high flavanol (326 mg/d) cocoa powder dissolved in 100 mL water for 12 weeks led to increases in blood flow of cutaneous and subcutaneous tissues, and to increases in skin density and skin hydration (Heinrich et al., 2006). No statistically significant changes were found upon intake of low flavanol cocoa (Neukam et al., 2007). There are limited studies on its use topically.

7.12. Antimalarial Prophylaxis

Malaria is a pre-eminent tropical parasitic disease. Diglycosides of flavanols (a specific type of flavonoids) have been shown to retard life cycle of malaria parasites, whereas monoglycosides completely inhibited proliferation of trophozoite stage of parasites (Murakami, 2003). Evidence exists that high plasma concentration of antioxidants coincides with less severe malaria (Metzger et al., 2001). Five mechanisms which could underpin cocoa's anecdotal antimalarial effect were: (i) increased availability of antioxidants in plasma, (ii) membrane effects in general and erythrocyte membrane in particular, (iii) increased plasma levels of NO, (iv) antimalarial activity of cocoa flavonoids and their derivatives, and (v) boosted immune system mediated by components of cocoa including cocoa butter, polyphenols, magnesium, and zinc (Addai, 2010).

7.13. Pain Tolerance

It has been found that the sweet taste of sucrose acts as an analgesic, whereas the bitterness decreases pain tolerance. Twenty-four male participants were exposed to cold pressor tests (CPTs) while holding 1 of 3 tastants in their mouth (Table 1; Eggleston et al., 2010). These tastants were water, sucrose, or sucrose with cocoa added. After each CPT, the participants rated pain intensity and tastant qualities. The results suggested that the addition of a bitter substance reduces cues to the nutritive value of sucrose that may drive its analgesic effect. The bitterness ratings of cocoa were higher than the ratings of sucrose (corrected for water) by a 16.9% which, in turn, produced a 30% reduction in the duration of pain tolerance (Eggleston et al., 2010).

7.14. Summary

During the last few decades, consumption of cocoa has increased and this may be attributed in part to its potential beneficial effects on human health. Cocoa flavonoids have shown powerful antioxidant activity providing protection against oxidation (including preventing oxidative damage to LDL) and helping to prevent oxidative stress-related diseases. If dietary cocoa polyphenol intake is due to chocolate, its high energy content must be taken into account. The evidence presented here establishes that the PC oligomer dimer B2, as well as the flavanol monomers epicatechin and catechin, can be absorbed into the circulation. Additional work is needed to determine whether even larger PCs can be absorbed. Future investigation is needed to determine whether the reported in vitro effects of these PC oligomers could also occur *in vivo* (Holt et al., 2002). There is the need to be established whether the consumption of products with lower polyphenol content is associated with any health benefits in humans. In order to determine potential health benefits of cocoa polyphenols, large-scale, long-term, randomized, placebo-controlled studies (ideally with a cross-over design), as well as prospective studies are warranted. Furthermore, the food industry is encouraged to indicate the flavonoid content on the labels of their cocoa-derived products (Rimbach et al., 2009).

The body of research supports the conclusion that cocoa flavanols may provide a dietary approach to improving antioxidant status, maintaining LDL cholesterol, activating platelets, reduced inflammation and heart disease as well as interventions for cardiovascular complications associated with cancer,

attenuating the inflammatory process in atherosclerosis, reducing thrombosis, promoting normal endothelial function, and blocking expression of cellular adhesion molecules, promoting sensitivity to insulin, cognitive performance and skin health, and averting age-related blood vessel dysfunction.

Cocoa polyphenols have been investigated predominantly for their effect on the vascular system, with NO concentrations being a central target. A systematic review of 136 publications revealed that the body of short-term randomized feeding trials suggested that cocoa and chocolate may exert beneficial effects on cardiovascular risk via effects on lowering blood pressure, antiinflammation, antiplatelet function, higher HDL, and decreased LDL oxidation. The maintenance or restoration of vascular NO production and bioavailability and the antioxidant effects are the mechanisms most consistently supported by experimental data. The facilitation of vasodilation and attenuating exercise-induced increases in blood pressure by cocoa flavanols may decrease cardiovascular risk and enhance the cardiovascular benefits of moderate intensity exercise in at-risk individuals. Based on scientific findings, acute doses of chocolate- derived PCs can induce transient cardioprotective effects in humans. Further studies are required to determine whether or not similar effects would result from chronic intakes or large amounts of cocoa/chocolate for multiple weeks and whether the effects become attenuated or amplified with time. Thus, although flavonoid-rich foods and beverages are likely to protect the cardiovascular system, further research is needed to characterize the mechanism of action on flavanol-rich foods. Also, it is necessary to identify the amount and specific class or composition of flavonoids in cocoa/chocolate that exhibit beneficial health effects (Keen, 2001). In a critical review of polyphenols from cocoa, human studies regarding the effect of cocoa polyphenols on vascular health often lack a rigorous study design. The need for conclusive evidence in the form of large-scale randomized clinical trials is still lacking with respect to the ability of flavanol-rich cocoa to confer cardiovascular health benefits (Chaitman et al., 2004). Long-term clinical trials are also needed to definitively clarify the benefits deriving from long-term consumption of flavanol-rich foods, particularly focusing on the lowest effective levels as well as synergism or antagonistic actions between different classes of flavonoids commonly found in foods. Clearly, further *in vivo* studies are needed to document the efficacy of cocoa flavanols as a cardiovascular modulator (Mao et al., 2003).

The antiinflammatory, antioxidant, antiplatelet aggregation and positive vascular effects may contribute to the overall benefits of cocoa consumption against atherosclerosis (Monagas et al., 2009). Milk chocolate was associated with a trend toward reduced (not of statistical significance) platelet-rich plasma aggregation but had no significant effect on platelet NO production. Dark chocolate consumption on ex vivo platelet function acutely reduced collagen-stimulated platelet aggregation and reduced plasma NO production. Cocoa was shown to have regulatory effects on the acquired immune response in both *in vitro* and *in vivo* experiments. The flavan-3-ols and PCs isolated from cocoa can potentially modulate the level and production of several signaling molecules associated with immune function and inflammation, including several cytokines and eicosanoids. Flavanol-rich cocoa enhanced several measures of endothelial function to a greater degree among older than younger healthy subjects since cocoa is accepted as a dietary source of polyphenols, future studies should focus on specific mechanisms of action, i.e. inflammatory pathways and not direct antioxidant effects, with more diversification on nonvascular end-points (Cooper et al., 2008b). Further research is needed to shed light on the interactions between cocoa and cell physiology, contributing to the health benefits.

The flavanol-rich cocoa increased the cerebral blood flow to brain gray matter, suggesting the potential of cocoa flavanols in the treatment of vascular impairment, including dementia and stroke.

Future nutritional trials need to assess a larger number of biomarkers that may be relevant for cancer risk, whereas epidemiologic studies require valid dietary assessment methods to examine the association between cocoa products and cancer risk in larger populations and to distinguish possible cancer protective effects of cocoa products from those due to other polyphenolic compounds (Maskarinec, 2009).

8. COCOA FLAVOUR—SENSORY, PROCESSING, AND MICROBIAL ASPECTS

8.1. Cocoa Flavor Components

More than 500 flavor compounds have been identified from cocoa products (Reed, 2010). The flavor of cocoa bean and cocoa liquor could be based on origin practices, shipping and storage environments and processing conditions (Reed, 2010). A guide to the flavor profiles of cocoa beans of different origins was reported (Reed, 2010). The chocolate characters which originate as flavor precursors in cocoa beans are generated during postharvest treatments and change into pleasing odor notes in processing (Afoakwa et al., 2008). Chocolate flavor is influenced by volatile aroma compounds, nonvolatiles, and the behavior of the continuous fat phase (Afoakwa et al., 2009).

The isolation of the volatile fraction from cocoa powder by extraction/distillation process followed by application of an aroma extract dilution analysis revealed 35 odor-active constituents in the flavor dilution (Frauendorfer and Schieberle, 2006). Among them were 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (caramel-like), 2-and 3-methylbutanoic acid (sweaty, rancid), dimethyl trisulfide (cooked cabbage), 2-ethyl-3,5-dimethylpyrazine (potato-chip-like), and phenylacetaldehyde (honey-like) showed the highest flavor dilution factors. Quantitation of 31 key odorants by means of stable isotope dilution assays, followed by a calculation of their odor activity values (OAVs) (ratio of concentration to odor threshold) revealed OAVs $>$ 100 for the five odorants acetic acid

(sour), 3-methylbutanal (malty), 3-methylbutanoic acid, phenylacetaldehyde, and 2-methylbutanal (malty) (Frauendorfer and Schieberle, 2008).

The aroma of cocoa liquor extracted by using solid phase micro extraction with polydimethylsyloxane-divinylbenzene polymer was analyzed in gas chromatography-olfactometry (Misnawi and Ariza, 2011). Most of the odor-active compounds were alcohols, carboxylic acids, aldehydes, ketones, esters, pyrazines, and amines. Cocoa-specific aroma attributed in terms of sweet, nutty, caramel, and chocolate-like notes were associated with trimethylpyrazine, tetramethylpyrazine, 2,3-butanediol, dodecanoic acid, phenylethyl alcohol, ethanone, benzeneacetaldehyde, and 1,4-bis (morpholinoacetyl) piperazine (Misnawi and Ariza, 2011). The defective cocoa aroma such as smoky and rancid was generated by benzeneacetaldehyde, alphaethylidene, trimethylpyrazine, and trans-2,3-dimethyloxirane. These compounds generated cocoa aroma attributes of sweet, nutty, caramel, and chocolate-like notes as well as defective cocoa aroma such as smoky and rancid.

A total number of 33 volatile compounds belonging to the chemical classes of alcohols, ketones, aldehydes, esters, acids, alkanes, sulfur compounds, aromatic hydrocarbons, pyrazines, terpenes, and furans were indentified in dark chocolate with hazelnuts on day 0. After 12 months of storage, an increase in concentration of aldehydes, ketones, alcohols, and alkanes with a parallel decrease in pyrazines were observed especially in case of least protected products after 6 and 12 months of storage (Mexis et al., 2010).

Influences of matrix particle size distribution and fat content on flavor release of dark chocolate volatiles were quantified by static headspace gas chromatography using GC–MS (Afoakwa et al., 2009). Sixty-eight flavor compounds were identified, comprising alcohols, aldehydes, esters, ketones, furans, pyrans, pyrazines, pyridines, pyroles, phenols, pyrones, and thiozoles. From GC–olfactometry, 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal had chocolate notes. With cocoa/roasted/nutty, notes were trimethyl-, tetramethyl-, 2,3-dimethyl-, 2,5-dimethyl-, 3(or 2),5- dimethyl-2(or 3)-ethyl-, and 3,5(or 6)-diethyl-2-methylpyrazine and furfuralpyrrole. Compounds with fruity/floral notes included 3,7-dimethyl-1,6-octadien-3-ol and 5-ethenyltetrahydro-R,R,5-trimethylcis-2-furanmethanol. Caramel-like, sweet and honey notes were conferred by 2-phenylethanol, phenylacetaldehyde, 2-phenylethylacetate, 2,3,5-trimethyl-6-ethylpyrazine, 2-carboxaldehyde-1H-pyrrole, furancarboxaldehyde, furfuryl alcohol, and 2,5-dimethyl-4-hydroxy-3(2H)furanone. Fat content was directly related to head space concentrations of compounds characterized by cocoa, chocolate, praline, fruity, and roasted notes: trimethypyrazine, 3-methylbutanal,2,3-dimethylpyrazine, 2,5-dimethylpyrazine, tetramethylpyrazine, linalool oxide and 2,3,5-triethyl-5-methylpyrazine, at all particle size distributions (Afoakwa et al., 2009).

Evaluation of cocoa with and without added organic acids revealed that oxalic acid was important in chocolate flavor. The reduction in the levels of acetic and lactic acids in cocoa beans may not be sufficient to produce a desirable flavor balance (Holm et al., 1993).

Chocolates were differentiated according to geographical origin using chemometrics (Cambrai et al., 2010). There was a clear separation of the volatile content of Caribbean chocolates from independent groups for both Africa and Madagascar. Volatile compounds of (E,E)-2,4-nonadienal and (E,E)-2,4-decadienal were identified as characteristic for the Caribbean group. The study revealed that hydrodistillation, gas chromatography analysis, and statistical analysis may improve the control of the geographical origin of chocolate during its long production process.

8.2. Sensory Assessment linked to Flavor Components

In international marketing, cocoa beans are categorized as "bulk" or "ordinary" and "fine or flavor" cocoa types. An optimized protocol for sensory assessment of cocoa liquors was developed and used for determining differences in flavor and sensory attributes between Ghanaian beans (classified as "bulk") and cocoa bean samples from commercial clones and estates (classified as "fine or flavor") in Trinidad (Sukha et al., 2008). This method was appropriate in delineating quantitative differences in flavor attributes among cocoa genotypes and in determining the influence of the environment on cocoa flavor profiles. This protocol involves a complete description of the various stages in cocoa bean processing, liquor preparation selection, and training of panelist and data analysis.

The sensory quality of fermented and roasted cocoa seeds was assessed by their desirable bitterness, slight sour taste, as well as typical astringent mouth-feel, which is perceived as a long-lasting puckering, shrinking, and drying sensation in the oral cavity (Stark and Hofmann, 2005). The application of chromatographic separation and taste dilution analyses revealed that besides PCs, a series of *N*-phenylpropenoyl amino acids were the key contributors to the astringent taste of nonfermented cocoa beans as well as roasted cocoa (Stark and Hofmann, 2005). The oral sensation imparted by the *N*-phenylpropenoyl amino acids was described as mouth-drying and puckering astringent, with threshold concentrations ranging from 26 to 190ímol/L. The taste thresholds obtained for the aspartic acid derivatives showed that the sensory activity was not strongly influenced by the hydroxyl groups at the cinnamoyl moiety.

The application of molecular science approach to alkalized cocoa in the structure determination and sensory analysis showed that alkalization of cocoa induced the nonenzymatic *C*-glycosylation of flavan-3-ols to form new identified flavan-3-ol- *C*-glycosides (Stark and Hofmann, 2006). A trained sensory panel indicated that the glycosides modified the bitter taste profile, decreased the bitter taste intensity of cocoa beverages and theobromine solutions while aglycones or other *C*-glycosides, such as apigenin-8-*C*-β-D-glucopyranoside and apigenin-6-*C*-β-D-glucopyranoside, did not show any significant activity (Stark and Hofmann, 2006). The molecular definition of taste

of roasted cocoa nibs was provided by quantitative studies and by a trained sensory panel (Stark et al., 2006). A total of 84 putative taste compounds were quantified in roasted cocoa beans and then rated for the taste contribution on the basis of dose-over-threshold factors to bridge the gap between pure structural chemistry and human taste perception. The key taste compounds in roasted cocoa nibs were bitter-tasting alkaloids theobromine and caffeine, seven bitter-tasting diketopiperazines, seven bitter- and astringent-tasting flavan-3-ols, six puckering astringent *N*-phenylpropenoyl-L-amino acids, four velvety astringent flavanol glycosides, γ-aminobutyric acid, β- aminoisobutyric acid, and six organic acids.

Brain function techniques (location, nature and characteristics) of brain activity during detection and identification of odors are of significant value to sensory systems. Steady-state probe topography has been used to record steady-state visual evoked potentials which have established cognitive task-related changes in a variety of testing paradigms response to olfactory stimulation (Patterson et al., 1998). The steady-state visually evoked topography changes associated with cocoa flavanol consumption were investigated (Camfield et al., 2011). In a randomized, double-blind placebo controlled trial, 63 middle-aged volunteers aged between 40 and 65 years were administered a daily chocolate drink containing 250 mg or 500 mg cocoa flavanols versus a low cocoa flavanol (placebo) drink over a 30-day period. Steady-state probe topography was used to assess neurocognitive changes associated with cocoa flavanol supplementation during the completion of the Spatial Working Memory task (Camfield et al. 2011). Differences in brain activation could be interpreted as evidence of increased neural efficiency in spatial working memory function associated with chronic cocoa flavanol consumption (Camfield et al., 2011).

In a study which assessed the shapes and speech sounds which people associate with chocolates of varying cocoa content demonstrated that certain chocolates were more strongly associated with angular shapes and "sharp" inflected, high-pitched meaningless words, such as "tuki" and "takete" (Ngo et al., 2011). Participants were given paper-based line scales, anchored at either end with either a nonsense word or simple outline shape. They tasted the chocolates and indicated whether their perception of the flavor better matched one or other of the items anchoring the scales by marking the appropriate point along the scale. Lindt extra creamy milk chocolate (30% cocoa) and Cadbury's Koko milk chocolate truffles were both more strongly associated with rounded shapes and softer sounding, lower-pitched pseudowords, such as "maluma" the phenomenon of sound symbolism extended beyond the visual modality into the domain of flavor perception where, in particular, speech sounds carry meaning in terms of the taste/flavor of chocolates.

8.3. Processing Effects on Cocoa Flavor

A critical review of flavor formation and character in cocoa and chocolate identified bean composition and precursor formation, effect of genotype on cocoa bean flavors, development of chocolate flavor during post-harvest treatments, processing, and chocolate manufacture, and the key flavor compounds in milk and dark chocolate (Afoakwa et al., 2008).

The sensory quality of cocoa nibs, cocoa liquor, and chocolates was modified by the addition of aromatic fruit pulps to wet cocoa beans during fermentation (Eskes et al., 2009). The aroma pulp of two tropical fruits, *Theobroma grandiflorum* and Anonna muricata was added to cocoa beans during fermentation. The results suggested that the cocoa cotyledon absorbed flavors from the aromatic pulp. The cocoa bean flavor environment could affect the expression of flavor in cocoa products.

Cocoa polyphenols undergo a series of transformations during cocoa processing leading to the characteristic cocoa flavor. Exogenous polyphenol oxidase (PPO) was applied to reduce the polyphenol content in cocoa nibs (de Brito et al., 2002). Total phenol content was reduced in PPO or water treatments, but when associated with air there was an increase in phenol content.

Using a panel of 15 qualified tasters, the overall aroma intensity and cocoa flavor of the chocolates made with dry cocoa beans from Guianan trees were found to be statistically superior to those of the industrial reference, the West African Amelonado (Assemat et al., 2005). Cocoa almonds (the fermented and dried cocoa seeds) are raw materials which are used for chocolate production. Poor-quality cocoa almonds were enzymatically treated to improve the chocolate flavor precursors in the production of liquors (Oliveira et al., 2011). The enzymatically treated samples were evaluated by difference-from-control and ranking preference sensory tests. There was an improvement of 50% in chocolate flavor with flavorzyme, which contained aspartic proteases and carboxypeptidases of microbial origin.

A study of fermented cocoa beans which were dried in a direct solar dryer at 3 levels of loading (20, 30, and 60 kg) found no significant difference ($p > 0.05$) among the treatments for cocoa astringency, bitterness, and sourness flavor notes by a trained sensory panel (Hii et al., 2006). However, a better flavor was observed for beans from the 20 kg treatment.

The key aroma compounds of 3-methylbutanoic acid, ethyl 2 methylbutanoate, and 2-phenylethanol were already present in unroasted, fermented Criollo cocoa beans which did not increase during roasting (Frauendorfer and Schieberle, 2008). A strong increase in the concentrations of the Strecker aldehydes 3-methylbutanal and phenylacetaldehyde and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone suggested that these odorants contributed to most of the overall aroma after roasting. Various compounds such as 3-methylbutanoic acid, ethyl 2-methylbutanoate, and 2-phenylethanol were present in unroasted, fermented cocoa beans and did not increase during roasting. The basic sensory perceptions (undesirable, bitter pungent, repulsive, fruity, nutty, floral, vegetal, and sweet chocolate) of roasted cocoa powder were explained by aroma compounds with R2-adjusted values of 0.85 and greater (Bonvehí, 2005). The major components of cocoa bean aroma were 2,3,5,6-tetramethylpyrazine, benzaldehyde, 2-phenylacetaldehyde, acetophenone, 3-methylbutyric

acid, 5-methyl-2-phenyl-2-hexenal, ethyl phenylacetate, and 3-hydroxy-2-methyl-4-pyrone (mean greater than 1.30 mg kg^{-1}).

A study investigated the influence of polyphenol and pH on flavor quality of cocoa beans during roasting (Noor-Soffalina et al., 2009). The response surface methodology been shown to be a useful tool to investigate the optimum concentration of polyphenol and pH on the production of cocoa Maillard-related flavor precursors. Reducing sugars or amino acids were mixed with 55% cocoa butter with 4% water (w/w). Polyphenols were added and pH was adjusted according to the central composite design. The response surface methodology indicated that a lower concentration of amino acids and reducing sugars was obtained at higher polyphenol concentration (120 g kg^{-1}) and lower pH value (4.5). The best polyphenol concentration for the development of the Maillard-related cocoa flavor precursors was between 43 g kg^{-1} and 58 g kg^{-1} and pH 7.0–7.5 (Noor-Soffalina et al., 2009).

The manufacture of chocolate liquor involves cleaning of cocoa beans, nib, and shell separation (micronization through winnowing), alkalization (dutching), heat treatment (roasting), grinding to a desired particle size, and the standardization of the product's fat. Chemical composition and sensory analysis of Ecuadorian cocoa liquor were conducted by a panel of Nestlé France composed of seven trained tasters (Luna et al., 2002). Seven attributes of Ecuadorian cocoa liquor were: cocoa flavour, bitterness, astringency, acidity, fruitiness, floral note and green note that were all scored from 1 to 5: (1) absent, (2) weak, (3) moderate, (4) strong, and (5) very strong. Polyphenols contributed to the overall perception of cocoa liquor characteristics and were correlated positively with bitterness, astringency, and the green note, and negatively with fruitiness. The composition and sensory quality of cocoa liquor were the result of both the genotypic contribution and the conditions of fermentation and roasting (Luna et al., 2002). Using a line scale, eight trained panelists conducted sensory analysis with a Ghanaian fermented cocoa liquor as a reference. The sensory attributes were cocoa flavor, astringency, bitterness, acidity/sourness, fruity/floral/bouquet, raw/green, smoky, mouldy/earthy, and viscosity. An increase in roasting duration of cocoa liquors containing 58 to 143 g kg^{-1} polyphenol increased to 170 g kg^{-1} polyphenols which have a negative effect on flavor (Misnawi et al., 2004).

A laboratory scale conch was developed with the purpose of testing new formulations using small amounts of chocolate mass. The resulting chocolate was tempered and then evaluated by a sensory panel. The sensory analysis showed a perceptible flavor difference between the laboratory scale and industrial scale conch processing (Schumacher et al., 2009).

Tea seed oil was used for cocoa butter replacer (CBR) after enzymatic modification (lipase from *Thermomyces lanuginosus*) up to 3.5% by weight of chocolate product (Zarringhalami et al., 2010). Adding up to 10% enzyme interesterified sample reduced bloom development without affecting the desirable crystal formation and sensory qualities in chocolate samples.

Full fat soy flour (FFSF) was used as a replacer for whole-milk powder (WMP), stevia–mannitol blend as replacer for sugar and soybean oil as replacer for cocoa butter in chocolate manufacture (Pandey and Singh, 2011). On the basis of overall acceptability scores, 40% replacement of WMP by FFSF in chocolate mix was found optimum. Lecithin at 0.32% (w/w) level of chocolate mix improved the overall acceptability of the product.

A study which investigated the effect of active and modified atmosphere packaging on quality retention of dark chocolate with hazelnuts revealed that after 12 months of storage, sensory odor changes were the least in products packaged with the oxygen absorber irrespective of packaging material permeability (score 7.6/9) while the lowest scores were recorded for dark chocolate commercially packaged at 20°C (score 4.19) using a consumer panel (Mexis et al., 2010). After 12 months of storage, smaller taste changes were recorded in products packaged with the oxygen absorber stored at 4°C (score 5.9/9) while the lowest scores were recorded for the commercially packaged product stored at 20°C (scores 3.8/9).

8.4. Microbial Associations to Cocoa Flavor

Differences in microbial activity were linked with the flavor of chocolates made from the corresponding dried, fermented cocoa beans. Sensory analysis performed by a trained panel showed that flavor profiles varied by heaps (Camu et al., 2008). The polyphenol and alkaloid contents of cocoa beans were crop- and heap-dependent, epicatechin and theobromine levels decreased during fermentation due to diffusion out of the bean cotyledons and polyphenol oxidation and condensation. Residual levels were responsible for the degree of bitterness of the final chocolates. Hence, fermentation control was necessary to control the flavor of chocolate (Camu et al., 2008).

Sensorial acceptability tests of the chocolate obtained from beans fermented with the *Kluyveromyces marxianus* inoculation was more accepted by panellists in comparison with the one from cocoa obtained through natural fermentation (Leal et al., 2008). The increase in mass aeration during the first 24 hours seemed to be fundamental for the improvement of fermentation quality, demonstrating the potential application of this improved hybrid yeast strain with superior exogenous pectinolytic activity.

8.5. Summary and Future Work

Flavor is critical to acceptability of cocoa and cocoa products. However, the extent to which the inherent bean constituents from the cocoa genotype, environmental factors, post-harvest treatment, and processing technologies influence chocolate flavor formation and relationships with final flavor quality is still not clear (Afoakwa et al., 2008). The analysis of cocoa aroma has been carried out by extract dilution and distillation, gas chromatography-olfactometry, static headspace gas chromatography using GC, and stable isotope dilution assays followed by calculation of odor activity values. Cocoa liquor extracts

were analyzed by flow-injection electrospray-mass spectrometry as a method for sensory quality prediction. A wide range of odor active compounds have been identified in cocoa liquor, such as alcohols, carboxylic acids, aldehydes, ketones, esters, pyrazines, and amines. Some cocoa-specific aroma attributes were described as sweet, nutty, caramel-like and chocolate-like, sweaty, rancid, cooked cabbage, potato-chip-like, smoky, and honey-like. Cocoa flavor compounds composed of alcohols, aldehydes, esters, ketones, furans, pyrans, pyrazines, pyridines, pyroles, phenols, pyrones, and thiozoles. PCs and a series of *N*-phenylpropenoyl amino acids were the key contributors to the astringent taste of nonfermented cocoa beans as well as roasted cocoa. Strecker aldehydes 3-methylbutanal and phenylacetaldehyde and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone were the odorants which contributed most to overall aroma after roasting. The response surface methodology is a useful tool to investigate the optimum concentration of polyphenol and pH on the production of cocoa Maillard-related flavor precursors. Polyphenols contributed to the overall perception of Ecuadorian cocoa liquor characteristics which were correlated positively with bitterness. Poor-quality cocoa beans were enzymatically treated to improve the chocolate flavor precursors in the production of liquors. The shapes and speech sounds which people associate with chocolates of varying cocoa content demonstrated that certain chocolates were more strongly associated with angular shapes and "sharp" inflected, high-pitched meaningless words. Differences in microbial activity were linked to the flavor of chocolates made from the corresponding dried, fermented cocoa beans. Matrix effects on flavor release in dark chocolate merit attention as new product development occurs and consumers demand a wider range of origins and defined products, and their influence on sensory effects with particle size distribution and fat content remains unclear (Afoakwa et al., 2009).

9. MICROBIOLOGICAL ASPECTS

9.1. Cacao Diseases

Phytophthora megakarya, Phytophthora capsici, and *Phytophthora citrophthora* have been responsible for damaging cacao pod rot (Guest, 2007; da Silva do Nascimento et al., 2010) in West Africa and Central and South America (Guest, 2007). Frosty pod is caused by *Moniliophthora roreri* (Guest, 2007) and witches' broom by *Moniliophthora perniciosa* (previously *Crinipellis perniciosa*) (Ploetz, 2007). Cacao breeding programs have been challenged by minimal resistance to some of the diseases. However, the spread of the pathogens could be prevented by effective quarantine barriers.

9.2. Cocoa Fermentation

Raw cacao has an astringent, unpleasant taste and flavor which is fermented, dried, and roasted in order to obtain the characteristic cocoa flavor and taste (Nielsen et al., 2008). The microbial activity during cacao fermentation causes physical and chemical changes that impact on bean physiology and biochemistry and ultimately on the chocolate quality (Schwan and Wheals, 2004) and in particular flavor (Camu et al., 2008).

The sugary-acid properties of the cacao pulp provide an excellent medium for the proliferation of yeast to 10^7 to 10^9 CFU/g (Fleet and Dirks, 2007), whereby the yeasts metabolize the pulp sugars to produce ethanol, and an array of secondary metabolites (Schwan and Wheals, 2004). Some yeasts could utilize the citric acid of the pulp, and thus contribute to the equilibration of cotyledon pH which impacts on endogenous enzyme activity (Hansen et al., 1998). A study of the microflora and biochemistry of cocoa fermentation in the Dominican Republic demonstrated a significant decrease in glucose, fructose, and citric acid in the pulp over 48 hours with a maximum in lactic acid after around 120 hours of fermentation (Lagunes Gálvez et al., 2007). The degradation of the pulp during cocoa fermentation might be due to a combined action of pectinolytic enzymes produced by both yeast and *Bacillus* strains (Ouattara et al., 2008). The pectate lyases isolated from fermented cocoa were synthesized by *Bacillus pumilus* BS22, *Bacillus subtilis* BS66, and *Bacillus fusiformis* BS90 (Ouattara et al., 2010).

In cocoa fermentation, yeasts displayed maximum development after 24 hours (6.1×10^7 CFU g^{-1} of dry matter), whilst for lactic (*Lactobacillus*) and acetic acid (*Acetobacter*) bacteria it occurred after 48 hours (7.3×10^7 and 1.5×10^8 CFU g^{-1} of dry matter, respectively) (Lagunes Gálvez et al., 2007). The *Bacillus* species are more tolerant at the higher temperatures (45–50°C), which prevail in the later stages of fermentation (Ardhana and Fleet, 2003; Schwan and Wheals, 2004). Spontaneous cocoa bean fermentation processes carried out on farms in different cocoa-producing regions, *viz*., of Ivory and Brazil indicated that species diversity and community dynamics were influenced by local operational practices, in particular pod/bean selection which has impact on the quality of fermented cocoa beans (Papalexandratou et al., 2011a). At three commercial fermentaries in Indonesia, the microbial ecology of cocoa bean fermentation (Forastero and Trinitario) revealed that during the first —two to three days of fermentation, succession growth *of* various species of filamentous fungi, yeasts, lactic acid bacteria, and acetic acid bacteria occurred (Papalexandratou and De Vuyst, 2011c). The yeast species composition of 12 cocoa bean fermentations conducted in Brazil, Ecuador, Ivory Coast, and Malaysia indicated that the most frequent yeast species were *Hanseniaspora* sp., followed by *Pichia kudriavzevii* and *Saccharomyces cerevisiae*, independent of the origin of the cocoa (Papalexandratou and De Vuyst, 2011c). The Ivorian cocoa bean box fermentation samples showed a wider yeast species composition, with *Hyphopichia burtonii* and *Meyerozyma caribbica being the main ones* (Papalexandratou and De Vuyst, 2011c). Also yeasts of the genera *Hanseniaspora* and *Candida* were isolated (Lagunes Gálvez et al., 2007). *Hanseniaspora* (anamorph, *Kloeckera*) which dominated during the first stage of fermentation were not particularly ethanol tolerant and die off as more fermentative,

ethanol producing species started to grow and dominate the middle stage of fermentation (Schwan and Wheals, 2004). The yeast isolates in Ghanaian cocoa fermentations based on the genetic and phenotypic revealed three novel species were *Candida halmiae*, *Candida Awuaii*, and *Geotrichum ghanense* (Nielsen et al., 2010). An assessment of the microbial community of cocoa bean heap fermentations in Ghana revealed 91 yeast isolates. Using PCR fingerprinting with the primer M13, 91 yeast isolates were identified in cocoa bean heap fermentation (Daniel et al., 2009). The major yeasts were: *Pichia kudriavzevii* (*Issatchenkia orientalis*—30% of total isolates) *Saccharomyces cerevisiae* (24%), and *Hanseniaspora opuntiae* (20%) (Jespersen et al., 2005; Nielsen et al., 2005, 2007; Daniel et al., 2009). Other less frequently encountered species were *Candida carpophila*, *Candida orthopsilosis*, *Kodamaea ohmeri*, *Meyerozyma* (Pichia) *caribbica*, *Pichia manshurica*, *Saccharomycodes ludwigii*, and *Yamadazyma* (Pichia) *mexicana* (Daniel et al., 2009).

Of the various lactic acid bacterial strains tested during laboratory fermentation on cocoa pulp simulation bacteria, *Lactobacillus fermentum* was best adapted to the cocoa pulp ecosystem in the fermentation of glucose to lactic acid and acetic acid, reduced fructose to mannitol, and converted citric acid into lactic acid (Lefeber et al., 2010). The capacity to use citric acid was exhibited by *L. fermentum* 222 and the formation of mannitol was dependent not only on the lactic acid strain but also on environmental conditions. A mixed-strain starter culture of *Lactobacillus plantarum* 80, *L. fermentum* 222, and *Acetobacter pasteurianus* 386B was considered for better controlled and more reliable cocoa bean fermentation processes (Lefeber et al., 2010). The determination of a well-fermented cocoa bean would depend on mannitol, lactic acid, and acetic acid production and overoxidation (Lefeber et al., 2010).

For the Ivorian farm, the species diversity of lactic acid bacteria and acetic acid bacteria was narrow (Papalexandratou et al., 2011b). *L. fermentum* and *Leuconostoc pseudomesenteroides* were the predominant lactic acid bacteria species. A wider microbial species diversity throughout the fermentation process was seen in the case of the box fermentations on the selected Brazilian farms. *L. plantarum*, *L durianis*, *L. fermentum*, *L. mali*, *L. nagelii*, *L. pseudomesenteroides*, and *Pediococcus acidilactici*, as well as *Bacillus* subtilis were present at late fermentation, when the temperature inside the fermenting mass reached values higher than 50°C. On two different farms in Brazil using spontaneous organic cocoa bean box fermentations, the prevailing species at the initial phase of cocoa bean fermentation were *Fructobacillus pseudoficulneus*, *L. plantarum*, and *Acetobacter senegalensis* (Papalexandratou et al., 2011b). The four main lactic acid bacteria clusters identified in spontaneous heap fermentation were: *L. plantarum*, *L. fermentum*, *Leuconostoc pseudomesenteroides*, and *Enterococcus casseliflavus* (Camu et al., 2007). Of a total of 193 lactic acid bacteria strains isolated, 40 (20.7%) were hetero-fermentative and comprised either *L. brevis* or *L. fermentum* strains (Kostinek et al., 2008). The majority of the isolates were homo-fermentative rods (110 strains; 57% of isolates), which were characterized as *L. plantarum* strains. The homo-fermentative cocci consisted predominantly of 35 (18.1% of isolates) *Pediococcus acidilactici* strains (Kostinek et al., 2008).

The acetic acid bacteria clusters were: *A. pasteurianus*, *A. syzygii*-like bacteria, and two small clusters of *Acetobacter tropicalis*-like bacteria. There was no significant influence of heap and farm, season, and size of the heap (<500 kg) thus indicating the significance of the microorganisms (Camu et al., 2007).

A study investigated the effects of fermentation methods (wooden box, plastic box and in heaps) with or without turning on the chemical and physical quality of raw cocoa beans (Guehi et al., 2010). Cocoa fermented in boxes during four days without turning resulted in pH values above 5.0 while cocoa fermented in heaps had a pH 4.92. Generally, cocoa beans fermented with turnings were less acidic than beans fermented without turning except for fermentation in boxes. Cocoa beans fermented with turnings had about 10% of defectives beans irrespective of the process. Percentage of purple beans decreased to about 12% for cocoa fermented in wooden box. Among the three cocoa fermentation methods, fermentation in heaps appeared to be better for the production of a good quality raw cocoa. The turning of the cocoa beans after 48 and 96 hours of fermentation improved the physical quality of raw cocoa material by providing greater aeration of the cocoa mass, disappearance of the cocoa bean mucilage, and providing for the growth of acetic acid bacteria (Guehi et al., 2010).

9.3. Some Identification Techniques

The fermentation of cocoa by correlating Denaturing Gradient Gel Electrophoresis (DGGE) profiles and Near Infrared spectra (Vis/NIR) showed that correlation of microbial DGGE profiles with Vis/NIR spectra could offer a tool for correlating microbial activity with biochemical changes in the cacao beans during fermentation (Nielsen et al., 2008).

The lactic acid bacteria dynamics during spontaneous fermentation of cocoa beans were verified by culture-independent denaturing gradient gel electrophoresis (Santos et al., 2011). The DNA was used as a template for amplification with Lac1-Lac2 and Lac3-Lac2 for denaturing gradient gel electrophoresis analyses. There were variations between the numbers of operational taxonomic units throughout the process and the greatest similarity in amplified samples was obtained with the primers Lac3-Lac2 (Santos et al., 2011). *L. fermentum* and *A. pasteurianus* were the predominating bacterial species of the fermentations as revealed through (GTG)5-PCR fingerprinting of isolates and polymerase chain reaction-*denaturing gradient gel electrophoresis methods* (PCR-DGGE) of 16S rRNA gene PCR amplicons of DNA directly extracted from fermentation samples (Papalexandratou et al., 2011c). During spontaneous cocoa bean fermentation in vessels, lactic acid bacteria isolates belonged to two main (GTG)5-PCR clusters, namely *L. plantarum* and *L. fermentum*, with *Fructobacillus pseudofilculneus* occurring occasionally (Lefeber et al., 2011).

A study of the microbial diversity of spontaneous fermentations of Ghanaian cocoa beans, phylogenetic analysis based on 16S rRNA gene sequences allocated the isolates to the genus *Acetobacter* and revealed *A. lovaniensis*, *A. ghanensis*, and *A. syzygii* to be nearest neighbors (Cleenwerck et al., 2008). Also, the results proved that the isolates should be classified as representatives of a novel *Acetobacter* species, for which the name *A. fabarum* sp. nov. is proposed. 16S rRNA-PCR-DGGE revealed that *Lb. plantarum* and *Lb. fermentum* dominated the fermentations from day two until the end and *Acetobacter* was the only acetic acid bacteria species present at the end of the fermentations (Lefeber et al., 2011). The $(GTG)_5$-PCR fingerprinting differentiated four major clusters among Ghanaian-fermented cocoa bean isolates, namely: *A. pasteurianus* (cluster I, 100 isolates), *A. syzygii*- or *A. lovaniensis*-like (cluster II, 23 isolates), and *A. tropicalis*-like (clusters III and IV containing 4 and 5 isolates, respectively) (De Vuyst et al., 2008).

The presence related to *Erwinia/Pantoea/Tatumella* of the Enterobacteriaceae was revealed in fermentation (Garcia-Armisen et al., 2010; Lefeber et al., 2011). The prevailing enterobacterial species as revealed by 16S rRNA gene-PCR-DGGE were *Tatumella ptyseos* and *Tatumella citrea* (Papalexandratou et al., 2011a).

9.4. Processing Effects

The environment, including the presence of vectors, intense handling and storage conditions appear to be the main critical points during pre-processing of cocoa contributing to contamination by these enteropathogens. The concentration of total coliforms and *Escherichia coli* was highest during drying and storage, with percentages of up to 100% and 89% of positive samples (da Silva do Nascimento et al., 2010). The presence of *Salmonella* was detected in only one of the 119 samples of stored beans.

In a study of mycobiota of cocoa from farm to chocolate, a total of 1132 potentially toxigenic fungi were isolated from the following species or species groups: *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. niger* group, *A. carbonarius*, and *A. ochraceus* group (Copetti et al., 2011). The highest percentage of toxigenic fungi was found at the drying and storage stages.

In the treatment of the cocoa powder suspensions from 100 to 170°C for 10 minutes, revealed the presence of thermoresistant spores in 36% of the samples (Lima et al., 2011). In total 61 thermoresistant strains were isolated, of which the majority belonged to the *Bacillus subtilis* complex (65.6%).

Cocoa beans (the Ivory Coast variety) were subjected to convective roasting at various air temperatures and relative humidity at constant air flow rate (v) of 1 m s^{-1} (Stobińska et al., 2006). The most effective bactericidal temperature was 150°C independently from relative humidity of air, applied as a heating medium (Stobińska et al., 2006). Satisfactory microbial safety at roasting was at 140°C, at RH of 0.4%, for 45 minutes. Roasting for 45 minutes, at 135°C, and RH of 2%, reduced indigenous microflora of cocoa beans to 10^4 cfu per g. Conching [50–90°C (Krapf and Gantenbein-Demarchi, 2009)] did not assure the thermal inactivation of *Salmonellae* in different chocolate products. A study to demonstrate that thermal inactivation during conching process alone does not ensure the inactivation of *Salmonella* spp. in different chocolate masses and that an additional decontamination step at the beginning of the process as well as an HACCP concept is necessary during chocolate production to guarantee the absence of *Salmonella* spp. in chocolates and related products (Krapf and Gantenbein-Demarchi, 2009). Results of the thermal treatment showed approximate D-values for cocoa butter from $D_{50°C} = 245$ minutes to $D_{60°C} = 306$ minutes, for cocoa liquor from $D_{50°C} = 999$ minutes to $_{90°C} =$ 26 minutes and for dark chocolate of $D_{50°C} =$ 1574 minutes. z-values were found to be 20°C in cocoa liquor and 14°C in dark chocolate.

A study was conducted with *Bacillus cereus* vegetative cells inoculated in skim milk (SM) and liquid whole egg/skim milk mixed beverages with or without antimicrobial cocoa powder cocoanOX 12% with an aim to extend the shelf-life (Pina-Pérez et al., 2009). After 15 days of storage, the antimicrobial compound lowered *Bacillus cereus* survival rates in the samples supplemented with CocoanOX 12% by a 4 log cycle reduction, as compared to the untreated samples without CocoanOX 12%. This could indicate that the PEF-antimicrobial combination has a synergistic effect on the bacterial cells under study, increasing their sensitivity to subsequent refrigerated storage.

9.5. Contamination

Three Chrysosporium spp. [*C. xerophilum* Pitt, *C. inops* (Carmichael), and *C. farinicola* (Burnside) Skou], being xerophilic fungi, were isolated from commercial chocolate bars with a water activity (a_w) of approximately 0.28 (Kinderlerer, 1997).

Cocoa polyphenol dimer, tetramer, and pentamer inhibited the growth of *Streptococcus sanguinis*, whereas the growth of *S. mutans* was unaffected. However, pretreatment of surfaces with cocoa polyphenol pentamer (35 μM) reduced biofilm formation by *S. mutans* at 4 and 24 hours, whereas the effects on *S. sanguinis* were less consistent (Percival et al., 2006).

The main contamination of cocoa beans appears in the development of mycotoxins. Mycotoxins are secondary metabolites of moulds are very significant as they can be injurious to public health (Varga et al., 2005). Mycotoxins of major importance include fumonisins by *Fusarium* species, aflatoxin B1 (*Aspergillus flavus*), and ochratoxin A from *Penicillium* and *Aspergillus ochraceus* species (Tagne et al., 2000, Tjamos et al., 2004, Varga et al., 2005). Ochratoxin A has been reported to be a dangerous, nephrotoxic, and carcinogenic mycotoxin (Vrabcheva et al., 2004). Cocoa beans have thin testa or shell, in which ochratoxin A tends to accumulate. Cocoa powder is used for the preparation of drinking chocolate—sweetened cocoa powder. Ochratoxin A has been reported in cocoa powder in

Ivory Coast, Guinea, Nigeria and Cameroon up to 4.4 μg/kg higher than the EU regulatory level (Serra Bonvehi, 2004). About 1800 ng/kg ochratoxin A has been found in German cocoa powder between 1996 to 1999 (Petzinger and Weidenbach, 2002).

Raters and Matissek (2005) proved that mycotoxins are not homogenously distributed in cocoa beans. The same authors (Raters and Matissek 2007) detected ochratoxin A, aflatoxins B1, B2, G1, and G2 in low concentrations in cocoa beans and cocoa products. They determined at what stages of the cocoa production process a contamination with the mycotoxin-producing moulds and the formation of mycotoxins takes place and suggested that in the case of cocoa the contamination does not involve only the individual beans, but the fermentation units. They also suggested different ways of transporting ochratoxin A (OTA) and aflatoxin to the cocoa kernel caused by different polarities of toxins (Raters and Matissek 2007). Raters and Matissek (2008) also reported that deoxynivalenol (DON) is more likely to be found in cocoa shells, suggesting that the separation of shells during processing can reduce potential DON content. A similar conclusion derived by Amezqueta et al. (2005) was that improvements in the shelling process can prevent OTA occurrence in final cocoa products.

Sanchez-Hervas et al. (2008) studied mycobiota and mycotoxins from cocoa beans and provided significant information on the key fungal species responsible for mycotoxins such as ochratoxin A, aflatoxins, and cyclopiazonic acid (CPA).

Aroyeun and Adegoke (2007) published a paper on the reduction of ochratoxin A in cocoa powder and beverages using aqueous extracts and essential oils of *Aframomum danielli*. In a subsequent paper, Aroyeun et al. (2009) suggested that the active components of *Aframomum danielli* such as mono-terpenes, alkaloids, and phenolic acids in the essential oils might be responsible for the reduction of aflatoxin and ochratoxin A.

Serra Bonvehi (2004) studied the occurrence of ochratoxin A in 170 samples of cocoa products from different geographical origins. Ochratoxin A was detected in cocoa beans at levels of 0.1 to 3.5 μg/kg. The results indicate that roasted cocoa powder is not a major source of ochratoxin A in the diet. Tafuri et al. (2004) reported that the quality of cocoa powder sold in the Italian market can be improved and that 22% of the samples studied contained ochratoxin A in quantities above the permissible limits.

Mounjouenpou et al. (2008) studied filamentous fungi producing ochratoxin A during cocoa processing in Cameroon and concluded that fermented, dried cocoa from poor quality pods were the main source of this toxin.

Ochratoxin A (OTA) was the main mycotoxin occurring in cocoa. A Canadian study analyzed 32 samples of cocoa powder (16 alkalized and 16 natural) for ochratoxin A. An incidence of 100%, with concentrations ranging from 0.25 to 7.8 ng $^{g-1}$, six samples exceeded 2 ng $^{g-1,}$ the previous European limit for cocoa (Turcotte and Scott, 2011). More ochratoxin A in cocoa prepared using alkali (Dutch process) was found than in natural cocoa. The incidence of ochratoxin A in 28 chocolate samples (21 dark or baking chocolate and 7 milk chocolate) was also 100%, with concentrations from 0.05 to 1.4 ng $^{g-1}$ which was greater than the European limit for chocolate of 1 ng $^{g-1}$. A toxigenesis study showed that *Aspergillus carbonarius* was the main ochratoxin A producing strain isolated in post-harvest treatment in boxes or heap in the Cameroon (Mounjouenpou et al. 2008). *A. niger* strains did not always produce OTA.

9.6. Cocoa Waste

Aspergillus niger was used as an inoculum to analyze and quantify the kinetic activity of enzymes ligninases laccase, lignin peroxidase, and manganese peroxidase, produced by solid state fermentation using cocoa bran as a waste material, (dos Santos et al. 2011). The maximum enzyme activity occurred within 72 hours of fermentation and 50% water content, at 30°C for all the enzymes

9.7. Summary

One of the challenges of cacao breeding program is the minimal resistance of planting some material to cacao pod diseases. Cocoa fermentation is a spontaneous process involving a succession of microbial activities starting with yeasts, followed by lactic and acetic bacteria which have a major impact on cocoa quality. Although it is well-known that lactic acid bacteria were responsible for flavor and aroma production in cocoa, there is the need for the characterization and selection of starter cultures which may positively influence the quality of cocoa fermentation. Of the various lactic acid bacterial strains tested, *L. fermentum* was best adapted to the cocoa pulp ecosystem in the fermentation of glucose to lactic acid and acetic acid, reduced fructose to mannitol, and converted citric acid into lactic acid. For controlled cocoa bean fermentation, acid tolerant, ethanol-tolerant, and citrate-utilizing strains of lactobacilli and acid-tolerant, ethanol-tolerant, and heat-resistant strains of acetic bacteria are desirable. A mixture of *L. plantarum, L. fermentum*, and *L. pasteurianus* can be considered as a mix-starter culture for better controlled and more reliable cocoa bean fermentations. *A. pasteurianus* was one of the main acetic acid bacteria in cocoa fermentation. All microbiological studies about cocoa bean fermentation have been based on culture-dependent but more recently the culture-independent (polymerase chain reaction denaturing gradient gel electrophoresis methods) are being used. Cocoa beans fermented with turnings had about 10% of defectives beans irrespective of the method (wooden box, plastic box and heaps) of fermentation.

It seems that mycotoxins including fumonisins, aflatoxin B1, and ochratoxin A are the major threat in cocoa beans and the cocoa industry. Very careful measures should be taken in order to eliminate this health hazard.

It is essential to control the raw material and cocoa processing as in roasting and conching (50–90°C) to assure the thermal

inactivation of *Salmonellae*. The xerophytic fungi of *Chrysosporium* spp. were isolated from commercial chocolate bars with low water activity.

10. CONCLUSIONS AND FUTURE MICROBIOLOGICAL TRENDS

1. The recognized major classes of cacao, *viz.*, Criollo, Forastero, and Trinitario, can be identified in terms of observable traits including flavor as well as by using molecular techniques.
2. The selection criteria employed in cocoa improvement programs are based on the characters of economic importance. Among traits, which superior varieties should have, are good bean weight, a high number of beans per pod, good yield potential, and resistance to diseases. Other selection criteria include vigor, uniform plant type, precocity (early flowering), drought tolerance, and bean quality (flavor and butterfat content). Characters that can be selected at an early stage of plant development are preferred.
3. Much progress has been made in the last decade in cacao genomics. The sequencing of the cacao genome has elucidated the connection between genes and traits of interest and will facilitate scientists and breeders in more easily developing superior cacao plants in terms of disease and pest resistance, yield, flavor, antioxidant content, and other traits of economic interest or with potential health benefits.
4. Cocoa pulp flavor traits migrate from the pulp to the cotyledons during the fermentation process. Future studies could focus on the selection of cocoa bean varieties for flavor quality based on the evaluation of cocoa pulp profiles. The additions of various aromatic fruit pulp during cocoa bean fermentation could modify the flavor profiles of cocoa products.
5. Pharmacological and health beneficial uses for cocoa and cocoa by-products were increasingly investigated over time, not unexpectedly since the earliest consumers of cocoa attributed to it "strengthening, restorative, and aphrodisiac" properties.
6. The emergence of trade in cocoa certified as organic cocoa is noteworthy. The organic chocolate market is experiencing a strong growth in demand. The turnover of companies producing organic chocolate can increase by 10% per annum. However, despite such strong growth, the share of organic cocoa remains small in the cocoa market, representing less than 0.5% of total cocoa production. The labeling of cocoa and cocoa products should be considered to differentiate by geographical and single-origin and if organically produced.
7. Cocoa beans are a rich source of dietary polyphenols contributing about 10% of the dry weight of the whole bean and its derivative chocolate, particularly dark chocolate.
8. The concentration of polyphenols in any chocolate would depend on both their content in the cacao plant and the procedures used for transforming the cocoa into chocolate.
9. Cocoa contains much higher levels of total phenolics and flavonoids per serving than black tea, green tea, and red wine.
10. The antioxidant activity and flavan-3-ol levels of milk and dark chocolates were found to be stable over typical shelf lives of one year under controlled storage and over 2 years in ambient storage in the laboratory.
11. Milk may interfere with the absorption of antioxidants from chocolate *in vivo* and may therefore negate the potential health benefits that can be derived from eating moderate amounts of dark chocolate.
12. The health benefits of a chocolate bar are controversial due to the large amounts of saturated fats contained despite the strong antioxidant activity exhibited. Chocolate processing in the future should look at low calorie and low fat products having texture and flavor corresponding to a normal chocolate.
13. In terms of product development, the industry should consider the international influence on the flavor of cocoa products such as the addition of exotic flavors.
14. Dark chocolate improves insulin resistance and sensitivity as well as decreases systolic blood pressure, whereas white chocolate exerts no effect.
15. Chocolate contains tryptophan, which is a chemical the brain uses to produce serotonin, a proven anti-depressant generating feelings of euphoria. In addition, the theobromine and phenylethylamine in cocoa have a stimulating effect.
16. Cocoa has been reported to exert cardio-protective effects with respect to antioxidant, vascular function and platelet reactivity and decreases inflammation. The future research on the potential health benefits of cocoa could focus on the underlying mechanisms, the active compounds and the dosage. In addition, screening of cacao germplasm should be undertaken to identify cultivars with elevated antioxidant (flavonoid) content in their dried beans. This could facilitate the development of nutraceutical products.
17. Mycotoxins, the main contaminants of cacao beans include fumonisins, aflatoxin B1 and ochratoxin A. Raw cocoa bean quality after harvest is influenced by a wide variety of abiotic and biotic factors such a temperature, moisture, endogenous fungal species, processing, and storage conditions. Drying could reduce the moisture content raw cocoa bean from 60 to 8% in limiting fungal growth.
18. Efforts should be directed to eliminate or minimize the risks of contamination during cocoa processing by introducing preventive measures based on the Hazard Analysis and Critical Control Points system and the adherence to Good Manufacturing Practices.

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