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S.S.D: AGR/16

CHARACTERIZATION OF THE MICROBIAL BIODIVERSITY IN FERMENTED COCOA BEANS

Candidate: Cristian Bortolini
Matr. n.: 4212104

Academic Year 2015/2016

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Preface

The work described in this thesis comprises the overall results of my 3-years industrial-PhD studies performed at the Institute of Microbiology of Università Cattolica del Sacro Cuore, Faculty of Agricultural Sciences of Piacenza in collaboration with Ferrero S.p.A. The research was performed with the purpose to give new insights concerning fundamental aspects of cocoa beans microbial ecology trough a High Throughput Sequencing (HTS) approach. Firstly, the methodological approach has been evaluated to identify the most suitable method for microbiota description. Once detected the appropriate method for our purposes, a detailed analysis of the bacterial populations in processed cocoa beans of different geographic origins has been performed, defining the principal species involved in cocoa post-harvest processing. The same approach was undertaken to evaluate the total fungal community through the comparison of two different genomic regions used as molecular markers. Lastly, we tried to assess the potential correlation between fungal populations and the amount and type of aromatic volatile compounds detected in cocoa bean samples. The methodologies used involved state of the art, analytical methods and development of bioinformatics and statistical background.

Overall, the opportunity of being involved in these projects has been a joy and an incredible learning experience.

The candidate Ph.D.

Cristian Bortolini

Summary

The quality of commercial cocoa beans, the principal raw material for chocolate production, depends on several factors including type of plantations, the agricultural practices and the post-harvest processing. Among these, fermentation and drying are generally considered the most relevant, since during these phases cocoa flavors precursors are formed and fixed. Furthermore, they represent crucial steps during which filamentous fungi contamination might occur. Fermentation is characterized by a well-defined succession of yeasts, lactic acid bacteria and acetic acid bacteria, so that, the aim of the described studies was to explore total bacterial and fungal communities involved in cocoa bean fermentation and to evaluate if geographical origin and fermentation method might affect their composition. To achieve these results, 16s rRNA gene was used as marker to assess the total bacterial community by using High Throughput Sequencing (HTS), indicating that this approach has the ability to provide a comprehensive view of the cocoa bean microbiota at the species level. In a second approach, Internal Transcribed Spacer 1 (ITS1) and the D1/D2 domain of the Large subunit (LSU) of the nuclear ribosomal RNA (26S rRNA) were screened to assess the total fungal community. Results revealed the ability of these two genomic regions to describe reliably the general composition, even if D1/D2domain was able to go deeper into the fungal composition resulting in a higher resolution. In the last approach the same samples subjected to HTS investigation were analyzed through SPME-GC-MS in order to underline the principal key-aroma compounds formed during the post-harvest processing.

Overall, results point out clearly that HTS approach has the ability to provide a comprehensive view of the total bacterial and fungal communities, and statistical analyses have shown how analyses of ITS1 sequences and volatile compounds might be useful for the geographical traceability of the processed cocoa beans samples.

Keywords: Cocoa bean, Fermentation, Bacterial community, Fungal community, High-throughput sequencing, SPME-GC-MS.

Table of contents

Chapter 1. Introduction

1.1. General introduction

Probably originated in Mesoamerica (De La Cruz et al., 1995), chocolate or cacao had already been used as food, beverage, medicine and even as a currency by the Mayan and Aztec populations; that is the reason of the first name of cocoa: *Amygdalae pecuniariae*, literally "almond money". It was the Swedish botanist Linnaeus who replaced the name in "Theobroma" from the Greek "Theos", meaning God and "broma", meaning food, literally "food of the gods", to stress the special status of this food in human culture (Lima et al., 2011; Schwan and Wheals, 2004).

The genus *Theobroma* (family *Sterculiaceae*) is composed by twenty-two species, among these *Theobroma cacao L.* is the most important under a commercial point of view, due to the value of its seeds (Bartley, 2005; Lima et al., 2011; Wood, 1975). The seeds or cocoa beans are the principal raw material for chocolate production and the distinctive flavor of chocolate is due these beans. Cocoa beans are used not only for chocolate production; other products are cocoa powder, largely used in food industry, and cocoa butter with several applications in confectionary, cosmetic and pharmaceutical industry.

The cocoa tree is a perennial tree, which is able to reach 8 to 15 m in height, limited to 2.5 - 3 m by pruning, under intensive cultivation, to guarantee a better phytosanitary control (Lima et al., 2011; Wood, 1975). The fruit of the cocoa tree is an indehiscent drupe called pod with an oval shape, about 12 – 30 cm long, and contains 30 to 40 beans embedded in a mucilaginous pulp, which represents approximately 40% of the bean fresh weight (Lass, 1999; Lima et al., 2011; Schwan and Wheals, 2004; Wood, 1975). The pulp is characterized by a high acidity due to the presence of several organic acids, mainly citric acid, a protein content between 0.4 to 0.6% ($^{w}/_{w}$) and a sugar content between 9 to 13% ($^{w}/_{w}$) (Lima et al., 2011). Cocoa beans are composed by two cotyledons, called "nibs", and a small embryo, all enclosed in a skin, called "shell". Cotyledons are composed by two different types of cells, i.e. parenchyma cells, deputed to storage, and pigmented cells. Parenchyma cells contain starch granules, protein bodies and fat globules. Fat is the most important nutrient and represents about half of the dry seed mass (54%), proteins represent about 12.5% and starch 6% (Table 1). Pigmented cells contain methylxantines and polyphenols (Biehl et al., 1977; Lima et al., 2011). Among the methylxantines, theobromine and caffeine are the most representative with an average percentage of 1.3%

and 0.1-0.2% in the dried nibs, respectively, and are responsible for the bitter taste of cocoa beans (Table 1). On the other side, polyphenols may range between 12 and 20% ($^{w}/_{w}$) in dried cocoa beans and contribute to give astringency and bitterness as well.

Anthocyanins (4%), flavan-3-ol (catechins) (37%) and proanthocyanidins (58%) represent the three main groups of polyphenols found in the cotyledons, among the catechins group the (-)-epicatechin is the predominant fraction (35%) of the total polyphenols content (Kim and Keeney, 1984; Lima et al., 2011; Wollgast and Anklam, 2000).

The quality of commercial cocoa is influenced by several factors, including good agricultural and post-harvest processing practices, each of which is essential for the development of the characteristic flavors and sensorial profile of chocolate. Lima et al. (2011) have well defined the different stages that contribute to the quality of commercial cocoa beans (Figure 1). The former includes the appropriate maintenance of plant population and phytosanitary state, soil and climate conditions, fruit maturation and harvest. Good post-harvest procedure includes optimized pod opening, fermentation, drying and storage conditions (Lima et al., 2011).

Table 1. Average chemical composition of roasted nibs (g.100 g^{-1}).

Composition	Roasted nibs	
Fat	54.0	54.0
Protein	12.5	-
Starch	6.0	6.0
Water*	3.0	3.7
Fiber	2.5	2.5
Ash	3.0	2.8
Theobromine	1.3	1.3
Caffeine	0.2	0.1
References	(Valiente et al., 1994)	(Minifie, 1980)

*varies depending on the degree of drying and roasting

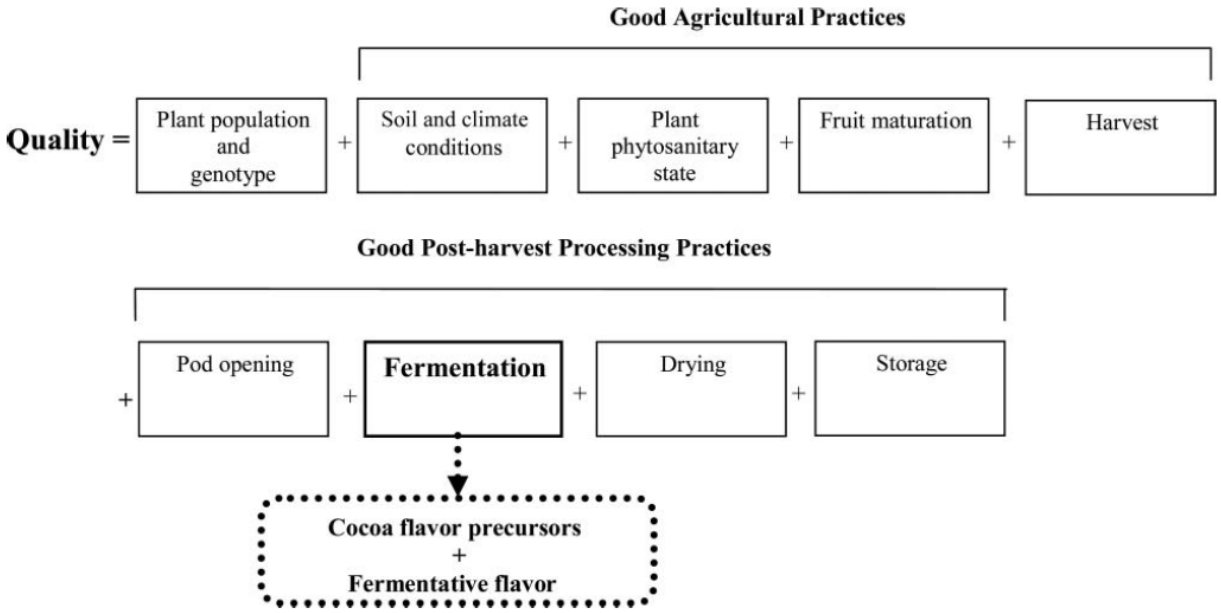

Figure 1. Stages that contribute to the quality of commercial cocoa beans (Lima et al., 2011)

1.2. Plant population and genotype

Within the genus *Theobroma cacao L.*, different subspecies can be identified. According to literature these subspecies are classified in four cultivars: Criollo, Forastero, Trinitario and Nacional (Kongor et al., 2016; Saltini et al., 2013). It Is well known that cocoa beans coming from different geographical origins and with different genotypes have characteristic flavor profiles (Table 2) (Kongor et al., 2016). The four principal cultivars show distinctive differences in pod shapes, flavor profiles and pests/diseases resistance (Afoakwa, 2010; Afoakwa et al., 2008; Kongor et al., 2016; Motamayor et al., 2008).

Criollo is mostly farmed in south and central America. This variety usually undergoes a very short or absent fermentation step. A subgroup of this variety is named Sanchez and is farmed especially in the Dominican Republic (Saltini et al., 2013). The beans are white to ivory due to an anthocyanin inhibitor gene (Kongor et al., 2016). Criollo beans contain a high amount of pyrazines and have a low pH that may affect the flavor profile. Forastero is native to the Amazon region and includes two subgroups named Amelonado and Amazon, this latter could be further divided in Lower Amazon and Upper Amazon depending on the geographical origin. However there are no significant differences under the chemical and genotypic point of view among these subgroups. Forastero represents 95% of the world cocoa production and is commonly known as "bulk cocoa" in trade and is predominantly cultivated in West Africa (Côte d'Ivoire, Ghana,

Nigeria and Cameroon). Beans belonging to this cultivar usually are flat, astringent and purple in color due to the presence of anthocyanins (Kongor et al., 2016; Saltini et al., 2013).

Table 2. Flavor profile of cocoa beans from different origins (Kongor et al., 2016)

Origin	Cocoa type	Flavor profile
Côte d'Ivoire	Forastero	Good cocoa impact, low bitterness, low acid, fruity, nutty
Ghana	Forastero	hybrids Strong chocolate flavor
Nigeria	Forastero	hybrids Medium cacao, occasional off-notes
São Tomé & Principe	Forastero	Good cocoa flavor, bitter, spicy, fruity, earthy
Madagascar	Criollo	Winey, putrid, citrus
Venezuela	Criollo "Porcelana"	Mild chocolate, slightly bitter, distinct fruity notes (plum and cherry)
Brazil	Forastero	Cocoa impact, bitter, acid, astringent (sometimes rubber, hammy, smoky), some fruitiness, no nutty notes
Colombia	Trinitario and Criollo	Fruity, bitter, cacao
Peru	Forastero	Slightly bitter and fruity
Ecuador	Forastero (Nacional)	Balanced profile, low chocolate, floral, fruity, grass, earthy notes
Mexico	Criollo/Forastero hybrids	Low chocolate, strong acid, low fruitiness
Panama	Forastero	Moderate chocolate, acidic, fruit and nut notes
Jamaica	Forastero	Fruity
Dominican Republic (Sanchez)	Criollo/Forastero hybrid	Low cacao, flavorless, bitter
Dominican Republic (Hispaniola)	Criollo/Forastero hybrid	Winey, earthy, can have tobacco notes
Costa Rica	Forastero	Fruity, balanced cocoa flavor
Trinidad & Tobago	Trinitario (birthplace)	High cacao, nutty and winey notes, aromatic
Grenada	Trinitario	Chocolate, fruity, floral, grassy, woody
Indonesia	Criollo/Forastero hybrid	Low chocolate, acidic, fruity
Sulawesi	Criollo/Forastero hybrid	High bitter, low sour, low cacao, astringent
Java	Criollo/Forastero hybrid	Mild, bland profile, acid, low cacao, light color
Papua New Guinea	Hybrid/pure Criollo and Forastero	Variable strong acid, floral, mild, nutty
Malaysian	Forastero hybrids	Low to medium cacao, medium to high acidity, astringent (due to fermentation level) phenolic

Forastero beans have a higher pH after fermentation and drying compared with Criollo beans, thus the chocolate produced with Forastero beans comes out with less bitterness, less acidity and less astringency compared with the chocolate produced with either Criollo or Trinitario beans (Kongor et al., 2016). Trinitario is a hybrid cultivar resulting from Criollo and Amelonado Forastero, originated in Trinidad. The beans are variable in color and this cultivar is known to have a characteristic winery type of aroma not found in other varieties (Afoakwa et al., 2008; Kongor et al., 2016; Saltini et al., 2013). Both Trinitario and Criollo are considered the "fine" cacao and account about 5% of the total world production. Nacional is a small cultivar grown in Ecuador, with few genetic differences from Criollo. Nacional cacao is considered as the finest variety producing the famous Arriba beans with typical floral and spicy flavor notes (Counet et al., 2004; Kongor et al.,2016).

Cocoa bean genotype influences several features including the type and quantity of bean storage proteins, carbohydrates and polyphenols (Afoakwa et al., 2008). Several studies have analyzed the chemical composition of cocoa beans.

The fresh cocoa beans are composed approximately of 32-39% water, 30-32% fat, 10-15% proteins, 5-6% polyphenols, 4-6% starch, 4-6% pentosans, 2-3% cellulose, 2-3% sucrose, 1-2% theobromine, 1% acids and 1% caffeine. The principal sugars in cocoa beans are sucrose (90% of the total sugars), glucose and fructose (6%) (Afoakwa, 2010; Bertazzo et al., 2011; Kongoe et al., 2016). Cocoa bean fat contains about 95% triacylglycerols, 2% diacylglycerols, 1% monoacylglycerols, 1% polar lipids, and 1% free fatty acids (as percentages of lipids) (Biehl and Ziegleder, 2003). Fatty acids in cocoa butter are divided principally in saturated (stearic; 18:0, 35% and palmitic; 16:0, 25%), monounsaturated (oleic; 18:1, 35%) and polyunsaturated (linoleic; 18:2, 3%) (Bracco, 1994). Regarding polyphenols, as mentioned before, three main groups that can be found in cocoa beans are anthocyanins (4%), flavan-3-ol (catechins) (37%) and proanthocyanidins (58%). The anthocyanin fraction is composed mainly of cyanidin-3-α-L-arabinoside and cyanidin-3-β-D galactoside (Wollgast and Anklam, 2000), while proanthocyanidins are predominantly flavan-3,4-diols. The main catechin in the cocoa bean is (-)-epicatechin with about 35% of polyphenol content, (+)-catechin, (+)-gallocatechin and (-)-epigallocatechin have been found in smaller amounts (Wollgast and Anklam, 2000; Afoakwa, 2010). Other polyphenols that could be detected in cocoa beans are the flavonol glycosides such as quercetin-3-O-α-D-arabinoside and quercetin-3-O-β-D-

glucopyranoside (Kongor et al., 2016; Wollgast and Anklam, 2000). Biehl and Ziegleder, (2003) have reported the presence of 17 phenolic acids and esters and the total amount of seven of them reach not more than 23 ppm of the seed dry weight (phloroglucinol, protocatechuic acid, vanillic acid, ohydroxyphenylacetic acid, p-coumaric acid, caffeic acid, ferulic acid). Epicatechin and the smaller procyanidins made of up to three subunits are soluble and cause the astringent taste of cocoa whereas molecules that are composed of more than three subunits are insoluble and cause no astringency (Ziegleder, 2009). The protein fraction in cocoa beans is divided in four main groups that represents 95% ($^{w}/_{w}$) of total seed proteins; albumins (water-soluble), globulins (salt-soluble), traces of prolamins (alcohol-soluble) and glutelins (dilute acids/ alkali-soluble). Albumin represents about 52% of total proteins, has a molecular weight of 21 kDa and is not degraded during fermentation. Globulin fraction accounts for 43% of total proteins and is composed by three polypeptides subunits, respectively 47 kDa, 31 kDa, 16 kDa, of vicilin-type globulin (VCG), a glycoprotein degraded during fermentation into peptides and amino acids which are fundamental for cocoa flavor formation through Maillard reactions during drying and the industrial process of roasting. (Kongor et al., 2016).

1.3. Soil and climate conditions

The natural habitat of the cocoa tree is the narrow equatorial zone, lower evergreen rain forest in the Amazon basin and other tropical areas of South and Central America (Lima et al., 2011). Most of the world's cocoa is produced in West Africa countries: Ivory Coast (37% of the worldwide production), Ghana, Nigeria and Cameroun. Also other tropical areas like Southern Asia contribute significantly to the global production of cocoa beans (Saltini et al., 2013). Cocoa trees are very sensible and extremely selective about soil and climate conditions compared to other tropical crops, they need a soil containing coarse particles, a good quantity of nutrients and a depth of 1.5 m to develop a good root system (Kongor et al., 2016). Since the cocoa tree is sensitive to water availability, the soil must have good water retention and drainage characteristics. The soil should also have a high content of organic matter, including plants, animal and microorganisms in all stages of decomposition (Kongor et al., 2016; Ololade et al., 2010). Cocoa is able to growth in soils with pH in the range of 5.0-7.5, thus moderate acid/alkaline soils can support cocoa trees growth, but extreme pH conditions must be avoided (International Cocoa Organization, ICCO, 2013). Soils for cocoa crop should have a good anionic and cationic balances. Availability of exchangeable cations (e.g. Ca^{2+}, Mg^{2+}, K^{+}) is one of the information

provided by the soil pH, while exchangeable bases should amount to at least 35% of the total cation exchange capacity (CEC) in order to avoid nutritional problems. The optimum total nitrogen / total phosphorus ratio should be around 1.5 (ICCO 2013). The CEC is a measure of the soil's capacity to adsorb and release cations (Ololade et al., 2010) and is fundamental to understand the ability of a certain soil to transport contaminants and to adsorb nutrients (Kongor et al., 2016).

Regarding the climate conditions cocoa plants respond well to relatively high temperatures, with a maximum annual average of 30 - 32ºC and a minimum average of 18 - 21ºC. Variations in the yield of cocoa trees from year to year are affected more by rainfall than by any other climatic factor. Rainfall should be profuse and well distributed throughout the year. An annual rainfall range between 1,500 mm and 2,000 mm is generally preferred and dry spells (rainfall is less than 100 mm per month) should not exceed three months. A hot and humid atmosphere is essential for the optimum development of cocoa trees. In cocoa producing countries, relative humidity is generally high, often as much as 100% during the day, falling to 70-80% during the night. Cocoa trees are able to use every light available and traditionally have been grown under shade (ICCO 2013).

1.4. Plant phytosanitary state

Pests and diseases are responsible for great economic losses in cocoa production, this problem is particularly relevant in West Africa, where a very large number of small and isolated cocoa farms have inadequate pest and disease control procedures. In addition, the origin of cocoa trees may affect pests and disease susceptibility. Forastero cocoa trees are very productive and are moderately resistant to pests and diseases (Bartley 2005; Kongor et al., 2016; Lima et al., 2011), Criollo cocoa trees presents a low yields and a high susceptibility to many diseases, at last Trinitario cocoa trees show a susceptibility intermediate to Forastero and Criollo cultivars (Bertley, 2005; Kongor et al., 2016). The two major diseases of cocoa trees in South America are Witches' Broom and Frosty Pod.

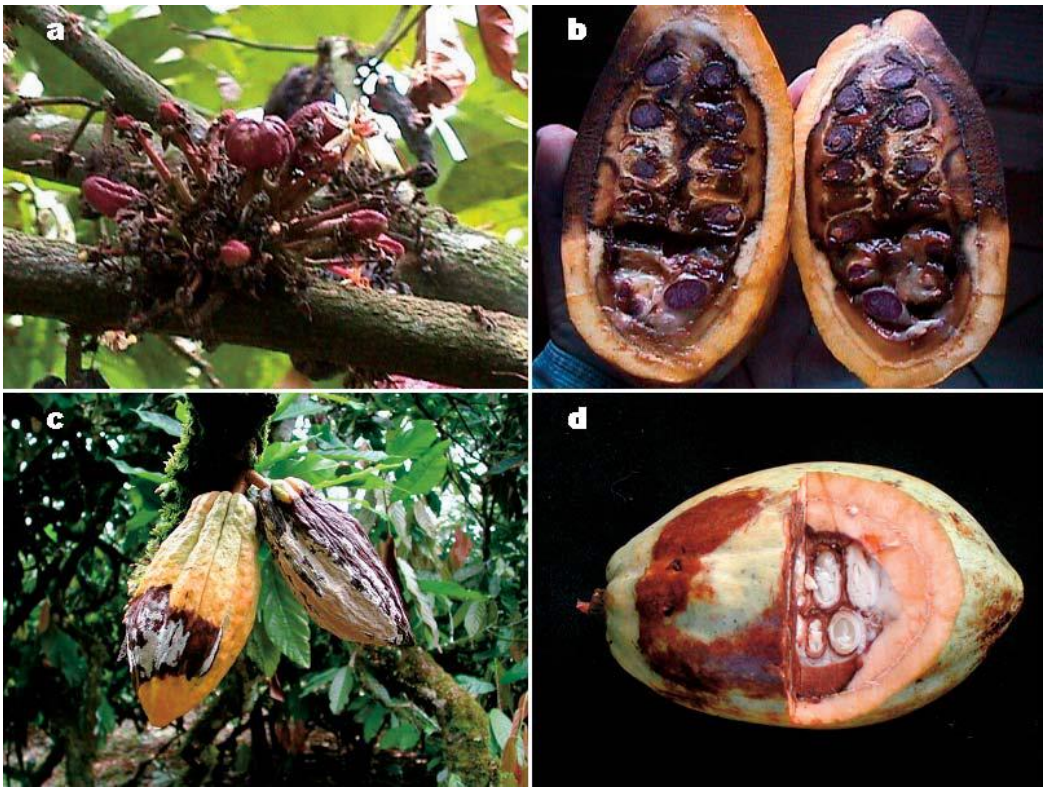

Fig.2. Infection of the chocolate (*Theobroma cacao*) tree and pods by cacao pathogens *Moniliophthora (Crinipellis) perniciosa* and *Moniliophthora roreri*. a) Witches' broom of plant stems caused by *M. perniciosa* infection. b) Chocolate pods and seeds infected with *M. perniciosa*. c, d) Frosty pod rot caused by *M. roreri* on pods and seeds. (Aime and Phillips-Mora, 2005)

Moniliophthora (Crinipellis) perniciosa (Fig. 2 a,b) is the pathogen responsible for Witches' Broom disease. During the last century the mushroom spread throughout South America, Panama and the Caribbean, causing great losses in production. The most visible effect can be seen in Brazil where the introduction of the disease in the region of Bahia caused a decrease in production of almost 70% during a period of 10 years (ICCO 2015). *M. perniciosa* infects only growing tissue such as shoots, flowers and pods with the result that cocoa trees produce branches with no fruit and ineffective leaves (Evans et al., 2013). The pods show distortion and present green patches similar to an uneven ripening. The life cycle of the fungus is synchronized with the phenology of the host. One of the crucial factors for the reproduction *M. perniciosa* is water. Basidiospores are released during the night with a level of humidity of 80% and temperature range between 20 and 30ºC (Evans, 2016; ICCO, 2015). The spores are able of being disseminated by water and cover long distances thanks to the wind. Basidiospores have a short viability period and are sensitive

to light and drying but are produced in large numbers (each basidiocarp can produce 2-3.5 million spores). The pathogen is also spread in infected seeds or buds. Until now phytosanitary pruning is the only effective way to control the Witches' Broom. Complete removal of all infected material is necessary, but it is a result not so easy to achieve because hidden inoculum sources always remain. (Evans, 2016; ICCO 2015).

The basidiomycete *Moniliophthora roreri* is the pathogen responsible for Frosty Pod Rot disease (Fig. 2 c,d). First reports of this disease date back to the end of the 19th century, where it caused devastation among Colombian and Ecuadorian cocoa cultivars (Evans et al., 2013; ICCO 2015). The fungus infects only growing pod tissues, especially young pods, and it takes a period of 1-3 months from the infection to the appearance of symptoms. The most visible symptom is the white fungal film on the pod surface. The great amount of spores produced (44 million spores per cm^2) and the genetic variability allow a considerable adaptability of this fungus (ICCO 2015). Disease incidence varies with cultivar, pod age and rainfall. Generally the greatest production is when rainfall is high. The use of copper and some organic protectors has proved to reduce the incidence of the disease. Systematic fungicides such as Flutolanil have showed some positive effects, although the use of agrochemicals is not economically sustainable because of the low price of cocoa. Crop sanitation involving the removal of infested pods is the principal method of control of the disease. This activity has to be done with extreme care due to the fact that healthy pods can be infected during the process. Frosty Pod accounts for about 5% of total annual crop loss (ICCO, 2015).

West African cocoa growing regions may be affected by the Cocoa Swollen Shoot Virus Disease (CSSVD). Especially in the past, many cocoa plantations have been destroyed by this virus, the disease was controlled by replacing millions of infected trees with an hybrid, virus-tolerant, coming from Upper Amazon, but this technique is not able to prevent new outbreaks in the newly planted areas (Dzahini-Obiatey et al., 2010; Wessel and Quist-Wessel, 2015). Given the extended area of the cocoa plantations and the fact that trees often do not show any symptom despite they carry the virus, it is clear that the removal of infected trees does not prevent virus spreading. One of the best solution that can be adopted against CSSVD contamination is a preventive strategy based on surrounding the newly planted fields by non-CSSVS host crop such as citrus, palm oil or coffee. Farmers turned against this concept, although the “barrier crops” may represent an

extra incoming (Dzahini-Obiatey et al., 2010; Kouakou et al., 2012; Wessel and Quist-Wessel, 2015).

In West Africa another important disease is the Black Pod disease or *Phytophthora* Pod Rot (PPR)(Fig. 3). PPR is caused by two pathogen species *P. palmivora* and *P.megakaria*. In Ghana, Côte d'Ivoire and above all in Cameroun these pathogens cause a mean pod losses of about 40% (Opoku et al, 2000; Wessel and Quist-Wessel, 2015). Regular pruning of infected pods and humidity control through shade reduction can decrease pod losses but usually fungicides are needed. However, due to the high costs of the fungicides, many farmers can't adopt this solution (Mpika et al., 2011; Wessel and Quist-Wessel, 2015).

Lastly, the most important insect pest of cocoa in West Africa are Mirids (*Distantiella theobroma* and *Sahlbergella singularis*), that cause annual crop losses of about 25% in Ghana and 30-40% in Côte d'Ivoire (Wessel and Quist-Wessel, 2015). The greatest damage occurs in lightly shaded and unshaded cocoa trees. Although proper pruning and shade removal can prevent pests outbreaks, control with insecticides is often needed. A pest control program has been financed by the Ghanaian government due to the high costs of equipment for each farmer. This program provided mass pesticides spraying of entire areas with consequent problems for human health and environment, for this reason the use of plant-based instead of synthetic pesticides was investigated (Wessel and Quist-Wessel, 2015).

Fig.3. Black pod infected by PPR. Image credit: Nathan Palmer-Royston

1.5. Age of cocoa tree, Fruit maturation and Harvest

The cocoa tree goes through four different productive stages during its entire life cycle (Mahrizal et al., 2013; Kongor et al., 2016).

The first stage consists in an early period of no yield which usually corresponds to the first three years of life, afterwards in the second stage there is a period of increasing yield at an increasing rate, followed by a third stage of increasing yield at decreasing rate, finally a period of decreasing yield. After four years from the planting the cocoa tree becomes productive and the yield rate increase annually until approximately 18 years, then the tree enters in the last stage of its life cycle and the yield begins to decline due to erosion, the increasing of occurrence of plant diseases and nutrient impoverishment from soil (Binam et al., 2008; Kongor et al., 2016). Pods containing cocoa beans grow from the trunk and branches of the cocoa tree. During the harvest, ripe pods are removed from trees and opened to extract the wet beans. The pods are harvested manually by making a clean cut through the stalk with a blade (ICCO, 2012).

1.6. Pulp pre-conditioning

Cocoa pulp is the substrate metabolized by a sequence of microorganisms during fermentation (see chapter 1.8), thus pulp pre-conditioning, that involves some changes before the fermentative microbial succession, may significantly affects the final result in terms of flavor profile and quality. These changes could occur in the moisture content of the pulp, sugar content, quantity of pulp per seed, and pH or acidity as well. According to the literature available, removing a certain quantity of pulp or reducing the fermentable sugar content may decrease acid production during fermentation, resulting in less acid beans (Afoakwa et al., 2012; Kongor et al., 2016). Three basic methods are principally used for pulp pre-conditioning: enzymatic or mechanical depulping of cocoa beans, pod storage and bean spreading (Afoakwa et al., 2011; Kongor et al., 2016). Cocoa pulp characteristics could be changed directly inside the pod (pod storage), before the pulp-bean mass is extracted from the pods or outside the pods (mechanical/enzymatic depulping and beans spreading).

1.6.1. Depulping of cocoa beans

Excessive pulp fraction in the cocoa pulp-bean fermenting mass leads to high acidity production and influences flavor quality resulting in an excessive sourness (Kongor et al., 2016). Removal of a fraction of the pulp, or reduction in the sugar content available for the fermentation process, leads the formation of less acid beans. It has been shown that removal of 10-20% of the total fresh weight of beans (pulp and beans) significantly increase the flavor profile of cocoa beans produced (Shwan and Wheals , 2004). Depulping can be done mechanically or enzymatically. Presses (Afoakwa et al., 2012) or modified domestic washing machines (centrifuges) (Shwan and Wheals, 2004) may be used for a partial removal (20%) of cocoa pulp resulting in an accelerated fermentation, a reduced substrates availability and therefore a decreased acidity production during fermentation. This process may destroy cell structures leading to the activation of enzymes which may affect several biochemical transformation during the fermentation. Moreover the excess pulp is used in the manufacture of jam, pulp juices, wines or cocoa soft drinks (Afoakwa, 2010; Kongor et al., 2016).

Enzymatic depulping through the addition of pectinolytic enzymes to the pulp-bean mass directly before fermentation or coupled with mechanical pulp extractor has been shown to help the subsequent fermentation. Because of the enzymes that break pectin

chains, pulp had a lower viscosity, leading to a better aeration of the mass. Since the addition of enzymes on a large scale farming process is prohibitive due to the cost, two different strategies may be undertaken. On one hand, it is possible to increase the pectinolytic action at the beginning of the fermentation (e.g. selected strains, above all yeasts, with high pectinolytic activity), on the other hand one a source of enzymes can be obtained directly from yeast cultures.

1.6.2 Pod storage

Pod storage before their opening is recommended for cocoa beans which are difficult to ferment. Pod storage reduces the sugar content and subsequently the amount of ethanol and acetic acid formed during fermentation, furthermore it increases the pH in fermented cocoa beans (Saltini et al., 2013). Pod storage appears to have beneficial effect on the chemical composition and flavor profile of chocolate. Studies have shown how pod storage decreases non-volatile acid and polyphenol compounds, especially –(-)epicatechin and (+)-catechin, with a general reduction of bitterness and astringency in cocoa (Kongor et al., 2016; Shwan and Wheals , 2004). However pod storage does not only give benefic effect, in fact an extended storage significantly increases mold contamination, thus pod storage is useful only in certain conditions and only if it is well controlled. In summary, different cultivars, farm practices and the healthy status of the cocoa pods, depending on crop cultivation management, affect the final composition in terms of flavor and quality of chocolate. Harvest time, storage time, maturation degree and separation between healthy and infected pods affect the final results as well (De Vuyst and Weckx, 2016; Saltini et al., 2013).

1.7. *Pod opening*

The post-harvest processing of cocoa beans starts by opening the fruits, usually with a cutting tool, such as a machete, during a period of three/four days to two weeks after the harvest (Lima et al., 2011). When pods are opened in the planting areas, the discarded shells can be distributed throughout the fields to return nutrients to the soil. The pulp and the beans within ripe healthy pods are supposed to be microbiologically sterile except for a few hundred of yeast per gram (De Vuyst and Weckx, 2016). The pods may be opened either manually or mechanically, but smallholders in general carry out the process manually. When the cocoa pulp-beans mass is removed from the inside of the pods they become immediately inoculated with different types of microorganisms coming from the

environment, many of which constitute the initial wild starter inoculum that contribute to the subsequent spontaneous fermentation of the pulp-bean mass (De Vuyst and Weckx, 2016; Nielsen et al., 2007; Schwan and Wheals, 2004). It clearly appears that pod opening is a crucial step, especially regarding molds contamination, if the tools and the environmental conditions for pod opening are not well maintained and defined, some molds contamination may occur with great losses in the production.

1.8. Fermentation

Raw cocoa beans, after pod opening, are inedible because of their bitter, astringent and unpleasant taste. For this reason fresh cocoa pulp-bean mass undergoes a natural fermentation to obtain a full-flavored cocoa and chocolate. Therefore is without any doubt that the process of cocoa bean fermentation plays a crucial role in the entire chocolate-making process. Inside the cocoa pods, the beans are embedded in a white pulp with an high presence of pectin, saccharides and citric acid (pH 3.0-4.0). Ripe pods contain mainly glucose and fructose due to the hydrolytic action of the invertases on sucrose and guarantee a correct cocoa beans fermentation process (De Vuyst and Weckx, 2016). Fermentation removes the viscous pulp around the beans, moreover contributes to color and flavor development of beans and reduces bitterness and astringency.

1.8.1. Fermentation process

Nowadays cocoa beans fermentation is still a spontaneous on-farm process, with the consequence that the end product may have variable quality. It could be carried out in heaps, boxes, baskets or directly on soil, usually surrounded with banana or plantain leaves, depending on the producing region, and lasts for about 2-10 days depending on cocoa quality and farm practices (De Vuyst and Weckx, 2016; Lima et al., 2011; Schwan and Wheals, 2004), even if fermentation process lasting more than 5-6 days does not improve the flavor potential of cocoa beans, on the contrary may cause deleterious effect related to mould contamination (Saltini et al., 2013). Natural cocoa beans fermentation is a very heterogeneous process and shows great variations concerning the course of microbial species and the metabolite compounds. These differences are mainly due to the different microbial environmental contaminations of the initial cocoa pulp-bean mass and the agronomical and farm practices applied. The initial inoculum of the pulp-bean mass is produced by the surrounding soil, air, dust and insects as well as by the cocoa pod shells itself, in fact pod surfaces are assumed to be the most important inoculum source.

Nevertheless banana and plantain leaves used to cover the fermenting mass, tools and equipment utilized is an important source for the initial microbial contamination (Camu et al., 2007; 2008; De Vuyst and Weckx, 2016; Nielsen et al., 2007). This heterogeneous background requires chocolate manufactures to use fermented dry cocoa bean blends to overcome the variable flavor composition of spontaneously fermented cocoa beans from different geographical origins and obtained with different farm practices in order to obtain a standardized flavor profile (De Vuyst and Weckx, 2016).

1.8.2. Fermentation methods

Since cocoa fermentation is a spontaneous on-farm process, different methods are used depending on farmers, producing countries and geographical areas. In general cocoa beans fermented in boxes have shown low sugars, ethanol and acetic acid concentration and a high pH; in addition, size, shape and material of the box may influence pH, sugar content and initial microbial populations in the cocoa pulp-bean mass. When fermentation is performed using the heap method, at the beginning of the process the temperature has been found to increase faster than in the box, this is probably the reason why less purple beans are found at the end of the fermentation, pointing out a better homogeneity of the process (Saltini et al., 2013). The platform method is considered obsolete especially because of its low fermentation rate, but due the low cost is still widely used for example in West Africa. Low fermentation rate is probably also the reason why this method has been historically used for the Criollo variety that needs only 2-3 days of fermentation and is considered inappropriate for Forastero beans which require a longer fermentation time (5-8 days). Another limit of this method is the higher incidence of mould growth, with the consequent bean-mass losses and off-flavor formation, compared with the other traditional fermentation methods (Saltini et al., 2013).

Independently of the fermentation method used, the mass size and the consequent aeration have a great influence on the whole process. If a little mass quantity is subjected to fermentation, it would result in a low quantity of free amino acids, peptides, fructose, glucose and total sugar available for the microbial metabolic activities during fermentation. The optimal quantity, about 55-60 kg, leads to an increase of these compounds, while a further enhancement of the mass quantity causes again a decrease of substrate availability due to the system saturation. On one hand fermenting a very high volume would reduce the aeration during the fermentation process, resulting in a reduced microflora activity, that means lower temperature and lower proteolytic activity. One the other hand, fermenting a

very small volume of cocoa beans allows a better aeration, but causes a loss of heat, thus reduction of the temperature and of the metabolic rates of the microflora activity. (Saltini et al., 2013).

1.8.3. Dynamics of microbial population during fermentation

Cocoa bean fermentation requires a particular succession of well-defined microbial populations, in particular indigenous yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB, Fig.4a). This particular succession is one of the most important pillars reached during years of studies about cocoa fermentation and has been found to be the same in most of the cocoa producing regions, although, obviously, specific strains were recovered from different geographical regions. These consecutive microbial activities are strictly dependent on different parameters such as microbial load and species, and physicochemical conditions of the fermenting mass in particular pH, oxygen tension, available nutrients and metabolites. Under optimal conditions fermentation should not exceed four days (Papalexandratou et al., 2013; De Vuyst and Weckx, 2016). When the fermentation process lasts more than four days bacilli and filamentous fungi may participate and/or contaminate the fermenting pulp-bean mass (Ardhana and Fleet 2003; Ho et al. 2014;Papalexandratou et al. 2011a; Pereira et al. 2012; 2013; Schwan and Wheals 2004). Enterobacteriaceae may intervene as well, with a positive effect during the initial phase of fermentation (De Vuyst and Weckx, 2016; Illeghems et al., 2012; 2015).

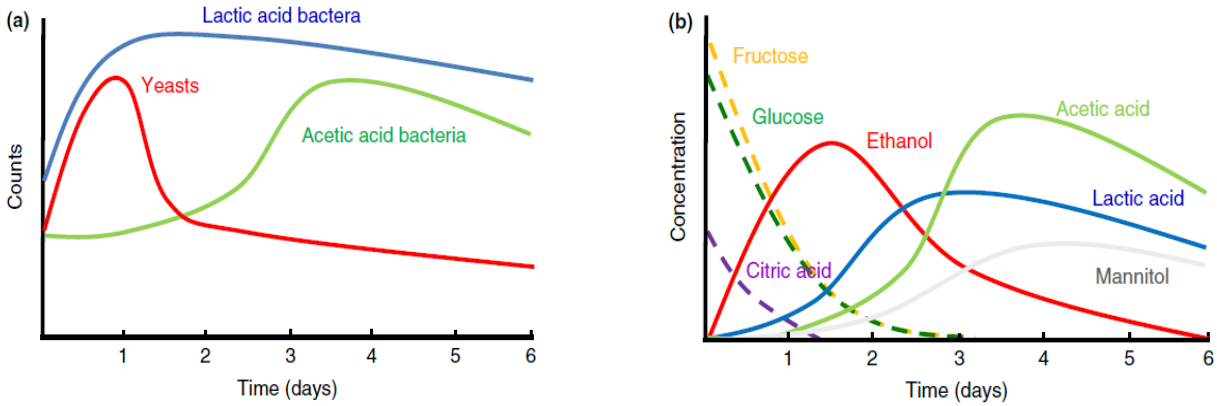

Fig.4. a) Community dynamics during spontaneous fermentation. b) Substrates degradation and metabolite production during spontaneous fermentation (De Vuyst and Weckx, 2016).

1.8.3.1. Yeast phase

The initial 24-48 hours of the fermentation are characterized by the predominance of yeasts (Fig. 4a), which, during this anaerobic phase, , are responsible for pulp degradation through pectinolysis, with the consequent releasing of sweating and reduction of pulp viscosity that allows air ingress in the fermenting mass. An optimal action of pectinolytic enzymes is important not only for the air ingress but also for the control of the thickness of the bean shells resulting after fermentation and drying (Crafack et al., 2013; De Vuyst and Weckx, 2016). In order to speed up the fermentation process and/or improve the quality of the final product, exogenous pectinases or starter strains overproducing pectinolytic enzymes may be added (Crafack et al., 2013; De Vuyst and Weckx, 2016; Schwan and Wheals 2004). Yeasts are able to produce ethanol from carbohydrates, mainly glucose (Fig. 4b), moreover carbon dioxide and glycerol are produced as side-products (Camu et al., 2007; 2008; Nielsen et al., 2007; Papalexandratou et al., 2011a;b;c; De Vuyst and Weckx, 2016). Sucrose, the main carbohydrates in the ripe pulp, is cleaved by the invertase activity of yeasts in glucose and fructose; during this initial phase of the fermentation fructose is almost not used. Ethanol production takes place under specific conditions: a carbohydrates-rich, acid medium, due to the presence of sucrose, glucose, fructose and citric acid in the pulp and an anaerobic environment due to tight packing of the fermenting mass in the heaps/box and the production of carbon dioxide as side-product from yeasts and LAB. The resulting ethanol will be partially diffused in the cocoa beans cotyledons, or will be used as substrate during the following steps of the fermentation. Ethanol production is an exothermic process during which temperature increases in the fermenting cocoa-bean mass from ambient temperature (25-30°C) to 35-40°C within 48 hours. Furthermore yeasts are able to produce (in addition to alcohol and side-products such as carbon dioxide and glycerol) organic acids, such as acetic acid and succinic acid (buffer effect in fermenting pulp) and volatile compounds such as higher alcohols, aldehydes, ketones and fatty acid esters, that may act as precursors to the production of cocoa flavor profile (De Vuyst and Weckx, 2016; Ho et al., 2014; Lima et al., 2011; Schwan and Wheals 2004).

Recent studies with different approaches have shown a great species diversity in the yeasts population, although several key species may be identified (De Vuyst and Weckx, 2016; Ho et al., 2014; Illeghems et al., 2012; Meersman et al., 2013; Moreira et al., 2013; Nielsen et al., 2007; Papalexandratou et al., 2011c; 2013). In the very first phase of the

fermentation process, *Hanseniaspora opuntiae/uvarum* has been identified as the predominant yeast due to its non-competitiveness with LAB species (citrate and fructose metabolizers), low tolerance to ethanol and high temperature and good tolerance to citrate (Hamdouche et al., 2015; Papalexandratou and De Vuyst, 2011). Furthermore other yeast species have been reported to play a role during the initial phase of the fermentation process: *Hanseniaspora guillermondii* (anamorph *Kloeckera apis*), *Hanseniaspora thailandica*, *Kluyveromyces marxianus*, *Pichia anomala* (reclassified as *Wickerhamomyces anomalus*), *Pichia fermentans*, *Pichia kluyveri*, *Pichia kudriavzevii* (formerly *Issatchenkia orientalis*, anamorph *Candida krusei*), *Pichia manshurica* and *Pichia membranifaciens* (Crafack et al., 2013; Daniel et al., 2009; De Vuyst and Weckx, 2016; Ho et al., 2014; Meersman et al., 2013; Moreira et al., 2013; Nielsen et al., 2007; Papalexandratou and De Vuyst, 2011; Papalexandratou et al., 2011c; 2013). The latter stage of the yeasts phase (24-48 hours) is often dominated by *P. kudriavzevii* that shows a high tolerance to ethanol, acidic environment and heat. Since the diversity of yeast species is much larger compared to that of bacterial populations involved in the process, yeasts may have a deep influence on the fermentation efficiency and final cocoa quality (De Vuyst and Weckx, 2016; Meersman et al., 2013). At the end of the first phase of the fermentation, the increase in pH values, the development of microaerobic conditions, the increase of the temperature above 45°C due to the exothermal reaction and nutrients depletion, all contribute to determine the optimal conditions for the growth of AAB, which start to oxidize the ethanol with the consequent decrease in the yeast population (Camu et al., 2007; Daniel et al., 2009; De Vuyst and Weckx, 2016; Shwan and Wheals, 2004).

1.8.3.2. LAB phase

Between 24-72 hours after fermentation starting, the increasing amount of air entering in the cocoa pulp-bean mass creates ideal conditions for the growth of a succession of enterobacteria, LAB and AAB (Fig. 4a). According to literature (De Vuyst and Weckx, 2016; Ho et al., 2014; Illeghems et al., 2015; Papalexandratou et al., 2011a ; b ; c ; Pereira et al., 2012 ; 2013) facultative *Enterobacteriaceae*, such as *Tatumella* species occur frequently, but transiently; these type of microorganisms, coming from the environment (especially soil and plants) may play a role in the production of glucuronic acid from glucose, an unwanted process yielding acidity and reducing the quantity of glucose available for yeasts and LAB (Illeghems et al., 2015; Papalexandratou et al., 2011a; b; c).

At this point of the fermentation microaerophilic, acid/ethanol-tolerant, fructophilic, LAB species come into play, in particular *Leuconostoc pseudomesenteroides*, *Fructobacillus pseudoficulneus*, *Fructobacillus tropeaoli*, *Lactobacillus cacaonum*, *Lactobacillus fabifermentans* and *Lactobacillus plantarum*. This is the very first group of LAB species that occurs during the fermentation process, followed by strictly heterofermentative *Lactobacillus fermentum*, that persists upon further fermentation. Many studies have addressed the presence, the functional role and the impact on cocoa flavor profile of LAB species in the fermentation pulp-beans mass, but, as reported by Ho et al., (2015) their contribution to obtain cocoa beans that give the typical chocolate flavor after the whole production process, may be not essential, although further studies in a large scale and under controlled conditions have to be performed. Anyway, they contribute to the fermentation process by controlling bacterial growth and pH (De Vuyst and Weckx, 2016). After yeast growth a certain quantity of glucose is still available as substrate for homo-fermentative or hetero-fermentative LAB to be converted in lactic acid, acetic acid, carbon dioxide and/or ethanol (Fig. 4b), while fructose (that is still abundant in the fermenting mass because is not metabolized by yeast) is also fermented to lactic acid, acetic acid, carbon dioxide and/or ethanol by fructose-loving LAB, or it is used as alternative external electron acceptor by strictly homo-fermentative LAB, being occasionally reduced to mannitol (De Vuyst and Weckx, 2016). In the early 24 hours during fermentation when yeasts anaerobically convert glucose into ethanol, citrate-positive LAB species are able to perform a heterolactic fermentation using citric acid as co-substrate to obtain lactic acid, or acetic acid and flavor precursors from pyruvate metabolism such as diacetyl, acetoin and 2,3 butanediol. Citric acid replacement by lactic acid and/or acetic acid allows a slight increase in the pH that promotes bacterial growth (Camu et al., 2007; De Vuyst and Weckx, 2016; Lefeber et al., 2011; Papalexandratou et al., 2011a,b,c). This heterogeneous situation covering all these fermentation end-products is crucial for the composition of the fermenting cocoa pulp-beans mass and is able to modulate the microbial succession. As mentioned before, the last period of the LAB phase during spontaneous fermentation is dominated by *L. fermentum* strains due to their citrate-converting, mannitol-producing and ethanol-, acid- and heat-tolerant capacities (Adler et al., 2013; Camu et al., 2007; 2008; De Vuyst and Weckx, 2016). As in the case of the yeast phase, changing conditions, nutrients depletion, increase of temperature and ethanol concentration lead to a decline, of LAB species.

1.8.3.3. AAB phase

During the aerobic phase (48-112 hours) caused by air ingress, the fermentation process is dominated by AAB species (Fig. 4a). *Acetobacter ghanensis* and *Acetobacter senegalensis* dominate the first part of the AAB phase, then *Acetobacter pasteurianus* takes over and persist upon further fermentation, presumably because of its ethanol-, mannitol- and lactic acid-oxidizing ability and acid and heat tolerance (Camu et al., 2007; 2008; Crafack et al., 2013; De Vuyst and Weckx, 2016; Illeghems et al., 2013; Lefeber et al., 2011; Meersman et al., 2013; Moens et al., 2014; Nielsen et al., 2007; Papalexandratou et al., 2011a; b; c). Also *Acetobacter syzygii* and *Acetobacter tropicalis* have been reported frequently as the predominant AAB species (Crafack et al., 2013; De Vuyst and Weckx, 2016; Pereira et al., 2012; 2013). Several studies have also reported the presence of *Gluconobacter* species, which are less common compared to *Acetobacter* species and may associated to poor fermentation conditions. *Gluconobacter* species are able to oxidize glucose into glucuronic acid and may cause another late yeast growth with the consequent production of off-flavors (De Vuyst and Weckx, 2016; Ho et al., 2014; Moens et al., 2014; Papalexandratou et al., 2011b). If the fermentation is performed under the right conditions, *Acetobacter* species oxidize ethanol (produced by yeasts in the very first part of the fermentation process) into acetic acid and lactic acid (produced by LAB) into acetic acid and acetoin. A further over-oxidation turns acetic acid into carbon dioxide and water (Adler et al., 2014; Camu et al., 2007; 2008; De Vuyst and Weckx, 2016; Moens et al., 2014). Acetic acid concentration decreases near the end of the fermentation process, due to its evaporation at the high temperature of the fermenting cocoa pulp-bean mass. A general overview of the fermentation process shows how temperature increases from 25-30°C to 45-50°C because of the exothermal reactions happening during the yeasts, LAB, AAB phases, resulting in a general decline of all microorganism populations at the end of fermentation.

Although the microflora acting during fermentation has been widely studied, and the principal key players are well-known, this spontaneous on-farm process may be influenced by several factors leading to a very heterogeneous picture. A very comprehensive work has been done by Saltini et al. (2013), which collected most of the data regarding the occurrence of different microbial species from previous studies together with their activity, when available, during cocoa bean fermentation (Appendix A).

Several molecules are produced during fermentation as a result of the biochemical degradation of sugars present in the pulp surrounding cocoa beans; among these components, propanoic acid, 2-methylpropanoic acid, 3- methylbutanoic acid and acetic acid have an enhanced role as odour-active compounds in cocoa (Kongor et al., 2016). Unfermented cocoa beans tend to develop little cocoa and chocolate flavour when roasted while beans subjected to an over-fermentation produce undesirable putrid flavours (Afoakwa et al., 2008; Afoakwa, 2015; Kongor et al., 2016). Notably, flavour-active components produced during fermentation include ethyl-2 methylbutanoate, tetramethylpyrazine and other pyrazines (Afoakwa et al., 2008; Afoakwa, 2015). Theobromine and caffeine, together with diketopiperazines formed during roasting (industrial processing) through thermal degradation of proteins, are responsible for the bitter notes (Afoakwa, 2015; Kongor et al., 2016). Other components derived from amino acids, released during fermentation include 3- methylbutanol, phenylacetaldehyde, 2-methyl-3-(methyldithio)furan, 2-ethyl-3,5-dimethyl- and 2,3 diethyl-5-methylpyrazine and represent important flavour precursors (Afoakwa, 2015; Kongor et al., 2016).

1.9 Drying

After fermentation, cocoa beans are subjected to a drying stage in order to reduce the moisture content from about 60% to 6-8% (w/w) (Kongor et al., 2016; Saltini et al., 2016). Drying allows to reduce mould contamination during storage and promotes chemical changes which contribute to improve flavour profile. Nowadays drying is based on an empirical method; each farmer, based on his own criteria and experience, decides when the cocoa beans are ready, thus even if farmers are well trained and experienced, the result in terms of well-dried cocoa beans may vary considerably (range from 5% to 24% in moisture) between different farms, even within the same region. During the drying stage, the biochemical oxidation of acetic acid, started during fermentation, keeps on, leading to the reduction of astringency, bitterness and acidity; moreover polyphenol oxidase initiates the oxidation of the polyphenolic component, giving rise to new flavor precursors and loss of the membrane integrity, inducing the characteristic brown color formation of well-fermented cocoa beans (Afoakwa, 2010; Kongor et al., 2016; Saltini et al., 2013). Reduced sugars participate in the Maillard reactions (non-enzymatic browning reactions) to form volatile fractions of pyrazines (Kongor et al., 2016). Drying rate during drying process is crucial for the final quality of cocoa beans. On one hand if the drying process is too fast, cocoa beans tend to retain an excessive amount of acid, including acetic acid, with

deleterious effects in terms of flavour. On the other hand, if the drying process is too slow it could result in low acidity, poorer color and high presence of moulds (Kongor et al., 2016; Saltini et al., 2013). In cocoa producing regions natural sun drying is still largely used, even though artificial driers have gained considerable attention because of the possibility to standardize the drying conditions and to obtain a more homogeneous product.

Once cocoa beans are correctly dried, they are bagged and marketed. After drying the cocoa beans are collected by local buyers, local and international traders, logistic companies, etc., thus many actors are present between the cocoa farmers and the chocolate manufacturers. No studies analyzing the influence of the storage conditions during transportation on cocoa beans were found. However, since improper storage conditions might be deleterious to the cocoa beans quality, the conditions of transportation should be taken into consideration when assessing the quality of cocoa beans (Saltini et al., 2013).

1.10. Fungi and mycotoxins in farm processing

Cocoa beans, during farm processing, pass through different steps, many of which, especially in West Africa, are based on rural practices. For this reason cocoa beans are susceptible to fungal contamination during many of these processing steps. Fungal growth is affected by several parameters of the cocoa-bean mass such as pH, water activity and organic acid produced during fermentation. The presence of filamentous fungi represents a crucial problem under two aspects; on one hand, moulds produce deteriorative alteration to sensorial properties and may cause great economic losses. On the other hand, fungal contamination may lead to mycotoxins production. Both aflatoxin and ochratoxin A (OTA) have been detected in cocoa and chocolate.

Pulp-bean mass inside healthy cocoa pods is microbiologically sterile (Fig. 5, 1A-C), but after pod opening it becomes soon contaminated by different microorganism above all those which will be responsible for the subsequent fermentation process (Copetti et al., 2014). As mentioned before, during spontaneous fermentation, yeast, lactic acid bacteria and acetic acid bacteria occur in a well-defined succession. The high amount of alcohol produced by yeast, and of lactic acid and acetic acid produced by bacteria, together with environmental factors such as low pH, high temperature and microaerophilic conditions, reduce significantly fungal growth (Schwan and Wheals, 2004; Copetti et al., 2014). Filamentous fungi have been reported to occur especially in the last days of fermentation (Fig.5, 2A-C) on the surface or when the pulp-bean mass is not turned regularly (Nielsen et

al., 2013). Although the role of moulds during fermentation is not well defined, it is known that some species are able to hydrolyze with the production of acids and off-flavour compounds which may alter cocoa beans quality. An extensive fungal growth may increase commodities deterioration and losses (Ardhana and Fleet, 2003; Schwan and Wheals, 2004). Studies carried out on the fermentation wooden box in Indonesia have shown the presence of *Penicillium citrinum* and an unidentified basidiomycete in the first 36 hours of fermentation, both fungi showing a strong polygalacturonase activity, suggesting their role in the degradation of pulp in the early stages of fermentation. The presence of *Aspergillus versicolor*, *Aspergillus wentii* and *Penicillium purpurogenum* was reported as well (Ardhana and Fleet, 2003; Copetti et al., 2014).

Fig.5. 1A, cocoa pod; 1B, cocoa beans surrounded by pulp; 2A, 2B, filamentous fungi during fermentation; 3A, sun drying of cocoa beans; 3B, mouldy cocoa beans at drying; 4A, storage of cocoa beans; 4B, mouldy cocoa beans in storage; 1C, 2C, 3C, 4C, mycological evaluation of cocoa beans by direct plating in DG18, 1C, before fermentation; 2C, during fermentation; 3C, during drying; 4C, during storage (Copetti et al., 2014)

Filamentous fungi contamination, in particular ochratoxin A-producing species, were compared between heap and box fermentation in Cameroun and no significant differences were found between the two fermentation methods in relation to the fungal species found: *Aspergillus fumigatus*, *Aspergillus tamarii*, *A. versicolor*, *Aspergillus carbonarius*, *Aspergillus niger*, *Penicillium sclerotiorum*, *Penicillium paneum*, *Penicillium crustosum*, *Mucor* spp., *Rhizopus* spp., *Fusarium* spp. and *Trichoderma* spp. (Copetti et al., 2014; Mounjouenpou et al., 2008). Damaged pods often showed proliferation of toxigenic fungi such as *A. carbonarius*, *A. niger*, and *Fusarium* species. This study showed that fungal contaminations during pre-processing greatly affect end-quality and that good pod condition and immediate pod opening can partly reduce these risks. Aflatoxigenic fungi such as *Aspergillus flavus* and *A. parasiticus* have been isolated from samples collected during fermentation; *A.niger* and *A. carbonarius*, ochratoxin A producers, have been isolated as well (Copetti et al; 2010; 2014; Mounjouenpou et al., 2008). In summary species producing mycotoxins were present in a minimum amount during fermentation, due to the strong competition with yeast, LAB and AAB (Copetti et al., 2014) but this initial inoculum might contribute to fungal spreading in subsequent processing steps when the competition decreases.

At the end of fermentation cocoa beans contain approximately 40-60% in moisture, depending on fermentation method and have to be dried to 6-7%. Sun drying on wooden platforms, the most used method, usually takes between 7 days and 2-4 weeks depending on weather conditions; mechanical driers could be used as well. During drying water activity is reduced from 0.99 to 0.85, effecting firstly bacterial growth and then yeasts growth which have a higher tolerance to low water availability (Copetti et al., 2014). As the final water activity of the beans is about 0.50, xerophilic fungi become dominant in the last stages of drying (Copetti et al., 2010) (Fig.5, 3A-C). Wooden platforms represent a source of fungal contamination and the thin layers in which cocoa beans are disposed for sun drying increase oxygen tension and decrease the concentration of inhibitory acid produced during fermentation, due to volatilization, allowing filamentous fungi to grow. Fungal species established during fermentation are overtaken by genera adapted to lower water availability; toxigenic species such as *A. flavus*, *A. parasiticus*, *A.niger* and *A. carbonarius* may increase during the last part of drying (Copetti et al., 2014). Copetti et al. (2010) found a correlation between the occurrence of ochratoxin A and the presence of *A.carbonarius*, indicating that this species is the principal OTA-producer in cocoa beans. In general, scientific evidence indicates that the drying stage, especially sun drying, is a

crucial point for fungal contamination due to the development of environmental conditions favorable to the fungal growth but inadequate for the competitors (yeasts, AAB, LAB). Nevertheless there was a weak correlation between fungal contamination and the presence of the toxins, suggesting the existence of anti-toxigenic compounds in cocoa such as tannins and caffeine (Copetti et al., 2011).

Dried beans are usually stored in bags at farms before being marketed. Fungal spores present at the end of the drying period remain viable for long times, so that good storage conditions are crucial to maintain the quality of the beans and avoid contamination spreading. If cocoa beans are stored in an improper way, high humidity may cause rapid increase of water activity, providing favorable conditions for spore germination, fungal growth and spoilage (Copetti et al., 2014). Toxigenic fungal species (*A.niger*, *A.flavus*) have been reported in several studies on stored cocoa beans (Copetti et al., 2011; 2014; Mounjouenpou et al., 2008). Xerophilic species, above all *Eurotium amstelodami*, *Eurotium chevalieri*, *Eurotium rubrum* and *Aspergillus penicillioides* may grow when cocoa beans are stored under improper conditions (Copetti et al., 2011) (Fig.5, 4A-C). Dried cocoa beans are hygroscopic, so cocoa will absorb moisture from the environment under high humidity conditions, leading to germination of spores.

In summary, all the steps foregoing industrial processing (pre-harvest, fermentation, drying and storage) may increase fungal contamination, hence mycotoxins production, if performed under poor conditions. Standardization of the whole production chain could sensibly reduce contamination risks (Fig.6).

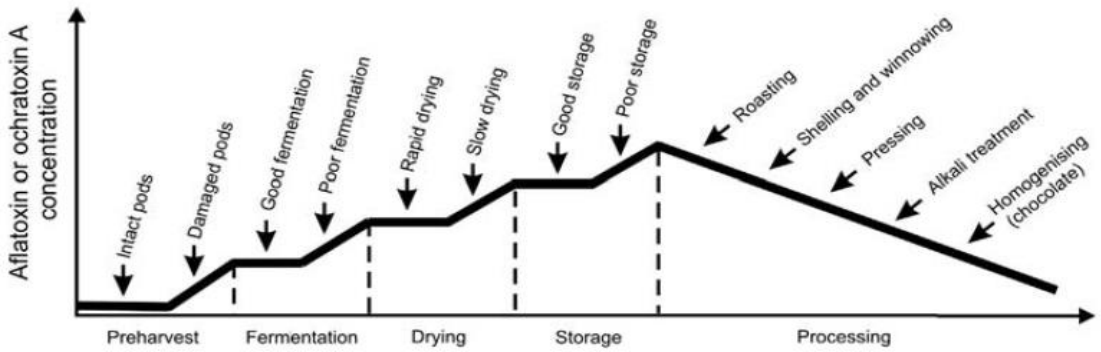

Fig. 6. Schematic of formation and reduction in aflatoxin and ochratoxin A during stages of production of cocoa powder and chocolate from cocoa beans. The diagram is qualitative (Copetti et al., 2014).

1.11. Analysis through Next-Generation Sequencing (NGS)

Nowadays polyphasic studies have dealt with both culture-dependent methods, which include classical microbiological methods alone or coupled with molecular identification techniques, and culture-independent methods, which are mainly based on the PCR amplification of 16S rRNA (bacteria) and 26S rRNA (yeast) genes, in combination or not with metabolite target analyses (Camu et al., 2007; 2008; De Vuyst and Weckx, 2016; Hamdouche et al., 2013; Ho et al., 2014; Moreira et al., 2013; Nielsen et al., 2007; Papalexandratou and De Vuyst, 2011; Papalexandratou et al., 2011a; b; c). Next-Generation Sequencing (NGS) has introduced a new molecular tool to deeply analyze the microbial flora of cocoa beans during farm processing, especially during fermentation (Garcia-Armisen et al., 2010; Illeghems et al., 2012; 2015).

Among the first studies on bacterial biodiversity through analysis of PCR-derived 16S rRNA gene amplicons, Garcia-Armisen et al. (2010) have extracted total DNA from spontaneous heap (Ghana) and box (Brazil) cocoa bean fermentation samples, using it to generate a 16S gene clone library. This approach has confirmed the low bacterial species diversity in the fermenting cocoa-bean mass, although a more wide range of species has been detected, in particular *Gluconacetobacter* and *Erwinia*, *Pantoea*, *Tatumella* species (De Vuyst and Weckx, 2016), as compared to results obtained by culture-dependent methods.

Total metagenomic DNA, extracted from a representative single sample coming from a spontaneous box fermentation, has been used for a shotgun sequencing to perform both phylogenetic analysis of the microbial diversity and functional bacterial meta-pathway analysis to deeper understand community capacities (Illeghems et al., 2015; De Vuyst and Weckx, 2016).

So that understanding the functional role and the dynamics of microbial communities involved in the production of fermented cocoa beans requires insight into their members' metabolism and interactions. It is however difficult to explore and understand these natural microbial ecosystems due to their complexity and their interactions, indeed, microbial communities may be very different in terms of composition, abundance, and functional roles along the fermentation process and uncultivable species represents an obstacle to the comprehensive characterization of the microbioma involved during fermentation (Illeghems et al., 2015). Therefore, the

use of next-generation sequencing methods, based on the sequencing of whole microbial community (metagenomic) DNA has been applied in recent years to assess the microbial community structure of complex food fermentation ecosystems and recently this approach has been applied to characterize the community composition of the fermented cocoa beans (Illeghems et al., 2012), in which yeasts, LAB and AAB are the key players (Schwan and Wheals, 2004).

1.12. Cocoa trade market

Cocoa beans production represent a massive trade market which account for 10.434.201 ha of harvestad area and 4.450.263 tonnes of world production (Fig.7).

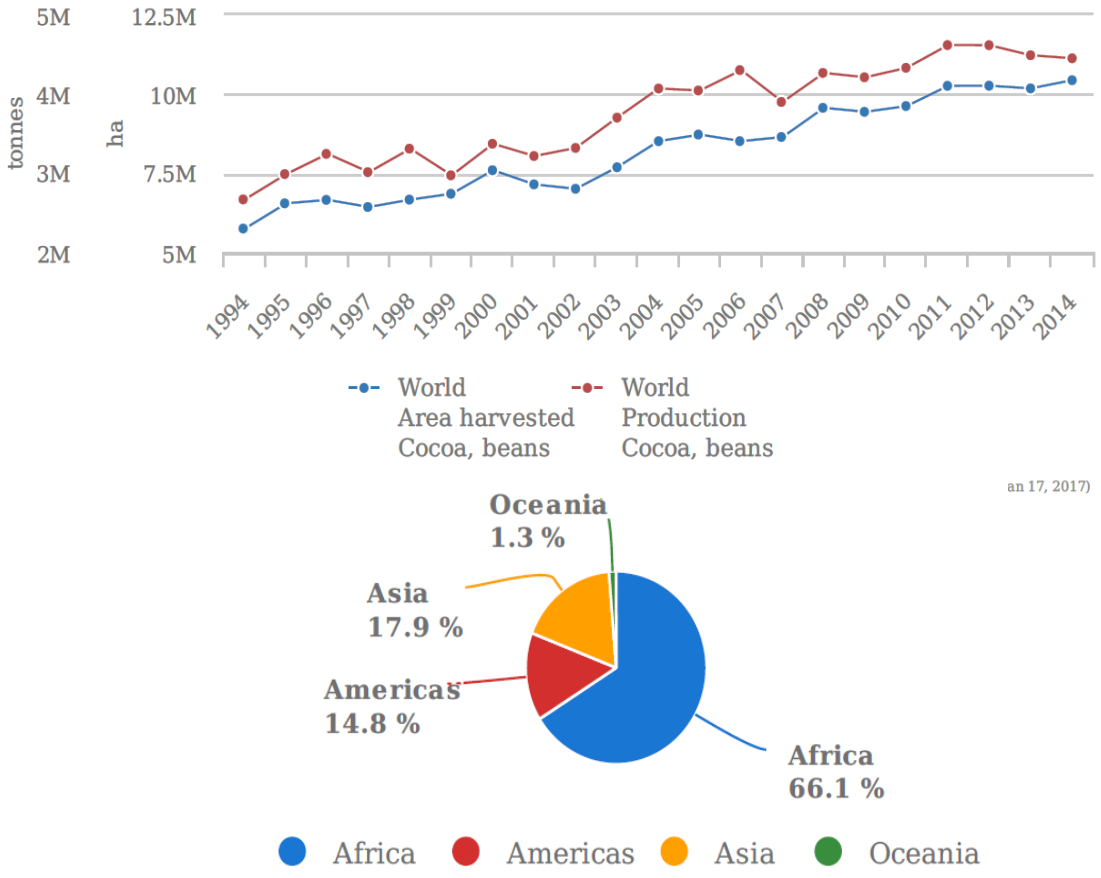

Fig.7. Production/yeald quantities of cocoa beans world production and production share by region (FAOSTAT,Jan-2017).

Trade in cocoa is complex and involves many players: farmers produce fermented beans, warehouses store the beans, traders and dealers ship principally to North America and Europe and manufacturers convert the beans into consumable products (Shwan and Wheals, 2004). Due to this complicated supplies chain, cocoa beans price may undergoes strong fluctuations: after reaching a peak of well over US$3,000/tonne in 1977 the price of roasted beans has fallen to an average about US $2,000/tonne during the last years (ICCO, 2017; Shwan and Wheals, 2004).

Table 3. Top ten global confectionery companies that manufacture some form of chocolate, by net confectionery sales value in 2015 (ICCO, 2016).

Company	Net Sales 2015 (US$ millions)
Mars Inc (USA)	18,400
Mondelēz International (USA)	16,691
Nestlé SA (Switzerland)	11,041
Ferrero Group (Luxembourg / Italy)	**9,757**
Meiji Co Ltd (Japan)	8,461*
Hershey Co (USA)	7,422
Chocoladenfabriken Lindt & Sprüngli AG (Switzerland)	4,171
Arcor (Argentina)	3,000
Ezaki Glico Co Ltd (Japan)	2,611*
Yildiz Holding (Turkey)	2,144

* This includes production of non-confectionery items

In this economic scenario Ferrero is one of the principal players (Table 3), so that the understang of the pillars, driving the chocolate quality, represent a core business for this company.

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Appendix A. Microbes involved in cocoa fermentation (Saltini et al., 2013)

Microbe		Notes	Main activity	Specifications
Yeasts	*Candida spp.*	Increased in number after 24 h.	Ethanol and lactic acid assimilation. Assimilation of glucose and sucrose. Able to assimilate citrate.	*Brazil, Ghana, Malaysia, Belize, Dominican republic*
	Candida krusei	Dominant species. Detected in different fermentation methods.	Ethanol fermentation, citric and lactic assimilation. Acetic acid production.	*Ghana, Dominican republic*
	Candida tropicalis	Important presence. Pathogen.	Assimilation of glucose and sucrose.	*Indonesia*
	Hansenula spp.		Largely used in difference food and pharmaceuticals productions. Assimilation of glucose and sucrose.	*Ghana, Malaysia*
	Hanseniaspora guilliermondii	Detected in several fermentation methods. Dominating yeast during early stages.	Ethanol fermentation, acetoin and 2,3-butanediol production. Already used in wine production.	*Ghana, Dominican republic*
	Kloeckera spp.	Disappeared after 24 h.	Already used in wine production.	*Brazil, Ghana, Malaysia, Belize*
	Kluyveromyces marxianus	Grow slowly and disappeared. Artificially inoculating a hybrid of this strain improves flavour acceptability.	Pectinolytic activity.	*Brazil.*
	Kluyveromyces thermotolerans	Found when temperature higher than 50 C.		*Brazil.*
	Kodamaea ohmeri		Assimilation of glucose and sucrose.	
	Lodderomyces elongisporus	Disappeared after few hours.		*Brazil.*
	Meyerozyma		Assimilation of glucose and sucrose.	
	Pichia spp. e Pichia membranifaciens	Disappeared after few hours. Detected in different fermentation methods. Dominating yeast in late stages. Detected in early stages.	Ethanol fermentation, lactic and citric acids assimilation. Assimilation of glucose and sucrose.	*Dominican republic.*
	Rhodotorula spp.			*Malaysia.*
	Saccharomyces spp.	Dominant strain during the	Well known and used	*Brazil, Ghana,*

		whole process.	in wine production.	*Malaysia, Belize,*
			Assimilation of	*Trinidad, Indonesia.*
			glucose and sucrose.	
	Saccharomycopsis spp.			*Ghana, Beliza.*
	Schizosaccharomyces spp.			*Ghana, Beliza.*
	Torulaspora pretoriensis	Found when temperature higher than 50 C.		*Brazil.*
	Torulopsis spp			*Ghana.*
	Trichosporon asahii			*Ghana.*
	Yamadazyma		Assimilation of glucose and sucrose.	
Lactic acid bacteria (LAB)	*Lactobacillus acidophilus*		Degrading glucose to lactic acid.	*Brazil, Africa.*
	Lb. brevis	Present between 48 and 96 h.	Most of them	*Brazil, Belize,*
			already used in	*Dominican republic.*
	Lb. buchneri		various industrial	*Belize.*
	Lb. casei		fermentations,	*Brazil, Belize.*
	Lb. cellobiosus	Principal spices.	like cheese, beer or wine production.	*Belize, Indonesia.*
	Lb. delbrueckii			*Brazil, Belize.*
	Lb. fermentum	Most abundant in first 24 h e dominating LAB strain.		
	Lb. fructivorans			*Belize.*
	Lb. gasseri			*Belize.*
	Lb. kandleri			*Belize.*
	Lb. plantarum	Principal spices.		*Brazil, Ghana, Malaysia, Belize, Indonesia, Africa, Indonesia,*
	Lb. paracasei	Present after 48 h.		*Dominican republic.*
	Lb. pentosus	Present after 48 h.		*Dominican republic.*
	Lb. collinoides			*Ghana, Malaysia.*
	Lb. lactis			*Brazil.*
	Lb. mali			*Ghana.*
	Lactococcus lactis	Most abundant in first 24 h.		*Brazil, Africa.*
	Leuconostoc mesenteroides	Most abundant in first 24 h.		
	Leuconostoc oenos			*Belize.*
	Leuconostoc paramesenteroides			*Belize, Ghana.*
	Leuconostoc pseudoficulneum	Might be important.		*Ghana.*
	Leuconostoc pseudomesenteroides			*Ghana.*
	Pediococcus acidilactici			*Brazil, Africa.*
	P. dextrinicus			*Brazil.*
	Weissella			*Africa, Ghana.*

Acetic acid bacteria (AAB)	*Acetobacter spp.*	Most common species. Its metabolism is improved by the presence of oxygen.	Oxidation of ethanol to acetic acid and further oxidation of the latter to carbon dioxide and water. They are obligatory aerobic.	*Belize.*
	A. aceti			*Brazil, Indonesia.*
	A. ascendens			*Ghana.*
	A. ghanensis			*Ghana.*
	A. lovaniensis	Present between 72 and 96 h.		*republic.*
	A. pasteurianus	Predominant AAB.		*Brazil, Indonesia, Ghana, Indonesia.*
	A. peroxydans			*Brazil.*
	A. rancens			*Ghana, Malaysia.*
	A. senegalensis			*Ghana.*
	A. syzygii	Predominant AAB.		*Ghana.*
	A. tropicalis	Predominant AAB.		*Ghana.*
	A. xylinum			*Ghana, Malaysia.*
	A. xylinum			*Malaysia.*
	Gluconobacter oxydans			*Brazil, Malaysia, Belize, Ghana.*
Spore forming bacteria	*Bacillus spp.*	Dominant in late stages.	Under fermentative conditions they might produce 2,3-butanediol, pyrazines, acetic and lactic acid, off-flavours.	*Brazil, Trinidad, Ghana, Malaysia, Indonesia.*
	B. brevis			*Brazil.*
	B. cereus	Development of off-flavours.		*Brazil, Trinidad.*
	B. circulans			*Brazil.*
	B. coagulans			*Brazil, Trinidad.*
	B. firmus			*Brazil.*
	B. laterosporus			*Brazil.*
	B. licheniformis			*Brazil, Trinidad, Ghana, Malaysia, Indonesia.*
	B. macerans			*Brazil.*
	B. megaterium	Development of off-flavours.		*Brazil.*
	B. pasteurii			*Brazil.*
	B. polymyxa			*Brazil.*
	B. pumilus			*Brazil, Trinidad,*
	B. subtilis	Development of off-flavours.		*Brazil, Trinidad,*