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Ph.D. Thesis

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October 2006

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Preface

The present Ph.D. thesis is presented to fulfil the requirements for a Ph.D. degree at The Royal Veterinary and Agricultural University (KVL), Frederiksberg, Denmark. The research forming the basis for the thesis has been carried out at The Department of Food Science, KVL and Institute für Hygiene und Toxikologie, Bundesforschungsanstalt für Ernährung und Lebensmittel, Karlsruhe, Germany. Field work in Ghana was carried out in collaboration with The Food Research Institute (Dr. Wisdom Amoa-Awua), Accra, Ghana; and The Cocoa Research Institute of Ghana (Dr. Jemmy Takrama), New Tafo, Ghana. My main supervisor has been Professor Mogens Jakobsen, with Associate Professor Lars Nørgaard, Professor Lars Munck and Professor Wilhelm H. Holzapfel being connected to the project as co-supervisors.

The project has mainly been funded by a grant from KVL, but parts of the research has also been funded by the European Union financed INCO project: "Developing biochemical and molecular markers for determining quality assurance in the primary processing of cocoa in West Africa – COCOQual" (ICA4-CT-2002-10040). The financial support of both sources is highly appreciated.

I would like to thank my main supervisor Professor Mogens Jakobsen for excellent guidance and support throughout the project. Furthermore I would like to thank all my co-supervisors for sharing their knowledge on their special field of expertise with me. Especially I would like to thank Professor Wilhelm H. Holzapfel for excellent guidance and for giving me the opportunity to spend 4 months with his group in Karlsruhe. During the project I have enjoyed the company and help of a lot of people – here at KVL, in Ghana and in Karlsruhe. Especially I would like to thank Peter Lange Møller for introducing me to the World of molecular biology, and all the people being directly involved in the project for shorter or longer periods – Debrah, Louis, Margaret, David, Susanne, Tanja, Pia, Lene, Janne, Dirk, Ulrich and Charles.

Last but not least a special thank to my family for sharing good and bad times – and especially to Gitte and Selma for just being there all the time!

KVL, October 2006

Summary

Two-thirds of the World's cocoa is produced in West Africa with Ghana being the World's second largest producer of cocoa beans accounting for approximately 20 % of the World annual production. Being the largest export commodity cocoa is of great economical importance for Ghana as a country and of even bigger socio-economic importance in the cocoa growing farms and villages throughout the country. Cocoa beans are the principal raw material of chocolate and originate as seeds in fruit pods of the tree *Theobroma cacao*. Raw cocoa beans have an astringent, unpleasant taste and flavour and have to be fermented, dried and roasted to obtain the characteristic cocoa taste and flavour. Despite the importance of the fermentation for the quality of the final product, our knowledge of the microbiology of the process is still inadequate. Especially there is a lack of studies on fermentation of cocoa in West Africa.

The overall objective of the present Ph.D. thesis was to conduct a detailed microbiological investigation of Ghanaian cocoa fermentations using culture-based and culture-independent techniques. Consequently another objective was to develop a culture-independent method suitable for the investigation of cocoa fermentations based on Denaturing Gradient Gel Electrophoresis (DGGE). The present study represents the first investigation of cocoa fermentations taking advantage of recent year's development in molecular biology for grouping and identification of the microorganisms participating in the process. Furthermore, the present study represents the first culture-independent investigation of cocoa fermentations.

The microbiology of a number of Ghanaian cocoa fermentations representing tray, small heap and large heap fermentations have been thoroughly investigated using culture-dependent and culture-independent methods. Furthermore, the microbiology of cocoa fermentations representing different harvesting periods and geographic locations in Ghana were investigated using culture independent methods only.

Samples were collected at 12-24 hour intervals during 72-144 hour tray and traditional heap fermentations. Yeast, Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and *Bacillus* spp. were enumerated on suitable substrates and more than 1500 isolates identified using phenotypic and molecular biology based methods. Samples for culture-independent

investigation of the process were freeze dried and DNA extracted using a DNA extraction protocol developed for the purpose.

A microbiological succession was observed during the fermentations. At the onset of fermentation yeasts were the dominating microorganisms. Lactic acid bacteria became dominant after 12-24 hours of fermentation and remained predominant throughout the fermentations with AAB reaching high counts in the mid phase of fermentation. *Bacillus* spp. were only detected during heap fermentations, where they reached high numbers during the later stages of fermentation. *Hanseniaspora guilliermondii* was the predominant yeast during the initial phase and *Pichia membranifaciens* during the later phases of fermentation. A number of other yeast species including *Issatchenkia orientalis*, *Candida zemplinina*, *Saccharomyces cerevisiae* and three putatively undescribed yeast species were isolated during the fermentations. Apparently *C. zemplinina* and *Sc. cerevisiae* played a more prominent role during the investigated tray fermentations compared to the heap fermentations. *Lactobacillus fermentum* was the dominant LAB in most samples. Several other LAB including *Lactobacillus plantarum*, *Leuconostoc pseudomesenteroides*, *Leuconostoc pseudoficulneum*, *Pediocococcus acidilactici* and a putatively undescribed LAB were detected during the fermentations. *Acetobacter pasteurianus*, *Acetobacter syzygii* and *Acetobacter tropicalis* were the predominant AAB in all investigated fermentations. During the later stages of heap fermentation *Bacillus licheniformis* and occasionally other *Bacillus* spp. were detected in high numbers.

Chromosome Length Polymorphism among yeasts involved in the fermentation was determined using Pulsed Field Gel Electrophoresis. Chromosome length polymorphism was evident within all investigated species showing that not only a range of different yeast species were involved in the fermentation, but among the different species also different strains were involved.

A DGGE based method for culture-independent investigation of the yeast and bacterial micro-populations involved in the cocoa fermentation was developed. The culture-based and culture-independent results yielded comparable, but slightly different results. Among the yeasts *H. guilliermondii* could be detected using DGGE several days after it became undetectable using

culture-based methods. *Trichosporon asahii* yielded on the other hand only faint bands in the denaturing gels despite the fact that it was detected using culture-based methods. Analysis of pure cultures showed that the targeted region of the 26S rRNA gene was poorly amplified in *T. asahii*, whereas all other investigated isolates were amplified equally efficiently using the chosen PCR approach. Among the bacteria DGGE indicated that *Lc. pseudoficulneum* plays a more important role during the fermentation of cocoa than expected from the culture-based findings where it was only infrequently detected, whereas it yielded a strong band in most DGGE profiles. Cluster analysis of the DGGE profiles revealed that the profiles clustered according to fermentation site and in the case of the profiles representing the yeast community also with fermentation method. Within each fermentation site the profiles clustered according to fermentation time. Given the results obtained during the present study DGGE seems to offer a relatively fast and reliable tool for studying yeast and bacterial dynamics during cocoa fermentations.

The putatively undescribed LAB was thoroughly characterised pheno- and genotypically. It was revealed 16S rRNA gene sequence analysis that the isolates phylogenetically belong to the genus *Lactobacillus* and were closely related to *Lactobacillus nagelii*, *Lactobacillus vini* and *Lactobacillis satsumensis*. Low DNA-DNA reassociation values were obtained between the isolates and the phylogenetically closest neighbours. Furthermore, a number of phenotypic tests differentiated the isolates and the phylogenetically closest neighbours. Based on the genetic and phenotypic results, the isolates were considered to represent a novel species, for which the name *Lactobacillus ghanaensis* was proposed.

Phylogenetic analyses of the 26S rRNA gene (D1/D2-region) revealed that the 3 putatively undescribed yeast species were distantly related to all known yeast species. The phylogenetically closest relatives of the 3 yeast species based on 26S rRNA gene (D1/D2-region) similarity were; Species A, *Saturnispora mendoncae* (92.4 % similarity); Species B, *Dipodascus geniculatus* (81.8 % similarity); and Species B, *Candida rugopelliculosa* (92.3 % similarity), respectively.

The work has been presented in 4 papers published in, accepted for publication in or submitted for publication to international scientific journals.

Resumé

To-trediedele af al kakao produceres i Vestafrika. Ghana, der er verdens andenstørste producent af kakaobønner, står for ca. 20 % af verdensproduktionen. Eftersom kakaobønner er den største eksportartikel har kakao stor økonomisk betydning for Ghana som land, og endnu større socio-økonomisk betydning i de kakaodyrkende områder og landsbyer rundt omkring i landet. Kakaobønnen, der er den primære råvare i chokolade, stammer fra træet *Theobroma cacao*, hvor de udgør frugtens frø. Rå kakaobønner har en bitter, ubehagelig smag og skal fermenteres, tørres og ristes for at opnå den karakteristiske kakaoduft og -smag. På trods af, at fermenteringen har stor betydning for kvaliteten af det færdige produkt, er vor viden om de mikrobiologiske aspekter af fermenteringen stadig mangelfuld. I særlig grad er der en mangel på viden omkring fermentering af kakao i Vestafrika.

Det overordnede mål for denne Ph.D.-afhandling var at gennemføre en detaljeret mikrobiologisk karakterisering af ghanesiske kakaofermenteringer med brug af såvel dyrkningsbaserede som dyrkningsuafhængige metoder. Da kakaofermenteringer ikke tidligere er blevet undersøgt vha. dyrkningsuafhængige metoder, var et yderligere mål at udvikle en dyrkningsuafhængig metode baseret på Denaturing Gradient Gel Electrophoresis (DGGE) egnet til at undersøge kakaofermenteringer mikrobiologisk. Dette studie repræsenterer den første mikrobiologiske undersøgelse af kakaofermenteringsprocessen, der drager nytte af de seneste års udvikling indenfor molekylærbiologien til at gruppere og identificere de involverede mikroorganismer. Ligeledes repræsenterer dette studie den første dyrknings-uafhængige undersøgelse af kakaofermenteringsprocessen.

En række ghanesiske kakaofermenteringer, repræsenterende 3 forskellige fermenterings-systemer ("bakke" samt små og store "bunke" fermenteringer), er blevet detaljeret mikrobiologisk karakteriseret vha. dyrkningsafhængige og dyrkningsuafhængige metoder. Ydermere er et antal fermenteringer, der stammer fra forskellige høsttidspunkter og områder i Ghana blevet karakteriseret udelukkende vha. dyrkningsuafhængige metoder.

Med 12-24 timers interval blev prøver udtaget gennem 72-144 timers "bakke" og traditionelle "bunke" fermenteringer. Gær, mælkesyrebakterier, eddikesyrebakterier og *Bacillus* spp. blev kvantificeret og isoleret på egnede substrater, og mere end 1500 isolater identificeret vha.

fæno- og genotypiske metoder. Til den dyrkningsuafhængige undersøgelse af processen blev prøverne frysetørret og DNA ekstraheret vha. en til formålet udviklet protokol.

En mikrobiologisk succession blev observeret gennem fermenteringerne. Indledningsvist var gær de dominerende mikroorganismer. Efter 12-24 timer blev mælkesyrebakterier dominerende, hvilket de, med få undtagelser, forblev hele vejen gennem fermenteringerne. Eddikesyrebakterier voksede til høje antal i midterfasen af fermenteringerne, mens *Bacillus* spp. udelukkende blev detekteret i de senere faser af bunkefermenteringerne. *Hanseniaspora guilliermondii* var den dominerende gær i den indledende fase af fermenteringerne, mens *Pichia membranifaciens* var den dominerende gær i de senere faser. En række andre gær, bl.a. *Issatchenkia orientalis*, *Candida zemplinina*, *Saccharmomyces cerevisiae* og 3 gærarter, der tilsyneladende ikke er blevet beskrevet tidligere, blev isoleret gennem fermenteringerne. Tilsyneladende spillede *C. zemplinina* og *Sc. cerevisiae* en større rolle i de undersøgte bakke-fermenteringen i forhold til de undersøgte bunkefermenteringer. *Lactobacillus fermentum* var den dominerende mælkesyrebakterie i de fleste undersøgte prøver. Flere andre mælkesyrebakterier, bl.a. *Lactobacillus plantarum*, *Leuconostoc pseudomesenteroides*, *Leuconostoc pseudoficulneum*, *Pediocococcus acidilactici* og en tilsyneladende ikke tidligere beskrevet mælkesyrebakterie blev isoleret gennem fermenteringerne. *Acetobacter pasteurianus*, *Acetobacter syzygii*, og *Acetobacter tropicalis* var de dominerende eddikesyrebakterier i alle undersøgte fermenteringer. Gennem de senere faser af bunkefermenteringerne blev *Bacillus licheniformis* og lejlighedsvist andre *Bacillus* spp. detekteret i høje antal.

Kromosomlængdepolymorfisme blandt gær involveret i kakaofermenteringerne blev undersøgt vha. Pulsed Field Gel Electrophoresis. Kromosomlængdepolymorfisme var tydelig blandt alle undersøgte arter. Det vil sige, at ikke blot var en række forskellige gær involveret i fermenteringen af kakao, men indenfor de enkelte arter var endvidere forskellige stammer involveret.

En DGGE-baseret metode blev udviklet med det formål dyrkningsuafhængigt at undersøge gær og bakterier involveret i kakaofermenteringerne. De dyrkningsbaserede og dyrkningsuafhængige metoder gav sammenlignelige, men dog lidt forskellige resultater. I den

DGGE-baserede undersøgelse af gær involveret i fermenteringerne kunne *H. guilliermondii* detekteres vha. DGGE flere døgn efter, at den ikke længere kunne detekteres vha. af dyrkningsbaserede metoder. På den anden side gav *Trichosporon asahii* kun svage bånd i DGGE-gelerne på trods af, at *T. asahii* blev detekteret vha. de dyrkningsbaserede metoder. Analyse af renkulturer viste, at 26S rRNA genet i *T. asahii*, i forhold til alle andre undersøgte isolater, blev dårligt amplificeret med den valgte PCR metode. Den DGGE-baserede undersøgelse af bakterier involveret i fermenteringen af kakao viste, at *Lc. pseudoficulneum* tilsyneladende spiller en større rolle i processen, end det var forventet ud fra de dyrkningsbaserede undersøgelser, hvor *Lc. pseudoficulneum* kun blev detekteret lejlighedsvist og i lave antal, hvorimod den gav et kraftigt bånd i de fleste DGGE profiler. Cluster analyse af DGGE profilerne viste, at profilerne grupperede sig efter, hvor fermenteringerne blev udført, mens gærprofilerne også grupperede sig efter fermenteringsmetode. Inden for hvert fermenteringssted grupperede profilerne sig efter hvor lang tid fermenteringen havde forløbet. Den udviklede DGGE metode synes at være et relativt hurtigt og pålideligt redskab til at undersøge betydningen af gær og bakterier gennem kakaofermenteringer.

Den tilsyneladende ikke tidligere beskrevne mælkesyrebakterie blev grundigt karakteriseret fæno- og genotypisk. Sekventering og analyse af 16S rRNA genet viste, at isolaterne phylogenetisk tilhørte slægten *Lactobacillus* og var tæt beslægtede med *Lactobacillus nagelii*, *Lactobacillus vini* og *Lactobacillis satsumensis*. Bestemmelse af DNA-DNA re-associering mellem isolaterne og de phylogenetisk tættest beslægtede arter gav kun lave DNA-DNA re-associerings-værdier. Ydermere kunne isolaterne adskilles fra de phylogenetisk tættest beslægtede arter på grundlag af en række fænotypiske test. På grundlag af de geno- og fænotypiske resultater blev det konkluderet, at isolaterne tilhører en ikke tidligere beskrevet art, for hvilken navnet *Lactobacillus ghanaensis* blev foreslået.

Phylogenetisk analyse af 26S rRNA genet (D1/D2-regionen) viste, at de 3 tilsyneladende ikke tidligere beskreve gærarter var fjernt beslægtet med alle kendte gærarter. De tættest beslægtede slægtninge til de 3 gærarter baseret på sammenligning af 26S rRNA gen sekvenser (D1/D2-region) var henholdsvis; Uidentificeret art A, *Saturnispora mendoncae* (92,4 % overensstemmelse); Uidentificeret art B, *Dipodascus geniculatus* (81,8 % overensstemmelse); og Uidentificeret art C, *Candida rugopelliculosa* (92,3 % overensstemmelse).

Det udførte arbejde er blevet præsenteret i 4 artikler, publiceret, accepteret til publikation eller indsendt til publikation i internationale, videnskabelige tidsskrifter.

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Appendix I:

Jespersen, L.; **Nielsen, D.S.**; Hønholt, S.; Jakobsen, M. (**2005**). Occurence and diversity of yeasts involved in fermentation of West African cocoa beans. *FEMS Yeast Research* **5**:441-453.

Appendix II:

Nielsen, **D.S.**; Hønholt, S.; Tano-Debrah, K.; Jespersen, L. (**2005**). Yeast populations associated with Ghanaian cocoa fermentations analysed using Denaturing Gradient Gel Electrophoresis (DGGE). *Yeast* **22**:271-284.

Appendix III:

Nielsen, D.S.; Teniola, O.D.; Ban-Koffi, L.; Owusu, M.; Andersson, T.; Holzapfel, W. (**2006**). The microbiology of Ghanaian cocoa fermentations analysed using culture dependent and culture independent methods. *International Journal of Food Microbiology*, Accepted for publication.

Appendix IV:

Nielsen, D.S.; Schillinger, U.; Franz, C.M.A.P.; Bresciani, J.; Amoa-Awua, W.; Holzapfel, W.H.; Jakobsen, M. (**2006**). *Lactobacillus ghanaensis;* A novel motile lactic acid bacteria isolated from Ghanaian cocoa fermentations. *International Journal of Systematic and Evolutionary Microbiology,* Submitted for publication.

1. Introduction

Cocoa beans originate as seeds in the fruit pods of the tree *Theobroma cacao*. They are the principal raw material of chocolate – a luxury commodity loved by most people and sold all over the World (Schwan and Wheals, 2004). Approximately 2/3 of the World's cocoa is produced in West Africa. Ghana is the World's second largest producer accounting for around 20 % of the World production (Anon., 2005). Being the largest export commodity cocoa is of great economical importance for Ghana as a country and of even bigger socio-economic importance in the cocoa growing farms and villages around the country .

Raw cocoa has an astringent unpleasant taste and has to be fermented, dried and roasted to obtain the characteristic cocoa taste and flavour. Cocoa beans are normally sold and exported as fermented, dried beans. The obtainable World market price is partly determined by the quality of the beans produced and the ability to produce cocoa beans of a consistent high quality is thus of great economical importance for the cocoa producing farmers and countries. Ghana is in general being considered as a premium producer of cocoa but variations in the quality are frequently encountered (Baker et al., 1994; Anon., 2005; Takrama, 2006; Aneani and Takrama, 2006). Beans of sub-optimal quality include under-fermented beans with a low flavour potential and over-fermented beans causing off-flavours in the final product. Furthermore, inadequate control of the fermentation and drying steps may lead to formation of mycotoxins such as ochratoxin A (OTA), causing among other things severe liver and kidney damage in humans (Höhler, 1998; Meister, 2004; Lindblom, 2006; Anon., 2006). This is not only a health problem for the consumers, but might also turn out to be a major economical problem for the cocoa producing countries in West Africa. The European Commission has not defined permitted limits for levels of OTA in cocoa beans yet. But depending on the limits (if any) to be set by the Commission it has been estimated that up to 20 % of the cocoa bean production could be excluded from human consumption (Brera et al., 2003; Anon., 2003; Lindblom, 2006; Anon., 2006).

Despite the importance of the fermentation for the quality of the final product and even though the process has been studied for more than 100 years, our knowledge of the microbial species participating in and contributing to the fermentation is still inadequate. Especially

there is a lack of studies on fermentation of cocoa in West Africa. As a result efficient quality management measures (e.g. Good Manufacturing Practice, GMP; and Hazard Analysis Critical Control Point, HACCP) have only been introduced to a limited extent in the primary steps of cocoa processing.

The fermentation of cocoa is a spontaneous microbiological process involving a large number of different microorganisms. The overall *objective* of the present Ph.D. thesis was to conduct a detailed microbiological investigation of Ghanaian cocoa fermentations hereby documenting the succession of microorganisms participating in the process. For the first time a combination of phenotypic and genotypic methods will be used for grouping and identification of the microorganisms being involved in the fermentation of cocoa.

Molecular biology based culture-independent methods are a promising supplement to traditional culture-based methods for the investigation of complex microbial processes such as the cocoa fermentation. Another *objective* of the present study was thus to develop a culture-independent method suitable for the investigation of cocoa fermentations using Denaturing Gradient Gel Electrophoresis (DGGE) as a rapid alternative to the traditional culture-based methods. Furthermore DGGE is offering the possibility to investigate the potential role of non-cultureable microorganisms in the process.

Finally, given the heterogeneous and spontaneous nature of the cocoa fermentations the possibility to isolate hitherto unknown microorganisms contributing to our understanding of the biodiversity of traditional African fermentations is another *objective* of the investigations carried out.

The results from the investigations carried out have been published in; accepted for publication in; or submitted to international scientific journals – see Appendixes I-IV. In chapters 2-7 the existing knowledge in the literature related to the aims and subject of the present thesis is described. The results obtained during the present study is presented and discussed in relation to the exiting literature where appropriate. Chapters 8-9 contain conclusions and perspectives for future research within the subject.

2. Cocoa

The cocoa tree (*Theobroma cacao*, family Sterculiaceae) grows wild in the Amazons and other tropical areas of South and Central America. Two subspecies are recognised within *T. cacao*: Criollo and Forastero, which are further divided into various varieties such as Amelonado, a Forastero variety. Trinitario is a third group often referred to in the literature. It is a hybrid between Criollo and Forastero varieties. (Wood and Lass, 1985; Fowler et al., 1998).

Criollo have been cultivated in South and Central America as far back as 600 AD. The beans were used to make a spicy drink "chocolatl" based on roasted and grounded cocoa beans, maize meal, vanilla and chili. The drink was apparently consumed in large amounts by the Aztec royal courts and the beans were thus relatively valuable. Cocoa beans are easy to count, uniform in size and were due to their value used as a sort of currency until the Spanish conquest of the Aztec empire (Wood and Lass, 1985; Fowler et al., 1998).

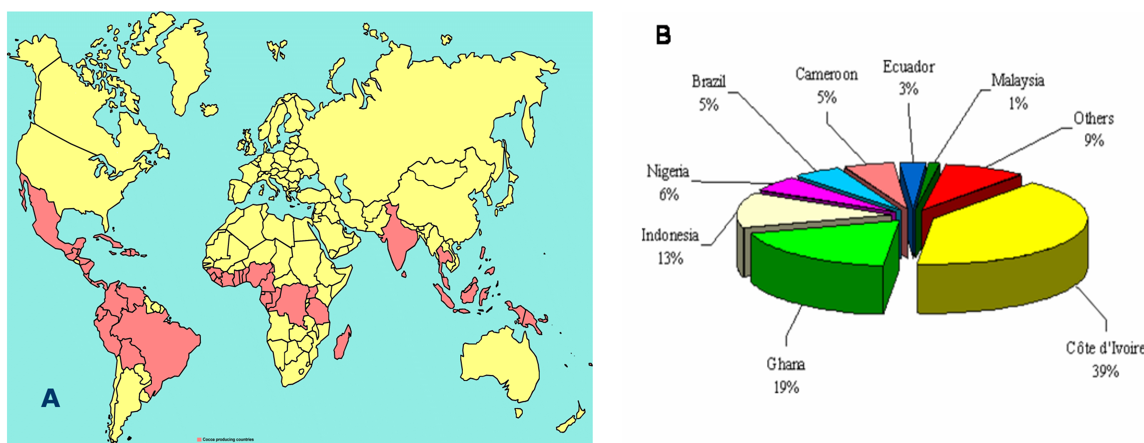


Fig. 1, A: Cocoa producing countries (red). **B:** Major cocoa producers, pct. of World production (2003/2004). Source: www.icco.org.

Following the Spanish conquest of Mexico cocoa cultivation was spread to the Caribbean Islands, parts of South America and later taken across the Pacific to the Philippines, Sulawesi and Java. Until the eighteenth century the majority of the cocoa cultivated was Criollo, but during the eighteenth and nineteenth century Forastero varieties began to predominate. Towards the end of the nineteenth century cocoa of the Amelonado type was taken from Brazil and across the Atlantic to Ghana (then a English colony under the name "Gold Coast")

where it formed the basis for cocoa production in West Africa (Wood and Lass, 1985; Fowler et al., 1998).

Today cocoa is grown in a 20° belt north and south of equator (Fig. 1A). The minimum mean (over a month) temperature in most cocoa growing regions is 18 °C, and the mean maximum temperature 32 °C. Temperatures as low as 2-6 °C can be tolerated, but if the temperature is 10 °C or lower for several consecutive days it will have a significant impact on yield with losses of 50 % or more. A high rainfall of 1000-4000 mm/year is required. Furthermore the distribution of the rainfall is important, as the dry season preferably should be shorter than 3 months and not totally dry. In the Tafo-region, Ghana, where a significant part of the present study has been carried out, the mean minimum temperature is 20-21.5 °C, the mean maximum temperature is 27.5-32.5 °C and the annual rainfall is 1600 mm. January is the driest month, with an average rainfall of 30-40 mm (Wood and Lass, 1985; Fowler et al., 1998).

Theobroma cacao grows well in a wide range of soils. The climate and soil in parts of West Africa is ideal for growth of the cocoa tree and since the introduction of cocoa in the region approximately 100 years ago Ivory Coast has become the Worlds largest producer followed by Ghana (Fig. 1B) (Wood and Lass, 1985; Fowler et al., 1998; Anon., 2005).

2.1 Composition of the mature cocoa fruit

The fruit of the cocoa tree is a pod containing 20-30 (Criollo) or 30-40 (Forastero and Trinitario) beans (or seeds, as the fresh, unfermented bean is often referred to in the literature) embedded in a mucilaginous pulp. The pods develop from pollinated flowers emerging directly out of the bark on the stem or trunks of the cocoa tree (Fig. 2 and Wood and Lass, 1985; Thompson et al., 2001).

Raw Forastero beans are violet in cross-section and produce a strong cocoa flavour upon proper processing. Criollo beans in the raw state are white, ivory or very pale purple coloured and produce cocoa with a weaker but very aromatic flavour (Wood and Lass, 1985). The Criollo beans tend to be bigger and rounder and have a lower fat content compared to Forastero (Wood and Lass, 1985).

Fig. 2: Cocoa fruit pod growing directly out of the bark on a trunk of a cocoa tree (Forastero-type). Source: Louis Ban-Koffi, private pictures, reprinted with permission.

Criollo and Trinitario are considered “fine” cocoas often sold at a higher price than Forastero. On the other hand Forastero is much less prone to various diseases giving a more stable crop from year to year and is thus preferred by farmers over most of the World. Today Forastero varieties account for approximately 95 % of the World production of cocoa. In West Africa almost exclusively varieties of Forastero (Amelonado, Amazon and Hybrid cultivars) are planted (Wood and Lass, 1985; Baker et al., 1994; Fowler et al., 1998; Takrama, 2006). For this reason focus will be on Forastaro cocoa in the following unless stated otherwise.

2.1.1 The pulp

The actual substrate for the fermentation of cocoa beans is the pulp surrounding the beans and the composition of the pulp is thus a decisive factor on the outcome of the fermentation step.
The pulp is rich in sugars with a total content of glucose, fructose and sucrose of 10-15 % (Roelofsen and Giesberger, 1947; Pettipher, 1986b; Thompson et al., 2001; Ardhana and Fleet, 2003). The glucose/fructose to sucrose ratio changes with the degree of maturity with unripe pods containing a higher proportion of sucrose and ripe pods containing mainly fructose and glucose (Packiyasothy et al., 1981). The pH is relatively low (3.3-4.0) mainly due to the content of 0.5-2 % citric acid. A relatively high content of pectin and other polysaccharides (1-2 %) makes the pulp viscous (Roelofsen and Giesberger, 1947; Pettipher, 1986b). In the present study it has been found that the main sugars of the fresh pulp were glucose (5.4-6.6 %) and fructose (6.3-7.4 %) with only small amounts of sucrose (less than 0.3 %) present. The citric acid content was 0.6-0.7 % and no or only low amounts (less than

0.2 %) of acetic acid, lactic acid and ethanol were detected in the fresh pulp; the pH of the fresh pulp was 3.94-4.12 (Appendix III).

2.1.2 The bean

The fresh, unfermented cocoa bean ("the seed") basically comprises two parts. An outer part comprising the testa (seed coat) surrounding the bean; and an inner part comprising the embryo (germ) and cotyledons contained within the testa. The testa of the cocoa seed is impermeable to larger molecules, whereas smaller molecules such ethanol and acetic acid are capable of diffusing into the seed (Roelofsen, 1958; Lopez and Dimick, 1995). The cotyledons are basically made up of different types of storage cells – e.g. lipid and polyphenol containing cells - with a plasma forming a grid between the two types of cells (see Fig. 3 and Lopez and Dimick, 1995).

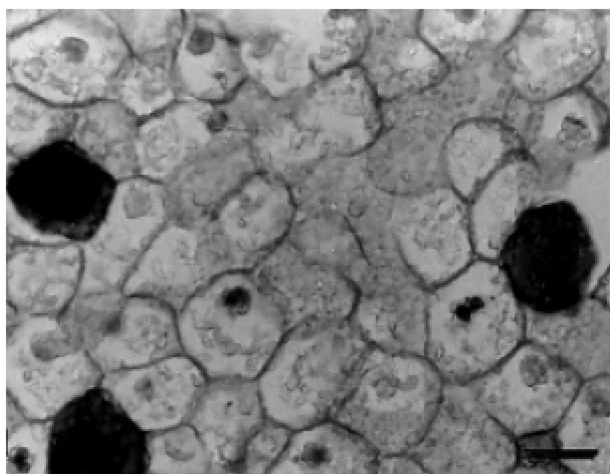


Fig. 3: Light microscopy of the inner of an unfermented cocoa bean fixated and cut lengthwise. Note the dark-stained phenolic compounds containing cells. Bar represents 20 µm (de Brito et al., 2000).

The cocoa seed has an approximate composition of 32-39 % water, 30-32 % fat, 8-10 % proteins, 2-3 % cellulose, 4-6 % starch, 4-6 % pentosans, 2-3 % sucrose, 5-6 % polyphenols, 1 % acids (mainly citric, oxalic and malic acid), 1-3 % theobromine and 0.2-1 % caffeine (Wadsworth, 1922; Forsyth and Quesnel, 1963; Weissberger et al., 1971; Lopez and Dimick, 1995; Bucheli et al., 2001; Goto et al., 2002 and Nielsen, D.S. unpublished results). Three groups of polyphenols can be distinguished in Forastero beans: Catachins (ca. 37 %), procyanidins (58 %) and anthocyanins (4 %) (Wollgast and Anklam, 2000). The main catechin is (-)-epicatechin constituting up the 35 % of the total polyphenol content (Kim and Keeney, 1984; Wollgast and Anklam, 2000). Beans of the Forastero variety owe their

characteristic violet colour to the content of anthocyanins (Roelofsen, 1958; Lopez and Dimick, 1995; Wollgast and Anklam, 2000). Beans of the Criollo type contain approximately 2/3 the amount of polyphenols found in Forastero and no anthocyanins have been detected (Wollgast and Anklam, 2000).

3. The primary processing of cocoa

Raw cocoa has an astringent unpleasant taste and has to be fermented, dried and roasted to obtain the characteristic "cocoa" taste and flavour. The primary processing steps of cocoa production include harvesting, pod breaking, fermentation and drying. The fermentation and drying steps are often referred to simply as "curing". During the fermentation various biochemical processes important for taste and flavour development are initiated in the beans (see chapter 4); processes that continue during the drying step. A further purpose of the fermentation is to facilitate removal of the mucilaginous pulp surrounding the beans as the pulp inhibit drying of the beans to a microbiological stable water content (Roelofsen, 1958; Wood and Lass, 1985; Lopez and Dimick, 1995).

The ripe pods are harvested by cutting down the fruits with varying forms of knives. Pods within reach are often harvested with a cutlass (Fig. 4A) whereas pods on the branches higher in the tree are harvested using special harvesting knives on long poles (Wood and Lass, 1985; Lopez and Dimick, 1995).

Fig. 4, A: Harvesting of ripe cocoa pods; pods within reach are harvested with a cutlass; **B & C:** The harvested pods are broken open with a cutlass or any other convenient tool. A and C: Louis Ban-Koffi, private pictures; B: Susanne Hønholt, private pictures, all reprinted with permission.

It is common practice in many cocoa producing regions including Ghana to harvest the pods over some days before the collected pods are transported to a place at the farm suitable for subsequent handling. Storing the pods for some days before opening is considered beneficial for the fermentation, as it results in a more rapid increase in temperature during fermentation – and thus a faster fermentation – presumably because sucrose is converted to glucose and

fructose (Rohan, 1963; Berbert, 1979; Dougan, 1980; Tomlins et al., 1993). The pods are broken open with e.g. a cutlass (Fig. 4B) or any other convenient tool and the beans scoped out of the broken pod (Fig. 4C) (Wood and Lass, 1985; Baker et al., 1994).

3.1 Fermentation

Following opening of the pods the cocoa beans are spontanously inoculated with a variety of microorganisms (See Chapter 5.1, Appendix I and Roelofsen, 1958; Thompson et al., 2001). During the fermentation various yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) and possibly *Bacillus* spp. develop in a form of succession carrying out the fermentation (Roelofsen and Giesberger, 1947; Roelofsen, 1958; Carr et al., 1979; Carr and Davies, 1980; Schwan et al., 1995; Thompson et al., 2001; Ardhana and Fleet, 2003; Schwan and Wheals, 2004). The microbiology of the fermentation will be dealt with in detail in chapters 5 and 6 but in summary the microorganisms metabolises the fermentable pulp sugars to ethanol. Subsequently some of the ethanol is further oxidised to acetic acid through an exothermal process. The ethanol and acetic acid penetrate the beans. This, in combination with the heat produced kill the germ and break down the cell walls in the bean initiating the processes leading to well fermented beans (Roelofsen and Giesberger, 1947; Roelofsen, 1958; Thompson et al., 2001).

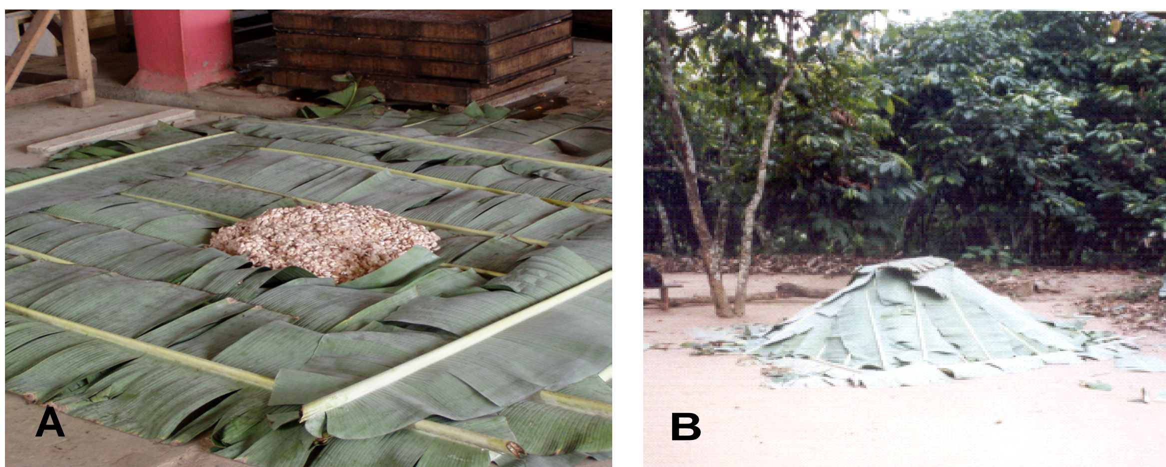


Fig. 5, A: Heap under construction; the beans are placed on plantain leaves **B:** Heap covered with plantain leaves. A: Own pictures, B: Susanne Hønholt, private pictures, reprinted with permission.

Various cocoa fermentation systems have been developed. The heap fermentation system dominates in Ghana and other West African countries (Wood and Lass, 1985; Baker et al.,

1994; Aneani and Takrama, 2006). In a heap fermentation the beans are piled on and covered with plantain leaves (Fig. 5A and B) or plastic tarpaulin. The cover protects the fermenting mass against insects and conserve heat (Wood and Lass, 1985; Aneani and Takrama, 2006; Zaouli, 2006).

It is recommended to turn/mix the heaps every 24-72 hours during the fermentation to ensure a uniform fermentation, enhance growth of beneficial microorganisms and limit the growth of unwanted microorganisms (Lehrian and Patterson, 1983; Baker et al., 1994). However, turning of the heaps is tedious (see Fig. 6A) and according to Baker et al. (1994) 57 % of the Ghanaian farmers do not turn their heaps. A recent investigation by Aneani and Takrama (2006) confirmed this finding as they reported that 62 % of Ghanaian farmers do not turn their heaps.

Fig. 6, A: Turning of a heap fermentation; **B:** Heap fermented for 48 hours; the beans of half the heap have been pushed aside. Three zones with varying degree of fermentation marked, 1: Outer zone; 2: Middle zone; 3: Central zone. Own Pictures.

The fermentation progresses faster in the outer, well aerated parts of the fermenting mass as seen in Fig. 6B, where a heap fermentation has been opened after 48 h of fermentation and the beans of half the fermentation pushed aside. Three zones with varying degrees of fermentation can be recognised: A thin, outer layer (1), where the beans are almost fully fermented and the pulp surrounding the beans have been broken down and drained away. A middle zone (2) where the fermentation have progressed some, and a central zone (3) where the beans are surrounded by a white mucilaginous pulp and have the same appearance as when they were removed from the pod. Turning is in other words necessary to achieve a final product with a uniform degree of fermentation.

To circumvent the laborious process of turning the heaps an experimental tray system claimed to give high quality beans in shorter time than the traditional heap system has been developed at the Cocoa Research Institute of Ghana (CRIG). In the tray system the raw cocoa beans are placed in 10 cm deep trays and 8-10 trays stacked on top of each other as illustrated in Fig. 7A. Air is allowed to circulate between the trays ensuring aeration of the fermenting mass without turning the beans (Allison and Rohan, 1958; Allison and Kenten, 1963).

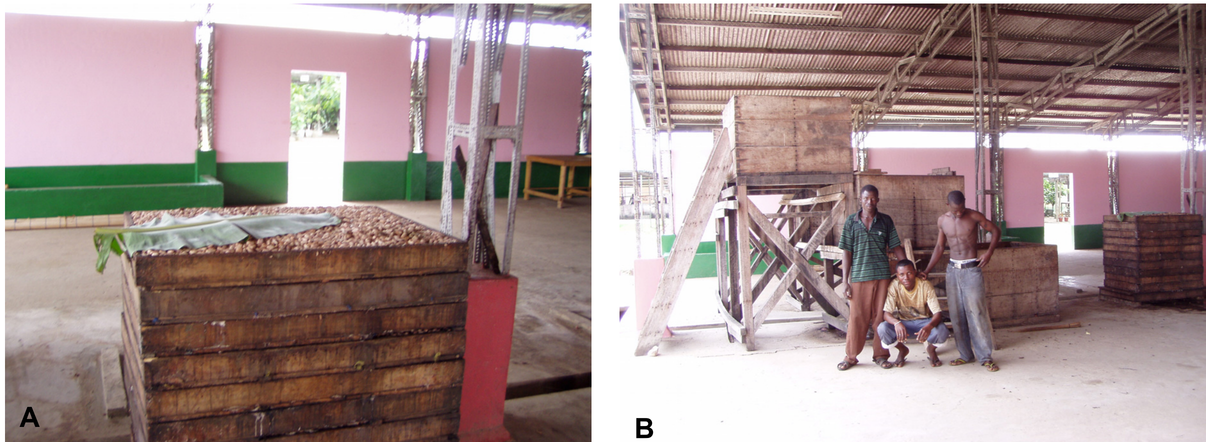


Fig. 7, A: Tray fermentation; **B:** Box fermentation of cocoa beans. Own pictures

A third system widely used in e.g. Brazil, Indonesia and Malaysia is the box fermentation system (Wood and Lass, 1985). As illustrated in Fig. 7B a number of boxes (here 3) are stacked in a stairwise manner on top of each other. Following pod breaking the beans are placed in the top box. After 1-2 days of fermentation the beans are moved to middle box and finally after 2-3 days of further fermentation into the lowest. The box fermentation systems facilitates turning, as the movement of the beans is aided by gravity and cocoa of good quality can be produced (Lehrian and Patterson, 1983; Wood and Lass, 1985). However, a problem occasionally encountered during box fermentation is uneven temperature and oxygen distribution through the fermenting mass with the corners and areas around aeration holes being better aerated and occasionally colder and less acidic than the rest of the fermenting mass enabling moulds to grow abundantly. Care must be taken with respect to adequate design and fermentation practice to avoid this (Maravalhas, 1966; Lehrian and Patterson, 1983; Schwan et al., 1995; Senenayake et al., 1997).

Several other fermentation systems including fermentation on drying platforms, in bags, barrels, baskets and holes dug in the ground have been used over the last century (Rohan, 1963; Lehrian and Patterson, 1983; Wood and Lass, 1985; Thompson et al., 2001). However, these methods are not widely used and will thus not be dealt with in detail here.

Unfortunately a firm and easy-to-follow definition of when to stop the fermentation have never been developed. As a consequence the fermentation time varies widely from country to country and even from farmer to farmer. As an example it can be mentioned that in Ghana some farmers ferment their heap fermentations for 3 days whereas others ferment for up to 7 days (Baker et al., 1994; Aneani and Takrama, 2006). However, even though no strict definition of when to terminate the fermentation has been developed, the experienced farmer still have a good idea of when to stop fermenting and start drying. This is based on the smell of the fermenting mass (i.e. development of acetic odour from the activity of acetic acid bacteria), the internal and external appearance of the beans and falling temperature of the fermenting mass (Forsyth and Quesnel, 1956; Forsyth and Quesnel, 1963).

3.2 Drying

The fermentation is stopped by drying the beans. The moisture content of the beans must be brought from the initial 40-60 % to 6-7 % to avoid growth of moulds. Furthermore, biochemical processes important for flavour and colour development of the cocoa beans take place during drying and the drying process is thus essential for the production of high quality cocoa. From the point of avoiding mould growth it is desirable to dry as fast as possible, whereas from the point of proper flavour development drying should not be too fast. It has been established that drying should take at least 48 hours to allow proper flavour development (Wood and Lass, 1985; Faborode et al., 1995; Thompson et al., 2001; Nganhou et al., 2003).

Two systems are used for drying: Sun drying and mechanical drying. During sun drying the beans are spread in a thin layer on e.g. bamboo mats raised from the ground (Fig. 8A), plastic sheds or concrete floors. It is important to ensure uniform drying by mixing the beans regularly, breaking up clumps of beans etc. Furthermore the beans should be protected from rewetting due to rain and dew during night by covering the beans adequately as seen in Fig.

8B or by collecting and moving the beans indoor during rain and at night (Wood and Lass, 1985; Thompson et al., 2001).

Fig. 8, A: Sun drying on bamboo mats; **B:** Beans covered to protect against rain. Own pictures.

Under sunny conditions the beans dry within a week, but under cloudy or rainy conditions drying may take up to 3-4 weeks. Obviously prolonged drying increases the risk of mould growth and spoilage (Wood and Lass, 1985; Thompson et al., 2001).

Mechanical drying is another option. Generally hot air dryers driven by wood or oil are employed. Numerous designs have been developed, but normally indirect heating using heat exchangers are preferred. The initially drying rate must be slow and with frequent mixing to achieve uniform removal of water and allow time for the flavour and aroma precursor producing biochemical reactions to complete. This is achieved by keeping the temperature at 60 °C or lower and dry for at least 48 hours (Wood and Lass, 1985; Thompson et al., 2001).

Sun drying is the most widely used method for several reasons: First and foremost sun drying is cheap with no need to invest in expensive equipment and consumables (fuel). Furthermore cocoa is very prone to contamination with smoke. If smoke from the heating source of the mechanical dryer reach the cocoa due to badly constructed or poorly maintained equipment it will result in cocoa with smoky off-flavours severely limiting the value of the product (Wood and Lass, 1985; Thompson et al., 2001).

3.3 Further handling

Following proper drying the beans are ready for sale. Normally the beans are stored and transported in jute bags or as bulk goods. Some cocoa producing countries have established industry for further processing of the beans into cocoa butter, cocoa powder, cocoa mass and chocolate but typically the fermented and dried beans are exported directly to Europe, USA, Japan and other parts of the industrialised world (Wood and Lass, 1985; Anon., 2005). Here the beans are roasted, de-shelled and further processed into cocoa butter (the fat containing part), cocoa powder (the de-fatted part) or used directly for production of chocolate. Cocoa butter is an important ingredient in chocolate, but has found a range of other uses as well – e.g. in the cosmetic industry. Cocoa powder is used in numerous confectionaries and beverages (Wood and Lass, 1985).

4. Changes in the bean during fermentation and drying

The fermentation of cocoa facilitate drying of the beans by removing the pulp, but the main reason for fermenting cocoa is to induce biochemical reactions within the bean that lead to formation of precursors of chocolate aroma, flavour and colour. The formation of aroma, flavour and colour precursors continue during the drying process as mentioned previously (Quesnel, 1965; Lopez and Dimick, 1995; Thompson et al., 2001).

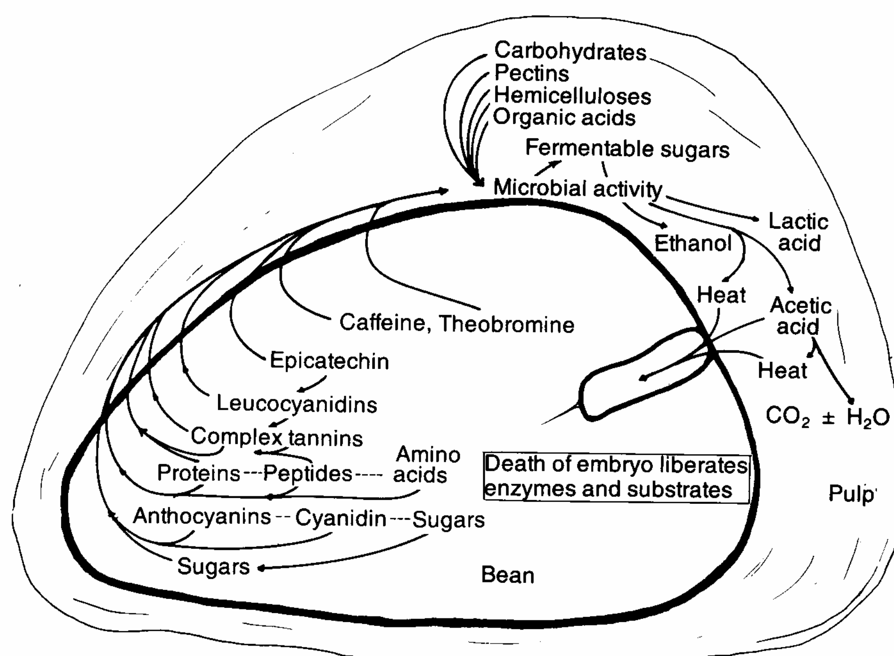


Fig. 9: Biochemical changes in the cocoa bean during fermentation (Lopez and Dimick, 1995).

The combined effect of increasing temperature and acetic acid and ethanol penetrating the testa and entering the bean as the fermentation progresses kill the embryo (germ) and causes breakdown of the protein-lipid and polyphenol containing storage cells in the cotyledon (Fig. 9 and Roelofsen, 1958; Biehl, 1973; Lopez et al., 1987; de Brito et al., 2000; Thompson et al., 2001). The penetrating acetic acid cause the bean pH to drop from the initial value of 6.3-6.8 to 4-4.5 (Forsyth and Quesnel, 1956; Lehrian and Patterson, 1983; Lopez and Dimick, 1995).

Following breakdown of the cell walls in the bean numerous biochemical processes take place leading to the breakdown of proteins to peptides and amino acids (Rohan and Stewart, 1967a; Zak and Keeney, 1976; Biehl and Passern, 1982; Amin et al., 1997; Amin et al., 1998;

Hashim et al., 1998; Lerceteau et al., 1999; Buyukpamukcu et al., 2001); and breakdown of sucrose to fructose and glucose (Rohan and Stewart, 1967b; Hansen et al., 1998; Hashim et al., 1998; Goto et al., 2001; Goto et al., 2002). The theobromine and caffeine content of the beans slightly decreases during fermentation due to diffusion out of the beans (Knapp and Wadsworth, 1924; Timbie et al., 1978; Aremu et al., 1995 and Nielsen, D.S., unpublished results). Apparently there is no change in the fat content during fermentation and drying (Roelofsen, 1958; Forsyth and Quesnel, 1963; Lehrian and Patterson, 1983).

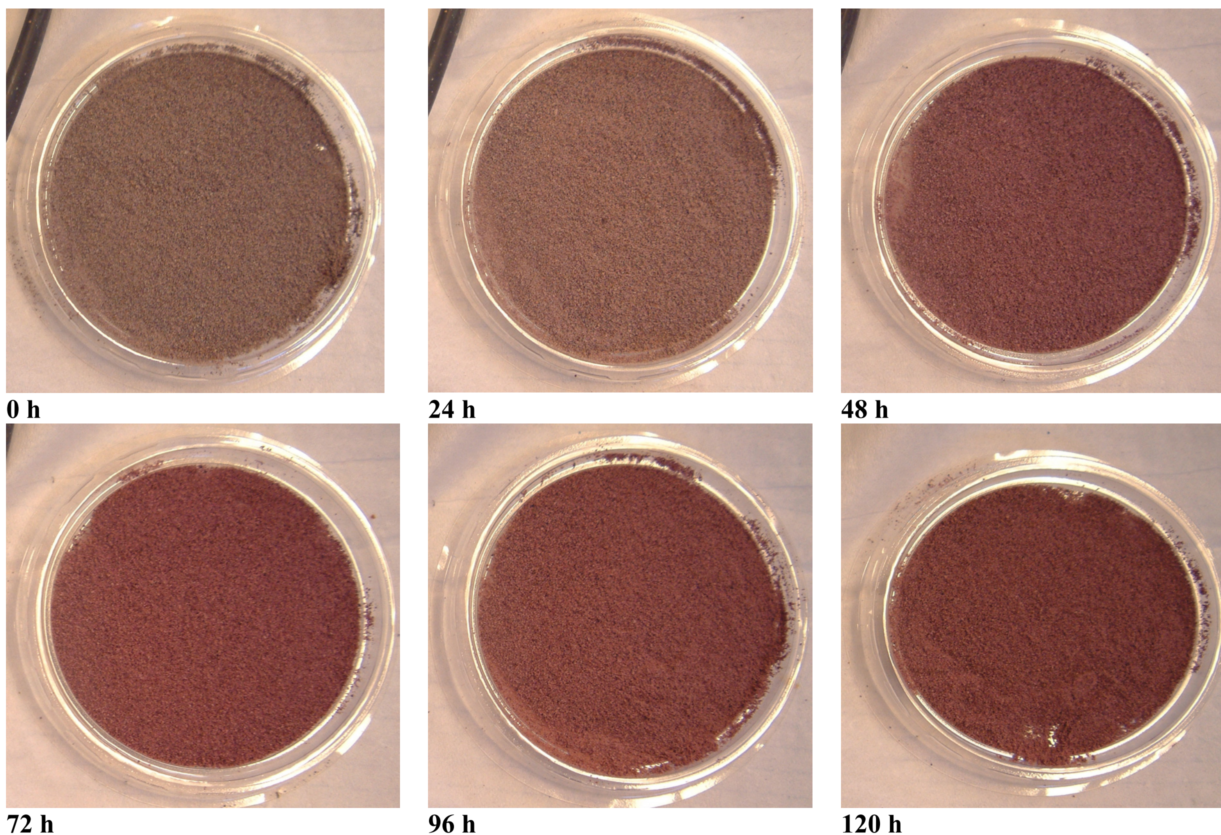


Fig. 10: Changes in bean colour during fermentation. Cocoa beans were sampled with 24 hour intervals during 120 h of heap fermentation. Still adhering pulp was manually removed and the beans sundried. The beans were subsequently de-shelled and grinded (P. Aculey, Unpublished results).

Anthocyanins rapidly break down to anthocyanidins and sugars (galactose and arabinose); reductions of 93 % after 4 days of fermentation have been reported (Forsyth and Quesnel, 1957; Lehrian and Patterson, 1983; Pettipher, 1986a; Lopez and Dimick, 1995; Wollgast and Anklam, 2000). The polyphenols (including the anthocyanidins) are oxidised and polymerize to insoluble high-molecular-weight compounds (tannins) during fermentation and drying. Furthermore polyphenols diffuse out of the beans during fermentation. All in all these processes lead to a significant decrease in the polyphenol content of the beans. The amount of

the main cocoa polyphenol, (-)-epicatechin, have been reported to decrease with 90 % or more during fermentation and drying (Kim and Keeney, 1984; Villeneuve et al., 1985; Pettipher, 1986a; Wollgast and Anklam, 2000; Kealey et al., 2001). This leads to changes in the internal colour of the dried cocoa beans from the grey colour of the unfermented beans over violet to the brown colour of well fermented cocoa beans (Fig. 10 and Pettipher, 1986a; Wollgast and Anklam, 2000). This is exploited in a simple method for determining the degree of fermentation called the cut test. In short, a number of beans are cut lengthwise and the internal colour assessed. Brown beans are considered well fermented, violet beans partly fermented and grey (slaty) beans unfermented. The method is widely used due to its simplicity but is not without problems (de Witt, 1953; Lopez, 1984; Wood and Lass, 1985).

Finally, during the roasting process the flavour and aroma precursors formed during fermentation and drying react to produce the characteristic "cocoa flavour" (Rohan and Stewart, 1966a; Rohan and Stewart, 1966b; Lehrian and Patterson, 1983; Thompson et al., 2001).

5. The microbiology of cocoa fermentation

Early investigations by Preyer-Buitenzorg (1901), Koeppen (1907) and Bainbridge and Davies (1912) all showed that microorganisms were involved in the fermentation of cocoa. Over the last century a number of studies have investigated the microbiology of cocoa fermentations, but unfortunately a great number of these studies have larger or smaller limitations:

- A great number of these studies only present qualitative information about the species present and in general there is a lack of studies presenting quantitative *and* qualitative information about the microbiology of cocoa fermentations
- The difficulties of conducting field work under tropical and often primitive conditions have led to some studies being carried out on samples transported for several days or even weeks before analysis and/or on small scale fermentations carried out in the laboratory. It is very likely that the microbial composition of these samples differs significantly from the composition immediately after sampling and the real fermentation
- Microbiological taxonomy has changed significantly over the years and earlier identifications may not necessarily be correct today
- Beside the present study, no studies taking advantage of recent years developments within molecular biology based methods for identifying the microorganisms associated with the fermentation of the cocoa have been published to date.

Despite the above mentioned limitations there is a fairly good understanding of the process today, as it also will be presented and discussed in the following.

5.1 Origin of inocula

The fermentation of cocoa is a spontaneous microbiological process. The interior of undamaged, healthy pods are sterile or almost sterile containing no more than a few hundred microorganisms/g (Appendix I and Hoynak et al., 1941; Roelofsen, 1958; Faparusi, 1974).

Faparusi (1974) investigated the occurrence of yeasts associated with cocoa at different stages from flower to ripe pods. A number of yeasts often present in high numbers during the actual fermentation such as *Candida krusei* (imperfect form of *Issatchenkia orientalis*)*, Kloeckra apiculata* (imperfect form of *Hanseniaspora uvarum*) and *Pichia membranifaciens* were detected at the different stages of maturation (Faparusi, 1974). The incidence of yeasts isolated from mature, aseptically opened pods differed from farm to farm. The incidence of *P. membranifaciens* infected pods differed from 25 to 50 % among the 5 farms investigated, whereas the incidence of *Saccharomyces* spp. infected pods ranged from 2 to 7.5 % of the pods (Faparusi, 1974). Maravalhas (1966) mention the occasional isolation of *C. krusei* from the interior of aseptically opened pods. During the present study it was found that the interior of the investigated Ghanaian cocoa pods contained around 10^2 yeast/g with *I. orientalis* constituting 80 % of the isolates and *P. membranifaciens* 20 % (Appendix I).

From the surface of healthy Ghanaian cocoa pods a wide range of yeasts have been isolated during the present study (% of isolates in brackets): *Hanseniaspora guilliermondii* (53 %), *Pichia guilliermondii* (22 %), *Candia intermedia* (7 %), *Candida parapsilosis* (6 %), *Cryptococcus laurentii* (4 %), *Candida silvicola* (2 %), *P. membranifaciens* (2 %), *Rhodotorula glutinis* (2 %) and *Cryptococcus humicola* (2 %). Additionally *I. orientalis* was isolated from pods infected with black pod disease (Appendix I).

Rombouts (1952) and Ostovar and Keeney (1973) isolated a wide range of yeasts involved in the fermentation of cocoa from knives used for pod breaking, fermentation boxes, pod surfaces, dried pulp and workers hands. Similar observations were reported by Rombouts (1952). During the present study *P. membranifaciens, I. orientalis* and *Trichosporon asahii* have been isolated from fermentation trays at the Cocoa Research Institute of Ghana (Appendix I). Plantain leaves used for covering the fermenting mass have been suggested as a source inoculation as well (Grimaldi, 1978).

The fruit fly *Drosophila melanogaster* and other insects such as ants are another possible (and possibly underestimated) source of inoculation (Ostovar and Keeney, 1973; Gilbert, 1980). During cocoa fermentations *D. melanogaster* is present in numbers so high, that it is even referred to as “the cocoa fly” in a few early publications (Bainbridge and Davies, 1912;

Nicholls, 1913). Early experiments by Nicholls (1913) suggested that *D. melanogaster* plays an important role in inoculating the cocoa pulp with yeasts and Bainbridge and Davies (1912) state that *D. melanogaster* is the main responsible for inoculating the fermenting mass with acetic acid bacteria. Ostovar and Keeney (1973) isolated 3 different AAB species, 4 different *Bacillus* spp., 4 different LAB species and various yeasts from 4 fruit flies collected at a cocoa farm on Trinidad. All isolates were isolated from fermenting cocoa at the farm as well. In Brazil it has been found that *Drosophila* spp. normally carries *C. krusei* and is an important vector for transferring microorganisms between ecological nieces (Dobzhansky and Da Cunha, 1955; Maravalhas, 1966; Gilbert, 1980).

5.2 Overall microbial development during fermentation

In the initial phases of the fermentation growth of yeasts are favoured due to the high sugar content, low pH (due to the relatively high content of citric acid) and limited oxygen availability in the pulp (Thompson et al., 2001). During the first 24-36 hours of fermentation the yeast population increase to 10^7-10^8 CFU/g normally followed by a steady decline through the rest of the fermentation (Roelofsen and Giesberger, 1947; Rombouts, 1952; Carr et al., 1979; Schwan et al., 1995; Ardhana and Fleet, 2003). Similar results have been found during the present study (Table 1 and Appendix III). However, in a few fermentations yeast growth was slower not peaking until after 48-72 hours of fermentation (Appendix I). These fermentations were carried out in December, one of the dry months in Tafo, Ghana (Wood and Lass, 1985). It has previously been reported that microbial growth and the progress of fermentation varies with season and local climate offering a possible explanation of the delayed yeast growth during these fermentations (Koeppen, 1907; Roelofsen and Giesberger, 1947; Rombouts, 1952; Wood and Lass, 1985).

One of the large heap fermentations investigated was turned after 48 and 96 hours of fermentation, which seems to influence the yeast growth as seen from Table 1, where a decrease in the yeast cell count is observed in the outer part of the fermentating mass after turning followed by renewed growth.

Table 1: Growth [log(CFU/g), standard deviations in brackets] of yeast, Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and *Bacillus* spp. during fermentation of cocoa in the outer (15 cm from the surface) and central parts of a large (500 kg) heap fermentation, a small (50 kg) heap fermentation (15 cm from surface) and a tray fermentation. All fermentations carried out in October. The large heap fermentation was turned after 48 and 96 hours. Appendix III.

	Fermentation time (hours)												
	0	12	24	36	48	60	72	84	96	108	120	132	144
Yeast, $\log(CFU_{yeast}/g)$													
Large heap, outer part	6.97 (0.04)	7.24 (0.03)	6.60 (0.21)	4.90 (0.43)	5.38 (0.25)	5.08 (0)	6.87 (0.02)	6.67 (0.16)	7.32 (0.34)	5.40 (0.02)	7.86 (0.12)	5.65 (0.14)	5.55 (0.14)
Large heap, central part	7.23 (0.21)	7.47 (0.12)	5.73 (0.02)	6.34 (0.06)	5.19 (0.16)	5.25 (0.07)	4.76 (0.06)	5.82 (0.06)	4.30 (0)	5.48 (0.04)	4.40 (0.71)	5.85 (0.10)	4.30 (0)
Small heap	7.38 (0)	8.03 (0.05)	6.68 (0.10)	5.53 (0.04)	5.88 (0.14)	3.75 (0.21)	5.96 (0.03)	4.99 (0.55)	4.30 (0)				
Tray	7.45 (0)	7.57 (0.10)	7.18 (0.38)	6.33 (0.17)	6.49 (0.16)	4.25 (0.07)	5.55 (0.07)	6.58 (0.03)	4.29 (0.12)				
LAB, $\log(CFU_{LAB}/g)$													
Large heap, outer part	5.99 (0.12)	8.09 (0.04)	8.86 (0.15)	9.08 (0.11)	9.03 (0.10)	8.47 (0.02)	9.09 (0.27)	9.25 (0.03)	9.23 (0.12)	6.72 (0.03)	9.16 (0.06)	8.73 (0.07)	8.88 (0.03)
Large heap, central part	5.53 (0.04)	7.02 (0.03)	9.16 (0.13)	9.18 (0.04)	9.41 (0.05)	9.40 (0.01)	9.29 (0.08)	8.17 (0)	6.76 (0.05)	4.90 (0)	6.43 (0.05)	8.29 (0.12)	5.58 (0.04)
Small heap	6.25 (0.07)	8.35 (0.21)	8.36 (0.18)	9.06 (0.16)	9.15 (0.18)	8.93 (0.18)	8.60 (0.60)	6.37 (0.10)	N.D.[1]				
Tray	7.23 (0.21)	8.62 (0.03)	9.71 (0.10)	9.54 (0.34)	9.33 (0.04)	8.27 (0.04)	9.07 (0.18)	8.45 (0.04)	8.58 (0.19)				
AAB, $\log(CFU_{AAB}/g)$													
Large heap, outer part	5.60 (0)	N.D.	7.14 (0.02)	7.33 (0.17)	7.76 (0.02)	6.94 (0.01)	No data[2]	7.54 (0.06)	7.38 (0)	5.41 (0.05)	8.14 (0.09)	5.84 (0.34)	5.99 (0.12)
Large heap, central part	N.D.	N.D.	5.60 (0)	6.99 (0.12)	4.60 (0)	7.13 (0.02)	6.29 (0.12)	4.90 (0.43)	4.60 (0)	6.47 (0.04)	3.70 (0)	N.D.	N.D.
Small heap	N.D.	6.19 (0.16)	7.76 (0.02)	7.88 (0.06)	7.60 (0)	6.26 (0.04)	6.25 (0.07	6.60 (0)	N.D.				
Tray	N.D.	N.D.	7.05 (0.21)	7.70 (0.14)	7.34 (0.06)	7.49 (0.16)	5.96 (0.03)	3.99 (0.55)	N.D.				
***Bacillus* spp.** $\log(CFU_{Bacillus}/g)$[3]													
Large heap, outer part	N.D.	N.D.	N.D.	N.D.	5.49 (0.15)	7.74 (0.09)	N.D.	3.60 (0)	9.10 (0.71)	5.75 (0.21)	7.99 (0.12)	7.75 (0.04)	7.81 (0.19)
Large heap, central part	N.D.	N.D.	N.D.	N.D.	N.D.	7.84 (0.34)	N.D.	4.14 (0.09)	4.66 (0.08)	6.96 (0.26)	5.57 (0.10)	7.43 (0.10)	6.83 (0.32)
Small heap	N.D.	N.D.	N.D.	N.D.	N.D.	4.05 (0.21)	N.D.	5.75 (0.21)	5.75 (0.21)				

[1] N.D.: None detected

[2] No data, plates partly overgrown by slimy non-AAB. CFU_{AAB} approximately as for 60 hours of fermentation.

[3] No *Bacillus* spp. detected during tray fermentation

The primary activity of the yeasts is assumed to be production of ethanol from carbohydrates. Consequently a sharp increase in the ethanol concentration and a decrease in the concentration of fermentable sugars are observed during the first 24-36 hours of fermentation (Roelofsen and Giesberger, 1947; Schwan et al., 1995; Lopez and Dimick, 1995; Ardhana and Fleet, 2003). Maximum pulp ethanol concentrations as high as 6.5 % (Ardhana and Fleet, 2003) and as low as 1 % or less (Carr et al., 1979; Schwan et al., 1995) have been reported in the literature. However, as different sampling preparation and extraction methods have been used

it is difficult to directly compare the figures. Using basically the same experimental approach as Ardhana and Fleet (2003) who investigated Indonesian box fermentations it was in the present study found that the pulp ethanol concentration peaked around 2 % after 24-36 hours of fermentation (Appendix III). The lower values found in the present study possibly reflects differences in fermentation method (box versus heap) and/or e.g. different cocoa cultivars used in Indonesia and Ghana.

During the fermentations investigated in the present study approximately 80 % of the sugars (glucose, fructose and sucrose) were metabolised within the first 24 hours. The exception here is the centre of the large heap fermentation, where a slower development was observed (Appendix III). Carr et al. (1979) also observed that the fermentation progressed slower in the central parts of large heap fermentations.

The conversion of glucose and fructose to ethanol is an exothermic process producing 93.3 kJ/mol reaction (Knapp, 1937; Forsyth and Quesnel, 1963; Carr, 1982). Consequently the production of ethanol in the initial phases of fermentation is accompanied by a moderate increase in temperature as also seen in Table 2 and Appendix III.

Table 2: Development in temperature and pulp pH during fermentation of cocoa in the outer (15 cm from the surface) and central parts of a large (500 kg) heap fermentation, a small (50 kg) heap fermentation (15 cm from surface) and a tray fermentation. The large heap fermentation was turned after 48 and 96 hours. Appendix III.

	Fermentation time (hours)												
	0	12	24	36	48	60	72	84	96	108	120	132	144
Temperature, °C													
Large heap, outer part	28	29.5	34	42	43	48	48	44	42	44.5	44	46	44
Large heap, central part	28	29	30.5	34	33.5	39	44	46	44.5	43	43.5	43.5	44
Small heap	28.5	31	35	42	46	47	45	44	44.5				
Tray	28.5	33.5	34	37.5	45	46	46	46	45.5				
pH, pulp													
Large heap, outer part	4.10	4.50	4.24	3.93	3.98	4.03	4.21	4.37	4.40	4.35	4.49	4.58	4.55
Large heap, central part	3.95	4.69	4.21	3.94	3.97	4.04	4.12	4.25	4.34	4.45	4.35	4.46	4.41
Small heap	3.94	4.55	3.97	3.91	4.06	4.12	4.12	4.29	4.22				
Tray	4.12	4.28	3.85	3.82	3.98	4.06	4.20	4.29	4.26				

During the early phases of fermentation abundant growth of Lactic Acid Bacteria (LAB) also fermenting the sugars producing lactic acid is observed (Roelofsen and Giesberger, 1947; Schwan et al., 1995; Thompson et al., 2001; Ardhana and Fleet, 2003; Schwan and Wheals, 2004). Lactic acid bacteria have been reported to reach 10^8-10^9 CFU/g during the first 24-48 hours of fermentation (Carr et al., 1979; Schwan et al., 1995; Ardhana and Fleet, 2003). In Brazilian, Indonesian and Trinidadian box fermentations LAB have been found to decrease to

low (10^4 CFU/g) or non-detectable numbers during the later stages of fermentation (Rombouts, 1952; Schwan et al., 1995; Ardhana and Fleet, 2003) whereas Carr et al. (1979) found that LAB remained a significant part of the micropopulation constituting 10-50 % in most samples throughout Ghanaian heap fermentations. In comparison with Malaysian box fermentations investigated during the same study LAB in general constituted a smaller portion of the micropopulation during the box fermentations, but LAB was detectable in most samples (Carr et al., 1979). In the present study LAB reached numbers similar to those reported in the literature; i.e. reaching more than 10^9 CFU/g during the first 36 hours in all investigated fermentations (Table 1 and Appendix III). In agreement with the observations by Carr et al. (1979), but contrary to most published studies on box fermentations the LAB remained a predominant part of the micropopulation throughout all fermentations investigated. Whether this observation reflects differences between heap and box fermentations or local differences (climate, cocoa cultivar etc.) cannot be judged on the basis of the information available at present, but deserves further investigation in future studies.

The role of yeast and LAB during the fermentation of cocoa is not restricted to producing ethanol and lactic acid. Breakdown of the pulp through pectinolytic activity by the yeast and assimilating citric acid by the yeast and LAB are other important functions (Sanchez et al., 1984; Hugenholtz, 1993; Schwan and Rose, 1994; Schwan et al., 1997; Thompson et al., 2001; Schwan and Wheals, 2004). The assimilation of citric acid causes the pH to increase. This is also observed in the present study, where the citric acid concentration in the pulp decreased from 0.6-0.7 % to low or non-detectable levels within the first 12 hours of fermentation accompanied by a pH-increase from 3.94-4.12 to 4.28-4.69 in the investigated fermentations (Table 2 and Appendix III). Following the initial increase in pH, pulp pH decreased again between 12 and 36 hours of fermentation followed by a steady increase from 36 hours and onwards (Table 2 and Appendix III).

In a recent study Ardhana and Fleet (2003) found that highly pectinolytic filamentous fungi grew well during first 36 hours of fermentation and suggested that filamentous fungi play a key role in the degradation of pulp pectin during the early phases of fermentation. On the contrary Roelofsen and Giesberger (1947) stated that moulds play no role during normal cocoa fermentations on Java (Indonesia).

The breakdown of the pulp cause some of the pulp to drain away and increase aeration of the fermenting mass favouring growth of aerobic AAB (Schwan et al., 1995). Acetic acid bacteria have been reported to reach anywhere between 10^5 and 10^9 CFU/g during box fermentations conducted in Indonesia, Belize, Brazil and Trinidad (Roelofsen and Giesberger, 1947; Rombouts, 1952; Ostovar and Keeney, 1973; Carr et al., 1979; Schwan et al., 1995; Thompson et al., 2001; Ardhana and Fleet, 2003). According to Carr et al. (1979) AAB reached around 10^8 CFU/g during Ghanaian heap fermentations. In the present study AAB grew from low or non-detectable levels at the onset of fermentation to 10^7 -10^8 CFU/g after 36 hours of fermentation (Table 1 and Appendix III). Aeration of the fermenting mass by turning clearly influenced AAB growth in the large heap fermentation. Following turning the AAB counts decreased in the outer part the heap followed by renewed AAB growth. In the centre of the fermenting mass the picture was reversed (Table 1 and Appendix III). Carr et al. (1979) also observed that AAB growth in the central, less aerated part of heap fermentations were positively influenced by turning. The effect was less clear in the outer part of the fermenting mass, but as Carr et al. (1979) only took samples with 24 hour intervals it is likely they missed some of the effects of turning on microbiological growth.

In general it is believed that AAB growth requires oxygen, but some strains are seemingly capable of growing under conditions with very low oxygen tension as Carr and Davies (1980) found that AAB from cocoa fermentations could be isolated from plates incubated anaerobically. The ability of AAB to spoil bottled wine further underline their potential for growing under very limited oxygen conditions (Drysdale and Fleet, 1988).

The AAB metabolises the ethanol initially formed by the yeasts to acetic acid through an exothermal process. Furthermore some AAB species are capable of completely oxidising acetic acid to CO_2 and H_2O through an exothermal process (Forsyth and Quesnel, 1963; Kersters et al., 2003). The AAB activity leads to a further increase in temperature reaching 45-50 °C or even higher (Roelofsen and Giesberger, 1947; Kenten and Powell, 1960; Forsyth and Quesnel, 1963; Quesnel, 1965; Biehl, 1969; Carr et al., 1979; Carr, 1982; Schwan et al., 1995). In the present study maximum temperatures of 46-50 °C were observed in all investigated fermentations (Table 2 and Appendix I and III). It is interesting to note that the

temperature development in the centre of the large heap fermentation is relatively slow the first 48 hours of fermentation compared to the other fermentations and sampling sites. Following turning after 48 hours the temperature increases rapidly in the central parts, too (Table 2). A similar trend has been observed during Brazilian and Indonesian box fermentations (Roelofsen and Giesberger, 1947; Biehl, 1969; Passos et al., 1984a) and by Rohan (1957) and Carr et al. (1979) during Ghanaian heap fermentations.

In the present study the acetic acid concentration peaked around 2 % after 60-72 hours of fermentation followed by a decline to 0.7-1 % by the end of fermentation (Appendix III). As mentioned previously it is difficult to directly compare values between different studies due to differences in methodology, but using a comparable experimental approach as in the present study Ardhana and Fleet (2003) obtained comparable results during Indonesian box fermentations. The decline in acetic acid concentration observed towards the end of fermentation were probably due to a combination of evaporation and metabolisation (Lehrian and Patterson, 1983; Schwan and Wheals, 2004).

The ethanol and acetic acid diffuse into the beans. This in combination with the heat produced by the activities of the AAB leads to bean death and induces the biochemical changes leading to well-fermented cocoa beans as reviewed in chapter 4 (Quesnel, 1965; Thompson et al., 2001).

The high temperatures in combination with the ethanol and acetic present is a severe stress factor limiting the growth or killing vegetative cells of many microorganisms (Roelofsen and Giesberger, 1947; Lehrian and Patterson, 1983; Thompson et al., 2001). In the later phases of fermentation conditions with increasing pH and aeration becomes favourable for growth of spore-forming *Bacillus* spp. often reaching 10^8 CFU/g or more in the later stages of the fermentation (Rombouts, 1952; Carr et al., 1979; Schwan et al., 1995; Ardhana and Fleet, 2003). Similar observations were made in the large heap fermentation investigated in the present study. In the investigated small heap and tray fermentations no or limited growth of *Bacillus* spp. were observed presumably because these fermentations were completed after 4 instead of 6 days of fermentation (Table 1 and Appendix III).

The role of *Bacillus* spp. in the fermentation of cocoa has not been fully elucidated. However, due to their high enzymatic activity and production of e.g. short chain fatty acids, pyrazines and 2,3-butanediol the growth and activity of *Bacillus* spp. is likely to influence the final product possibly causing off flavours (Zak et al., 1972; Lopez and Quesnel, 1973; Schwan et al., 1986; Romanczyk et al., 1995).

Towards the end of fermentation filamentous fungi grows well in the cooler and more aerated parts of the fermenting mass (Maravalhas, 1966; Lehrian and Patterson, 1983). Mould growth has been associated with internal moulding of the beans (a serious commercial defect), various off flavours such as “mouldy odour” and increased free fatty acids levels (Ciferri, 1927; Dade, 1928; Ciferri, 1931; Hansen et al., 1973; Anon., 2003; Anon., 2006). Furthermore mould growth may lead to production of mycotoxins such as ochratoxin A, aflatoxins and citrinin (Höhler, 1998; Meister, 2004). It has recently been shown that ochratoxin A is produced not only during the drying step, but also during the actual cocoa fermentation (Petithuguenin, 2002; Lindblom, 2006; Anon., 2006). It is furthermore likely that excessive mould growth during the later parts of fermentation will enhance mould growth and thus potential mycotoxin production during the subsequent drying phase (Bunting, 1928; Roelofsen, 1958; Anon., 2003; Lindblom, 2006; Anon., 2006).

5.2.1 Yeast involved in the fermentation

The distinct smell of alcohol apparent during the early stages of cocoa fermentation soon led early investigators of the cocoa fermentation to conclude that yeasts were involved in the process (Preyer-Buitenzorg, 1901; Loew, 1907; Nicholls, 1913; Rombouts, 1953). These early studies reported the occurrence of a *Saccharomyces*-like yeast species for which Preyer-Buitenzorg (1901) and later Nicholls (1913) suggested the name *Saccharomyces theobromae*. However, as stated by Rombouts (1953) it is most likely that *Sc. theobromae* was a mixture of different yeasts species. Furthermore a fission yeast, possibly *Schizosaccharomyces pombe*, an apiculate yeast, possibly *K. apiculata* and *Saccharomyces* spp. were reported frequently (Preyer-Buitenzorg, 1901; Loew, 1907; Bainbridge and Davies, 1912; Nicholls, 1913; Rombouts, 1953).

The involvement of *Saccharomyces* spp., *Schizosaccharomyces* spp. and apiculate yeasts in the fermentation of cocoa in all the important cocoa producing regions of the World, including South America, Indonesia and West Africa were confirmed by later studies (Steinmann, 1928; Busse et al., 1929; Ficker and von Lilienfeld-Toal, 1930; Ciferri, 1931). According to Knapp (1937) H.A. Dade isolated a number of yeasts from Gold Coast (Ghana) cocoa fermentations and deposited these isolates at the Central Bureau voor Schimmelcultures (CBS), Holland, where they were later identified as *K. apiculata*, *C. krusei*, *Pichia fermentans*, *Candida mycoderma* [now *Candida vini* (Meyer et al., 1998)] and *Saccharomyces cerevisiae* (Knapp, 1937; Rombouts, 1953).

Roelofsen and Giesberger (1947) carried out a comprehensive study on box fermentation of cocoa in Java (Indonesia). Unfortunately, except for the identification of a few isolates to genus-level (*Pichia* spp. and *Saccharomyces* spp.) no serious attempts were made to identify the yeasts isolated during the study. Rombouts (1952) studied box fermentations on Trinidad and found that yeasts dominated the fermentations during the first 24 hours. The isolates were not identified in the original publication but according to Roelofsen (1958) the isolates were later identified and *C. krusei* was found to be the predominating yeast. Among others *Sc. cerevisiae* [then named *Sc. cerevisiae* var. *ellipsoides* (Vaughan-Martini and Martini, 1998)] *K. apiculata* and *P. membranifaciens* were isolated frequently as well.

More recent studies of box fermentations in Belize, Brazil, Côte d'Ivoire, Malaysia and Indonesia have confirmed that the yeast community associated with cocoa fermentations is complex involving a number of species with *Sc. cerevisiae*, *K. apis*, *K. apiculata*, *P. membranifaciens*, *P. fermentans*, *I. orientalis* (and the imperfect form *C. krusei*), and various *Candida*, *Torulopsis* and *Schizosaccharomyces* spp. being isolated frequently (Ostovar and Keeney, 1973; Gauthier et al., 1977; Sanchez et al., 1985; Ravelomanana et al., 1985; Schwan et al., 1995; Thompson et al., 2001; Ardhana and Fleet, 2003).

Only one systematic study dealing with the microbiology of Ghanaian cocoa fermentations have been published: Carr et al. (1979) reported the involvement of *Hansenula*, *Kloeckra*, *Torulopsis*, *Saccharomyces*, *Candida*, *Pichia* and *Schizosaccharomyces* spp. in Ghanaian

heap fermentations. Unfortunately the isolates were only identified to genus level (Carr et al., 1979; Carr and Davies, 1980).

Table 3: Composition of the yeast community sampled 15 cm from the surface (outer part) and in the center of a large (500 kg) heap cocoa fermentation turned after 48 and 96 hours. Abbreviations: *H.*: *Hanseniaspora*, *S.*: *Saccharomycopsis*, *P.*: *Pichia*, *I.*: *Issatchenkia*, *C.*: *Candida*, *Sc.*: *Saccharomyces*, *Schiz.*: *Schizosaccharomyces* (Appendix III).

	Fermentation time (hours)												
	0	12	24	36	48	60	72	84	96	108	120	132	144
Outer part, % of yeast population													
H. guilliermondii	79	67	71										
S. crataegensis	7												
P. pijperi	9	2											
Unidentified Species C	3												
I. hanoiensis	2												
C. zemplinina		19	14										
C. michaelii		3											
C. diversa		9	14										
P. membranifaciens			Det.[1]	100	70	50	100	100	100	90	100	100	100
C. ethanolica					15						Det.	Det.	
Sc. cerevisiae					15	17							
Schiz. pombe						33							
I. orientalis										10			
Central part, % of yeast population													
H. guilliermondii	78	53	21		25								
P. kluyveri	11												
C. diversa	11				13								
Unidentified Species C	Det.	14											
S. crataegensis	Det.												
C. zemplinina		33	57										
P. membranifaciens				9	13	11	31	97	50	100	100	67	100
Sc. cerevisiae			22	91	25	89	45						
I. orientalis				Det.									
I. occidentalis				Det.									
C. ethanolica					13			3	50			33	
T. delbreuckii					13								
Schiz. pombe						Det.	24						

[1] Detected sporadically

Except for the studies published in connection with the present study (Appendix I-III) no studies on the microbiology of cocoa fermentations taking advantage of recent year's development in molecular biology have been published to date. In the present study approximately 750 yeasts isolates from cocoa fermentations were grouped and identified using a combination of pheno- and genotypic methods including determination of micro- and macromorphology, carbohydrate assimilation patterns, Intragenic Transcribed Spacers (ITS)-PCR, Repetive Element Palndromic (rep)-PCR and sequencing of the D1/D2-region of the 26S rRNA gene. Over recent years rep-PCR has won increasing use as a tool for grouping bacteria and more recently also yeasts (Gevers et al., 2001; Capece et al., 2003; Andrade et al., 2006). The method proved to be an efficient and reliable tool for grouping the yeast isolates during the present study (Appendix III).

Table 4: Composition of the yeast community during tray fermentations carried out in October and December, respectively, and a small (50 kg, samples taken 15 cm from the surface) heap cocoa fermentation carried out in October. Abbreviations: *H.*: *Hanseniaspora*, *S.*: *Saccharomycopsis*, *P.*: *Pichia*, *I.*: *Issatchenkia*, *C.*: *Candida*, *Sc.*: *Saccharomyces*, *Schiz.*: *Schizosaccharomyces*; *R.*: *Rhodotorula*. (Appendix I and III).

	Fermentation time (hours)								
	0	12	24	36	48	60	72	84	96
Small heap, % of yeast population									
H. guilliermondii	42	82	71		Det.[1]				
Unidentified Species A	8								
C. quercitrusa	8								
Unidentified Species B	8	Det.							
C. diversa	25								
C. sorboxylosa	8								
S. crataegensis		9	Det.						
P. pijperi		2							
Unidentified Species C		7							
C. zemplinina			13						
C. cylindracea			8						
P. membranifaciens			8	13	66	67	92	71	33
I. orientalis				25	3		4		67
Sc. cerevisiae				62	31	33	4	29	
Tray, October, % of yeast population									
H. guilliermondii	18	47	33						
I. occidentalis	9								
I. hanoensis	9								
C. zemplinina	37	11	11	20					
C. sorboxylosa	18								
C.silvae	9	37							
C. cylindracea		5							
P. kluyveri			22						
C. diversa			34						
P. membranifaciens				40	94		100	95	100
Sc. cerevisiae				40	6	67			
Schiz. pombe						33			
I. orientalis								5	
Tray, December, % of yeast population									
H. guilliermondii	31								
P. kluyveri	31		4						
C. stellimalicola	16								
I. orientalis	11						8		
Sc. cerevisiae	11		57		90		4		
C. quercitrusa			4						
R. glutinis			8						
P. membranifaciens			27		10		88		

[1] Detected sporadically

The yeast community associated with Ghanaian cocoa fermentations was complex with 4-6 different species detected in each sample during the first 24 hours of fermentation (Table 3 and 4 and Appendix I-III). *Hanseniaspora guilliermondii* was the predominant yeast in all heap fermentations during the first 24 hours with the exception of the central part of a large heap fermentation, where it was only detected at the onset of fermentation (Appendix I). Later in the fermentations, *H. guilliermondii* was only detected more occasionally (Table 3, 4 and Appendix III). The declining numbers of *H. guilliermondii* have been explained by the relatively low ethanol tolerance of *Hanseniaspora* spp. and the imperfect form, *Kloeckra*

(Schwan et al., 1995; Ardhana and Fleet, 2003). The increasing temperatures during the fermentation probably influences the growth and death of the various yeast species present during the fermentation as well (Kurtzmann and Fell, 1998; Ardhana and Fleet, 2003).

Ardhana and Fleet (2003) reported the involvement of high numbers of *K. apis* (imperfect form of *H. guilliermondii*) during the first 24 hours of cocoa fermentations in Indonesia. Also Thompson et al. (2001) mention the involvement of *K. apis* during cocoa fermentations in Belize. Other studies report the involvement of the closely related *Hanseniaspora uvarum* (or its anamorph *K. apiculata*) in cocoa fermentations and according to Sanchez et al. (1985) and Schwan et al. (1995) *K. apiculata* shows the same growth pattern as observed for *H. guilliermondii* during the present study (de Camargo et al., 1963; Sanchez et al., 1985; Schwan et al., 1995).

The newly described yeast *Candida zemplinina* (Sipiczki, 2003) was detected in several fermentations, most notably in the centre of a large heap fermentation and in the tray fermentation carried out in October (Table 3, 4 and Appendix III). Apparently this is the first reported isolation of *C. zemplinina* from other sources than botrytised wine fermentations.

Compared to the heap fermentations *H. guilliermondii* was less dominant during the initial phases of the tray fermentations. *Candia silvae*, *C. zemplinina*, *Candida diversa*, *Candida stellimalicola* and *Pichia kluyveri* were among the other predominant yeasts during this phase of the tray fermentations possibly reflecting the higher oxygen availability during tray fermentations.

After 36-48 hours of fermentation a switch in the yeast population was observed in all fermentations with *Sc. cerevisiae* and *P. membranifaciens* becoming the predominant yeasts. The only exception was a large heap fermentation where *Issatchenkia orientalis* became the predominating yeast (Appendix I). However, after 72 hours of fermentation a switch towards *P. membranifaciens* was observed here as well. Due to local farmer practices a few fermentations were only allowed to progress for 3 days (Appendix I). In all fermentations allowed to progress for 4 (small heap and tray fermentations) to 6 days (large heap fermentation) *P. membranifaciens* became the predominating yeast constituting up to 100 %

of the yeast population during the later phases of fermentation with the occasional appearance of *S. cerevisiae*, *I. orientalis* and *C. ethanolica* (Table 3-4 and Appendix III).

Investigation of Chromosome Length Polymorphism (CLP) using Pulsed Field Gel Electrophoresis (PFGE) among of a number of *H. guilliermondii*, *P. membranifaciens*, *P. kluyveri*, *Sc. cerevisiae* and *I. orientalis* strains isolated during cocoa fermentations revealed that CLP was evident within all species investigated (Appendix I). This finding further adds to the complexity of cocoa fermentations as not only is a number of different species involved in the fermentation, but within the different species different strains are involved as well.

Three putatively undescribed yeast species tentatively named Unidentified Species A, B and C were isolated during the investigated fermentations (Table 3 and 4 and Appendix III). The contribution to the actual fermentation of Unidentified Species A and B is probably limited as the two species apparently did not grow during the fermentation. Unidentified Species C did on the other hand grow during the initial phases of the small heap and in the centre of a large heap fermentation possible playing some sort of role in the implicated fermentations (Table 3 and 4). The 3 putatively undescribed species will be described more detailed in chapter 7.2.

5.2.2 Lactic Acid Bacteria involved in the fermentation

The involvement of LAB in the fermentation of cocoa was established relatively late compared to the other major microbiological groups involved. Using microscopy Busse et al. (1929) and Ficker and von Lilienfeld-Toal (1930) both observed LAB-like microorganisms in the pulp from fermenting cocoa, but LAB was not cultivated from fermenting cocoa until the work by Roelofsen and Giesberger (1947) and Rombouts (1952).

Only a very limited number of studies have investigated the importance of LAB in West African cocoa fermentations. Carr *et al.* (1979) and Carr and Davies (1980) found *Lactobacillus collinoides*, *Lactobacillus fermentum*, *Lactobacillus mali* and *Lactobacillus plantarum* to be the dominant LAB during Ghanaian heap fermentations. However, only a limited number of isolates were fully identified (Carr and Davies, 1980).

Ardhana & Fleet (2003) found *Lactobacillus cellobiosus* [later synonym of *Lb. fermentum* (Dellaglio et al., 2004)] and *Lb. plantarum* to be the dominant LAB involved in Indonesian box fermentations. Also Roelofsen and Giesberger (1947) found *Lb. fermentum* to dominate the LAB community during Indonesian cocoa fermentations.

The LAB population of Brazilian and Caribbean box fermentations have been reported to be considerably more complex involving a number of *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Pediococcus* species, represented by species such as *Lb. fermentum*, *Lactobacillus brevis*, *Lb. plantarum*, *Leuconostoc mesenteroides*, *Lactococcus lactis* and *Pediococcus acidilactii* (Ostovar and Keeney, 1973; Passos et al., 1984b; Thompson et al., 2001; Schwan and Wheals, 2004).

In the present study more than 500 LAB isolates were identified using a combination of phenotypic and genotypic methods. Initially the isolates (all Gram-positive and catalase-negative) were grouped on the basis of cell shape, gas production from glucose and growth at cardinal temperatures. After initial grouping the isolates were genotypically grouped using rep-PCR. Rep-PCR has previously been shown to be an effective method for rapid grouping of LAB isolates (Gevers et al., 2001; Kostinek et al., 2005) as was also the case in the present study. Relevant type strains were included in the rep-PCR and subsequent cluster analysis yielded a tentative identification of some of the isolates already at this stage (Appendix III).

Following grouping by rep-PCR a large number of representative isolates were further characterised and identified on the basis of lactate isomer produced, production of NH_3 from arginine, carbohydrate fermentation profile, 16S rRNA gene sequencing and in the case of all homofermentative isolates the presence of D-*meso*-diaminopimelic acid (*m*DAP) in the cell wall. Isolates belonging to the *Lb. plantarum/Lactobacillus pentosus*-group were unambiguously identified using multiplex PCR (Appendix III and Torriani et al., 2001).

Table 5: Composition of the Lactic Acid Bacteria (LAB) community during a large (500 kg) heap fermentation [samples were taken from 2 positions: 15 cm from the surface (outer part) and in the centre of the fermenting mass], a small (50 kg) heap fermentation (samples taken 15 cm from the surface) and a tray fermentation. The large heap fermentation was turned after 48 and 96 hours. Abbreviations: *Lb.*: *Lactobacillus*; *Lc.*: *Leuconostoc*; *Pd.*: *Pediococcus* (Appendix III).

	Fermentation time (hours)												
	0	12	24	36	48	60	72	84	96	108	120	132	144
Large heap, outer part, % of LAB population													
Lb. plantarum	60	11	5		9		9				21	85	63
Lb. fermentum	40	81	76	100	86	100	81	89	92	69	74	15	37
Unidentified Species D		8						11	8		5		
Lc. pseudoficulneum			19										
Lb. hilgardii[1]					5								
Pd. acidilactici										31			
Large heap, centre, % of LAB population													
Lc. pseudomesenteroides	82												
Lb. rossii[1]	18												
Lb. fermentum		100	98	47	87	100	100	100	100	100	52	75	4
Lc. pseudoficulneum			2										
Lb. plantarum				53	9						42	25	76
Unidentified Species D					4								
Pd. acidilactici											6		
Lb.hilgardii[1]													20
Small heap, % of LAB population													
Lc. pseudomesenteroides	100	83		5									
Lb. fermentum		17	63	90	77	100	100	100					
Lb. plantarum			2										
Lc. pseudoficulneum			28										
Lac. lactis			7										
Pd. acidilactici				5	23								
Tray, % of LAB population													
Lb. plantarum	89	30	35	5					10				
Lc. pseudoficulneum	11		3										
Lb. fermentum		64	57	95	100	100	100	89	90				
Lc. pseudomesenteroides		6	2										
Lb. brevis			3										
Pd. acidilactici								11					

[1] Isolates did not grow upon purification. Identified directly by rep-PCR grouping and 16S rRNA gene sequencing (see Appendix III for details)

In the present study *Lc. pseudomesenteroides* and *Lb. plantarum* dominated the LAB community at the onset of fermentation but after 12-24 hours, *Lb. fermentum* took over as the dominant LAB (Table 5 and Appendix III). Despite differences in size and fermentation method, *Lb. fermentum* dominated throughout all fermentations investigated, a position only challenged by *Lb. plantarum* towards the end of the large heap fermentation (Table 5). Carr et al. (1979) also isolated *Lb. plantarum* and *Lb. fermentum* during Ghanaian heap fermentations but did not state which organisms dominated during the different stages of fermentation (Carr et al., 1979; Carr and Davies, 1980). Ardhana and Fleet (2003) found *Lb. fermentum* to be the predominating LAB during Indonesian cocoa fermentations. In agreement with the present study *Lb. plantarum* was isolated regularly and *Lb. hilgardii* more occasionally during the Indonesian cocoa fermentations (Ardhana and Fleet, 2003).

The involvement of *Leuconostoc*, *Pediococcus* and *Lactococcus* spp. in West African cocoa fermentations has not been reported before. However, their involvement in cocoa fermentations have been reported from Brazilian and Belizian fermentations previously (Passos et al., 1984b; Thompson et al., 2001).

A putatively undescribed *Lactobacillus* spp. (Unidentified Species D in Table 5) was isolated mainly from the outer part of the large heap fermentation and will be described more detailed in chapter 7.1. The recently described *Leuconostoc pseudoficulneum* was isolated from all fermentations but in general it constituted a small part of the LAB community (Table 5). Apparently this is the first report of *Lc. pseudoficulneum* being isolated outside its original habitat, ripe fig (Chambel et al., 2006).

Lactobacillus plantarum and in most cases also *Lb. fermentum* have been isolated during a wide range of cocoa fermentations around the World (Roelofsen and Giesberger, 1947; Ostovar and Keeney, 1973; Carr et al., 1979; Carr and Davies, 1980; Passos et al., 1984b; Schwan, 1998; Thompson et al., 2001; Ardhana and Fleet, 2003). Together with the results obtained during the present study this indicates that *Lb. plantarum* and possibly also *Lb. fermentum* are indigenous to fermentation of cocoa Worldwide.

5.2.3 Acetic Acid Bacteria involved in the fermentation

The ethanol formed by the yeasts in the early phase of fermentation is metabolised to acetic acid by AAB as mentioned previously. The smell of acetic acid associated with fermenting cocoa is easily recognisable and soon led early investigators to conclude that acetic acid producing microorganisms were involved in and significant contributors to the fermentation of cocoa (Loew, 1907; Bainbridge and Davies, 1912; Nicholls, 1913; Ashby, 1925; Knapp, 1937).

The first comprehensive study on AAB associated with cocoa was carried out by Eckmann (1928) who grew AAB cultures in Germany from pulp sweatings, dry beans and fresh fruits transported from the tropics. Eckmann (1928) mainly isolated *Gluconacetobacter xylinus* [then named *Bacterium xylinus* (Euzéby, 2006)], *Acetobacter orleanensis* [then named

Bacterium orleanese (Euzéby, 2006)] and *Acetobacter pasteurianus* [then named *Bacterium ascendens* (Euzéby, 2006)]. However, as the samples were transported for several weeks before being examined it is impossible to conclude anything about the significance of the isolated species on actual fermentations. Roelofsen and Giesberger (1947) found that *A. pasteurianus* [then named *Acetobacter rancens* (Euzéby, 2006)] and *Gluconobacter oxydans* [then named *Acetobacter melanogenum* (Euzéby, 2006)] were the dominant AAB during Indonesian (Java) cocoa fermentations. More recently Ardhana and Fleet (2003) isolated *A. pasteurianus* and *Acetobacter aceti* during Indonesian cocoa fermentations.

In their study on Trinidadian box fermentations Ostovar and Keeney (1973) found that *Gluconobacter oxydans* [then named *Acetobacter suboxydans* (Euzéby, 2006)] was the predominant AAB during the first 24-32 hours of fermentation whereas *A. pasteurianus* predominated the AAB community from 40 hours of fermentation and onwards. *Gluconobacter oxydans*, *A. aceti* and *A. pasteurianus* have been found to dominate the AAB community in a number of other studies on cocoa box fermentations in Malaysia, Belize and Brazil (Carr et al., 1979; Passos and Passos, 1985; Schwan, 1998; Thompson et al., 2001).

Besides the study by Carr at al. (1979) data regarding AAB involved in the fermentation of West African cocoa are scarce. Carr et al. (1979) and Carr and Davies (1980) identified a limited number of AAB isolated from fermenting cocoa and reported the involvement of *A. pasteurianus* [then named *A. rancens* and *Acetobacter ascendens* (Gosselé et al., 1983; Euzéby, 2006)], *Gluconacetobacter xylinus* [then named *Acetobacter xylinum* (Yamada et al., 1997; Euzéby, 2006)] and *Gluconobacter oxydans* in Ghanaian cocoa fermentations.

In the present study AAB were isolated on GYC (Glucose Yeast extract Carbonate) agar (Appendix III). Acid production dissolves the carbonate in the GYC agar causing a clearing zone around positive isolates (Drysdale and Fleet, 1988). Gram negative and Gram variable, catalase positive isolates causing clearing of GYC agar were considered presumptive AAB and were identified by Restriction Fragment Length Polymorphism analysis of 16S rRNA gene and 16S-ITS-23S rRNA gene fragments (PCR-RFLP) as described by González et al. (2006). Approximately 250 AAB isolates were identified using this approach. The PCR-RFLP results were confirmed by sequencing of the 16S rRNA gene of selected isolates.

Sequencing and PCR-RFLP analysis gave identical results in all cases. The identification of *Gluconobacter oxydans* was further strengthened by the formation of water-soluble pigments on GYC agar and the absence of acid production from D- and L-arabitol (Appendix III and Kersters et al., 2003).

Table 6: Composition of the Acetic Acid Bacteria (AAB) community during a large (500 kg) heap fermentation [samples were taken from 2 positions: 15 cm from the surface (outer part) and in the centre of the fermenting mass], a small (50 kg) heap fermentation (samples taken 15 cm from the surface) and a tray fermentation. The large heap fermentation was turned after 48 and 96 hours. Abbreviations: *A.*: *Acetobacter*, *G.*: *Gluconobacter* (Appendix III).

	Fermentation time (hours)												
	0	12	24	36	48	60	72	84	96	108	120	132	144
Large heap, outer part, % of AAB population							No data[1]						
A. pasteurianus	100			55		22				62			
A. syzygii			57			33		72					
A. tropicalis			29	45	100	45		14	100	38	100	100	100
A. malorum			14										
G. oxydans								14					
Large heap, centre, % of AAB population													
A. pasteurianus			100			22	80			13			
A. syzygii				100		45	20			34			
A. tropicalis					100	33		100	100	53	100		
Small heap, % of AAB population													
A. syzygii		100	83	89	100	100	22	75					
A. pasteurianus			17				33	25					
A. malorum				11									
A. tropicalis							45						
Tray, % of AAB population													
A. syzygii			50	100									
A. malorum			50										
A. pasteurianus					73	100							
A. tropicalis					27		100	100					

[1] No data, plates partly overgrown by slimy non-AAB. Composition of the AAB micropopulation approximately as after 60 hours of fermentation

Acetobacter pasteurianus, *Acetobacter syzygii* and *Acetobacter tropicalis* were the dominating AAB during the fermentations. Occasionally *A. malorum* and *G. oxydans* were detected as well (Table 6 and Appendix III). *Acetobacter pasteurianus* and *G. oxydans* have previously been reported to form a significant part of the AAB community during cocoa fermentations in Ghana (heap), Malaysia (box), Indonesia (box), Trinidad (box) and Brazil (box) (Ostovar and Keeney, 1973; Carr et al., 1979; Carr and Davies, 1980; Passos and Passos, 1985; Ardhana and Fleet, 2003; Schwan and Wheals, 2004) whereas the involvement of *A. syzygii* in cocoa fermentations not has been reported before. The finding of previous studies indicating the involvement of *A. aceti* in the fermentation of cocoa could not be confirmed here (Ostovar and Keeney, 1973; Carr et al., 1979; Ardhana and Fleet, 2003; Schwan and Wheals, 2004).

5.2.4 *Bacillus* spp. involved in the fermentation

Bainbridge and Davies (1912) were the first to observe growth of aerobic spore-forming bacteria during the later stages of cocoa fermentation and identified the bacteria as being of the *Bacillus subtilis* type. Later H.A. Dade (mentioned in a private communication to Knapp, 1937) isolated *Bacillus undulatus* [*B. undulatus* is not a validly published name; furthermore it is not clear what the present name of *Bacillus undulates* would be (Euzéby, 2006)] and *Bacillus megaterium* during Gold Coast (Ghana) cocoa fermentations. In a later study by Carr et al. (1979) and Carr and Davies (1980) all identified isolates from Ghanaian heap fermentations were identified as *B. subtilis*. However, the number of isolates identified to species level was limited.

Ostovar and Keeney (1973) and Schwan et al. (1986) reported that the *Bacillus* community associated with box fermentations in Trinidad and Brazil were complex involving 8-14 different *Bacillus* spp. Carr and Davies (1980) reported the involvement of *Bacillus licheniformis* and *Bacillus subtilis* during Malaysian box fermentations. During an investigation of Indonesian box fermentations Ardhana and Fleet (2003) isolated *Bacillus pumilus* from all 3 estates investigated.

Table 6: Composition of the *Bacillus* spp. community during a large (500 kg) heap fermentation and a small (50 kg) heap fermentation. The large heap fermentation was turned after 48 and 96 hours and samples were taken from 2 positions: 15 cm from the surface (outer part) and in the centre of the fermenting mass. From the small heap fermentation samples were taken 15 cm from the surface. Abbreviations: *B.*: *Bacillus* (Appendix III).

	Fermentation time (hours)												
	0	12	24	36	48	60	72	84	96	108	120	132	144
Large heap, outer part, % of *Bacillus spp.* population													
B. licheniformis					100				50	100	72	96	80
B. cereus						Det.[1]							
B. pumilus											18	4	11
B. megaterium						100		100	50		10		
B. subtilis													9
Large heap, centre, % of *Bacillus spp.* population													
B. licheniformis								29	35	17	68	60	34
B. pumilus						Det.			22	66	32	40	66
B. megaterium						100		71					
B. sphaericus										17			
B. subtilis									43				Det.
Small heap, % of *Bacillus spp.* population													
B. megaterium						100							
B. licheniformis								100	100				

[1] Detected sporadically

In the present study *Bacillus* spp. were identified using a using a combination of phenotypic and genotypic methods. Following micro- and macro-morphological examination all endospore-forming isolates were grouped and partly identified on the basis of 16S-ITS-23S rRNA gene polymorphism and rep-PCR (GTG_5-primer) (Appendix III and Daffonchio et al., 1998; de Clerck et al., 2004). Representative isolates were picked from all groups and subjected to 16S rRNA gene sequencing and API 50 CHB carbohydrate assimilation profiling. The different methods yielded similar results, identifying the major group of *Bacillus* isolates as *B. licheniformis* with *B. megaterium* and *B. pumilus* constituting two other significant groups. A smaller number of isolates were identified as *B. subtilis*, *B. cereus* and *B. sphaericus* (Table 6 and Appendix III).

In general, *B. megaterium* was detected in the middle phase (60-96 hours), and *B. licheniformis* and *B. pumilus* in the mid and late phases of heap fermentation (Table 6 and Appendix III). No *Bacillus* spp. were isolated from the tray fermentation (Table 1 and Appendix III). It is interesting to note, that Ostovar and Keeney (1973) also mainly isolated *B. megaterium* during the middle phase of Trinidadian cocoa fermentations. Contrary to the results reported here Carr and Davies (1980) reported *B. subtilis* as the only *Bacillus* spp. involved in Ghanaian heap fermentations (Carr et al., 1979; Carr and Davies, 1980). *Bacillus megaterium* has been isolated from Ghanaian cocoa fermentations previously (H.A. Dade, personal communication to Knapp, 1937), but no data on the growth of this bacterium during heap fermentations have been published before. The involvement of *Bacillus licheniformis*, *B. pumilus*, *B. cereus* and *B. sphaericus* in heap fermentations have not been reported before, but their association to Indonesian, Malaysian, Brazilian and Trinidadian box cocoa fermentations have been established previously (Ostovar and Keeney, 1973; Carr and Davies, 1980; Schwan et al., 1986; Ardhana and Fleet, 2003).

It is difficult to conclude which – if any – *Bacillus* spp. in general are predominating during cocoa fermentations on the basis of the results obtained during the present study and previously published literature, as data seems to be somewhat contradictive. A few examples: As mentioned above Carr and Davies (1980) only isolated *B. subtilis* during Ghanaian cocoa fermentations. In the present study *B. licheniformis* predominated among the *Bacillus* spp., whereas *B. subtilis* was isolated very infrequently. *Bacillus licheniformis* has also been found

to be among the predominating *Bacillus* spp. during cocoa box fermentations by Carr and Davies (1980), Schwan et al. (1986) and Ardhana and Fleet (2003) – whereas Ostovar and Keeney (1973) also investigating box fermentations only isolated *B. licheniformis* during the drying process and not during the actual fermentations.

5.2.5 Other bacteria involved in the fermentation

A wide range of bacteria other than LAB, AAB and *Bacillus* spp. have been isolated during cocoa fermentations, but the importance of any given species is difficult to judge, as also stated by Lehrian and Patterson (1983). However, some species are encountered frequently and probably play some role in the fermentation. In Indonesia Ardhana and Fleet (2003) isolated *Micrococcus kristinae* and various *Staphyloccus* spp. during cocoa fermentations carried out at 3 different plantations, and *Pseudomonas cepacea* was isolated from 2 out of 3 fermentations. Also Roelofesen and Giesberger (1947) and Ostovar and Keeney (1973) mention the occasional isolation of *Micrococcus* spp. during Indonesian and Trinidadian box fermentations.

During an investigating of cocoa box fermentations carried out at 2 different estates in Trinidad Ostovar and Keeney (1973) observed that *Zymomonas mobilis* constituted 40-80 % of the micropopulation after 16-48 hours of fermentation in the central part of a box fermentation on an estate where the beans were tightly packed in the boxes and the oxygen availability thus limited. On the contrary *Z. mobilis* accounted for a much smaller percentage of the microorganisms at the other estate, where the beans were packed more loosely and the oxygen availability higher. *Zymomonas mobilis* grows well under anaerobic conditions and is an efficient producer of ethanol from glucose, fructose and sucrose. Consequently it has been suggested that *Z. mobilis* along with the yeasts play a role in converting sugars to ethanol during the first days of fermentation (Ostovar and Keeney, 1973; Lin and Tanaka, 2006). The role (if any) of *Z. mobilis* in Ghanaian cocoa fermentations is not known, but deserves investigation in a future study.

Hoynak et al. (1941) isolated *Flavobacterium* spp., *Achromobacter* spp. and *Proteus* spp. during laboratory scale cocoa fermentations. However, as the fermentations were carried out

using cocoa pods transported for 3 weeks and subsequently sterilised it is questionable whether the mentioned species play any role during role during real cocoa fermentations.

5.2.6 Moulds involved in the fermentation

Filamentous fungi can be readily observed on the surface of fermenting cocoa if left undisturbed (i.e. no turning) for a few days, as seen in Fig. 11. Fungal growth is rarely observed below the outer layers of the fermenting mass (Dade, 1928; Bunting, 1928; Roelofsen and Giesberger, 1947; Roelofsen, 1958; Maravalhas, 1966; Lehrian and Patterson, 1983), with the exception of the study by Ardhana and Fleet (2003) who observed fungal growth in samples taken from the centre of Indonesian box fermentations during the first 36 h of fermentation.

Fig. 11: Visible mould growth on the surface of an unturned Ghanaian heap fermentation. Susanne Hønholt, private pictures, reprinted with permission.

According to Bunting (1928) and Dade (1928) the moulds most frequently isolated during Gold Coast (Ghana) cocoa heap fermentations were members of the *Aspergillus fumigatus* group and *Mucor* spp. Maravalhas (1966) observed that moulds appear in succession with *A. fumigatus* appearing first followed by *Mortierella gamsii* [then named *Mortierella spinosa* (Gams, 1977)] and later *Paecilomyces varioti*. In the later stages of fermentation the temperature of the fermenting mass decreases and this is accompanied by growth of *Penicillium citrinum* and *Aspergillus glaucus*.

Penicillium citrinum produces the mycotoxin citrinin (Malmstrom et al., 2000). Citrinin was recently reported detected in cocoa bean shells, but has so far not been detected in the actual bean (Meister, 2004). However, according to Meister (2004) the relatively few reports of citrinin in food and feed stuff may be due to analytical difficulties in determining citrinin and not due to actual absence of the mycotoxin. If this is the case it is worrying that Ardhana and Fleet (2003) reported good growth of *P. citrinum* during the initial phases of Indonesian cocoa fermentations. Ardhana and Fleet (2003) furthermore reported growth of *Penicillium purpurogenum*, *Penicillium ochrochloron*, *Aspergillus versicolor* and *Aspergillus wentii* during the fermentations.

5.3 Microbial growth during drying

The ultimate goal of the drying process is to obtain microbiologically stable cocoa beans by drying to an extent where microbial growth becomes impossible. A water content of 7.2 %, has been established as a safe limit (Roelofsen and Giesberger, 1947; Roelofsen, 1958). As the water availability decreases during the drying process fewer and fewer microorganisms are capable of growing and a pronounced reduction in the number of viable microorganisms is observed (Carr et al., 1979; Schwan and Wheals, 2004). At the onset of drying yeast, AAB, LAB and *Bacillus* spp. were isolated by Carr et al. (1979) in Ghana. During the solar drying process all but sporeforming *Bacillus* spp. died out. A similar picture has been observed in Brazil (Schwan and Wheals, 2004).

Many moulds are capable of growing at low water activities and if the reduction in moisture content during drying is too slow moulds will grow (Roelofsen, 1958; Wood and Lass, 1985). Various *Aspergillus* spp., *Penicillium* spp. and *Mucor* spp. have been frequently isolated from mouldy cocoa beans. Mould growth during drying should be avoided as it can result in the formation of off flavours, internal moulding of the beans and formation of mycotoxins (Dade, 1928; Bunting, 1928; Roelofsen, 1958; Wood and Lass, 1985; Anon., 2003; Lindblom, 2006; Anon., 2006).

5.4 Use of starter cultures for fermentation of cocoa

Soon after it was realised that microorganisms were responsible for the fermentation of cocoa beans the first attempts of controlling the fermentation using starter cultures were made, as Preyer-Buitenzorg (1901) inoculated cocoa fermentations with his so-called *Sc. theobromae* (see section 5.2.1). According to Preyer-Buitenzorg (1901) the resulting cocoa was of a better quality than cocoa fermented without the addition of *Sc. theobromae*. Subsequently a number of attempts have been made to improve or control the quality of fermented cocoa by using yeast as a starter culture (Ficker and von Lilienfeld-Toal, 1930; Knapp, 1937; Roelofsen and Giesberger, 1947; Sanchez et al., 1985; Samah et al., 1992; Dzogbefia et al., 1999). In general the effect of using yeast as a sole starter culture was limited (Knapp, 1937; Roelofsen, 1958).

According to Busse et al. (1929) the quality of Cameroonian cocoa suffered from being too acidic and it was unsuccessfully attempted to retard the growth of AAB by adding *Lactobacillus delbrueckii* [then named *Bacillus delbrückii* (Euzéby, 2006)].

More recently Schwan (1998) used a defined microbial inoculum consisting of a mixture of *Sc. cerevisiae*, *Lb. plantarum*, *Lactobacillus delbrueckii* subsp. *lactis* [then named *Lactobacillus lactis* (Weiss et al., 1983; Euzéby, 2006)], *A. aceti* and *G. oxydans*. The first 2-3 days of fermentation the inoculum dominated the fermentation, but during the later phases of fermentation the inoculum was outgrown by naturally occurring microorganisms. The chocolate produced from the inoculated bean was of a quality comparable to chocolate produced from a spontaneously inoculated control fermentation.

So far the aim of the different attempts to use starter cultures for the fermentation of cocoa has been to improve the quality of the resulting beans in terms of flavour potential, absence of excess acidity etc. and/or to increase the speed of fermentation. Work of Masoud et al. (2005) and Masoud and Kaltoft (2006) point at another potential use of artificial inoculation of cocoa fermentations, as strains of *Pichia anomala*, *P. kluyveri* and *H. uvarum* isolated from coffee fermentations have been found to inhibit growth and ochratoxin A production by *Aspergillus ochraceus*. The primary processing of cocoa and coffee has a number of similarities (Thompson et al., 2001) and it is indeed possible that the potential of some yeasts to inhibit

mould growth and mycotoxin production could be exploited in the primary processing of cocoa as well.

6. Culture-independent investigation of cocoa fermentations

Due to complexity of the cocoa fermentation culture-based investigations of cocoa fermentations are tedious and time consuming. Furthermore, due to the spontaneous nature of the process it is desirable to investigate more than just a few fermentations to get a clear picture of which organisms are consistently present during the fermentations and which are just coincidently present in a few fermentations. Molecular biology based finger-printing methods such as Terminal-Restriction Fragment Length Polymorphism (T-RFLP), Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE), offer rapid, semi-quantitative alternatives for investigating the fermentation process also enabling the detection of organisms difficult to cultivate by conventional methods (Muyzer et al., 1993; Muyzer and Smalla, 1998; Giraffa and Neviani, 2001).

Denaturing Gradient Gel Electrophoresis is based on sequence-specific separation of PCR-derived rRNA gene amplicons in polyacrylamide gels containg a linearly increasing concentration of denaturant (urea and formamide) as described by Muyzer and Smalla (1998). The technique has been most widely applied to study complex microbiota like the intestinal colon (Tannock, 1999; Nielsen et al., 2003) and soil (Muyzer and Smalla, 1998), but the method has during recent years also been applied to study the bacterial dynamics of various fermented products like; sausages (Cocolin et al., 2001b; Cocolin et al., 2004b), probiotic milk products (Fasoli et al., 2003), sourdough (Meroth et al., 2003b), fermented cassava (Ampe et al., 2001; Miambi et al., 2003), fermented maize products (Ampe et al., 1999; Ampe and Miambi, 2000; Omar and Ampe, 2000), cheese (Cocolin et al., 2004a), balsamic vinegar (De Vero et al., 2006), rice vinegar (Haruta et al., 2006), wine (Lopez et al., 2003; Bae et al., 2006) and whisky (van Beek and Priest, 2002). Furthermore the DGGE method has been further developed to investigate yeast population dynamics in milk (Cocolin et al., 2002a), sourdough (Meroth et al., 2003a; Gatto and Torriani, 2004) and during cassava (Miambi et al., 2003), coffee (Masoud et al., 2004) and wine fermentations (Cocolin et al., 2000; Cocolin et al., 2001a; Mills et al., 2002; Cocolin et al., 2002b; Cocolin and Mills, 2003; Prakitchaiwattana et al., 2004).

6.1 Development of a Denaturing Gradient Gel Electrophoresis (DGGE) based method for investigation of cocoa fermentations

Sampling in the field raises the problem of preserving the samples. Freeze drying has previously been shown to be a suitable method for preserving DNA in various matrixes (Huckenbeck and Bonte, 1992; Verkooyen et al., 2003) and was thus chosen as preservation method with good results (Appendix II).

Initially DNA was attempted extracted from the freeze dried cocoa pulp using 2 commercially available DNA extraction kits, but unfortunately with unsatisfying results (Appendix II). To overcome this, a protocol for DNA extraction from freeze dried cocoa pulp was developed. Efficient cell lysis was achieved using a combination of enzymatic, chemical and mechanical treatments (Appendix II and Zhou et al., 1996; Schabereiter-Gurtner et al., 2001). Cocoa pulp contains several potentially PCR-inhibiting compounds like polysaccharides, enzymes and other proteins that have to be removed during DNA extraction and purification (Pettipher, 1986b; Rossen et al., 1992; Wilson, 1997). This was achieved through addition of cetyltrimethyl ammonium bromide [CTAB; at high ionic strength CTAB binds proteins and polysaccharides that otherwise might inhibit subsequent PCR reactions (Sambrook and Russell, 2001)], chloroform/isoamyl extraction and spin column purification (Appendix II).

Choice of primer is an important parameter in DGGE mediated analysis of complex microbial communities. For bacteria primers most often target the 16S rRNA gene, but also primers targeting e.g. the *rpo*B gene have been developed (Giraffa and Neviani, 2001; Peixto et al., 2002). For yeast and other eukaryotic organisms primers often target the 26S rRNA gene (Cocolin et al., 2000). Universal bacterial primers are in principle capable of amplifying DNA from all bacteria, whereas species- or group-specific primers selectively amplify bacteria from a specific group of microorganisms – e.g. lactic acid bacteria (Heilig et al., 2002; Nielsen et al., 2003). However, apparently some universal primers are not truly universal or at least some primers have a lower detection limit than others, as seen from Fig. 12. Two sets of universal primers were used to amplify the same samples. On average twice as many bands appeared when using the primers targeting the 16S rRNA gene V3-region compared to the primers targeting the V6-V8-region of the 16S rRNA gene. A similar observation was made by Nielsen et al. (2005a).

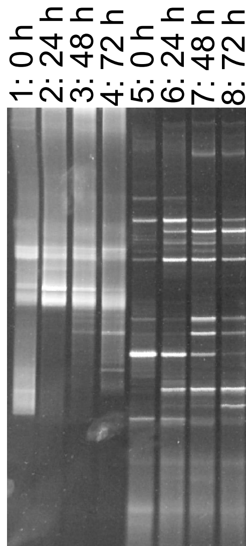


Figure 12: DGGE profiles (35-70 % denaturant) representing 16S rRNA gene fragments of cocoa pulp sampled after 0, 24, 48 and 72 hours of tray fermentation (carried out in January and amplified using PCR and 2 different primer sets: Lane 1-4 amplified using primers 968fGC and 1401r (V6-V8-region); Lane 5-8 amplified using primers PRBA338fGC and PRUN518r (V3-region). Appendix III.

Various gradients between 20 and 80 % denaturant were tested. It was found that a 35-65 % gradient was optimal for DGGE analysis of the yeast and bacterial communities associated with cocoa fermentation (Appendix II and III).

Bands of interest in the DGGE gels can be identified by sequencing. In short the band is excised from the gel, the DNA eluted from the gel fragment and re-amplified using PCR and sequenced (Appendix II). Identification of DGGE bands using this approach should however be interpreted with caution, as formation of heteroduplexes during PCR, single-stranded DNA forming a “back-ground smear” in the gels and co-migration of different species with identical melting behaviour complicates the picture (Sekiguchi et al., 2001; Zhang et al., 2005; Nikolausz et al., 2005).

To confirm the electrophoretic mobility of the re-amplified fragment relative to the fragment from which it was excised the re-amplified PCR products were re-analyzed by DGGE using the original conditions. During the present and other studies occasionally a re-amplified fragment did not migrate as a single band, probably because the excised DNA fragment was contaminated with a back ground smear of single stranded DNA (Appendix II and III and Zhang et al., 2005; Nikolausz et al., 2005; Bae et al., 2006). If this is the case it is necessary to purify the band before sequencing to obtain a reliable sequence. In the present study this was

done by repeating the above procedure until a pure, single band was obtained (Appendix II and III). Other methods have been proposed, for instance treating the excised DNA fragment with mung bean nuclease to degrade contaminating single-stranded DNA before re-amplification (Zhang et al., 2005).

Figure 13 represents DGGE profiles of 26S rRNA gene fragments of cocoa pulp sampled after 3 and 24 h of fermentation during a tray fermentation carried out at the Cocoa Research Institute of Ghana. Selected fragments were identified by sequencing and the results compared to culture-based isolations and subsequent identification of the isolates (Fig. 13 and Table 7).

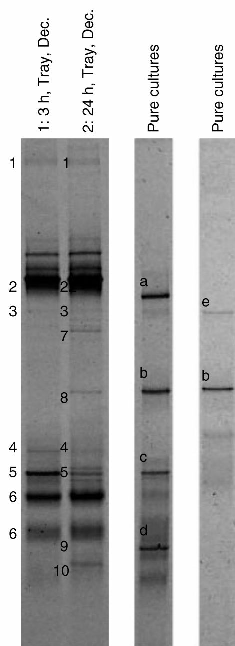


Fig. 13: DGGE profiles (35-65 % denaturant) representing 26S rRNA (yeast) gene fragments of cocoa pulp sampled after 3 h and 24 h of tray fermentation and mixtures of DNA originating from pure cultures of a) *Candida friedrichii* b) *Saccharomyces cerevisiae* c) *Issatchenkia hanoiensis* d) *Pichia membranifaciens* and e) *Trichosporon asahii*.

The closest relatives of the fragments sequenced (% identical nucleotides compared to sequences retrieved from the GenBank database) are as follows: *Candida amapae* (1, 100 %); *Hanseniaspora guilliermondii* (2, 100 %); *Trichosporon asahii* (3, 99.3 %); *Candida stellimalicola* (4, 100 %); *Pichia kluyveri* (5, 99.5%); Single stranded DNA/smear (6); ; *Candida zemplinina* (7, 100 %); *Saccharomyces cerevisiae* (8, 100 %); *Pichia membranifaciens* (9, 100 %); *Issatchenkia orientalis* (10, 100 %). Appendix II.

The molecular biology and traditional methods yielded comparable, but slightly different results as seen in Table 7. For both samples more species were detected using the DGGE method compared to the traditional culture-based method. A fragment closest related to the wine-associated yeast *Candida zemplinina* (Sipiczki, 2003) was only detected using DGGE (band 7, Fig. 13 and Table 7). It has been suggested that the presence of *C. zemplinina* in wine fermentations is underestimated as it is difficult to cultivate (Mills et al., 2002), thus offering a possible explanation why *C. zemplinina* was only detected using DGGE and not using culture based methods. This finding underlines that the ability to detect organisms difficult to

cultivate is one of the DGGE techniques main advantages (Muyzer and Smalla, 1998). However, it should be noted that in a later investigation it was possible to detect *C. zemplinina* using DGGE as well as culture-based methods (Fig. 14, Table 3, Table 4 and Appendix III). Possibly *C. zemplinina* constituted a higher percentage of the yeast population during these fermentations, and was thus detectable using culture-based methods even though it was possibly underestimated (Mills et al., 2002).

Table 7: Composition of yeast populations after 3 and 24 h, respectively, of a tray fermentation investigated using culture based isolations and DGGE. Appendix II.

	3 h Tray fermentation (CFU/g (yeast)[1] = 4.3×10^6)		24 h Tray fermentation (CFU/g (yeast)[1] = 1.0×10^5)	
Yeast	**% of yeast population**	**Detected by DGGE**	**% of yeast population**	**Detected by DGGE**
Candida amapae	9	Yes		Yes
Hanseniaspora guilliermondii	50	Yes	18	Yes
Trichosporon asahii	18	Yes	9	Yes
Candia stellimalicola		Yes	9	Yes
Pichia kluyveri	23	Yes	4	Yes
Candida zemplinina		No		Yes
Saccharomyces cerevisiae		No	4	Yes
Pichia membranifaciens		No	4	Yes
Candida krusei		No	52	Yes

[1] Malt agar with antibiotics incubated at 30 °C for 72 h.

Candida stellimalicola was only detected by DGGE after 3 h of fermentation and by DGGE as well as culture based isolations after 24 h (band 4, Fig. 13 and Table 7). The lower limit of detection using DGGE has been estimated to comprise 1 % of the yeast population (Prakitchaiwattana et al., 2004). For the culture based isolations 25 colonies were randomly picked and identified giving an approximate limit of detection of 4 % of the yeast population (Appendix I). Possibly *C. stellimalicola* constituted too little a fraction of the yeasts present after 3 h to allow detection using the culture-based approach. On the other hand *Trichosporon asahii* yielded only a relatively weak band in the denaturing gels (band 3, Fig 13) even though it formed 18% and 9% of the yeast population after 3 and 24 h of fermentation, respectively (Table 7). Estimation of the size and amount of PCR product by agarose gel electrophoresis showed that *T. asahii* pure cultures yielded a relatively weak band compared to other isolates. A possible explanation may be that the amplified fragment of the *T. asahii* 26S rRNA gene was less efficiently amplified compared to other yeast species present during the fermentation of cocoa (Appendix II and Kanagawa, 2003). DNA originating from different pure cultures

representing *Candida friedrichii*, *Sc. cerevisiae*, *Issatchenkia hanoiensis*, *I. orientalis*, *T. asahii* and *P. membranifaciens* isolated from cocoa fermentations were mixed and the mixtures used as template in PCR to investigate if the 26S rRNA gene fragment of some isolates were preferentially amplified. DGGE analysis of the mixtures showed that the 26S rRNA gene of *T. asahii* was less efficiently amplified compared to the other isolates investigated (results partly shown in Fig. 13) explaining why *T. asahii* only yielded a weak band in the denaturing gels. All other tested isolates were amplified equally effective (results partly shown in Fig. 13).

Despite the above mentioned limitations DGGE offers a rapid and valuable alternative to the traditional culture-based investigation of cocoa fermentations.

6.2 Investigation of Ghanaian cocoa fermentations using DGGE

Samples representing cocoa fermentations carried out in different parts of Ghana [at the Cocoa Research Institute of Ghana (CRIG), New Tafo, and at farmers in New Tafo, Bompata, and Mampong], at different times during the season (August, October, December, January) and using different fermentation methods (small heap, large heap and tray fermentations) were investigated using DGGE (Appendix II and III).

The yeast community associated with the fermentation of cocoa was investigated by DGGE analysis of PCR amplicons amplified using eukaryotic universal primers targeting a fragment of the D1/D2-region of the 26S rRNA gene (see 6.2.1); whereas the bacterial community was investigated by DGGE analysis of PCR amplicons amplified using prokaryotic universal primers amplifying the V3-region of the 26S rRNA gene (see 6.2.2). Fragments (bands) of interest were excised and identified using sequencing (Appendix II and III). Microbial dynamics during the fermentations were furthermore investigated by cluster analysis (see 6.2.3).

6.2.1 The yeast community

Some of the fermentations have been investigated using culture-based methods as well as DGGE, for instance the fermentations represented by Fig. 14 carried out at CRIG, and Fig. 15

carried out at New Tafo (Appendix II and III). The DGGE and culture-based findings corresponded well, but in general *H. guilliermondii* was detected longer using DGGE compared to culture-based findings. This is for instance seen from Fig. 14, where *H. guilliermondii* yields as strong band until after 84 hours of fermentation. According to the culture-based findings *H. guilliermondii* constituted 67-79% of the yeast population during the first 24 hour of fermentation but was not detected afterwards.

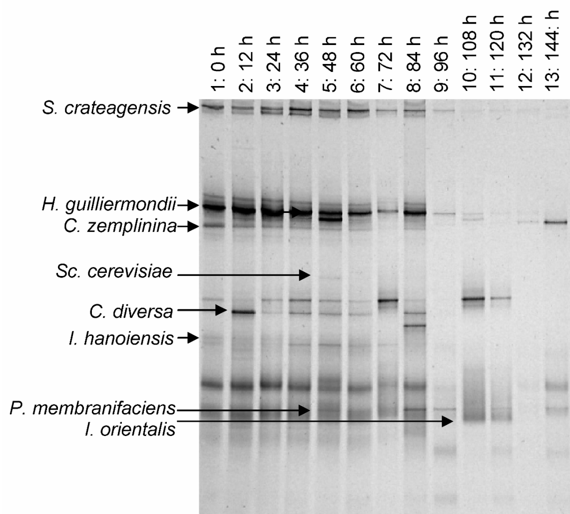


Fig. 14: DGGE profiles (35-65 % denaturant) representing 26S rRNA gene fragments (yeast) of cocoa pulp sampled with 12 hour intervals during 144 hours of fermentation in a large heap (500 kg) carried out in October at the Cocoa Research Institute of Ghana. Identity of identified fragments indicated, see arrows. Abbreviations: *H.*: *Hanseniaspora*, *S.*: *Saccharomycopsis*, *P.*: *Pichia*, *I.*: *Issatchenkia*, *C.*: *Candida*, *Sc.*: *Saccharomyces*. Appendix III.

The lower limit of detection using DGGE has as mentioned above been estimated to comprise 1 % of the yeast population (Prakitchaiwattana et al., 2004). It can be speculated that the presence of a strong band representing *H. guilliermondii* at sampling times where *H. guilliermondii* was not detected using culture-based isolations, is due to the fact that *H. guilliermondii* was present in numbers below the detection limit of the culture-based isolations but above the detection limit of the DGGE method until 84 hours of fermentation. Alternatively *H. guilliermondii* possibly enters an uncultureable state after 36 hours of fermentation (Appendix II and III).

Hanseniaspora guilliermondii was detected in the early stages of all fermentations investigated including the fermentations carried out in Mampong and Bompata (Fig. 14 and 15; Appendix II and III). Taken together with the culture-based findings this indicates that *H. guilliermondii* is the dominating yeast in the early phases of Ghanaian cocoa fermentations. Later in the fermentations in particular *P. membranifaciens* but also *I. orientalis* yielded

strong bands in DGGE profiles representing all investigated fermentations (Fig. 14 and 15; Appendix II and III). Again this is supported by the culture-based findings indicating their predominant role among the yeast during the later phases of fermentations.

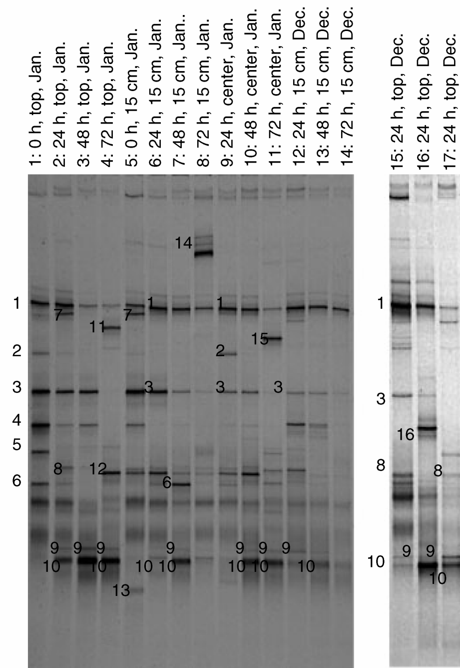


Fig. 15: DGGE profiles (35-65 % denaturant) representing 26S rRNA gene fragments of cocoa pulp sampled after 0, 24, 48 and 72 h of heap fermentation at New Tafo, Ghana. Lanes 1-11 represents cocoa pulp from the same fermentation (carried out in January) but sampled in different depths of the fermenting mass (surface, 15 cm from surface and centre of the heap). Lanes 12-17 represents cocoa pulp from a fermentation carried out in December sampled at the surface and 15 cm from the surface of the heap. The closest relatives of the fragments sequenced (% identical nucleotides compared to sequences retrieved from the GenBank database) are as follows: *Hanseniaspora guilliermondii* (1, 100 %); *Candida neodendra* and *Candida diddensiae* (2, 99.5 %); *Ceratocystis paradoxa* (3, 100 %); *Scytaladium hyalinum* (4, 95.1 %); *Candida stellimalicola* (5, 100 %); *Penicillium sclerotium* (6, 96.2 %); *Candida friedrichii* and *Candida tammaniensis* (7, 99.5 %); *Pichia kluyveri* (8, 99.0 %); *Pichia membranifaciens* (9, 100 %); *Issatchenkia orientalis* (10, 100 %); *Rhizomucor pusillus* (11, 99.0 %); *Issatchenkia hanoiensis* (12, 98.0 %); *Aspergillus japonicus* (13, 99.5 %); *Saccharomycopsis vini* (14, 99.5 %); *Candida zemplinina* (15, 100 %); No sequence obtained (16). Appendix II.

It was surprising to detect fragments closest related to the moulds *Penicillium sclerotium*; *Scytaladium hyalinum* and *Ceratocystis paradoxa*, the later causing rotting of bananas, pineapples and other tropical fruits, during the early phases of the New Tafo heap fermentations (Fig. 15 and Appendix II). This indicates that the beans were heavily infected with moulds from the onset of fermentation. The pods for these fermentations were harvested over 2-3 weeks. If the pods were damaged during harvest, it is likely that moulds will have penetrated into the pulp and developed during pod storage increasing the risk of mycotoxin production (Anon., 2003; Lindblom, 2006; Anon., 2006).

The New Tafo fermentations were fermented for only 3 days (72 hours) and without turning (Fig. 15 and Appendix II). This fermentation schedule is practiced by some farmers, but generally not recommended (Baker et al., 1994). After 72 hours a mould, closest related to *Rhizomucor pusillus*, was detected in the outer part of the January fermentation (Fig. 15 and Appendix II) possibly reflecting the higher oxygen availability in the outer part of the fermenting mass.

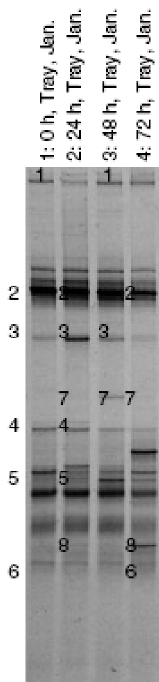


Fig. 16: DGGE profiles (35-65 % denaturant) representing 26S rRNA gene fragments of cocoa pulp sampled with 24 h intervals during tray fermentation carried out in January at the Cocoa Research Institute of Ghana. The closest relatives of the fragments sequenced (% identical nucleotides compared to sequences retrieved from the GenBank database) are as follows: *Candida amapae* (1, 100 %); *Hanseniaspora guilliermondii* (2, 100 %); *Candida zemplinina* (3, 100 %); *Candida stellimalicola* (4, 100 %); *Pichia kluyveri* (5, 99.0 %); *Issatchenkia orientalis* (6, 100 %); *Saccharomyces cerevisiae* (7, 100 %); *Pichia membranifaciens* (8, 100 %). Appendix II.

Compared to the heap fermentations *Sc. cerevisiae* and *C. zemplinina* apparently play are more important during role during the investigated tray fermentations as they were detected during all investigated tray fermentations (Fig. 16), and only more occasionally during the heap fermentations (Appendix II and III).

6.2.2 The bacterial community

Denaturing gradient gel electrophoresis analysis showed that in the initial phases of cocoa fermentation LAB dominated the bacterial community with *Lb. fermentum* yielding a strong band throughout the fermentations. After 24-36 hours of fermentation, bands representing AAB becomes visible, and *B. licheniformis* and occasionally other *Bacillus* spp. are detected during the later stages of heap fermentation (Fig. 17 and 18 and Appendix III). A relatively complex AAB micro-population was observed during the Mampong fermentation as *G. oxydans*, *A. syzygii*/*A. pasteurianus* and *A. tropicalis* all yielded relatively strong bands during this fermentation (Appendix III). Unfortunately *A. syzygii* and *A. pasteurianus* migrate to the same position in the DGGE gels. As a consequence it was not possible to discriminate between these two species using DGGE (Fig. 17 and 18 and Appendix III).

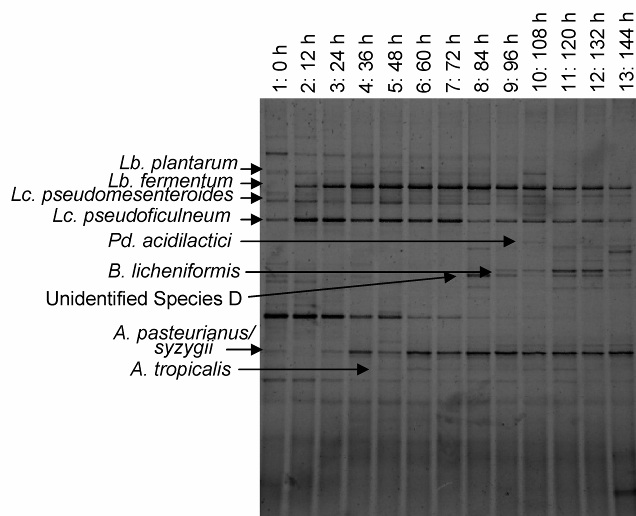


Fig. 17: DGGE profiles (35-65 % denaturant) representing 16S rRNA gene fragments (bacteria) of cocoa pulp sampled with 12 hour intervals during 144 hours of fermentation in a large heap (500 kg) carried out in October at the Cocoa Research Institute of Ghana. Identity of identified fragments indicated, see arrows. Appendix III.

The DGGE and the culture-based findings corresponded well with a few exceptions. *Leuconostoc pseudoficulneum* was occasionally identified during the culture-based part of the study (Table 5 and Appendix III). Surprisingly, this species yielded a strong band during all fermentations except the Mampong heap fermentation (Fig. 17 and 18 and Appendix III). Given these results, it is indeed possible that *Lc. pseudoficulneum* plays a more important role during the fermentation of cocoa than anticipated from the culture-based findings.

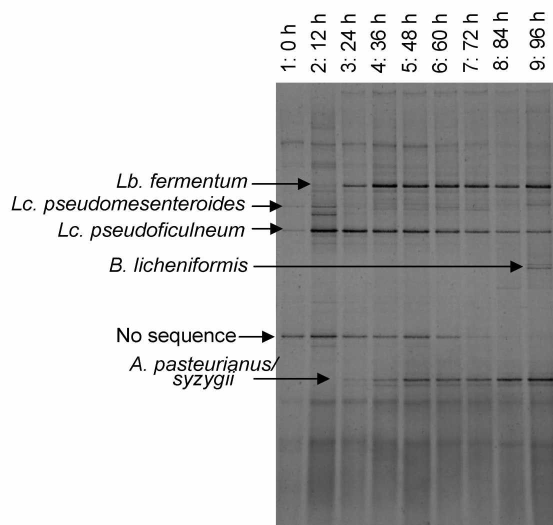


Fig. 18: DGGE profiles (35-65 % denaturant) representing 16S rRNA gene fragments (bacteria) of cocoa pulp sampled with 12 hour intervals during 96 hours of fermentation in a small heap (50 kg) carried out in October at the Cocoa Research Institute of Ghana. Identity of identified fragments indicated, see arrows. Appendix III.

A band migrating to approximately the middle of the gel in all fermentations yielded a strong band in the initial phases of all fermentations including the fermentations carried out in Mampong and Bompata (indicated with an arrow and “No sequence” in Fig. 18).

Unfortunately, it was not possible to obtain a satisfying sequence from this band, and no pure isolates had the same electrophoretic mobility. As a consequence, the band remained unidentified (Appendix III).

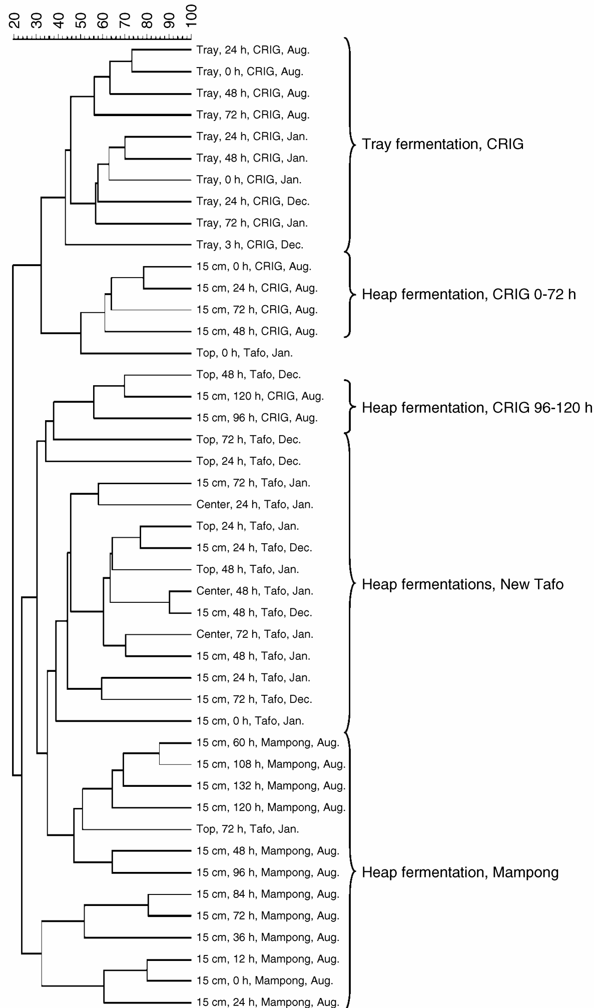
A weak fragment detected in the initial phase of one of the tray fermentations investigated using bacterial universal primers (V3-region) was surprisingly identified as the yeast *H. guilliermondii* (results not shown). The unintended amplification of eukaryotic DNA using prokaryotic specific primers has been reported before (Lopez et al., 2003). The problem is probably due to amplification of 18S rRNA gene fragments in PCR reactions where eukaryotic DNA is present in excess amounts compared to the amounts of prokaryotic DNA. This is likely to be the case in the initial phases of cocoa fermentations where *H. guilliermondii* as seen from Table 1 reaches high counts. However, as the fragment was weak and only detected in the initial phase of a single fermentation the problem was considered negligible but underlines that DGGE profiles always should be interpreted with caution (Appendix III).

6.2.3 Cluster analysis

Cluster analysis of DGGE profiles has previously been shown to yield valuable information about the microbial dynamics in various microbial communities including fermented foods (Ampe and Miambi, 2000; Nielsen et al., 2003). The dendrograms (Fig. 19 and 20) were calculated on the basis of Dice's coefficient of similarity. Data were weighted to take into account the different intensities of the bands (Appendix II and III and Eichner et al., 1999; Ampe and Miambi, 2000).

Cluster analysis of the DGGE profiles representing the yeast community associated with cocoa fermentations showed that the profiles grouped according to fermentation method and site (Fig. 19A). The tray fermentations formed one group in the dendrogram. The heap fermentations carried out at Mampong formed another group while the CRIG and New Tafo heap fermentations grouped between the tray and Mampong fermentations (Fig. 19A).

A



B

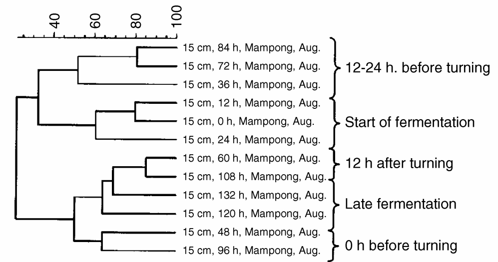


Fig. 19, A: Dendrogram derived from DGGE analysis of the yeast populations associated with the fermentation of cocoa based on Dice's coefficient of similarity (weighted) with the unweighted pair group method with arithmetic averages clustering algorithm. Tray: Tray fermentation. Top: Heap fermentation, surface sample. 15 cm: Heap fermentation, sample taken 15 cm below surface. Center: Heap fermentation, sample taken in the centre of the fermenting mass. Samples taken during 0-132 h of fermentation at Cocoa Research Institute of Ghana (CRIG); a farm in New Tafo (Tafo) and a farm in Mampong, respectively, in December, January and August. Appendix II.

Figure 19, B: Detailed representation of the part of the dendrogram in Fig. 19A representing the Mampong fermentation. The heap fermentation was turned at 48 and 96 h, respectively. Appendix II.

If the dendrograms were calculated on the basis of the DGGE profiles from one fermentation site at a time grouping with progress in the fermentation was observed. Figure 19B represents a dendrogram calculated on the basis of the DGGE profiles originating from the heap fermentation carried out in Mampong. Five groups in the dendrogram were recognized each representing a specific stage in the fermentation process. The impact of turning the heaps was also observed: The profiles representing samples taken immediately before turning (48 and 96 h) group together, while the profiles representing samples taken 12 h after turning (60 and 108 h) form another group (Fig. 19B). The same pattern was observed for the heap fermentation carried out at CRIG. Within the heap fermentations carried out at New Tafo grouping with fermentation time was also observed. However, the grouping was less clear cut compared to the Mampong and CRIG fermentations, probably because sampling position in the heap (surface, below surface and center) also influences the DGGE profiles (Fig 19A and

Appendix II). The tray fermentations grouped with season (August or December/January) but also with early and late fermentation within the seasons (Fig. 19A and Appendix II). Similar results were obtained when yeast derived DGGE profiles representing another set of fermentations were examined (Appendix III).

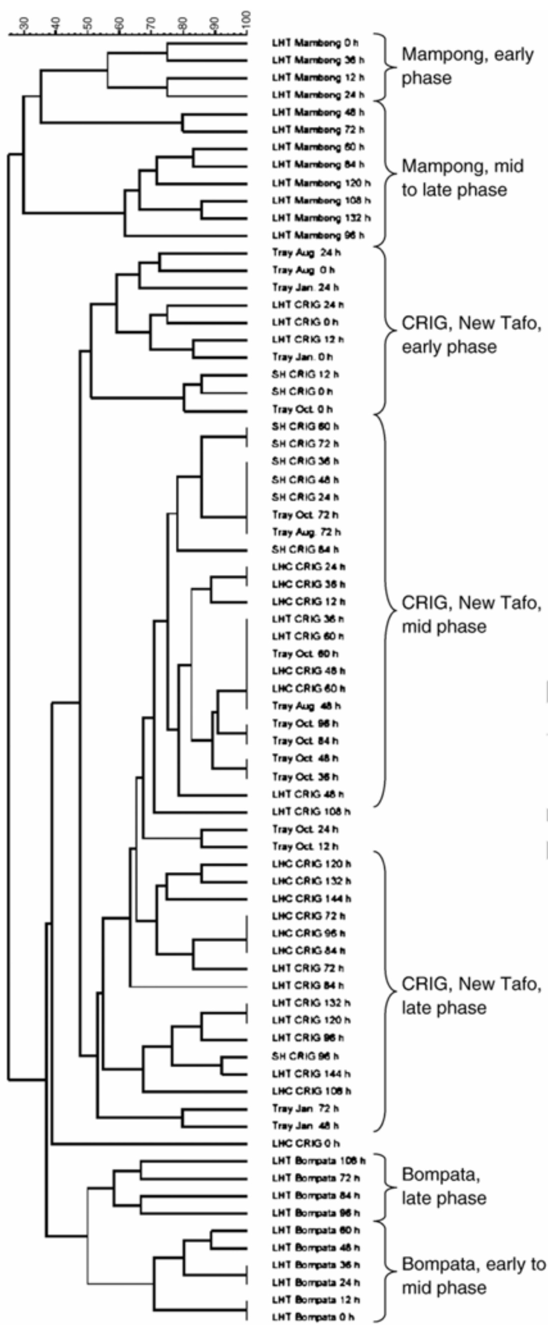


Fig. 20: Dendrogram derived from DGGE analysis of the bacterial populations associated with heap and tray fermentation of cocoa in Ghana. The dendrogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA). Samples were taken during 0-144 hours of fermentation at the Cocoa Research Institute of Ghana and at farms in Mampong and Bompata, respectively. Abbreviations: LHT: Large Heap Top fermentation (i.e. pulp sampled 15 cm from the surface of the heap); LHC: Large Heap Centre fermentation (i.e. pulp sampled in the centre of the heap); SH: Small Heap fermentation; Tray: Tray fermentation; CRIG: Cocoa Research Institute of Ghana. Appendix III.

Cluster analysis of the DGGE profiles representing the bacterial community associated with cocoa fermentations revealed that the profiles grouped according to fermentation site (Fig.

20). In contrast to the cluster analysis of the DGGE profiles representing the yeast community the profiles representing the bacterial community did not cluster according to fermentation method. This indicates that the composition of the bacterial community associated with cocoa fermentation is more influenced by the fermentation site, than the fermentation method.

Within each fermentation site the profiles clustered according to fermentation time (Fig. 20 and Appendix III). This was also the case for the profiles representing the yeast community as mentioned previously (Fig. 19 and Appendix II and III). Construction of a library of DGGE profiles combined with cluster analysis seems in other words to provide a possible tool for monitoring the progress of fermentation at specific production sites (Appendix II and III).

It is likely that the ability to monitor the progress of fermentation using DGGE can be used to obtain further insight into the processes leading to well fermented cocoa by correlating DGGE profiles with physical and/or chemical changes in the beans during fermentation. Recently it has been shown that Near Infra Red (NIR) and fluorescence spectroscopy offer efficient tools for monitoring some of the changes taking place in the cocoa bean during fermentation and drying (Nielsen, 2006). Preliminary results have indicated that correlating NIR spectra of dried and grounded cocoa beans with DGGE profiles offers a promising novel method for investigating the connection between microbial activity in the pulp and the changes taking place inside the beans during fermentation (D.S. Nielsen, P.S. Nielsen and F. van den Berg, unpublished results).

7. Cocoa fermentations as a source of microbial diversity

Detailed microbial characterisation of spontaneously fermented indigenous foods and feed products have revealed a range of previously undescribed microorganisms during recent years (Holzapfel, 2006). During the present study a previously undescribed LAB (see section 7.1) and 3 putatively undescribed yeast species (see section 7.2) have been isolated (Appendix III).

7.1 Lactobacillus ghanaensis *sp. nov.; a novel lactic acid bacterium isolated from Ghanaian cocoa fermentations*

From a large heap fermentation carried out at CRIG a number of isolates with unusual properties were isolated from MRS agar and tentatively identified as *Lactobacillus* spp. (Section 5.2.2 and Appendix III). Three of the isolates, designated L486, L489^T and L499, were subjected to a detailed morphological, biochemical and molecular characterisation.

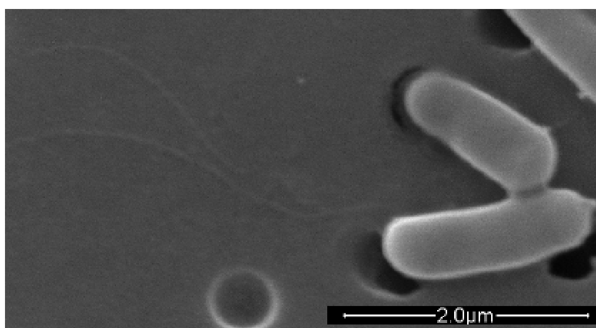


Fig. 21: Cells and flagella of *Lactobacillus ghanaensis* sp. nov. L489^T grown over night in MRS broth at 30 °C. Scanning Electron Microscopy, 40000×magnification. Bar represents 2 µm. Appendix IV.

All isolates were Gram-positive, catalase negative and did not produce gas from glucose. Under the microscope the isolates were rod-shaped and highly motile. Scanning Electron Microscopy revealed the presence of peritrichous flagella (Fig. 21 and Appendix IV).

Sequencing of the 16S rRNA gene showed that L486 and L489^T had 100 % identical 16S rRNA gene sequences, whereas L499 differed in one nucleotide from the former two (Appendix IV). The 16S rRNA gene sequences of L486, L489^T, L499 and sequences of the closest phylogentic relatives retrieved from the GenBank database were aligned and a phylogenetic tree was constructed by the neighbour-joining method (Fig. 22). Comparison between the 16S rRNA gene sequences of L486, L489^T and L499 with type strains in the

GenBank database revealed highest similarities with *L. nagelii* DSM 13675^T (98.0 %), *L. satsumensis* DSM 16230^T (95.5 %) and *L. vini* DSM 20605^T (93.7 %) (Table 8).

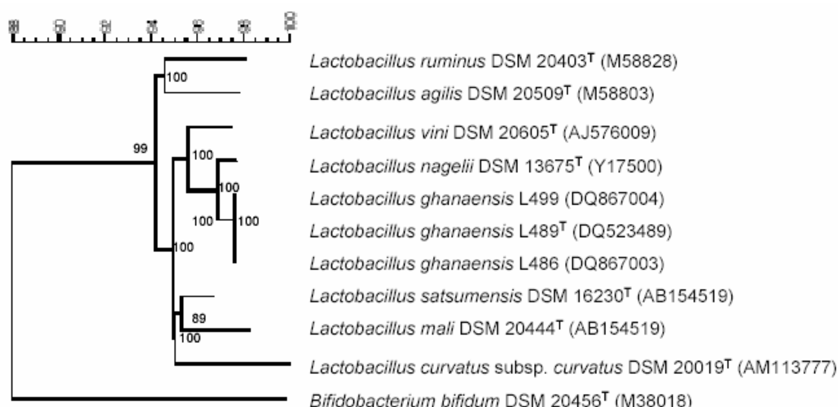


Fig 22: Phylogenetic tree (Neighbour-joining method) based on almost complete 16S rRNA gene sequences showing the phylogenetic position of *Lactobacillus ghanaensis* sp. nov. among closely related *Lactobacillus* spp. Bootstrap values (%) based on 1000 replications are stated on each node. Appendix IV.

DNA from the strains L489^T and L499 was hybridised to each other and to *L. nagelii* DSM 13675^T and *L. satsumensis* DSM 16230^T. L486 was not included in the analysis as it had 100 % 16S rRNA gene homology to L489^T. The DNA-DNA reassociation values between L489^T and L499 were 92.5 % indicating that the strains are con-specific. Reassociation values of 18-44 % were obtained with *L. nagelii* DSM 13675^T and 0-12 % with *L. satsumensis* DSM 16230^T. All values for DNA-DNA hybridisation studies with the closest relatives *L. nagelii* DSM 13675^{T}and *L. satsumensis* DSM 16230^T were thus well below the 70 % cut-off value that indicates separate species (Wayne et al., 1987). DNA reassociation values between L489^T and L499 and the phylogenetically closely related *L. vini* were not determined, as *L. vini* does not contain *m*DAP in the cell wall (Table 8). The G+C content of strains L489^T and L499 was 37.8 mol% compared to the 37.7 mol% of *L. nagelii* DSM 13675^T and 40.2 mol% of *L. satsumensis* DSM 16230^T, respectively (Table 8).

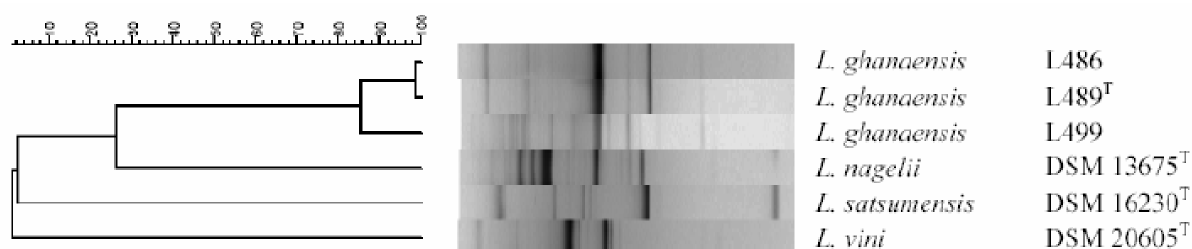


Fig. 23: GTG_5-PCR fingerprints and corresponding dendrogram, derived from UPGMA linkage of correlation coefficients of *Lactobacillus ghanaensis* and related lactobacilli. Appendix IV.

Rep-PCR analysis underlined that strains L486, L489[T] and L499 were genotypically very similar to each other, and different from the phyllogenetically closest neighbours, as they clustered together, away from *Lb. nagelii, Lb. satsumensis* and *Lb. vini* (Fig. 23 and Appendix IV).

Table 8: Phenotypic differentiating features of motile *Lactobacillus* species. *Lactobacillus mali* is non-motile, but phylogenetically closely related to *Lb. ghanaensis* sp. nov. and is thus included for comparison. Appendix IV.

Species: 1. *Lb. ghanaensis* sp. nov.; 2. *Lb. nagelii*; 3. *Lb. satsumensis*; 4: *Lb. vini*; 5: *Lb. agilis*; 6: *Lb. ruminis*; 7: *Lb. curvatus* subsp. *curvatus*; 8: *Lb. mali*. Data partially adopted from Torriani et al. (1996), Edwards et al. (2000); Kato et al. (2000); Hammes and Hertel (2003); Endo and Okada (2005) and Rodas et al. (2006). +, positive; -, negative; w, weak; d, strain dependent; ND, no data.

Characteristic	1	2	3	4	5	6	7*	8§
Growth in MRS at 15 °C	w	+	+	-	-	-	+	+
Growth in MRS at 45 °C	+	+	+	+	+	d	-	-
Growth in MRS with 6.5 % NaCl	-	+	+	+	ND	ND	ND	-
Growth at pH = 3.9 (MRS)	w	+	+	+	ND	ND	ND	ND
Growth at pH = 8.0 (MRS)	-	+	ND	+	ND	ND	ND	-
Lactate isomer	DL	DL	L	DL	L	L	DL	L
Acid from								
L-Arabinose	-	-	-	+	-	-	-	+
D-Cellobiose	+	+	-	+	+	+	d	+
Mannitol	+	+	+	-	+	-	-	-
Raffinose	-	-	-	-	+	+	-	-
L-Rhamnose	+	+	+	-	-	-	-	d
Sorbitol	-/w	+	-	-	d	-	-	-
Dextran from sucrose	-	+	+	-	ND	ND	ND	+
*m*DAP in cell wall	+	+	+	-	+	+	-	+
DNA G+C content (mol%)	37.8	37.7	40.2	39.4	43-44	44-47	42-44	32-34
Percentage 16S rRNA gene similarity to *L. ghanaensis* L489[T]	99.9-100	98.0	95.5	93.7	90.3	92.3	89.9	91.9

* Some strains motile, but lose their motility when subcultured (Torriani *et al.*, 1996).
§ Some strains reported motile (Kaneuchi *et al.*, 1998)

Based on the above data it was concluded that the strains L486, L489[T] and L499 form a homogenous genetical distinct group closest related to *L. nagelii* and *L. satsumensis*. Phenotypically, strains L486, L489[T] and L499 are closely related to *L. nagelii* but the absent growth at pH 8.0 and 6.5 % NaCl; the absent to weak growth at 15 °C and pH 3.9, the inability to produce acid from sorbitol and tagatose (although delayed positive reactions were observed in strain L499) and the absence of dextran-production from sucrose differentiates strains L486, L489 and L499 from *L. nagelii* (Table 8 and Appendix IV).

The results obtained clearly indicated that the strains studied represented a new species in the genus *Lactobacillus*, for which the name *Lactobacillus ghanaensis* sp. nov. has been proposed (Appendix IV).

7.2 Three putatively undescribed yeast species isolated during Ghanaian cocoa fermentations

During the identification of yeasts isolated during cocoa fermentations it turned out that 3 small groups of yeasts all had very low 26S rRNA gene D1/D2-region sequence similarity (81.8-92.4 %) to sequences deposited in Genbank. Neither was it possible to identify these isolates on the basis of their carbohydrate assimilation profile (Kurtzmann and Fell, 1998). Consequently the isolates were tentatively named Unidentified Species A, B and C. Unidentified Species A and B were only isolated from the small heap fermentation, whereas Unidentified Species C was isolated from the outer and central part of a large heap fermentations as well as from the small heap fermentation (Table 3, Table 4 and Appendix III).

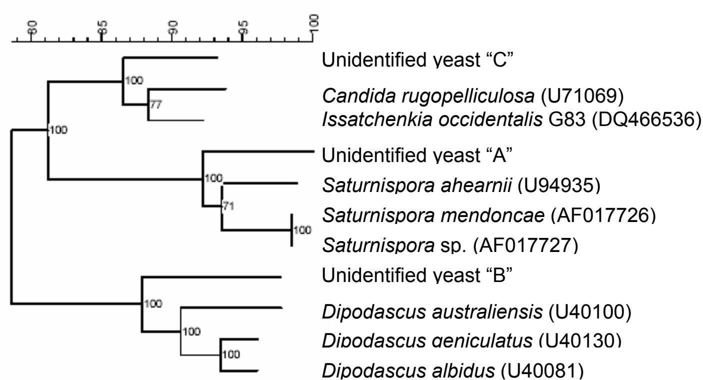


Fig. 24: Phylogenetic tree (Neighbour-joining method) based on almost complete 26S rRNA gene D1/D2 region sequences showing the phylogenetic position of 3 putatively undescribed yeast species isolated from cocoa among closely related yeast species. Bootstrap values (%) based on 1000 replications are stated on each node. D.S. Nielsen, unpublished results.

The 26S rRNA gene D1/D2-region sequences of isolates belonging to Unidentified Species A, B and C and the phylogenetic closest neighbours retrieved from the GenBank database were aligned and a phylogenetic tree was constructed by the neighbour-joining method (Fig. 24). Unidentified Species A was closest related to [26S rRNA gene (D1/D2-region) similarity in brackets] *Saturnispora mendoncae* (92.4 %) and *Saturnispora ahearnii* (88.5 %); Unidentified Species B was closest related to *Dipodascus geniculatus* (81.8 %), *Dipodascus*

albidus (81.0 %) and *Dipodascus australiensis* (80.2 %); and Unidentified Species C was closest related to *Candida rugopelliculosa* (92.3 %) and *Issatchenkia occidentalis* (91.6 %).

The low 26S rRNA gene D1/D2-region sequence similarity to other known species is a very strong indication that Unidentified Species A, B and C represents separate previously not described species (Peterson and Kurtzmann, 1991; Kurtzmann and Robnett, 1998).

At present Unidentified Species A, B and C are undergoing a detailed morphological, bio-chemical and molecular characterisation with the aim giving a detailed description of the 3 putatively new species in a future publication.

8. Conclusion

The present study represents the first investigation of cocoa fermentations taking advantage of recent year's development in molecular biology based methods for typing and identification of microorganisms. Furthermore, using DGGE, the yeast and bacterial communities associated with cocoa fermentation have been investigated using a culture-independent approach for the first time in the present study. Two different fermentation systems (heap and tray) and fermentations carried out at different geographic locations and at different times during the season have been investigated and the succession of microorganisms being involved in the fermentation has been demonstrated.

More specifically the present study has shown that during Ghanaian cocoa fermentations:

- Yeast and LAB reached high cell counts during the first 24-36 hours of fermentation. Later in the fermentation the yeast cell counts decreased, whereas the LAB remained among the dominant organisms throughout the fermentations. Acetic Acid Bacteria showed good growth in the middle phase of the fermentations and in the later phases of the heap fermentations *Bacillus* spp. reached high numbers.
- *Hanseniaspora guilliermondii* and *P. membranifaciens* were the predominating yeasts during early and late fermentation, respectively. Various other yeasts including *C. zemplinina*, *I. orientalis* and *Sc. cerevisiae* played prominent roles as well.
- Three putatively previously undescribed yeast species were isolated during the early phases of fermentation.
- Chromosome Length Polymorphism was evident within all yeast species investigated.
- *Lactobacillus fermentum* was the dominating LAB during all investigated fermentations, with among others *Lc. pseudomesenteroides* and *Lb. plantarum* being isolated frequently as well. Based on the present study and other published studies *Lb. plantarum* and possibly *Lb. fermentum* seems to be indigenous to cocoa fermentations around the World.
- A previously undescribed LAB was isolated from a large heap fermentation and named *Lactobacillus ghanaensis* sp. nov.
- *Acetobacter pasteurianus*, *A. syzygii* and *A. tropicalis* were the predominant AAB during the investigated fermentations.

- *Bacillus licheniformis*, *B. pumilus* and *B. megaterium* reached high numbers during the later phases of heap fermentation.
- No *Bacillus* spp. were detected during tray fermentation
- Turning of heaps influences microbial growth during the fermentations.
- Denaturing Gradient Gel Electrophoresis and culture-based investigations in general corresponded well and DGGE seems thus as an efficient tool for investigating cocoa fermentations.
- Judged from the DGGE-based results *Lc. pseudoficulneum* possibly plays a more important during the fermentations than anticipated from the culture-based results.
- Combined with cluster analysis DGGE offers a tool for monitoring the progress of cocoa fermentations.

9. A broader perspective - recommended future studies

During the present study a good understanding of the microbiology of Ghanaian cocoa fermentations has been obtained through the combined use of culture-dependent and culture-independent methods. It would add a lot to our understanding of cocoa fermentations as such, if fermentations in the other major producing countries such Côte d'Ivoire, Nigeria, Brazil, Indonesia, Malaysia etc. were investigated using the same approach. Furthermore it would be highly interesting to link such investigations to the quality of the final product, chocolate.

The use of culture-independent techniques for the investigation of cocoa fermentations should be further exploited. The present study has pointed at DGGE as an effective tool for the investigation of cocoa fermentations. However, being only semi-quantitative DGGE should be supplemented with quantitative culture-independent methods. For instance DGGE could be used to investigate which organisms are present – and Real Time (RT)-PCR used to quantify these organisms.

Leuconostoc pseduoficulneum was detected using DGGE in most fermentations investigated during the present study. The possible importance of *Lc. pseudoficulneum* during the fermentations could be investigated using RT-PCR. Alternatively a substrate better suited for isolation of *Lc. pseudoficulneum* than MRS should be identified or developed.

Zymomonas mobilis has previously been found to constitute a significant part of the micropopulation during Trinidadian cocoa fermentations. Similarly it should be investigated whether *Z. mobilis* plays a role during Ghanaian cocoa fermentations as well as in cocoa fermentations in other parts of the World.

Batches of cocoa showing an inhomogeneous degree of fermentation is a problem frequently encountered. Based on the more thorough knowledge of Ghanaian cocoa fermentations now obtained it should be considered to investigate the potential use of starter cultures to ensure an uniform degree of fermentation. Furthermore, the potential of using starter cultures for controlling mould growth and mycotoxin production during the fermentation should be investigated.

Tray fermentation is a promising method for uniform fermentation of cocoa, but our microbiological knowledge of the process is still inadequate. The present study has indicated that the microbiology of tray fermentations at least with regards to the yeast community differ from the traditional heap fermentations. How or if this influences the quality of the final product is not known. Furthermore it would be interesting to investigate possible differences between for instance the top tray, a tray in the middle of the stack and the bottom tray.

As pointed out above there are issues left, that should be investigated, but our present knowledge of the primary processing of cocoa is adequate to develop and implement quality management measures based on e.g. the Good Manufacturing Practices (GMP) and Hazard Analysis Critical Control Point (HACCP) principles. However, implementing such quality management measures will not be easy. Alone the process of communicating the principles to the thousands of farmers in Ghana (not to mention the rest of West Africa) is a huge task. Even worse there is no real incentive for the farmers to use the principles as it will mean extra work without any benefits as the system is today, where for instance Ghana operates with 3 standards for cocoa beans: Grade I, Grade II and sub-standard. Grade I is paid better than Grade II etc. The problem is that the beans are graded on the basis of absence of defects – i.e. no visible mould growth, no slaty beans etc. – not on the basis of positive attributes such as a high percentage of well-fermented brown beans with a high flavour potential. As the beans cannot be graded higher than Grade I there is no economical incentive for the farmer already producing Grade I cocoa to further improve the process. The solution is to develop a grading system rewarding beans of good quality – not just beans without defects. Alternatively chocolate producers may consider buying directly from the farmers or groups of farmers that have accepted to follow a given quality management system in the primary processing of cocoa.

Should important markets like the EU or USA decide to impose regulatory limits on the amount of e.g. ochratoxin A in cocoa and chocolate products many cocoa producing farmers and countries will find themselves in troubles with selling a significant portion of their cocoa beans. However, if sound quality management measures are implemented the problem can be controlled if not completely avoided.

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Due to restrictions from the publishers of the journals in which the papers in appendix I-III have been published, these papers are not present in this PDF. The papers can be found in:

Appendix I:
Jespersen, L., Nielsen, D. S., Hønholt, S., & Jakobsen, M. (2005). Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. *FEMS Yeast Research, 5*, 441-453.
DOI: 10.1016/j.femsyr.2004.11.002

Appendix II:
D.S.Nielsen, S.Hønholt, Tano, D., & L.Jespersen (2005). Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). Yeast, 22, 271-284.
DOI: 10.1002/yea.1207

Appendix III:
Nielsen, D. S., Teniola, O. D., Ban-Koffi, L., Owusu, M., Andersson, T. S., & Holzapfel, W. H. (2007). The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. International Journal of Food Microbiology, 114, 168-186.
DOI: 10.1016/j.ijfoodmicro.2006.09.010

Appendix IV:
The article in appendix IV is now published. It is available in a pre-print edition in this PDF. The published article is available here:
Nielsen, D. S., Schillinger, U., Franz, C. M. A. P., Bresciani, J., Amoa-Awua, W., Holzapfel, W. H. et al. (2007). Lactobacillus ghanensis sp. nov., a motile lactic acid bacterium isolated from Ghanaian cocoa fermentations. International Journal of Systematic and Evolutionary Microbiology, 57, 1468-1472.
DOI: 10.1099/ijs.0.64811-0

Appendix I

Occurrence and diversity of yeasts involved in fermentation of
West African cocoa beans

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Appendix II

Yeast populations associated with Ghanaian cocoa fermentations analysed using Denaturing Gradient Gel Electrophoresis (DGGE)

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Appendix III

The microbiology of Ghanaian cocoa fermentations analysed using culture dependent and culture independent methods

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Appendix IV

Lactobacillus ghanaensis sp. nov., a novel, motile lactic acid bacterium isolated from Ghanaian cocoa fermentations

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Lactobacillus ghanaensis sp. nov., a novel, motile lactic acid bacterium isolated from Ghanaian cocoa fermentations

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The Genbank accession no. for the 16S rRNA gene sequences determined in this study are: L486 (DQ867003), L489^{T} (DQ523489) and L499 (DQ867004).

Abstract

Three Gram-positive, catalase-negative, motile, rod-shaped strains L486, L489^T and L499, were isolated from fermenting cocoa. The organisms produced DL-lactic acid from glucose without gas formation. Ammonia was not produced from arginine. Acid was produced from amygdalin, D-cellobiose, esculin, D-fructose, D-glucose, D-galactose, D-mannitol, D-mannose, N-acetylglucosamine, L-rhamnose, D-sucrose, salicin and D-trehalose. The cell wall contained peptidoglycan of the D-*meso*-diaminopimelic acid (*m*DAP)-type. 16S rRNA gene sequence analysis revealed that the isolates phylogenetically belong to the genus *Lactobacillus* and are closely related to *Lactobacillus nagelii*, *Lactobacillus vini* and *Lactobacillis satsumensis*. Low DNA-DNA reassociation values were obtained between the isolates and the phylogenetically closest neighbours. Based on the genetic and phenotypic results, the isolates are considered to represent a novel species, for which the name *Lactobacillus ghanaensis* is proposed. The type strain is L489^T (= DSMZ 18630^T = CCUG 53453^T).

Cocoa beans, the principal raw material of chocolate, have to be fermented, dried and roasted to obtain the characteristic cocoa flavour and taste. The fermentation of cocoa is a microbiologically complex process involving the activities of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (Schwan and Wheals, 2004). During an investigation of the microorganisms involved in the fermentation of cocoa beans, a number of isolates with unusual properties were isolated from MRS agar and tentatively identified as *Lactobacillus* spp. (Nielsen *et al.*, 2006). This study presents the morphological, biochemical and molecular characterisation of 3 of these isolates designated L486, L489^T (= DSMZ 18630^T = CCUG 53453^T) and L499 (= DSM 18631 = CCUG 53454).

Strains L486, L489^T, L499 and the reference strains *Lactobacillus nagelii* DSM 13675^T, *Lactobacillus satsumensis* DSM 16230^T and *Lactobacillus vini* DSM 20605^T were grown in MRS-broth at 30 °C (Merck, Darmstadt, Germany) for 2-3 days, added 20 % glycerol and stored at -80 °C.

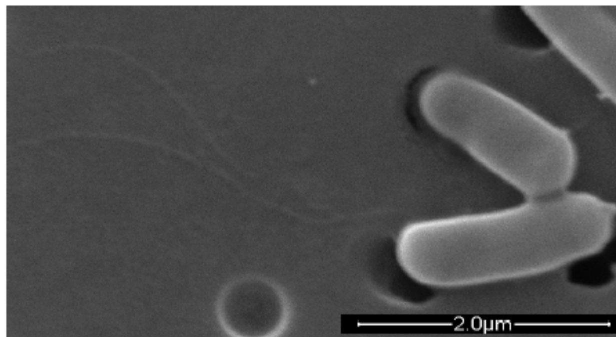

Fig. 1: Cells and flagella of *Lactobacillus ghanaensis* sp nov. L489^T grown over night in MRS broth at 30 °C. Scanning Electron Microscopy, 40000×magnification. Bar represents 2 μm.

Cell morphology of cultures grown over-night in MRS (30 °C) was determined using phase-contrast microscopy and scanning electron microscopy (SEM). For SEM, 30 μl of a culture diluted 10 times with sterile MilliQ-water was filtered through a polycarbonate filter (pore size 0.2 μm), exposed to osmium vapour for 1 h and coated with gold-palladium. Cells were observed in a FEI Quanta 200 SEM at 15 kV. Cells were rod-shaped (0.7-0.8 μm × 1.4-2.5 μm) occurring either singly, in pairs or in short chains of 3-4 cells (Fig. 1). The cells were observed to be highly motile under the phase contrast microscope. Peritrichous flagella were observed using SEM (Fig. 1).

Colonies grown on MRS-agar (Merck) incubated anaerobically for 4 days at 30 °C were 2-3 mm in diameter, white to creamish white, smooth, circular, convex and with entire or slightly uneven margin. If incubated aerobically (4 days, 30 °C), colonies were pin-point sized.

Gram reaction and catalase activity were tested using standard methods. Growth at 15 and 45 °C (MRS broth), in the presence of 6.5 % NaCl, at pH 3.9 and pH 8.0, gas production from glucose (MRS broth with inverted Durham tubes, determined at 30 °C), production of NH_3 from arginine and the presence of D-*meso*-diaminopimelic acid (*m*DAP) in the cell wall were tested following the protocol of Schillinger and Lücke (1987). The configuration of the lactic acid enantiomer produced was determined enzymatically (Boehringer Mannheim GmbH, Mannheim, Germany) (Schillinger and Lücke, 1987).

Carbohydrate fermentation patterns of L486, L489^T and L499 were determined in microtitre plates following the protocol of Jayne-Williams (1976) and in addition, using the API50 CHL identification system (bioMerieux, Marcy-L'Etolie, France).

For 16S rRNA gene sequencing, DNA was extracted using the method of Björkroth and Korkeala (1996) and the almost complete 16S rRNA gene was amplified using primers 7f and 1510r (Lane, 1991; Björkroth and Korkeala, 1996). All reactions were carried out in a 50 µl volume containing 1.25 U *Taq* DNA polymerase (Amersham Biosciences, Piscataway, NJ, USA), 5 µl 10×PCR reaction buffer (Amersham Biosciences), 200 µM of each deoxynucleotide triphosphate (Amersham Biosciences), 3.0 mM $MgCl_2$ (Amersham Biosciences), 0.1 µM of each primer (DNA Technologies, Aarhus, Denmark), 1 % (vol/vol) formamide (Merck), 0.1 % (wt/vol) Bovine Serum Albumin (BSA, New England Biolabs, Beverly, MA, USA), 20 ng of DNA template and sterile MilliQ water for adjustment of the volume to 50 µl. The PCR reaction was performed under the following thermocycling program: 5 min of initial denaturation at 94 °C, 30 cycles of 94 °C for 90 s, 52 °C for 30 s, 72 °C for 90 s followed by a final elongation step of 72 °C for 7 min. Following purification (Qiagen PCR Purification kit) the PCR products were sequenced in both directions using a CEQ 2000 Automated Sequencer (Beckmann Coulter, Fullerton, CA, USA), and a CEQ 2000 Dye Terminator Cycle Sequencing Quick Start kit (Beckmann Coulter) or sent to a commercial sequencing facility (DNA Technology). Sequences were manually corrected and aligned using Vector NTI Suite 7 (Informax, Bethesda, MD, USA). Closest phylogenetic relatives

were determined by aligning the corrected sequences to 16S rRNA gene sequences in GenBank Database using the BLAST algorithm (Altschul *et al.*, 1997). Strains (GenBank/EMBL/DDBJ accession no. in brackets) L486 (DQ867003) and L489^T (DQ523489) had 100 % identical 16S rRNA gene sequences, whereas L499 (DQ867004) differed in one nucleotide from the former two. The 16S rRNA gene sequences of L486, L489^T, L499 and sequences of the closest phylogentic relatives retrieved from the GenBank database were aligned and a phylogenetic tree was constructed by the neighbour-joining method using Bionumerics version 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). Unknown bases were discarded for the analysis. The statistical reliability of the topology of the neighbour-joining tree was evaluated using bootstrap resampling of the data (1000 resamplings) (Fig. 2). Comparison between the 16S rRNA gene sequence of L489^T with type strains in the GenBank database revealed highest similarities with *L. nagelii* DSM 13675^T (98.0 %), *L. satsumensis* DSM 16230^T (95.5 %) and *L. vini* DSM 20605^T (93.7 %) (Table 1).

Table 1: Phenotypic differentiating features of motile *Lactobacillus* species. *Lactobacillus mali* is non-motile, but phylogenetically closely related to *L. ghanaensis* sp. nov. and is thus included for comparison.

Species: 1. *L. ghanaensis* sp. nov.; 2. *L. nagelii*; 3. *L. satsumensis*; 4: *L. vini*; 5: *L. agilis*; 6: *L. ruminis*; 7: *L. curvatus* subsp. *curvatus*; 8: *L. mali*. Data partially adopted from Edwards *et al.* (2000); Endo and Okada (2005); Hammes and Hertel (2003); Kato *et al.* (2000); Rodas *et al.* (2006) and Torriani *et al.* (1996). +, positive; -, negative; w, weak; d, strain dependent; ND, no data.

Characteristic	1	2	3	4	5	6	7*	8§
Growth in MRS at 15 °C	w	+	+	-	-	-	+	+
Growth in MRS at 45 °C	+	+	+	+	+	d	-	-
Growth in MRS with 6.5 % NaCl	-	+	+	+	ND	ND	ND	-
Growth at pH = 3.9 (MRS)	w	+	+	+	ND	ND	ND	ND
Growth at pH = 8.0 (MRS)	-	+	ND	+	ND	ND	ND	-
Lactate isomer	DL	DL	L	DL	L	L	DL	L
Acid from								
L-Arabinose	-	-	-	+	-	-	-	+
D-Cellobiose	+	+	-	+	+	+	d	+
Mannitol	+	+	+	-	+	-	-	-
Raffinose	-	-	-	-	+	+	-	-
L-Rhamnose	+	+	+	-	-	-	-	d
Sorbitol	-/w	+	-	-	d	-	-	-
Dextran from sucrose	-	+	+	-	ND	ND	ND	+
*m*DAP in cell wall	+	+	+	-	+	+	-	+
DNA G+C content (mol%)	37.8	37.7	40.2	39.4	43-44	44-47	42-44	32-34
Percentage 16S rRNA gene similarity to *L. ghanaensis* L489^T	99.9-100	98.0	95.5	93.7	90.3	92.3	89.9	91.9

* Some strains motile, but lose their motility when subcultured (Torriani *et al.*, 1996).
§ Some strains reported motile (Kaneuchi *et al.*, 1998)

For determination of the G+C content and DNA-DNA hybridisation levels, DNA was extracted from L489^T, L499, *L. nagelii* DSM 13675^T and *L. satsumensis* DSM 16230^T and purified following

the protocol of Marmur (1961) as modified by Stackebrandt and Kandler (1978). Strain L486 was not included in the DNA-DNA hybridisation studies, as it has 100 % 16S rRNA gene homology with L489^T. The G+C content of DNA was determined from the thermal melting temperature (T_m) of DNA using a Varian Cary 100 Bio UV-Visible spectrophotometer (Varian, Palo Alto, CA, USA). DNA-DNA relatedness was determined spectrophotometrically from renaturation rates (de Ley *et al*., 1970). DNA from the strains L489^T and L499 was hybridised to each other and to *L. nagelii* DSM 13675^T and *L. satsumensis* DSM 16230^T. The reassociation values between L489^T and L499 were 92.5 % indicating that the strains are con-specific. Reassociation values of 18-44 % were obtained with *L. nagelii* DSM 13675^T and 0-12 % with *L. satsumensis* DSM 16230^T. All values for DNA-DNA hybridisation studies with the closest relatives *L. nagelii* DSM 13675^{T}and *L. satsumensis* DSM 16230^T were thus well below the 70 % cut-off value that indicates separate species (Wayne *et al*., 1987). DNA reassociation values between L489^T and L499 and the phylogenetically closely related *L. vini* were not determined, as *L. vini* does not contain *m*DAP in the cell wall (Table 1). The G+C content of strains L489^T and L499 was 37.8 mol% compared to the 37.7 mol% of *L. nagelii* DSM 13675^T and 40.2 mol% of *L. satsumensis* DSM 16230^T, respectively (Table 1).

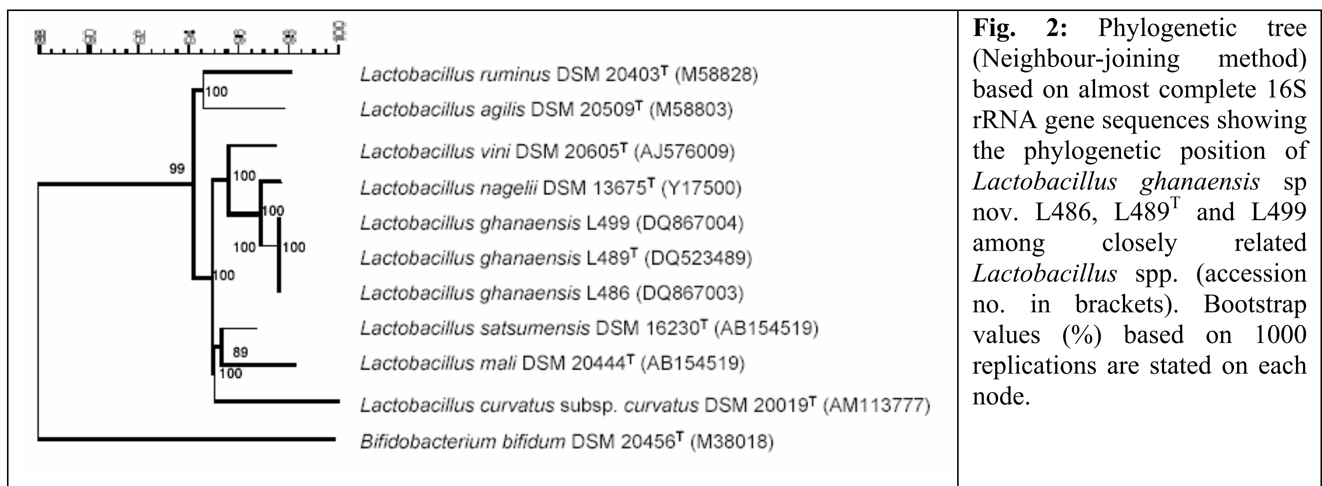

Fig. 2: Phylogenetic tree (Neighbour-joining method) based on almost complete 16S rRNA gene sequences showing the phylogenetic position of *Lactobacillus ghanaensis* sp nov. L486, L489^T and L499 among closely related *Lactobacillus* spp. (accession no. in brackets). Bootstrap values (%) based on 1000 replications are stated on each node.

The three strains were also genotypically investigated by Repetitive Element Palindromic (rep)-PCR using the primer GTG_5 (5'-GTG GTG GTG GTG GTG-3') following the method of Gevers *et al.* (2001) for lactic acid bacteria as previously described (Franz *et al*., 2006). Strains L486, L489^T and L499 clustered closely (r = 85%) and were only distantly related to the type strains of *L. nagelii, L. satsumensis* and *L. vini* (Fig. 3).

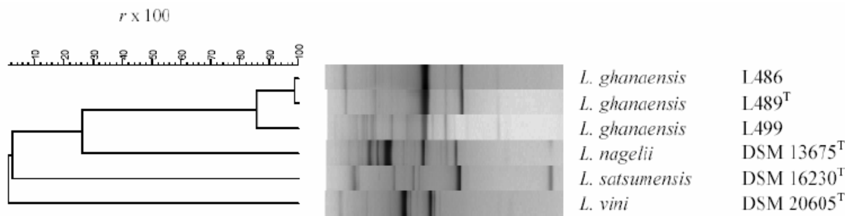

Fig. 3: $(GTG)_5$-PCR fingerprints and corresponding dendrogram, derived from UPGMA linkage of correlation coefficients (*r*, expressed as a percentage value for convenience) of *Lactobacillus ghanaensis* sp. nov. and related lactobacilli.

Based on the above data it can be concluded that the strains L486, L489 and L499 form a homogenous genetical distinct group closest related to *L. nagelii* and *L. satsumensis*. Phenotypically, strains L486, L489^T and L499 are closely related to *L. nagelii* but the absence of growth at pH 8.0 and 6.5 % NaCl and the absent to weak growth at pH 3.9, the inability to produce acid from sorbitol and tagatose (although delayed positive reactions were observed in strain L499) and the absence of dextran-production from glucose differentiates strains L486, L489 and L499 from *L. nagelii* (Table 1).

The results obtained in the present study clearly indicate that the strains studied represent a new species in the genus *Lactobacillus*, for which we propose the name *Lactobacillus ghanaensis* sp. nov. with strain L489^T (= DSMZ 18630^T = CCUG 53453^T) as the type strain.

Description of *Lactobacillus ghanaensis* sp. nov.

Lactobacillus ghanaensis (gha.na.en'sis, N.L. gen. n. *Ghana* named after the country where it was first isolated).

Cells are rod-shaped 0.7-0.8 µm × 1.4-2.5 µm, occurring singly, in pairs or short chains of 3-4 cells. They are Gram-positive, catalase-negative, motile with peritrichous flagella and non-spore forming. Colonies are 2-3 mm in diameter, white to creamish white, smooth, circular, convex and with entire or slightly uneven edges after 3-4 days of anaerobic growth. Weak growth at 15 °C, good growth at 45 °C. No growth occurs at 6.5 % NaCl. Weak growth at pH 3.9 and no growth at pH 8.0. Ammonia is not produced from arginine. No gas is produced from glucose. D(-)- and L(+)-lactic acid is produced as the end product from glucose metabolism. Acid is produced from amygdalin, D-cellobiose, esculin, D-fructose, D-glucose, D-galactose, D-mannitol, D-mannose, N-acetylglucosamine, L-rhamnose, D-sucrose, salicin and D-trehalose. Acid is not produced from D-adonitol, amidon, D- and L-arabinose, arbutin, dulcitol, erythritol, D- and L-fucose, gentiobiose, gluconate, glycogen, inositol, inulin, D-lyxose, D-melezitose, D-melibiose, methyl ß-xyloside, D-raffinose, D-ribose, D-sorbitol, D-turanose, xylitol, D- and L-xylose, 2- and 5-ketogluconate. Acid

production from glycerol, D-lactose, D-maltose, methyl α-D-glucoside (delayed reaction), D-sorbitol (delayed reaction), L-sorbose, and D-tagatose (delayed reaction) is strain-dependent. Does not produce dextran from sucrose. The cell wall contains peptidoglycan of the D-*meso*-diaminopimelic acid (*m*DAP)-type. The G+C-content of the DNA is 37.8 mol%.

The type strain is L489^T (= DSMZ 18630^T = CCUG 53453^T). The type strain and all other known strains of the species have been isolated from cocoa fermentations in Tafo, Ghana. The description of the type strain corresponds to the description the species except that no acid is produced from glycerol, maltose, methyl α-D-glucoside, D-lactose, sorbitol and tagatose.

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