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Full Length Research Paper

Microbial populations during maize storage in Cameroon

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Key microbiota of maize kernels from two farms in Cameroon were characterised at harvest and during five months of drying / storage in firewood kitchens. Moulds populations shifted from *Fusarium* sp. to *Penicillium* and *Aspergillus* during drying, and aflatoxigenic moulds were absent. Lactic acid bacteria (*Lactobacillus plantarum*, *Leuconostoc citreum*) were only present earlier on, and *Enterobacteriaceae* became dominant. Common yeasts were *Candida quercitrusa* (early storage) and *Meyerozyma guilliermondii* (late storage). Strains of *L. plantarum* and *M. guilliermondii* are known to inhibit mould growth and could be used in an energy-efficient system for moist-storage of maize kernels.

Key words: Maize, mycotoxins, mould, yeast, lactic acid bacteria, biocontrol.

INTRODUCTION

Maize is the most widely cultivated cereal crop in Cameroon and other places in sub-saharan Africa. It is a staple food for humans and animals. Together with groundnuts, it is also one of the two crops in Africa at very high risk of mycotoxin contamination in the field and during storage, which is detrimental to human and animal health (Wagacha and Muthomi, 2008). Effective drying of maize and control of insect damage can minimise the risk of mould infection and mycotoxin formation during storage. The primary maize harvest in Cameroon occurs during the rainy season (August to October), after which maize is often dried in huts using the smoke from small fires (firewood kitchens). This technique uses up precious resources of firewood, and can aggravate breathing difficulties among farm workers. In addition, drying facilities may be too small and/or located at distance away from maize fields; thus, space and transport limitations are a 'bottleneck' for any potential increased maize production per hectare, which would otherwise be of great benefit for the small-holding farmers.

It would be advantageous to omit the drying step and store high-moisture maize safely, preferably utilising land near harvest sites. Indeed, such techniques have been extended from silage production and applied in Sweden for the storage of moist-harvested grain in plastic bales or loaves, relying on the preservative activity of lactic acid bacteria (LAB) and/or yeasts naturally present or inoculated onto the grain (Olstorpe and Passoth, 2011). The authors reported that the yeast, *Wickerhamomyces anomalus* (syn. *Hansenula anomala*, formerly *Pichia anomala*) is a particularly effective biocontrol agent in this setting, producing ethyl acetate that inhibits mould growth, inhibiting *Enterobacteriaceae* by an unspecified mechanism, and producing phytase that degrades phytic acid (phytate), which in turn releases soluble phosphorus as well as the trace metals chelated by phytate (calcium, zinc, iron). The value of phytase activity from natural microbiota is already recognised in Cameroon, where dried maize is pre-soaked overnight to allow an initial 'fermentation' before feeding to poultry.

Characterisation of the natural microbiota present in maize is the first step in assessing the potential for nutritional improvement and innovative biocontrol of mould spoilage during storage. This study surveys the LAB, yeasts and moulds associated with maize stored in two farms in Cameroon.

MATERIALS AND METHODS

Maize sampling: Storage, moisture content and climate

The yellow maize cultivar 'acid-tolerant population' (ATP) was

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grown in a farm in Nforya-Bamenda (NB) at 1239 m above sea level, approx. latitude 6° north, longitude 10° east, whereas the white maize cultivar 'Kasai' was grown in a farm in Dschang (D) at 1385 m above sea level, approx. latitude 5° north, longitude 10° east. Maize was 'smoke-dried' and stored according to common local practice by hanging the ears from the rafters of the firewood kitchen. Triplicate samples of kernels were shucked from freshly harvested maize in August 2010, after two weeks, two months and five months of storage. The moisture content was calculated by weighing 10 g samples of maize before and after drying at 85°C for 48 h.

Rainfall and temperature at both locations during harvest and storage were fairly typical for the region, viz, 490, 592, 400 mm in NB, and 232, 357, 474 mm in D during the rainy season in the months of August, September and October, 2010 (two month sampling point). Some amounts of rain fell during November (59 mm in NB and 28 mm in D), but December and January (five month sampling point) were the warmest months because there was no rain. Minimum temperatures were 14 to 17°C, and maximum temperatures were slightly cooler in NB (21°C in August to September, and 24 to 26°C in October to January) than in D (25 to 29°C).

Microbial quantification

Maize samples were pre-soaked in sterile peptone diluent (1% w/v +0.01% Tween) at 2°C overnight, prior to serial dilution, plating/incubation, and enumeration on appropriate media as follows: presumptive LAB on man-rogosa-sharp agar (MRSA) anaerobically at 30°C for three days; *Enterobacteriaceae* on violet red bile glucose (VRBG) agar pour plates at 30°C for 24 h; total aerobic bacteria on tryptone glucose extract agar (TGEA) pour plates at 30°C for three days; and yeasts on yeast peptone dextrose agar at 25°C for two days (Olstorpe et al., 2008).

Endogenous mould infection was assessed by surface disinfection of kernels in sodium hypochlorite solution (equivalent 0.4% active chlorine) for 2 min, and plating 20 kernels on dichloran rose bengal chloramphenicol agar (DG18; 5 kernels/plate) held at 25°C for six days.

Microbial identification

Fingerprinting and rRNA gene amplification of presumptive LAB and yeast isolates

Presumptive LAB and yeasts were identified according to the method of Olstorpe et al. (2008). Briefly, up to 10 representative colonies of LAB or yeast from each sample were purified and polymerase chain reaction (PCR)-typed using the microsatellite primer GTG (5'-GTGGTGGTGGTG GTG-3'). PCR of LAB isolates was performed directly from pure colonies, whereas yeast template DNA (1 µl) was extracted by boiling a single colony in 50 µl of nuclease-free water for 5 min. PCR fingerprint patterns were generated on a 1% agarose gel and compared visually. Strains representing each unique profile were identified by amplifying the 16S rRNA gene in LAB (primers 16Ss / 16Sr) or D1 / D2 region of the 26S rRNA in yeasts (primers NL1 / NL4). Amplicons were sequenced at Macrogen, Korea, and isolates were identified by sequence comparison against the Genbank database.

Mould identification

Representative isolates were purified and preliminary identifications performed based on macro- and micro-morphology. DNA was extracted by the method of Cenis (1992), and selected genes for each isolate amplified as follows: translation elongation factor 1 α in *Fusarium* spp. with primers EF1 / EF2 (O'Donnell et al., 1998); β-tubulin gene in *Penicillium* subgenus *Penicillium* spp. with primers bt2a / bt2b (Glass and Donaldson, 1995); rDNA internal transcribed spacer in all other species with primers ITS1F / ITS 4 (White et al., 1990). Isolates were identified by searching sequence databases in the *Fusarium* database (http://isolate.fusariumdb.org/index.php), Genbank and Centraalbureau voor Schimmelcultures (CBS), with confirmation by microscopy. *Penicillium* subgen. *Penicillium* isolates were further cultivated at 25°C for seven days on malt extract agar (MEA), creatine sucrose agar, yeast extract sucrose agar and Czapek yeast extract agar (also at 30°C) for examination of colony size and appearance (Samson and Frisvad, 2004). These data and the DNA sequences were combined for polyphasic identification using the CBS database (http://www.cbs.knaw.nl/penicillium/BioloMICSID.aspx).

RESULTS AND DISCUSSION

Bacterial counts on maize decreased generally between harvest and two months of storage, probably because the decreasing moisture contents in the maize were unfavourable for survival of these organisms (Figure 1). The reason for the increase in bacterial and yeast counts from NB farm after five months of storage is not apparent, as the maize would be very dry by this stage (2% moisture) and relative humidity would be low during the dry months. The final hygienic status of the grain with regard to bacterial counts was better on D farm (total aerobic bacteria and *Enterobacteriaceae* < log 2 c.f.u./g) than on NB farm (approx. log 5 c.f.u./g); levels on NB farm were slightly above the recommended limits for *Enterobacteriaceae* contamination in dried cereal grains (< log 5 c.f.u./g) (Swedish Code of Statutes, 2006). The majority of bacteria present after five months on both farms appeared to be *Enterobacteriaceae*, as the counts for total aerobic bacteria, presumptive LAB and *Enterobacteriaceae* were roughly equivalent at the final sampling point, and all presumptive LAB isolated on MRSA plates were subsequently identified as *Enterobacteriaceae* (Table 1). Thus, MRSA should not be considered to be solely selective for LAB, and results must be interpreted with care. True LAB such as *Lactobacillus plantarum* and *Lactobacillus citreum* were only present in freshly harvested maize and during the early storage period (up to two months). These species are common on grain (Olstorpe et al., 2010) and if grain moisture is sufficiently high, they can initiate silage-style fermentation in anaerobic conditions (Sundberg, 2007; cited in Olstorpe and Passoth, 2011). Organic acids produced by LAB decrease the pH which preserves the grain from microbial spoilage for a long period, as long as anaerobic conditions are maintained.

Yeast counts on both farms decreased from harvest time until two months later, and then increased again in the five month samples (Figure 1). This could represent a shift in population to species that are more suited to the conditions on the dried maize, or which are best able to compete with the other microbiota present on the surface

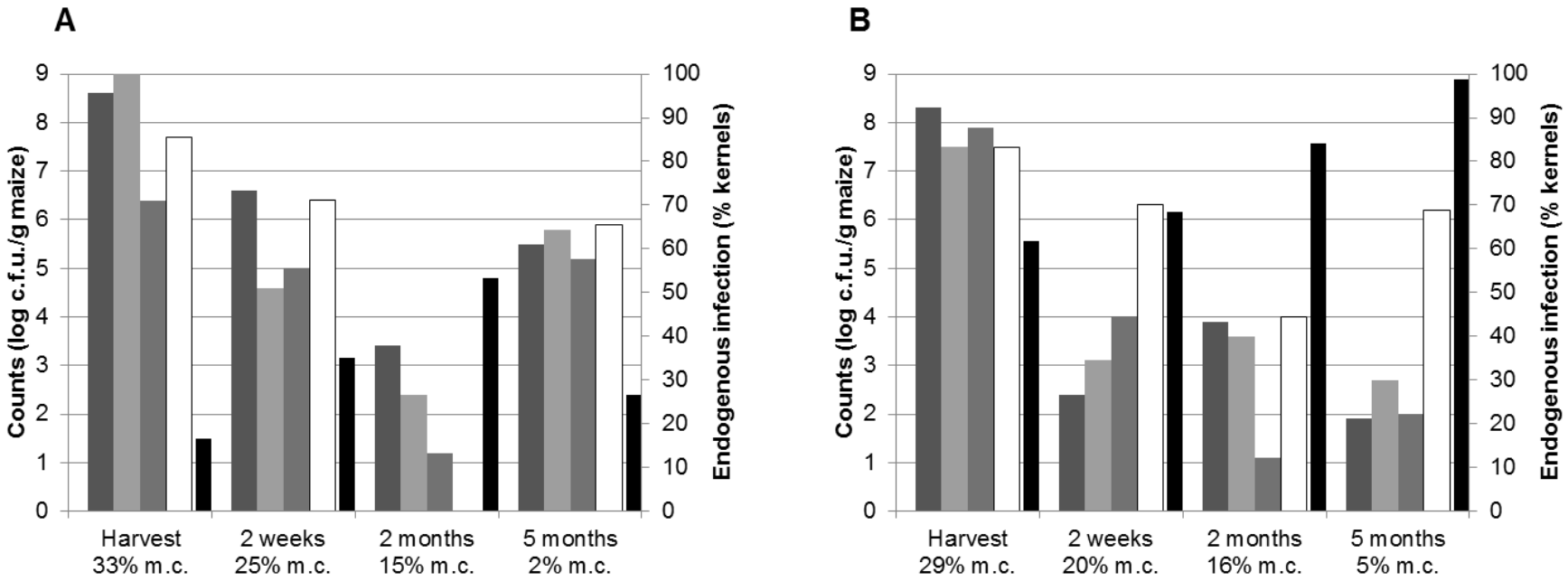

Figure 1. Microbial counts on maize from Nforya-Bamenda farm (A) and Dschang farm (B) during storage. At each sampling point, grey columns from left to right represent: total aerobic bacteria; presumptive lactic acid bacteria isolated on MRSA and *Enterobacteriaceae*, respectively. Yeast counts are shown in white columns. Endogenous infection of kernels with moulds is denoted by narrow black columns, with reference to the secondary *y*-axis. The moisture content (m.c.) of kernels is noted on the *x*-axis.

of the dry kernels. At harvest, *Candida quercitrusa* was dominant on both farms, and other species were also found throughout the early stages of storage (Table 2). The dry-tolerant yeast, *Debaryomyces hansenii* was isolated between two weeks and two months, and *Meyerozyma caribbica* was also common at these stages. The final samples were dominated by the mould-like yeast, *Hyphopichia burtonii* on farm D, and *Meyerozyma guilliermondii* (formerly *Pichia guilliermondi*) on farm NB (also present on farm D). This latter species is of interest because it is a biocontrol agent against mould spoilage on fruits, and marginal inhibition of mould growth on wheat has been observed in a model grain storage system (Schnürer and Jonsson, 2011). A strain of *M. guilliermondii* has been accessioned as J698 into the culture collection at the Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala.

Although, stored maize from D farm had better 'hygiene' than maize from farm NB, that is, D had lower final bacterial counts, the opposite was true with regard to mould infection. This was lower on NB farm and < 55% of kernels remained for the entire storage period, whereas D farm started with 62% of kernels endogenously infected with mould, increasing to 100% at the end of storage. At harvest, farm D had > 25% of kernels infected with *Fusarium verticillioides* and/or *Fusarium meriodionale. Fusarium* sp. commonly infect maize in the field, where *F. verticillioides* can produce fumonisin and *F. meridionale*, which is a close relative of *F. graminearum*, produces the trichothecene toxin, nivalenol (Wagacha and Muthomi, 2008; Sampietro et al., 2011). These species were also present on farm NB, but infection with *Fusarium* was less severe. On both farms, *Fusarium* infection decreased during storage, whereas more xero-tolerant (dry-tolerant) genera such as *Penicillium* were common after two months. Xerophilic *Aspergillus* species, *Aspergillus wentii* and *Eurotium herbariorum* are well suited to growth on dried commodities, and contributed to the 100% infection of kernels on D

Table 1. Presumptive lactic acid bacteria growing on MRSA and yeasts, isolated from maize in Cameroon at harvest (h) and after storage for 2 weeks (2w), 2 months (2m) and 5 months (5m).

Nforya-Bamenda	Presence[a]	Dschang	Presence
Bacteria growing on MRSA			
Lactobacillus plantarum	**H**, **2W**, 2m,–	*Lactobacillus plantarum*	h, 2w, 2m, –
Leuconostoc citreum	**H**, **2W**, –, –	*Leuconostoc citreum*	h, –, – , –
Leuconostoc holzapfelii	(h), –, –, –	*Enterobacter* sp.	h, **2W**, –, 5m
Lactococcus lactis	–, (2w), –, –	*Weissella cibaria*	h, –, –, –
Pediococcus pentosaceus	–, (2w), –, –	*Weissella kandleri*	h, –, –, –
Klebsiella pneumoniae	–, (2w), –, –	*Leuconostoc fallax*	–, (2w), –, –
Weissella cibaria	–, (2w), –, –	*Paenibacillus polymyxa*	–, (2w),**2M**, –
Enterobacter sp.	–, –, **2M**,5m	*Klebsiella pneumoniae*	–, –, –,**5M**
Staphylococcus epidermidis	–, –, **2M**, –	*Klebsiella* sp.	–, –, –,5m
Enterococcus casseliflavus	–, –, –, 5m		
Klebsiella pneumoniae	–, –, –, 5m		
Enterococcus sp.	–, –, –, 5m		
Klebsiella sp.	–, –, –, (5m)		
Enterobacter aerogenes	–, –, –, (5m)		
Citrobacter sp.	–, –, –, (5m)		
Klebsiella oxytoca	–, –, –, (5m)		
Klebsiella variicola	–, –, –, (5m)		
Yeast			
Candida quercitrusa	**H**, 2w, –, –	*Candida quercitrusa*	**H**, 2w, –, –
Candida sorboxylosa	h, (2w), –, –	*Meyerozyma caribbica*	h, **2W**, –, –
Candida cylindracea	h, (2w), –, –	*Candida intermedia*	(h),–, –, –
Candida silvae	h, –, –, –	*Debaryomyces hansenii*	–, 2w,2m, –
Candida intermedia	(h),2w,–,5m	*Acremonium zeae*	–, 2w, –, –
Hanseniaspora sp.	(h),–, –, –	*Candida leandrae*	–, (2w), –, –
Pichia membranifaciens	(h),–, –, –	*Candida zeylanoides*	–, –, 2m, –
Hanseniaspora opuntiae	(h),–, –, –	*Candida pyralidae*	–, –, 2m, –
Candida leandrae	–, 2w, –, –	*Candida intermedia*	–, –, (2m), –
Debaryomyces hansenii	–, 2w, –, –	*Hyphopichia burtonii*	–, –, –, **5M**
Acremonium zeae	–, (2w), –, –	*Meyerozyma guilliermondii*	–, –, –, (5m)
Yamadazyma mexicana	–, (2w), –, –		
Pseudozyma sp.	–, (2w), –, –		
Meyerozyma caribbica	–, –,**2M**, –		
Meyerozyma guilliermondii	–, –, –, **5M**		
Candida pseudointermedia	–, –, –, (5m)		

[a]Dominant species constituting > 40% of isolates have the time point denoted in **bold uppercase**. Common species constituting 10 to 39% of isolates have the time point denoted in normal lowercase. Less common species constituting < 10% of isolates have the bracketed time point. MRSA, Man-rogosa-sharp agar.

farm after five months storage. Aflatoxin-producing species, *Aspergillus flavus* and *Aspergillus parasiticus* were not isolated from any samples in this study, although such species were commonly isolated from stored maize in Uganda (Kaaya and Kyamuhangire, 2006); furthermore, infection of poorly conserved (moist) maize with these fungi has led to outbreaks of acute aflatoxicoses in Africa, causing hundreds of deaths (Lewis et al., 2005).

Conclusions

Mould infection of the maize samples at harvest and during storage varied between the two farms, and probably were affected by cultivar and individual storage conditions. Potentially toxigenic *Fusarium* spp., typically infecting maize kernels in the field, were present at harvest and during the early stages of storage; however, toxigenic storage moulds, for example, *A. flavus*, were

Table 2. Moulds endogenously infecting maize kernels in Cameroon at harvest (h) and after storage for 2 weeks (2w), 2 months (2m) and 5 months (5m).

Nforya-Bamenda	Presence[a]	Dschang	Presence
Fusarium udum	h, (2w), –[b], –	*Fusarium meridionale*	**H**, **2W**, **2M**, –
Fusarium meridionale	(h), –, 2m, –	*Fusarium verticillioides*	**H**, 2w, (2m), (5m)
Fusarium equiseti sp. compl.	(h), (2w), (2m), –	*Fusarium equiseti* sp. compl.	h, 2w, –, (5m)
Fusarium oxysporum	(h), (2w), –, –	*Nigrospora oryzae*	(h), –, 2m, **5M**
Epicoccum nigrum	(h), –, –, –	*Mucor fragilis*	(h), –, –, –
Mucor fragilis	(h), –, –, –	*Fusarium pseudograminearum*	–, 2w, –, –
Fusarium verticillioides	–, 2w, 2m, 5m	*Penicillium crustosum*	–, 2w, –, (5m)
Gibberella fujikuroi sp. compl.	–, 2w, –, 5m	*Penicillium variabile*	–, (2w), –, –
Cladosporium cladosporioides	–, (2w), –, –	*Fusarium boothii*	–, –, 2m, –
Eupenicillium shearii	–, (2w), (2m),–	*Stenocarpella macrospora*	–, –, 2m, –
Penicillium simplicissimum	–, (2w), –, –	*Gibberella fujikuroi* sp. compl.	–, –, (2m), –
Penicillium steckii	–, –, 2m, –	*Penicillium steckii*	–, –, (2m), (5m)
Penicillium citrinum	–, –, 2m, –	*Penicillium fellutanum*	–, –, (2m), (5m)
Penicillium variabile	–, –, (2m), (5m)	*Penicillium citrinum*	–, –, (2m), –
Penicillium glabrum	–, –, (2m), –	*Penicillium glabrum*	–, –, (2m), –
Trichoderma atroviride	–, –, (2m), –	*Aspergillus ostianus*	–, –, (2m), (5m)
Nigrospora oryzae	–, –, (2m), –	*Aspergillus wentii*	–, –, –, **5M**
Stenocarpella macrospora	–, –, (2m), –	*Eurotium herbariorum*	–, –, –, **5M**
Aspergillus caesiellus	–, –, (2m), –	*Hypocrea viridescens*	–, –, –, 5m
Cladosporium tenuissimum	–, –, –, (5m)	*Aspergillus caesiellus*	–, –, –, (5m)
Eurotium herbariorum	–, –, –, (5m)		
Chaetomium sp.	–, –, –, (5m)		

[a]Dominant species endogenously infecting 26 to 50% of kernels have the time point denoted in **bold uppercase**. Common species infecting 5 to 25% of kernels have the time point denoted in normal lowercase. Less common species infecting < 5% of kernels have the time point bracketed.
[b]Additional species infecting single kernels in single samples are not listed in the table. These included *Ampellomyces* sp., *Arthroderma* sp., *Cladosporium herbarum, Eurotium repens, Neiodeightonia phoenicum, Penicillium olsonii, Penicillium paxilli, Penicillium raistrickii* and *Rhizomucor variabilis.*

not isolated in this study. LAB were present on maize kernels during early storage (up to two months), of which *L. plantarum* may be of future interest as a biocontrol agent, because the strains of this species have been reported to display broad spectrum anti-fungal activity (Schnürer and Magnusson, 2005). Among the yeasts, *M. guilliermondi* was dominant during late storage; however, the biocontrol yeast (*W. anomalus*) was not naturally present. Both of them could be inoculated into moist-stored maize, for evaluation and comparison as potential biocontrol agents against mould spoilage in this system.

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