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Abstract

Today one third of all produced food is thrown away and one reason for this is microbial spoilage. Hunger and malnutrition in Africa could be reduced if the food loss is reduced. *Wickerhamomyces anomalus* is a possible biocontrol agent in food science that has shown antifungal and antibacterial effects on, for example, stored barley. The yeast produces ethyl acetate which suppresses mould growth. The mechanism for its antibacterial effect is still unknown.

Sorghum bicolor is the second most important food crop for humans in Africa, which would benefit from a safe, stable storage method. In this experiment, 2 month-stored samples from three different storage methods were compared with each other and with 0 month samples from an earlier analysis, to see which method yielded the safest product, from a microbial hygiene perspective. Storage methods included traditionally open air dried, moist airtight with the yeast *W. anomalus* inoculated, and moist airtight as a control. Quantification and identification of *Enterobacteriaceae*, yeast, moulds and lactic acid bacteria (LAB) were performed by serial dilution on microbiological media, PCR, electrophoresis, DNA sequencing and database comparison.

The result shows clear reductions in mould and *Enterobacteriaceae* amount in both control and inoculated samples compared to the dried sample. The inoculated sample had a significantly reduced amount of moulds in this study. It was also the only sample where the dominant yeast was *W. anomalus*. *W. anomalus* seems to be an efficient biocontrol agent in 2 month moist airtight stored sorghum. No sample was entirely free from mycotoxigenic moulds, and *Aspergillus flavus* and *Penicillium citrinum* were identified in both dried and inoculated samples. *Enterobacteriaceae* amounts decreased during 2 months storage, and previous studies show that there could be further reductions during extended storage. LAB had reduced in all samples and was not detected in the dried sample. Further studies should investigate if harvesting at a higher moisture content and crimping the sorghum could support a higher amount of LAB, which could be favorable for a more rapid reduction of undesirable microorganisms.

Keywords: Wickerhamomyces anomalus, sorghum, storage, biocontrol, biopreservation, food loss

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Abbreviations

C	Control sample
CREA	Creatine Sucrose agar
D	Dried sample
MC	Moisture content
MEAC	Malt extract agar with chloramphenicol
MO	Microorganism
MRS	Man Rogosa Sharp agar
NA	Nutrient agar
VRB	Violet Red Bile agar
W	Sample inoculated with *W. anomalus*
YES	Yeast Extract Sucrose agar

1 Introduction

1.1 Current status in food challenges

We are obliged to increase food production by 60-80% during the next four decades if we want to feed the world's population (*FAO Statistical Yearbook 2012 Africa*). Absurdly, we only consume about two thirds of what we grow today (FAO, 2011). If food loss is reduced, the pressure to increase food production would decrease. Food loss occurs for many reasons, one of them is the presence of unfavorable microorganisms (MOs) or products produced by them during storage. These MOs cause decreased durability of the food, lowered nutritional values or make the food inedible by breaking down food texture or producing unwanted odors. While affluent people can discard spoiled products and buy new food, other people either cannot afford to buy more or do not have food available (food insecurity). This leads to hunger, nutrition deficiency, illness and in worst case scenario, death. In Africa, hunger is a big issue; one fifth of the population suffers from undernourishment. Problems with hunger are more concentrated to the dry parts of the continent where food production is limited (FAO, 2015).

1.2 Microorganisms and their properties

Most MOs are harmless but some of them can be devastating. Besides food spoilage, certain bacteria can be pathogenic or produce enterotoxins and some fungi can produce mycotoxin, causing either acute or progressive illness (Tham & Danielsson, 2014). MOs could arrive from soil, air, water, animals or humans either pre or post storage. Nutrition, pH, water activity (a_w), temperature and gas proportions are the prime factors affecting MO growth. Most bacteria require a neutral pH around 7 while most fungi grow well at pH 3-8. Lactic acid bacteria (LAB) are exceptions, since they can grow at lower pH. A_w below 0.95 is unfavorable for most bacteria while common spoilage fungi – such as *Eurotium* spp. - can grow at a_w down to 0.75. A_w is reduced when the free water is decreased inside the food.

This occurs by binding water to solutes or by lowering the moisture content (MC). MOs relevant for food spoilage have a temperature optimum between 8 and 45°C; the temperatures at which we store most of our food. Lower temperatures decrease the growth rate while higher temperatures can kill MOs or destroy spores. Many yeast and bacteria are facultative anaerobes while all filamentous fungi require oxygen. The different requirements of these groups of MOs make it possible to develop effective preserving methods. Changing more than one environmental factor usually increases the preservative effect when developing food safety methods (Pitt & Hocking, 1997).

1.3 Biopreservation of grain

Biopreservation includes all kinds of preservation methods using the natural microflora and/or their products. One potential biocontrol agent is LAB. During moist airtight storage of crimped barley in Sweden, they produce lactic acid which is unfavorable for most bacteria. LAB requires a higher MC than fungi, around 30-45%, to be able to grow and suppress other bacteria. The antibacterial effect is derived both from the lowered pH and the fact that LAB overgrow unfavorable bacteria, such as *Enterobacteriaceae* (Olstorpe *et al.*, 2010).

Wickerhamomyces anomalus (*Hansenula anomala*, *Pichia anomala)* is a potential biocontrol agent since it tolerates low a_w, high and low pH, high osmotic pressure and is facultatively anaerobic. The yeast is a common spoilage yeast in silage, since it can consume lactic acid and thereby the preservative effect of lowered pH in silage is lost (Walker, 2011). *W. anomalus* produces ethyl acetate which has been shown to have an antifungal effect, with a production peak during limited oxygen access (Druvefors *et al.*, 2005). Biopreservation with *W. anomalus* in airtight stored moist crimped barley has been shown to reduce the amount of toxigenic moulds. The yeast also suppresses *Enterobacteriaceae* though the mechanism for this is still not understood (Olstorpe *et al.*, 2012). The antibacterial effect on bacterial genera other than *Enterobacteriaceae* has not been investigated during airtight moist grain storage.

The effects of adding *W. anomalus* before airtight storage, combined with the naturally present LAB, reduced undesirable microbial growth in maize from Cameroon. The study showed a more rapid reduction of moulds compared to the moist airtight stored control sample (Niba *et al.*, 2014). Investigations of biopreservation with the yeast *W. anomalus* in grains is still under progress, but the method seems to be efficient on more than one type of grain.

1.4 Sorghum

Sorghum bicolor is the second most important crop after maize in Africa, seen in a quantitative aspect (FAO, 1995). In Africa, sorghum is mainly used as human food compared to developed areas where it is used as animal feed (Hulse, 1980). What characterizes sorghum and makes it a good crop in the poor and arid parts of Africa is its tolerance to drought and water logging (Taylor, 2003). Compared to maize, sorghum has a lower yield but is more resistant to drought because of smaller stomata and the ability to penetrate further down in the soil (Assefa *et al.*, 2013). Spoilage fungi in sorghum grains usually derive from the mould genera *Aspergillus*, *Fusarium* and *Penicillium* and differ pre and postharvest (FAO, 1995). The moulds are present when the moisture content is above 13% inside the grain (Pitt & Hocking, 1997). Bacteria are not considered to cause much food loss in stored sorghum (FAO, 1995) but the presence of *Enterobacteriaceae* could give an indication of the hygiene of the grains (van Schothorst & Oosterom, 1984).

1.5 Hypothesis and aims

Adding *W. anomalus* to sorghum before moist airtight storage could be a way to improve food safety of the grain and thereby secure food availability for developing parts of Africa. During this experiment, the same biopreservation method previously tested for maize will be applied to sorghum from Cameroon. The aim of this experiment is to test the effect of *W. anomalus* on stored sorghum and evaluate if it is a possible biocontrol agent when applied at the same inoculum levels as on maize. The MOs in focus are bacteria and fungi. The experiment will enumerate yeast, mould, LAB and *Enterobacteriaceae* on 2 month samples from dried, airtight, and inoculated airtight storage. The result will be also compared with already analyzed data from 0 month stored samples. This project is part of a continuing trial in which the sorghum grains are stored and analyzed after 5 and 8 months storage as well (Swedish Science Council Uforsk project “Secure and sustainable cereal storage for small-holding farmers based on biopreservation and nutritional improvement by microorganisms” SWE-2012-099). The analysis is made from a biopreservative efficiency perspective only, no complete food safety or storage costs are considered.

2 Material and Methods

2.1 Sample preparation

Harvested sorghum of cultivar *Sorghum bicolor*, locally called Jigari, had been stored in three different ways in Cameroon. The crop was stored in batches of 33 kg. In the 'traditionally dried' sorghum, grains were spread out in the open air (D) then stored in a tarpaulin bag. Moist airtight inoculated sorghum was mixed by hand with 1×10^5cells of *W. anomalus* J121 per g sorghum as described in (Niba *et al.*, 2014) (W). Moist airtight control (C) was prepared as W but inoculated with 0.1 % peptone water instead of yeast. C samples were prepared first to avoid contamination. C and W samples had been divided into triplicates of 11 kg each and stored in 20 liter airtight plastic drums. After 2 months storage the samples was collected, their moisture content was measured using a handheld Wood Moisture Meter (MD 7820, Sanpometer, Guangdong, China), and they were sent to Sweden for analysis. The water content of the samples after 2 month storage was 16.5, 29.5 and 30.0% for D, C and W respectively.

2.2 Sample pH

A layer of kernels was put into a 60 ml flask and 20 ml water was added for each of the airtight stored samples, C and W. The suspension was left for 30 minutes and the pH was read in a pH meter (PHM 92 LAB, Radiometer Analytical, Villeurbanne Cedex, France). pH was not measured for D samples since it had not been stored in moist conditions, and so growth and acid production by LAB was not expected.

2.3 Quantification

20 g from each sample triplicate was mixed with 180 g of peptone water (1% Bacto^{BM} Peptone; BD Bionutrients, New Jersey, USA) in double Stomacher bags. The bags were homogenized in a Stomacher 400 Laboratory blender (Seward, Medical, London, UK) for 120 seconds at normal speed. Dilution series were made for each sample and spread onto different media. To quantify yeasts and moulds, 100 µl of expected suitable dilutions were spread onto Malt Extract Agar (MEA, Merck, KGaA., Darmstadt, Germany) with 0.1% chloramphenicol (SIGMA, Merck, KGaA., Darmstadt, Germany) to suppress bacterial growth. The MEAC plates were incubated inverted at 25°C for 2 days for yeast growth, and upright another 5 days for mould growth. For LAB quantification, 100 µl of different dilutions were spread on Man Rogosa Sharp (MRS, Merck, KGaA., Darmstadt, Germany), supplemented with 0.01% of Delvocid (DSM, Heerlen, Netherlands) to suppress fungal growth. The MRS plates were incubated inverted and anaerobically at 30°C for 48 hours. For *Enterobacteriaceae* quantification, pour plating was used with 1.0 ml of different dilutions with Violet Red Bile agar (VRB, Merck, KGaA., Darmstadt, Germany). The VRB plates were incubated inverted at 37°C for 24 hours. C samples were inoculated for an extra 24 hours after counting to see if bacteria without halo developed a halo. Bacteria, yeasts and moulds were enumerated and expressed as cfu/g sorghum.

2.4 Purification

Twenty yeast colonies each from C, D and W were randomly selected for purification on new MEAC plates. Five colonies were purified on each plate and incubated inverted at 25°C for 2-4 days until the colonies were of a suitable size. Four randomly picked VRB-bacteria colonies with halo were plated on nutrient agar (NA, Oxoid, Thermo Fisher Scientific Inc., Waltham, USA) and incubated inverted at 37°C for 24 hours. Two of the picked bacteria colonies were with a distinct halo and two without halo, with the intention to investigate if they all were genera of *Enterobacteriaceae*.

2.5 Polymerase Chain Reaction (PCR)

The bench and all equipment were wiped with 0.4% sodium hypochlorite solution (diluted from 14%, VWR, Radnor, Pennsylvania) to degrade any DNA contaminating the surface. Template suspensions were made for the 20 purified colonies of yeasts, the VRB bacteria and for 20 randomly selected LAB colonies from each sample. An Eppendorf tube was filled with 100 µl of autoclaved water and colony cells were transferred with a sterile toothpick into each tube. The Eppendorf tubes

were mixed thoroughly in the vortex machine. One PCR tube was prepared for every colony. The PCR tube was filled with 24 µl mixture (1:1:12:12) of forward primer and reverse primer [NL1/NL4 (Kurtzman & Robnett, 1997) for yeasts and 16Ss/16Sr for bacteria (Pedersen *et al.*, 2004)], sterile H_2O, and DreamTaq Green PCR Master Mix (Thermo Fisher Scientific Inc., Waltham, USA) containing a buffer, Taq polymerase, dNTPs and loading dye. After mixing, 2 µl sterile water was added into the control PCR tube and 2 µl template suspension was added into each sample PCR tube. The PCR tube was mixed by inverting it several times and centrifuged to collect the PCR mixture in the bottom of the tube. PCR (GeneAmp PCR Sytstem 9700, Thermo Fisher Scientific Inc., Waltham, USA) was performed as follow: The program had an initial denaturation for 5 min at 95°C and then 35 or 30 cycles (for yeasts and bacteria, respectively) with denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C, 1 minute for yeast and 2 min for LAB. The final extension was at 72°C for 10 minutes and the samples were held at 16°C until electrophoresis.

2.6 Electrophoresis

40 ml 1% agarose (Agarose; Sigma-Aldrish Co. LLC., Saint Louis, USA) gel in 0.5× TBE (Appendix 1) was mixed with 40 µl staining Gelred (GelRed™ Nucleic Acid Gel Stain, 10,000× in Water; Biotium, Hayward, USA), poured into a tray and solidified with column shapers for 15 minutes. The agar was put into the 0.5× TBE-filled electrophoresis tank and 3 µl of Generuler (Thermo Scientific GeneRµler 1kb Plus DNA Ladder, ready-to-use; Thermo Fisher Scientific Inc., Waltham, USA), samples and controls were loaded. The electrophoresis (EC 105, Thermo Fisher Scientific Inc., Waltham, USA) was run at 100 V for 30 min. The bands were visualized and documented with a UV-camera (Bio-Rad Gel Doc™ 2000,Bio-Rad Laboratories, California, USA). The yeast and bacteria primers used in this study yielded bands at approximately 600 bp and 1420 bp, respectively. For uncontaminated samples with bands of correct size, 5 µl was transferred into 96-well plates together with 5 µl reverse primer and was sent for sequencing at Macrogen Europe, Amsterdam, The Netherlands. The DNA sequences were compared with data from Genbank (using BLAST, National Library of Medicine, Bethesda, USA) and the MO species were identified based on > 99% sequence identity.

2.7 Mould identification

MEAC plates were screened and all different mould colony types were sub-cultured on suitable agar plates, namely MEA for most colony types, except those

suspected to be *Eurotium* spp. Those were plated on MA20S (Malt Extract Agar with additional 20 % sucrose), a reduced water activity medium which favours growth of *Eurotium* spp. which otherwise grow poorly on high water activity media (Pitt & Hocking, 1997). Moulds that were suspected to be *Penicillium* spp. were additionally plated on Yeast Extract Sucrose agar (YES, Appendix 1) and Creatine Sucrose agar (CREA, Appendix 1). The plates were incubated at 25°C for 7 days. A small sample of both spores and mycelia was transferred to Eppendorf tubes, filled with 500 µl Glucose Yeast medium (GY, Appendix 1). The tubes were left to incubate on a shaker table at 25°C for 3 days and then without shaking at room temperature for another 2 days. The moulds were prepared for PCR according to the method from (Cenis, 1992). PCR, electrophoresis and gene comparison were made according to (Leong *et al.*, 2012). Primers used were EF1/EF2 for suspected *Fusarium* spp., bt2a/bt2b for suspected *Penicillium* spp. and the universal ITS1F/ITS4 for all the other mould types. The specific primers were used to get a better differentiation between the species during sequence comparison.

2.8 Statistical analysis

The quantified microbial counts were statistically analyzed using a t-test to calculate the p value. A p value<0.05 on mean values was considered as significantly different (Englund, Engstrand & Olsson, 2005).

3 Results

Analysis of samples at the beginning of the storage trial (0 months) was performed by Dr Albina Bakeeva. Those results are included here for the purposes of comparison and discussion.

3.1 Quantification

After 2 months storage, the biggest quantified variation was seen between dried and moist stored samples (Table 1). Yeast were significantly different ($p<0.05$) between 0 and 2 month storage within all samples, higher for C and W, lower for D. LAB in dried samples was below detection limit of log 2.0 cfu/g. LAB amounts in C and W samples were reduced ($p<0.05$). *Enterobacteriaceae* counts was reduced in all samples ($p<0.02$). W sample was the only treatment with a significant difference in mould reduction between 0 and 2 month storage ($p<0.001$)

Table 1. Quantification of microbes in log cfu/g grain at 0 and 2 months storage for dried (D), control airtight (C) and inoculated airtight (W) samples

Storage time	Treatment	Microbes			
		Yeast	LAB	*Enterobacteriaceae*	Moulds
0 month	D	6.73±0.21	6.87±0.15	6.19±0.13	5.99±0.07
	C	6.80±0.16	7.24±0.19	6.59±0.31	5.62±0.24
	W	6.49±0.27	7.40±0.24	6.72±0.05	5.48±0.15
2 month	D	5.03±0.05	<2.00[a]	4.36±0.08	6.90±0.17
	C	8.06±0.09	5.08±0.14	3.51±0.09	4.72±0.94
	W	7.86±0.23	4.30±0.36	3.41±0.07	3.10±0.25

[a]No colonies present with detection level (100 cfu/g grain) log 2.0.

3.2 pH

The pH of the airtight stored control and inoculated sample was similar, around pH 6. The results of the pH measurement can be seen in Appendix 2.

3.3 Lactic Acid Bacteria

All colonies present on MRS were counted as presumptive LAB. The non-lab species, *Staphylococcus gallinarum* was the most frequently found bacteria in all harvest samples (Table 2). The only LAB species found in both control and inoculated samples after 2 months was *Weissella paramesenteroides* (*Leuconostoc pseudomesenteroides*).

Table 2. Presumptive lactic acid bacteria present at 0 and 2 months storage for inoculated (W), control (C) and dried (D) samples. Isolate rates are given from the identification of 20 randomly chosen colonies from MRS plates.

	Treatment					
	W		C		D	
Species/Sample month	0	2	0	2	0	2
Staphylococcus gallinarum	20		12		20	
Weissella cibaria			3			
Weissella paramesenteroides		20	2	20		0[a]

[a]No colonies present with detection level (100 cfu/g grain) log 2.0.

3.4 Yeasts

After 2 months, *W. anomalus* was the most frequently found yeast in the inoculated sample only. *Meyerozyma guilliermondii* and *Cryptococcus flavescens* were the most frequently yeasts found in control and dry samples, respectively.

Table 3. Yeast species present at 0 and 2 months storage for inoculated (W), control (C) and dried (D) samples. Isolate rates are given from the identification of 20-30 randomly chosen colonies

	Treatment					
	W		C		D	
Species/Sample month	0	2	0	2	0	2
Cryptococcus flavescens				1	17	13
Cryptococcus flavus					1	
Cryptococcus rajasthanensis			1		10	6
Hyphopichia burtonii				3		
Kodamaea ohmeri			2	3		
Meyerozyma guilliermondii[a]			9	9		

Pichia mexicana				3		
Pseudozyma hubeiensis			1			
Pseudozyma vetiver			3			
Rhodosporidium fluviale						1
Sporisorium lepturi					1	
Ustilago escμlenta			2		1	
Wickerhamomyces anomalus	20	20		1		

[a]Alternatively, the closely related species *M. caribbica* based on >99% similar sequence identity. Additional sequencing is needed to differentiate these two species.

3.5 Moulds

The W samples had no particular mould colony-type as dominant. On C samples, *Hypoxylon duranii* and an unknown *Fusarium* spp. were the most frequently isolated species. The dried sample moulds were already visible and interfering with the yeast colonies after 2 days of incubation, and *Aspergillus niger* was much more frequent than the other species.

*Table 4. Mould species at 0 and 2 months storage for inoculated (W), control (C) and dried (D) samples. All the different colony-morphologies on the agar plates were identified, and if certain colony-types in the 2 month samples were frequently observed, these are noted with *.*

	Treatment		
Storage time	W	C	D
0	*Alternaria longissima*	*Fusarium oxysporum*	*Fusarium oxysporum*
	Fusarium oxysporum	*Fusarium thapsinum*	*Phoma herbarum*
	Fusarium thapsinum	*Phoma herbarum*	*Penicillium citrinum*
	Phoma herbarum	*F. chlamydosporum*	*Phoma glomerata*
	Setosphaeria rostrata	*Fusarium andiyazi*	*Eurotium amstelodami*
		Fusarium circinatum	*Colletotrichum sublineola*
		Penicillium citrinum	*Bipolaris setariae*
2	*Aspergillus flavus*	*Aspergillus niger*	*Aspergillus flavus*
	Aspergillus niger	*Clonostachys rosea*	*Aspergillus niger**
	Fusarium oxysporum	*Fusarium* spp.*	*Aspergillus terreus*
	Fusarium thapsinum	*Hypoxylon duranii**	*Curvularia aeria*
	Geosmithia lavendula		*Eurotium amstelodami*
	Hypoxylon duranii		*Fusarium oxysporum*
	Penicillium citrinum		*Penicillium citrinum*
			Phoma herbarum

3.6 Enterobacteriaceae

Bacterial identification suggested that all colonies with halo were of *Enterobacteriaceae* spp., including the colony-types that developed a halo after an extra 24 hours incubation. Theses colonies (‘late-halo’ colonies) were not included in the enumeration (Table 1), since the method should be consistent to be able to be comparable between and within studies. For example, such colonies were not counted as *Enterobacteriaceae* in the 0 month samples.

4 Discussion

4.1 LAB

The counts of presumptive LAB decreased and the pH did not reach a low level during 2 months' storage, indicating that the storage environment was unfavorable for LAB growth. This could be due to the MC which was on the lower acceptable limit for LAB growth, just around 30%. The non-detectable level of LAB in the dried sample is expected since LAB cannot grow when the MC is 16%.

The amount of LAB after 2 months storage was lower than in previous studies; log 3.10 and log 4.72 cfu/g grain compared to amounts around log 5-6 and log 8 cfg/g grain (Olstorpe *et al.*, 2010; Niba *et al.*, 2014). One method difference from recent studies and this sorghum study is that the grains were not crimped before storage, which could have made the nutrition less accessible for LAB. Maize was not crimped before storage either, but instead the kernels have a thinner shell, making them softer and therefore easier to penetrate. In other words, nutrition availability could be another reason for poor LAB growth.

The pH of the airtight stored samples was around 6 which is equivalent to previous studies on barley, even though the amount of LAB differs (Olstorpe *et al.*, 2010). Adding LAB in the beginning of the storage could be beneficial and lead to lowered pH which was the case in another Swedish study (Borling Welin *et al.*, 2015). Inoculation of LAB into the moist grains before storage showed a pH decrease from 6.0 to 4.6 in 6 weeks. The same study also showed the same pH decrease in the samples without inoculated LAB and no differences in LAB amounts after 2 months. Borling Welin *et al.*'s proposal regarding the beneficial impact of adding LAB early during storage needs further investigations before any conclusions can be made.

S. gallinarum was isolated on MRS plates from the 0 month samples as a presumptive LAB, but it is not a LAB. The enumeration of presumptive LABs from 0 month samples is therefore not reliable – the counts suggest that LAB are quite abundant, but actually, on W samples they form less than 5% of the population

isolated on MRS (less than 0 of 20 randomly chosen colonies). Assuming that most of the 'presumptive LAB' counted on the MRS plates from 0 month samples were not LAB is logical. In previous studies, the proportions of randomly picked and identified colonies from 0 month stored MRS plates have frequently been LAB (Olstorpe *et al.*, 2010; Niba *et al.*, 2014). In this experiment, they most frequently were not. The low amount of naturally occurring LAB from harvest indicates less attractive growth conditions for LAB on the sorghum grains used. *S. gallinarum* seems to be a common bacteria in the microbial flora of grains since it has been isolated from grains in different contexts; southern Chinese *Daqu* and wheat flour from Italy (Minervini *et al.*, 2015; Zheng *et al.*, 2015).

The most frequent isolates from sorghum on MRS plates changed from being *S. gallinarum* to *W. paramesenteriodes* during 2 month storage. *W. paramesenteroide* is typically found in crops (Fusco *et al.*, 2015). The same LAB species has been found in earlier studies. It was present at relatively high amounts before storage of maize, and one colony was identified after a few months storage in barley (Olstorpe *et al.*, 2010; Niba *et al.*, 2014). The most frequently found LAB in maize after 2 months moist airtight storage was *Lactobacillus plantarum* (Niba *et al.*, 2014). It would be interesting to investigate whether *L. plantarum* is better adapted to and more tolerant of the *W. anomalus*-based biopreservation method, or if other factors lead to a higher amount LABs in maize than in sorghum after 2 months moist airtight storage.

4.2 Yeast

The amount of yeast in D samples compared to C and W samples was considerably lower. This was expected because of the low MC in the dried sample. The inoculated sample was the only one with *W. anomalus* as most frequently identified yeast and also had the lowest amount of mould after 2 month storage. This indicates that inoculation of *W. anomalus* on grains before moist airtight storage is effective for reduction in mould amounts in sorghum grains.

4.3 Moulds

As stated, the mould amount declined substantially in both control and inoculated samples after 2 months. Mould amount after further storage of sorghum will be interesting to see, since earlier studies showed both continued reduction and increases after extended storage (Olstorpe *et al.*, 2010; Niba *et al.*, 2014; Borling Welin *et al.*, 2015). Common field flora in our stored sorghum samples included genera previously reported from sorghum from Mexico, e.g. *Fusarium* spp., *Curvularia* spp. and *Alternaria* spp., where *Fusarim thapsinum* was the most frequent-

ly found species (Montes-Belmont *et al.*, 2003). It is therefore logical that this mould was found in our 0 month samples and that some is present after 2 month storage. *F. thapsinum* is not associated with spoilage (Montes-Belmont *et al.*, 2003). Storage mould genera are most commonly *Aspergillus* spp. and *Penicillium* spp. which also was the case in this experiment, seeing that *A. niger* was frequently found in the dried sample. *A. niger* is commonly found on air dried food in warmer climates and recent studies state that the mould possibly produces both ochratoxin A and fumonisins (Palencia *et al.*, 2010).

Even though the amount of mould was reduced with *W. anomalus* present, some of the species present after 2 months were toxigenic. *Aspergillus flavus*, found in both inoculated and dried samples after 2 months storage, is able to produce aflatoxin, which is a hepatotoxin causing liver cirrhosis and inducing tumours in humans and animals, even in very low doses (Bennett & Klich, 2003). Due to the toxicity of aflatoxin, many countries have regulations or guidelines regarding the level of aflatoxins and other mycotoxins in human food and animal feed. Regulations do not seem to exist in Cameroon (FAO, 2003). *Aspergillus terreus* and *Penicillium citrinum* were also identified in the samples and they could produce the nephrotoxin citrinin. Fortunately, none of these toxigenic moulds were dominant in the samples, so toxin-production is only likely to be a problem if the storage conditions are disrupted. If water leaks onto dried stored sorghum, the moisture content could increase, which is favorable for further mould growth. If there is a hole causing oxygen access into the drums or if the inoculated *W. anomalus* dies during airtight storage, moulds could thrive and contaminate the batch. Permissive growth conditions for moulds can give rise to high contamination rates, but not necessarily mycotoxin production. Grains with the same mould amount can have different mycotoxin levels and thus have completely different toxic effects. High humidity and MC speeds up the mycotoxin production (Adams & Moss, 2008). Moist storage in a warm country such as Cameroon is favorable for mould growth, and the airtight storage is an important barrier. The degree of toxicity from the toxigenic moulds in the 2 month stored inoculated sorghum, should be analyzed in further studies, to see if the grains are safe to eat. For quantification of aflatoxins, citrinin, fumonisins, ochratoxin A and other common grain mycotoxins, liquid chromatography/electrospray ionization tandem mass spectrometry (LC-MS/MS) could be used (Sulyok *et al.*, 2007). The quantified mycotoxins should then be compared with values known to be toxic (Bennett & Klich, 2003) and regulation levels from authorities (Regulation (EC) No 1881/2006).

4.4 Enterobacteriaceae

The amount of *Enterobacteriaceae* decreased after 2 months storage for all samples. In the case of control and inoculated samples, LAB could be the reason, since they produce antibacterial compounds, lower the pH and they also overgrow *Enterobacteriaceae*. The dried sample had no detected LABs but instead lower moisture content, giving rise to an unfavorable environment for most bacteria. Storage of sorghum using a moist airtight strategy could be a way to maintain a higher hygienic standard of the grains, as the *Enterobacteriaceae* counts were significantly lower ($P<0.05$) after 2 months storage in moist airtight storage compared with traditional air-dried storage.

It is not surprising that *Enterobacteriaceae* were still detected in all samples after 2 months. In the Swedish study on barley grains, a reduction was shown after 1 month moist airtight storage and then there were no detected *Enterobacteriaceae* after 5 months' storage (Olstorpe *et al.*, 2010). When analyzing the 5 month samples of sorghum, the result will hopefully be similar to Olstorpe *et* al 2010 with a complete reduction in *Enterobacteriaceae* counts in both control and inoculated airtight stored sorghum.

4.5 Methodological evaluation

The size of the batches and the amount of added yeast in this sorghum trial was similar to previous studies on maize in Cameroon (Niba *et al.*, 2014), and seemed to be suitable for favorable yeast growth during this experiment.

The moulds were hard to count and distinguish because of the yeast growth on the agar plates. This could have led to suppression of moulds in the higher concentrated dilutions and a less accurate quantification result. In future studies, moulds could be enumerated on separate MEA plates supplemented with both chloramphenicol and cycloheximide for bacterial and yeast suppression, respectively. Yeast could be grown on MEAC plates only, without any mould suppression since the yeast could be quantified without any interference from the moulds.

The counting of *Enterobacteriaceae* on VRB plates was difficult. Since there was a low concentration of *Enterobacteriaceae*, particles from the sample could interfere with bacterial growth, making it hard to distinguish bacteria from particles. The colonies with clear halos after 24 hours incubation were counted as *Enterobacteriaceae* according to the media manufacturer's instructions (Merck, KGaA., Darmstadt, Germany). The 'late-halo' colonies later shown to be *Enterobacteriaceae* were not included in the counting since the method should be consistent to be able to be comparable between and within studies. Since *Enterobacteriaceae* were not the most central MO family investigated during in this study, this probably did not affect the interpretation of results too much.

4.6 Storage options for Sorghum

Today sorghum is traditionally air-dried, and in this experiment the resulting moisture content of it was 16%; a MC too high for mould growth suppression. One alternative to inoculated moist airtight stored storage could be to dry the grains down to a MC below 13%. The benefit would be that humidity could be kept on a lower level and therefore reduce the risk for mycotoxin production from moulds that are possibly present. Unfortunately, traditional drying did not reach the safe MC level in this trial.

Storing sorghum in moist airtight conditions seems to be effective in reducing both *Enterobacteriaceae* and mould growth. To further optimize the food safety, additional preservative effects from LAB could be combined with the preserving effects of the yeast. LAB was naturally found in the yeast-inoculated treatment, and according to previous studies, it does not suppress the efficiency of *W. anomalus* biopreservation in any way (Borling Welin *et al.*, 2015). A higher LAB concentration could likely give sorghum grains a more rapid drop in the levels of *Enterobacteriaceae*. The storage method could be improved for LAB growth if the grains were crimped and moisture content was increased to around 40 %, by harvesting the grains earlier if possible.

5 Conclusions

There seems to be a positive correlation between adding the yeast *W. anomalus* to sorghum before moist airtight storage and decreased mould growth. The hygienic status – measured in amount of *Enterobacteriaceae* – was also improved with moist airtight storage. The air dried storage treatment is an uncertain method, since it is dependent on weather conditions and the MC might therefore not attain the necessary safety levels. Further studies could try to crimp the grains and have an increased moisture content at the beginning of storage to promote growth of LAB, and investigate if there would then be a more rapid decrease in unfavorable MOs during storage.

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Appendix 1

Media formulations

CREA

In 1000 ml

1.0 g	Creatine (unknown manufacturer)
30.0 g	Sucrose (VWR, Radnor, Pennsylvania)
0.5 g	KCl (Merck, KGaA., Darmstadt, Germany)
0.5 g	$MgSO_4*7H_2O$ (Scharlab, Barcelona, Spain)
0.01 g	$FeSO_4*7H_2O$ (Sigma-Aldrish Co. LLC., Saint Louis, USA)
1.3 g	$K_2HPO_4*3H_2O$ (Merck, KGaA., Darmstadt, Germany)
0.05 g	Bromocresol purple (Merck, KGaA., Darmstadt, Germany)
15 g	Agar (Merck, KGaA., Darmstadt, Germany)

Mix and autoclave at 121°C for 15 minutes.

YES

In 1000 ml

20 g	Yeast extract (Thermo Fisher Scientific Inc., Waltham, USA)
150 g	Sucrose (VWR, Radnor, Pennsylvania)
0.5 g	$MgSO_4*7H_2O$ (Scharlab, Barcelona, Spain)
20 g	Agar (Merck, KGaA., Darmstadt, Germany)

Mix and autoclave at 121°C for 15 minutes.

GY

In 1000 ml

1,0 g	$NH_4(H_2PO_4)$ (Sigma-Aldrish Co. LLC., Saint Louis, USA)
0.20 g	KCl (Merck, KGaA., Darmstadt, Germany)
0.20 g	$MgSO_4*7H_2O$ (Scharlab, Barcelona, Spain)
10.0 g	Glucose (Merck, KGaA., Darmstadt, Germany)
5.0	Yeast extract (Thermo Fisher Scientific Inc., Waltham, USA)
1.0 ml	Trace metals solution (0.5% CuSO4, 1 % ZnSO4) (unknown manufacturer)

Mix and autoclave at 121°C for 15 minutes.

5× TBE (1000 ml)

54 g	Trisbase (unknown manufacturer)
27.5 g	Boric acid (unknown manufacturer)
20 ml	0.5 M EDTA (Merck, KGaA., Darmstadt, Germany)

Mix and autoclave at 121°C for 15 minutes.

Appendix 2

Average pH on 2 month inoculated (W) and control (C) samples.

Sample	W	C
pH	5.97	5.98