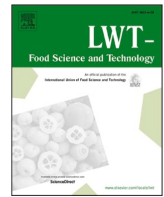
LWT - Food Science and Technology 171 (2022) 113997

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt



Microbiological safety of traditionally processed fermented foods based on raw milk, the case of Mabisi from Zambia

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ARTICLE INFO

Keywords:
Challenge tests
Field survey
Dairy
Traditional uncontrolled fermentation
Raw non-sterile milk

ABSTRACT

For centuries, perishable raw materials such as raw milk have been processed through traditional fermentation. For many, microbiological safety has not been assessed systematically. Here, we study the microbiological safety of an archetypical traditional fermented raw milk product with high cultural importance: Mabisi from Zambia. We focus on traditional processing without the use of defined starter culture – a method that is employed by local small-scale processors at home or at farmer cooperatives. In a field survey, we found that food associated pathogenic bacteria such as *Salmonella* sp. and *Staphylococcus aureus* were present in raw milk used for processing. In processed Mabisi these pathogens were found to be present albeit at levels that fall below common limits of microbiological safety for dairy products. In standardized laboratory experiments, we investigated the microbiological safety of traditionally processed Mabisi using challenge tests with important pathogens related to raw milk and Mabisi. Strains of *S. aureus*, *Salmonella* and *Listeria monocytogenes* could survive fermentation and were present in the end-product after 48 or 72 h of fermentation, yet below legally set thresholds of acceptability. Our work shows that use of traditional processing methods for fermentation of raw milk can result in products that are microbiologically safe.

1. Introduction

Fermentation is a process of transformation of raw materials into processed foods through microbial activity. It has been used for centuries around the world as a way to preserve perishable foods, turning raw materials in products with improved microbiological safety and to achieve desirable organoleptic properties (Marco et al., 2017; M. J. R. Nout, 1994; Smid & Hugenholtz, 2010; Voidarou et al., 2020). Over the last centuries, traditional (or artisanal) fermentation processes have been formalized and upscaled for various products, such as yoghurt, cheese, beer and wine. At the same time, many fermented foods are still produced using traditional processing techniques, where most rely on spontaneous fermentation, while most industrial processes are driven by a defined mix of a few bacterial strains (M.J.R. Nout, Darkar, & Beuchat, 2007).

Fermented foods, including the ones that are traditionally produced, are considered as generally microbiologically safe products. A drop in pH below 4.5 is known to inhibit growth of or kill most pathogens (M.J. R. Nout et al., 2007). The acidification of raw materials and/or the *in situ* production of alcohols and other antimicrobial compounds prohibits the proliferation of pathogenic bacteria (Dimidi, Cox, Rossi, & Whelan, 2019). In addition, fermentation with complex microbial communities may provide intrinsic compositional stability due to interacting species that prevent the proliferation of invader strains, including pathogenic strains. The complex microbial community likely contains natural probiotic strains enhancing the health benefits for their consumption (Butler & O'Dwyer, 2018; Marco et al., 2021). However, spontaneous fermentation using raw materials that may contain pathogenic bacteria poses potential risks for microbiological safety. To protect consumers of fermented foods that are processed through traditional methods, and to inform legal bodies who may consider developing guidelines or standards of traditional processing practice using non-sterile milk and undefined starters, assessments of microbiological safety are essential to ensure safe foods.

Here, we present an assessment of the microbiological safety of Mabisi from Zambia, which is produced at household and -to-medium

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https://doi.org/10.1016/j.lwt.2022.113997
Received 3 March 2022; Received in revised form 26 July 2022; Accepted 16 September 2022
Available online 26 September 2022
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enterprise levels by spontaneous fermentation of raw milk using traditional methods. Mabisi is a non-alcoholic traditional fermented dairy product from Zambia with a high cultural importance (Schoustra, Kasase, Toarta, Kassen, & Poulain, 2013). The traditional process involves spontaneous fermentation of raw milk in gourds over 48 h resulting in a viscous, sour, yoghurt-like product with variations in processing methods in various regions of Zambia (Moonga et al., 2019). The final Mabisi product is usually dominated by 6–12 species of lactic acid and acetic acid bacteria, including *Lactococcus lactis, Leuconostoc mesenteroides* and species of the genus *Acetobacter* (Moonga et al., 2020). Raw milk may be contaminated by pathogenic bacteria (Phiri, Sakumona, Hang'ombe, Fetsch, & Schaarschmidt, 2021) and since traditional Mabisi is made from raw milk (Moonga et al., 2019), there are concerns about its microbiological safety. In the current practice, many small-holder farmers in Zambia deliver their raw milk for sale at a Milk Collection Centre (MCC) where it is subjected to an alcohol test to analyse the freshness quality, which results in accepting or rejecting the raw milk. The rejected raw milk may be partly used to produce Mabisi (Materia, Linnemann, Smid, & Schoustra, 2021; Moonga, Schoustra, Linnemann, Shindano, & Smid, 2022).

In this study, we evaluate the microbiological safety of traditionally processed Mabisi produced by small-scale processors, using a field survey and a laboratory study. Following an earlier survey (Phiri et al., 2021), we conducted a field survey to establish which food-borne pathogens were present in the raw milk that was presented at a Milk Collection Centre and used for Mabisi fermentation. Thereafter, we analysed the levels of pathogens found in traditional Mabisi made from that raw milk. The field survey was used to inform which pathogenic bacteria to include in a controlled laboratory study.

To investigate the risks of contamination of these pathogens in processing contaminated raw milk into Mabisi, we assessed the survival of these pathogens by performing challenge tests (based on the ISO protocol 20976–1:2019) in the laboratory. We chose to use a worst-case-scenario of Mabisi processing and pathogen survival, by adding pathogenic bacteria to the raw milk prior to fermentation at high levels of up to 4 log CFU/mL. We chose a low inoculum (1%) of mature Mabisi from an earlier batch (traditional Mabisi starter culture) leading to a slow acidification rate with pH remaining above pH 5 for at least 24 h. This approach is of relevance to numerous other traditional fermented foods that use a range of non-sterile raw materials and rely on acidification for microbiological safety of final products. It has scientific significance in understanding microbial dynamics during fermentation and its microbial ecology and can help to promote the development of safe Mabisi processing based on traditional processing techniques at house-hold and SME-level.

2. Materials and methods

2.1. Field survey: microbiological quality of raw milk and mabisi

2.1.1. Synopsis and sample collection

To determine the freshness of the milk, the Milk Collection Centre uses an alcohol (75–85%) test. The test is based on the instability of proteins, which can be caused by the acidification of the milk as well as salt concentrates due to mastitis and other causes (Tessema & Tibbo, 2009). In the alcohol test, milk that contains lactic acid in greater than normal amounts coagulates when alcohol is added, and is rejected as not being fresh. Sour milk (rejected raw milk) combined with raw milk left overnight without refrigeration is used for Mabisi production at the Milk Collection Centre using a Tonga-type processing method (Moonga et al., 2019), while fresh milk is used for yoghurt production or direct sales as fresh raw milk.

As the raw milk used for Mabisi production is not heat-treated prior to fermentation (Moonga et al., 2019), it may contain pathogens that can present a food safety concern. We thus conducted a survey at a selected Milk Collection Centre in Zambia to determine the concentration of pathogens in raw milk in Zambia. We focussed on four pathogen groups; Enterobacteriaceae, *Staphylococcus aureus*, *Salmonella* sp., and *Listeria* sp. Over the course of five days in August 2019, ten samples of raw milk were tested, as well as four samples of the Mabisi that was produced using that raw milk (Table 1) (see Table 2).

Full details on methods used for the field survey are in the Methods Supplement.

2.2. Laboratory study: challenge tests

2.2.1. Synopsis

In the laboratory, challenge tests were performed following an ISO protocol 20976–1:2019 to assess the microbiological safety of Mabisi in relation to five food-borne pathogens found in raw milk and mabisi during the field survey, complemented by strains commonly associated to raw milk based products. The challenge test was performed over one Mabisi production cycle of 48 h by adding the food borne pathogens at the start of each fermentation cycle at three levels of abundance and tracking their density during the fermentation cycle. Mabisi used in the challenge test was processed in the laboratory using sterile UHT full fat milk (3.5% fat).

To prepare a microbial starter culture for all fermentations, one representative Mabisi sample was taken from the −80 °C freezer and defrosted. The sample of 1 mL was stored with 0.5 mL 85% glycerol. A defrosted 0.75 mL Mabisi sample was added to 49.5 mL UHT milk. The Mabisi inoculated UHT milk was incubated at 25 °C for 48 h with the cap of the flask slightly left open to ensure oxygen was present in the headspace. After fermentation, the Mabisi was distributed over Eppendorf tubes, by adding 2 mL Mabisi to 0.4 mL 85% glycerol to obtain a final glycerol concentration of 15%. The Mabisi samples were kept frozen at −80 °C until further use and will in this article be referred to as "starter culture". The Mabisi starter culture consists of lactic acid and acetic acid bacteria (Moonga et al., 2020; Schoustra et al., 2013).

For the challenge test, the pathogens we used were *Campylobacter jejuni*, *Salmonella* spp., *Yersinia enterocolitica*, *Listeria monocytogenes* and *Staphylococcus aureus* (Table 3). The pathogens were inoculated in the milk at three different concentrations, low, middle and high (Table 4). These levels are worst case scenario's and not representative for an actual level of contamination in milk, which is expected to be lower. After 0, 7, 24, 31 and 48 h of fermentation the concentration of the

Table 1
Samples used for the field survey.

Sample #	Collection day	Collection time	Type	Alcohol test (75%) result
1	Day 1	Morning	Raw milk	Rejected
2			Raw milk	Accepted
3		Afternoon	Raw milk	Rejected
4			Raw milk	Accepted
5	Day 2	Morning	Mabisi	
6	Day 3	Morning	Raw milk	Accepted
7			Raw milk	Rejected
8			Raw milk	Rejected
9			Mabisi	
10	Day 4	Morning	Raw milk	Accepted
11			Raw milk	Accepted
12			Mabisi	
13			Raw milk	Mixed Rejected + Accepted
14	Day 5	Morning	Mabisi	

Table 2
Media used in this study per bacterium, with its full name and abbreviation.

Bacterium (group)	Medium used	Abbreviation
Campylobacter spp.	RAPID′ Campylobacter agar (Biorad 3564295 + 3564296)	RCA
	Modified Charcoal-Cefoperazone-Deoxycholate agar (Oxoid CM0739 + SR0155E)	mCCDA
Salmonella spp.	Xylose-Lysine-Desoxycholate agar (Oxoid CM0469)	XLD
	Brilliant green agar (Oxoid CM0263)	BGA
Yersinia entercolitica	Cefsulodin-Irgasan-Novobiocin agar (Oxoid CM0990+SR0181)	CIN
Staphylococcus aureus	Baird-Parker agar (Oxoid CM0275+BD 233475)	BP
	Baird-Parker agar with Rabbit plasma fibrinogen (Oxoid CM0275+Biotrading K121F020TW)	BP-RPF
Listeria monocytogenes	Agar *Listeria* according to Ottaviani and Agosti (Biomérieux AEB520079)	ALOA
Total viable counts	Plate count agar (Oxoid CM0325)	PCA
Lactic acid bacteria	De Man, Rogosa and Sharpe agar (Merck VM853561)	MRSA
Enterobacteriaceae	Violet red bile glucose agar (Oxoid CM1082)	VRBGA
Yeasts	Dichloran Rose-Bengal Chloramphenicol agar (Oxoid CM1148+SR0078E)	DRBCA

Table 3
Strains used in experiments. Strains were obtained from the Food Microbiology strain collection at Wageningen University.

Species	Strain name	Reference strain	Source
Campylobacter	*Campylobacter jejuni*	ATCC 33560	Bovine faeces
	Campylobacter jejuni 146	ATCC 29428 WDCM 00156	Human faeces
	Campylobacter jejuni 193	ATCC 33291 WDCM 0005	Human faeces
	Campylobacter coli 194	ATCC 43478 WDCM 0004	Marmoset faeces
Yersinia	*Yersinia enterocolitica 1268/11 6.30+*	Ye0001	Unknown
	Yersinia enterocolitica 1272/11 6.30+	Ye0004	Unknown
	Yersinia enterocolitica	ATCC 9610	Unknown
Salmonella	*Salmonella choleraesuis* subsp. *choleraesuis*	ATCC 13076 DSM 9898	Unknown
	Salmonella typhimurium	Sa0189	Meat - Bovine animal
	Salmonella enteritidis	ATCC 14028 DSMZ 19587	Heart and liver tissues from 4-week-old chicken
	Salmonella enteritidis	Sa0261	Human faeces, RIVM
	Salmonella enteritidis	Sa0303	Milkpowder (animal origin), RIVM
Listeria	*Listeria monocytogenes*	ATCC 23074	Stanford university
	Listeria monocytogenes	ATCC 35152 DSM 12464	Guinea pig, England
	Listeria monocytogenes	ATCC 191188	Unknown
	Listeria monocytogenes	NCTC 11994 DSM 15675	Soft cheese
Staphylococcus	*Staphylococcus aureus*	DSM 346 ATCC 6538 P	Unknown
	Staphylococcus aureus	ATCC 14458	Human faeces
	Staphylococcus aureus	Sc0231	VWA cheese from raw milk (“rauwmelkse kaas”
	Staphylococcus aureus	ATCC 29213	VWA reference strain

pathogen was measured on selective plates, the pH was measured, and after 0 and 48 h the background microbiota (Total viable count) was examined. For relevant comparisons, observed differences were statistically tested for significance using two-sided t-tests.

Table 4
Inoculation density of pathogens of the UHT milk with pathogens at the start of fermentation.

Inoculation level	Pathogen density at start of fermentation (log CFU/mL)
High	4
Middle	3
Low	2
Control	0 (no pathogens added)

Full details on the methods used for the challenge tests are in the Methods Supplement.

3. Results

3.1. Field survey

All samples contained detectable levels of enterobacteria, *Staphylococcus aureus* and lactic acid bacteria (Fig. 1 and Supplement S1). *Salmonella* was not detected in 25 mL of any of the samples. *Listeria monocytogenes* was suspected to be present in 25 mL in sample 8, a sour milk from day 3 and *Listeria* spp was present in sample 12-Mabisi of day 4. Samples 6 and 10 gave unclear results and in the remaining samples *Listeria* was not detected in 25 mL.

For enterobacteria, we expected to find a correlation between bacterial density and the quality of the milk as determined by the alcohol test (Supplement S2 shows experimental results on the correlation of alcohol test values and pH of the raw milk). However, results do not show such correlation, since similar viable cell counts were found in accepted milk, rejected milk and Mabisi. Levels of *S. aureus* counts ranged from 4 log (CFU/mL) in accepted and rejected raw milk to 5 log (CFU/mL) in Mabisi. When *S. aureus* levels exceed 5 log (CFU/mL), there may be a concern of production of toxins, which poses risks for microbiological safety. Thus, our survey suggests *S. aureus* is a safety concern for the production of Mabisi from raw milk. *L. monocytogenes* may have been present in some of the samples, this also points to a potential microbiological safety concern since according to Regulation EC No 1441/2007 the levels of this pathogen should be below 100 CFU/g at consumption (See Supplement S3). We used internationally accepted criteria such as those defined by the European Union for the results interpretation, since the Zambia Bureau of Standards does not specify

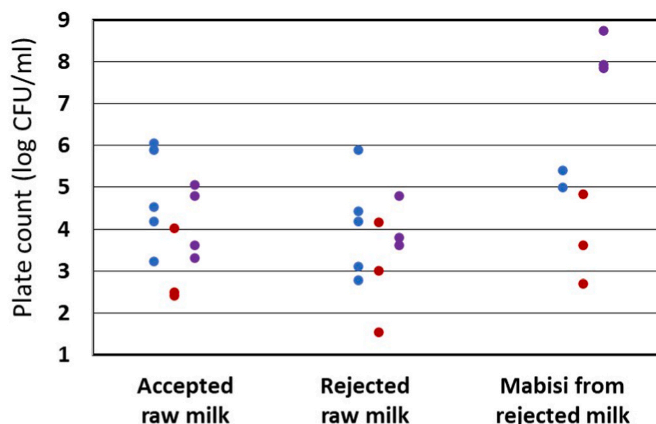

Figure:1. Bacterial counts in samples (n=14) collected during the field survey. Bacterial counts(CFU/mL of enterobacteria (blue symbols), Staphylococcus aureus (red symbols) and lactic acid bacteria(LAB; purple symbols) from samples taken as part of the field survey. Detection limit was 1 CFU/mL). Thegraphs shows bacterial counts clustered per sample type based on the alcohol test: accepted raw milk,rejected raw milk and Mabisi made from the rejected milk. Full results are in Supplement S1.

criteria for products derived from raw milk. These criteria for raw milk are set by the European Commission for mandatory compliance by every country in the European Union. However, countries are allowed to set their own stricter limits. The microbial criteria for various dairy products set by the European Commission can be found in Table S3 (See Supplement S3).

3.2. Challenge tests in the laboratory

3.2.1. Selection of relevant pathogens

Five pathogens were selected for the challenge tests. The results of the field survey showed that *S. aureus* and *L. monocytogenes* are potentially a microbiological safety concern in the processing of Mabisi from raw milk. While our field survey did not detect any *Salmonella*, this bacterium has been reported to be a safety concern in comparable food products, as are *Campylobacter* spp. and *Yersinia* spp. ((Msalya, 2017), Supplement S3; Supplement S4).

3.2.2. pH during fermentation

During fermentations performed in this study, the pH decreased on average from 6.6 to a value of 3.9 in a period of 48 h (Fig. 2). Lactic acid bacteria present in the starter culture starts producing organic acids, which results in a pH decrease. In our experiments, pH remained relatively high for the first 24 h, potentially allowing pathogenic strains to proliferate before pH drops below levels of 4.5. The observed levels of pH reached after 48 h, aligns with previously found pH values in traditional Mabisi made by local processors (Moonga et al., 2019).

3.2.3. Bacterial counts of pathogen persistence

Fig. 2 shows abundance of pathogens used in the challenge test at various time points over the fermentation cycle.

3.2.4. Yersinia enterocolitica

The pH of the milk inoculated with the cocktail of *Y. enterocolitica* strains, decreased from 6.8 to 3.8 during 48 h of fermentation. Our results show that *Yersinia* was able to grow during the first 24 h of fermentation, independent of the inoculum concentrations (3.2, 2.2, 1.2 log (CFU/mL)). Between 24 and 31 h of fermentation, the concentration of viable cells decreased until a final concentration of 0.8, 0.1 and 0.4 log(CFU/mL) was reached at 48 h for a high, middle and low inoculum size, respectively.

The exact dose-response relationship for *Yersinia enterocolitica* is unknown, but it was estimated to be higher than 4 log(CFU/mL) (Ahmed, Tahoun, Abou Elez, Abd El-Hamid, & Abd Ellatif, 2019). The residual concentrations found in Mabisi after fermentation were on average 0.7, 0.1 and 0.3 log(CFU/mL) for the high, middle and low inoculum, respectively, which is lower than 4 log (CFU/ml) (*t*-test, $n = 3$, $P < 0.001$). A consumer with high Mabisi intake would consume at

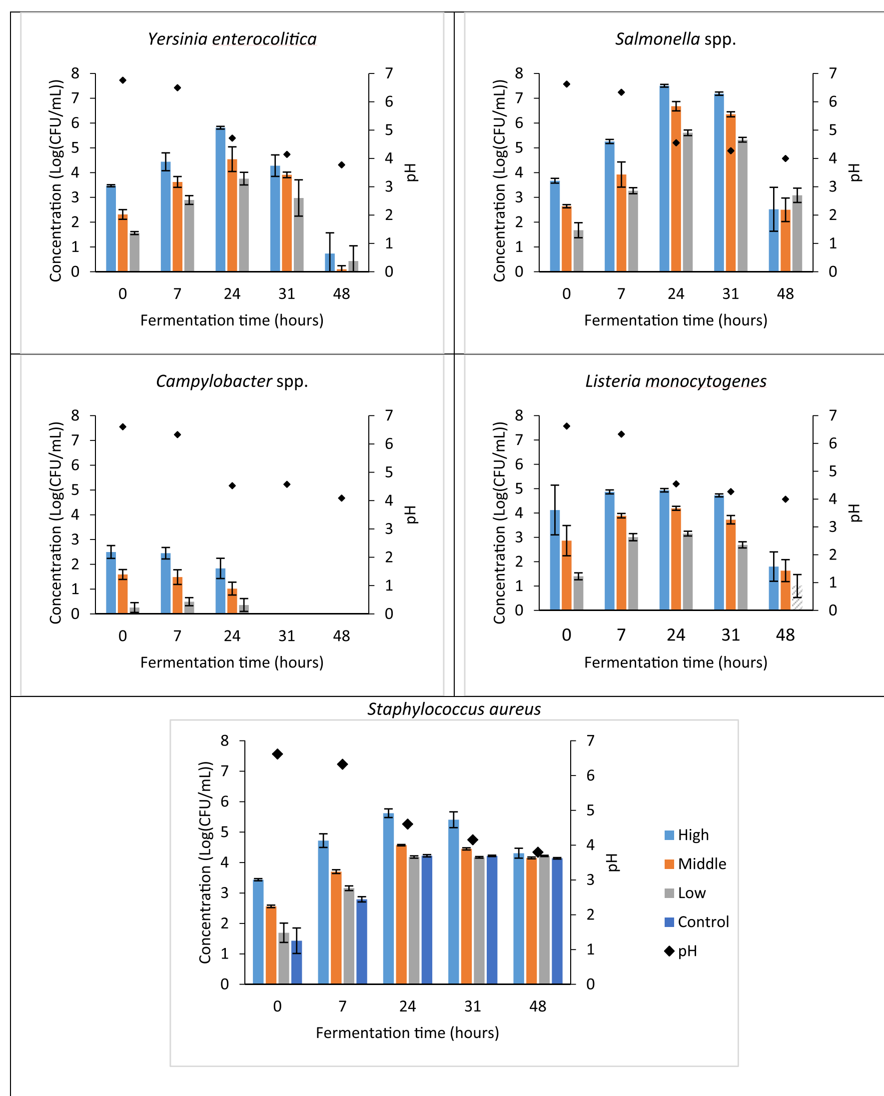

Fig. 2. Concentration of each of the pathogens used for challenge tests (left y-axis) and pH **of Mabisi (right y-axis) during 48 h of fermentation**. Blue bars indicate the high inoculum, orange the middle, grey the low inoculum concentration and dark blue the control treatment (Table 4).Limit of detection was 1 CFU/mL. Error bars show standard error of the mean over 3 replicate measures. A patterned bar indicates samples below the detection limit of 1.3 log CFU/mL (L. monocytogenes); for Campylobacter spp the detection limit was 1 CFU/ml). Black dots depict average pH (right y-axis), error bars of pH were <0.26. n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

maximum 500 g per day, equal to 5000 CFU. This therefore does not seem to be a risk for the consumer, since the infectious dose is higher than the *Y. enterocolitica* concentration and prolonged exposure is not expected. Additionally, there are no regulations laid down in the EU on the presence of *Yersinia* spp. in foodstuffs.

3.2.5. Salmonella spp.

During the fermentation of milk inoculated with the mixture of *Salmonella* strains, the pH decreased from 6.6 to 4.0 and the pathogens were able to grow during the first 24 h (Fig. 2). The low inoculum increased from 1.7 to 5.6, the middle from 2.6 to 6.7 and the high inoculum from 3.7 to 7.5 log(CFU/mL). After 24 h, the concentration of *Salmonella* cells decreased until a final concentration of 2.5, 2.5 and 3.1 log(CFU/mL) for a high, middle and low inoculum concentration after 48 h, respectively.

Outbreak studies calculated the infective doses of *Salmonella* to be between 10^3 and 10^5 (Blaser & Newman, 1982). This is however dependent on various factors like strain, temperature and food matrix. In addition, fat in food products, e.g. fatty foods protect *Salmonella* sp. during stomach transit (Greig, Todd, Bartleson, & Michaels, 2010; Kothary & Babu, 2001). The average concentrations of *Salmonella* after fermentation were 2.5, 2.5 and 3.1 log(CFU/mL) which can possibly be a hazard for consumers.

Regulations in the EU about *Salmonella* spp. in dairy products made from raw milk state that *Salmonella* should be absent in five samples of 25 g (European Commission, Regulation No 2073/2005). The Mabisi produced in this study would not be able to meet this requirement. However, when the fermentation is continued for 72 h, the low and high inoculum concentration samples are below the detection limit of 1 log (CFU/mL) (Supplement S5). To verify if Mabisi would comply to this regulation, the detection limit was lowered to 0 log (CFU/mL) by plating 1 mL of sample, distributed over 3 plates. After 79 h of fermentation some cells were still found in the Mabisi inoculated with 1.4 log(CFU/mL) of the *Salmonella* strain mix, or 22 cells/mL (Supplement S5).

To have a higher probability for a safe Mabisi regarding *Salmonella*, it is recommended to apply a processing method where pH drops soon after start of fermentation for instance by adding a larger amount of Mabisi from a previous batch to start fermentation (Mabisi starter culture), thus preventing outgrowth of *Salmonella* sp. Alternatively, an approach could be to ferment the milk for 3 days instead of 2, since it was shown that after 48 h there were considerable concentrations of *Salmonella* present. After 72 h these concentrations were decreased to either below the detection limit or to only a few cells.

3.2.6. Campylobacter spp

None of the *Campylobacter* strains in the test mix survived during fermentation of raw milk into Mabisi. After 31 h of fermentation the *Campylobacter* viable cell count was below the detection limit of 0 log (CFU/mL) (Fig. 2). This observation was made for all tested inoculum concentrations (2.4, 1.4 and 0.4 log(CFU/mL) at t0. It is therefore not likely that C*ampylobacter* is an issue with regards to the safety of Mabisi, if contaminated raw milk is used for fermentation at 25 °C. It is however recommended to ferment for at least 31 h to ensure safe Mabisi concerning *Campylobacter*.

It should be noted that when a temperature higher than 30 °C is reached during fermentation *Campylobacter* might be able to grow if Mabisi is contaminated (Snelling, Matsuda, Moore, & Dooley, 2005) and become a potential hazard for consumers of Mabisi. This can arise when a fermentation vessel is positioned in the sun in the hot season and not put in the shade, inside the house or in a water bath. However, Moonga et al. (2019) found that 91% of the Mabisi produced by interviewed producers was put inside the house during fermentation (Moonga et al., 2019), suggesting that it is unlikely that temperatures higher than 30 °C will be reached in Mabisi fermentation vessels (Moonga et al., 2021).

3.2.7. Staphylococcus aureus

During 48 h of fermentation the pH decreased from 6.6 to 3.8 and the cell counts of *Staphylococci* increased within the first 24 h and were stable after 48 h around 4.2 log(CFU/mL). These observed levels are in line with levels observed in Mabisi during the field survey (Fig. 1).

The selective medium we used detects not only *S. aureus* but also other *Staphylococcus* species from various sources. These other *Staphylococcus* species are present in the Mabisi bacterial community since these were detected when plating the control treatment on the selective medium. Amplicon sequencing of DNA from Mabisi from various sources to profile the species composition (Moonga et al., 2020) did not reveal presence of staphylococci, supporting the assumption that *S. aureus* is not present at levels exceeding 10^5 (0.1% abundance in the total bacterial community) in Mabisi processed from raw milk. In addition, people milking the cows can be carriers of *S. aureus* on their skin thus

adding these bacteria to the raw milk used for subsequent Mabisi processing. The *S. aureus* estimates from the challenge tests may be an overestimation of the actual number of *S. aureus*. Coagulase negative *Staphylococci* from animal skin are in some cases able to form dark colonies which can be wrongly detected as typical *S. aureus* colonies (Devriese, 1981). Well-known coagulase negative staphylococci were found to be *S. epidermidis* and *S. haemolyticus*. Potentially, these coagulase negative *Staphylococci* are present in Mabisi and give false positive results on Baird-Parker (BP) plates. Notably, in the control sample dark colonies were found on the BP plates used for examination of *S. aureus* in Mabisi.

Observed levels of *S. aureus* of up to 4.2 log (CFU/mL) are below, yet close to the commonly used safety threshold of 5 log CFU/g (*t*-test, $n = 4$, $P < 0.001$) (Bryan, Guzewich, & Todd, 1997; Le Marc, Valík, & Medveďová, 2009) at which *S. aureus* is known to potentially produce toxins (Bhunia, 2018). Criteria laid down in Regulation EC NO 1441/2007 b y the EU are in Supplement S3. Since no specific regulations are available for yoghurt-like products produced from raw milk similar to Mabisi, food Category 2.2.3 is most applicable. It states that in a sampling plan of 5 samples, 2 samples are allowed to be between 4 log and 5 log CFU/mL. Due to slow acidification, in the Mabisi inoculated with *S. aureus*, all inoculum concentrations reached levels higher than 4 log CFU/ml after 24 h of fermentation. Therefore, if the samples were taken at this moment, they should have been tested for enterotoxins. This calls for processing protocols that ensure fast acidification to limit the outgrowth of *S. aureus* to levels where toxins could be formed. Further, low levels of pH need to be maintained for at least 24 h to limit the numbers of *S. aureus* and ensure safe Mabisi.

3.2.8. Listeria monocytogenes

During the fermentation, the pH decreased from 6.6 to 4.0 over 48 h (Fig. 2). An initial L. *monocytogenes* cell count of 4.1, 2.9 and 1.4 log (CFU/mL) was measured and a final concentration of 1.8, 1.6 and < 1.0 log(CFU/mL) was found for respectively a high, middle and low inoculum size.

Nero et al. (2009) isolated naturally occurring microorganisms from raw milk and tested the antagonistic effects of these on *L. monocytogenes* (Tamanini et al., 2012). Twenty-five percent of the isolated strains were found to inhibit growth of *L. monocytogenes.* Additionally, a study by Conner et al. (1990) showed that *L. monocytogenes* was inhibited by lactic acid at pH 4.5. In the first 24 h of fermentation, *L. monocytogenes* was able to grow, but after 24 h the viable plate count decreased (Fig. 2). This is as expected since *L. monocytogenes* has a minimum pH for growth of 4.2. Between 24 and 31 h of fermentation, the pH drops below 4.5, suppressing the growth of *L. monocytogenes (*Fig. 2*)*.

Regulation EC No 1441/2007 lays down microbiological criteria for various food matrices in different stages in the food chain. A distinction is made between foods able and unable to support growth of *L. monocytogenes*, where a food unable to support growth has a pH $\leq$ 4.4. Therefore Mabisi can be categorized as a food considered to be unable to

support growth of *L. monocytogenes*. The limits for *L. monocytogenes* are laid down in Category 1.3 of Regulation EC No 1441/2007 (See Supplement S3). A sample taken to test for the presence of *L. monocytogenes* should not contain more than 2 log(CFU/mL). If the product contains more cells, the Mabisi should be considered as unsafe for consumption. In this study, the *L. monocytogenes* cell counts after 48 h of fermentation were on average below 2 log(CFU/mL) for the low inoculum treatment (*t*-test, n = 3, P < 0.001) and the samples can thus be considered safe. To ensure the safety of Mabisi, it is recommended to ferment the milk for at least 48 h to reduce the *L. monocytogenes* concentrations to lower than 2 log(CFU/mL).

3.2.9. Background flora

For each of the challenge tests, we assessed densities of bacteria (total viable count and lactic acid bacterial count) and yeast at the start and at the end (t = 48 h) of each fermentation cycle. Since similar trends were observed for the background flora found for every sample, the results of fermentations with inoculated *S. aureus* are shown here (Fig. 3) as an illustration for all tests we performed. The results for counts of background flora in experiments for the challenge tests with other pathogens can be found in the Supplement S6.

During the fermentation cycles, there is an increase in total viable counts (TVC), lactic acid bacteria (LAB) and yeasts. Similar final concentrations were found for TVC and LAB, which might suggest that LAB represent the most important class of all bacteria present in the Mabisi (Moonga et al., 2020; Schoustra et al., 2013). Relatively high numbers of LAB were found in this study, around 9.2 log(CFU/mL) after fermentation. As was mentioned before, this is an important factor in the production of lactic acid and the accompanying drop in pH, which is comparable to numbers found for Mabisi in previous studies. In previous work, Mabisi samples from Zambia were found to have a TVC concentration of approximately 7 log(CFU/mL) when incubating the Mabisi at 25 °C for 24 h during fermentation using the same growth media (Schoustra et al., 2013). The difference between previous results and results in this study could be due to a higher incubation temperature (30 °C) and longer incubation time (48 h) (Moonga et al., 2021). Yeasts were also present in the starter culture of Mabisi we used, reaching a final density of 5.3–5.5 log(CFU/mL).

4. Discussion

The main aim of this study was to assess the microbiological safety of a fermented milk product that had been processed by traditional processors using traditional methods, using a two-step approach. Firstly, we assessed the microbiological quality of raw milk used for traditional fermentation and the resulting final product (Mabisi) in a field survey to select potential pathogenic bacteria that are relevant for microbiological safety of the final product. Secondly, we tracked the fate of relevant pathogens during fermentation under laboratory conditions that mimic the traditional process.

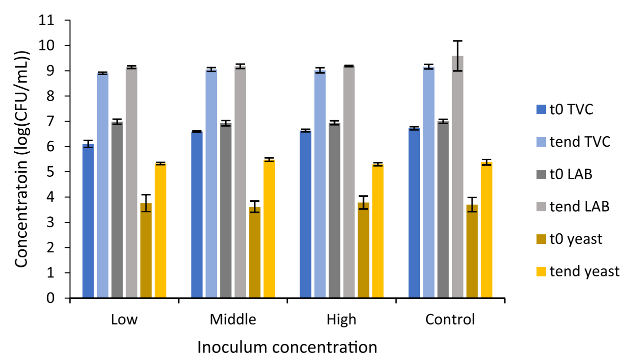

Fig. 3. Background flora measured before and after fermentation in the sample inoculated with a mixture of *Staphylococcus aureus* strains at three levels of abundance (Table 4). Blue bars indicate concentration of total viable counts (TVC), grey bars lactic acid bacteria (LAB) and yellow bars yeasts. Dark coloured bars indicate the concentration before fermentation (t0) and light coloured after fermentation. Error bars show standard error of the mean over three replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The field survey focussing on raw milk supplied to Milk Collection Centres and the Mabisi processed from this milk, revealed that both products contain levels of *S. aureus* that are below, yet approach, acceptable limits. In some samples, *L. monocytogenes* was detected, which is a potential microbiological safety concern. Levels of *Salmonella* sp. were below the detection limit.

The laboratory challenge tests show that *S. aureus* and *Salmonella* sp. are a potential concern for microbiological safety of traditionally processed Mabisi. These tests were done under a worstt-case scenario of pathogen abundance of up to 4 log CFU in raw milk and a high fermentation temperature of 30 °C. Having found that all potential pathogens do not exceed unacceptable levels after 48 h of fermentation (and 72 h in the case of *Salmonella* when present at high inocula of 4 log CFU/g), traditional Mabisi made from raw milk can be considered a safe product if the fermentation is carried out for at least 48 h (or preferably 72 h) at temperatures of 30 °C. The laboratory challenge tests were performed under a worst-case scenario for two reasons. Firstly, the pH remained high (above 5.5) for 24 h, reaching 4.5 after 31 h and 3.9 after 48 h. In our experiments, Mabisi mixed starter was added from a frozen stock, increasing the lag-time of the fermenting microbes in our tests. During traditional processing, fermentation vessels are re-used without cleaning providing a microbial starter, usually leading to a drop in pH below 4.5 within the first 12 h (Groenenboom, Shindano, Cheepa, Smid, & Schoustra, 2020). Secondly, we inoculated pathogens at very high density compared to expected densities of these pathogenic strains in the raw milk used. In addition, contrary to the fermenting microbes from the Mabisi mixed starter, the pathogens were pre-cultured and added from a live culture, giving them a further growth advantage over the fermenting community.

Various interventions can contribute to higher levels of microbiological safety in traditional processing of raw milk to Mabisi. First, while our results suggest that raw milk that is rejected at milk collection centres can result in a microbiologically safe Mabisi product, setting standards for the quality of the raw milk will enhance microbiological safety. Second, a swift decrease in pH is very important since low pH greatly contributes to microbial safety. Swift drop in pH could be achieved by adding a substantial inoculum of a previous Mabisi batch to kick-start fermentation. This would be backslopping that is common for many traditional fermentation processes but not very commonly used for Mabisi (Groenenboom et al., 2020). In that way, pathogens that are present in the raw milk will have less time to proliferate and will be exposed to low pH for a part of a typical 48 h fermentation cycle, both aspects will enhance microbiological safety. At a low pH (under pH 4.5), lactic acid can enter the bacterial cell and dissociate, releasing protons and thus decreasing the internal pH. The protons can be secreted by proton pumps, but this is an energy demanding process, temporarily reducing cell growth. At prolonged exposure to this low pH, this can lead to cell death, since all energy is used up by the organism. This was exemplified in our work by the decline of *Salmonella* after prolonged exposure to low pH conditions. It was shown that most pathogens are not able to grow below a pH of 4.2. However, some more resistant organisms might be able to survive and can continue growth if conditions become more favourable, posing a potential hazard for the consumer. For example, the growth of *Salmonella* sp. is limited at a pH of 3.7, and *S. aureus* at a pH of 4.0 (U.S. Food and Drug Administration, 2011), showing that these bacteria are more resistant to low pH conditions compared to other pathogens.

The applied challenge tests have limitations. The ISO protocol for

challenge tests (ISO 20976–1:2019) mentions that stress can be applied to strains to mimic the environment before and during contamination of the milk. It is known that some pathogens, for example *L. monocytogenes*, can activate an adaptive stress response resulting in more resistance to additional stresses (Wesche, Gurtler, Marks, & Ryser, 2009). This could result in a more resistant pathogenic strain and a higher concentration in Mabisi. Further, while likely common for Mabisi, the fact that our Mabisi starter culture contained bacteria that can grow on the selective medium we used for *S. aureus,* resulted in a less accurate assessment of the risk of this particular pathogen, potentially leading to an overestimation of the microbiological safety risk.

In conclusion, the present work suggests that current traditional Mabisi processing practice using raw milk and spontaneous fermentation leads to a microbiologically safe product, although some potential pathogens approach critical limits. The setting of a minimum quality of the raw milk used and more rapid acidification during fermentation are expected to further have a positive impact. Exact quality criteria and ways to enhance rapid acidification need to be validated in future work (Rampa, Lammers, Linnemann, Schoustra, & de Winter 2020). Results of our work can be important for recognizing that traditional processing of raw milk into Mabisi results in a microbiologically safe food. This can help competent authorities towards legalizing the use of raw milk in traditional fermentation allowing for formalized sales (Materia et al., 2021). (Supplement S7). This would allow current traditional processors to engage in formal sales of Mabisi from traditional small scale processing, which that can enhance the setting up of small-to-medium level enterprises while promoting local culture, nutrition and livelihoods (Moonga et al., 2022). More generally, our approach of showing that traditional fermentation can ensure microbial safety of end products could be applied to similar small-scale traditional fermented foods that rely on spontaneous fermentation.

CRediT authorship contribution statement

Sijmen Schoustra: Writing – original draft, reviewing, Conceptualization, Supervision. **Charlotte van der Zon:** Investigation, Formal analysis. **Anneloes Groenenboom:** Investigation, Formal analysis. **Himoonga Bernard Moonga:** Writing – review & editing, Conceptualization. **John Shindano:** Writing – review & editing, Conceptualization. **Eddy J. Smid:** Writing – review & editing, Conceptualization, Supervision. **Wilma Hazeleger:** Writing – review & editing, Conceptualization, Supervision.

Declaration of competing interest

The authors declare no conflict of interest exist.

Acknowledgements

We thank Dechen Wangmo and Liv van de Ven for their preliminary data and Mr Anthony Munyenyembe at the Zambia Bureau of Standards for his input. We thank various technicians at the University of Zambia and Wageningen University. We further thank the Choma Dairy Cooperative and the Choma District Hospital for their help and willingness to participate in this study.

This work was funded by the Netherlands Organisation for Scientific Research NWO-WOTRO, a Marie Curie Fellowship from the European Union and the Integrated Research Fund (INREF) at Wageningen University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113997.

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