



## Inactivation of bacterial pathogens in yoba *mutandabota*, a dairy product fermented with the probiotic *Lactobacillus rhamnosus* yoba



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### ABSTRACT

*Mutandabota* is a dairy product consumed as a major source of proteins and micronutrients in Southern Africa. In this study the microbial safety of traditional and a variant of *mutandabota* fermented with the probiotic *Lactobacillus rhamnosus* yoba (*yoba mutandabota*) was investigated by challenging the products with five important food pathogens: *Listeria monocytogenes*, *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Bacillus cereus*. Pasteurized full-fat cow's milk was used for producing traditional and *yoba mutandabota*, and was inoculated with a cocktail of strains of the pathogens at an inoculum level of 5.5 log cfu/mL. Survival of the pathogens was monitored over a potential consumption time of 24 h for traditional *mutandabota*, and over 24 h of fermentation followed by 24 h of potential consumption time for *yoba mutandabota*. In traditional *mutandabota* (pH 3.4 ± 0.1) no viable cells of *B. cereus* and *C. jejuni* were detected 3 h after inoculation, while *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* spp. significantly declined ( $P < 0.05$ ), but could still be detected (<3.5 log inactivation) at the end of the potential consumption time. This indicated that consumption of traditional *mutandabota* exposes consumers to the risk of food-borne microbial infections. In *yoba mutandabota*, *L. rhamnosus* yoba grew from 5.5 ± 0.1 log cfu/mL to 9.1 ± 0.4 log cfu/mL in the presence of pathogens. The pH of *yoba mutandabota* dropped from 4.2 ± 0.1 to 3.3 ± 0.1 after 24 h of fermentation, mainly due to organic acids produced during fermentation. Only *Salmonella* spp. was able to grow in *yoba mutandabota* during the first 9 h of fermentation, but then decreased in viable plate count. None of the tested pathogens were detected (>3.5 log inactivation) after 3 h into potential consumption time of *yoba mutandabota*. Inactivation of pathogens in *mutandabota* is of public health significance because food-borne pathogens endanger public health upon consumption of contaminated food, especially in Southern Africa where there are many vulnerable consumers of *mutandabota* such as children, elderly and immuno-compromised people with HIV/AIDS. The findings of this study demonstrate that *mutandabota* fermented with *L. rhamnosus* yoba has antimicrobial properties against the tested pathogens and it is safer compared to the traditional *mutandabota*.

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### 1. Introduction

Probiotic bacteria and their health effects are a focus of international food research. Incorporation of selected strains of the genera *Bifidobacterium* and *Lactobacillus* in milk products and lately in non-dairy products has been studied in detail (McMaster et al., 2005; Østlie et al., 2003; Van Tienen et al., 2011). The beneficial effects of probiotic strains on the host and their mechanism of action have also been demonstrated quite well (Guandalini et al., 2000; Kankainen et al., 2009; von Ossowski et al., 2010). However, little information is available on the survival and growth of pathogens in dairy foods containing probiotic bacteria. Not only good survival of the probiotic bacteria in food

products during their specified shelf life is essential, but also the potential antimicrobial action of the probiotic bacteria against contaminating pathogens during the production process and shelf life is relevant.

*Mutandabota* is a non-fermented, milk-based food consumed daily as a major source of proteins and micronutrients, and it is also sometimes used as a weaning food for infants in Southern Africa (Zimbabwe Ministry of Agriculture, 2001). The product is made by mixing raw cow's or goat's milk 79% (wt/wt), dry baobab (*Adansonia digitata* L.) fruit pulp 14% (wt/wt) and sugar 7% (wt/wt) (Mpfu et al., 2014a). *Mutandabota* has a thick, yoghurt-like consistency, a sour taste and a pH of 3.4 ± 0.1. Generally, low pH products are regarded as microbiologically stable and safe to eat (ICMSF, 2002). However, observations on preparation of traditional *mutandabota* evoked questions about its potential role as a vehicle for food-borne microbial infections. The traditional method utilizes raw milk, which raises a food safety concern since

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the milk may contain pathogenic bacteria like *Salmonella* spp., *Listeria monocytogenes* and *Campylobacter jejuni*, which can cause illness in humans (Kumbhar et al., 2009; Nanu et al., 2007). Coliforms and enterotoxigenic *Escherichia coli* have been isolated in raw milk in Zimbabwe and South Africa (Gran et al., 2002; Ibtisam et al., 2008; Mhone et al., 2011). Preparation of *mutandabota* is carried out at household level in a shaded open space and does not use aseptic techniques. When *mutandabota* is contaminated by pathogens and then consumed, it might cause microbial infection amongst its consumers.

On the basis of *mutandabota*, a variant of *mutandabota* fermented with the probiotic *Lactobacillus rhamnosus* yoba (referred to as yoba *mutandabota*) was developed to enable resource-poor populations in Southern Africa to benefit from a functional food (Mpofu et al., 2014b). *L. rhamnosus* yoba was isolated from a commercially available product, containing *L. rhamnosus* GG. The identity of the isolate was confirmed by 16S rRNA sequencing and the isolate was deposited at the Belgian Co-ordinated Collections of microorganisms/Laboratorium voor Microbiologie Gent (BCCM/LMG) culture collection under the name of *L. rhamnosus* yoba (Kort and Sybesma, 2012). There is evidence of beneficial effects of *L. rhamnosus* GG based on clinical trials with double-blind and placebo-controlled cross-over designs for prevention and treatment of diarrhea and gastrointestinal and upper respiratory tract infections in children (Grandy et al., 2010; Hojsak et al., 2010; Guandalini et al., 2000). For the production of yoba *mutandabota*, a new process was designed based on traditional *mutandabota* preparation procedures. Two major steps were incorporated into the traditional procedure, namely the boiling of raw milk and fermentation with *L. rhamnosus* yoba. Contamination of the product with pathogenic bacteria may occur after the heat treatment; bacterial pathogens have been isolated from pasteurized milk and products from pasteurized milk (Beukes et al., 2001; Gran et al., 2002; Nyatoti et al., 1997). Producing yoba *mutandabota* through fermentation might enhance its microbiological safety. This study was performed to investigate the survival of bacterial pathogens in traditional and yoba *mutandabota*.

## 2. Materials and methods

### 2.1. Preparation of *L. rhamnosus* yoba inoculum

An isolate of the probiotic bacterium *L. rhamnosus* GG, under the name *L. rhamnosus* yoba (Kort and Sybesma, 2012), was used in this study. The bacterium was obtained from Yoba for Life Foundation (<http://www.yoba4life.com>), Amsterdam, The Netherlands. It was stored at  $-80^{\circ}\text{C}$ , before being freeze-dried for long-term storage at  $4^{\circ}\text{C}$  in 50 mL tubes (Greiner Bio-One, BV, Alphen a/d Rijn, The Netherlands). To prepare the inoculum, baobab fruit pulp was added to UHT full-fat cow's milk to a concentration of 4% (wt/wt). Freeze dried *L. rhamnosus* yoba was dissolved in this medium, to a concentration of  $5 \log \text{cfu/mL}$ , in a fermentation vessel that was incubated at  $37^{\circ}\text{C}$  for 24 h. This gave a concentration of approximately  $9 \log \text{cfu/mL}$  *L. rhamnosus* yoba. This culture was sequentially diluted in peptone physiological saline (PPS) with the final dilution  $10^{-2}$  done in UHT full-fat cow's milk to give approximately  $7 \log \text{cfu/mL}$  *L. rhamnosus* yoba. This inoculum was then used for producing yoba *mutandabota*.

### 2.2. Preparation of traditional and yoba *mutandabota*

Traditional *mutandabota* (100 g) was prepared based on the local practice in Binga district, Zimbabwe ( $17^{\circ} 36' \text{S}$ ,  $27^{\circ} 32' \text{E}$ ) (Mpofu et al., 2014a). This was done by gradually adding, while continuously shaking, 14 g of baobab fruit pulp and 7 g of crystalline sucrose to 79 g of UHT full-fat cow's milk in a 250 mL sterile bottle. Manual stirring was continued for 7 min or until a homogeneous mixture was achieved. Yoba *mutandabota* was prepared as illustrated in Fig. 1 (Mpofu et al., 2014b). To prepare 100 g of yoba *mutandabota*, *L. rhamnosus* yoba inoculum (prepared earlier) was added to 78 g of UHT full-fat cow's milk to a

concentration of  $5.5 \log \text{cfu/mL}$ . Dry baobab fruit pulp was then added to give a concentration of 4% (wt/wt) and vigorously mixed. This mixture was left to ferment for 24 h at  $37^{\circ}\text{C}$  in a stationary incubator. After the fermentation step, an additional 10 g of baobab fruit pulp and 7 g crystalline sucrose were added and mixed for 7 min to obtain a homogeneous mixture of yoba *mutandabota*. This procedure enabled attainment of approximately  $9 \log \text{cfu/mL}$  *L. rhamnosus* yoba in yoba *mutandabota*. The product was ready for consumption.

### 2.3. Selection of bacterial pathogens

Five bacterial pathogenic species were selected to evaluate the food safety risk of traditional and yoba *mutandabota*, namely *Campylobacter jejuni*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Bacillus cereus* and *Salmonella enterica*. The pathogen selection was based on expert advice and scientific literature on pathogens identified as causing food-borne microbial illnesses in Southern Africa, particularly Zimbabwe. The selected pathogenic strains were five strains each of *Campylobacter jejuni*, *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Bacillus cereus*. Then three strains of *Salmonella* Enteritidis, a strain each of *Salmonella* Paratyphi B and *Salmonella* Typhimurium. All pathogenic strains except four, were obtained from the Laboratory of Food Microbiology culture collection, Wageningen University, Wageningen, The Netherlands. Four of the *E. coli* O157:H7 strains were obtained from The Netherlands National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

### 2.4. Preparing cocktails of bacterial pathogens

All strains were stored in cryovials in a freezer at  $-80^{\circ}\text{C}$  and were revived by plating. *L. monocytogenes*, *Salmonella* spp., *E. coli* O157:H7 and *B. cereus* strains were plated individually on Brain Heart Infusion (BHI) agar [37 g BHI broth (Oxoid, Basingstoke, UK) and 12 g agar bacteriological (Oxoid), in 1 L of distilled water]. *C. jejuni* strains were plated individually on blood agar [40 g blood agar base (Oxoid) and 7% sterile blood in 1 L of distilled water]. BHI agar plates with either *E. coli*, *L. monocytogenes* or *Salmonella* spp. were incubated aerobically at  $37^{\circ}\text{C}$  for 24 h. Plates with *B. cereus* were incubated aerobically at  $30^{\circ}\text{C}$  for 24 h. Plates with *C. jejuni* were incubated microaerobically at  $41.5^{\circ}\text{C}$  for 48 h. To prepare the inoculum, a colony of each strain was picked from a respective plate and inoculated into BHI broth, with heart infusion (HI) broth (Oxoid) being used for each *C. jejuni* strain. Incubation was done in stationary incubators for 24 h, reaching a bacterial broth culture concentration of approximately  $9 \log \text{cfu/mL}$ . A cocktail of five strains of each species in suspension was produced by mixing equal portions (2 mL) of each strain in a sterile test tube. The three strains of *Salmonella* Enteritidis, a strain each of *Salmonella* Paratyphi B and *Salmonella* Typhimurium were mixed together in equal portions (2 mL) of each strain in a sterile test tube. Each pathogenic bacterial cocktail was then ready for inoculation into *mutandabota*. In total 5 pathogenic bacterial cocktails were produced.

### 2.5. Artificial contamination of traditional and yoba *mutandabota*

A pathogenic bacterial cocktail in the respective broth was serially diluted in PPS, with the final dilution  $10^{-2}$  done in UHT full-fat cow's milk. Of this, 1 mL was inoculated into 79 g UHT full-fat cow's milk that was used to prepare traditional *mutandabota*, and 1 mL was inoculated into 78 g UHT full-fat cow's milk that was used to prepare yoba *mutandabota*. In both traditional and yoba *mutandabota*, the initial concentration of the pathogenic cocktail suspension was approximately  $5.5 \log \text{cfu/mL}$ . For yoba *mutandabota*, inoculation of the pathogenic cocktail suspension was done simultaneously with *L. rhamnosus* yoba inoculation (Section 2.1). Independent triplicate experiments were done with each pathogenic bacterial cocktail. Non inoculated samples (negative controls inoculated with 1 mL full-fat UHT milk) were also

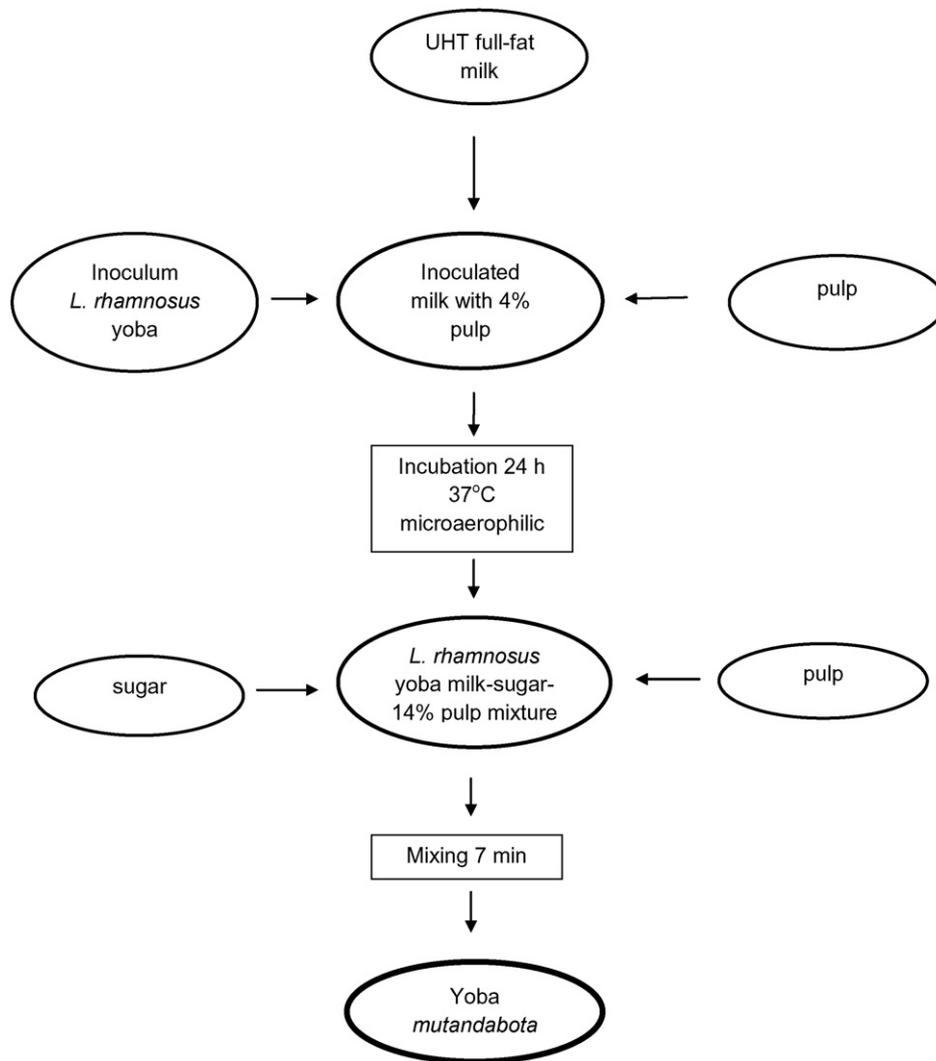


Fig. 1. Flow chart for the production of yoba *mutandabota*.

tested using the above procedure to confirm that no naturally occurring bacterial pathogens under test, or organisms giving similar colonial morphologies to those added to the inoculated products, were present.

#### 2.6. Sampling, incubation and enumeration of bacterial strains

In Southern Africa *mutandabota* is consumed within 24 h after preparation. Therefore the survival of pathogens in both traditional and yoba *mutandabota* was determined over a potential consumption time of 24 h at 25 °C, to simulate the average ambient temperature in Southern Africa where the product is consumed. For traditional *mutandabota*, sampling was immediately done after mixing of ingredients and the inoculum (defined as  $t = 0$ ), and at approximately 3 h intervals until the end of the 24 h storage at  $t = 24$ . For yoba *mutandabota* (Fig. 1), sampling was immediately after mixing of ingredients and the inoculum (defined as  $t = -24$ ), and at approximately 3 h intervals throughout the 24 h fermentation time and the subsequent 24 h storage time until  $t = 24$ . Sequential serial dilutions were made in PPS and plating was done on selective media followed by incubation under appropriate conditions. For *L. monocytogenes*, plating was on Agar Listeria Ottavani & Agosti (bioMerieux, Marcy l'Etoile, France) and incubation was aerobic at 37 °C for 24 h. For *Salmonella* spp., plating was on Xylose–Lysine–Desoxycholate Agar (Oxoid) and incubation was aerobic at 37 °C for 24 h. For *E. coli* O157:H7, plating was on MacConkey agar (Oxoid) and incubation was aerobic at 37 °C for 24 h. For *B. cereus* plating was on

Mannitol Egg Yolk Polymyxin Agar (Oxoid) and incubation was aerobic at 30 °C for 24 h. For *C. jejuni* plating was on Campy Food Agar (bioMerieux) and incubation was microaerobic at 41.5 °C for 48 h. For *L. rhamnosus* yoba, plating was on de Man, Rogosa and Sharpe Agar [MRSA: 12 g agar bacteriological (Oxoid), added to 52.2 g de Man, Rogosa and Sharpe broth (Merck, Darmstadt, Germany) in 1 L distilled water] and incubation was under microaerobic conditions at 37 °C for 24 h. Colonies were enumerated and results were expressed in log cfu/mL of either traditional or yoba *mutandabota*. The detection limit of the plating method was 100 cfu/mL.

#### 2.7. Lactic acid determination

Lactic acid was determined by HPLC. Briefly, samples of traditional and yoba *mutandabota* were deproteinated by adding 0.25 mL cold Carrez A solution (42.2 g  $K_4Fe(CN)_6 \cdot 3H_2O$  per 1 L demineralized water) (Sigma-Aldrich, Steinheim, Germany) to 0.5 mL sample of traditional or yoba *mutandabota* in an Eppendorf tube and mixed, then 0.25 mL of cold Carrez B solution (57.5 g  $ZnSO_4 \cdot 7H_2O$  per 1 L demineralized water) (Sigma-Aldrich, Steinheim, Germany) was added and mixed. The Eppendorf tubes with samples were centrifuged and the supernatant was taken for HPLC analysis (Ultimate 3000, Dionex), using an Aminex HPX-87 H 300 × 7.8 mm column with a pre-column (Biorad). The eluent was 5 mM  $H_2SO_4$  at a flow rate of 0.6

ml/min at 40 °C. Detection was done by refractive index (Shodex RI 101). Sample volume was 10 µL and the run time was 30 min.

### 2.8. pH measurement

The pH values during the experiments were measured using a combined glass electrode pH meter (WTW, Weilheim, Germany) that was calibrated using standard buffer solutions (Merck, Darmstadt, Germany) at pH 4.0 and 7.0. The pH was determined each time a sample was taken for microbial analysis at times indicated above.

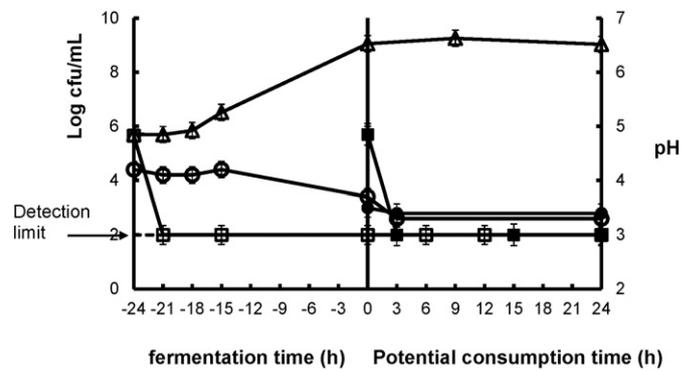
### 2.9. Statistical analysis

Independent experiments including inoculum preparation, product making and storage were performed in triplicate. Data points were represented by the mean, with the standard deviation indicated by error bars. The mean values of pH of all samples as well as mean log counts of the bacterial pathogens and *L. rhamnosus* yoba were compared using one-way ANOVA and Tukey's post-hoc tests. Statistical analysis was done using SPSS 13.0 for Windows (Apache Software Foundation, Forest Hill, Maryland, USA) and Microsoft Excel.

## 3. Results and discussion

### 3.1. pH changes in traditional and yoba mutandabota

The time course of acidification in traditional *mutandabota* was similar in all experiments with the 5 cocktails of bacterial pathogens (Figs. 2 to 6). The pH was 3.5 ( $n = 15$ ) immediately after preparation when *mutandabota* was ready for consumption at time 0 h ( $t = 0$ ). This pH-value remained rather constant throughout the 24 h potential consumption time, also regarded as the storage period, and at  $t = 24$  the pH was  $3.4 \pm 0.1$ . The low pH could be attributed to the acidic nature of dry baobab fruit pulp. Airan and Desai (1954) and Carr (1955) first highlighted the presence of organic acids in baobab fruit pulp. Later reports by Nour et al. (1980) and Vertuani et al. (2002) confirmed the presence of citric, tartaric, malic, succinic and ascorbic acids in baobab fruit pulp. For yoba *mutandabota* (Figs. 2 to 6) at the moment of simultaneous inoculation with *L. rhamnosus* yoba and the pathogenic bacteria cocktail into the 4% pulp–milk mixture ( $t = -24$  h), the pH was  $4.2 \pm 0.1$  ( $n = 15$ ). After 9 h the pH remained stable at 4.2. At  $t = 0$ , signaling the end of the fermentation stage, the pH was  $3.7 \pm 0.1$ . The lowering of pH from 4.2 to 3.7 during the fermentation phase could be attributed to organic acids such as lactic acid produced by the fermenting *L. rhamnosus* yoba. When the remaining 10% pulp and 7% sugar were added to get the standard constituents of *mutandabota*, the pH remained stable at  $3.7 \pm 0.1$ . Yoba *mutandabota* was then ready for



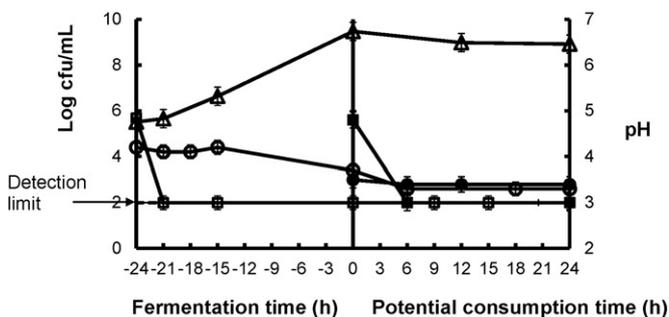
**Fig. 3.** Kinetics of *Lactobacillus rhamnosus* yoba and *Campylobacter jejuni* during production of traditional and yoba *mutandabota*. Fermentation takes 24 h, 0 h is the start of potential consumption time; (open triangle) *Lactobacillus rhamnosus* yoba in yoba *mutandabota*, (closed square) *C. jejuni* in traditional *mutandabota*, (open square) *C. jejuni* in yoba *mutandabota*, (closed circle) pH changes in traditional *mutandabota*, (open circle) pH changes in yoba *mutandabota*.

consumption. The pH remained rather constant at  $3.3 \pm 0.1$  throughout the storage period of yoba *mutandabota* resulting in a pH of  $3.3 \pm 0.1$  at  $t = 24$ .

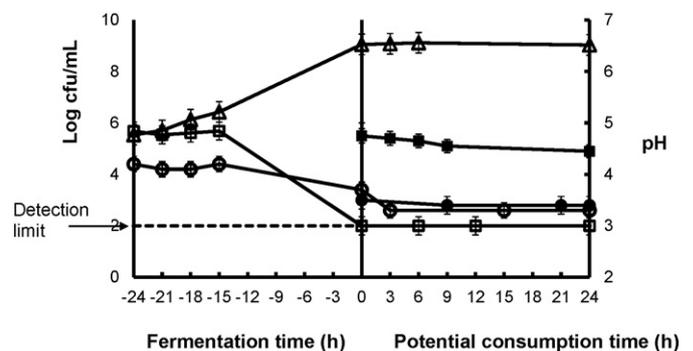
In preliminary experiments to determine growth of *L. rhamnosus* yoba at different pH values obtained by mixing different concentrations of baobab fruit pulp and UHT full fat cow's milk, it was shown that a fruit pulp content of 2% gave a pH of 5. This pH value allowed growth of *L. rhamnosus* yoba but was not enough to sustain growth of the bacterium over a 24 h period due to substrate limitation. Doubling the amount of fruit pulp to 4% resulted in a pH of 4.2. This pulp concentration of 4% and pH value of 4.2 allowed sustained growth of *L. rhamnosus* yoba over a 24 h period. Further doubling of the fruit pulp concentration to 8% resulted in a further decrease in pH (3.9), which allowed survival of *L. rhamnosus* yoba, but not its growth (Mpfu et al., 2014b).

### 3.2. Growth of *L. rhamnosus* yoba in yoba mutandabota with pathogens

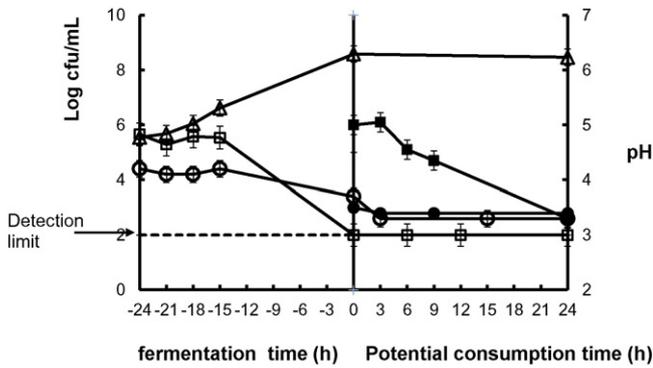
*L. rhamnosus* yoba, an isolate of the probiotic bacterium *L. rhamnosus* GG (Kort and Sybesma, 2012) was chosen for this study. *L. rhamnosus* GG, originally cultured from a healthy human intestinal source, has been thoroughly studied and used safely as a probiotic strain in a variety of probiotic foods (Bernardeau et al., 2006; Hatakka et al., 2001; Kalliomäki et al., 2001). *L. rhamnosus* GG is widely prescribed for treatment of acute diarrhea in children, its efficacy was evaluated by in vivo studies (Canani et al., 2007; Grandy et al., 2010; Hojsak et al., 2010). The growth of *L. rhamnosus* yoba in yoba *mutandabota* followed a similar pattern in each experiment (Figs. 2 to 6). Generally, from an inoculation



**Fig. 2.** Kinetics of *Lactobacillus rhamnosus* yoba and *Bacillus cereus* during production of traditional and yoba *mutandabota*. Fermentation takes 24 h, 0 h is the start of potential consumption time; (open triangle) *Lactobacillus rhamnosus* yoba in yoba *mutandabota*, (closed square) *B. cereus* in traditional *mutandabota*, (open square) *B. cereus* in yoba *mutandabota*, (closed circle) pH changes in traditional *mutandabota*, (open circle) pH changes in yoba *mutandabota*. Points represented on the detection limit indicate results below the detection limit (no detection).



**Fig. 4.** Kinetics of *Lactobacillus rhamnosus* yoba and *Listeria monocytogenes* during production of traditional and yoba *mutandabota*. Fermentation takes 24 h, 0 h is the start of potential consumption time; (open triangle) *Lactobacillus rhamnosus* yoba in yoba *mutandabota*, (closed square) *Listeria monocytogenes* in traditional *mutandabota*, (open square) *Listeria monocytogenes* in yoba *mutandabota*, (closed circle) pH changes in traditional *mutandabota*, (open circle) pH changes in yoba *mutandabota*.

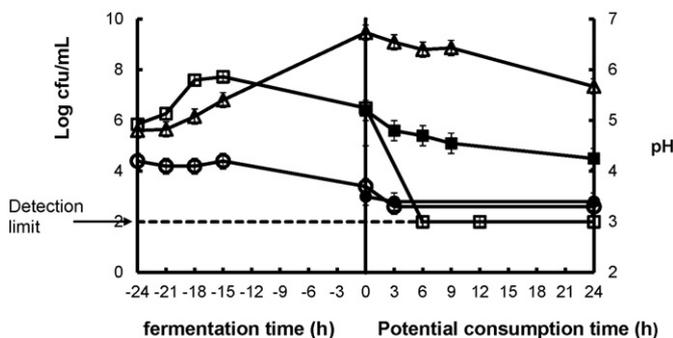


**Fig. 5.** Kinetics of *Lactobacillus rhamnosus* yoba and *Escherichia coli* O157:H7 during production of traditional and yoba *mutandabota*. Fermentation takes 24 h, 0 h is the moment of start of potential consumption time; (open triangle) *Lactobacillus rhamnosus* yoba in yoba *mutandabota*, (closed square) *E. coli* O157:H7 in traditional *mutandabota*, (open square) *E. coli* O157:H7 in yoba *mutandabota*, (closed circle) pH changes in traditional *mutandabota*, (open circle) pH changes in yoba *mutandabota*.

level of  $5.5 \pm 0.1$  log cfu/mL, *L. rhamnosus* yoba showed robustness and increased counts in the presence of pathogens, in some instance, even with increasing pathogen concentration, such as *Salmonella* (Fig. 6). *L. rhamnosus* yoba reached  $6.6 \pm 0.2$  log cfu/mL at  $t = -15$ , and further increased to  $9.1 \pm 0.4$  log cfu/mL at  $t = 0$ , which was the end of the fermentation process. At this time the remaining baobab fruit pulp and sugar were added to reach the standard 14% pulp and 7% sugar in yoba *mutandabota*. At the end of the storage time,  $t = 24$ , *L. rhamnosus* yoba viable counts were at  $8.5 \pm 0.9$  log cfu/mL.

### 3.3. Survival and decline of pathogens in traditional and yoba *mutandabota*

The loss in viability of *B. cereus* and *C. jejuni* inoculated into traditional *mutandabota* and yoba *mutandabota* is depicted in Figs. 2 and 3 respectively. The harsh effect of the acidic environment with a pH of 3.5 was pronounced. From an inoculation level of 5.6 and 5.7 log cfu/mL *B. cereus* and *C. jejuni* respectively, in both traditional and yoba *mutandabota*, both pathogens could not be detected 3 h after inoculation. *B. cereus* does not have a marked tolerance for environments with pH values below 4.5. Hassan et al. (2010) noted that vegetative cells of *B. cereus* rapidly die in yoghurt, a comparable product to yoba *mutandabota*, hence acidification is a common method of preservation. Working on yoghurt, Ayoub et al. (2003) found that 20% of examined yoghurt samples were tested positive for *B. cereus*. Lower incidence of *B. cereus* was reported by Hassan et al. (2010), who found 2% (1 out of 50) of yoghurt samples positive for *B. cereus*. Conversely, Khalil (1997) and Abdel-Khalek (2002) did not detect *B. cereus* in yoghurt. The



**Fig. 6.** Kinetics of *Lactobacillus rhamnosus* yoba and *Salmonella* spp. during production of traditional and yoba *mutandabota*. Fermentation takes 24 h, 0 h is the moment of start of potential consumption time; (open triangle) *Lactobacillus rhamnosus* yoba in yoba *mutandabota*, (closed square) *Salmonella* spp. in traditional *mutandabota*, (open square) *Salmonella* spp. in yoba *mutandabota*, (closed circle) pH changes in traditional *mutandabota* over a period of 24 h, (open circle) pH changes in yoba *mutandabota*.

lower incidence or absence of *B. cereus* in yoghurt was attributed to the inhibitory effect of lactic acid bacteria on *B. cereus*. Simango and Rukure (1991) observed a similar trend for *C. jejuni* inactivation in *mahewu*, a traditional fermented cereal beverage in Zimbabwe. They showed that none of the 4 strains of *C. jejuni* tested survived for 30 min in *mahewu* (pH 3.6) although high inocula of 6 to 7 log cfu/mL were used. *C. jejuni* was also shown to die rapidly within 30 min in yoghurt (Cuz et al., 1987) and within 2 h at pH 4 in the presence of formic acid (Chaveerach et al., 2003). Rahimi et al. (2013), investigating the prevalence of *Campylobacter* spp. in milk and dairy products, isolated *C. jejuni* from raw cow's milk, goat's milk and traditional cheese made from raw milk. No *C. jejuni* was isolated from pasteurized milk, yoghurt and commercial dairy products. Boiling milk or pasteurization is believed to be sufficient to inactivate *C. jejuni* (D'Aoust et al., 1988). The combination of organic acids and low pH of both types of *mutandabota* could explain the failure of *B. cereus* and *C. jejuni* to survive in the products during production and the period of potential consumption. In conclusion, when stored, both traditional and yoba *mutandabota* are unlikely to be sources of *B. cereus* and *C. jejuni* infection.

In traditional *mutandabota* (Figs. 4 and 5), *L. monocytogenes* and *E. coli* O157:H7 from inoculation levels of 5.5 and 5.9 log cfu/mL, decreased to 5.1 and 4.7 log cfu/mL respectively after 9 h. At the end of the period of potential consumption at  $t = 24$ , they were 4.9 and 2.6 log cfu/mL respectively, a significant ( $P < 0.05$ ), but limited decline. Although contamination is unlikely to reach the levels used in this study, it is evident that low pH alone ( $3.5 \pm 0.1$ ) will not completely inactivate the two bacterial pathogens and will not guarantee microbiological safety of traditional *mutandabota*. It must be accompanied by other measures, such as controlled fermentation, to produce a fermented product with antimicrobial properties. In yoba *mutandabota* (Figs. 4 and 5), *L. monocytogenes* and *E. coli* O157:H7 remained rather constant at the inoculated level of 5.7 and 5.9 log cfu/mL respectively, for the first 9 h. However, no *L. monocytogenes* or *E. coli* O157:H7 could be detected at  $t = 0$ , the end of fermentation. The decrease of *L. monocytogenes* and *E. coli* O157:H7 corresponded to an increase in *L. rhamnosus* yoba from 5.5 log cfu/mL at  $t = -24$  to 9.1 log cfu/mL at  $t = 0$  at the end of the fermentation (Figs. 4 and 5). When the remaining pulp and sugar were added to prepare *mutandabota* for consumption, yoba *mutandabota* was microbiologically safe to consume.

Similar observations were made by Dalu and Feresu (1995), working on traditionally fermented unpasteurized and pasteurized milk and on an industrially fermented milk marketed in Zimbabwe. Their results indicated that *L. monocytogenes* was inactivated at different levels during fermentation and storage of all the three fermented milk products (pH 4.5 and stored at 20 °C). In a study to investigate the survival of bacterial pathogens that had been associated with childhood diarrhea in Zimbabwe, Simango and Rukure (1991), showed that starting with a high inoculum of 6 to 7 log cfu/mL in *mahewu*, all strains of enteropathogenic and enterotoxigenic *E. coli* were detected in *mahewu* (pH 3.6) after 24 h of storage at 25 °C. Most of the *E. coli* strains showed very little change in numbers of surviving cells. The current study demonstrated that inactivation of *L. monocytogenes* and *E. coli* O157:H7 in yoba *mutandabota* was probably due to a combined effect of a low pH of 3.4, organic acids and possibly other fermentation products produced by the fermenting *L. rhamnosus* yoba, since in traditional *mutandabota* (pH 3.5), the two pathogens survived rather well.

In traditional *mutandabota*, there was a decrease of *Salmonella* spp. throughout the potential consumption period of 24 h (Fig. 6). From an inoculation level of 6.4 log cfu/mL, the *Salmonella* counts decreased to 5.1 log cfu/mL at  $t = 9$ , and 4.5 log cfu/mL at the end of the potential consumption time,  $t = 24$ , a significant decrease ( $P < 0.05$ ) by 2 log units. However, the level still remained above the detection limit. This reflects that once contaminated with *Salmonella* spp., traditional *mutandabota* could be a health hazard. Meanwhile in yoba *mutandabota*, *Salmonella* spp. increased from an inoculation level of 5.8 log cfu/mL at  $t = -24$  to 7.7 log cfu/mL at  $t = -15$ , giving higher levels than *L.*

*rhamnosus* yoba during the first 9 h of fermentation (Fig. 6). This suggests that with a pH of 4.2, the milk–pulp mixture provided a conducive environment for proliferation of *Salmonella*. The *Salmonella* spp. then decreased to 6.5 log cfu/mL at the end of fermentation at  $t = 0$ . The remaining 10% pulp and sugar were then added, subsequently within 6 h, no *Salmonella* could be detected (Fig. 6). This suggests that *Salmonella* spp. could not withstand the additional hurdle due to the added pulp. It should be noted that the other tested pathogens in this study did not need this extra hurdle to be inactivated below the detection threshold. Mufandaedza et al. (2006) observed a similar trend with *S. Enteritidis* in naturally fermented milk and industrially fermented milk (pH 4.4). *S. Enteritidis* grew from 7 log cfu/mL to reach high populations of about 9 and 8.8 log cfu/mL respectively, after 18 h. But *S. Enteritidis* could not be recovered from the cultures after 48 h. The inhibitory effect was associated with fast acid production by the fermenting lactic acid bacteria, which resulted in a pH reduction. Several investigations have demonstrated that *Salmonella* spp. can survive in acidic foods at lower pH values for longer periods of time. Indeed, declining numbers of viable cells have been detected up to 12 weeks in apple, orange, pineapple and white grape juice concentrates (Oyarzabal et al., 2003; Parish et al., 1997) and 10 weeks in yoghurt (El-Gazzar and Marth, 1992). Mugochoi et al. (1999) found that within 30 min of inoculation at 6 to 7 log cfu/mL, there were no viable *Salmonella* group B and *Salmonella* Enteritidis in the fermented *mapfura* (*Sclerocarya birrea* subsp. *caffra*) juice (pH 3.4), while in the unfermented juice, more than 4 log cfu/mL were still viable after 8 h (pH 3.4). However, none were still present after 24 h. They attributed the disappearance of *Salmonella* to antimicrobial substances in the fermented *mapfura* juice.

Control of pathogens in *mutandabota* and other dairy products is important because these pathogens or their toxins, endanger public health upon consumption of contaminated foods. *C. jejuni* is one of the most important causes of diarrhea in infants under 2 years of age in Southern Africa (Simango and Rukure, 1991; Kotloff et al., 2013). The disease caused by *C. jejuni* usually manifests itself as diarrhea, fever, malaise and severe abdominal pain. More recent studies suggest that *C. jejuni* infections can lead to inflammatory bowel diseases such as Crohn's disease (Horrocks et al., 2009). The ability of *L. monocytogenes* to survive in traditional *mutandabota* is of public health significance. *L. monocytogenes* can cause meningitis, encephalitis, abortion, premature birth, stillbirth, and gastroenteritis (Seeliger and Jones, 1986; Siegman-Igra et al., 2002). The poor inactivation of *E. coli* in traditional *mutandabota* coupled with the low infective dose of verotoxin-producing *E. coli* (VTEC), the increase in incidence of infection by *E. coli* O157:H7 and emergence of other VTEC sero-groups such as O111 and O26 (Baylis et al., 2004), does give cause for concern, especially for susceptible consumer groups, such as children and elderly. In the control challenge experiments no growth of pathogens was observed, this confirmed that no naturally occurring bacterial pathogens under test, or organisms giving similar colonial morphologies to those added to the inoculated products, were present.

The inactivation of pathogens in yoba *mutandabota* was clearly enhanced by fermenting with *L. rhamnosus* yoba. The bacterial pathogens could not survive in yoba *mutandabota* during the potential consumption time. This was probably due to lactic acid and other organic acids produced by *L. rhamnosus* yoba during the fermentation process. Yoba *mutandabota* is thus safer than traditional *mutandabota* during the potential consumption time. In producing yoba *mutandabota*, the inoculum was diluted approximately 1:80, this greatly reduces the chances, but does not exclude the inhibition being caused by a compound present in the pre-inoculum, further work on this aspect would be of value. No lactic acid was detected in traditional *mutandabota*, however in yoba *mutandabota* the amount of lactic acid was  $2.2 \pm 1.2$  g/L. This is on the lower side when compared with studies done by Østlie et al. (2003), in which lactic acid produced by *L. rhamnosus* GG (ATCC 53103) was 7 g/L after 24 h of fermentation in UHT milk supplemented

with 0.75% (w/v) fructose. So far, in literature the role of lactic acid in the antimicrobial activity of *L. rhamnosus* GG is controversial with hypotheses being formulated that range from minimal or no role at all (Silva et al., 1987; Fayol-Messaoudi et al., 2005) to lactic acid being the major antimicrobial agent (Makras et al., 2006; Marianelli et al., 2010; Hutt et al., 2006). While some *Lactobacillus* strains such as *L. acidophilus* and *L. casei* produce bacteriocins, there is still controversy on production of bacteriocins by *L. rhamnosus* GG (Avonts et al., 2004; Lu et al., 2009). Bacteriocins have specific inhibitory activity against Gram positive bacteria (Abee et al., 1995), whereas organic acids are known to have specific inhibitory activity against both Gram negative and Gram positive pathogens (Alakomi et al., 2000; De Keersmaecker et al., 2006; Fayol-Messaoudi et al., 2005). Lu et al. (2009) isolated and characterized 7 peptides produced by *L. rhamnosus* GG, the peptide NPSRQERR showed the highest antibacterial properties, both on Gram negative and Gram positive bacteria. Kankainen et al. (2009) described the presence of several bacteriocin related genes in the genome of *L. rhamnosus* GG. It is of value to investigate further the antimicrobial action of *L. rhamnosus* yoba in yoba *mutandabota*.

#### 4. Conclusion

This study focused on the question whether five important pathogens, namely, *L. monocytogenes*, *Salmonella* spp., *C. jejuni*, *E. coli* O157:H7 and *B. cereus*, could pose a foodborne risk to consumers of both traditional and yoba *mutandabota*. In traditional *mutandabota* (pH 3.5) no viable *B. cereus* and *C. jejuni* were detected 3 h after inoculation. However, *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 significantly declined ( $P < 0.05$ ), but could still be detected at the end of the storage period. This indicates that consumption of traditional *mutandabota* exposes consumers to the risk of food-borne microbial infections. In yoba *mutandabota*, the pH dropped from  $4.2 \pm 0.1$  to  $3.7 \pm 0.1$  after 24 h of fermentation, and remained rather constant at pH  $3.7 \pm 0.1$  throughout the 24 h storage period. *L. rhamnosus* yoba showed robustness in yoba *mutandabota* and grew from  $5.5 \pm 0.1$  to  $9.1 \pm 0.4$  log cfu/mL, even in the presence of pathogens during fermentation. None of the bacterial pathogens tested survived during production and/or storage of yoba *mutandabota*. Yoba *mutandabota* was made from pasteurized milk, and in practice, this will also largely reduce the risk of contaminated milk with pathogens. Our findings demonstrate that yoba *mutandabota* fermented with *L. rhamnosus* yoba has antimicrobial properties against the tested bacterial pathogens and can thus be regarded as a safer product compared to its traditional counterpart. Inactivation of pathogens in *mutandabota* is of public health significance because food-borne pathogens endanger public health upon consumption of contaminated *mutandabota*. Improving safety of *mutandabota* should also be achieved by providing information to food handlers and consumers on food hygiene to reduce the risk of contamination of *mutandabota*.

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