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Antimicrobial activity of novel *Lactococcus lactis* strains against *Salmonella* Typhimurium DT12, *Escherichia coli* O157:H7 VT^{-} and *Klebsiella pneumoniae* in raw and pasteurised camel milk

Esben Bragason [a], Tesfemariam Berhe [b], Dakalo Dashe [b], Kim Ib Sørensen [c], Mituku Eshetu Guya [b], Egon Bech Hansen [a, *]

[a] Technical University of Denmark, National Food Institute, 2800 Kgs. Lyngby, Denmark
[b] Haramaya University, School of Animal and Range Sciences, P.O. Box 138, Dire Dawa, Ethiopia
[c] Chr. Hansen A/S, Bøge Alle 10-12, 2970 Hørsholm, Denmark

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ABSTRACT

Bad hygiene and lack of cooling facilities have resulted in spontaneously fermented African camel milk with high incidence of contaminants. Starter cultures promote food safety through fermentation control. Commercial cultures developed for bovine milk acidify poorly in camel milk and cultures optimised for camel milk with inhibitory effects against pathogens are therefore needed. Inhibition of multiple food related pathogens in raw and pasteurised camel milk during fermentation with four novel *Lactococcus lactis* strains was investigated. All pathogens alone in camel milk reached 8.0 log cfu mL^{-1}. When the pathogens were cultivated with *L. lactis* MS22333 or MS22337 they were reduced between 0.9 and 6.0 log cfu mL^{-1}. *L. lactis* MS22314 and MS22336 showed no antimicrobial activity. To our knowledge, we have for the first time demonstrated that some *L. lactis* strains isolated from camel milk can inhibit the growth of food related pathogens in both raw and pasteurised camel milk.

1. Introduction

The quality and safety of milk is highly influenced by the rate of contamination by pathogens. *Staphylococcus* spp., *Escherichia coli*, *Salmonella* spp., *Klebsiella* spp., *Campylobacter jejuni, Bacillus cereus* and *Listeria monocytogenes* have been reported multiple times with high frequency of contamination in milk (Ismaili et al., 2019; Oliver et al., 2005; Tetili et al., 2017).

A study by Abera et al. (2016) demonstrated bacterial contamination in 108 (85.7%) samples from camel milk. Samples were collected in Ethiopian Somali region where poor water quality, bad sanitation, uncooled preservation and transportation traditions were some of the main factors promoting contamination.

Food poisoning is relatively harmless in developed countries where a medical system can support fast recovery and hydration. In African undeveloped countries, diarrhoea, salmonellosis, dehydration and vomiting can be fatal. In Africa food poisoning is estimated to kill 137,000 persons yearly (Bisholo et al., 2018; Rebgui et al., 2013). *Camelus dromedarius* is an important milk producer in sub-Saharan and East African countries, where raw and non-heat-treated dairy products are consumed. Camel milk is mainly consumed directly after milking or as sour milk, often due to spontaneous fermentation (Abera et al., 2016).

The acidification of camel milk is a complicated procedure due to relative higher concentrations of antibacterial and antiviral substances compared with bovine milk (El Agamy et al., 1992). Commercial starter cultures designed for bovine milk have been tested in camel milk and they were able to acidify camel milk to comparable final pH, but with slower acidification rates (Berhe et al., 2018). Starter cultures designed specific for camel milk with optimum acidification rates to both increase the food quality and inhibit growth of spoilage and pathogenic microorganisms remain to be developed.

Lactococcus lactis are used in many starter cultures to ferment foods and thereby prevent growth of pathogens or spoilage bacteria by producing multiple antimicrobial substances (Cizeikiene et al., 2013; Jay, 1982; Kondrotiene et al., 2018; Mufandaedza et al., 2006; Roessland et al., 2003).

* Corresponding author. Tel.: +45 3588 6203.
E-mail address: egbh@food.dtu.dk (E.B. Hansen).

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Studies of inhibition of several common food pathogens by *L. lactis* strains have been reported in cow milk and cheese products (Kasra-Kermanshahi & Mobarak-Qamsari, 2015; Kondrotiene et al., 2018; Roessland et al., 2003; Tetili et al., 2017). To our knowledge, only few studies have described inhibition of food pathogens using lactic acid bacteria (LAB) isolated from camel milk (Benmechernene et al., 2013; Rahmeh et al., 2019). Rahmeh et al. (2019) characterised antimicrobial activity of multiple genera within lactic acid bacteria and the distribution of LAB in raw camel milk from western Asia. Benmechernene et al. (2013) have shown that two strains of *Leuconostoc mesenteroides* subsp. *mesenteroides* isolated from Algerian camel milk could be used as a bioactive strain against *L. monocytogenes* and *Staphylococcus aureus.*

The aim of this study was to identify potential *L. lactis* candidates for a starter culture with antimicrobial properties demonstrated in camel milk to enhance food safety in African countries.

2. Materials and methods

2.1. Bacterial strains

L. lactis strains were obtained from the strain collection at the National Food Institute at the Technical University of Denmark. Originally, the strains MS22314, MS22333, MS22336 and MS22337 were isolated from spontaneous fermented camel milk in Ethiopia (Fugl et al., 2017). Genome sequences of the strains can be accessed at the Genbank under BioSample numbers: SAMN13701540, SAMN13701541, 76 SAMN13701542, SAMN13701543 (Bragason, Svendsen, Guya, Berhe, & Hansen, submitted for publication). The cultures were grown for 24 h at 30 °C in M17 broth (Oxoid, Thermo Scientific, Hampshire, UK) containing 0.5% lactose.

Klebsiella pneumoniae (MS22380) was obtained from DSMZ – German Collection of Microorganisms and Cell Cultures (DSM 30104).

Salmonella Typhimurium DT12 (MS20842) and *E. coli* O:157 VT$^-$ (MS21811) were obtained from the strain collection at the National Reference Laboratory for *Salmonella* and zoonotic *E. coli* at the Technical University of Denmark. *S.* Typhimurium DT12 was originally isolated in cow faeces. All pathogens were grown for 24 h at 37 °C in brain-heart infusion (BHI) broth (Oxoid, Thermo Scientific, Hampshire, UK).

2.2. Media preparation

Unpasteurised camel milk provided by Kamelenmelkerij Smits (Berlicum, Netherlands) was sent within 24 h after bottled on 0.5 L plastic bottles and immediately stored at −40 °C until use. The milk was placed in the fridge 2 days before use at 5 °C to thaw. Enzymatic inactivation of the milk was carried out by heating the milk for 10 min at 96 °C in a VWR water bath (Grant Instruments Ltd., Cambridge, UK) followed by cooling to room temperature before further use.

Raw bovine milk was obtained from a farmer on Zealand (Denmark) and stored in the fridge at 5 °C until use.

Preparation of Prussian blue (PB) agar plates were made following the formulation described by Saito et al. (2007). M17-lac agar were made using M17 agar provided by SSI Diagnostica (Hillerød, Denmark) in 200 mL bottles and sterile 10% lactose solution were added to a final concentration of 0.5%. Xylose-lysine-deoxycholate (XLD) agar plates were made following the directions by the manufacturer (Oxoid, Thermo Scientific, Hampshire, UK). MacConkey no. 3 agar plates were provided by SSI Diagnostica ready to use. All agar plates kept at 5 °C until use.

2.3. Antimicrobial activity of L. lactis

Aliquots of 50 mL raw or pasteurised camel milk were stored in 50 mL falcon tubes. Milk samples were inoculated with overnight cultures of each pathogen (5 μL) and *L. lactis* strain (50 μL). Falcon tubes were homogenised using a Vortex mixer and incubated at 30 °C for 75 h. Samples were withdrawn every 10–20 h over a period of three days.

2.4. Viable bacteria count

At each sampling interval the milk was homogenised and 100 μL were removed for serial dilutions. Maximum recovery diluent provided by SSI Diagnostica was used to dilute samples. Adequate dilutions were chosen and 40 μL was pipetted on a half agar plate. M17-lac agar plates were incubated for 48 h at 30 °C and used for *L. lactis* strains, XLD agar plates incubated for 24 h at 37 °C and used for *S.* Typhimurium DT12 and MacConkey No. 3 incubated for 24 h at 37 °C and used for *K. pneumoniae* and *E. coli* O:157 VT$^-$. Colonies were counted using a Stuart SC6+ Colony Counter (Cole-Parmer, Staffordshire, UK) and colony-forming units (cfu) per millilitre were calculated using a weighted mean.

2.5. Measurement of hydrogen peroxide

Determination of H_2O_2 concentration in samples was carried out using CDR FOODLAB® (CDR s.r.l, Florence, Italy) photometric analyser according to manufacturer's instructions. Samples were taken from an overnight culture containing either MS22333 or MS22337. The *L. lactis* strains were grown in both bovine milk and camel milk for evaluation of possible differences.

3. Results

In this study *L. lactis* strains were inoculated into both raw and pasteurised camel milk at around 10^6–10^7 cfu mL^{-1} to simulate the fermentation. Results are presented in Figs. 1 and 2. All *L. lactis* strains reached levels of 10^8–10^9 cfu mL^{-1} both in raw and pasteurised milk with pH ranging between 4.0 and 4.5.

3.1. Inhibition of E. coli O:157 VT$^-$ in raw camel milk

The inhibitory effect varied between *L. lactis* strains MS22314, MS22333, MS22336 and MS22337. The inhibition is shown in Fig. 1. The pathogenic *E. coli* O:157 VT$^-$ was inoculated at about 4.7×10^4–5.0×10^5 cfu mL^{-1} and after 8 h inhibition effects were detected. MS22333 and MS22337 decreased the concentration of *E. coli* O:157 VT$^-$ with 0.9–4.8 log cfu mL^{-1} within 54 h at 30 °C. In contrast, MS22314 and MS22336 did not inhibit the growth of *E. coli* O:157 VT$^-$ at any stages of fermentation. After 8 h of incubation the concentration of *E. coli* O:157 VT$^-$ was 0.5–1.0 log cfu mL^{-1} higher in milk samples containing either MS22314 or MS22333 compared with *E. coli* O:157 VT$^-$ growing alone in raw camel milk. After 54 h, all three samples reached a stable *E. coli* O:157 VT$^-$ concentration of 5.5–7.5×10^7 cfu mL^{-1}.

In the absence of *L. lactis* strains and *E. coli* O:157 VT$^-$ the raw camel milk sample showed both growth on M17-lac and MacConkey no. 3 agar plates indicating both natural LAB and unknown *Enterobacteriaceae.*

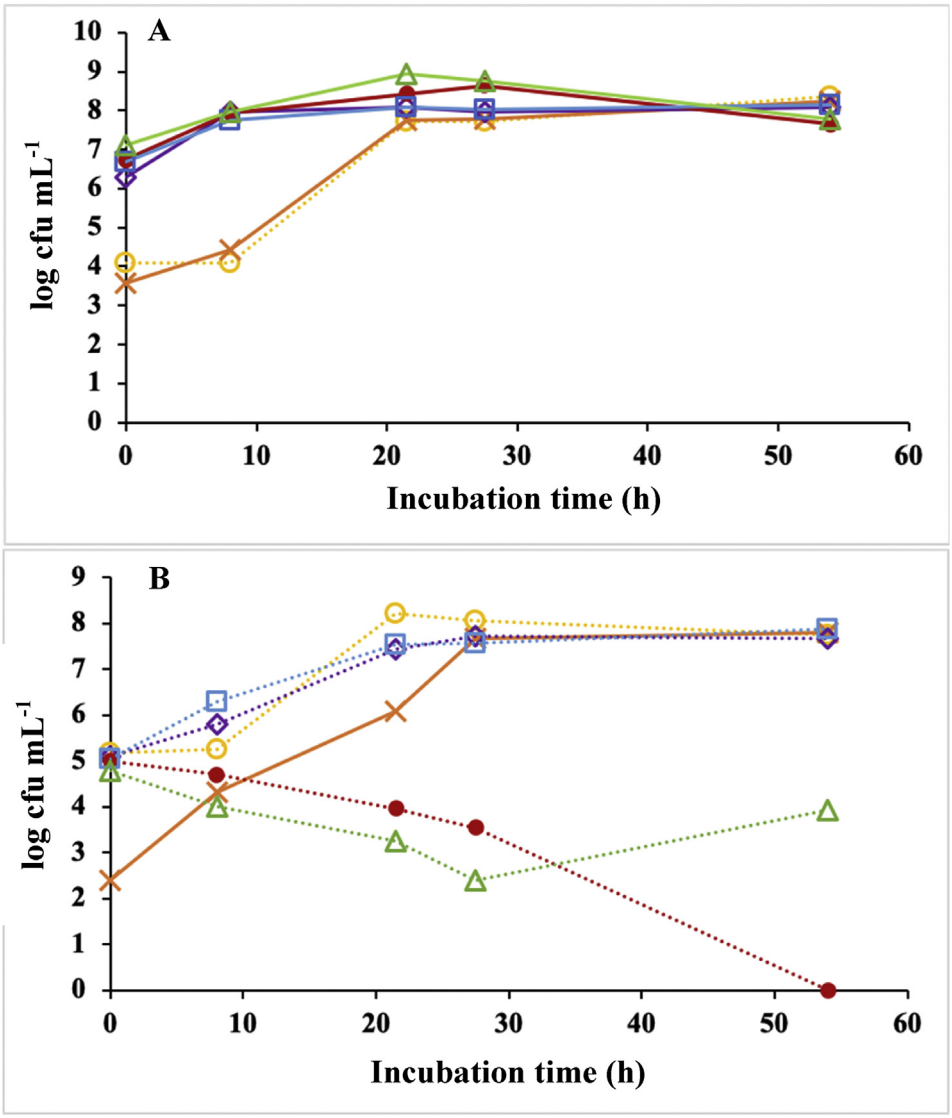

Fig. 1. Evolution of novel *L. lactis strains* and Gram-negative bacteria in raw camel milk: growth curves of lactose fermenting strains on M17 agar with 0.5% lactose (A) and of Gram-negative strains on MacConkey agar no. 3 (B) from raw camel milk fermentations at 30 °C with *Lactococcus lactis* strains inoculated: *L. lactis* MS22314 (◇), *L. lactis* MS22333 (●), *L. lactis* MS22336 (□) and *L. lactis* MS22337 (△) were inoculated in raw camel milk with *E. coli O:157* VT⁻ (·····) at 10:1 cfu mL^{-1}. Control samples without inoculation of *L. lactis* strains in raw camel milk (—×—) or with *E. coli O:157* VT⁻ (···○···) inoculated.

3.2. *Inhibition of* Salmonella *Typhimurium DT12 and* K. pneumoniae *in pasteurised camel milk*

The effect of *L. lactis* MS22333 and MS22337 to inhibit the pathogens in pasteurised camel milk is presented in Fig. 2B and C. Both *S.* Typhimurium DT12 and *K. pneumoniae* were inoculated at concentrations about 6.0 log cfu mL^{-1}, while *L. lactis* strains were about 7.0–7.5 log cfu mL^{-1}. Overall, *L. lactis* MS22333 and MS22337 inhibited both pathogens and a mixed starter culture composed of both did not show a significant difference. No inhibitory effect on *S.* Typhimurium DT12 were seen the first 21 h of the fermentation. After 47 h, it was impossible to detect *S.* Typhimurium DT12 in milk samples containing either MS22333 or MS22337 or a 50:50 mixed culture.

Similar results were seen with *K. pneumoniae* at 21 h. At 47 h no growth on MacConkey no. 3 agar was detected in the *L. lactis* MS22337 inoculated milk. The mixed *L. lactis* culture and *L. lactis* MS22333 showed concentrations of, respectively, 4.5 and 3.8 log cfu mL^{-1} at same sampling interval. After 75 h, *K. pneumoniae* were impossible to detect in all milk samples inoculated with a *L. lactis* strain.

3.3. *Production of hydrogen peroxide*

Characterisation of H_2O_2 producing *L. lactis* strains were carried out using PB agar. Prussian blue colour formation was seen as a blue halo around positive colonies. MS22333 showed strong blue colour formation around each colony, while MS22337 had small light blue halos around most colonies (data not shown). *L. lactis* MG1363 were included as a negative control. *L. lactis* MS22314 and MS2236 looked like *L. lactis* MG1363 with no clear colour formation.

Quantification of H_2O_2 levels in camel milk is shown in Table 1. Overall, none of the *L. lactis* strains showed high concentrations of H_2O_2 <24 ppm. The concentration of H_2O_2 in both raw and pasteurised camel milk were at the beginning below detection level (DL) <1.5 ppm. Similar results were obtained for bovine milk. After 24 h at 30 °C, all samples showed an increased H_2O_2 concentration.

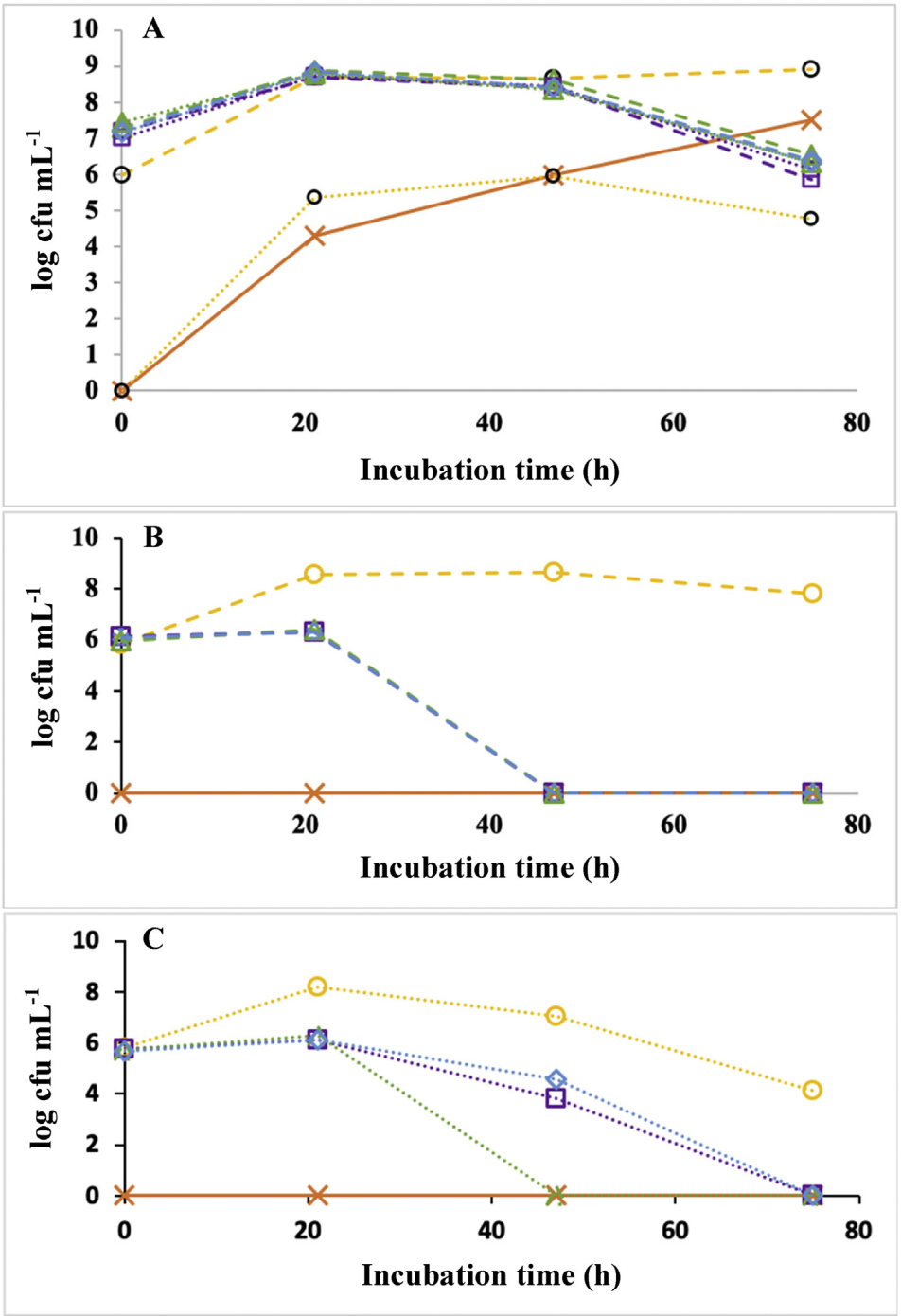

Fig. 2. Evolution of novel *L. lactis strains* and pathogenic bacteria in pasteurised camel milk. Panel A: growth curves of *Lactococcus lactis* strains in pasteurised camel milk with *Salmonella* Typhimurium DT12 or *Klebsiella pneumoniae* present. *L. lactis* MS22333 (□), *L. lactis* MS22337 (△), a mixed culture of *L. lactis* MS22333 and *L. lactis* MS22337(◇) were inoculated with either *S.* Typhimurium DT12 (– – –) or *K. pneumoniae* (·····) at 10:1 cfu mL^{-1}. Control samples without inoculation of *L. lactis* strains in pasteurised camel milk (—×—) inoculated with either *S.* Typhimurium DT12 (- -○- -) or *K. pneumoniae* (· ·○· ·). Panel B: growth curves of *Salmonella* Typhimurium DT12 on XLD agar from pasteurised camel milk fermentations at 30 °C with *Lactococcus lactis* strains inoculated. *L. lactis* MS22333 (□), *L. lactis* MS22337 (△) and a mixed culture of *L. lactis* MS22333 and *L. lactis* MS22337 (◇) were inoculated with *S.* Typhimurium DT12 (– – –) at 10:1 cfu mL^{-1}. Control samples without inoculation of *L. lactis* strains in pasteurised camel milk (—×—) inoculated with *S.* Typhimurium DT12 (- -○- -). Panel C: growth curves of *Klebsiella pneumoniae* on MacConkey agar no. 3 from pasteurised camel milk fermentations at 30 °C with *Lactococcus lactis* strains inoculated. *L. lactis* MS22333 (□), *L. lactis* MS22337 (△) and a mixed culture of *L. lactis* MS22333 and *L. lactis* MS22337 (◇) were inoculated with *K. pneumoniae* (·····) at 10:1 cfu mL^{-1}. Control samples without inoculation of *L. lactis* strains in pasteurised camel milk (—×—) inoculated with *K. pneumoniae* (- -○- -).

Table 1
H_2O_2 levels in raw and pasteurised camel and bovine milk fermented with *L. lactis* strains MS22333 or MS22337 at 30 °C.[a]

Sample	H_2O_2 (ppm)		
	0 h	3 h	24 h
Raw camel milk (RC)	<DL	–	–
Raw bovine milk (RB)	<DL	–	–
Pasteurised camel milk (PC)	<DL	<DL	4.2
Pasteurised bovine milk (PB)	<DL	<DL	4.1
RB_MS22333	2.9	<DL	15.0
RC_MS22333	1.5	<DL	3.6
RB_MS22337	<DL	<DL	14.2
RC_MS22337	<DL	<DL	4.3
PB_MS22333	<DL	<DL	17.3
PC_MS22333	<DL	<DL	7.4
PB_MS22337	<DL	<DL	6.6
PC_MS22337	<DL	<DL	23.8

[a] RC and RB were only measured at t = 0 h for baseline; DL, below detection limit (1.5 ppm).

23.8 ppm were the highest concentration detected, which was in pasteurised camel milk inoculated with *L. lactis* MS22337.

4. Discussion

Milk and processed dairy products are consumed globally by all social classes. Contaminants can occur through the food chain from milking of the animal to the final consumers. Bacterial contamination of milk should be minimal, as contaminated milk is a threat to public health. As the majority of camel milk producers lack cooling facilities, the camel milk often acidifies by spontaneous fermentation (Holzapfel, 2002). We have been studying LAB in spontaneous fermented camel milk to isolate the most beneficial strains based on antimicrobial activity and rate of fermentation. Our findings of two strains inhibiting *E. coli* O:157 VT$^-$ in raw camel milk, demonstrates antimicrobial activity of *L. lactis* strains with comparable acidification rates and pH levels as commercial starter cultures (Berhe et al., 2018).

A study by Charlier et al. (2008) demonstrated that low-acidifying *L. lactis* strains efficiently could inhibit the growth of *S. aureus* in milk. The inhibitory effect seen in *L. lactis* MS22333 and MS22337 and not in MS22314 and MS22336 is thereby not due to lactic acid production as they all had similar acidification curves (data not shown) and similar pH levels.

The same strains showed H_2O_2 production on PB agar, but only low levels <24 ppm could be detected using the CDR FOODLAB analyser. Multiple *L. lactis* strains isolated from food samples have earlier been reported to accumulate more than 300 ppm H_2O_2 when the suspension were aerated (Ito et al., 2003). The results shown in Table 1 could be residue levels, from an incomplete utilisation of hydrogen peroxide potential caused by anaerobic conditions. Furthermore, H_2O_2 is known to have a short lifetime in milk, and in raw milk, H_2O_2 may activate the naturally occurring lactoperoxidase (LP) enzyme system (Martin et al., 2014). Detection of H_2O_2 in a complex media such as milk can be a challenge. H_2O_2 concentrations as low as 60 mg L^{-1} administered along with 28 mg L^{-1} thiocyanate have been reported to activate the LP system and extend the shelf life of raw ovine, bovine and caprine milk for several days (Boulares et al., 2011).

To our knowledge, inhibition of pathogens in pasteurised camel milk have not been demonstrated before. *L. lactis* MS22333 and MS22337 showed complete inhibition of both *S.* Typhimurium DT12 and *K. pneumoniae* within 75 h (Fig. 2B and C). The reason for the 4.07 log cfu mL^{-1} decrease of *K. pneumoniae* in fermented camel milk is unknown, but may be due to growth of unknown bacteria inhibiting and utilising lactose, which was seen as growth on M17-lac agar plates reaching 6.0 log cfu mL^{-1} at 47 h (Fig. 2A).

Pasteurisation of camel milk inactivates the LP system (Sharma & Rajput, 2014). The inhibition of *S.* Typhimurium DT12 and *K. pneumoniae* by *L. lactis* MS22333 and MS22337 indicates that other antimicrobial mechanisms are happening during fermentation. According to the literature, *L. lactis* strains have been shown to inhibit pathogens by producing bacteriocins and other low molecular compounds (Armas et al., 2017; Cardinal et al., 1997; Enan et al., 2013; Loh et al., 2017; Millette et al., 2004).

We have previously published the genome sequences of *L. lactis* MS22314, MS22333, MS22336 and MS22337 (Bragason, Svendsen, Guya, Berhe, & Hansen, submitted for publication), where annotation of the contigs showed that MS22333 were the only strain without any genes coding for antibiotic resistance. Starter cultures containing resistance genes can possibly be a critical source of spreading antibiotic resistance, and studies have found multiple starter cultures with resistance genes (Kastner et al., 2006; Katla et al., 2001). Future studies should explore the mechanism of inhibition and develop *L. lactis* MS22333 into a starter culture specific for camel dairy.

5. Conclusions

Our present study shows that *L. lactis* MS22333 and *L. lactis* MS22337 isolated from spontaneous fermented camel milk have antimicrobial abilities and can be applied as a starter culture to promote food safety in African countries. We have demonstrated for the first time that *S.* Typhimurium and *K. pneumoniae* can be eliminated in pasteurised camel milk by *L. lactis* strains. Further work has to be done to explain the mechanism of inhibition.

CRediT author statement

Esben Bragason: Conceptualization, Investigation, Writing; **Tesfemariam Berhe**: Resources; **Dakalo Dashe**: Resources; **Kim Ib Sørensen**: Validation; **Mituku E. Guya**: Resources; **Egon Bech Hansen**: Supervision, Funding acquisition.

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