Characterization of *E. coli* and other Enterobacteriaceae in producer-distributor bulk milk

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INTERPRETIVE SUMMARY

Characterization of *E. coli* and other Enterobacteriaceae in producer-distributor bulk milk, *Ntuli***,** The direct sale of milk from producer-distributor (PD) to consumers is a common practice in many countries. We characterized *E. coli* and other Enterobacteriaceae in PD bulk milk sold at retail points in South Africa (SA). The study highlighted that there is a potential risk of transferring drug resistant pathogenic *E. coli* to consumers through the food chain. Information obtained from the study will be used to model the risk of pathogenic *E. coli* in PD milk and the proposal of most potentially effective mitigation efforts towards enhancing food security.

ABSTRACT

The current study was undertaken to characterize *Escherichia coli* and other Enterobacteriaceae in raw and pasteurized producer-distributor bulk milk (PDBM). A total of 258 samples were collected from purchase points in 8 provinces in South Africa. The samples were tested for

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antibiotic residues, phosphatase, total aerobic bacteria, coliforms and *E. coli* counts. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used for identification of isolates. *E. coli* isolates were characterized for virulence factors, antimicrobial resistance, serotypes and presumptive *E. coli* O157:H7. Antibiotic residues and alkaline phosphatase were detected in 2 % (n=258) and 21 % (n=104) of the samples respectively. A total of 729 isolates belonging to 21 genera and 59 species were identified. *E. coli, Enterobacter cloacae, Klebsiella oxytoca* and *Raoultella ornithinolytica* were the most abundant species. Spoilage Enterobacteriaceae species exceeded 50 % of the total isolates. *E. coli* was detected and isolated from 36 % of the milk samples. Thirty one *E. coli* isolates harbored virulence genes *stx1/stx2* and 38 % (n=121) were presumptive O157:H7. Prevalence of samples with presumptive shigatoxin producing *E. coli* (STEC) was 10 %. Antimicrobial resistant *E. coli* isolates were detected in 70 % of the milk samples with 36 % of *stx1/stx2* positive *E. coli* showing multi-drug resistance. Information obtained from the study will be used for modelling the public health risk posed by milkborne pathogens in PDBM, which in many cases is consumed by the poor and vulnerable population.

Key words: producer-distributor milk, Enterobacteriaceae, *E. coli*, shigatoxin, antibiotic resistance.

INTRODUCTION

Members of the Enterobacteriaceae family have been implicated in many of such safety and spoilage problems (Baylis et al., 2011). Enterobacteriaceae can enter an inadequately handled dairy chain and cause enzymatic breakdown of proteins or lipids, instigating spoilage which

contribute to substantial economic losses and wastage (Baylis et al., 2011). More so, some Enterobacteriaceae have emerged as potential opportunistic pathogens due to acquisition (via horizontal gene transfer (**HGT**) among different strains or bacterial species) of virulence and antibiotic resistance factors carried on mobile genetic elements such as plasmids, transposons and bacteriophage (Baylis et al., 2011). Enterobacteriaceae including *E. coli* (STEC), *Salmonella* spp., and *Yersinia enterocolitica* have been detected frequently in milk borne disease outbreaks (Jayarao and Henning, 2001, Oliver et al., 2009).

 Among members of Enterobacteriaceae which are most implicated in serious fatalities and illnesses in most food borne outbreaks, including milkborne disease outbreaks around the world, include several proficient *E. coli* pathotypes. Numerous outbreaks associated with Enteropathogenic *E. coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) in milk and other foods has been recently reported (Oliver et al., 2009, EFSA-ECDC, 2012, EFSA, 2015). Pathogenic *E. coli* can cause disease in humans and animals due to an assortment of virulence factors (Nataro and Kaper, 1998). One such *E. coli* pathotype which has been reported in milk outbreaks is the STEC which is a causative agent of haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Oliver et al., 2009). The main route of infection with STEC in humans is via consumption of contaminated foods including milk, which is commonly associated with asymptomatic dairy cattle reservoirs (Iweriebor et al., 2015). Important STEC virulence encoding genes, which make *E. coli* an insidious threat to food safety, are the phage-encoded shigatoxin genes (*stx*1 and *stx*2) which inhibit protein synthesis of host cells and leads to cell death and intimin (*eae*) which mediate colonization. Enterohemolysin gene (*ehxA*) encodes for cell lyses whereby the membranes of target cells including erythrocytes, leukocytes and renal tubular cells are perforated (Nataro and Kaper, 1998). The most implicated STEC in human

illnesses associated with foods is the *E. coli* serotype O157:H7, however, current studies have revealed the global emergence of non-O157 STEC serotypes (O111, O26, O145 and O103) associated with food borne outbreaks (Constantiniu, 2002). Recent milk-borne outbreaks associated with STEC O157:H7 have been reported in 2005, 2006 and 2007 in USA (CDC, 2008), 2008 in Germany and 2012 in Finland (EFSA, 2015). The presumptive route of O157 STEC in milk is through fecal contamination during milking and also direct shedding from infected udder (King, 2007).

The potential occurrence of pathogenic *E. coli* in food is not the only risk. In addition to the outbreaks, treatment of *E. coli* infections is now threatened by a rapid evolution of antimicrobial resistance strains (da Silva and Mendonça, 2012). Resistance of *E. coli* and other bacteria to antimicrobials has been reported worldwide and is one of the most pressing public health issues of our time. Inappropriate selection, intensive usage and abuse of antimicrobials may have led to antimicrobial resistance in bacteria (da Silva and Mendonça, 2012). Concerns have been raised about the potential spread of antibiotic resistance genes such as beta-lactams from food animal products such as milk to humans through the food supply (Iweriebor et al., 2015). Research has also revealed that there is an association between resistance to drugs and virulence capacity (da Silva and Mendonça, 2012). Acquisition of antibiotic resistance and virulence factor determinants in *E. coli* can be though lateral gene transfer in the environment or in the human and animal gut; and the determinants can be found either inserted on the same plasmid or separately on bacterial chromosomal DNA and plasmids. These determinants might be co-selected by antibiotic selection pressure, hence, exacerbating the risk posed by *E. coli* to food safety (da Silva and Mendonça, 2012). Outbreaks of antibiotic resistance pathogenic bacteria have been recently reported in different foods (CDC, 2013).

Occurrence of most milk-borne disease outbreaks has been implicated to consumption of raw milk at farms or milk which is sold directly from producer-distributor (**PD**) or from farm to consumer in bulk tanks (Jayarao et al., 2006, Oliver et al., 2009, EFSA-ECDC, 2012, EFSA, 2015). However, there are few reports on outbreaks from pasteurized milk (CDC, 2008). In developing countries, there are no available epidemiological statistics on milkborne outbreaks even though the sale of producer-distributor bulk milk (**PDBM**) is common. Suffice to say, in developing countries, the direct sale of milk to consumers plays an important role as it contributes to food security and nutrition as well as social and economic benefits (Swai and Schoonman, 2011). South Africa (**SA**) is one such country where the dairy industry is characterized by a growing number of PDs selling milk directly from producers to consumer in form of bulk tank milk. The milk is mostly consumed by poor and vulnerable members of the population.

Recent reports have highlighted microbiological inadequacy in the quality of PDBM sold to in South Africa. However, these studies have not characterized spoilage microbiota and possible pathogens in the milk. Therefore, this study focuses on characterization of *E. coli* and other Enterobacteriaceae in PDBM. It is envisaged that the information from this research will be useful in modelling the risk from pathogenic *E. coli* in retail producer-distributor bulk milk and the proposal of most potentially effective mitigation efforts towards enhanced food security through reduction in incidences of milk-borne zoonosis.

MATERIALS AND METHODS

Milk Sample Collection

Sampling of PDBM was done from purchase points according to ISO standards (ISO, 1997) within 8 different geographical provinces (P1- P8) in SA by environmental health practitioners (EHP's). An average of 30 retail bulk milk samples were collected per province adding up to a total of 258 samples (154 raw and 104 pasteurized). Each of the 500 ml milk sample was collected in sterile containers which were kept chilled and analyzed at Lactolab Irene, Pretoria, SA, within 48 h of collection

Antibiotic Residue and Phosphatase Test

 Alkaline phosphatase (**ALP**) test was carried out using PhosphatesmoMI test kit (MACHEREY-NAGEL GmbH & Co.KG, Germany). A CMT Antibiotic test kit (CHR HANSEN, Hoersholm, Denmark) (code number 611683) was used to detect the presence of antibiotic residues in the milk.

Microbiological Analyses

$3M^{TM}$ petrifilm^{TM} (Minisota, USA) were used for enumeration of total aerobic plate count, *E. coli* and coliforms. Isolation and characterization of *E. coli*, coliforms and other Enterobacteriaceae was carried out at the Department of Food Science, University of Pretoria. Five colonies from each *E. coli*/coliform petriflm plate were selected based on colour, size and morphology. Colonies were then streaked and purified on nutrient agar (Oxoid, UK) (18-24 h at

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37°C±0.5°C). Eosin methylene blue and sorbitol MacConkey agar (Oxoid) were used for further presumptive confirmation of *E. coli* and coliforms (37°C±0.5°C for 24 h±2 h). Potassium hydroxide (3 %) was used for Gram stain reactions (Halebian et al., 1981).

Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) Identification and Characterization of the Bacterial Isolates

A method by Mazzeo et al. (2006) and Pinto et al. (2010) was used for identification of the isolates. Bacterial cultures consisting of 8 wells per isolate in nutrient agar were extracted and transferred directly to the MALDI-TOF steel-polished target plate and overlaid with alpha-cyano-4 hydroxycinnamic acid matrix (Bruker, Germany) according to manufacturer instructions (Bruker Daltonik, Bremen, Germany). Mass spectra were generated with the Microflex LT mass spectrometer operated by the MALDI-Biotyper automation control and recorded by Flex Control software (Bruker Daltonics, Bremen, Germany). Three hundred shots per sample spot were acquired using the instrument settings consisting of linear positive mode, 60 Hz laser frequency, 20 kV acceleration voltage, 16.7 kV IS2 voltage, 170 ns extraction delay, and 2,000 to 20,137 m/z range as recommended for bacterial identification. The peak lists generated were used to directly obtain matches against the reference library using the integrated pattern matching algorithm of the software (MALDI-TOF 3.0, Bruker Daltonics). MALDI Biotyper 3.0 software was used to analyze raw spectra of the bacterial isolates, with default settings. The software compares acquired sample spectra to reference spectra in the provided database and compiles a list of best matching database records. MALDI-TOF identification results were recorded as score values (SV) and used to determine the identity of the organism as proposed by the manufacturer. A score <1.700 indicates no identification, 1.700-1.999 indicate identification to genus level,

≥2.000 denotes identification to genus and probable species and scores above 2.3 indicate highly probable species identification (Cherkaoui et al., 2010).

Antimicrobial Agent Susceptibility Testing of **E. coli** *Isolates*

The Kirby-Bauer disk diffusion method (Bauer et al., 1966) was used to determine antibiotic sensitivity profiles of the isolated *E. coli*. A total of 11 antimicrobial agents (Oxytetracycline, Cephalothin, Nitrofurantoin, Amoxicillin, Ampicillin, Neomycin, Amikacin, Gentamicin, Nalidixic acid, Chloramphenicol, Norfloxacin) (Mast Diagnostics, UK) were chosen on the basis of their common usage in the medical and agricultural fields for treatment or as feed additives to promote growth in animals (Sayah et al., 2005). Isolates were categorized as susceptible or resistance to each antimicrobial agent (CLSI, 2011).

Serotyping

Serotyping of the *E. coli* isolates was performed by agglutination tests using polyvalent antisera (Antiserum *E. coli* TRIVAKENT III; BIO-RAD, Paris, France) against eleven common O antigens O111, O55, O26, O114, O124, O142, O125, O126, O128, O86 and O127.

Molecular Detection of virulence genes stx and eae in **E. coli**

Multiplex real time PCR was used to detect virulence factors; *shiga* toxin 1 (*stx 1*), s*higa* toxin 2 (*stx 2*) and intimin (*eae*) in *E. coli* isolates. The thermocycler (C1000 Touch ThermalCycler CFX96TM Real Time System, Bio-Rad) and software (CFX 127 Manager IDE, Bio-Rad) were

set up for analysis using iQ-Check™ STEC VirX catalogue # 128 357-8139 (Bio-Rad, Hercules, California).

Presumptive Detection of Shigatoxin O157:H7 **E. coli**

Presumptive detection of O157:H7 *E. coli* was carried out using three selective media: BBL™ CHROMagar™ O157 agar (CHROMagar, Paris, France) with cefixime-tellurite (CT) selective supplement (Fluka analytical); BBL™ CHROMagar™ STEC with STEC ST162(S) supplement (CHROMagar Paris France); and Sorbitol MacConkey Agar (Merck, SA) supplemented with CT. *E. coli* isolates were incubated at 37°C±0.5°C for 24 h±2 h. *E. coli* O157:H7 (ATCC 35150) and *E. coli* ATCC (25922) was used as a positive and negative control respectively.

Statistical Analysis

The data was analyzed with STATA$^{®}$ version 11 (StataCorp, Texas, USA). Chi-square test (P <0.05) was used to test for significant differences in *E. coli*, coliforms and total aerobic counts between raw and pasteurized milk samples. Two-sided Fisher's exact test was used to test for correlation between virulence factor and antibiotic resistance capacity. Analyses of variance (one way) was performed to test for significant differences in antibiotic resistance patterns for *E. coli* isolated from the PDBM in different provinces at 95% confidence interval. Raw spectra of the *E. coli*, *S. liquefaciens* and *K. oxytoca* strains were analyzed using MALDI Biotyper 3.0 software in order to determine the association (clustering) of the isolates with source province/region. Cross-wise minimum spanning tree (**MSP**) matching was used to create a dendrogram. Similar MSPs result in a high matching score value. Each MSP is matched against all MSPs of the analyzed set.

The list of score values was used to calculate normalized distance values between the analyzed species resulting in a matrix of matching scores. The visualization of the respective relationship between the MSPs was displayed in a dendrogram using the standard settings of the MALDI Biotyper 3.0 software (Dubois et al., 2010). Distance values in a dendrogram were relative and normalized to a maximal value of 10. Species with distance levels under 1.5 have been described as reliably classified into distinct strains (Sauer et al., 2008).

RESULTS AND DISCUSSION

Total Plate Count, Coliforms and **E. coli** *Counts in retail PDBM*

There was a significant difference between raw and pasteurized milk (P< 0.05) in mean $\log_{10}$ counts of total plate count, coliforms and *E. coli* (Fig. 1). Total aerobic plate count, coliforms and *E. coli* counts for raw milk ranges were $3.4 - 6$, $2.4 - 2.7$ and $2 - 2.1$ $\log_{10}$cfu/ml respectively (Fig. 1a). Prevalence of raw milk samples above SA national standards (SA, 2001 Act (54), (1972)) were 42 %, 88 % and 68 % for *E. coli*, coliforms and total aerobic plate counts, respectively. Our results on microbial counts from raw PDBM, where more than 60 % of the sample were above national standard, were consistent with previous studies carried out in SA (Lues et al., 2003, O'Ferrall-Berndt, 2003, Lues et al., 2010) and concur with studies in other countries, Torkar and Teger (2008); Slovenia, Pyz-Łukasik et al. (2015) Poland and Van Kessel et al. (2004) USA. These studies highlighted, animal and equipment cleanliness, feeding and housing strategies, rinsing water for milking machine and milking equipment washing, mastitis in animals, milk transportation and storage conditions, as reasons for high microbial counts in raw milk. High prevalence of coliforms and *E. coli* in raw milk is a cause of concern due to their association with contamination of fecal origin and the consequent risk of enteric pathogenic
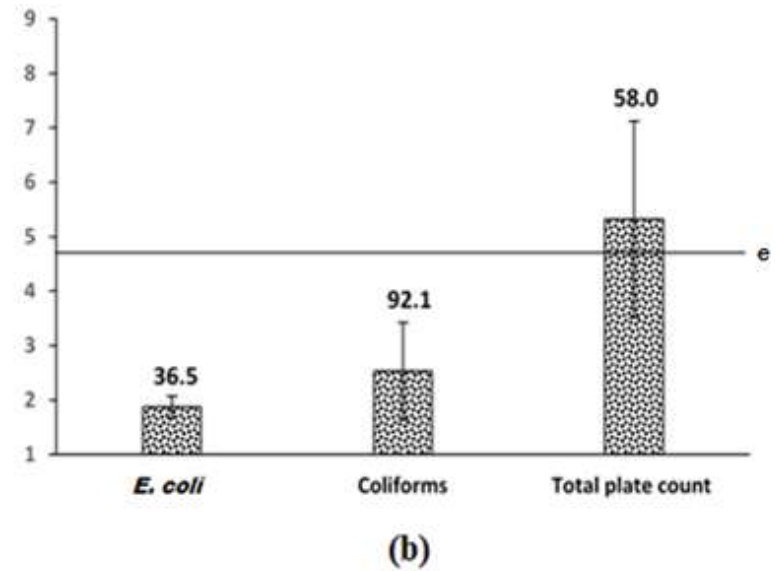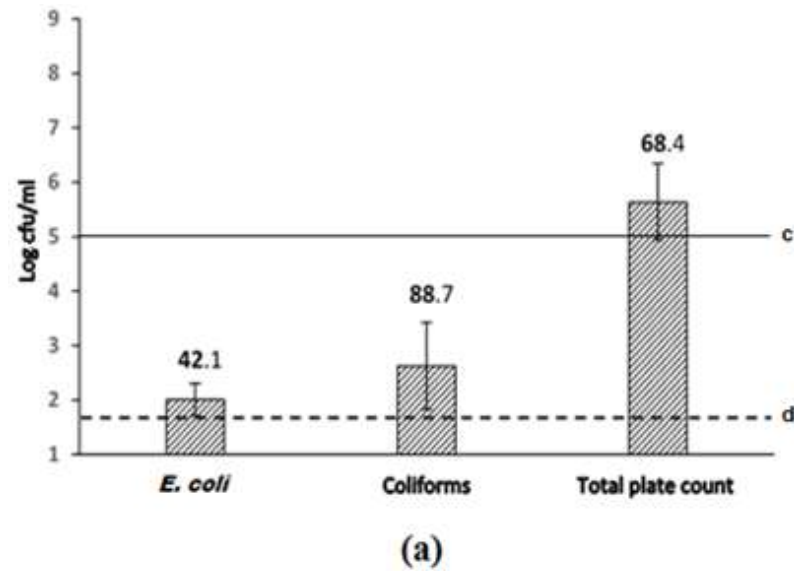
Fig 1: Mean *E. coli*, coliform and total aerobic plate count (log cfu/ml) for raw bulk-milk (**a**) and pasteurised bulk milk (**b**) from producer-distributors in 8 provinces in SA. n = 258, SD – standard deviation and LS means – Least square means.

Solid and broken horizontal lines designated (c) and (d) on the graph, represent maximum limit for total plate count and coliforms in raw milk, respectively. Solid line designated (e) represent maximum limit for total plate count in pasteurised milk. Numbers above the graphs represent percentage of samples above Foodstuffs, Cosmetics and Disinfectants Act (54), (1972) limit for raw milk (1a) and pasteurised (1b). Coliforms should not be detected in pasteurised milk and *E coli* should not be detected in both raw and pasteurised milk.

organisms in food. Total aerobic plate counts, coliforms and *E. coli* counts for pasteurized milk ranges were $2.2 - 4.8$, $0 - 2.5$ and $0 - 2$ $\log_{10}$cfu/ml respectively (Fig. 1b). The presence of high aerobic plate counts in pasteurized milk can be attributed to cross-contamination, however, the issue of some thermoduric microorganisms which are reported to resist heat treatment in food cannot be ignored (Washam et al., 1977). There were 21 % of the pasteurized milk samples which were positive for ALP. The presence of ALP can be due to ineffective pasteurization or poor milk handling after pasteurization. Conversely, bacterial counts greater than 1.2×10^7 cfu/ml, as experienced in our study, can contribute sufficient microbial ALP that cause a false positive ALP test (Knight and Fryer, 1989), again, ALP reactivation also results in positive ALP test (Whitaker et al., 2003). The finding of microbiological counts in both raw and pasteurized milk in 60 % of the milk samples exceeding SA regulatory standards suggest the need to assess public health risk posed to consumer by retail PDBM in the country.

Identification and Characterization of bacterial species

 A total of 729 Enterobacteriaceae and other bacterial isolates were detected and identified from 258 PDBM samples. The isolates belonged to 21 genera and 59 species. Prevalence of the dominant Enterobacteriaceae species isolated from raw and pasteurized PDBM (coded 1-59) is depicted in Table 1 and their distribution in raw and pasteurized PDBM in the respective provinces is presented in Figure 2. Raw and pasteurized retail bulk milk samples from all the 8 provinces were contaminated with a wide diversity of Enterobacteriaceae species. More than 50 % of the Enterobacteriaceae isolated and detected in our study were spoilage psychrotrophic microbiota. *Pseudomonas* spp., was the dominant psychrotrophic bacteria in all the provinces. A

Table 1: Prevalence of dominant Enterobacteriaceae and other bacterial species isolated from raw and pasteurised producer-distributor bulk milk in SA (n=258)

Species code	Species name	RM (%)	PM (%)	Total number of isolates
3	*Acinetobacter guillouiae*	0.6	0.5	4
13	*Buttiauxella agrestis*	-	2.3	5
14	*Buttiauxella. gaviniae*	0.4	1.9	6
15	*Buttiauxella noackiae*	-	3.2	7
17	*Citrobacter braakii*	3.3	3.2	23
18	*Citrobacter freundii*	2.1	2.3	15
21	***Escherichia coli***	**22.8**	**5.6**	**121**
23	*Enterobacter amnigenus*	3.1	4.2	24
24	***Enterobacter asburiae***	**7.5**	**3.2**	**43**
25	***Enterobacter cloacae***	**7.5**	**7.4**	**52**
26	*Enterobacter kobei*	0.8	1.4	7
27	*Enterobacter ludwigii*	2.5	1.4	15
28	*Ewingella Americana*	0.4	1.4	5
29	***Hafnia alvei***	**4.4**	**14**	**52**
30	***Klebsiella oxytoca***	**10.2**	**9.7**	**70**
31	*Klebsiella pneumonia*	2.9	0.9	16
33	*Kluyvera intermedia*	1	0.9	7
35	*Pantoea agglomerans*	1.5	0.9	9
36	*Proteus mirabilis*	0.4	-	2
37	*Proteus vulgaris*	0.2	0.5	2
39	*Pseudomonas aeruginosa*	0.2	0.5	2
43	*Pseudomonas fluorescens*	0.2	1.4	4
47	*Pseudomonas lundensis*	0.4	-	2
48	*Pseudomonas nitroreducens*	-	0.9	2
52	*Rahnella aquatilis*	0.8	2.3	9
53	***Raoultella ornithinolytica***	**16.1**	**17.1**	**114**
54	*Raoultella terrigena*	0.4	0.5	3
55	*Serratia fonticolla*	0.4	-	2
56	***Serratia liquefaciens***	**6.3**	**8.3**	**48**
57	*Serratia marcescens*	1.5	1.9	11
58	*Stenotrophomonas maltophilia*	0.2	0.5	2
59	*Yersinia enterocolitica*	1.7	1.9	12
	Total number of isolates	479	216	697

RM – Raw milk,

PM – Pasteurised milk,

(-) not present,

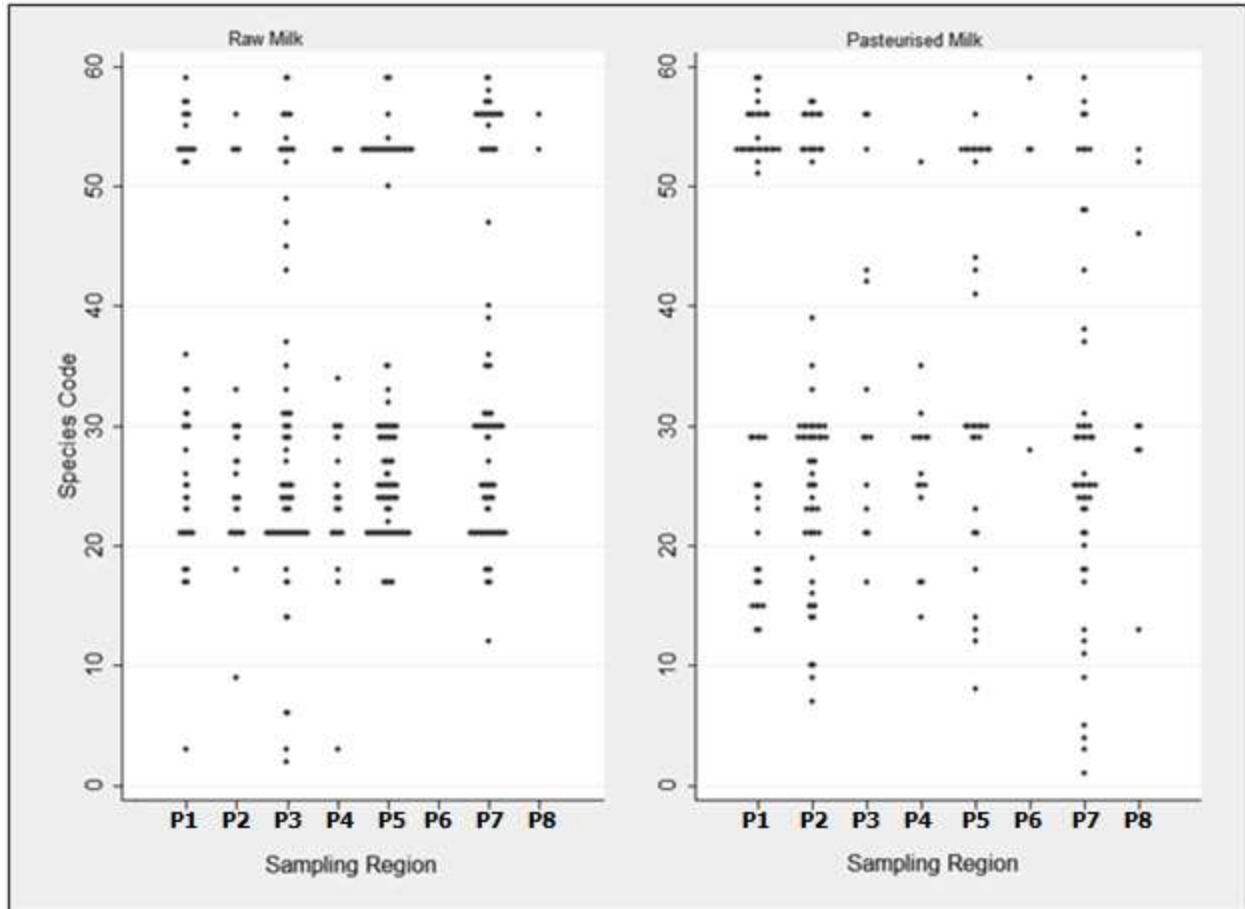Figures in bold represent the most abundantly isolated species.

Fig 2: Distribution of Enterobacteriaceae and other bacterial species (n=729) from raw and pasteurised producer- distributor bulk milk from 8 different provinces in SA

Isolated species were coded 1 to 59. Width of the band represents the abundance of a particular species in the province. Species names for the dominant Enterobacteriaceae species are given in Table 1.

commercially important characteristic of psychrotrophs that were isolated in our study, is their abilities to grow at low temperatures (3–7 $^{\circ}$C) and release of enzymes that hydrolyze and use large protein molecules and lipids for growth ultimately causing milk spoilage (Ledenbach and Marshall, 2010). The released enzymes are heat stable and are not inactivated by pasteurization or by other heat treatments and may continue to degrade milk and its products, even when the bacterium is destroyed (Ledenbach and Marshall, 2010).

The most prevalent Enterobacteriaceae species in our study from PDBM, in decreasing order, were *E. coli*, *R. ornithinolytica, K. oxytoca, E. cloacae, E. asburiae, Serratia liquefaciens and Hafnia alvei*. These bacteria were also the spoilage microbiota isolated in high numbers in food products as by Doulgeraki et al. (2011). The Enterobacteriaceae are also considered as opportunistic pathogens although their implication in diarrheal diseases is not clear with the exception for *E. coli* pathotypes. More so, Enterobacteriaceae species in other studies have been noted to harbor antibiotic resistance and multi-drug resistance capacities (Neu, 1992). Coque et al. (2008) reported Enterobacteriaceae as reservoirs of extended-spectrum β-lactamases (ESBLs) in bulk milk environments. *R. ornithinolytica* were the most prevalent species in pasteurized PDBM samples (Table 1). Most reports of *R. ornithinolytica* (formerly *Klebsiella ornithinolytica)* were associated with incidences of food poisoning (Lin et al., 2012). *R. ornithinolytica* has histamine-producing capabilities, due to *hdc* genes, which encode histidine decarboxylase (Lin et al., 2012). There are few studies on this bacteria in milk and its products and the real importance of *R. ornithinolytica* as a food spoilage organism or an enteric pathogen is underestimated (Lin et al., 2012). Detection of *S. liquefaciens, K. oxytoca* and *H. alvei* in our study is of commercial significance in milk. Their presence in milk has not only been linked to undesirable alteration in appearance and flavours but also to residual activity of enzymes such as proteases which have been associated with gelation of milk and coagulation of milk proteins during storage (Nornberg et al., 2010). We identified six different species of the genus Enterobacter from PDBM, however, *E. cloacae* and *E. asburiae* were detected at higher prevalence. *E. cloacae* and *E. asburiae* are emerging opportunistic pathogens causing nosocomial infections and are frequently detected in infant formula milk (Gaston, 1988).

More diverse species were isolated in the pasteurized milk than the raw milk in this study (Fig 2). A study done by Lindberg et al. (1998) isolated and detected *H. alvei, R. aquatilis, Citrobacter freundii and S. liquefaciens* in poorly stored pasteurized milk. These results are similar to those of our study where these species were also isolated at high frequency from pasteurized milk. These findings suggests cross contamination of pasteurized milk. *S. liquefaciens* and *K. oxytoca* were found in all milk samples from all the 8 provinces while 12 % of the species were isolated from at least 78 % of the samples (Fig. 3).
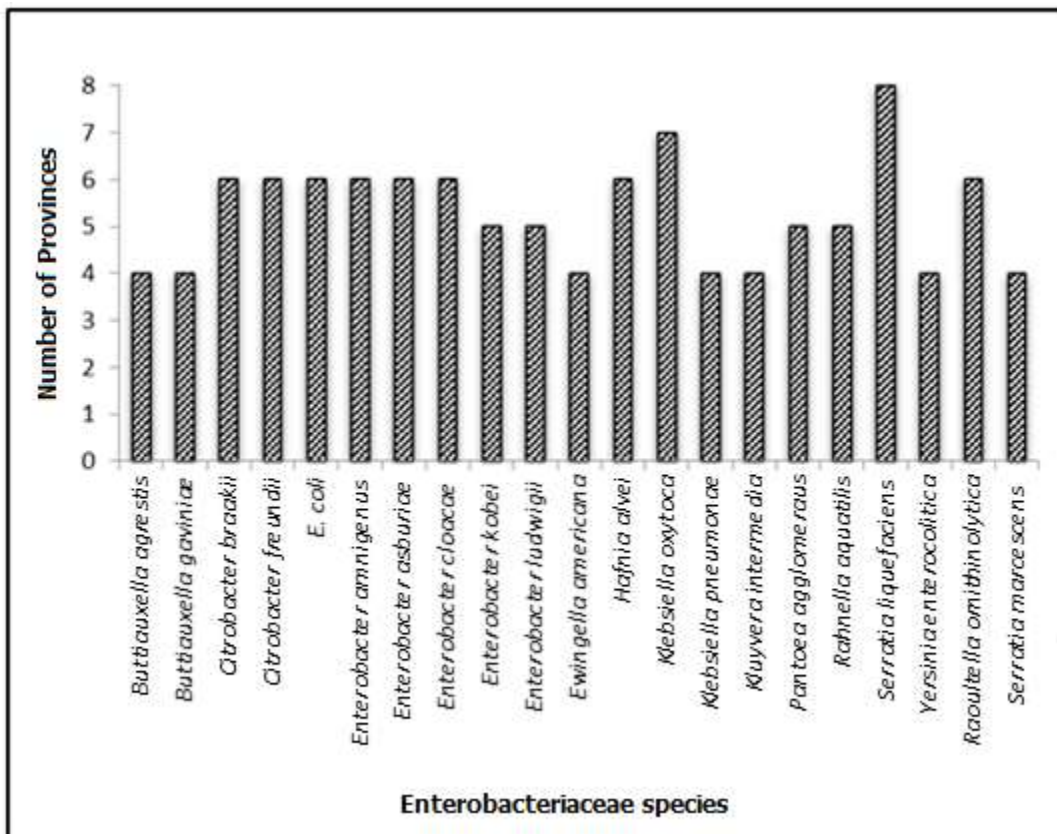

Fig 3: Frequency of dominant Enterobacteriaceae and other bacterial species isolated from raw and pasteurised producer-distributor bulk milk from 8 provinces in SA

We also identified *Yersinia enterocolitica* in 12 out of 258 PD milk samples. Two out of 12 isolates were isolated from 2 pasteurized PDBM samples. *Yersinia enterocolitica* has been

reported as an important public health hazard (Jayarao and Henning, 2001, Jayarao et al., 2006, Oliver et al., 2009) and has been frequently isolated in milk borne outbreaks in the USA and Europe (Oliver et al., 2009, EFSA, 2015). Jayarao and Henning (2001) in a survey in USA reported 8 pathogenic *Y. enterocolitica* isolated from 131 bulk tank milk samples. We however did not test the virulence capacity of *Y. enterocolitica* strains identified in the present study. Important to note is that *Yersinia enterocolitica* can also resist heat treatment and is able to grow to large numbers at below 15 ^{o}C (refrigeration temperatures) which makes contaminated milk a significant health risk (Jayarao and Henning, 2001).
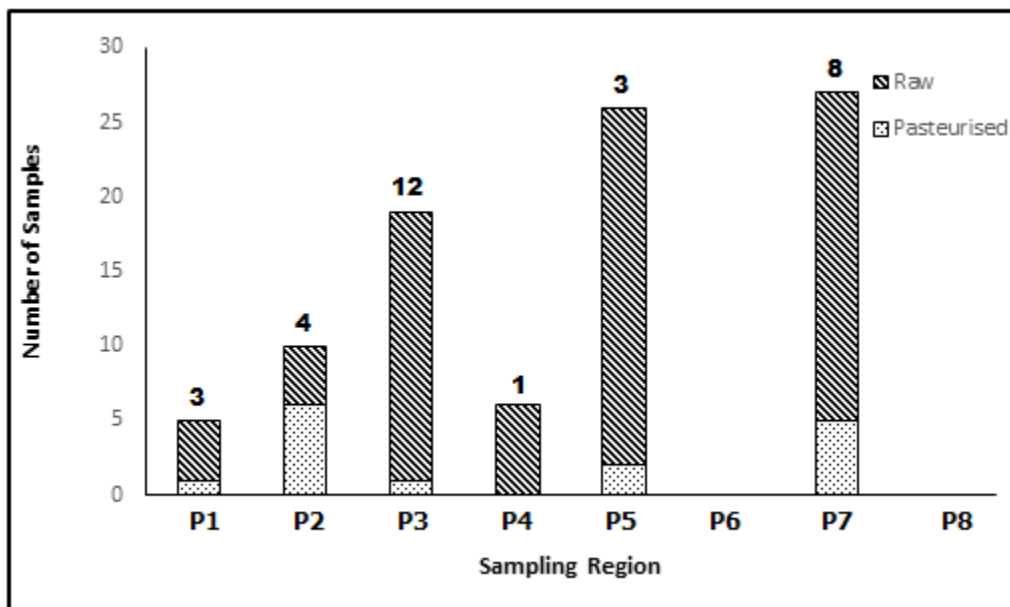

Fig 4: Number of samples which tested positive for *E. coli* from raw and pasteurised producer-distributor bulk milk from the 8 provinces in SA. Figures above the graphs represent number of *E. coli* isolates which tested positive for either *stx* 1/*stx* 2

Characterization of **E. coli**

E. coli was isolated from 36 % (n=258) of all the samples, with more raw milk samples (59 %) testing positive for *E. coli* than pasteurized milk (40 %) (Fig. 4). Only milk samples from two

provinces were negative for *E. coli*. All 121 *E. coli* isolates tested negative for the 11 tested serotypes. Van Kessel et al. (2004) reported that presence of *E. coli* in raw milk can be via intramammary secretion or via fecal contamination of udder or milking equipment.

In the present study, 2 % (n=258) PDBM samples tested positive for antibiotic residues. The risk of exposure to antimicrobial residues in milk has previously been reported (Kurwijila et al., 2006). Studies have reported a significant association between veterinary antibiotic residue in animal products and obesity in humans (Riley et al., 2007). However, there are disagreements when it comes to the association between antimicrobial residue in food and subsequent development of resistant by bacteria. Antimicrobial resistant *E. coli* isolates were detected in more than 70 % of the milk samples. Out of the 121 isolates tested, 73 % showed resistance to at least one of the 11 antibiotics (Table 2). Resistance to antibiotics in *E. coli* from provinces P2 and P7 was significantly higher ($p < 0.05$) than in all the other four provinces. Higher number of isolates were resistant to cephalothin. Approximately 60 % (n=89) of the antibiotic resistant isolates were confirmed multidrug resistant (MDR). The most prevalent MDR patterns were resistant to 2 and 3 of the antibiotic agents, however, more than 3 % of isolates in provinces P2 and P3 exhibited MDR resistance to either 8 or 9 antibacterial agents.

High resistance of the *E. coli* isolates to cephalothin (51 % isolates), neomycin (34 % isolates), ampicillin (24 % isolates), amoxicillin (23 % isolates) and oxytetracycline (17 % isolates) suggest the exposure of the isolates to the antibiotics. These antibiotics belong to commonly used classes of antibiotics including Cephalosporins, Tetracyclines, Aminoglycosides and Penicillins in SA for treatment, prophylaxis and as growth promoters in food-producing animals (Moyane et al., 2013). Kim et al. (2007) suggested use of antibiotics in treatment of cattle mastitis as a factor influencing increased prevalence of antibiotic-resistant pathogens though there is a lack of

consensus concerning the link between drug usage and subsequent resistance in bacteria. Human food safety concerns has been raised in cases where drug resistance bacteria is isolated in food since there is a potential transfer of resistance food borne pathogens to humans thought the food chain (Moyane et al., 2013). The highest resistance from the *E. coli* isolates in this study was noted in β-lactam antibiotics. β-lactam antibiotics resistance have low toxicity, a factor that has resulted in overuse of these drugs within animal husbandry and the medical fraternity (Moyane et al., 2013). Few studies have noted Enterobacteriaceae resistance to β-lactam antibiotic in milk samples, however, a study by (Geser et al., 2012) reported resistance in *E. coli* from milk

Table 2: Prevalence of antibiotic resistant *E. coli* isolated from producer-distributor bulk milk in SA

Antibiotic	P1 (n=10)	P2 (n=15)	Provinces			P7 (n=27)	Resistance (n =121)
			P3 (n=30)	P4 (n=7)	P5 (n=32)		
Oxytetracycline	10	33.3	23.3	12.5	15.6	7.6	**17.4**
Cephalothin	50	40	70	37.5	53.1	38.4	**51.2**
Nitroforantoin	0	6.7	10	0	3.1	15.3	**7.4**
Amoxicillin	40	6.7	36.7	12.5	21.9	15.3	**23.1**
Gentamicin	0	6.7	20	0	9.4	0	**8.3**
Neomycin	20	20	56.7	50	37.5	15.3	**34.7**
Amikacin	0	0	16.7	0	0	7.6	**5.8**
Norfloxacin	0	6.7	6.7	0	0	0	**2.5**
Nalidixic acid	0	0	6.7	0	0	0	**1.7**
Chloramphenicol	0	0	10	0	0	3.8	**3.3**
Ampicillin	20	6.7	46.7	12.5	25	15.3	**24.8**

Values in Table 4 represent % of resistant isolates. n- is number of *E. coli* isolates per province

samples to CTX-M β-lactam antibiotics. Resistance to β-lactam antibiotic and the aminoglycosides was detected in *E. coli* isolates (73 %, n=121) in PDBM sampled from all the provinces. This may be as a result of the *E. coli* from different provinces in SA being exposed to similar antibiotics despite the geographical difference.

There was a higher prevalence in multiple antibiotic resistant *E. coli* from province P2 and P3 than that of other provinces. Multiple resistance to antibiotics by bacteria may be linked to acquire resistance either by cross-resistance or co-selection of antibiotic resistance determinants (Baker-Austin et al., 2006). In cases of cross-resistance in bacteria, a single resistance mechanism may confer resistance to an entire class of antibiotics and co-selection means that, a bacteria can acquire additional resistance through mobile genetic elements such as plasmids in a large pool of antibiotic resistance bacteria in the environment, ultimately resisting more than one antibiotic (Baker-Austin et al., 2006). For this reason, PDBM in our study may serve as a reservoirs of antibiotic-resistant pathogens and can pose a food safety risk to the public as new antibiotic resistant pathogenic Enterobacteriaceae strain can emerge through gene transfer.

Thirty one *E. coli* isolates were positive for virulence genes *stx1*/*stx2* and 38% (n=121) were presumptive O157:H7. A total of 16 %, 13 % and 12 % (n=121) of the *E. coli* isolates were presumptively positive for O157:H7 on CT-SMAC, CHROMagar[TM] O157 and BBL[TM] CHROMagar[TM] STEC agar respectively. Out of the 46 isolates which were presumptive positive for O157:H7 *E. coli*, 10 % showed a positive result on at least two of the media. The intimin (*eae*) gene was absent in all the *E. coli* isolates (Table 3). A proportion of 28 % *stx1*/*stx2* positive *E. coli* were isolated in pasteurized PDBM samples. Six presumptive O157:H7 *E. coli* isolates also harbored *stx1*/*stx2* genes. Presence of virulence factors linked with enterohemorrhagic *E. coli* (EHEC) in PDBM suggested the ability to cause shigatoxin-related human infection and

disease. Milk borne outbreaks and hospitalization owing to this *E. coli* pathotypes has been well documented and reported around the world (CDC, 2005, Oliver et al., 2009, EFSA-ECDC, 2012).

Table 3: Occurrence of presumptive O157:H7 strains and virulence factors (stx_1, stx_2) in *E. coli* isolates from raw and pasteurised producer-distributor bulk milk in SA

Provinces	O157:H7 %	stx_1/stx_2 %	Total number of *E. coli* isolates
P1	40.0	30.0	10
P2	53.3	26.7	15
P3	50.0	40.0	30
P4	14.2	14.2	7
P5	12.5	9.2	32
P7	51.8	29.6	27
Total number of isolates	**46**(38.0%)	**31**(25.6)%	**121**

Shigatoxin producing *E. coli* was present in 7 % and 3 %, n=258 of the raw and pasteurized PDBM samples, respectively. Several studies around the world have reported the presence of STEC only in raw milk samples in Italy (5.7 %, n=123), USA (2.4 %, n=228) (Jayarao and Henning (2001), and France (21 %, n=205) Perelle et al. (2007). The main route by which STEC enters the raw milk supply is through fecal contamination or intramammary secretions from infected cow udder (King, 2007).

The sensitivity and specificity of the media used for the identification of presumptive *E. coli* O157:H7 was not evaluated, though CT-SMAC showed the highest number of O157:H7 characteristic colony appearance. Manafi and Kremsmaier (2001) evaluated sensitivity and

specificity of four different O157:H7 media SMAC, RainbowAgar O157:H7, Biosynth Culture Medium O157:H7 and Fluorocult HC and found Biosynth Culture Medium to be most sensitive media. However, they strongly recommended a confirmatory test either by immunological or PCR method for suspect EHEC colony, irrespective of whatever combination of media used.

Identification of *stx* genes in presumptive O157:H7 from PDBM in SA demonstrates that this milk may present a risk to public health. *E. coli* O157:H7 is of great significance to food borne diseases, because of its high virulence (it can cause disease at a dose of 5–50 cells) (Farrokh et al., 2013). Prevalence of STEC O157:H7 in bulk milk samples in our study (2 %, n=258) was in the range of studies, who worked on similar milk, carried in the USA, (0.02%, n=859) (Karns et al., 2007), (2.4% n=248) (Jayarao et al., 2006), Greece (0.7%, n = 950) (Solomakos et al., 2009) and Australian milk (varied from 1 - 3%) (Dairy Australia, 2006). All presumptive O157:H7 *E. coli* from PDBM which harboured *sxt1* and/or *stx2* lacked the *eae* gene. This is contrary to other studies which noted an association between *stx* and *eae* gene in *E. coli* (Omisakin et al., 2003). The EHEC O157:H7 strain contains a locus of enterocyte effacement which is characterized by presence of either *eae, stx1, stx2* genes or a combination of all the three (Karns et al., 2007). Pathogenic *E. coli* lacking the *eae* gene has been reported to use other adhesion factors for attachment and pathogenicity (Doulgeraki et al., 2011). In similar findings to our study, STEC isolates from raw milk (44 isolates) did not harbor the *eae* gene (Farzan et al. (2012). The 80 % (n=31) of *stx* positive *E. coli* which were not O157:H7 suggested that the PDBM was also contaminated with emerging non-O157:H7 EHEC strains. Non-O157 *E. coli* pathotypes have been well documented in food borne outbreaks which include milk and its products around the world (Constantiniu, 2002). Karns et al. (2007) reported a higher prevalence in non-O157 EHEC

than O157:H7and other serotypes in bulk tank milk. *E. coli* strains belonging to other serogroups (diarrheagenic) of public health concern may therefore pose health risk in PDBM in SA.

In both raw and pasteurized PDBM, 58 % of *stx1/stx2* positive *E. coli* were resistant to at least one antibiotic which was tested. Multi-drug resistant (MDR) to at least two antibiotics was recorded in 38 % (n=31) of the *stx1/stx2* positive isolates. More than 50 % of the *stx1/stx2* positive isolates were resistant to Cephalothin, a beta lactam first generation drug. Result of the two-sided Fisher's test revealed that there was no significant correlation between antibiotic resistance and presence of *stx1/stx2* gene. The dissemination of resistance is associated with genetic mobile elements such pathogenicity islands that may also carry virulence determinants (da Silva and Mendonça, 2012). Apart from virulence traits and antibiotic resistance genes being on the same genetic island such as plasmids, *E. coli* can also acquire virulence factor-encoding prophase (on chromosomal DNA) or plasmid bearing antibiotic resistance factors (Rasko et al., 2011).

We also isolated STEC (2 isolates) which were resistant to quinolones in PDBM from the current study. Quinolone resistance are reported to have an inverse relationship with virulence genes. Resistance to the quinolones is mediated by point mutation in DNA gyrases and topoisomerases (da Silva and Mendonça, 2012). Most researcher have argued the use of antibiotics such as quinolone in treatment of STEC infected patients because they have highlighted their administration to increase risk of HUS (Muniesa et al., 2012). In this current study, prevalence of potential pathogenic EHEC which has drug resistance capacity, and also possibility of the organisms to grow under improper storage can present a significant public health risk, especially to the vulnerable members of the community.

Hierarchical Cluster Analysis

Five main clusters (A to E) were observed from the *E. coli* dendrogram (Fig 5). The *E. coli* isolates were further subdivided into 8 sub-clusters defined by greater than 75 % similarity which was equivalent to 1.5 a distance level considered as demarcating similar strains. Isolates within the five main clusters grouped irrespective of the province they were isolated. Group A comprised of isolates which emanated from five provinces, however, isolates from province P5 (40 % n=22) were dominant. Suffice to note, all the five main clusters (A − E) observed contained at least one isolate from the 6 different provinces. The five main cluster groups contained at least 4 *stx1/stx2* positive *E. coli* isolates which came from different geographical locations. Isolates from P5 were found in all the sub-clusters. However, isolates from province P3 and P7 formed at least 80 % of isolates from sub-clusters C5 and C8. Sub-cluster C4 was the biggest group consisting of 21 % (n=121) of the *E. coli* isolates which were representative isolates from all the 6 provinces. This group consisted of isolates with common resistance to cephalothin, neomycin, ampicillin, amoxicillin and oxytetracycline. There was no particular trend with respect to origin of the isolates. The isolates grouped irrespective of the origin indicating that isolates from different provinces were related. Some of the isolates from certain provinces were found in all the cluster groups which indicates high genetic diversity among the isolates and wide distribution despite spatial differences in the milk sources. High genetic diversity of *E. coli* has been noted in isolates from human, animal, food and the environment (Aslam et al., 2003, Apun et al., 2006, Van Elsas et al., 2011). Reason for high genetic diversity in the *E. coli* could be attributed to its versatility and high degree of genomic plasticity, via gene loss or gain and through lateral gene transfer (Rasko et al., 2008). Internationally standardized phage typing, subtyping of *stx* and *eae* genes, molecular fingerprinting using PFGE or REP-PCR
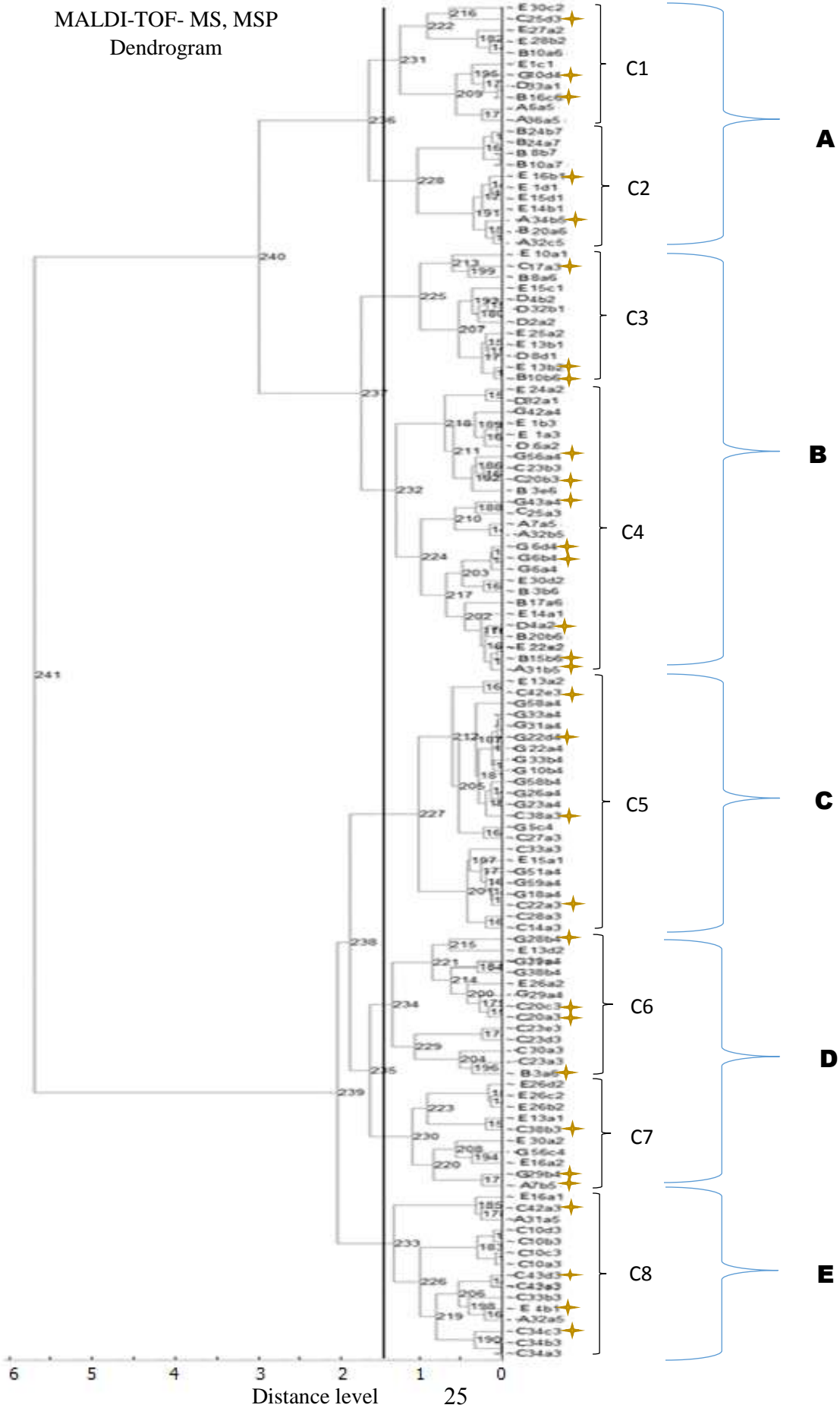
MALDI-TOF- MS, MSP
Dendrogram

Distance level

25

Fig 5: Two-dimensional hierarchical cluster analysis dendrogram for 121 *Escherichia coli* isolates from producer-distributor bulk milk in SA. Capital letters represent the different geographical provinces while numbers with small letters represent different strains. The vertical line at 1.5 distance level represent the minimum similarity considered to be the highest point which distinct strains were identified (>75% similarity). Cluster groups under distance level 1.5 were considered to be distinct strains. Major brackets enclose the five super clustering groups (A to E) while the minor brackets enclose the sub-clusters (C1 to C8). Provinces were labelled as follows: *P1-* (A), *P2-* (B), *P3-* (C), *P4-* (D), *P5-* (E), *P6-* (F), *P7-* (G), *P8-* (H) Isolates with the coloured asterisk ( ) were positive for *stx1/stx2*.

have been used in studying epidemiological relationships and diversity among *E. coli* pathotypes (Beutin et al., 2002). However, recent developments in mass spectrometry (MS) used in our study compares well to molecular tools and have made it possible to use whole-cell MALDI-TOF MS in elucidating relationship among bacteria isolates from food and the environment. Albeit, originating from different provinces, STEC from PDBM in the current study revealed genetic relatedness by grouping in unique class. *E. coli* clusters which had common antibiotic resistance patterns, even though they came from different regions, revealed possibility of common antibiotics use in different provinces in SA (da Silva and Mendonça, 2012).

S. liquefaciens and *K. oxytoca* were clustered because of their high occurrence and frequency of isolation in all the 8 provinces (Fig 3) and also their significance in food safety as opportunistic pathogenic and food spoilage organisms. *S. liquefaciens* strains showed three main distinct clusters (A, B and C) and 6 sub-clusters (C1 to C6) from the 8 provinces *S. liquefaciens* isolates were distributed irrespective of origin in all the three main clusters (Fig 6). Isolates from P1 were found in sub-clusters C2, C3, C4 and C6 and they also formed 70 % of the members of the sub-cluster groups C3 and C6. Sub-cluster group C1 was the biggest group which consisted of 25 % (n=47) of the *S. liquefaciens* isolates and majority of the isolates in C1 came from province P5.
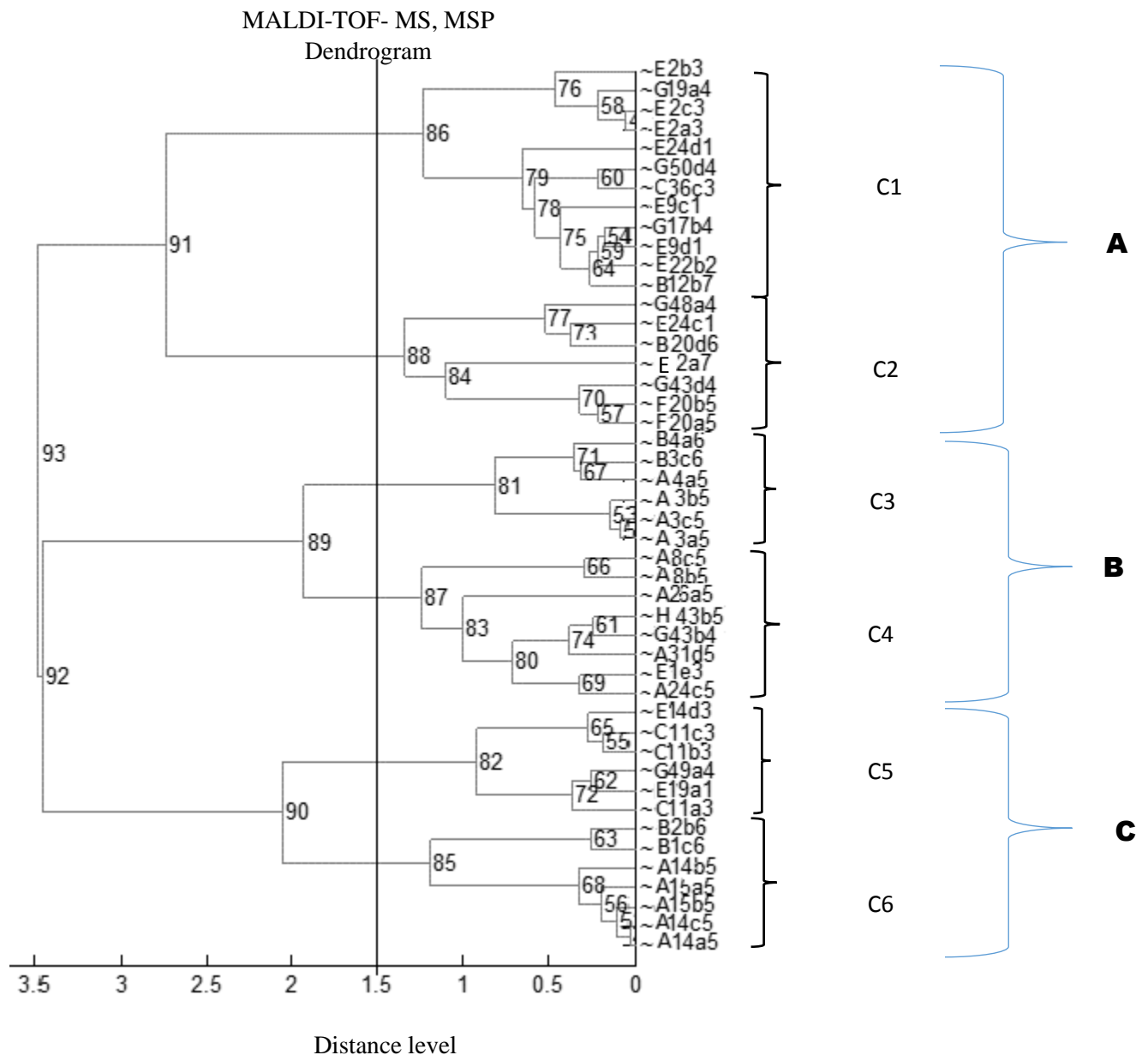
Fig 6: Two-dimensional hierarchical cluster analysis dendrogram for 47 *S. liquefaciens* isolates from producer-distributor bulk milk in SA.

Capital letters represent the different geographical provinces while numbers with small letters represent different strains. The vertical line at 1.5 distance level represent the minimum similarity considered to be the highest point which distinct strains were identified (>75% similarity). Cluster groups under distance level 1.5 were considered to be distinct strains. Major brackets enclose the three super clustering groups (A, B, C) while the minor brackets enclose the sub-clusters (C1 to C6). Provinces were labelled as follows: *P1*- (A), *P2*- (B), *P3*- (C), *P4*- (D), *P5*- (E), *P6*- (F), *P7*- (G), *P8*- (H).
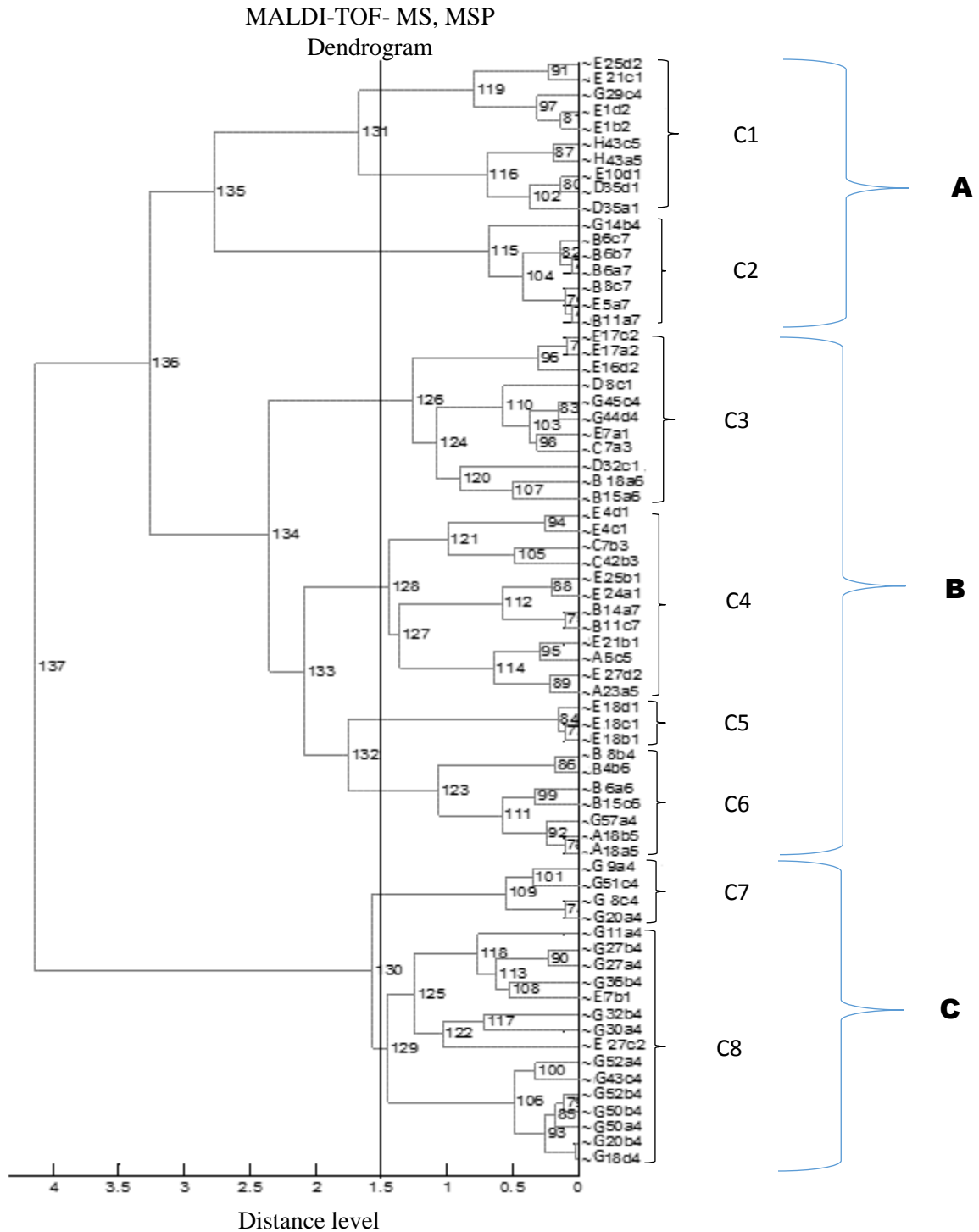
Fig 7: Two-dimensional hierarchical cluster analysis dendrogram for 70 *K. oxytoca* isolates from producer-distributor bulk milk in SA. Capital letters represent the different geographical provinces while numbers with small letters represent different strains. The vertical line at 1.5 distance level represent the minimum similarity considered to be the highest point which distinct strains were identified (>75% similarity). Cluster groups

under distance level 1.5 were considered to be distinct strains. Major brackets enclose the three super clustering groups (A, B, C) while the minor brackets enclose the sub-clusters (C1 to C8). Provinces were labelled as follows: *P1-* (A), *P2-* (B), *P3-* (C), *P4-* (D), *P5-* (E), *P6-* (F), *P7-* (G), *P8-* (H).

Sub-clusters from *S. liquefaciens* dendrogram consisted of isolates from provinces which are in close geographically proximity.

A two dimensional hierarchical cluster analysis for *Klebsiella oxytoca* showed 8 sub-clusters from 3 distinct main clusters (A, B, C). Isolates within the main cluster groups A and B were grouped irrespective of the geographical source. Cluster group B contained 4 sub-clusters with isolates mostly from GP (Fig 7). Isolates from P7 formed 90 % of the members of cluster group C. Sub-cluster C8 was the biggest consisting of 21 % of the *K. oxytoca* isolates and 86 % (n=15) were from province P7. Isolates from province P5 where found in all the sub-clusters. Isolates originating from provinces in close proximity, grouped together into sub-clusters.

S. liquefaciens and *K. oxytoca* isolates also showed no particular trend with respect to origin of the isolates. The isolates, like in *E. coli* cluster analysis, grouped irrespective of the origin indicating high genetic diversity as well. Due to genomic plasticity of Enterobacteriaceae through lateral gene transfer, plasmid-encoding genetic determinants give rise to genetic and ecological diversity in this family of bacteria (Brisse and Verhoef, 2001, Iguchi et al., 2014a). Iguchi et al. (2014b) characterized genetic diversity of *Serratia* spp., using complete genome sequences and found a remarkable intra-species genetic diversity, both at the sequence level and with regards to genome flexibility, which may reflect the diversity in members of this species. Brisse and Verhoef (2001) noted high degree of genetic heterogeneity and phylogenetic diversity of *K. oxytoca* from 120 clinical isolates using randomly amplified polymorphic DNA (RAPD) analysis. The authors noted that molecular techniques such as RAPD and 16S rRNA PCR have

limitation in deducing strain relationship of Enterobacteriaceae species. Such shortcomings can be overcome by use of MALDI-TOF-MS. The MALDI-TOF-MS techniques compares well with minimal limitations in bacteria characterization (Maja et al., 2013), source tracking (Siegrist et al., 2007) and strain relationship (Pignone et al., 2006) between isolates from food and the environment. Based on this study, the analysis of level of diversity among strains originating from different location using MALDI-TOF-MS can be helpful in typing possible clonal relationship among Enterobacteriaceae strains and to monitor the pathway of transmission of pathogenic strains that may pose threat to public health and also the spoilage microbiota in food.

CONCLUSION

This study report poor microbiological quality characterized by the occurrence of a high microbial counts which may reduce shelf life and can be a reservoir of pathogenic bacteria that potentially pose public health risks in PDBM. A wide diversity of Enterobacteriaceae species belonging mostly to spoilage microbiota was detected in PDBM. Considerable genetic diversity was found among the *E. coli, S. liquefaciens* and *K. oxytoca* strains. We detected multidrug resistant pathogenic *E. coli* in both raw and pasteurized retail PDBM which is a cause of concern since these strains are a potential source of antimicrobial resistant food borne pathogens to humans though the food chain. These findings call for further assessment of the potential risk posed to public health by the milk-borne pathogens through quantitative microbial risk assessment as a prerequisite to formulation of situation specific mitigation strategies.

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