Pit latrines in a peri-urban South African community - a hygiene challenge and a health risk owing to current desludging practices and biofilm-forming, multi-drug resistant bacteria

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Declaration 2 - Publications and Conference Presentations

Details of publications and conference presentations that form part and/or include research presented in this thesis:

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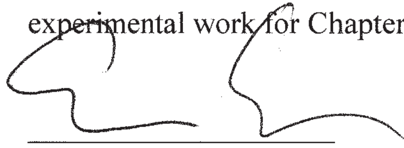
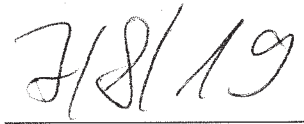
Ms Lorika Beukes performed the experimental work and drafted the manuscripts for the publications and drafted the posters and talks for the conference presentations. Ms T. L King assisted with the experimental work for publication 1 and Ms T. Beharielal assisted with the experimental work for Chapter 6.





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This thesis represents a compilation of manuscripts where each chapter is an individual entity therefore, some repetition between chapters has been unavoidable.

List of Abbreviations

ABC	ATP binding cassette
ABR	antibiotic resistance
AIEC	adherent-invasive *Escherichia coli*
AMR	antimicrobial resistance
ATP	adenosine triphosphate
BRICS	Brazil, Russia, India, China and South Africa
BSAC	British society for antimicrobial chemotherapy
cAmpC	chromosome-encoded AmpC (β-lactamase)
CFB	Cytophaga-Flavobacterium-Bacteroides
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
CNS	coagulase negative *Staphylococcus* spp.
CPS	coagulase positive *Staphylococcus* spp.
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
CV	crystal violet
DAEC	diffusely adherent *Escherichia coli*
DAPI	4’,6-diamidino-2-phenylindole
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EAEC	enteroaggregative *Escherichia coli*
EC	*Escherichia coli*
EcoSan	ecological sanitation
eDNA	extracellular DNA
EHEC	enterohemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
ENT	ear, nose and throat
EPEC	enteropathogenic *Escherichia coli*
EPS	extracellular polymeric substance
ESBL	extended spectrum β-lactamase
ETEC	enterotoxigenic *Escherichia coli*
EUCAST	European committee on antimicrobial susceptibility testing

FA	fossa alterna
GAPPD	global action plan for the prevention and control of pneumonia and diarrhea
GAS	group A *Streptococcus*
GI	gastrointestinal
GM	gut microbiota
HDPE	high-density polyethylene
HIV	human immunodeficiency virus
HOCl	hypochlorous acid
HRMO	highly resistant microorganism
HUS	haemolytic uraemic syndrome
ISO	international organization for standardization
LDPE	low-density polyethylene
MATE	multi-drug and toxic compound efflux system
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MBL	metallo-β-lactamase
MDR	multidrug resistant
MF	major facilitator
MGE	mobile genetic element
MG-RAST	metagenome rapid annotation subsystem technology
MIC	minimum inhibitory concentration
MLSB	macrolides, lincosamides and streptogramin B
MPN	most probable number
MRSA	methicillin resistant *Staphylococcus aureus*
MSSA	methicillin susceptible *Staphylococcus aureus*
NaCl	sodium chloride
NaOCl	sodium hypochlorite
NCBI	national center for biotechnology information
NGS	next generation sequencing
NRCS	national regulator for compulsory specifications
NS	not sampled
OMP	outer membrane protein
ORSA	oxacillin resistant *Staphylococcus aureus*
OTU	operational taxonomic unit

pAmpC	plasmid-mediated AmpC (β-lactamase)
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PDR	pandrug-resistant
PETE/PET	polyethylene terephthalate
PP	polypropylene
PPE	personal protective equipment
PS	polystyrene or styrofoam
PVC	polyvinyl chloride
RDP	ribosomal database project
RND	resistance-nodulation-division
rRNA	ribosomal ribonucleic acid
RTE	ready-to-eat
SA	South Africa
SCCmec	staphylococcal cassette chromosome mec
SCFA	short chain fatty acid
SDS	sodium dodecyl sulphate
SMR	small multidrug resistance (efflux systems)
SNP	single nucleotide polymorphism
SOP	standard operating procedures
STEC	shiga toxin-producing *Escherichia coli*
TB	tuberculosis
TSB	tryptic soy broth
UDDT	urine diverting dry toilet
USA	Unites States of America
UTI	urinary tract infection
UV	ultra-violet (light)
VBNC	viable but not culturable
VIP	ventilated improved pit latrine
VRE	vancomycin resistant *Enterococcus*
VTEC	verotoxigenic *Escherichia coli*
WASH	water, sanitation and hygiene
WWTP	wastewater treatment plant
WHO	world health organization

XDR	extensively drug-resistant
ZOI	zone of inhibition

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Abstract

In South Africa there is a lack of data on the screening and emergence of antibiotic resistant bacteria from pit latrines and the potential health risks faced by community members and municipal workers who manually empty these sanitation systems. Therefore, the aim of this study was to initially determine the presence of bacterial contamination from ventilated improved pit latrine (VIP) fecal sludge on peri-urban household surfaces (KwaZulu-Natal, South Africa), on municipal workers' skin, their personal protective equipment (PPE) and the municipal vehicle before and after manual pit latrine emptying. Thereafter, the bacterial diversity in the pit latrine fecal sludge was determined using next generation sequencing (NGS). Identifying all pathogens present in fecal sludge is complex and tedious, therefore selected indicator microorganisms are often used in risk assessments to predict the presence of other fecal pathogens. For the purpose of this study, the well-established Gram negative hygiene indicator *Escherichia coli* and a representative Gram positive genus (*Staphylococcus* spp.) were targeted. The antimicrobial resistance profiles of *Escherichia coli* and *Staphylococcus* spp. were determined using the European committee on antimicrobial susceptibility testing (EUCAST) disk diffusion method. The British society for antimicrobial chemotherapy (BSAC) procedure was used for oxacillin sensitivity in staphylococcus isolates. β-Lactamase activity was then confirmed in *E. coli* pit latrine isolates displaying resistance or intermediate resistance to β-lactam antibiotics using the chromogenic cephalosporin, nitrocefin. Finally, the ability of selected antibiotic resistant *E. coli* and *Staphylococcus* spp. isolates to form biofilms on household surfaces was determined via the crystal violet (CV) assay and confirmed by microscopy. Additionally, the bacteriostatic activity of a commercially available sporicidal bedscreen curtain was tested against multi-drug resistant (MDR) and non-MDR coagulase positive and negative *Staphylococcus* spp. (CPS, CNS) and *E. coli* pit latrine isolates, as bedscreen curtains in healthcare facilities within peri-urban communities can be involved in the transmission of disease from patients infected with potential pathogens found in pit latrines to non-infected patients.

The results obtained indicate that the frequency of detection of *E. coli* and *Staphylococcus* spp. on surfaces surrounding households and on municipal workers hands occasionally increased after the pits were emptied. In addition, it was also evident that the hands of municipal workers emptying these pit latrines were frequently contaminated even before pit emptying. NGS revealed that the five most abundant bacterial genera in the pit latrine fecal sludge microbiome

belonged to *Pseudomonas*, *Bacillus*, *Escherichia, Candidatus Aquiluna* and *Candidatus Rhodoluna*. In addition, the genus *Staphylococcus* was among the genera representing <1% of the bacteria in the pit latrine fecal sludge. Using a most probable number (MPN) method, the abundance of *E. coli* in pit latrine samples was established in the range of one to 6.2 $\log_{10}$ MPN per gram of fresh pit latrine fecal sludge. In addition, an average presumptive *Staphylococcus* spp. count of 2.1×10^5 colony forming units (CFU) per gram of pit latrine fecal sludge was established from two randomly selected pit latrine fecal sludge samples. Fourteen percent of the *E. coli* pit latrine isolates were categorized as MDR, all showing resistance to four or more antibiotics. The 68 selected *Staphylococcus* spp. pit latrine isolates comprised of 49% coagulase positive (CP) and 51% coagulase negative (CN) strains with 65% (36% CP, 91% CN) categorized as MDR. The nitrocefin test proved useful in the detection of β-lactamase activity in *E. coli* pit latrine isolates displaying resistance to ampicillin. It also highlighted that in the case of isolates displaying resistance to β-lactam antibiotics using antibiotic disk diffusion assays, more than one mechanism of resistance can be expected.

A coagulase positive MDR *Staphylococcus* spp. strain was identified as the most prolific biofilm former among the tested pit latrine isolates. Electron microscopy revealed that a 5 minute treatment with 3% formaldehyde was most effective in reducing the metabolic activity of cells within *Staphylococcus* and *E. coli* biofilms. In addition, a commercial household bleach was able to reduce the metabolic activity within both *Staphylococcus* and *E. coli* biofilms while a biocide-free, commercial household dishwashing liquid was least effective in reducing the metabolic activity within *Staphylococcus* biofilms. The tested bedscreen curtain inhibited MDR and non-MDR CPS, CNS and *E. coli* pit latrine isolates but was more effective against *Staphylococcus* spp. isolates. Overall, the results obtained indicate that the manual emptying of pit latrines in peri-urban communities presents a hygiene challenge due to the presence of bacterial contamination on certain household surfaces and on municipal workers' skin after pit emptying and a potential health risk due to the presence of biofilm forming, MDR bacteria present in pit latrine fecal sludge.

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Chapter 1

Literature review

Introduction

Adequate sanitation, acceptable hygiene practices and the provision of safe drinking water are the three main factors contributing to good health as well as social and economic development (Lane et al. 2010). According to the South African National Sanitation Policy (2016), the domestic sanitation objective is to raise the proportion of families with access to functional sanitation from 84% in 2013 to 90% by 2019. Despite the effort to increase basic sanitation facilities, diarrhea remains the second leading cause of child deaths under the age of five globally, with the majority of these deaths in South Asia and Sub-Saharan Africa (WHO-MCEE 2018). In 2017 intestinal infectious diseases (which include diarrhea) were ranked third among the ten leading underlying natural causes of death for infants and children below the age of 5 in South Africa (Stats SA 2019a).

About 13.6% of South African households lived in informal dwellings and 5.5% in traditional dwellings in 2017 (Stats SA 2019b). According to Statistics South Africa (Stats SA 2019b), the percentage of households in South Africa with access to improved sanitation facilities increased from 61.7% to 82.2% from the year 2002 to 2017. Despite this increase many households are still without any sanitation facilities. Some of the major challenges facing these communities, include borehole/groundwater pollution mainly due to the leaching of chemical and/or bacterial contaminants from pit latrines into the surrounding soil and groundwater system, water depletion, access to proper infrastructure related to water supply, poor sewerage systems and improper disposal of solid wastes (Parkinson and Tayler 2003). The factors that aggravate the above circumstances are a lack of public awareness, a lack of education and poor attitudes towards proper hygiene and sanitation (Parkinson and Tayler 2003; Sibiya and Gumbo 2013). Although pit latrines are a cheap and basic means of sanitation in peri-urban settings, there are problems associated with these systems. Some of the major challenges associated with pit latrines include the addition of solid and liquid wastes other than feces and urine to the pits (Bakare et al. 2012; Brouckaert et al. 2013), the method in which the pits are emptied (O'Riordan 2009), the time delays between pit emptying (EWS 2011) and the limited access of disposal vehicles and pit emptying equipment to peri-urban communities due to poor road

infrastructures (Montangero and Strauss 2002). In addition, traditional pit latrines tend to easily collapse due to the effects of underground water and sandy soils, they also require sufficient land space to dig new pits as they are not permanent structures and their contents cannot be directly reused for agriculture (Grimason et al. 2000). In a study by Jenkins et al. (2015), residents from peri-urban areas in Dar Es Salaam, Tanzania, complained of pit latrines that were flooded, full and of poor quality and about a lack of pit emptying services and government assistance with these systems. Municipal workers involved in emptying pit latrines have a higher risk of exposure to toxic chemicals and pathogenic bacteria (Lewin et al. 2007) found in pit latrine fecal sludge. Poor hygiene practices, such as a lack of hand washing after handling fecal material or desludging/emptying of pit latrines, adopted by municipal workers or household members, can lead to fecal contamination on surfaces inside and/or surrounding peri-urban households as well as on the PPE (personal protective equipment) of workers. This in turn can lead to the spread of infectious diseases unless proper hygiene measures are implemented.

The microorganisms found in pit latrine fecal sludge are usually determined by the individuals who make use of these systems (WHO 2008). Therefore, if individuals are infected with pathogenic bacteria, these pathogens may also be present in the pit latrine fecal sludge and can therefore present a health risk to other individuals who make use of these systems. Exposure to these microorganisms can take place via inhalation of bioaerosols in close proximity to the pit latrines (Douwes et al. 2003; Farling et al. 2019), ingestion of contaminated groundwater, accidental ingestion of fecal sludge and through poor hygiene practices.

Escherichia coli (*E. coli*), a Gram negative, facultative anaerobic bacterium of the Enterobacteriaceae family, is frequently encountered in humans and livestock, but also in other settings such as soil and water (Escherich 1886; Malcolm 1938; Byappanahalli et al. 2006). As *E. coli* is commonly associated with human and animal feces, it is used as a typical hygiene indicator for fecal contamination. *E. coli* is part of the natural gut microflora and is usually not pathogenic. However, pathogenic *E. coli* strains do exist such as EPEC (enteropathogenic), ETEC (enterotoxigenic) and EIEC (enteroinvasive) strains, which have been implicated in cases of morbidity and mortality. Another bacterial genus associated with human or animal feces is *Staphylococcus* spp. Members of this genus are Gram positive cocci, which often grow in clusters (Chambers 2001) and are comprised of more than 60 recognized species and sub-species. Most are members of the normal skin flora, others colonize the gut of humans and

animals and some strains are potential pathogens (Acton et al. 2009; Lamers et al. 2012). The most common pathogenic strains infecting humans belong to the species of *Staphylococcus aureus* and *Staphylococcus epidermidis*, which is commonly found on the skin where it is known to be harmless. However, *Staphylococcus epidermidis* can cause disease in unhealthy or immunocompromised individuals (Mack et al. 2004). Both *E. coli* and *Staphylococcus* spp. can be expected in pit latrine fecal sludge material and are able to survive for extended periods on biotic (Ayliffe et al. 1988; Fryklund et al. 1995) and abiotic surfaces (Neely and Maley 2000; Warnes et al. 2012) in the form of biofilms. Biofilms provide a protective barrier for these microorganisms against antimicrobial agents such as biocides and antibiotics (Kumar and Anand 1998; Bjarnsholt 2013), making them difficult to eradicate. Only recently, Cave et al. (2019) recovered multidrug resistant staphylococci on general public surfaces and on surfaces in hospital public areas in East and West London.

Research has shown that access to improved water, sanitation and proper hygiene reduces diarrhea, morbidity and mortality, particularly in children (Albonico et al. 2008; Montgomery and Elimelech 2007; Joshi and Amadi 2013). In addition, Navab-Daneshmand et al. (2018) found that the presence of handwashing facilities at urban households was associated with significantly lesser *E. coli* contamination of hands. Therefore, efficient handling and treatment of pit latrine systems and appropriate hygiene practices should be implemented in order to reduce the risk of contamination and thus the spread of infectious diseases within peri-urban communities.

A. Pit Latrines

In most developing countries, peri-urban households are usually allocated to a pit latrine, which is used by one or shared between several households. Shared sanitation facilities are beneficial as sanitation costs can be divided between households, making it a cheaper option than single pit latrines and they can also help reduce open defecation in these areas however, they do have some downfalls (Still and O'Riordan 2012). According to Heijnen et al. (2014), shared sanitation may lead to diarrhea, helminth infections, enteric fevers, other fecal-oral illnesses, trachoma and maternal or birth complications. However, according to a report by McGinnis et al. (2019), pit latrines shared by community members may be as clean as or even cleaner than household latrines provided they are well maintained. Therefore, the use of community or

shared latrines is encouraged to improve access to sanitation infrastructure in developing countries. The number of shared sanitation facilities in developing countries has steadily increased since 1990 to more than 437 million (Templeton 2015). According to Thye et al. (2011), 18% of sub-Saharan Africa and 19% of Eastern Asia make use of shared sanitation facilities with the highest percentage of users in Ghana (59%), Congo and Gabon (both 34%).

1. Pit latrine characteristics

Approximately 16.6% of the South African population makes use of ventilated improved pit latrines (VIP's) and 14.5% use non-ventilated pit latrines (Stats SA 2016). Pit latrines are basically holes dug to a specific depth in the soil for the purpose of defecation and urination and are usually covered with a concrete slab (Graham and Polizzotto 2013). Ventilated improved pit latrines (VIP's) are permanent single-pit structures with the following dimensions and features: a minimum depth of 1.5m, a foundation, cover slab and a framework with a vent pipe and a fly screen (Fig. 1) (Mara 1984). The structure of a pit latrine affects factors such as the content of the latrine and the frequency at which it fills (Thye et al. 2011). There are two primary categories of pit latrines, i.e. those, which allow liquids to flow out of the pit and those which retain solids and liquids (Hawkins 1982). Pit latrine sludge in the first category is usually thicker and more compact and therefore not easily accessible to pit emptying machines such as vacuum tankers (Hawkins 1982). In this case, manual pit emptying would be the practical option.

2. Pit latrine fecal sludge characteristics

The characteristics of pit latrine sludge vary between households and communities. Additional waste other than excreta that is added to the pit latrines, the climate and soil characteristics and the diet of individuals making use of these systems, all impact on the characteristics of the sludge found in pit latrines (Pickford 1995; Still 2002; Thye et al. 2011). Over 50% of households using pit latrines in Tanzania, Dar Es Salaam added a variety of products to the pits in order to manage bad odors and insects and to reduce the sludge volume, a behavior which was common among households with full pit latrines (Jenkins et al. 2015). The products added to the pit latrines included salt, ashes, old batteries, diesel and paraffin, which were added to reduce the sludge volume by a process referred to as sludge sinking (Jenkins et al. 2015).

Figure 1 Ventilated improved pit latrine (VIP) structure. (A) Entrance to VIP, (B) area where pit latrine fecal sludge is collected when the pit is full.

Pit latrine fecal sludge consists of four layers; floating scum, liquid, a sludge layer and a sediment layer (Hawkins 1982). A layer of scum is present in pits with poor drainage and pits with multiple users and a well-defined sludge layer can be observed after six months of usage. Density is a major characteristic of pit latrine sludge and can range from 0.97 to 1.75 kg/dm^3 (Hawkins 1982). Moisture content affects sludge density, which in turn affects the fluidity of the sludge, thereby impacting on the method used to empty the pit latrine (Hawkins 1982; Still 2002). Pit latrine sludge high in moisture content is more fluid and can be easily removed by vacuum tankers, while manual pit emptying is more applicable for sludge with low moisture content. Apart from urine, rain water and household wastewater, refuse added to pit latrines impacts on the moisture content of the pit latrine sludge as it is high in organic content (Hawkins 1982). Moisture content can also provide an indication of the age of the sludge and/or the age of the pit latrine as moisture content decreases when decomposition takes place (Hawkins 1982).

3. Pit latrine filling rates

The rate at which pit latrines fill is determined by the size of the pit latrine, the area where the pit latrine is located, the number of people using the pit latrine and the amount of excreta produced by each individual (Pickford 1995; Still 2002). Individuals in low-income countries typically produce on average 250g wet weight or 38g dry weight of feces per individual per day (Rose et al. 2015). Other factors that contribute to the rate at which pit latrines fill relate to the characteristics of the surrounding soil, the depth of the groundwater table, the

presence/absence of a pit lining, material used for anal cleaning and the amount of waste - other than excreta - that is disposed into the pit such as food, clothing, garden waste, etc. (Pickford 1995; Zziwa et al. 2016). When determining the filling rate one must also consider the amount of decomposition that takes place in the pit (Zziwa et al. 2016). Taking all factors into consideration, pit latrines can take up to 25 years to fill (Still 2002; Brouckaert et al. 2013).

4. Fecal sludge management in peri-urban communities

Three common practices used to manage fecal sludge accumulation in pit latrines were identified in Dar Es Salaam, Tanzania. These included replacing the pit latrine, pit emptying and pit additives (Jenkins et al. 2015).

4.1 Pit latrine replacement

Forty one percent of households with pit latrines in Dar Es Salaam had their old pit latrines replaced with new ones. The main reason for pit latrine replacement was the pit latrine being full. Other reasons were due to the collapse or poor condition of the previous latrine or to install a more modern pit latrine (Jenkins et al. 2015).

4.2 Pit latrine emptying

A full pit latrine can have many undesirable consequences such as overflowing of fecal sludge into households, neighborhoods and water ways due to flooding during the rainy season as well as unsanitary and unpleasant conditions (bad odors) in the pit latrines. As a result, there is an increased risk of direct contact with fecal sludge, increased exposure to flies and insects and increased contamination of shallow groundwater (Jenkins et al. 2015). The choice of pit emptying method is not only influenced by affordability and ease of availability but also by the ability of the method to remove all the sludge from the pit latrine (Jenkins et al. 2015). There are three common pit latrine emptying methods: pure manual (Fig. 2), semi-mechanized (labor and machine) and fully mechanized (100% machine). These methods can be further separated into 'hygienic' or 'unhygienic' methods. Pit emptying using machinery is considered hygienic or safe as it separates waste from human contact (Thye et al. 2011) and after the fecal sludge is extracted from the pit latrines, it is safely transported to authorized treatment facilities (Jenkins et al. 2014). Unhygienic methods used to empty pit latrines include manual pit emptying, pit diversion and flooding out of pit latrines (O'Riordan 2009; Jenkins et al. 2014). These methods are considered as unhygienic or unsafe as sludge is usually left on the property

or released into the neighborhood where it can contaminate the environment and causes bad odors (Jenkins et al. 2015).

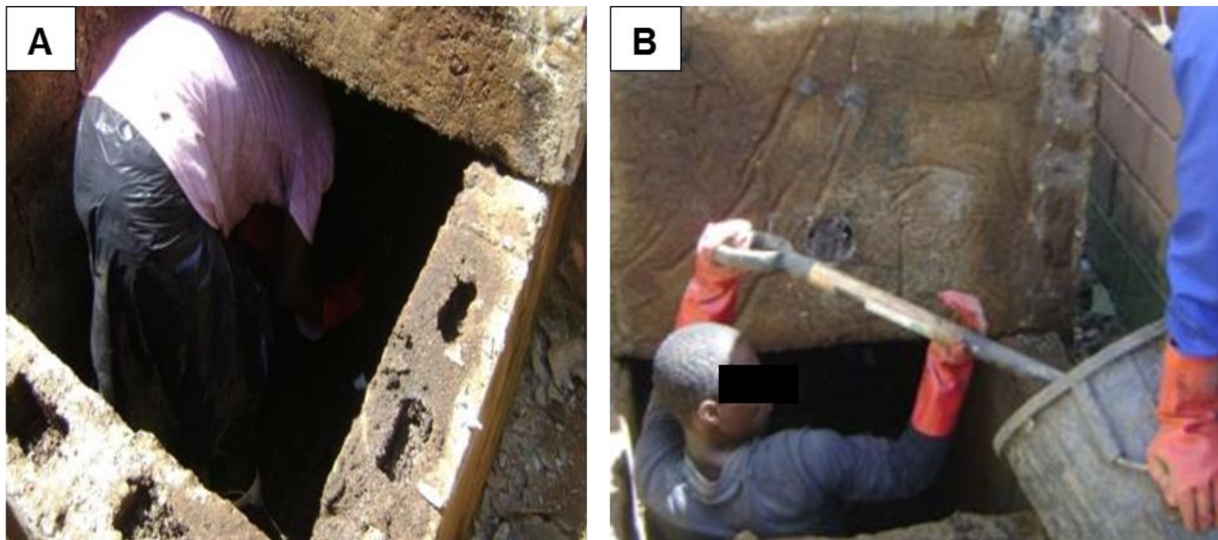

Figure 2 Images of municipal workers manually emptying pit latrines (A and B) in a peri-urban community in KwaZulu Natal, South Africa.

Two major reasons for the use of manual pit emptying are the difficulty of passing large pit emptying equipment through very tiny spaces between households and the affordability of this method. In addition, if sludge is left to accumulate in pit latrines, it eventually starts solidifying, making it difficult for vacuum pumps to remove (Tilley et al. 2014). Although a cheaper option than purchasing and maintaining expensive equipment, manual pit emptying is labor intensive and the riskiest option for workers and households, as workers must physically enter these pits to reach lower laying sludge (O'Riordan 2009). This directly exposes workers to potential pathogens present in the sludge, and in doing so they can cause contamination of the surrounding household environment by splashing sludge when they empty the pits (O'Riordan 2009).

Pit diversion entails slowly draining or flushing latrine sludge from an opening on the side of the pit into a hole dug adjacent to the pit latrine and flooding of pit latrines involves the deliberate release of fecal sludge into the neighborhood by means of an unplugged drain pipe, a technique often used during heavy rainfall (Jenkins et al. 2014). The above three methods involve the direct contact of workers or household members with fecal sludge and are therefore considered unhygienic and unsafe. The VIP's in the current study were emptied manually.

4.3 Pit latrine additives

In Malawi, residents added soil, ash, and used hot water and chemicals to kill maggots in pit latrines (Kumwenda et al. 2016). Previous studies have demonstrated that the addition of earthworms to pit latrines is an attractive alternative to the use of harmful chemicals to reduce sludge levels (Eastman et al. 2001; Parvaresh et al. 2004). The earthworms feed on the fecal sludge and produce a dry by-product known as vermi-compost. It is easier to handle and transport this material than the 'normal' pit latrine sludge (Eastman et al. 2001). In China, large-scale community worm-based systems have been tested for the treatment of sludge (Xing et al. 2011) and sewage (Wang et al. 2011). Furthermore, worm-based pit latrines linked to pour-flush toilets have shown promising outcomes in reduced filling rates for pit latrines and can be specifically designed as an affordable septic tank alternative for developing nations (Furlong et al. 2014).

5. Health risks associated with pit latrine fecal sludge and pit emptying

Sanitation workers handling sewage, refuse, wastewater or fecal sludge and peri-urban community members who make use of pit latrines can all be exposed to potential pathogens present in these waste materials. Very little or no information is documented for South African sanitation workers handling pit latrine fecal sludge. However, key exposure/transmission pathways related to sanitation technologies were previously identified in other countries (Brown 1997; Stenström et al. 2011). During pit emptying, workers can be exposed to microorganisms and chemicals in pit latrine sludge via several routes or entry points into the body. Inhalation is one of the major routes of exposure to microorganisms and chemicals during pit emptying (Brown 1997; Stenström et al. 2011; Farling et al. 2019). Microscopic droplets of water and particles also known as aerosols, which can carry pathogenic microorganisms, can be transferred to workers via inhalation (Farling et al. 2019). Aerosols and dust particles are usually found in close proximity to pit latrines and can also occur at sewage or waste water treatment plants during aeration and dewatering processes (Brown 1997). Upon inhalation of aerosols, bacteria and chemicals can become trapped in mucous and swallowed, leading to respiratory and gastrointestinal infections (Brown 1997). Parasites such as worm eggs can also be carried in dust particles and be inhaled by community members and workers where they can proliferate in their guts (Brown 1997). In addition, Kumwenda et al. (2017) established that the risk for ingestion of *E. coli* was 5.4×10^{-1} CFU's/g of *E. coli* for pit latrine fecal sludge in Malawi.

Pre-existing cuts, bruises or open wounds in the skin are also possible entry points for microorganisms (Stenström et al. 2011). Some of the common injuries to workers result from accidental falls into the pit latrines or cuts from sharp objects found in the pits (Stenström et al. 2011). In addition, some opportunistic microorganisms can enter the body through the surfaces of the eyes, nose, and mouth (Tiwari 2008). The skin is not only an entry point for microorganisms but also for chemicals and worms, which can be absorbed by the skin through contact with wastewater or pit latrine sludge (Brown 1997; Kumwenda et al. 2017). Microorganisms in pit latrine sludge can also enter the body through the accidental ingestion of fecal sludge (Kumwenda et al. 2017). This can occur via direct splashes into the mouth during pit emptying or indirectly to the mouth via items contaminated with fecal sludge such as hands, gloves, facial masks, cellphones, eating utensils, etc. (Stenström et al. 2011). Microorganisms in pit latrine fecal sludge can also leach into the surrounding soil from pit latrines without linings and cause contamination of the groundwater (Stenström et al. 2011). Produce such as vegetables can become contaminated with potential pathogens if they are irrigated with water that is contaminated with excreta or if they are fertilized with fecal sludge that has not been properly treated or processed (Stenström et al. 2011). Microorganisms can therefore enter the body through the consumption of contaminated produce.

Insects such as flies can function as vectors for the transfer of potential pathogens contained in pit latrine sludge to individuals and food items and are thus potential vectors for the spread of antimicrobial resistance (AMR) (Onwugamba et al. 2018). In addition, bites from mosquitoes carrying disease causing microorganisms can also cause the transfer of pathogens from pit latrine sludge to workers and household members (Stenström et al. 2011).

Overflowing pit latrines are usually a result of flooding, which can be due to heavy rains or due to the intentional flooding out of pit latrines, groundwater intrusion, or the general malfunctioning of the pit latrine (Stenström et al. 2011). Thus, pit latrine contents can enter houses and the surrounding environment, contaminating small-scale crops and drinking water sources. Workers or community members who encounter overflowing pit latrine contents are at a higher risk of becoming infected with potential pathogens contained in this waste (Stenström et al. 2011). Municipal workers or members of the community may also be exposed to enteric pathogens in fecal sludge by ingestion of water contaminated due to improper disposal of pit latrine/toilet contents, poor hand hygiene or direct contact with contaminated pit latrine/toilet surfaces (Montgomery and Elimelech 2007; Flores et al. 2011; McGinnis et al.

2019). Furthermore, Kumwenda et al. (2017) observed that individuals can be exposed differently to fecal sludge and pathogens contained therein, depending on how they empty the pits, store the sludge, use the sludge and whether they wear shoes or personal protective equipment (PPE).

5.1 Diarrheal disease burden attributed to poor sanitation and hygiene in Africa

The incidence of diarrhea in children is substantially lower for households or communities with access to the more 'advanced' water, sanitation and hygiene (WASH) technologies, such as flush toilets in comparison to those with basic WASH technologies such as pit latrines or those with a lack of access to any WASH technology (Gunther and Fink 2010). There are limited studies in South Africa focusing on the health risks faced by household members who make use of pit latrines. Health statistics for 2017 show that diarrheal diseases are still one of the top ten major causes of infant deaths in South Africa, where human immunodeficiency virus (HIV) and tuberculosis (TB) infection rates are high (Stats SA 2019a). Diarrheal diseases are ranked 3rd in the top ten leading underlying natural causes of death for children under 5 years of age, accounting for 7.3% of the total deaths and for 1.5% of all underlying causes of death in 2017 in South Africa (Stats SA 2019a). In KwaZulu-Natal, diarrheal diseases for the age group 1-14 is even ranked 2nd among the ten leading underlying natural causes of death, accounting for a total 5.9% of deaths in this group (Stats SA 2019a). Children are at a higher risk for contracting diarrheal diseases and their stools tend to carry a higher pathogen load than adults (WHO/UNICEF 2009). Deaths due to diarrheal diseases are further exacerbated in children living with HIV, causing an 11-fold increase in mortality in comparison to HIV negative children (Tindyebwa et al. 2004). In addition, a higher cumulative burden of diarrhea increases the risk of stunting in children 2 years of age (Checkley et al. 2008).

Previous studies show that access to proper water and sanitation technologies reduces the odds of children under five suffering from diarrhea by 7.3% (Gunther and Fink 2010). Chola et al. (2015) analyzed the cost and effect of scaling up 13 interventions to avoid and treat diarrhea in kids under the age of five in South Africa between 2015 and 2030, using 2014 as a baseline (13 million cases of diarrhea). Some of these interventions included improved sanitation, improved water sources, and proper hand washing with soap, etc. (Chola et al. 2015). Although scaling up of the 13 essential interventions could have a substantial impact on reducing diarrheal deaths, the disadvantage would be the high costs of implementation (Chola et al. 2015).

B. Microorganisms Present in Fecal Sludge

The type of microorganisms and especially pathogens present in fecal sludge usually depends on the health status of individuals living in the household or the community from where it originates (Bruce and Davis 1983). Many illnesses and infections can be verified by identifying the microorganisms that are shed during defecation (Berendes et al. 2017). The general microbial population present in solid waste and wastewater can be divided into 3 major groups i.e. prokaryotes (Bacteria and Archaea), eukaryotes (fungi, protozoa and algae) and viruses.

Typical viruses found in municipal waste and sewage sludge include Poliovirus (poliomyelitis), Coxsackievirus (meningitis, pneumonia, hepatitis and fever), Echovirus (meningitis, paralysis and encephalitis), Hepatitis A (infectious hepatitis), Reovirus (respiratory and gastroenteritis) and Astrovirus (gastroenteritis) viruses (Bibby and Peccia 2013). These viruses are considered potential reference pathogens and can contaminate water via human excreta from infected patients or human waste that contains high concentrations of virus infectious units (WHO 2011a).

Cryptosporidium (gastroenteritis and cryptosporidiosis), *Cyclospora cayetanensis* (watery diarrhea), *Entamoeba histolytica* (acute enteritis), *Giardia lamblia* (giardiasis, diarrhea and abdominal cramps), *Toxoplasma gondii* (toxoplasmosis) and *Balantidium coli* (diarrhea and dysentery) are the typical protozoa present in municipal waste and sewage sludge (Singh et al. 2011). Both *Cryptosporidium* spp. and *Balantidium coli* occur in animals and humans while *Cyclospora cayetanensis* and *Entamoeba histolytica* are known to only occur in humans (Fayer 2008). However, in a recent study by Nolan et al. (2017), gene sequences belonging to the genus *Entamoeba* were identified in humans as well as human-habituated mountain gorillas and livestock in Uganda. The two most common protozoa in municipal waste and sewage sludge are *Cryptosporidium* spp. and *Giardia lamblia*. *Cryptosporidium* spp. are smaller than *Giardia lamblia* and thus more difficult to remove by physical processes and are also highly resistant to oxidizing disinfectants such as chlorine (WHO 2011a).

Potentially pathogenic fungi such as *Aspergillus* spp., *Phialophora richardsii*, *Geotrichum candidum*, *Trichophyton* spp. and *Epidermophyton* spp. have been isolated from fecal sludge (Straub et al. 1993; Carrington 2001). *Aspergillus fumigatus* is a medically important

opportunistic fungal pathogen commonly present in sewage sludge, contaminating the atmosphere of WWTPS and composting plants while representing up to 75% of the airborne microflora (Milner et al. 1977).

Fecal sludge contains a high diversity of bacteria including some pathogenic bacterial species, which is a major health concern. There are six genera of Gram negative pathogenic bacteria that are of major concern in solid waste and wastewater treatment, which are known to cause infections in humans. The genera of bacteria and the diseases they cause are as follows: pathogenic *Salmonella* spp. (salmonellosis - food poisoning and typhoid fever - *S.* Typhi); *Shigella* spp. (bacillary dysentery); *Yersinia* spp. (acute gastroenteritis); *Vibrio cholerae* (cholera); *Campylobacter jejuni* (gastroenteritis) and pathogenic species of *Escherichia coli* (gastroenteritis) (Singh et al. 2011; Lu et al. 2015). For the purpose of this review pathogenic strains of a well-established Gram negative (*E. coli*) hygiene indicator and a representative Gram positive genus (*Staphylococcus* spp.) will be discussed. *Escherichia coli* is a typical gut commensal found in humans and animals but does include pathogenic strains such as Shiga toxin-producing *E. coli* (STEC) (Majowicz et al. 2014). *Staphylococcus* spp. are members of the normal skin flora but can also colonize the gut of humans and animals and are also found in the environment (Schulz et al. 2012; Schmidt et al. 2014; Kates et al. 2018). Several species of this genus such as *Staphylococcus aureus*, *S. lugdunensis*, *S. saprophyticus* and *S. haemolyticus* are pathogenic in nature (Kuroda et al. 2005; Frank et al. 2008; Becker et al. 2014).

1. *Escherichia coli*

Pit latrine fecal sludge typically contains *E. coli* in the range of 6-7 $\log_{10}$ CFU/g at sludge depths of between 0 and 1.5m, with higher counts typically present in the surface layers of the sludge (Nabateesa et al. 2017). Humans and animals are reservoirs for pathogenic *E. coli* strains. The pathogenic strains of *E. coli* have been divided based on their different virulence factors which include: enterohemorrhagic *E. coli* (EHEC) (includes Shiga toxin producing *E. coli* (STEC)), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and Adherent-invasive *E. coli* (AIEC) (Croxen et al. 2013). Tanih et al. (2014) screened diarrheal and non-diarrheal fecal samples from young children in a peri-urban community in South Africa (SA) for the presence of ETEC, EAEC, EPEC, STEC and EIEC. At least one *E. coli* pathotype was detected in 41% of the fecal samples tested. Interestingly, these authors found

that the majority of *E. coli* pathotypes were detected in non-diarrheal fecal samples and none of the diarrheal fecal samples were positive for STEC and EIEC. Overall, EAEC (57.3%) was identified as the most frequent pathotype present followed by EPEC (20.8%), ETEC (17.5%), STEC (3.8%) and EIEC (1.1%).

The major resulting infections of these pathotypes include among others diarrhea and haemolytic uraemic syndrome (Croxen et al. 2013). The common routes of exposure for this group of bacteria is from person-to-person, contact with animals, contaminated food, biological waste and consumption of contaminated water. For the purpose of this review, the major or common *E. coli* pathotypes will be mentioned. However, future work on the current study would include identifying some of the pathotypes present in pit latrine fecal sludge.

1.1 Enterotoxigenic *E. coli* (ETEC)

ETEC infections are the leading cause of diarrhea in children in developing countries and in those traveling to these countries (Lanata et al. 2013). The ETEC pathotype produces heat-labile and heat-stable enterotoxins (LT and ST) (Qadri et al. 2005). The two major subtypes of ST, which are important in humans, are STa (STI) and STb (STII) (Qadri et al. 2005; Taxt et al. 2010). The majority of ETEC strains isolated from humans with diarrhea produce STa, often together with LT. In addition, ETEC is of considerable economic importance as it also causes diarrhea in domestic animals, particularly calves and piglets (Nagy and Fekete 1999; Fairbrother et al. 2005). Khalil et al. (2018) estimated the global mortality due to ETEC diarrhea for the period 1990 to 2016 and found that the highest number of deaths for children under 5 years of age was located in eastern sub-Saharan Africa, with an average of 5485 deaths for the period 1990 to 2016.

1.2 Enteropathogenic *E. coli* (EPEC)

EPEC is predominantly found in developing countries and to a lesser extent in the developed parts of the world (WHO 2011a). In South Africa, EPEC strains were detected in fecal samples from 9% of patients experiencing diarrhea when tested at a healthcare facility in Cape Town (Kullin et al. 2015). This strain has been frequently associated with severe chronic non-bloody diarrhea, vomiting and fevers in infants. Infants infected with this pathotype suffer from malnutrition, weight loss and growth retardation (WHO 2011a).

1.3 Enteroinvasive *E. coli* (EIEC)

These *E. coli* strains have a similar pathogenic colon cell invasion mechanism to that of *Shigella* spp. EIEC and *Shigella* spp. both cause bacillary dysentery and both contain a key virulence determinant, a plasmid known as pINV, which is exclusive to these bacteria (Marteyn et al. 2012; Croxen et al. 2013). This plasmid/virulence determinant allows shigellae/EIEC to penetrate epithelial cells, move inside these cells and attack adjacent cells (Marteyn et al. 2012). EIEC is associated with infections such as watery and occasionally bloody diarrhea and the major reservoir for this pathotype is also humans (WHO 2011a). Transmission is mainly via the oral-fecal route and infections due to EIEC are common in developing countries due to poor sanitation facilities (Vieira et al. 2007).

1.4 Enteroaggregative *E. coli* (EAEC)

EAEC is characterized by a 'stacked-brick' form of adherence to tissue culture cells in vitro (Nataro et al. 1987), which is due to one of various hydrophobic aggregative fimbriae (Nataro et al. 1987). EAEC causes diarrhea in children in developing countries and in adults traveling to these countries (Okeke and Nataro 2001; Harrington et al. 2006). Aijuka et al. (2018) determined the routes of transmission for different *E. coli* pathotypes (EAEC, EPEC, ETEC, DAEC). Pathogenic *E. coli* strains where obtained from a bulk milk distributor (PDBM), irrigation water, irrigated lettuce and coleslaw from a street vendor in South Africa (Aijuka et al. 2018). The same authors showed that EAEC was the dominant diarrhea causing *E. coli* pathotype in irrigation water and food sources in South Africa, highlighting these mediums as possible sources of this group of pathogens.

1.5 Diffusely adherent *E. coli* (DAEC)

DAEC pathotypes harbor genes encoding for Afa/Dr adhesins (Meza-Segura and Estrada-Garcia 2016). These adhesins bind to cell receptors, which induce receptor clustering and finger-like cell projections, causing the bacteria to embed without complete internalization (Meza-Segura and Estrada-Garcia 2016). Infections with DAEC cause diarrhea and intestinal epithelia damage, which can contribute to the development of other intestinal ailments (Meza-Segura and Estrada-Garcia 2016). DAEC were identified in 23% of children under the age of 7 showing symptoms of diarrhea and in 11% of children without symptoms, in Maputo, Mozambique, a country neighboring South Africa (Rappelli et al. 2005).

1.6 Adherent-invasive *E. coli* (AIEC)

AIEC are an unusual group of *E. coli* pathotypes as they are not associated with diarrhea but are thought to contribute to the development of chronic inflammatory bowel disease, also known as Crohn's disease (Alhagamhmad et al. 2016). The AIEC phenotype is thought to possibly result from metabolic processes that enhance the growth of this pathotype in tissues affected by Crohn's disease (O'Brien et al. 2017). In addition, they have the ability to adhere to and invade epithelial cells and replicate within macrophages (Martinez-Medina et al. 2009).

1.7 Enterohemorrhagic *E. coli* (EHEC)

The major reservoirs for EHEC strains are in livestock such as cattle and sheep (Caprioli et al. 2005). The pathogen is shed in the manure of these animals where they can survive and contaminate surface and drinking water through run-off water contaminated with animal feces (Elder et al. 2000; Caprioli et al. 2005). EHEC strains produce either Shiga toxin or a Shiga-like toxin and are therefore also defined as Shiga toxin producing *E. coli* (STEC). The major reservoirs for STEC is the ruminants of cattle, sheep, goats and pets (Frank et al. 2011b; Slayton et al. 2011). The common routes of transmission for this group of bacteria is through direct person-to-person contact (smear infection), contact with animals, consumption of contaminated food and water or contact with biological waste (Frank et al. 2011b). Shiga toxins produce profound cytopathic effects in vero cells and bacterial strains producing these toxins are therefore also known as Verotoxigenic *E. coli* (VTEC) (Woodward et al. 2002). STEC/VTEC strains can be further divided into serotypes. The most common STEC/VTEC serotypes worldwide are *E. coli* O157:H7 and *E. coli* O104:H4 (McPherson et al. 2009). Each STEC/VTEC serotype varies in its virulence and infected individuals can display a range of symptoms, most often characterized by bloody diarrhea (Papaconstantinou and Thomas 2007).

Prevalence of STEC serotypes: *E. coli* O157:H7 and O104:H4

Escherichia coli O157:H7 was first isolated in the USA in 1975 from a sporadic case of hemorrhagic colitis (Riley et al. 1983; Wachsmuth et al. 1991). In Swaziland and South Africa in 1992, an outbreak of *E. coli* O157:H7 resulted in a sevenfold increase in diarrhea from the previous year within a 2-month period. Patients displayed symptoms of bloody diarrhea and severe abdominal pains (Effler et al. 2001). The outbreak was attributed to a combination of factors, which included among others, a prolonged drought and the subsequent death of cattle (Effler et al. 2001). A heavy onset of rain and the failure to remove dead cattle carcasses (possible reservoirs of *E. coli* O157:H7) from the land resulted in the contamination of surface

water in South Africa and sugar cane plantations in Swaziland with this pathogen (Effler et al. 2001). Abong'o and Momba (2008a) reported a 10.3% prevalence of STEC O157:H7 in vegetable samples from the Eastern Cape Province in South Africa. The first case of human infections with *E. coli* O157:H7 in South Africa was reported in 1990 in the city of Johannesburg (Browning et al. 1990). Studies that followed reported a prevalence of infections with *E. coli* O157:H75 at 6.5% and 43.5% for confirmed and non-confirmed HIV/AIDS patients respectively, in the Eastern Cape Province of South Africa (Abong'o et al. 2008b). Majowicz et al. (2014) reported 10 200 cases of STEC infections in Africa and an incidence rate of 1.4 cases per 100 000 person-years with STEC O157:H7 infections contributing to 10% of this burden.

Two serious outbreaks were reported in Germany and South Korea. In Germany, STEC serotype O104:H4 was the cause of prolific outbreaks of hemolytic uraemic syndrome (HUS) and bloody diarrhea (Frank et al. 2011a). This *E. coli* pathogen displayed an unusual characteristic combination such as: antimicrobial resistance, stx2 (shiga toxin) production and enhanced adhesion, all of which contributed to the extreme virulence of this outbreak (Karch et al. 2012). In South Korea, *E. coli* O104:H4 was responsible for an outbreak of HUS in 2004 (Kim et al. 2011). This strain was positive for both stx1 and stx2 shiga toxins and was resistant to ampicillin and trimethoprim/sulfamethoxazole with susceptibility to cefotaxime, ceftriaxone, tetracycline and nalidixic acid (Kim et al. 2011). The characteristics of the *E. coli* O104:H4 strain responsible for an outbreak of HUS in 2004 included the lack of an enteroaggregative *E. coli* determinant (aggR) and the absence of resistance to extended spectrum β-lactamases thereby differing from the *E. coli* O104:H4 strain responsible for the HUS outbreak in 2011 in Europe (Kim et al. 2011). HUS is a very serious complication, which is characterized by acute renal failure, haemolytic anemia and thrombocytopenia, typically preceded by bloody diarrhea (Frank et al. 2011a).

2. Staphylococcus

The ability of some species from the staphylococcus genus to cause disease depends on the expression of virulence factors, which allows them to colonize and evade the host's immune system (Yang et al. 2015). Until the late 1960's, the coagulase positive *Staphylococcus aureus* was considered the most pathogenic species of this group and therefore, coagulase positive *Staphylococcus* spp. (CPS) were mainly studied. As CNS constitute a large part of the human skin microbiome, their role as pathogens was undermined thus distinction between species was

not common or otherwise neglected (Eng et al. 1982). However, in 1977 infections caused by CNS were reported for the first time, after which CNS gained more recognition as possible pathogens (Liekweg and Greenfield 1977). In addition, *Staphylococcus saprophyticus* was associated with frequent urinary tract infections (John et al. 1978). Kloos and Schleifer (1975) designed biochemical tests to distinguish between CPS and CNS isolates, which was later consolidated by molecular techniques (Matthews et al. 1997) that enabled a more accurate identification of the known *Staphylococcus* spp. as well as new species. *Staphylococcus* spp. have also been isolated from human feces (McDonald et al. 1982).

2.1 Coagulase positive staphylococci

2.1.1 *Staphylococcus aureus*

Staphylococcus aureus is not only the most common coagulase positive species, it is also regarded as the most pathogenic of this genus, frequently causing illnesses such as nosocomial pneumonia, skin and wound infections, bloodstream infections, toxic shock syndrome, meningitis and foodborne illnesses (Waldvogel 2000; Chambers 2001). Pathogenic strains of *S. aureus* resistant to methicillin are termed MRSA (Chambers 2001). MRSA is one of the common infectious agents found in hospitals where staphylococcal outbreaks have occurred (CDC 2014). *S. aureus* was first suspected to cause antibiotic-associated diarrhea (AAD) in 1954 (Oeding and Austarheim 1954). Since then several studies reporting the occurrence of methicillin resistant *S. aureus* (MRSA) in the stool of patients with AAD (McDonald et al. 1982; Adesiyun et al. 1992; Gravet et al. 1999) have indicated that MRSA is a causative agent for AAD.

Some studies have also reported a higher incidence of *S. aureus* infections in low-income/developing countries. In South Africa, *S. aureus* was previously implicated as the causative agent for a range of infections in patients at hospitals in KwaZulu-Natal (Essa et al. 2009). *S. aureus* is commonly associated with blood stream infections (bacteremia) and has also been implicated as one of the causative agents for diarrhea. According to Naidoo et al. (2013), the annual incidence of *S. aureus* bacteremia infections in children from Cape Town, South Africa was 3.28 cases per 1000 hospital admissions, with a higher prevalence in children under the age of 5. Perovic et al. (2015) determined the geographical distribution of *S. aureus* bacteremia in South Africa from June 2010 until July 2012 and found that MRSA bacteremia infections were significantly higher in the Gauteng province (53%) of South Africa compared

to the provinces Free State (41%), KwaZulu-Natal (47%) and the Western Cape (37%). Furthermore, Fortuin-de Smidt et al. (2015) reported 442 cases of bacteremia from 2012 to 2013 in Gauteng in South Africa. In addition, Kullin et al. (2015) analyzed the presence of potential diarrhea-causing bacteria in fecal samples from patients with diarrhea at a healthcare facility in Cape Town, South Africa and found that *S. aureus* was present in 6% of fecal samples tested, possibly indicating the involvement of this pathogen in diarrhea.

Other coagulase positive staphylococci are primarily found in dogs and cats, namely *S. intermedius, S. schleiferi* subsp. *coagulans* (Morris et al. 2006) and *S. pseudintermedius* (Sasaki et al. 2007).

2.2 Coagulase negative staphylococci

2.2.1 *Staphylococcus epidermidis*

Staphylococcus epidermidis is not regarded as pathogenic to healthy individuals. However, it is one of the main causes of coagulase negative staphylococcal infections in unhealthy or immune compromised individuals (Mack et al. 2004). *S. epidermidis* is a strong biofilm former which enables it to resist the harsh impact of antimicrobial agents (Mack et al. 2004). These biofilms are a major problem in hospital settings, causing infections associated with the indwelling of medical devices (Foster and McDevitt 1994). *S. epidermidis* was identified as one of the most prevalent pathogens responsible for catheter-related bloodstream infections in patients from a hospital in Pretoria, South Africa (Ehlers et al. 2018).

2.2.2 *Staphylococcus lugdunensis*

Staphylococcus lugdunensis, first described by Freney et al. (1988), is a human skin commensal that can cause infections such as osteomyelitis, arthritis and septicemia in unhealthy or immune compromised individuals (Frank et al. 2008). In addition, it was also previously reported to cause acute endocarditis in prosthetic and native valves (Jones et al. 2002). In 2014, Al-Charrakh and Obayes reported for the first time the detection of Methicillin Resistant *S. lugdunensis* (MRSL) in clinical samples in Iraq.

2.2.3 *Staphylococcus haemolyticus*

Staphylococcus haemolyticus is the second most frequently isolated species, the first being *S. aureus*, from hospitalized patients with bloodstream infections, including sepsis (Silva et al.

2013; Becker et al. 2014) and owes its pathogenicity to the production of enterotoxins (Froggatt et al. 1989). *S. haemolyticus* can be isolated from fecal samples of infected humans (Froggatt et al. 1989) and recent studies have demonstrated its clinical significance as a multi-drug resistant species with the ability to acquire resistance against available antimicrobial agents (Czekaj et al. 2015). The unusual genome plasticity of *S. haemolyticus* strains established by a large number of insertion sequences and identified single nucleotide polymorphisms (SNPs) could contribute to its acquisition of antibiotic resistance genes (Czekaj et al. 2015). In addition, this species also plays an important role in the dissemination of resistance genes, via interspecies transfer of staphylococcal cassette chromosome mec (SCCmec) genes, having contributed to the emergence of more virulent *S. aureus* epidemic clones (Cavanagh et al. 2014).

2.2.4 *Staphylococcus saprophyticus*

Staphylococcus saprophyticus is a common pathogenic CNS known to cause urinary tract infections (UTIs) (Kuroda et al. 2005). It was only in the early 1970's that CN staphylococci became recognized as a frequent cause of UTIs, more than ten years after the original demonstration of *S. saprophyticus* in urine specimens (Hovelius and Mårdh 1984). The second most common cause of acute UTIs in young females, after *E. coli*, is *S. saprophyticus*. Patients with UTIs caused by *S. saprophyticus* usually present with symptomatic cystitis (Hovelius and Mårdh 1984) and similar to UTIs caused by *E. faecalis*, patients display kidney tropism (Kline et al. 2010).

C. Biofilm Formation, Survival and Treatment on Household Surfaces

1. Biofilm formation

Some bacteria such as *Campylobacter jejuni* have both an intrinsic and extrinsic ability to produce biofilms (Melo et al. 2017; Feng et al. 2018), while other *Proteobacteria* such as *Pseudomonas aeruginosa* and *E. coli*, produce biofilms as a survival mechanism when conditions become unfavorable (Bjarnsholt 2013; Rossi et al. 2018). Biofilms are matrix-enclosed, multi-layered structures of microorganisms, which adhere to biological or non-biological surfaces (Hall-Stoodley et al. 2004) and their formation is a dynamic process involving a series of steps, which may take hours or days (Kumar and Anand 1998). Fimbriae, flagella, cell wall components, and extracellular polymeric substances (EPS) facilitate the

colonization of the microorganisms. The EPS is comprised of polysaccharides, proteins, nucleic acids, and lipids and is responsible for the morphology, structure, and physicochemical properties of the biofilm (Flemming and Wingender 2010). Biofilm adhesion to surfaces is initially reversible and thereafter, the microorganisms produce exopolymers and adhesins that complex with surface materials and adhesion becomes irreversible, i.e. gentle rinsing will not dislodge the biofilm (Dunne 2002). Biofilm-associated cells can be differentiated from planktonic cells by the production of EPS, the regulation of specific genes and cell-to-cell communication. Biofilms are also crossed by channels and pores that support substrate distribution and facilitate horizontal gene transfer between bacteria (Donlan 2002).

Biofilms provide protection for bacteria against the impact of antimicrobial agents (Bridier et al. 2011). Bacterial cells within biofilms can become 10-1000 times more resistant to the effects of antimicrobial agents than their planktonic counterparts depending on the species-drug combination (Mah and O'Toole 2001). Microscopy techniques such as specialized light microscopy and electron microscopy allow for the direct observation of microbial communities on surfaces (Djordjevic et al. 2002). Microorganisms within biofilms can be monitored by staining. Stains such as 4',6-diamidino-2-phenylindole (DAPI) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) can be used to determine the presence and viability of bacteria within the biofilm and the crystal violet stain can be used to detect and quantify the biofilm. In addition, qualitative information of the biofilm can be determined using image analysis (Jun et al. 2010).

2. Survival of bacteria on typical household surfaces

Biofilms can develop on a wide variety of surfaces and within any environment in which viable microorganisms are present (Kumar and Anand 1998; da Silva Meira et al. 2012). However, depending on the surface type, differences have been reported in both the extent and rate of attachment of microorganisms to each surface (Boistier-Marquisa et al. 2000; Silva et al. 2008). Biofilm formation on food contact surfaces is of great concern in that it provides an ideal environment for microorganisms including potential pathogens to colonize and subsequently contaminate food products (Marques et al. 2007; Brooks and Flint 2008). Some of the microbes which are of concern in food production lines (e.g. dairies/bakeries) include *Listeria monocytogenes*, *Campylobacter jejuni*, *Salmonella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *E. coli* O157:H7 and *Bacillus cereus* (Cappitelli et al. 2014). Due to a lack of proper hygiene and sanitation, microorganisms including pathogens can be transferred from hands or

household surfaces to food products possibly causing foodborne infections/intoxification (Medeiros et al. 2001; van Asselt et al. 2008). In addition to food contamination and spoilage, biofilms can cause metal corrosion in pipelines and tanks and contamination of dairy products with nonstarter bacteria (e.g. cheese by nonstarter lactic acid bacteria) (Beech and Sunner 2004; Shi and Zhu 2009; Malek et al. 2012). Households typically contain surfaces that are made of plastic, glass and/or stainless steel, which can become contaminated with potential pathogens due to a lack of proper hygiene and sanitation.

2.1 Plastics

There are 7 common groups of plastics namely; Polyethylene Terephthalate (PETE or PET), High-Density Polyethylene (HDPE), Low-Density Polyethylene (LDPE), Polyvinyl Chloride (PVC), Polypropylene (PP), Polystyrene or Styrofoam (PS) and other miscellaneous plastics such as polycarbonate, polyctide, acrylic, acrylonitrile butadiene, styrene, fiberglass and nylon. Most plastics are usually cheap and durable polymers and PP and PS are commonly used in most kitchen appliances, utensils, cutting boards and work top surfaces. They come in various types or textures with different properties (Liu et al. 2011) and have a global production of 25 million tons per year (Asadinezhad et al. 2012). One of the major disadvantages of this material is that some plastics may release bisphenol-A (BPA)-like plasticizers into food and beverages, which at low doses of chronic exposure may contribute to cancer, low immune function, hyperactivity, diabetes, and obesity (Careli et al. 2009). Secondly, when heated, plastics become more hazardous, as the rate of transfer of toxic chemicals into food and beverages increases. Finally, plastics are subject to crevices and cracks, which allow for colonization by microorganisms (Careli et al. 2009) and combined with their surface properties such as unwanted protein adsorption, these materials support bacterial adhesion, which can lead to biofilm formation (Asadinezhad et al. 2012). However, to overcome these poor surface properties, plastic manufacturers have incorporated antimicrobial resins or chemicals such as polymethyl methacrylate-grafted halloysite nanotubes into specific types of plastics to reduce the risk of food contamination and thus the spread of foodborne illnesses (Liu et al. 2011).

2.2 Glass

Glass is also a common food contact surface in kitchens but is costlier than plastics (Schmidt and Ericksson 2014). Glass food contact surfaces include glass cutting boards, crockery and storage containers (Schmidt and Ericksson 2014). Although glass is used in many households, the use of glass as food contact surfaces in peri-urban households is unlikely due to the high

costs of glass items. It is similar to stainless steel in that the surface appears smooth and polished but is often subject to minute cracks or crevices allowing for microbial colonization (DeVere and Purchase 2007). Glass is also regarded as less durable as it has the potential of easily breaking (Schmidt and Ericksson 2014). Due to the use of glass in kitchens as food contact surfaces and the risk of food contamination by food pathogens, glass manufacturers have also included antimicrobial substances such as chlorhexidine, cetylpyridinium chloride, benzalkonium chloride and cetrimide, into specific types of glass with the aim of reducing microbial contamination (Schmidt and Ericksson 2014; Tüzüner et al. 2019).

2.3 Stainless steel

Stainless steel is commonly used as a food contact surface especially in industry but also in households due to its stability at various processing temperatures, its ease of cleaning and resistance to corrosion (Euro Inox 2007). However, it is expensive and therefore not commonly used in peri-urban households. Stainless steel material does not have a naturally smooth surface and upon close examination, it does contain cracks and crevices (Milledge 2010), which can allow for microbial colonization. Therefore, stainless steel surfaces or products are often subjected to polishing and coating to reduce the risk of microbial colonization (Zhang et al. 2011).

3. Biofilm treatment/removal

Biofilm removal often requires a combination of mechanical abrasion to destroy the biofilm, followed by treatment with antimicrobial agents such as disinfectants.

3.1 Soaps

Soaps are detergent based products and their activity is attributed to the detergent properties. They are commonly used in households for social hand wash purposes in bathrooms and toilet facilities (Kampf and Kramer 2004). Studies have shown that washing hands with water alone can reduce the microbial population on hands (Lowbury et al. 1964). Luby et al. (2011) found that hand washing with water alone before preparation of food reduced the incidence of diarrhea in children in peri-urban villages in Bangladesh, suggesting that hand washing with water alone can significantly reduce the incidence of childhood diarrhea. In addition, Burton et al. (2011) found that handwashing with soap and water was more effective in the removal of bacteria of potential fecal origin from hand surfaces than handwashing with water alone. Although hand washing with soap can be expensive, especially in impoverished communities,

studies have shown that hand washing with soap is more effective in the reduction of microorganisms present on the surfaces of hands in comparison to washing with water alone (Hoque and Briend 1991; Burton et al. 2011). Similarly, soap, like detergents, can be used to remove microbes from contact surfaces.

3.2 Disinfectants

It is of critical importance that surfaces in households are properly cleaned and disinfected to prevent the spread of disease. According to the national regulator for compulsory specifications (NRCS), disinfectants are defined as formulated chemicals used in healthcare facilities, in the food industry and many other industries to remove microorganisms from inanimate surfaces. Disinfectants contain a wide variety of active ingredients or combinations thereof that provide disinfecting and cleaning properties. These chemicals possess different modes of action, either killing or inhibiting the growth of microorganisms (Maris 1995; Fraise 2002). It is important to understand that the mode of action for a particular disinfectant may vary depending on the target microorganism and the state in which it is found, as well as the environmental conditions wherein the disinfectant is applied (Poole 2002). Physiochemical parameters such as temperature, pH and salinity affect the way in which a particular disinfectant works (McDonnell and Russell 1999). Disinfectants used in households are termed household disinfectants or sanitizers, which include soaps, alcohol-based hand sanitizers and sodium hypochlorite formulations, which consist of a number of different active agents as well as surfactants (McDonnell and Russell 1999). Industrial disinfectants include sodium hypochlorite, ethanol, hydrogen peroxide, phenol, and formaldehyde (Condell et al. 2012).

3.2.1 Household bleach (Active ingredient: Sodium Hypochlorite)

Household bleach (sodium hypochlorite, NaOCl) is a chlorine releasing agent which is used for antiseptic and disinfecting purposes and also for decontaminating non-biological surfaces in healthcare facilities (Rutala and Weber 1998). It has a broad antimicrobial spectrum over a wide range of temperatures and is easy to handle, non-toxic, relatively inexpensive, fast acting and compatible with anionic and non-ionic detergents (Rutala and Weber 1997; Penna et al. 2001). The antimicrobial activity of household bleach is due to both chlorine and oxygen, which are responsible for oxidizing thiol groups (Maris 1995). Chlorine is electronegative and therefore oxidizes peptide links and denatures proteins (Maris 1995). Chlorine dioxide acts on the permeability of the external membrane of Gram negative bacteria such as *E. coli*, causing a substantial leakage of potassium ions and sub-lethal doses inhibit cellular respiration due to

a nonspecific oxidizing effect (Maris 1995). Lethal doses of hypochlorous acid (HOCl) cause a decrease in adenosine triphosphate (ATP) production in bacterial strains such as *Escherichia coli*, *Pseudomonas* spp. and *Staphylococcus* spp. (Maris 1995).

Some health risks associated with exposure to household bleach include ocular irritation and gastric burns (Landau and Saunders 1964; Ward and Routledge 1988; Ingram 1990; Weber and Rutala 1998) and some disadvantages are corrosiveness to metals at high concentrations (>500 mg/L), discoloring or bleaching of fabrics, and release of toxic chlorine gas when mixed with ammonia or acid (e.g., household cleaning agents) (Mrvos et al. 1993).

3.2.2 Alcohols

Alcohol-based products have been used for many years as a disinfectant for both hands and household surfaces and their activity is based on the non-specific denaturing of proteins (Draelos 2012). The alcohols that are commonly used as disinfectants include ethanol and isopropanol (Larson and Morton 1991; McDonnell and Russell 1999). They are effective against a broad spectrum of Gram negative and Gram positive bacteria as well as fungi, but are not effective against bacterial spores and fungal conidia and have variable activity against enveloped viruses (Draelos 2012). Alcohols have very little tendency towards the development of antimicrobial resistance for most pathogens found on hands (Larson and Morton 1991). The mode of action of alcohols is through the denaturation of membrane proteins, resulting in altered membrane permeability, which causes vital cell constituents to leak out of the cell, leading to cell death. Alcohols are also known to cause cell death through dehydration (Larson and Morton 1991; McDonnell and Russell 1999). At concentrations of 60-70% alcohols have successfully been used as hand antiseptics, with the ability of producing greater reductions in microbial counts on hands than any other hand disinfectant (Lowbury et al. 1974; Rotter 1984). It is known that alcohols at concentrations of 70% or higher can produce rapid and broad-spectrum antimicrobial action although they do come with disadvantages when used as hand scrubs, such as the drying out of hands and the inability to remove dirt due to a lack of surfactant properties (Hobson et al. 1998). However newer formulations that contain surfactants and emollients have overcome these problems (Hobson et al. 1998).

3.2.3 Formaldehyde

Aldehydes are a group of chemical agents characterized by a formyl group. The two most common aldehydes, which are used as disinfectants in industrial settings are formaldehyde and

glutaraldehyde (McDonnell and Russell 1999). Aldehydes act on bacterial cells by reacting with the amine groups and forming a cytotoxic agent (McDonnell and Russell 1999). Formaldehyde is proposed to act as a mutagenic and alkylating agent through reactions with carboxyl, hydroxyl and sulfhydryl groups of the target organism (Maris 1995). Formaldehyde has long been used as a sterilizing agent in hospitals for heat-labile and electrical equipment (Nyström 1991). However, due to its highly toxic nature, it is not recommended for disinfection of household surfaces.

4. Resistance to biocides

The mechanism of resistance to biocides varies from agent to agent and is dependent on the bacterial species involved. Gram negative bacteria are generally less susceptible to biocides than Gram positive bacteria as the outer membrane acts as a protective barrier (Russell 1997). Bacterial resistance to biocides often involves one or a combination of three mechanisms, which are alteration of the target site in the bacterial cell, the destruction or modification of the agent via enzymes and the removal of the agent through either passive or active efflux systems (Poole 2002). Biocide resistance is not as common as antibiotic resistance, which may be due to the fact that most biocides are not specific in their mode of action and rather target a number of cellular constituents instead of just one (Poole 2002).

4.1 Biofilm formation and its contribution to biocide resistance

In addition to differences between bacterial species and their intrinsic resistance mechanisms, the physiological state of bacteria also affects their resistance to biocides (Bridier et al. 2011). Biofilms are one such state wherein bacteria are found to be less susceptible to both antibiotics and biocides (Marques et al. 2007; Bridier et al. 2011). Biofilms are much harder to eradicate than planktonic cells as the EPS surrounding the cells within the biofilm can act as a protection barrier. The resistance that biofilms confer to bacteria residing within them may be due to several different mechanisms. These mechanisms include decreased permeability or limited penetration of the biocide into the biofilm, chemical interactions of the biocide with components of the biofilm matrix (e.g. carbohydrates and proteins) and metabolic inactivation, which leads to reduced efficacy of the biocide, total inactivation of the biocide and physical adaptation of cells to the microenvironments within the biofilm (Kumar and Anand 1998).

D. Antibiotic Resistance

Antibiotic resistance is the ability of a microorganism to resist the inhibitory or killing effect of an antibiotic beyond the normal susceptibility of the specific microorganism (Acar and Röstel 2001). Resistance displayed by bacteria to antibiotics can be differentiated into 3 categories. Bacterial cells susceptible to antibiotics are inhibited by a concentration of an antibiotic that is likely to have therapeutic success; those that are intermediately resistant to antibiotics are inhibited by an antibiotic of uncertain therapeutic effect; and those resistant to antibiotics are inhibited by a concentration of an antibiotic that has a high chance of therapeutic failure (Turnidge and Paterson 2007; Rodloff et al. 2008). According to Magiorakos et al. (2012), the following antibiotic resistance categories can be used to define acquired resistance in bacterial isolates; multi-drug resistant (MDR), extensively drug resistant (XDR) and pan-drug resistant (PDR).

Antibiotic resistance predates the era of antibiotic use in agriculture and medicine, as antibiotic producing bacteria - such as members of the soil inhabiting bacterial genus *Streptomyces* that are responsible for the biosynthesis of most naturally produced antibiotics (Watve et al. 2001) - possess mechanisms providing resistance against these antibiotics (Hopwood 2007). However, the overuse of antibiotics has led to an increase in antimicrobial resistance (Ventola 2015). This is further aggravated by the growing demand for meat and dairy products, tempting farmers particularly in developing countries to use antibiotics such as amoxicillin and erythromycin - that are also used to treat infections in humans - in low doses for extended periods to promote the growth of animals (Sarmah et al. 2006).

In 2010, the global use of antibiotics in livestock production was estimated at between 60 000 and 65 000 metric tons with predictions of an increase by two-thirds globally in 2030 and a doubling in the so called "BRICS" group of countries (Brazil, Russia, India, China and South Africa) (van Boeckel et al. 2015). In addition to improper sanitation and hygiene, bacterial infections in humans due to antibiotic resistant strains can result from the consumption of contaminated food or the zoonotic spread of antibiotic resistant pathogens to humans (Fletcher 2015). According to the World Health Organization (2016), the critically important, highest priority antibiotics used in human medicine - again also used in animal husbandry - are the cephalosporins (3rd, 4th and 5th generation), glycopeptides, macrolides and ketolides,

polymyxins and quinolones, as these antibiotics are used as a last resort to treat infections caused by MDR strains. These antimicrobials are considered critically important based on their role in the treatment of human infectious diseases, including those transmitted to humans via zoonosis (Collignon et al. 2009).

1. Mobilization of antibiotic resistance in the environment

Factors involved in the acceleration of antibiotic resistance (ABR) dissemination include the increased and improper use of antibiotics and the increased migration of humans and animals between multiple countries. This contributes to the spread of resistant bacteria, thereby limiting traditional clinical and therapeutic treatment options (van der Bij and Pitout 2012). Direct animal-human contact is considered as the dominant route of transmission for antibiotic resistance. However, Dohmen et al. (2017) suggested air exposure as another possible route of transmission for extended spectrum β-lactamase (ESBL) carriage in pig farmers.

Traveling has also been suggested as a route of transmission for ABR. Freeman et al. (2008) found that patients who had recently travelled either to India or to central Africa, presented with community-associated genitourinary tract infections caused by the ESBL CTX-M-15 producing *E. coli* and lacked typical UTI symptoms. It was also found that consumption of antibiotics while travelling to high-risk countries was a supplemental risk factor for ESBL associated infections (Freeman et al. 2008; Kuster et al. 2010). Another example of community-associated resistance is traveler's diarrhea caused by ETEC. Tham et al. (2010) found that travelers to India had the highest risk of acquiring CTX-M producing *E. coli*, with approximately seven out of eight travelers testing positive upon return.

The use of antibiotics in hospital-settings is an essential, life-saving necessity but can also contribute to the dissemination of ABR. Medical facilities and nursing homes are considered reservoirs for the dissemination of oxacillin-resistant *Staphylococcus aureus* (ORSA), previously known as methicillin-resistant *Staphylococcus aureus* (MRSA) (Gruber et al. 2013). The transfer of patients between hospitals and other healthcare institutions can therefore aid in the spread of nosocomial infections caused by ABR pathogens such as ORSA, multi-drug resistant Gram negative bacteria and vancomycin-resistant enterococci (VRE) (Ciccolini et al. 2013). Additionally, most antimicrobial agents administered are not fully metabolized by patients and can therefore be discharged via human excrement into hospital sewage systems (Bruinsma 2003). The waste from hospital sewage systems then enters sewage treatment plants

and thereafter, the public water system (Bruinsma 2003). If not properly treated, it can contribute to the dissemination of antibiotic resistant genes in the environment and the community.

In addition to the spread of ABR by humans, animals (livestock/poultry) are also a major source for the occurrence of ABR. Antibiotic use in animals for the treatment of infections and as a feed additive to promote the growth of livestock is an established agricultural practice in many countries. However, this can have a negative effect on the welfare of the animal as well as humans who consume them (Soulsby 2005). These antibiotics are usually administered in high doses in water or feed and continued and repeated administration of these antibiotics can result in antibiotic resistance (van den Bogaard et al. 2001). Antibiotic resistant *E. coli* strains residing in the gut of poultry can contaminate the flesh of the poultry during slaughter and the eggs during laying (Lakhotia and Stephens 1973; Turtura et al. 1990). The consumption of such contaminated food aids in the spread of ABR from animals to humans (van den Bogaard et al. 2001). The use of 'last resort' antibiotics such as the fluoroquinolone enrofloxacin, for the treatment of infections in livestock (Soulsby 2005), is critical as enrofloxacin contributes to the increase in fluoroquinolone resistant *Campylobacter* spp. in humans (Piddock 1995) - a frequent cause of food poisoning (Soulsby 2005). Therefore, the use of enrofloxacin for any ailment in poultry was banned in the United States in 2005 by the Food and Drug Administration (Soulsby 2005). Detection of ORSA in meat from chickens, turkeys, pigs and bovine animals (EFSA and ECDC 2014), indicate that these animals can act as reservoirs for the dissemination of ORSA. In addition, a study by van de Sande-Bruinsma et al. (2015) shows evidence for the spread of ORSA from livestock to hospital settings via infected patients.

2. Antibiotic resistance mechanisms

Antibiotic resistance can be intrinsic or acquired. Intrinsic resistance is a naturally occurring phenomenon that predates antibiotic chemotherapy and is an inherent characteristic of a bacterial species or genus towards a certain antibiotic (Aarestrup 2006; Harbottle et al. 2006). On the other hand, bacteria can acquire antibiotic resistance through genome changes or horizontal transfer of resistance genes (Courvalin 1994; Davies and Davies 2010). Bacteria can also display transient resistance to antibiotics, which allows them to temporarily survive the effects of an antibiotic (El Meouche et al. 2016). The most important of these acquisition mechanisms is horizontal gene transfer (Munita and Arias 2016). Antibiotic use has always been considered as the main driving force for the emergence of antibiotic resistance other than

intrinsic resistance. However, a recent study by Knöppel et al. (2017) showed that antibiotic resistance can evolve coincidentally in response to other environmental selective pressures without exposure to antibiotics. The genetic adaptation of bacteria to natural environments generates a pool of resistance mutations that could act on enrichment of resistant mutants when exposure to antibiotics occurs (Knöppel et al. 2017).

2.1 Intrinsic resistance

Bacteria displaying intrinsic resistance against antibiotics dramatically limit antimicrobial therapy available for infections caused by such microorganisms (Verraes et al. 2013). There are two main mechanisms of intrinsic antibiotic resistance i.e. outer membrane permeability and efflux pumps (Cox and Wright 2013). In Gram-negative bacteria, the outer membrane effectively reduces the permeability of molecules of certain sizes and as a result reduces the susceptibility of the bacteria to antibiotics such as penicillin, vancomycin and polymyxin B (Cox and Wright 2013). However, the membrane cannot hinder the influx of all antimicrobial agents, as structures in the membrane called "porins" enable the influx of mostly small and non-charged molecules (Cox and Wright 2013). A synergy between the outer membrane permeability barrier and an active efflux mechanism is therefore necessary to reduce the intracellular concentration of an antimicrobial agent effectively (Nikaido 1994; Cox and Wright 2013). However, even intrinsic resistance can be mobilized horizontally through microbial populations in addition to being evolved independently (D'Costa et al. 2006).

Efflux pumps are widespread in both Gram positive and Gram negative microorganisms and can be divided into two groups, broad spectrum and substrate specific pumps (van Bambeke et al. 2000; Piddock 2006). Broad spectrum pumps export members of structurally distinct molecular classes, whereas substrate specific pumps only export one specific molecule (Cox and Wright 2013). There are five main classes of efflux pumps associated with bacterial membranes i.e. the ATP binding cassette (ABC) system, the major facilitator (MF), the resistance-nodulation-division (RND) family, the small multi-drug resistance (SMR) and the multidrug and toxic compound (MATE) efflux systems (Webber and Piddock 2003; Piddock 2006). With the exception of the ABC systems, all other pump systems utilize the proton motive force as an energy source for substrate dissemination (Paulsen et al. 1996; Webber and Piddock 2003).

2.2 Acquired resistance

Acquired resistance is typically due to genome changes that occur through mutation or gene transfer (Alekshun and Levy 2007). The mutation can be spontaneous or a specific resistance gene can be acquired by a susceptible strain via horizontal gene transfer (Verraes et al. 2013). The 3 primary mechanisms of horizontal gene transfer include conjugation, transformation and transduction (Taylor et al. 2004; Clewell and Francia 2004). Plasmids play a major role in horizontal gene transfer and can be exchanged via gene transfer between members of the same or different bacterial species and genera. Interestingly, Dubey and Ben-Yehuda (2011) demonstrated that bacteria can acquire hereditary and non-hereditary antibiotic resistances via nanotubes established between neighboring cells of different species at sufficiently high cell densities. This kind of scenario can be expected in pit latrine fecal sludge given the wide variety of bacteria present in this material. Resistance to the major classes of antibiotics such as β-lactams, aminoglycosides, tetracyclines, and quinolones is typically conferred by plasmids (Schultsz and Geerlings 2012).

2.3 Sub-inhibitory concentrations of antibiotics

In addition to spontaneous mutation, recombination, and horizontal gene transfer, recent studies have shown that sub-inhibitory concentrations of antibiotics, as would be the case in waste water and fecal sludge treatment plants, can also foster resistance as an undesirable side-effect (Rodríguez-Rojas et al. 2013; Laureti et al. 2013). Sub-inhibitory concentrations of antibiotics can produce genetic changes such as induction of error-prone DNA-polymerases mediated by SOS response and imbalanced nucleotide metabolism, among others (Fig. 3) (Rodríguez-Rojas et al. 2013). Another undesirable side effect is the selection of hypermutable clones, which can enhance the probability of resistance development (Fig. 3).

Alternatively, if the population is exposed to antibiotic concentrations well below the minimum inhibitory concentration (MIC), there is an increase in the genetic variability within the population, resulting in a low and high-level resistant population (Rodríguez-Rojas et al. 2013; Laureti et al. 2013). However, when a bacterial population is exposed to high concentrations of an antibiotic, only resistant strains can survive and are selected resulting in a highly resistant bacterial population (Fig. 3) (Rodríguez-Rojas et al. 2013). Therefore, different concentrations of antibiotics can cause mutagenesis, recombination, and/or horizontal gene transfer, which are key processes in the development and spread of resistance (Rodríguez-Rojas et al. 2013; Laureti et al. 2013). The choice of antibiotic concentration affects the percentage of mutator

strains in the population, increasing the likelihood of resistance even to non-related antibiotics (Rodríguez-Rojas et al. 2013).

2.4 Cross-protection mutualism

Enzymatic deactivation of antibiotics via production and release of resistance enzymes (Wright 2005) is a cooperative behavior that can allow resistant cells to protect sensitive cells from antibiotics by reducing the concentration of active antibiotics in the environment (Perlin et al. 2009; Yurtsev et al. 2013). Cross-protection mutualism has been observed in clinical pathogenic strains, which were sensitive to antibiotics but at the same time protected against them by neighboring microbes (Perlin et al. 2009). Cross-protection mutualism allows a bacterial population composed of multiple resistant strains to survive in a multi-drug environment such as pit latrine fecal sludge, albeit individual bacterial strains may not be resistant to every drug present in that particular environment (Yurtsev et al. 2016).

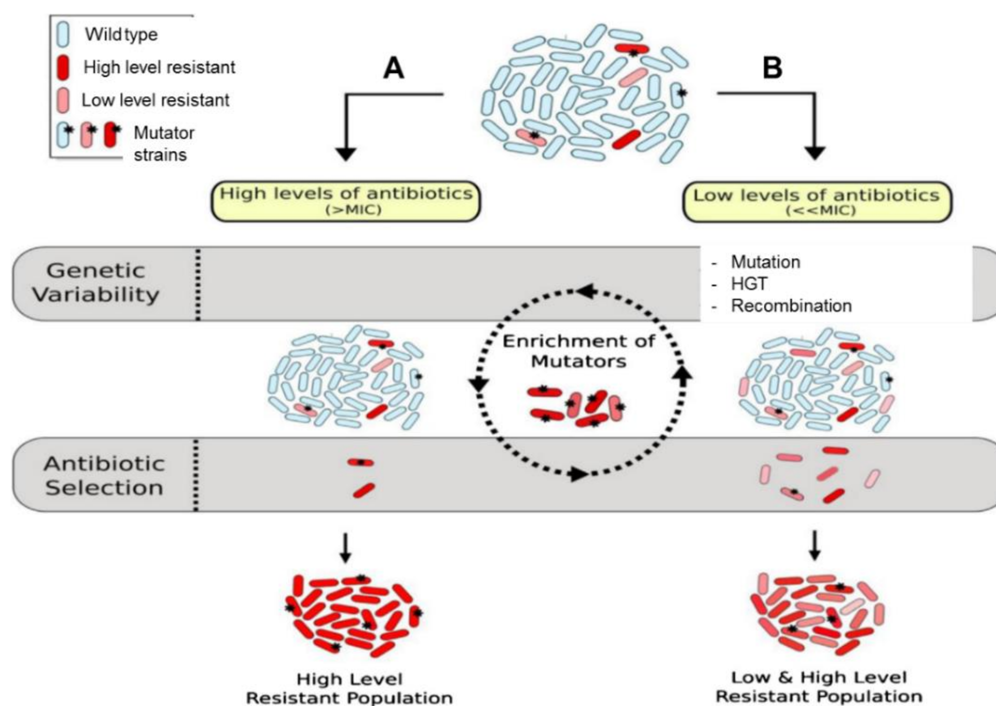

Figure 3 Diagram adapted from Rodríguez-Rojas et al. (2013), showing the possible effects of high (A) and low (B) concentrations of antibiotics on bacterial populations.

2.5 Transient resistance and bacterial persisters

Transient resistance allows microorganisms to temporarily survive lethal concentrations or the sudden appearance of antibiotics (Alekshun and Levy 2007). This resistance mechanism is best

illustrated by bacterial persistence, which can be achieved by stochastic processes, where individual cells within a population exhibit various phenotypes to block the appearance of an antibiotic (Rotem et al. 2010) or by transiently inducing antibiotic resistance at the population level (Piddock 2014). In bacterial persistence, a small group of bacterial cells in a population randomly enters a dormant, drug-tolerant state providing protection for the population against the sudden appearance of an antibiotic (Balaban et al. 2004).

This small group of cells is called persisters and ensure the survival of a population. Unlike resistant mutants, persister cells neither grow nor die in the presence of an antimicrobial agent but randomly in time switch back to a growing state once the antimicrobial agent is removed (Keren et al. 2004). Bacterial persistence is a non-inherited phenomenon with persister cells genetically identical to the rest of the bacterial population, unlike resistant bacteria that arise from heritable mutations (Helaine and Kugelberg 2014). In the past, transient stochastic resistance was linked to mechanisms where a subpopulation of cells enters a dormant, drug-tolerant state. However, Pu et al. (2017) recently described that in addition to 'passive defense' via dormancy, persister cells employ 'active defense' via enhanced efflux activity to expel drugs. Persister cells are thought to be responsible for many persistent bacterial infections, which pose a significant public health problem often resulting in the recurring use of antibiotics. Therefore, the interest in persister cells has increased dramatically over the past 5 years (Allison et al. 2011).

3. Antimicrobial resistance mechanisms in *Enterobacteriaceae* and resistance to major antibiotic categories

There are 4 major antimicrobial resistance mechanisms present in *Enterobacteriaceae* (Fig. 4). The first is related to the outer membrane permeability (Allen et al. 2010). Outer membrane proteins (Omp's), otherwise known as porins, allow for the transfer of essential molecules across the outer membrane into the bacterial cell (Harbottle et al. 2006). Common Omp's found in *E. coli* are OmpF, OmpC and OmpE (Poole 2002). If the function of one of the key porins is lost or altered via mutation, increased resistance to antimicrobials can occur (Poole 2002). OmpC and OmpF are the two most important porin proteins in *E. coli*, controlling the passage of small molecules into the cell (Lin et al. 2012). Mortimer and Piddock (1993) showed that mutations in OmpF caused increased resistance to antibiotics including tetracycline and cefoxitin and that mutations in OmpC resulted in a reduced accumulation of cephalothin and

cefoxitin. Certain antibiotics are too large to pass through porins, which explains the intrinsic resistance of Gram negative bacteria against these antibiotics.

The second mechanism relates to efflux pumps. Members belonging to the family *Enterobacteriaceae* possess multi-drug resistant efflux pumps, which are responsible for actively pumping antimicrobials out of the bacterial cell (Webber and Coldham 2010). Some efflux pumps, such as those of the resistance-nodulation-cell division (RND) family, can pump antibiotics directly outside the cell, while others like the major facilitator superfamily, secrete them into the periplasm (Allen et al. 2010). Efflux pumps which confer intrinsic resistance in Gram negative bacteria predominantly belong to the resistance-nodulation-division family (Fernando and Kumar 2013). A well-known example is the AcrAB-TolC RND efflux system, which is responsible for resistance to antibiotics including tetracyclines, macrolides, and fluoroquinolones in *E. coli* (Piddock 2006).

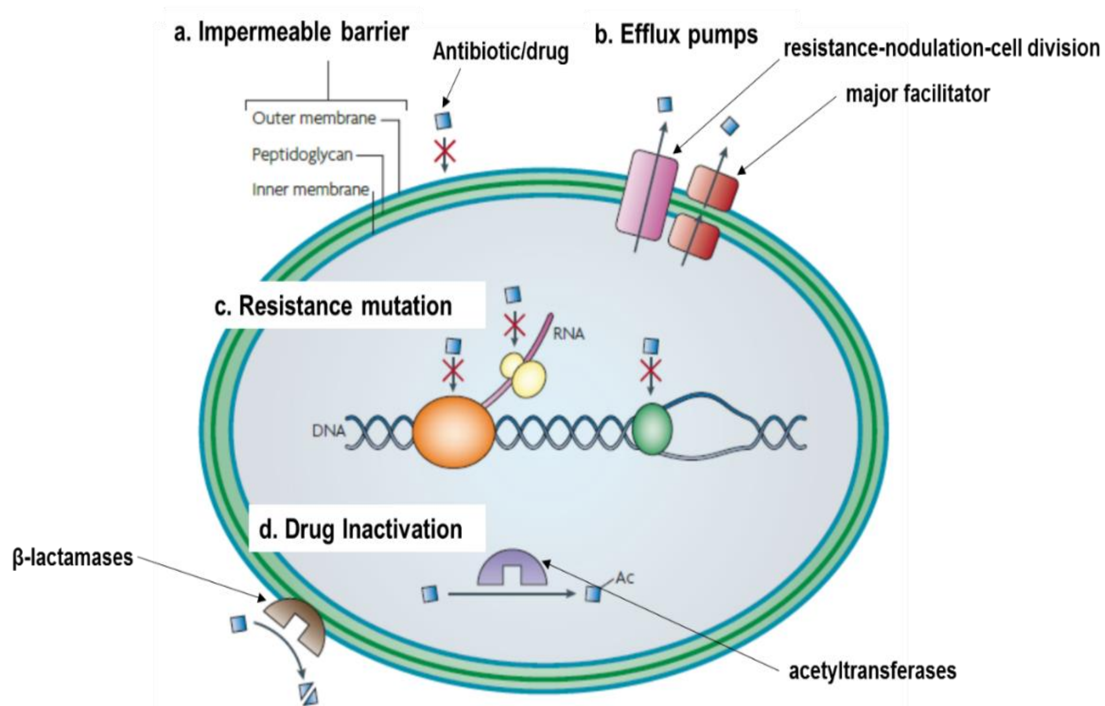

Figure 4 Diagram adapted from Allen et al. (2010), showing the possible mechanisms of antibiotic resistance in a typical Gram negative bacterium.

A single efflux pump has the ability to mediate resistance to multiple structurally discrete antibiotics. Therefore, inhibition of a certain pump could restore the antimicrobial effects of several antibiotics (Lomovskaya et al. 2001; Blair and Piddock 2009). This phenomenon was

demonstrated in *E. coli*, where phenothiazine drugs were found to indirectly inhibit efflux activity through reduction in the expression levels of the acrB gene, a key component of the pump (Chan et al. 2007; Blair and Piddock 2009).

A third resistance mechanism described by Allen et al. (2010) is through mutations, which modify the target protein by disabling the antibiotic-binding site but leaving the cellular functionality of the protein intact. The fourth mechanism involves inactivation of the antibiotic, which can occur by covalent modification of the antibiotic, e.g. acetyltransferases acting on aminoglycoside antibiotics, or by degradation of the antibiotic, e.g. β-lactamases acting on β-lactam antibiotics. In addition, *Enterobacteriaceae* can acquire resistance through plasmid transfer e.g. the acquisition of antibiotic resistant genes from extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* via horizontal gene transfer (Vaidya 2011).

3.1 β-lactam resistance

β-Lactamases are specialized enzymes produced by bacteria that confer resistance to β-lactam antibiotics through hydrolytic cleavage of the β-lactam ring present in the antibiotic (Pfeifer et al. 2010). These enzymes confer resistance to a wide range of antibiotics with resistance to the penicillins and cephalosporins being the most prevalent in MDR phenotypes (Allocati et al. 2013). In *E. coli*, the production of β-lactamase mediates resistance to broad spectrum β-lactam antibiotics and *E. coli* in particular produces the majority of the β-lactamase enzymes encoded on plasmids (Allocati et al. 2013). There are two primary classification schemes for β-lactamase enzymes; the first is the Ambler molecular classification scheme (Table 1), the second is the Bush-Jacoby-Medeiros functional classification system (Ambler et al. 1991; Bush et al. 1995). The Ambler classification scheme is based on protein homology (not phenotypic characteristics) and divides β-lactamases into 4 groups (A-D) (Ambler et al. 1991). β-Lactamases of groups A, C (Jaurin and Grundström 1981) and D (Ouellette et al. 1987) are serine β-lactamases and group B enzymes are metallo-β-lactamases, which need a bivalent cation, preferentially zinc, for hydrolysis (Ambler et al. 1991). The Bush-Jacoby-Medeiros classification scheme groups β-lactamases according to functional similarities (substrate and inhibitor profile) (Bush et al. 1995). Like the Ambler classification scheme, this scheme has 4 major groups of enzymes with multiple subgroups and is thought to have much more immediate relevance in diagnostic laboratories as it considers β-lactamase inhibitors and β-lactam substrates that are clinically relevant (Bush et al. 1995).

3.1.1 CTX-M type β-lactamases

The CTX-M type β-lactamases are named after the antibiotic on which they are active, which is Cefotaxime, and the location where they were first isolated, in Munich (Peirano and Pitout 2010). These enzymes have replaced the previously dominant Extended-spectrum beta-lactamases (ESBL's) and have proven highly successful in terms of diffusion into various epidemiological settings (Peirano and Pitout 2010). Resistance to specific cephalosporins of the third generation suggests the production of ESBLs in *E. coli* and *Klebsiella* spp., particularly if cefoxitin susceptibility is maintained (Livermore and Brown 2001). The genes involved in resistance within the CTX-M type β-lactamases are the blaCTX-M genes, which have played an important role in the dissemination of resistance in *E. coli* (Peirano and Pitout 2010). The source of these genes has been traced to the genus *Kluyvera*, family *Enterobacteriaceae* (D'Andrea et al. 2013).

Table 1 Modified classification scheme of β-lactamases (Ambler 1980).

	β-lactamase class	**Type of β-lactamases**	**Important examples**	**Preferential occurrence**	**Important phenotypical traits**[a]
Serine β-lactamases	A	Broad-spectrum	TEM-1, TEM-2, SHV-1, SHV-11	*Enterobacteriaceae* and nonfermenters[b]	Ampicillin, cephalotin
		ESBL TEM-type	TEM-3, TEM-52		Penicillins, 3rd generation cephalosporins
		ESBL SHV-type	SHV-5, SHV-12		
		ESBL CTX-M-type	CTX-M-1, CTX-M-15		
		Carbapenemases	KPC, GES, SME		All β-lactams[c]
	C	AmpC cephamycinases (chromosome-encoded)	AmpC	*Enterobacter* spp. *Citrobacter* spp.	Cephamycins (cefoxitin), 3rd generation cephalosporins
	D	AmpC cephamycinases (plasmid-encoded)	CMY, DHA, MOX, FOX, ACC	*Enterobacteriaceae*	Cephamycins (cefoxitin), 3rd generation cephalosporins
		Broad-spectrum	OXA-1, OXA-9	*Enterobacteriaceae*; *A. baumannii*	Oxacillin, ampicillin, cephalotin
		ESBL OXA-type	OXA-2, OXA-10		Penicillins, 3rd generation cephalosporins

		Carbapenemases	OXA-48; OXA-23,-24,-58		Ampicillin, imipenem; All β-lactams[c]
Metallo-β-lactamases	B	Metallo-β-lactamases (Carbapenemases)	VIM, IMP NDM-1	*Enterobacteriaceae* and nonfermenters	All β-lactams[c]

[a] characteristical resistances that are partially used for diagnostic purposes

[b] broad-spectrum β-lactamase TEM-1 frequently occurs in nonfermenters (*P. aeruginosa*, *A. baumannii*)

[c] broad hydrolytic spectrum including carbapenems

The mobilization of the blaCTX-M genes has been very successful, resulting in the dispersal of these genes in multiple plasmid groups within the family *Enterobacteriaceae*, contributing to the global dissemination of CTX-M type ESBLs (D'Andrea et al. 2013). A history of urinary tract infections, previous hospitalization, international travel, diabetes mellitus, and prior use of cephalosporin or fluoroquinolone antibiotics are all risk factors for the acquisition of infections caused by CTX-M producing *E. coli* (Laupland et al. 2008).

3.1.2 AmpC β-lactamases

Chromosome-encoded AmpC (cAmpC) β-lactamases

cAmpC β-lactamases confer resistance to β-lactams in many pathogenic and opportunistic bacteria. They are abundant in enterobacteria, except *Salmonella* spp., *Klebsiella* spp., *Proteus mirabilis*, *Shigella flexneri*, and *Shigella dysenteriae,* and their expression is inducible in all except *E. coli* and some *Shigella* spp. other than *S. flexneri* and *S. dysenteriae* (Livermore 1995). In inducible strains, ampC transcription is controlled by the transcriptional activator AmpR, which is absent in *E. coli* (Bartowsky and Normark 1991; Philippon et al. 2002). *E. coli* AmpC β-lactamases have a similar hydrolytic profile to other ESBLs with additional hydrolytic activity towards cephamycins such as cefoxitin and cefotetan (Bajaj et al. 2016). AmpC β-lactamases are typically inhibited by cloxacillin and boronic acid, but not by conventional β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Doi and Paterson 2007; Bajaj et al. 2016).

Plasmid-mediated AmpC (pAmpC) β-lactamases

The second most common cephalosporin resistance mechanism in *E. coli*, following CTX-M type β-lactamases, is that mediated by plasmid encoded AmpC β-lactamases (pAmpC) (Edquist et al. 2013). Six families of pAmpC β-lactamases - CIT, FOX, MOX, DHA, EBC, and ACC - were described by Pérez-Pérez and Hanson (2002). In *E. coli*, the most common pAmpC β-

lactamase is the CMY-2 type, which belongs to the CIT family and shares homology with the cAmpC from *Citrobacter freundii* (Helmy and Wasfi 2014). These enzymes hydrolyze all β-lactam antibiotics except the carbapenems and fourth generation cephalosporins (Gude et al. 2013). Isolates displaying resistance to both cefoxitin and extended spectrum cephalosporins are regarded as pAmpC producers, which can confer multi-resistance, as the ampC genes are often located on plasmids containing other antimicrobial resistance genes (Philippon et al. 2002; Gude et al. 2013). Gude et al. (2013) found that urinary tract infections were the most prevalent type of infection related to pAmpC-producing isolates and almost 57% of patients hospitalized with infections were caused by pAmpC isolates from external origin.

3.1.3 Metallo-β-lactamases

Metallo-β-lactamases (MBLs) have the ability to hydrolyze carbapenems and are resistant to commercially available β-lactamase inhibitors but are susceptible to inhibition by metal ion chelators (Koh et al. 2001; Queenan and Bush 2007). They have a broad substrate spectrum, hydrolyzing in addition to the carbapenems, cephalosporins and penicillins but not aztreonam (Koh et al. 2001). Their mechanism of hydrolysis relies on the interaction of β-lactams with zinc ions in the active site of the enzyme resulting in the unique trait of their inhibition by EDTA, a chelator of Zn^{2+} and other divalent cations (Koh et al. 2001). Acquired MBLs which belong to the IMP and VIM families are an increasing concern among *Pseudomonas* spp. and *Acinetobacter* spp. and some cases have also been reported for *Enterobacteriaceae* (Nordmann and Poirel 2002). In addition, some bacteria that produce MBLs remain almost fully susceptible to carbapenems in vitro, possibly because resistance, especially among *Enterobacteriaceae*, requires both membrane impermeability as well as the action of an enzyme (Koh et al. 2001; Yan et al. 2002).

In addition to the IMP- and VIM-type MBLs, the recently discovered NDM-1 (New Delhi metallo-beta-lactamase) β-lactamase, which was first detected in 2008 in *Klebsiella pneumoniae* and *E. coli* from a patient returning to Sweden from India, showed the potential for the dissemination of such MBLs (Yong et al. 2009). In addition, NDM-1 has been rapidly and widely spread between bacterial species from numerous countries (Yong et al. 2009; Nordmann et al. 2011). The dissemination of these β-lactamases is attributed to the bla_{NDM-1} gene, which is found on several plasmid types and can be transferred among Gram negative bacteria by conjugation (Yong et al. 2009). Unlike genes encoding IMP- and VIM-type MBLs, the bla_{NDM-1} gene has not been found in integron structures (Yong et al. 2009).

4. Antimicrobial resistance mechanisms in *Staphylococcus* spp. and resistance to major antibiotic categories

Staphylococcus aureus, the most common pathogen in this genus, is naturally susceptible to most antibiotics and resistance is often acquired by the horizontal transfer of genes located on mobile genetic elements (MGE's) such as plasmids or gene cassettes (Katayama et al. 2000). A common example of horizontal gene transfer in *S. aureus* is resistance to the antibiotic methicillin. *S. aureus* has also been reported to possess more than 20 multi-drug resistant efflux pumps (Fig. 5) which contribute to its resistance against antimicrobial agents (Schindler et al. 2015). Jang (2016) describes efflux pumps as membrane integrated proteins, which expel toxic compounds such as antibiotics, biocides and toxic metals from within the bacterial cell to the external environment (Fig. 5). These efflux pumps can be specific, recognizing a single antibiotic or antibiotic class or they can recognize several antibiotics with similar characteristics, making them multidrug efflux pumps (Jang 2016).

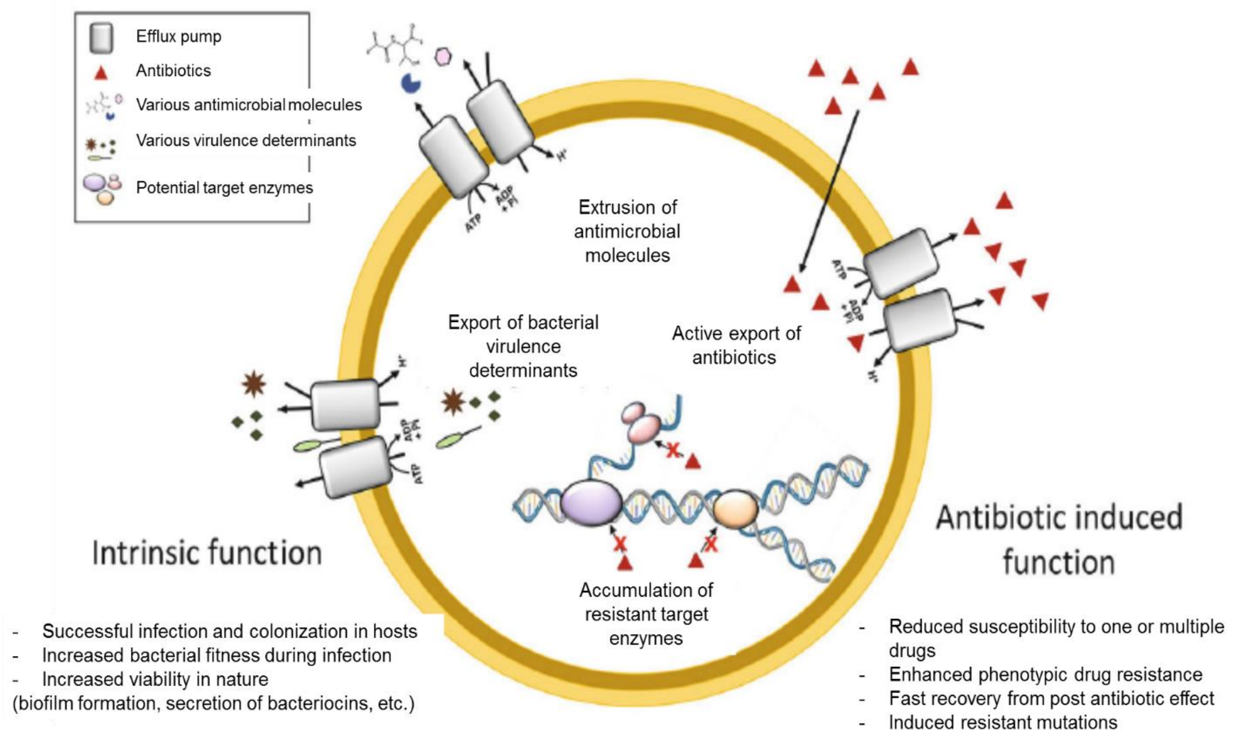

Figure 5 Diagram adapted from Jang (2016), showing the typical efflux pumps present in a Gram positive bacterium.

4.1 β-lactam resistance

The most important example of β-lactam resistance in *Staphylococcus* spp. is the mecA-mediated methicillin resistance, which confers resistance to broad-spectrum β-lactams and is responsible for the methicillin-resistant *Staphylococcus aureus* (MRSA) pandemics (Lindsay 2013; Otto 2013). The few β-lactams to which mecA does not confer resistance include ceftobiprole and ceftaroline. Penicillin G, ampicillin, and amoxicillin are active against penicillinase-negative MRSA strains (Entenza et al. 2002; Ishikawa et al. 2003).

Methicillin was first introduced as celbenin in 1959 for the treatment of infections caused by penicillin-resistant *S. aureus* strains and is now substituted by oxacillin (Jevons 1961). Methicillin/oxacillin was the preferred alternative to other penicillin derivatives and cephalosporins for the treatment of staphylococcus infections, with erythromycin and clindamycin recommended as a second-line therapy for patients with β-lactam allergies (Leclercq 2002; Gherardi et al. 2009). Oxacillin resistance in *Staphylococcus* spp. is due to the production of an alternative penicillin-binding protein (PBP2a) such as mecA, that has low affinity for almost all β-lactams (Ito et al. 2012) and whose presence can be predicted phenotypically using a screen for cefoxitin or oxacillin resistance (CLSI 2012).

MRSA can also acquire resistance plasmids from distantly related bacteria and some of these plasmids can replicate and also express their resistance genes without difficulty within the staphylococcal host (Abdelbary et al. 2017). An example of this is the acquisition of vancomycin resistance genes (vanA) by *S. aureus* from distantly related bacteria such as enterococci (Weigel et al. 2003). In addition to accepting foreign resistance genes and their associated mobile genetic elements, staphylococci may also pass plasmid- or transposon-borne resistance genes to other bacteria (Abdelbary et al. 2017). These mobile genetic elements can exchange useful information with other bacterial species, thereby making *S. aureus* strains one of the most skillful groups of microorganisms that are able to spread antimicrobial resistant genes (Kuroda et al. 2001).

4.2 Macrolides, lincosamides and streptogramin B (MLSB) resistance

Macrolides, lincosamides and streptogramin B (MLSB) are the preferred alternatives to penicillins and cephalosporins for the treatment of MRSA infections (Szczuka et al. 2016) and the macrolides, erythromycin and clindamycin, are recommended as a second-line of therapy for patients with β-lactam allergies (Leclercq 2002; Gherardi et al. 2009). MLSB antibiotics

are structurally different yet functionally similar as they inhibit the synthesis of proteins by binding to the 50S subunit (23S rRNA) of the bacterial ribosome (Leclercq 2002). Macrolides generally have a spectrum of activity limited to Gram negative cocci, Gram positive cocci (mainly staphylococci and streptococci) and bacilli and intracellular bacteria (*Chlamydia* and *Rickettsia* species).

4.3 Glycopeptide resistance

Glycopeptides, alone or in combination, are often the only therapy for infections caused by MDR strains of staphylococci, streptococci, and enterococci (Faron et al. 2016). They act on the bacterial cell wall, inhibiting peptidoglycan synthesis by binding with peptidoglycan units (at the D-alanyl-D-alanine dipeptide) and by blocking transglycosylase and transpeptidase activity (Kahne et al. 2005; Faron et al. 2016). This ultimately inhibits cell wall synthesis and the bound vancomycin (glycopeptide)-pentapeptide complexes accumulate within the cell (Barna and Williams 1984). Therefore, glycopeptides like teicoplanin and vancomycin generally act as steric inhibitors of peptidoglycan maturation and reduce cellular mechanical strength (Ge et al. 1999). The glycopeptide, vancomycin, is a drug of choice for the treatment of MDR *S. aureus* (Melo-Cristino et al. 2013) including severe MRSA infections (Sorrell et al. 1982). However, vancomycin resistant *Staphylococcus aureus* has been reported in South Africa (Ferraz et al. 2000; Fortuin-de Smidt et al. 2015).

5. The antibiotic resistance situation in South Africa

Despite having probably the most active surveillance for antibiotic resistance in Africa, the levels of antibiotic resistance in South Africa (SA) remain high and resistance to last-resort antibiotics is on the rise.

5.1 Antibiotic use

According to a recent report on the surveillance for antimicrobial resistance and consumption of antibiotics in South Africa for the period 2012-2017, antibiotic use in 2015 was 21 149 standard units per 1000 population, which was significantly higher than for most other countries in the world. For the period 2014-2015, antibiotic procurement for animal health contributed 23-36% and that for human health about 74-77% (DoH 2018). This is in contrast to reports from the United States of America (USA), China and India, where animal related consumption of antibiotics is higher than that for human consumption (DoH 2018). In addition, for the period 2014 to 2015 tetracyclines and macrolides, also used for human consumption,

were the major antibiotics used in animal husbandry (DoH 2018). In South Africa, a high human consumption of penicillins and streptomycins was reported, with broad-spectrum penicillin usage being higher than in other BRICS countries, the United Kingdom and the USA (DoH 2018). However, the consumption of broad-spectrum penicillins in South Africa declined from 19.6% to 18.2% between 2016 and 2017 (DoH 2018).

5.2 Resistance patterns in humans

Gram negative bacteria in South Africa are becoming increasingly resistant to last-resort antibiotics such as carbapenems, tigecycline, and colistin (polymyxin E) (Osei Sekyere 2016). The mcr-1 colistin resistance gene has been reported among *E. coli* isolates from both clinical and poultry samples in two provinces in South Africa (Osei Sekyere 2016). Moreover, NDM-1 and OXA-48-like carbapenemases are the most prevalent carbapenemases discovered among South Africa's Gram negative bacteria and have been involved in clinical infection outbreaks. The most common bacteria cultured from human blood specimens in South Africa for the period 2012-2017 were *Klebsiella pneumoniae*, *S. aureus*, followed by *E. coli* and *Acinetobacter baumannii* (DoH 2018). In a study based on the surveillance of antibiotic resistance in bacterial isolates from blood culture samples from 16 hospitals across South Africa (2015-2016), the highest number of antibiotic resistant *E. coli* and *S. aureus* isolates came from hospitals in the Gauteng province in South Africa (Perovic et al. 2018). While <30% of *E. coli* isolates were resistant to 3rd and 4th generation cephalosporins, about 30% were resistant to the fluoroquinolone ciprofloxacin (Perovic et al. 2018). However, 86% of *E. coli* isolates showed resistance to ampicillin/amoxicillin, reflecting the overuse/misuse of β-lactam antibiotics in South Africa. In addition, the same study reported that 30% of *S. aureus* isolates were resistant to cloxacillin, which was the only antibiotic with data reported for *S. aureus* for the period 2015-2016.

The report published by the South African Department of Health in 2018 on surveillance for antimicrobial resistance and consumption of antibiotics in South Africa for the period 2012-2017 states that 1 in 4 *E. coli* isolates from blood stream infections is an Extended Spectrum β-Lactamase (ESBL) producer with resistance to 3rd generation cephalosporins (DoH 2018). In 2017, 26% of *E. coli* isolates from blood stream infections were resistant to the fluoroquinolone ciprofloxacin and 25% were resistant to the 3rd generation cephalosporins cefotaxime and ceftriaxone. In South Africa, *E. coli* is believed to be the main cause of community-acquired infections such as urinary tract infections (UTI) and has displayed

increasing - from 19% in 2012 to 26% in 2017 - antibiotic resistance to the fluoroquinolones, which are the antibiotics of choice for the treatment of UTI's (DoH 2018).

Surveillance for antibiotic resistant *S. aureus* in South Africa conducted from 2010 to 2017 revealed that 92% of methicillin resistant *S. aureus* (MRSA) cases were healthcare-associated bacteremia and 8% community-associated bacteremia (Quan and McCarthy 2018). Similarly, Steinhaus et al. (2018) reported a high level of healthcare associated *S. aureus* bacteremia (68%) infections at a South African referral hospital, with 24% of all cases reported caused by MRSA. However, resistance levels of *S. aureus* reportedly declined from 36% to 23% over the past 6 years, with resistance levels varying across provinces in South Africa (DoH 2018). Again, 1 in 4 *S. aureus* isolates from blood stream infections is methicillin/oxacillin resistant and 1 in 3 is resistant to cloxacillin (MRSA) (DoH 2018).

5.3 Resistance patterns in animals

The transfer of antibiotic resistance to humans usually occurs via the food chain and the farming industry is apparently one of the major contributors of antibiotic resistance in healthcare settings in South Africa (Ekwanzala et al. 2018). High levels of antibiotic resistance have been detected in farm animals in South Africa. *S. aureus* isolated from meat carcasses and bovine milk in abattoirs and dairy farms of the Eastern Cape showed a high level of resistance to penicillin G (72%) and to tetracycline (39%) (Pekana and Green 2018). In addition, antibiotic resistant *S. aureus* strains were detected in chicken flesh and chicken fecal samples collected from different areas in Durban, KwaZulu-Natal, South Africa (Mkize et al. 2017). Game meat, which is farmed widely in South Africa, is a food source gaining increasingly in popularity (van den Honert et al. 2018). However, MRSA and antibiotic resistant *E. coli* have been found in these animals (van den Honert et al. 2018). The environment, including wildlife, is not isolated from the rest of the farm and can thus be a source of antibiotic resistance genes and bacteria or a transfer vector of these agents (EFSA 2018). Therefore, animals used for food production in South Africa are potential reservoirs for ABR and even MDR bacteria, potentially contributing to and can therefore cause the spread of antibiotic resistant pathogens and resistance genes to the environment and more importantly, to humans.

5.4 Resistance patterns in the environment

One of the most common environmental sources of antibiotic resistance is water. Wose et al. (2010) determined the antibiotic resistance profiles of *E. coli* isolates from different water

sources such as local wastewater- and water-treatment plants, the Modimola Dam and homes in the Mmabatho community in South Africa. Of the *E. coli* isolates tested, more than 70% were resistant to erythromycin, tetracycline, ampicillin, chloramphenicol and norfloxacin. Mulamattathil et al. (2014) analyzed water samples from five different sites (raw and drinking water) in Mafikeng in South Africa for the presence of antibiotic resistant fecal indicator bacteria as well as *Aeromonas* and *Pseudomonas* species. All tested isolates showed a high level of resistance to erythromycin, trimethoprim, and amoxicillin similar to the resistance levels determined by Wose et al. (2010) for erythromycin and amoxicillin. Malema et al. (2018) investigated the antimicrobial resistance patterns of *E. coli* from harvested rainwater tanks in the Eastern Cape Province in South Africa and found the highest level of resistance of *E. coli* isolates was to the broad-spectrum cephalosporin cephalothin (76%), with 52% of isolates showing multi-drug resistance. The above findings indicate a high level of antibiotic resistance in various water sources, highlighting that the use of untreated harvested rain or dam water for consumption purposes may pose a risk of transmission of pathogenic MDR *E. coli*.

5.5 Knowledge attitudes and perceptions of healthcare professionals and patients

Ncube et al. (2017) evaluated South African general practitioners' antibiotic prescription patterns in the treatment of chronic bronchitis and discovered that more than 70% of the prescribed antibiotics were cephalosporins, penicillins and other β-lactams. In addition, Ncube et al. (2017) discovered that antibiotics were more likely to be prescribed in patients with viral bronchitis than in patients with bacterial bronchitis, reflecting a clear lack in understanding of antibiotic prescription.

Farley et al. (2018) conducted a cross-sectional survey to determine knowledge, attitudes, and perceptions of primary healthcare providers in South Africa to antibiotic resistance. Approximately 96% of participants thought that antibiotic resistance is a major issue in the country and 67% felt patient pressure to prescribe antibiotics. Furthermore, at a regional hospital in KwaZulu-Natal, South Africa, Ramchurren et al. (2018) performed a survey to determine the knowledge, attitudes, and practices of patients concerning antibiotic use. This research showed that only 53% of patients had a decent amount of antibiotic use awareness and although 70% of patients knew that antibiotics were used to treat bacterial infections, 55% believed that antibiotics were also used to treat viral infections. In addition, only 44% of patients were aware of antibiotic resistance associated with the overuse of antibiotics, 42% believed that taking antibiotics for a common cold would help them recover faster, 46%

expected the doctor to prescribe more than one antibiotic for a severe cold, and 40% reported sharing antibiotics with friends or family (Ramchurren et al. 2018).

The above findings clearly indicate a need for more education on the use of antibiotics by healthcare workers and patients in South Africa and on antibiotic resistance in general, as Ramchurren et al. (2018) indicated that patients with good knowledge on antibiotic use were six times more likely to have good antibiotic practices.

5.6 Strategies to combat antibiotic resistance in South Africa

In South Africa, the identification of the first untreatable, pan-resistant *K. pneumoniae* strain, the emergence of colistin resistance genes in poultry and humans, and the recent detection of vancomycin-resistant enterococci in most South African ecological niches poses a serious threat to public health as the treatment options in case of these infections is limited (Tatsing Foka et al. 2018; DoH and DAFF 2018; DoH 2018). Therefore, the South African National AMR Strategy Framework for 2018-2024 (DoH and DAFF 2018) has developed a structure for managing AMR among humans and animals to limit further increases in AMR infections and improve the health of these populations. This structure is made up of 5 strategic objectives. Objective 1 is to strengthen, coordinate and institutionalize interdisciplinary and intersectoral efforts, objective 2 is the implementation of diagnostic stewardship programs, objective 3 is the optimization of surveillance and early detection of AMR and antibiotic use, objective 4 is to enhance infection prevention and control and biosecurity to prevent the spread of resistant microbes and lastly, objective 5 is the promotion of appropriate antibiotic use in human and animal health (DoH and DAFF 2018).

As many peri-urban communities in South Africa still make use of pit latrines as a basic means of sanitation, and these can contain pathogenic MDR bacteria that can infect humans due to unhygienic practices, the following study aimed to initially screen for the presence of hygiene indicators on household surfaces in a peri-urban community in KwaZulu-Natal, South Africa, as well as on the skin surface of municipal workers, their PPE (personal protective equipment) and municipal vehicle before and after manual pit latrine emptying. In addition, the bacterial community present in a ventilated improved pit latrine (VIP) fecal sludge sample was analyzed. Furthermore, the antibiotic resistance patterns of selected pit latrine isolates (*E. coli* and *Staphylococcus* spp.), their ability to form biofilms on household surfaces and the effect of selected biocides on these biofilms was determined.

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Chapter 2

Manual pit latrine emptying and hygiene - a baseline survey in a peri-urban community in KwaZulu-Natal (South Africa)

Abstract

Pit latrines offer a basic means of sanitation to many peri-urban communities in developing countries. However, due to hygiene challenges associated with these systems, they can also present a potential health risk to household members and municipal workers. Therefore, the following study aimed to determine the frequency of detection of *Escherichia coli* and *Staphylococcus* spp. on the skin surface, personal protective equipment (PPE), the municipal vehicle and on various surfaces at 10 households in a peri-urban community (KwaMashu, KwaZulu-Natal, South Africa) before and after manual pit latrine emptying. Surface samples were collected using sterile wet wipes and *E. coli* and *Staphylococcus* spp. were detected in samples using standard procedures. In addition, *E. coli* was enumerated in soil samples from areas where open defecation and geophagia occurred, using a most probable number (MPN) method. The MPN/g values established for *E. coli* in areas where geophagia took place ranged from $\log_{10}$ 2.7 to $\log_{10}$ 3.3 MPN/g. Open defecation occurred at one household and an MPN/g value of $\log_{10}$ 3.7 was established for soil in this area. The frequency of detection of the target microorganisms on household surfaces such as the walkway between the pit latrine and the municipal vehicle and on municipal workers' hands occasionally increased after the pits were emptied. It was also evident that the hands of municipal workers' emptying these pit latrines were frequently contaminated even before pit emptying. In addition, the presence of *E. coli* and *Staphylococcus* spp. in soil from areas where geophagia took place indicates a potential health risk to individuals who still partake in this practice, as other pathogens present may be ingested. Overall, the detection of the target microorganisms on household surfaces and on municipal workers' skin and PPE after pit emptying indicates that the presence of pit latrines, at peri-urban households, are a potential health risk to household members and municipal workers if manual pit emptying is practiced.

Keywords: Peri-urban communities, South Africa, pit latrines, municipal workers, pit emptying, contamination, *E. coli*, *Staphylococcus* spp.

Introduction

Of the sanitation systems available to South African households, approximately 16.6% are ventilated improved pit latrines (VIP's) and 14.5% are non-ventilated pit latrines (Stats SA 2016). VIP's are permanent single-pit structures with the following typical features: a minimum depth of 1.5m, a foundation, a cover slab and a framework with a vent pipe and a fly screen (Mara 1984). There are approximately 40 000 VIP's in the eThekwini Municipality in KwaZulu Natal, South Africa, which are emptied on a 5-year cycle by the municipality at no cost to the households (EWS 2011). VIP's can take up to 25 years to fill, depending on the contents and the size of the pit latrine (Brouckaert et al. 2013). Approximately 87% of VIP contents consist of fecal material, the rest of the pit contains urine and general solid wastes such as plastics, paper and fabrics (Bakare et al. 2012; Brouckaert et al. 2013). One of the problems with pit latrines is the lack of an impermeable physical barrier between pit latrine contents and the surrounding environment. Therefore, harmful substances and pathogenic bacteria can potentially leach into the groundwater (van Ryneveld and Fourie 1997). In addition, traditional pit latrines are often built on marginal land and tend to easily collapse and sufficient land space is required to dig new pits as they are not permanent structures (Grimason et al. 2000).

The type of microorganisms present in pit latrine fecal sludge is typically determined by the health status of the community or the households that generate this waste (Singh et al. 2011). Identifying all pathogens present in fecal sludge is complex and tedious, therefore selected indicator microorganisms are often used in risk assessments to predict the presence of other fecal pathogens. For the purpose of this study, one well-established Gram negative hygiene indicator - *Escherichia coli* - and a representative Gram positive genus (*Staphylococcus* spp.) were targeted. *Escherichia coli* is a typical gut commensal found in humans and animals and therefore a well-established hygiene indicator. However, it includes pathogenic strains such as Shiga toxin-producing *E. coli* (STEC) (Majowicz et al. 2014). *Staphylococcus* spp. are members of the normal skin flora. However, they can colonize the gut of humans and animals and are also found in the environment (Schulz et al. 2012; Schmidt et al. 2014; Kates et al. 2018), with several species such as *Staphylococcus aureus*, *S. lugdunensis*, *S. saprophyticus* and *S. haemolyticus* being pathogenic in nature (Kuroda et al. 2005; Frank et al. 2008; Becker et al. 2014).

Households or communities where pit latrines are located and municipal workers involved in the manual emptying thereof are at risk of exposure to potential pathogens contained in pit latrine fecal sludge (Lewin et al. 2007). Municipal workers or community members can be exposed to enteric pathogens in pit latrine fecal sludge through ingestion of water that is contaminated by improper desludging of pit latrines, poor hygiene practices or through direct contact with contaminated pit latrine surfaces (Montgomery and Elimelech 2007; Flores et al. 2011; McGinnis et al. 2019). In addition, Kumwenda et al. (2017) found that the main exposure scenario to microorganisms found in pit latrine fecal sludge for workers emptying pit latrines was inhalation of aerosols and dust particles, accidental ingestion of- and direct contact with fecal sludge. Additionally, when pit latrines become full, community members can defecate in areas surrounding households, creating greater risk of exposure to potential pathogens for those community members who practice geophagia.

Geophagia, defined as the deliberate consumption of earth (soil or clay) by both humans and animals (George and Ndip 2011), is commonly practiced by women (non-pregnant and pregnant) (Meel 2012; Mathee et al. 2014). In a study by Macheka et al. (2016), geophagia was reported by 54% of women visiting an antenatal clinic in Pretoria and of these women, 75% consumed at least 3 teaspoons of soil or clay per day. Macheka et al. (2016) found that education levels were not a contributing factor for geophagia as both literate and illiterate women consumed soil/clay. However, whether the women had spouses/partners or not was a contributing factor for geophagia as most soil consuming women were not married. Reasons for this practice ranged from unexplained cravings to the belief that soil/clay acts as an iron supplement (Macheka et al. 2016). In addition, in a survey conducted in one of the rural districts of the Eastern Cape province in South Africa, George and Ndip (2011) found that consumers of soil/clay believed that soils had the ability to reduce the symptoms of morning sickness and hunger pangs.

Due to a lack of space between peri-urban houses and narrow streets in many peri-urban communities, vacuum pump trucks cannot enter these areas and thus pit latrines have to be manually emptied (O'Riordan 2009). Manual pit emptying can be a messy task as workers have to physically enter these pits to remove the lower layers of fecal sludge (Jenkins et al. 2015). In addition, pit latrine contents can be accidentally sprayed or spilled onto household surfaces during pit emptying. *E. coli* and *Staphylococcus* spp. already present in pit latrine fecal sludge can - together with other bacterial pathogens present - be transferred to household surfaces,

where they are able to survive and if conditions are right, even thrive for extended periods in the form of biofilms (Galié et al. 2018).

The bacteria in these biofilms can display increased virulence and enhanced resistance to biocides and antibiotics due to the protective nature of the extracellular polysaccharide (EPS) matrix (Bjarnsholt 2013; Singh et al. 2017). *E. coli* is known to persist on hands for 6-90min (Fryklund et al. 1995) and on inanimate surfaces from 2h up to approximately 16 months (Maule 2000; Møretrø et al. 2010), thus highlighting the suitability of this microorganism as a hygiene indicator. In addition, studies have demonstrated that Gram positive bacteria such as *Staphylococcus aureus* can survive on hands for a minimum of 150min (Ayliffe et al. 1988) and up to 7 months on inanimate surfaces (Neely and Maley 2000). Unless these surfaces are treated in a timely and appropriate manner, bacteria involved in surface contamination can cause the transmission of disease. This study therefore investigated the presence of *E. coli* and *Staphylococcus* spp. as indicators for bacterial contamination on household surfaces and on municipal workers' skin and PPE before and after manual pit emptying at 10 households in a peri-urban community (KwaMashu, KwaZulu-Natal, South Africa).

Materials and methods

Sample collection

The 10 households situated in KwaMashu, KwaZulu-Natal, South Africa are represented by locations D1-D10 (Fig. 1). In total, 14 surface samples were collected from each of the 10 households with VIP's and for 5 of the surfaces, a before and after pit emptying sample was collected (Table 1). The remainder of the surfaces were only sampled after pit emptying (Table 2). As access to households during pit emptying was restricted to municipal workers, samples for subsequent analyses were collected by members of staff of the pit emptying group, who received basic training on sample collection. Samples were collected before and after pit emptying from the walkway between the municipal vehicle and the VIP, the pit cover, the household tap handle, the VIP door handle and the hands of a volunteer municipal worker. In addition, samples were collected from the face and inner mask, outer mask, outer gloves and the bottom of a boot of a volunteer municipal worker, the lip of the pit latrine, an area away from pit emptying activity and the municipal vehicle door handle after pit emptying.

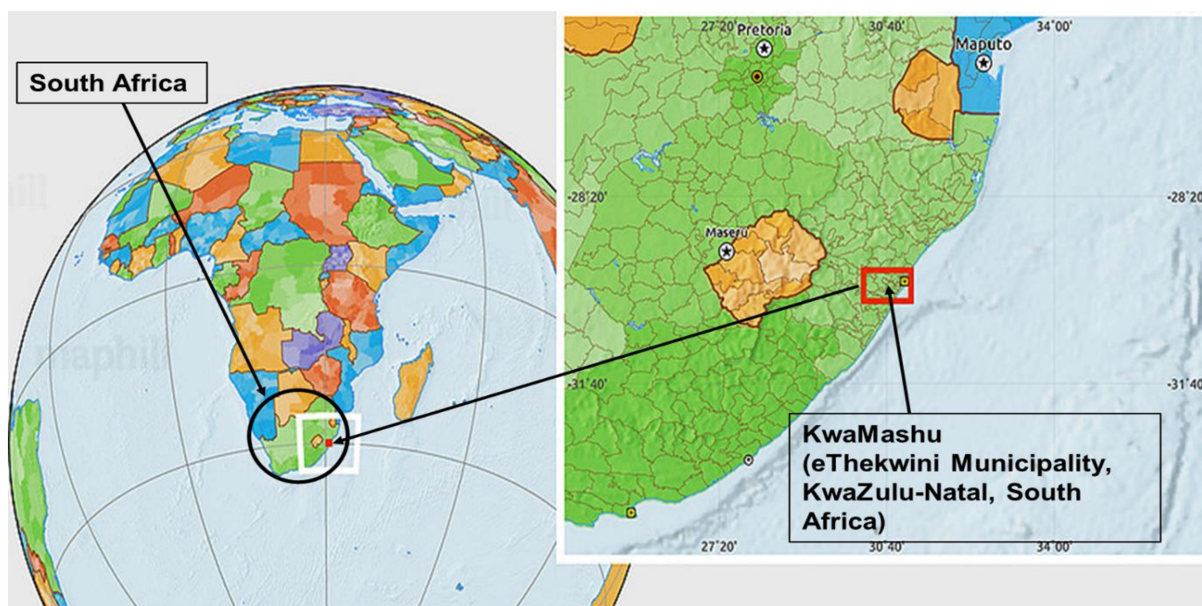

Figure 1 Amended map showing the sampling location in KwaMashu, KwaZulu-Natal, South Africa. http://www.maphill.com/south-africa/kwazulu-natal/ntuzumao/kwamashu/location maps/political-map/

Soil samples were collected from areas where open defecation occurred and from areas where soil was ingested. Inner hand gloves were used as hand surface samples as these gloves made direct contact with the hands of volunteers and face surface samples were collected by wiping the face area under the inner facemask of the volunteers. Facemasks, inner hand gloves and soil samples were placed directly into sterile plastic bags and the remaining surfaces were wiped down using sterile wet wipes and thereafter placed in sterile plastic bags. 14×18cm Pieces of previously autoclaved cotton fabric was moistened with sterile saline before it was used to wipe down surfaces.

Detection of the target bacteria on household surfaces and on municipal workers

The presence of *E. coli* and *Staphylococcus* spp. on municipal workers' skin and PPE and on household surfaces was determined using culture based techniques. *E. coli* was only enumerated in soil samples and confirmed to genus level using polymerase chain reaction (PCR). Only an absence/presence test for the target bacteria was conducted for all other samples. Wipes, face masks and gloves were initially left to soak in 30ml of sterile saline solution for 15h at room temperature before analysis.

E. coli

E. coli was enumerated in a total of 4 soil samples from areas where soil was ingested and areas where open defecation occurred at households D2, D3 and D5, using a previously reported MPN method (Mdluli et al. 2013). However, one milliliter of saline solution from samples other than soil was pre-enriched in 9ml of LST broth for 24h at 35°C. Presumptive *E. coli* isolates obtained from soil samples, wet wipes, facemasks and inner gloves were confirmed using prescribed biochemical tests (GIMViC). In addition, presumptive *E. coli* isolates from soil samples were confirmed to genus level via PCR based amplification of the *gadA* gene (glutamate decarboxylase A) as reported previously (Gemmell and Schmidt 2012). MPN values for *E. coli* were established according to de Man (1983) and expressed as $\log_{10}$ MPN/g of soil.

***Staphylococcus* spp.**

Detection of *Staphylococcus* spp. essentially followed the ISO 6888-1 guideline (1999). One milliliter of saline solution from wet wipes, face masks and inner gloves was pre-enriched for an additional 15h in 9ml tryptic soy broth (TSB) (Oxoid) supplemented with 6.5% sodium chloride (NaCl) (Safdar et al. 2003). Thereafter, 100µl aliquots of each enrichment were spread plated in duplicate on the surface of pre-dried Baird-Parker agar plates (Merck). One gram soil samples from areas where soil was ingested and areas where open defecation occurred, were serially diluted with sterile saline (0.85% NaCl) to an appropriate level, and 100µl aliquots of these dilutions were spread plated in duplicate onto pre-dried Baird-Parker agar plates (Merck) to verify the presence of presumptive *Staphylococcus* spp. Colonies that were black or grey in color surrounded by a clear zone with or without the appearance of an opalescent ring, after 24h incubation at 37°C, were considered positive for the presence of presumptive *Staphylococcus* spp.

Chemicals

Unless otherwise stated all chemicals used were of the best quality commercially available.

Results and discussion

Detection of *Staphylococcus* spp. and enumeration of *E. coli* in soil samples

E. coli was not detected in the area where geophagia took place at household D2. However, *E. coli* was present at $\log_{10}$ 2.7 MPN/g in an area at household D3 where geophagia took place. At household D5, both open defecation and geophagia took place and *Staphylococcus* spp. was

present in soil samples from both these areas. The MPN/g values for *E. coli* in soil samples from household D5 were in the same range at $\log_{10}$ 3.7 MPN/g for the open defecation area and $\log_{10}$ 3.3 MPN/g for the area were geophagia took place. These *E. coli* counts obtained for soil samples from areas where geophagia took place fall in the range reported for urban household soil samples in Harare (Zimbabwe), which ranged from 0.89-4.76 CFU/g dry solids (Navab-Daneshmand et al. 2018). While pit latrine fecal sludge analyzed in Haiti contained $\log_{10}$ 6-7 MPN/g of *E. coli* (Berendes et al. 2015), fresh human feces in Sierra Leone contained *E. coli* in the range of $\log_{10}$ 4.38-8.60 MPN/g (Wright 1982). Fecal sludge from a public toilet in a peri-urban area in Ghana contained 3.4×10^6 CFU/100ml *E. coli* (Appiah-Effah et al. 2015) and fecal sludge from pit latrines and urine diverting dry toilets in Malawi contained 0-4100 and 0-5500 *E. coli* CFU/g respectively (Kumwenda et al. 2017).

As expected, the *E. coli* counts obtained for the soil area where open defecation occurred was lower than that obtained for pit latrine fecal sludge and fresh human feces. This could be due to dilution effects due to rain and die off of *E. coli* depending on the quantity of feces contaminating the soil as well as exposure of the feces to various weather conditions affecting survival (ultra-violet light (UV), temperature), predatory grazing and the ability of the *E. coli* strains to adapt and compete with indigenous soil microorganisms for nutrients (Lang et al. 2007; Brooks et al. 2012). However, Stocker et al. (2015) found that initial *E. coli* levels even increased in soil, after dairy cattle manure application, with increasing concentrations of simulated rainfall and the first 1cm layer of soil was found to contain more indicator bacteria than the layers beneath it. Brooks et al. (2012) found that risks from pathogenic *E. coli* decreased overtime after initial application of manure to the soil and the decrease in risk was typically over six orders of magnitude beyond 30 days. Following livestock manure application to soil, Nicholson et al. (2005) found that *E. coli* O157 generally survived for up to one month after application and die-off appeared to be more rapid in sludge applied in winter, with a drop in counts from 1.2×10^5 to <100/g in approximately 5.5 months as a result of factors such as sludge temperature, moisture, pH and microbial competition. Similarly, Lang et al. (2007) found that *E. coli* and *Salmonella* spp. introduced from sewage sludge declined rapidly in soil after application, with survival limited to 3 months, irrespective of the timing of application or the environment.

In light of the above findings, a model calculation for the decay/die-off rates of *E. coli* in soil contaminated with pit latrine fecal sludge at peri-urban households can be done to determine

the possible risk faced by household members who practice geophagia. Assuming an initial concentration of $\log_{10}$ 6-7 MPN/g *E. coli*, established by Berendes et al. (2015) for pit latrine sludge in Haiti, and that workers accidentally sprayed/splashed 10g pit latrine fecal sludge over an area of $1m^2$ during pit emptying, considering a soil depth of 0.05m matching the typical geophagia sampling depth and a consumption of approximately 1g of this soil by household members, a possible risk of intake can be calculated. Using a decay/die-off rate of 4.0-5.0 $\log_{10}$ CFU/g per 50 days employed by Brooks et al. (2012) for *E. coli* O157:H7, possible counts for *E. coli* in soil samples in the current study would be less than 1 CFU/g after ≥50 days exposure of the soil to pit latrine fecal sludge.

The data in the current study indicate that *E. coli* counts in soil samples from areas where geophagia took place are predicted to be lower than 1 CFU/g post 50 days exposure to pit latrine fecal sludge and that initial *E. coli* counts in these areas are similar to previously reported counts in other soil environments with no known input of human feces. However, the presence of *E. coli* and *Staphylococcus* spp. in these soils presents a potential health risk to individuals who practice geophagia as other pathogens might be present in the soil, especially if these strains are present in the viable but not culturable (VBNC) state. Even though *E. coli* is a commensal bacterial species colonizing the gastrointestinal tract of mammals, some pathogenic *E. coli* strains have evolved the ability to induce disease in humans (diarrhea-agenic *E. coli* DEC). In fact, Appiah-Effah et al. (2015) found that *Salmonella* spp. - typical bacterial pathogens - were present at concentrations similar to *E. coli* in fecal sludge from public toilets in rural areas in Ghana. In addition, the time interval between contamination and consumption of the soil may be less than 50 days, with the potential of higher *E. coli* counts present in the soil and some of these *E. coli* strains may be pathogenic. In addition, Brooks et al. (2012) found that the greatest one-time risks were from direct consumption of soil contaminated with fecal matter or exposure to fomites. Moreover, the South African department of health guidelines (2002) for assessing the microbiological safety of ready-to-eat (RTE) foods stipulate the absence of *E. coli* per gram of RTE food products. The *E. coli* counts obtained for areas where geophagia was observed were clearly higher than the above guidelines for RTE food products, indicating that the soil in these areas is clearly not suitable for consumption.

Kumwenda et al. (2017) estimated annual risks for inhalation, ingestion and dermal contact due to exposure to microorganisms in pit latrine fecal sludge. The risk for *E. coli* uptake through inhalation and ingestion was estimated at 6.0×10^{-1} and 5.4×10^{-1} CFUs/g for different toilet

types in Malawi (Kumwenda et al. 2017). In addition, exposure due to inhalation of fecal sludge aerosols while walking outside where humus was stored, where farming or pit emptying activity took place as well as application of fecal sludge in the field was estimated at a maximum of 0.1g fecal sludge material per day for 6 months in a year with an average dose for two different toilet types at 3.0g and 1.8g per year (Kumwenda et al. 2017). Exposure due to accidental ingestion of fecal sludge during pit emptying, transportation, application in the field without PPE, poor hygiene and through water and food contamination was estimated at a maximum of 0.1g per day for 4 days a year with an estimated average dose of 0.2g per year (Kumwenda et al. 2017). Exposure due to dermal contact with fecal sludge as a result of walking without shoes around the household where humus is stored, due pit emptying or transportation of fecal sludge and during application of fecal sludge in the field was estimated at 0.3g maximum per day for 6 months in a year with an average dose for two different toilet types at 18.0g and 10.8g per year (Kumwenda et al. 2017). However, Kumwenda et al. (2017) considered that the greatest risk from exposure to pit latrine fecal sludge was through dermal contact.

Detection of the target bacteria on household surfaces and on municipal workers

The monitoring data across all 10 households for areas sampled before and after pit emptying indicated that the frequency of detection of *E. coli* (B (before)-70%, A (after)-80%) and *Staphylococcus* spp. (B-60%, A-70%) on the walkway between the municipal vehicle and the pit latrine increased to some degree after the pits were emptied and remained the same before and after pit emptying on the pit cover, *E. coli* (B-60%, A-60%) and *Staphylococcus* spp. (B-50%, A-50%) (Table1). On the household tap, the frequency of detectable *E. coli* decreased (B-40%, A-20%) after pit emptying while the frequency of detection of *Staphylococcus* spp. remained the same before and after pit emptying (B-40%, A-40%) (Table 1). The detection frequency of *E. coli* remained the same on the VIP door handle before and after pit emptying (B-20%, A-20%) while the detection frequency of *Staphylococcus* spp. increased after pit emptying (B-30%, A-50%) (Table 1). Overall, 60% of volunteer municipal workers' hands were already contaminated with at least one of the two target bacteria before pit emptying (Table 1). In total, 10% of hands tested positive for *E. coli* before pit emptying and 30% after, while 50% tested positive for *Staphylococcus* spp. before pit emptying and 30% after (Table 1).

Samples were only collected after pit emptying from the face and inner mask (E (*E. coli*)-20%, S (*Staphylococcus* spp.)-50%), outer mask (E-50%, S-50%), outer gloves (E-60%, S-50%) and the bottom of the boot (E-50%, S-50%) of volunteer municipal workers, the lip of the pit latrine (E-50%, S-40%), an area away from pit emptying activity (E-33%, S-44%) and the municipal vehicle door handle (Table 2). However, no samples were collected for the area away from pit emptying activity at household D1 (Table 2) and the municipal vehicle door handle was only sampled at household D10 and both *E. coli* and *Staphylococcus* spp. were absent from this sample. It is also worth noting that *Staphylococcus* spp. was the most frequently detected target group of bacteria in samples across all households, and that household D5 had the highest frequency of detection of both *E. coli* and *Staphylococcus* spp. in all samples collected, either before or after pit emptying. An exception was the VIP door handle, where *Staphylococcus* spp. was absent before and after pit emptying (Table 1 and 2). The possible reason why *Staphylococcus* spp. was the most frequently detected target bacterial representative in samples across households is probably due to the fact that this bacterial genus comprises of resilient biofilm formers (Stepanović et al. 2000), enabling them to survive on household surfaces for extended periods of time (Mack et al. 1996; Foster et al. 2014). Overall, the data indicate that the hands of municipal workers were already frequently contaminated before pit emptying.

The presence of the target bacteria was expected in the following areas as they were potentially exposed to feces or pit latrine fecal sludge: the walkway between the vehicle and pit before and after pit emptying, the pit cover before and after pit emptying, soil areas where open defecation occurred, the bottom of the boot of a volunteer municipal worker after pit emptying and the lip of the pit latrine after pit emptying and the areas where soil was ingested. The detection of target bacteria on surfaces such as the VIP door handle, the household tap handle, the municipal vehicle door handle and the outer gloves and outer masks of volunteer municipal workers after pit emptying, was also not unexpected given that manual pit emptying can cause the spraying or splashing of fecal sludge onto surfaces surrounding households and on the PPE of municipal workers (Jenkins et al. 2015).

Table 1 Detection of *E. coli* and *Staphylococcus* spp. on surfaces at peri-urban households and on municipal workers' hands before and after pit emptying.

Absence/Presence		Households																			
		D1		D2		D3		D4		D5		D6		D7		D8		D9		D10	
Sample Description	**Target Microorganism**	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A
Walkway surfaces between vehicle and VIP	*E. coli*	+	+	+	+	-	+	-	+	+	+	+	+	+	+	-	-	+	-	+	+
	Staphylococcus spp.	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-
Pit cover	*E. coli*	+	+	+	-	-	-	+	-	+	+	-	+	+	+	-	+	-	-	+	+
	Staphylococcus spp.	+	+	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-
Household outside tap handle	*E. coli*	+	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	+
	Staphylococcus spp.	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-
VIP door handle	*E. coli*	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+
	Staphylococcus spp.	-	+	+	+	+	+	-	+	-	-	+	-	-	+	-	-	-	-	-	-
Hands of a volunteer municipal worker (inner hand gloves)	*E. coli*	-	+	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-
	Staphylococcus spp.	+	+	+	+	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-

– (ABSENCE), + (PRESENCE), B = Before, A = After, D1-D10 represent households that were sampled in the peri-urban community

However, the presence of these bacteria on the VIP door handle, the household tap handle, the municipal vehicle door handle and the outer gloves and outer masks of volunteer municipal workers before pit emptying is concerning, as this indicates improper hygiene practices by household members and municipal workers.

McGinnis et al. (2019) sampled surfaces such as the pit latrine slab/cover, spray handle/bucket or tap used for anal cleansing, the pit latrine door handle, the pit latrine wall and the sink handle at the handwashing station, at both community and household pit latrines for total coliforms and *E. coli*. Samples were taken before and after daily cleaning at community pit latrines, while household samples were collected at midday to reflect standard/normal conditions. The pit latrine slab/cover, the spray handle/bucket or tap used for anal cleansing and the door handle to the pit latrine were identified as the surfaces with the highest level of bacterial contamination (McGinnis et al. 2019). However, cleaning practices significantly reduced bacterial contamination on pit latrine surfaces at community pit latrine sites, especially on the pit latrine slab (McGinnis et al. 2019). In the current study, the pit cover was the second most contaminated surface both before and after pit emptying with *E. coli* (B-60%, A-60%), which was similar to the findings reported by McGinnis et al. (2019). However, the door handle to the VIP in the current study had a lower frequency of detection of *E. coli* than the pit cover before and after pit emptying (B-20%, A-20%).

Montangero and Strauss (2004) reported that poor hygiene practices such as a lack of hand washing by municipal workers and community members and improper fecal sludge management were the major causes for the spread of contamination and disease within peri-urban communities. The behavior of municipal workers during pit emptying in the current study was also observed on site and it was found that workers had a tendency of using household taps to wash their soiled hands and they also used household equipment during pit emptying. This behavior could have contributed to the contamination of the household taps with the target bacteria after pit emptying. Similarly, Kumwenda et al. (2016) observed pit emptying activities in Malawi and reported that during emptying, PPE such as gloves and gumboots/shoes were rarely used. It was expected that the face and inner mask and hands of volunteer municipal workers would be free of contamination before and after pit emptying provided that the workers wore their PPE correctly and practiced proper hygiene. The detection of *Staphylococcus* spp. on the hands and face of municipal workers was not unexpected, as representatives of this genus are commonly found on skin surfaces (Kloos and Schleifer 1975).

However, the detection of *E. coli* on the face and hands of workers is concerning as *E. coli* is a common gut commensal and some strains such as STEC are pathogenic and therefore pose a potential health risk (Galane and Le Roux 2001).

The presence of bacterial contamination on surfaces at households even before pit emptying might be due to previous pit emptying activity at these households or poor hygiene practices by household members. Depending on the level of contamination and die-off rates, the target bacteria might survive on inanimate surfaces for extended periods of time (Warnes et al. 2012), potentially resulting in some of the background contamination observed in the present study. Williams et al. (2005) found that *Escherichia coli* O157 from fresh cattle feces persisted for longer periods on moist wood samples under cooler temperatures with a large population present even after 28 days. Factors that affected the survival of *E. coli* O157 on these surfaces were high temperatures and dehydration resulting in a more rapid decline in *E. coli* O157 counts. Similarly, Taylor et al. (2013) demonstrated the survival of *Enterococcus faecalis* and *E. coli* on building materials such as brick, wood, and plaster in a flooded property, with a decline in growth observed only after 4 days.

However, the data in this study indicate that the pit emptying per se caused an increased presence of detectable target bacteria on surfaces at households after pit emptying. For example, the frequency of detection of *Staphylococcus* spp. on the VIP door handle and the frequency of detection of both target bacteria on the walkway between the municipal vehicle and the pit latrine increased after pit emptying.

Contamination due to defecating animals or similar events causing the transfer of fecal matter - albeit not documented in this study - are other possible reasons for the background contamination observed on surfaces outside households. The results obtained indicate that the workers or more specifically their PPE and equipment are regularly contaminated due to their exposure to fecal pit latrine sludge, a lack of cleaning after previous pit emptying activities or due to cross contamination from contaminated to clean PPE and equipment. Finally, the occasional detection of the target bacteria before and not after pit emptying, for example the presence of *Staphylococcus* spp. on the VIP door handle before pit emptying but not after (Table 1, D6), are unexpected. These surfaces could have been wiped down before pit emptying, thereby removing previous contamination and might have not been used during or after pit emptying, resulting in the absence of the target bacteria.

Table 2 Detection of *E. coli* and *Staphylococcus* spp. on surfaces at peri-urban households and on municipal workers' PPE after pit emptying.

Absence/Presence		**Households**									
		D1	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**
Sample Description	**Target Microorganism**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
Face and inner mask of a volunteer municipal worker	*E. coli*	-	-	-	-	+	-	-	-	-	+
	Staphylococcus spp.	-	+	+	+	+	+	-	-	-	-
Outer mask of a volunteer municipal worker	*E. coli*	+	-	-	-	+	+	+	+	-	-
	Staphylococcus spp.	+	-	+	-	+	-	+	-	+	-
Outer gloves of a volunteer municipal worker	*E. coli*	-	+	-	-	+	+	-	+	+	+
	Staphylococcus spp.	+	-	-	+	+	-	+	-	+	-
Bottom of boot of a volunteer municipal worker	*E. coli*	-	-	-	-	+	+	+	-	+	+
	Staphylococcus spp.	+	+	-	+	+	-	+	-	-	-
Lip of latrine	*E. coli*	-	-	-	+	+	+	-	+	-	+
	Staphylococcus spp.	+	-	+	-	+	+	-	-	-	-
Area away from pit emptying activity	*E. coli*	NS	-	-	-	+	-	+	+	-	-
	Staphylococcus spp.	NS	+	+	-	+	-	+	-	-	-
– (ABSENCE), + (PRESENCE), A = After, NS = Not sampled, D1-D10 represent households that were sampled in the peri-urban community											

In addition, cases where the target bacteria are noted as absent might be due to the true absence of these bacteria in samples, their presence at levels below the detection limit or the presence of viable but not culturable (VBNC) bacteria.

Unhygienic behavior/practices at peri-urban households

In the current study, both geophagia and open defecation were observed at household D5 and only geophagia was observed at households D2 and D3. According to the world health organization (WHO) sanitation fact sheet (2018), approximately 15% of the world's population still practice open defecation. As open defecation still takes place in South Africa, geophagia poses a serious health risk to community members, as some microorganism present in this fecal contaminated soil may be pathogenic. According to Montangero and Strauss (2004) the time delays between pit emptying can increase the spread of contamination and the risk of disease in peri-urban communities. In the current study, the pit latrines analyzed are emptied on a 5-year cycle, which may cause household members to resort to open defecation when pit latrines become full. In addition to open defecation, households can also resort to shared sanitation, which can result in adverse health outcomes such as diarrhea, helminth infections, enteric fevers, other fecal-oral diseases, trachoma and adverse maternal or birth outcomes (Heijnen et al. 2014). Additional problems associated with full pits include overflowing of pit latrine contents into houses, communities and the water table especially during the rainy season and increased exposure to vectors such as flies and often insects, which can transmit disease (Jenkins et al. 2015).

Previous studies have reported that gastrointestinal and respiratory tract infections are common among employees at sewage treatment plants and endotoxins in Gram negative bacteria have been implicated as one of the possible causative agents for these infections (Thorn and Kerekes 2001). The mere presence of pit latrines at households is a risk as pathogenic bacteria can even be inhaled through bioaerosols, posing a greater risk to pit emptiers than to household members (Farling et al. 2019). As highlighted by Arthurson (2008), human derived biosolids contain a variety of pathogenic bacteria and the majority of standard fecal sludge stabilization or treatment procedures do not effectively reduce the concentrations of these pathogens. Therefore, sanitization procedures with greater efficiency of pathogen elimination need to be applied to pit latrine fecal sludge before it can be handled by municipal workers or disposed of in land-fill sites.

Limitations of this study

Although basic training was given to the sample collectors, sampling variation and inaccuracies given the limited experience of the sample collectors were unavoidable. In addition, repeated delays to the start of the pit emptying program compromised the ability to collect surface samples from households over 2-3 pit emptying cycles, resulting in the lack of previous pit emptying data for comparison. Overall, despite the limitations and the difficulty in carrying out this study, the data obtained form a baseline for comparison in similar future studies, as it is the first study of its kind investigating the presence of bacterial contamination on household surfaces in a peri-urban community, in KwaZulu-Natal South Africa, where pit latrines are used as a basic means of sanitation and manual pit emptying is employed.

Conclusion

The data in this study indicate that the municipal workers possibly did not wear their PPE correctly and might have contaminated surfaces at households during pit emptying and other related activities. This was evident from the detection of the target bacteria on the hands, face, and PPE of municipal workers before pit emptying and on selected household surfaces i.e., the VIP door handle and the handle of a tap outside the household after pit emptying. While providing a basic, affordable means of sanitation to peri-urban communities, pit latrines can also present a potential health risk as manual pit emptying can introduce fecal contamination on surfaces at households causing the exposure of municipal workers, household members and the environment to potential pathogens. This study highlights an urgent need for education for both municipal workers and household members on personal hygiene, with emphasis on proper handwashing and cleaning of PPE, given that manual pit latrine emptying is taking place. In addition, municipal workers must be trained on the hygienic and safe emptying of pit latrines and the efficient and safe handling of pit latrine fecal sludge, in order to reduce the risk of contamination and the spread of disease within peri-urban communities.

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Chapter 3

A bacterial diversity snapshot of pit latrine fecal sludge from a peri-urban community in KwaZulu-Natal (South Africa)

Abstract

As data on the bacterial diversity of pit latrine fecal sludge are scarce, the current study employed a commercial next generation sequencing (NGS) kit to determine the bacterial diversity present in pit latrine fecal sludge from a peri-urban community in KwaZulu-Natal, South Africa. The metagenome rapid annotation subsystem technology (MG-RAST) was used to analyze the taxonomic abundance of bacteria in the pit latrine fecal sludge based on short sequence read libraries. Seventeen percent of the sequences in the pit latrine microbiome were identified as unclassified bacteria and the five most abundant bacterial genera were identified as *Pseudomonas*, *Bacillus*, *Escherichia*, *Candidatus Aquiluna* and *Candidatus Rhodoluna*. Furthermore, the genus *Staphylococcus* was among the genera representing <1% of the bacteria in the pit latrine fecal sludge. Overall, the pit latrine fecal sludge microbiome showed similarities to human and latrine fecal microbiomes from non-westernized societies at the phylum level, but differed at the lower taxonomic levels.

Keywords: Pit latrines, fecal sludge, next generation sequencing, Illumina MiSeq sequencing, MG-RAST

Introduction

The microbial community of pit latrines is a combination of microorganisms from human feces, household and animal waste and from the surrounding soil and groundwater (McLellan et al. 2010). Strictly anaerobic bacteria from the phyla *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, including uncultured microorganisms inhabit the guts of humans (Arumugam et al. 2011). Soil contains mainly members of the phyla *Proteobacteria* and *Acidobacteria* and together with other phyla such as *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi*, *Planctomycetes*, *Gemmatimonadetes* and *Firmicutes*, they make up approximately 92% of the soil bacteria (Janssen 2006). However, a wide range of heterotrophic *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* dominate groundwater ecosystems (Griebler and Lueders 2009).

The gastrointestinal tract (GI) harbors a vibrant population of microorganisms called the gut microbiota (GM) (Thursby and Juge 2017). The GM is a combination of bacteria, viruses, archaea and eukarya, which have co-evolved with the host forming an intricate and mutually beneficial relationship (Backhed 2005). These microorganisms vary in abundance and complexity between the different topographical sites of the bowel (Zoetendal et al. 2006) and between individuals and societies e.g. westernized versus non-industrialized societies (De Filippo et al. 2010; Yatsunenko et al. 2012). The GM is also known to contribute to non-communicable diseases and colon cancer (Koeth et al. 2013; Trompette et al. 2014). Westernized societies have a higher prevalence of colon cancer whereas non-westernized societies have a higher incidence of infectious diseases, including severe diarrhea (Pop et al. 2014). The host immune system and diet are the main drivers in shaping the GM throughout an individual's lifetime (Albenberg and Wu 2014; Belkaid and Hand 2014). Additionally, several environmental factors such as geographic location, surgery, smoking, depression and living arrangements (urban or rural) have an influence on the GM composition (Biedermann et al. 2013; Tyakht et al. 2013; Jiang et al. 2015). Lifestyle activities such as vaginal birth versus caesarian sections, antibiotic use and formula- versus breast-fed infants may also influence the GM assembly (Risnes et al. 2011; Conradi et al. 2013). An altered gut microbial composition (dysbiosis) has been correlated with several human diseases (Wu et al. 2013; D'Argenio et al. 2013).

Culture based techniques used for the detection of specific microorganisms are often laborious and may fail to detect microorganisms with complex nutritional and host specific requirements (Evangelista and Coburn 2010), including those which exist in a dormant but potentially infective state i.e., the viable but non culturable (VBNC) state (Ramamurthy et al. 2014). To overcome these problems, cultivation-independent molecular methods were developed e.g. 16S ribosomal RNA gene (rRNA) next-generation sequencing (NGS). This approach allows for the high throughput microbial identification of the majority of microorganisms present in a specific environment (Kim et al. 2013). Nevertheless, there are potential disadvantages to this approach such as PCR bias if employed, extraction bias, 16S rRNA gene copy number variation, naked or free DNA may also be detected and quantification of pathogenic bacteria based on biomarker DNA is unreliable and thresholds of "acceptable risk" need to be defined by risk assessment frameworks (Tan et al. 2015). Moreover, Plummer et al. (2015) found that 16S rRNA gene sequencing of short reads, due to nearly identical 16S rRNA gene sequences, cannot reliably differentiate between closely affiliated bacterial species at the genus and species level. Despite

the improvement of metagenomics in the understanding of the relationship between the human gut microbiome, health and diseases, the NGS based analysis has also generated a large number of unidentified sequences that may be correlated with microorganisms of known identity (Lagier et al. 2012b; Rinke et al. 2013).

To overcome the problems of culture-based and culture-independent molecular techniques, the microbial culturomics approach has recently been introduced. This approach combines multiple comprehensive culture conditions (different atmospheres, temperatures, pH, nutrients, minerals, antibiotics or phages), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry and 16S rRNA gene sequencing for the study of the human microbiota (Lagier et al. 2012a; Lagier et al. 2015). Culturomics has allowed for the culturing of microorganisms, which have not previously been assigned to known genera or species (Lagier et al. 2016). To date, culturomics have detected 247 new species and their genomes, including 174 species never mentioned in the human gut and 31 new species and genera, which have had their genomes sequenced (Lagier et al. 2012a). Interestingly, culturomics also broke records for the identification of the largest human-isolated bacterium and virus (Lagier et al. 2012a). Overall, the culturomics approach demonstrates that the microbial abundance in the human gut is greater than that estimated by genomic and metagenomic analyses (Fournier et al. 2007; Arumugam et al. 2011).

Depending on the choice of NGS platform, sequencing results may vary in terms of resolution and quantitative power as a result of differential sequence throughput and coverage. The Illumina MiSeq platform is a high-throughput, streamlined sequencing platform, which is able to determine the microbial community composition in clinical and environmental samples (Fadrosh et al. 2014; Goodwin et al. 2016). MiSeq's most popular sequencing approaches include a single- (Caporaso et al. 2012) or the lately established dual-indexing (Kozich et al. 2013) technique which targets the hypervariable V4 area of the 16S rRNA gene. The Illumina MiSeq platform has shown improved performance in terms of sequencing depth, coverage, and detection sensitivity (Sinclair et al. 2015).

Several open-source bioinformatics software packages exist to analyze paired-end sequence data generated from the Illumina MiSeq platform. For the purpose of the current study, the MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST) was used. The MG-RAST server is an open source platform for comparative genomics based on the SEED

framework (Overbeek et al. 2005; McNeil et al. 2007). Unlike some bioinformatics software packages, this server does not need a sophisticated computer to process various samples and acts as a public database for 16S rRNA gene and metagenomic shotgun datasets, allowing comparison and investigation of other publicly accessible datasets (Plummer et al. 2015). In addition, MG-RAST is useful for first-time users who are unfamiliar with metagenomic analysis output (D'Argenio et al. 2014) and contains highly robust standard operating procedures (SOPs) as well as affordable computational practices (Wilke et al. 2016). In the current study, NGS analysis of a pit latrine fecal sludge sample was done using a commercially available NGS kit. The Illumina MiSeq sequencing platform together with the MG-RAST bioinformatics pipeline were used to determine the bacterial diversity in pit latrine fecal sludge from a peri-urban community in KwaZulu-Natal South Africa.

Materials and methods

Sample collection

A pooled pit latrine fecal sludge sample was collected from a central storage point containing pit latrine fecal sludge from ventilated improved pit latrines (VIP's) in a peri-urban community (KwaMashu) in the eThekwini municipality (KwaZulu-Natal, South Africa).

16S rRNA gene sequencing

The uBiome gut sampling kit (San Francisco, USA) (Almonacid et al. 2017) was used to generate 4 paired-end read libraries using the Illumina MiSeq instrument. Thereafter, the Fast Q sequences generated by uBiome were analyzed using MG-RAST.
(http://metagenomics.anl.gov/index.htmlversion 4.0.3)

Metagenomic analysis

The 4 paired-end read libraries obtained from uBiome were submitted to MG-RAST (Meyer et al. 2008) for analysis of the taxonomic abundance under the project name: Pit latrine LSB and project ID: mgp20673. The choices for the MG-RAST pipeline used in this study were as follows: artificial replication reads were removed and host contamination in reads was screened using Homo sapiens as a reference in the NCBI v36 database. The Silva SSU database (Quast et al. 2013) and a 95% identity cut-off was used to cluster reads for taxonomic classification. The parameters used for the analysis were an e-value of $1e^{-5}$, sequence length of 50 and a minimum abundance of 10. In addition, a taxonomic classification was provided to reads using

the MG-RAST 'Best Hit Classification' option, which reports the top scoring annotation(s) for each read.

Results and discussion

A total of 87% of bacterial families had an abundance of ≤1% (Table 1). The rarefaction curve indicated that the number of short sequence reads analyzed partially covered the diversity of bacteria present in the pit latrine fecal sludge microbiome (Fig. 1). Similarly, in a study based on the microbial diversity in Tanzanian and Vietnamese pit latrines, Torondel et al. (2016) observed that the rarefaction curves had not reached saturation. The 5 most abundant phyla detected in the pit latrine fecal sludge were identified as *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Verrucomicrobia* (Fig. 2), the five most abundant bacterial genera were identified as *Pseudomonas, Bacillus, Escherichia, Candidatus Aquiluna and Candidatus Rhodoluna* (Fig. 3) and the 5 most abundant bacterial species were identified as *Bacillus subtilis*, *Pseudomonas stutzeri*, *Escherichia coli*, *Pseudomonas putida* and *Candidatus Aquiluna rubra* (Fig. 4). In addition, *Staphylococcus* was among the genera representing <1% of the bacteria and the only species represented by this genus was *Staphylococcus epidermidis*. The genus *Escherichia* was made up of 2 species, *Escherichia albertii* at 0.7% and *Escherichia coli* at an abundance of 10% in the pit latrine microbiome.

When feces leave the gut, the survival of microbes therein relies on their capability to react to environmental stress. This involves adaptations to varying nutrient availability such as low iron, variation in atmospheric oxygen, and the capacity to survive by altering their morphological characteristics such as spore formation (Leffler and Lamont 2012). The pit latrine fecal sludge at the central storage point might have been exposed to environmental elements such as rain and direct sunlight, which could have affected the microbial diversity and abundance of microorganisms such as *E. coli* and *Staphylococcus* spp. within fecal sludge samples. However, the NGS approach has a much lower detection sensitivity than the culturomics approach (Lagier et al. 2015; Lagier et al. 2016). As pit latrines receive in addition to human feces materials such as refuse (Bakare et al. 2012; Brouckaert et al. 2013), the pit latrine fecal sludge microbiome was compared to microbiomes from various environments and to human gut microbial community profiles from different geographical locations.

Metagenomic sequencing has shown that bacteria make up the vast majority of the human gut microbiota, with *Bacteroidetes* and *Firmicutes* as the two main phyla (Fernandes et al. 2014;

Jandhyala et al. 2015). Similarly, Newton et al. (2015) found that human-fecal microbiomes, detected in sewage treatment plants in the USA, were predominantly represented by the phyla *Firmicutes*, *Bacteroidetes* and to a lesser extent by other mostly anaerobic microbes, e.g., *Bifidobacteriaceae* and *Coriobacteriaceae*. At the genus level, Tap et al. (2009) identified *Faecalibacterium*, *Ruminococcus*, *Eubacterium*, *Dorea*, *Bacteroides*, *Alistipes* and *Bifidobacterium* as the dominant genera present in human fecal samples.

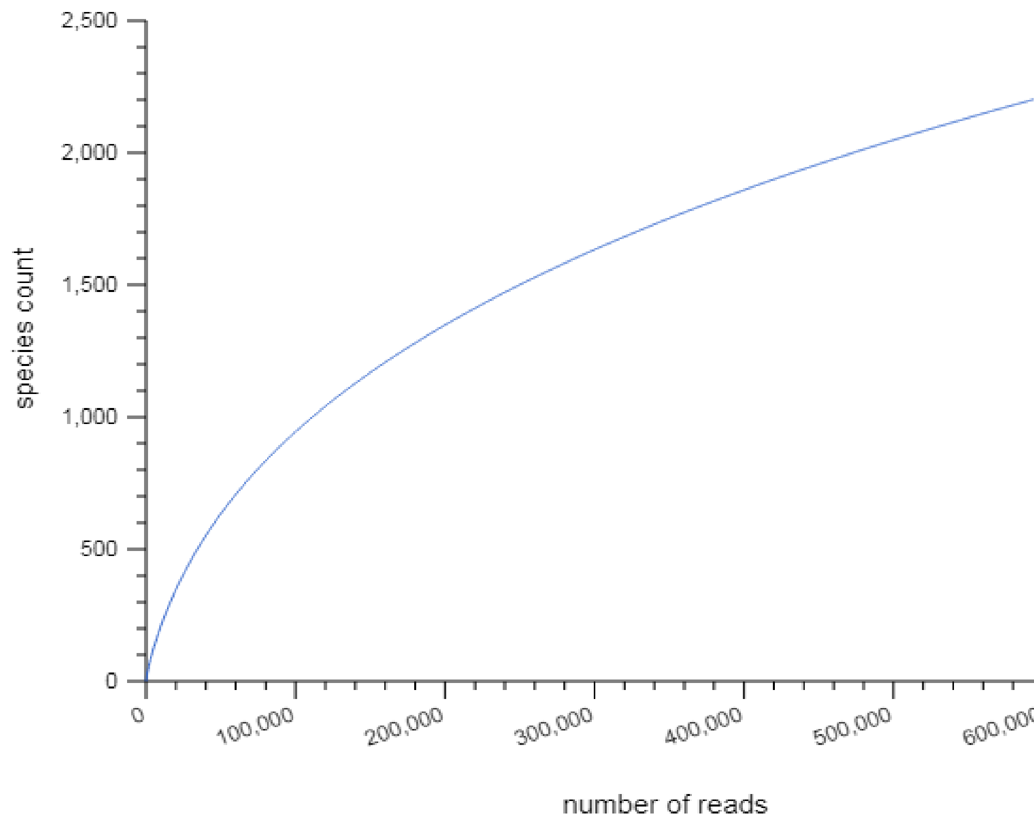

Figure 1 Rarefaction curve showing the relationship between the number of short sequence reads generated and the number of species detected, computed using MG-RAST.

In the current study, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Verrucomicrobia* were the most abundant phyla in the pit latrine microbiome (Fig. 2), which was similar to the findings of Jandhyala et al. (2015) and Newton et al. (2015) as *Firmicutes* and *Bacteroidetes* were among the 5 most abundant phyla in the pit latrine fecal sludge microbiome. Lastly, at the genus level, *Pseudomonas*, *Bacillus*, *Escherichia*, *Candidatus Aquiluna*, *Candidatus Rhodoluna*, *Flavobacterium*, *Clostridium,* and *Cytophaga* were among the dominant bacterial genera present in the pit latrine microbiome (Fig. 3), which was different

from the findings by Tap et al. (2009) as none of the most abundant genera found in human fecal samples was present among the 10 most abundant bacterial genera in the pit latrine fecal sludge microbiome.

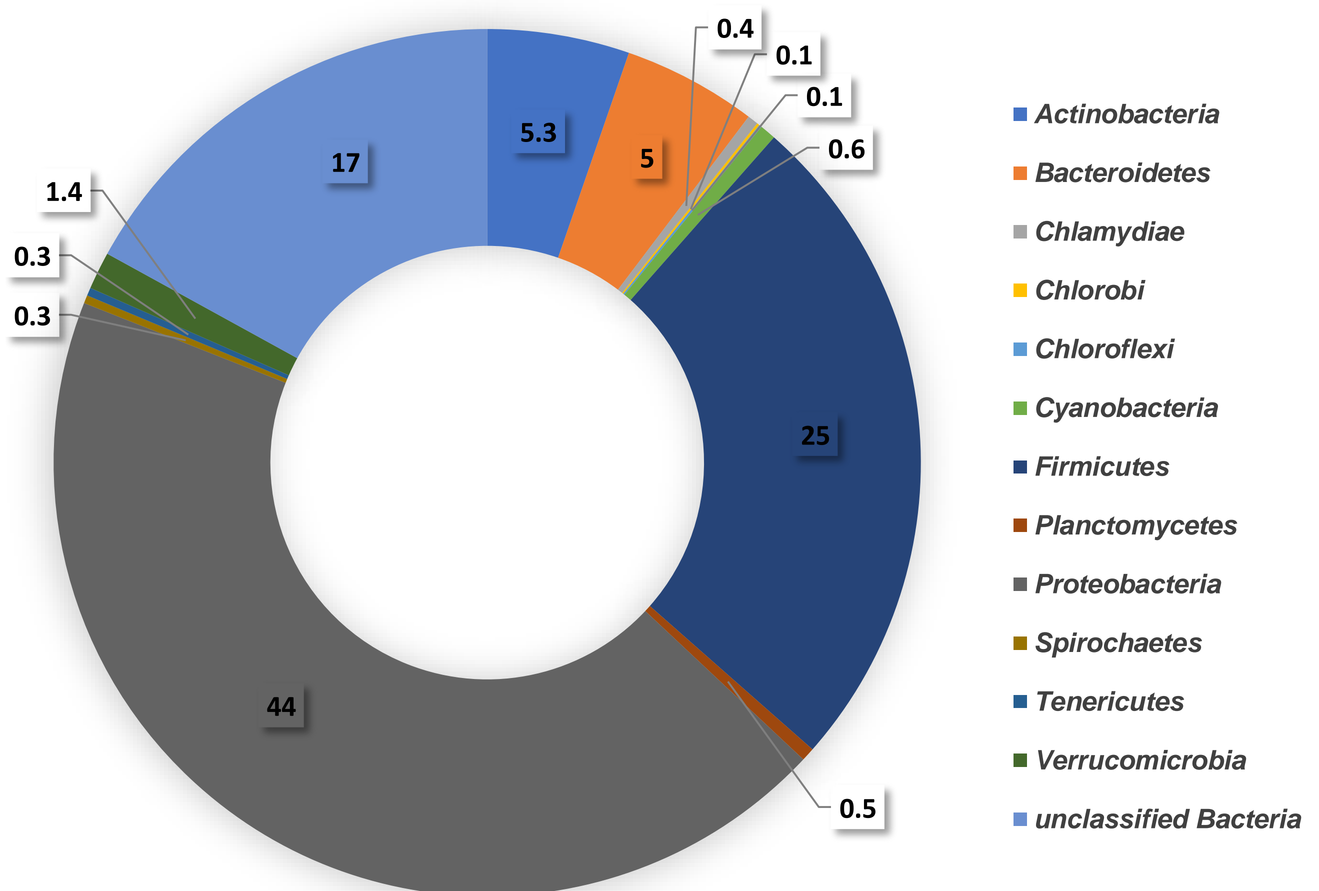

Figure 2 Abundance of bacterial phyla in the pit latrine fecal sludge microbiome. Data shown in the chart are percentage values of the total reads.

Pit latrines can also contain infant feces, which can affect the microbial diversity in pit latrine fecal sludge. In infants that are breast-fed, a common practice in peri-urban communities, a higher abundance of *Bifidobacterium longum* in the gut is typical as this bacterial species is able to utilize fucosylated oligosaccharides present in human milk and thus outcompetes other bacteria such as *E. coli* and *Clostridium perfringens* (Yu et al. 2013). The opposite is true for formula-fed infants who have an increased bacterial diversity and altered levels of *E. coli*, *Clostridium difficile*, *Bacteroides fragilis* and lactobacilli (Penders et al. 2006; Bezirtzoglou et al. 2011). Children from rural areas in Africa have a diet rich in starch, fiber, and plant polysaccharides and their gut microbiota is abundant in *Actinobacteria* and *Bacteroidetes* (De

Filippo et al. 2010). The abundance of these groups is decreased in European children whose diet is rich in sugar, starch, and animal protein (De Filippo et al. 2010). De Filippo et al. (2010) also discovered that some producers of short chain fatty acids (SCFA) like *Prevotella* were exclusive to the gut microbiota of African children. However, the microbial profile of the pit latrine fecal sludge did not match the findings of Yu et al. (2013) for breast-fed infants as the genus *Bifidobacterium* and the SCFA producer *Prevotella*, which was found to be exclusive in the microbiota of African children (De Filippo et al. 2010), was absent from the pit latrine microbiome (Fig. 3). Nevertheless, the high abundance of the phyla *Actinobacteria* and *Bacteroidetes* in the pit latrine fecal sludge microbiome (Fig. 2) was similar to the findings by De Filippo et al. (2010) for feces of African children with a diet dominated by starch, fibre, and plant polysaccharides.

Ou et al. (2013) described the difference in microorganisms and their metabolites in colons of African Americans with an elevated threat of colon cancer and rural Africans living outside the city of Empangeni in South Africa's KwaZulu-Natal province, with a low threat of colon cancer. Overall, the fecal microbial composition between rural South Africans and African Americans was very different. Total bacteria, *Succinivibrio* and oscillospira-microbes, such as *Prevotella*, which are potentially involved in starch, hemicellulose and xylan degradation (Walker et al. 2011) and short-chain fatty acid production were higher in abundance in native Africans. The findings by Ou et al. (2013) were similar to those by De Filippo et al. (2010) for central African children from Burkina Faso, showing a high abundance of *Prevotella* and other microbes likely involved in starch and cellulose degradation. The 'traditional African diet' is rich in coarse grains and vegetables, and correlated with a low threat of gastric diseases and colon cancer, according to Burkitt (1973). A greater abundance of potentially pathogenic *Proteobacteria* (*Escherichia* and *Acinetobacter*), *Bacteroides*, *Lactobacillus* spp., and elevated levels of secondary fecal bile acids defined the American gut microbiota (Ou et al. 2013). Interestingly, Ou et al. (2013) found that the American gut microbiota were higher in diversity than the African gut microbiota, which could reflect the consumption of a more diversified diet. The pit latrine microbiome in the current study was different to the gut profiles of individuals from Empangeni in the KwaZulu-Natal province of South Africa, as it did not contain the genus *Prevotella*. However, it did contain a high abundance of the genus *Escherichia* (Fig. 3), which was similar to the gut profiles of African Americans. The absence of *Prevotella* spp. could be due to the true absence of this genus from the guts of community members in KwaMashu, KwaZulu-Natal, South Africa. However, the current study is limited

as only one pooled sample was collected from a central fecal sludge storage site. More samples need to be analyzed to compare microbial communities between pit latrines from different peri-urban communities and over different seasons to rule out the absence of *Prevotella spp.* in pit latrine samples from KwaMashu, KwaZulu-Natal, South Africa.

Schnorr et al. (2014) demonstrated that the guts of Hadza of Tanzania in Africa have a higher level of microbial richness and biodiversity than the guts of Italians. While both the Hadza and Italian bacterial gut communities were largely dominated by *Bacteroidetes* and *Firmicutes*, the Hadza were characterized by a higher abundance of *Bacteroidetes* and a lower abundance of *Firmicutes*.

Table 1 Bacterial abundance at class, order and family level in the pit latrine fecal sludge microbiome.

Domain	Class	Order	Family	%
Bacteria	*Actinobacteria*	*Actinomycetales*	*Actinomycetaceae*	<1
Bacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	<1
Bacteria	*Alphaproteobacteria*	*Rickettsiales*	*Anaplasmataceae*	<1
Bacteria	*Bacilli*	*Bacillales*	*Bacillaceae*	22
Bacteria	*Deltaproteobacteria*	*Bdellovibrionales*	*Bacteriovoracaceae*	<1
Bacteria	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	<1
Bacteria	*Deltaproteobacteria*	*Bdellovibrionales*	*Bdellovibrionaceae*	<1
Bacteria	*Spirochaetes*	*Spirochaetales*	*Brachyspiraceae*	<1
Bacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	<1
Bacteria	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	<1
Bacteria	*Chlamydiae*	*Chlamydiales*	*Chlamydiaceae*	<1
Bacteria	*Chlorobia*	*Chlorobiales*	*Chlorobiaceae*	<1
Bacteria	*Clostridia*	*Clostridiales*	*Clostridiaceae*	1.4
Bacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	2.5
Bacteria	*Actinobacteria*	*Solirubrobacterales*	*Conexibacteraceae*	<1
Bacteria	*Cytophagia*	*Cytophagales*	*Cytophagaceae*	1
Bacteria	*Actinobacteria*	*Actinomycetales*	*Dermacoccaceae*	<1
Bacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobulbaceae*	<1
Bacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	11.5

Domain	Class	Order	Family	%
Bacteria	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	2.7
Bacteria	*Actinobacteria*	*Actinomycetales*	*Intrasporangiaceae*	<1
Bacteria	*Gammaproteobacteria*	*Legionellales*	*Legionellaceae*	<1
Bacteria	*Verrucomicrobia*	*Methylacidiphilales*	*Methylacidiphilaceae*	<1
Bacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	<1
Bacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	3.8
Bacteria	*Actinobacteria*	*Actinomycetales*	*Micrococcaceae*	<1
Bacteria	*Actinobacteria*	*Actinomycetales*	*Mycobacteriaceae*	<1
Bacteria	*Mollicutes*	*Mycoplasmatales*	*Mycoplasmataceae*	<1
Bacteria	*Betaproteobacteria*	*Neisseriales*	*Neisseriaceae*	<1
Bacteria	*Cyanobacteria*	*Nostocales*	*Nostocaceae*	<1
Bacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	<1
Bacteria	*Bacilli*	*Bacillales*	*Paenibacillaceae*	<1
Bacteria	*Chlamydiae*	*Chlamydiales*	*Parachlamydiaceae*	<1
Bacteria	*Clostridia*	*Clostridiales*	*Peptococcaceae*	<1
Bacteria	*Gammaproteobacteria*	*Thiotrichales*	*Piscirickettsiaceae*	<1
Bacteria	*Planctomycetacia*	*Planctomycetales*	*Planctomycetaceae*	<1
Bacteria	*Deltaproteobacteria*	*Myxococcales*	*Polyangiaceae*	<1
Bacteria	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	<1
Bacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	25
Bacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	<1
Bacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	<1
Bacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	<1
Bacteria	*Sphingobacteria*	*Sphingobacteriales*	*Rhodothermaceae*	<1
Bacteria	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	<1
Bacteria	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriaceae*	<1
Bacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	<1
Bacteria	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	<1
Bacteria	*Bacilli*	*Bacillales*	*Staphylococcaceae*	<1
Bacteria	*Actinobacteria*	*Actinomycetales*	*Streptomycetaceae*	<1
Bacteria	*Thermomicrobia*	*Thermomicrobiales*	*Thermomicrobiaceae*	<1

Domain	Class	Order	Family	%
Bacteria	*Gammaproteobacteria*	*Thiotrichales*	*Thiotrichaceae*	<1
Bacteria	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	1.2
Bacteria	*Alphaproteobacteria*	*Alphaproteobacteria* (UC)	*Alphaproteobacteria* (UC)	<1
Bacteria	*Bacteria* (UC)	*Bacteria* (UC)	*Bacteria* (UC)	17
Bacteria	*Betaproteobacteria*	*Betaproteobacteria* (UC)	*Betaproteobacteria* (UC)	<1
Bacteria	*Betaproteobacteria*	*Burkholderiales*	unclassified *Burkholderiales*	<1
Bacteria	*Deltaproteobacteria*	*Deltaproteobacteria* (UC)	*Deltaproteobacteria* (UC)	<1
Bacteria	*Epsilonproteobacteria*	*Epsilonproteobacteria* (UC)	*Epsilonproteobacteria* (UC)	<1
Bacteria	*Gammaproteobacteria*	*Gammaproteobacteria* (UC)	*Gammaproteobacteria* (UC)	<1
Bacteria	*Spartobacteria*	*Spartobacteria* (UC)	*Spartobacteria* (UC)	<1
Bacteria	*Sphingobacteria*	*Sphingobacteriales*	*Sphingobacteriales* (UC)	<1

UC - unclassified
% values are of the total reads

Other represented phyla were the *Proteobacteria* and *Spirochaetes*, which were more abundant in the Hadza and rare in the Italian GM (Turroni et al. 2009). Furthermore, the *Actinobacteria* class was almost entirely absent from the microbiome of the Hadza. *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Clostridiales Incertae Sedis* XIV, *Succinivibrionaceae*, *Spirochetaceae* and *Eubacteriaceae* were the most common families in the Hadza GM and the most common genera were identified as *Prevotella*, *Eubacterium*, *Oscillibacter* and *Butyricicoccus*, *Sporobacter*, *Succinivibrio* and *Treponema* (Turroni et al. 2009). The lack of *Actinobacteria*, especially *Bifidobacterium* in the Hadza GM was unexpected as *Bifidobacteria* are common in the guts of infants that are breast-fed, constituting a large majority of the GM in the first few months after birth (Turroni et al. 2009). *Bifidobacteria* make up 1-10% of the adult GM population and their absence may result from the post-weaning GM structure in the absence of agro-pastoral-derived foods (Schnorr et al. 2014). A similar observation was made by Zimmer et al. (2012) for vegans and Koreans, whose consumption of meat and/or dairy is low or absent.

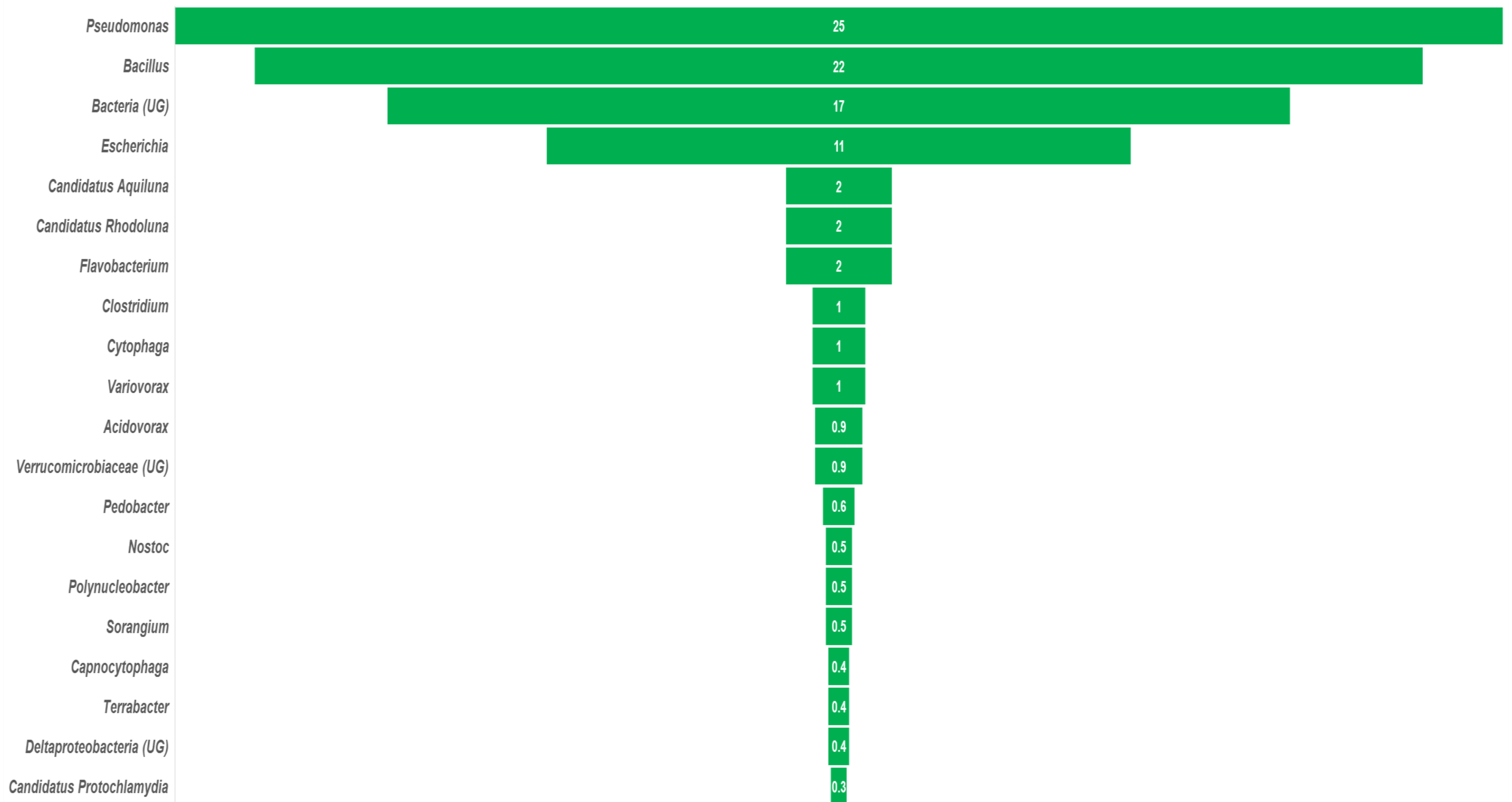

Figure 3 The twenty most abundant bacterial genera present in the pit latrine fecal sludge microbiome. Data shown in the chart are percentage values of the total reads. UG - unclassified genus.

Overall, the depletion in *Bifidobacterium* and enrichment in *Bacteroidetes* and *Prevotella* might enhance the Hadza's ability to digest and extract nutrients from fibrous plant foods (Flint et al. 2008). The results in the current study are similar to the findings of Schnorr et al. (2014) for the Hadza GM at the phylum level, as the pit latrine microbiome contained *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Fig. 2). However, the pit latrine microbiome contained a higher abundance of *Firmicutes* and a lower abundance of *Bacteroidetes*. At the family level, the pit latrine microbiome was abundant in *Pseudomonadaceae*, *Enterobacteriaceae*, *Microbacteriaceae*, *Flavobacteriaceae* and *Comamonadaceae* (Table 1), which was different to the Hadza GM. Another difference between the Hadza GM and the pit latrine microbiome is the lack of *Actinobacteria* in the Hadza GM, which was present in the pit latrine microbiome and unlike the Hadza GM and other African populations (De Filippo et al. 2010; Yatsunenko et al. 2012; Ou et al. 2013), the pit latrine microbiome lacked *Prevotella*, *Succinivibrio* and *Treponema*.

Torondel et al. (2016) used pyrosequencing of 16S rRNA genes to study bacterial diversity and structure in 30 latrines in Tanzania and Vietnam and linked them with a number of intrinsic environmental variables like pH, temperature, organic matter composition, and geographical factors. These authors found that the diversity of bacteria in individual latrine samples was higher when compared with human gut samples (Turnbaugh et al. 2010) and when comparing latrines from Vietnam and Tanzania, they found a lower microbial abundance in Tanzanian latrines. Overall, the Vietnamese latrines were largely represented by *Proteobacteria*, *Actinobacteria*, *Deinococcus-Thermus* and *Verrucomicrobia*, while the Tanzanian latrines were largely represented by *Firmicutes*, *Synergistetes* and *Spirochaetes*. The most abundant phyla in order of decreasing abundance were the *Firmicutes*, followed by the *Bacteroidetes*, *Proteobacteria* and *Actinobacteria*, comprising a total of 37% and 66% of the bacteria in Vietnamese and Tanzanian latrines respectively. In the Tanzanian latrines, *Synergistaceae* and the *Firmicutes* lines such as *Clostridiaceae*, *Ruminococcaceae*, *Incertae Sedis* XI and *Erysipelotrichaceae* were abundant, while in the Vietnamese latrines, *Xanthomonadaceae*, *Actinomycetales*, *Flavobacteriaceae* and *Trueperaceae* were in abundance (Torondel et al. 2016). In addition, Torondel et al. (2016) found that latrine identity and to a lesser extent the country where the latrine is located, had a major influence on the bacterial community structure within latrine samples at all taxonomic levels.

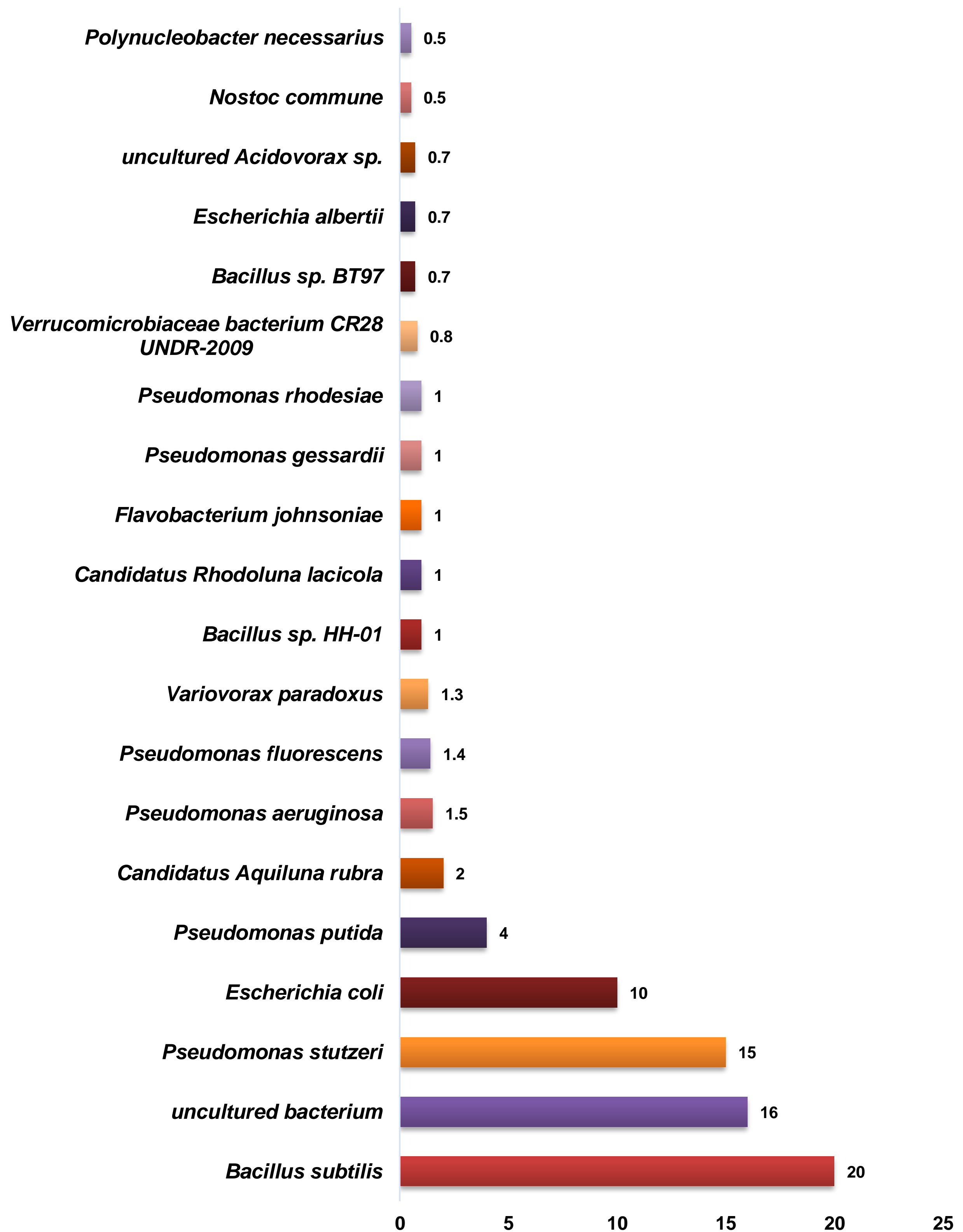

Figure 4 The twenty most abundant bacterial species present in the pit latrine fecal sludge microbiome. Data shown in the chart are percentage values of the total reads.

At the phylum level, the pit latrine contents in the current study were most abundant in *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Verrucomicrobia* (Fig. 2), which partly matched both Vietnamese and Tanzanian latrines. However, at the family level, there was a high abundance of *Pseudomonadaceae*, *Enterobacteriaceae*, *Microbacteriaceae*, *Flavobacteriaceae,* and *Comamonadaceae* in the KwaMashu pit latrine microbiome (Table 1), which was different to the results obtained by Torondel et al. (2016) for Tanzanian and Vietnamese latrines.

The bacterial populations present in full-scale wastewater treatment plants (WWTP) have been previously studied and the phylum *Proteobacteria* was found to dominate with *Betaproteobacteria* as the most abundant class, followed by the phyla *Bacteroidetes*, *Acidobacteria* and *Chloroflexi* (Nielsen et al. 2010; Nguyen et al. 2011; Wan et al. 2011; Hu et al. 2012; Wang et al. 2012). *Hyphomicrobium*, *Trichococcus*, *Tetrasphaera*, *Rhodoferax*, *Rhodobacter*, *Candidatus Microthrix*, p-55-a5 (*Firmicutes*) and P2CN44 and B45 (*Chloroflexi*) were the most abundant bacterial genera across 20 WWTPs (McIllory et al. 2015). Zhang et al. (2012) discovered that the composition of species showed geographic variation, although all WWTPs contained microbial genera *Tricoccus*, *Prosthecobacter*, *Zoogloea*, *Caldilinea* and *Dechloromonas*. In addition, Wang et al. (2012) found that the orders *Verrucomicrobiales*, *Myxococcales, Sphingobacteriales*, *Rhodocyclales*, *Burkholderiales*, *Rhizobiales*, *Anaerolineales*, *Xanthomonadales*, *Clostridiales* and *Planctomycetales* were most abundant in sludge from fourteen WWTPs, making up a total of 95% of all sequences. The results in the current study were similar to the results obtained for municipal WWTPs as *Proteobacteria* and *Bacteroidetes* were among the top 5 most abundant phyla present in the pit latrine microbiome.

Depending on their shared networks of co-and anti-correlating genera, human populations can be divided into 1 out of 3 enterotypes, *Bacteroides* (type 1), *Prevotella* (type 2), or *Ruminococcus* (type 3) (Arumugam et al. 2011). The pit latrine microbiome only somewhat resembled enterotype 1 as it contained the genus *Bacteroides* even though it was only present at <1%.

Limitations of this study

There are several factors that can affect the outcome of metagenomic studies such as the sequencing approach (Steven et al. 2012), the sequencing technology (Loman et al. 2012) and

the choice of bioinformatics tools (Narzisi and Mishra 2011). The interpretation of metagenomic results is also restricted and defined by bacterial species, which were earlier characterized and cultivated (Wesolowska-Andersen et al. 2014). Furthermore, the communities characterized are only estimates of the actual microbial composition, as they reflect a snapshot at a single point in time (Staley et al. 2018). In addition, the datasets generated are subject to the same biases and limitations as environmental studies, relating to sampling methods and sample storage conditions (Staley and Sadowsky 2016). Nevertheless, NGS was successful in providing a first insight into the bacterial diversity present within the pit latrine fecal sludge as reported for other engineered environments (Cydzik-Kwiatkowska and Zielińska 2016). However, the current study is limited as only one pooled sample was collected from the pit latrines in KwaMashu at a single point in time, thereby only providing a snapshot of the bacterial community present in peri-urban pit latrines. More samples are needed to compare microbial communities between pit latrines from different peri-urban communities and over different seasons.

Conclusion

The pit latrine fecal sludge microbiome in the current study was similar to that of human-fecal microbiomes detected in sewage treatment plants in the USA, which were dominated by the phyla *Firmicutes*, *Bacteroidetes* and to a lesser extent by other mostly anaerobic microbes, e.g., *Bifidobacteriaceae* and *Coriobacteriaceae*. In addition, the pit latrine microbiome showed some similarities at the phylum level to latrine and individual gut microbiota profiles from non-westernized societies i.e. central African children from Burkina Faso, other rural South African communities and the Hadza of Tanzania in Africa. However, the pit latrine microbiome differed from latrine and individual gut microbiota profiles from non-westernized societies at the lower taxonomic levels.

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Chapter 4

Assessment of pit latrines in a peri-urban community in KwaZulu-Natal (South Africa) as a source of antibiotic resistant *E. coli* strains

The majority of this chapter dealing with the antibiotic resistant Escherichia coli strains from pit latrines, has been published under the title: Assessment of pit latrines in a peri-urban community in KwaZulu-Natal (South Africa) as a source of antibiotic resistant E. coli strains in International Journal of Hygiene and Environmental Health (220 (2017) 1279-1284) and the published manuscript is reproduced on the following pages. However, additional data not included in the published manuscript are presented in an appendix following this chapter.

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Assessment of pit latrines in a peri-urban community in KwaZulu-Natal (South Africa) as a source of antibiotic resistant *E. coli* strains

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ABSTRACT

Due to the frequent use of antibiotics and recurring illnesses related to multidrug-resistant (MDR) bacteria in South Africa, we determined if MDR *Escherichia coli* were present in pit latrine fecal sludge samples obtained from a peri-urban community in KwaZulu-Natal, South Africa. The abundance of *E. coli* in pit latrine samples was established using a most probable number (MPN) method with species confirmation done using biochemical tests and polymerase chain reaction (PCR). Forty-four randomly selected *E. coli* pit latrine isolates were further characterized, using the European committee on antimicrobial susceptibility testing (EUCAST) disk diffusion method to establish antibiotic resistance profiles for these *E. coli* isolates. The resulting MPN values for *E. coli* ranged from one to 6.2 $\log_{10}$ MPN per gram of fresh pit latrine fecal sludge. While only 3 out of 44 *E. coli* pit latrine isolates showed no resistance to any of the 12 tested antibiotics, most isolates were resistant to two or more antibiotics. The majority of isolates showed resistance to at least one of the two tested aminoglycosides, one isolate showed resistance to the carbapenem ertapenem, and although resistance was not detected for tigecycline four pit latrine *E. coli* isolates showed intermediate resistance to this antibiotic. However, about 14% of the *E. coli* pit latrine isolates were categorized as MDR, all of which showed resistance to four or more antibiotics. The presence of MDR *E. coli* strains in pit latrine samples demonstrates that these facilities are potential sources for MDR bacteria.

1. Introduction

Such international organizations as the European Food and Safety Authority (EFSA, 2017), the World Health Organization (WHO, 2014) and the United Nations (Mushtaq, 2016) have acknowledged that antimicrobial resistance (AMR) poses a threat to global health and have therefore highlighted the need for its prevention, detection, and control. The WHO embarked on a survey in countries within the six WHO regions to determine whether structures were in place to address the problem of AMR, with responses received from the African region revealing existing knowledge gaps concerning AMR monitoring and surveillance on the African continent (WHO, 2015). In addition, the Organization for Economic Co-operation and Development highlighted in a recent report published jointly with other international organizations such as the Food and Agriculture Organization of the United Nations, that the spread of antimicrobial resistance must be contained as it is threatening the world economy in addition to global health (OECD, 2017).

It is generally acknowledged that the advent of acquired antimicrobial resistance has drastically increased due to the global misuse and overuse of antibiotics (Fletcher, 2015; Wellington et al., 2013) and that these resistances can spread via horizontal gene transfer (Davies and Davies, 2010). This is potentially exacerbated in developing countries like South Africa, with an estimated 11 million bacterial infections per year (OECD, 2017) and the incidence of HIV-related illnesses and tuberculosis caused by drug-resistant strains on the rise (Day and Gray, 2016; Ndihokubwayo et al., 2013; WHO, 2015). In addition, poor sanitation and hygiene are contributing to high mortality levels among children under the age of five due to diarrhea frequently caused by AMR strains (Chola et al., 2015; Day and Gray, 2016; Galane and Le Roux, 2001).

An estimated 21–40% of the South African population makes use of pit latrines (Graham and Polizzotto, 2013). According to the most recent data, 16.6% of South African households use ventilated pit latrines, and 14.5% use non-ventilated pit latrines (Stats SA, 2016). Pit latrines frequently lack a proper physical barrier between excreta and soil, which can lead to the leaching of bacterial contaminants into the surrounding terrain and groundwater (van Ryneveld and Fourie, 1997) or well water (Akoachere et al., 2013). In addition, pit latrines are mostly built on marginal land which – including the pit latrines – is potentially subjected to flooding (Todman et al., 2015). Furthermore, they are

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sources of odors and attract insects, which in turn are potential disease vectors (Nakagiri et al., 2016); while cleaning practices involving manual pit emptying procedures might expose workers and communities to potentially infectious fecal matter (Kwiringira et al., 2016).

Naturally, human excreta found in pit latrines contain diverse *Enterobacteriaceae* including the well-known hygiene indicator *Escherichia coli* (Graham and Polizzotto, 2013). However, pathogenic *Enterobacteriaceae*, including pathogenic strains of *E. coli,* are commonly associated with community- and healthcare-acquired infections (Galane and Le Roux, 2001; McKay and Bamford, 2015; Sievert et al., 2013), and treatment of these infections is becoming a challenge due to the threat of AMR. For South Africa, it was shown that a high percentage of individuals representing different age groups from urban and rural areas excreted ampicillin resistant microorganisms via feces, with *E. coli* identified as the most abundant bacterial species of the AMR isolates (Shanahan et al., 1993). The aim of this study was therefore to assess the presence of AMR and MDR *E. coli* strains in pit latrine fecal sludge samples collected in a peri-urban community in KwaZulu-Natal (South Africa), because data on the presence of such *E. coli* strains are scarce for these sanitation facilities in KwaZulu-Natal.

2. Materials and methods

2.1. Sample collection

Fecal sludge samples (one each) were collected from ten ventilated pit latrines in a peri-urban community (KwaMashu) in the eThekwini municipality (KwaZulu-Natal, South Africa). This was done in autumn 2014 when these ten pit latrines were due for manual pit emptying (Fig. S1) according to the municipality's pit emptying schedule. After collection, samples were transported on ice to the laboratory and stored at 4 °C until further use. Samples were analyzed within 12–18 h upon arrival in the laboratory.

2.2. E. coli enumeration and isolation

E. coli was quantified in pit latrine fecal sludge samples at the time of sampling using a previously reported most probable number (MPN) method (Mdluli et al., 2013). Presumptive *E. coli* isolates obtained from pit latrine fecal sludge samples were confirmed using prescribed biochemical tests (GIMViC) and PCR-based amplification of the *gadA* gene (glutamate decarboxylase A) as reported previously (Gemmell and Schmidt, 2012). MPN values for *E. coli* were established according to de Man (1983) and expressed as $\log_{10}$ MPN/g of fresh fecal pit latrine sludge.

2.3. EUCAST disk diffusion assay

Forty-four *E. coli* strains, randomly selected from all confirmed pit latrine fecal sludge *E. coli* isolates, were characterized using the EUCAST (2015) disk diffusion method to determine their antibiotic resistance profiles. Thus 100 µl cell suspension of overnight cultures (Nutrient broth (Merck), 120 rpm, 37 °C) of selected *E. coli* isolates adjusted to 2×10^8 cells/ml (equivalent to 0.5 McFarland standard) using a Helber-type bacterial counting chamber (Marienfeld, Germany) were spread plated onto 25 ml Mueller-Hinton agar (Oxoid) in 90 mm Petri dishes. Four equally spaced antibiotic test disks were aseptically placed on each agar plate. As recommended (EUCAST, 2016), the following antibiotic test disks (6 mm, Oxoid) with the specified antibiotic concentrations were used: ampicillin (AMP – 10 µg), amoxicillin/clavulanic acid (AMC – 20 µg/10 µg), cefotaxime (CTX – 5 µg), ceftazidime (CAZ – 10 µg), ertapenem (ERT – 10 µg), meropenem (MEM – 10 µg), aztreonam (ATM – 30 µg), ciprofloxacin (CIP – 5 µg), norfloxacin (NOR – 10 µg), gentamicin (GEN – 10 µg), tobramycin (TOB – 10 µg) and tigecycline (TIG – 15 µg). The disk diffusion assay was done in duplicate for all 12 antibiotics and for each *E. coli* isolate tested. Plates were sealed with parafilm, incubated at 35 °C for 20 h and inhibition zones were measured using a digital Vernier caliper (Marshal Tools, India). Average inhibition zone values established were compared to the EUCAST (2016) breakpoint table to determine the resistance profile for each isolate, using the recommended quality control strain *E. coli* ATCC 25922.

2.4. Chemicals

Unless otherwise stated, all media and chemicals used were of the highest quality commercially available.

3. Results and discussion

3.1. E. coli enumeration and isolation

At the time of sampling, the average MPN/g value established in this study for *E. coli* in fecal pit latrine sludge samples was $\log_{10}$ 5.29, with a median value of $\log_{10}$ 4.10. The lowest and highest value were established as $\log_{10}$ 1.04 and $\log_{10}$ 6.20 MPN/g. Comparable results were reported for latrine waste collected in Haiti (Berendes et al., 2015), with *E. coli* $\log_{10}$ MPN values of 6–7 per g dry weight. The dry weight of the pooled fecal sludge pit latrine samples from the eThekwini municipality was established as 0.19 g per g wet weight, thereby matching the moisture content of 79.1% reported for latrine waste in Haiti (Berendes et al., 2015) as well as the moisture content (53–92%) reported for human feces (Nishimuta et al., 2006).

A study conducted in a rural area in Sierra Leone quantified *E. coli* in freshly collected human feces in a range of $\log_{10}$ 4.38–8.60 MPN/g (Wright, 1982), while the average *E. coli* count in feces of 100 healthy French volunteers was reported as $\log_{10}$ 7.84 CFU/g (Smati et al., 2013). The MPN values determined in this study for *E. coli* in pit latrine fecal sludge samples were somewhat lower than values reported for fresh feces, which might be due to extended filling time and increased pit latrine fecal sludge age causing *E. coli* die-off over time, as was demonstrated for sewage sludge (Czajkowska et al., 2008; Edmonds, 1976). The *E. coli* burden of the pit latrine fecal sludge was therefore similar to values reported for raw secondary (*E. coli* present at $\log_{10}$ 3.48–6.46 CFU/g dry weight; Levantesi et al., 2015) and for raw sewage sludge (*E. coli* present at $\log_{10}$ 3.66–6.04 CFU/g of total solids; Carballa et al., 2009).

Dilution effects due to urine and rainfall as well as the use of disinfectants, bleach, and insecticides by members of the pit latrine using communities to control odor formation and insects can cause an additional reduction in viable counts for *E. coli*. Similarly, the disposal of unused antibiotics or their excretion via feces and urine might affect the viability of bacteria present in pit latrine fecal sludge and even select for antibiotic resistant cells. Furthermore, an apparent die-off of bacteria such as *E. coli* present in pit latrines over time might be due to the transformation of culturable bacterial cells into the non-culturable VBNC (viable but non-culturable) state. In such case, the VBNC state can be reverted to the culturable state as was shown for sewage sludge (Fu et al., 2014), thereby rendering such material a potential hazard even though the number of culturable bacterial cells is reduced.

3.2. Antimicrobial resistance among pit latrine fecal sludge E. coli isolates

Out of the fecal pit latrine sludge *E. coli* isolates tested, only one isolate (EC5) was susceptible to all antibiotics tested (Table 1), two isolates (EC3, EC16) showed only intermediate resistance, while 23 isolates were resistant to one (6/44) or two (17/44) antibiotics, with the latter being the largest resistance group among the 44 isolates (Fig. S2). The remaining 18 isolates were resistant to three or more antibiotics (Table 1), with six of these isolates (EC9, EC10, EC20, EC25, EC27 and EC41) categorized as MDR based on resistance to ≥3 antibiotics representing ≥3 antibiotic categories as suggested by

Table 1
Antibiotic resistance profiles of *E. coli* pit latrine sludge isolates.

Isolate	Antibiotic classes							Antibiotic resistance profile
	PC	CS	CP	MB	FQ	AG	TC	
EC 1	AMP AMC	-	-	-	-	gen TOB	-	AMP-AMC-TOB
EC 2	AMP	-	-	-	-	gen tob	-	AMP
EC 3	-	caz	-	-	-	gen tob	-	-
EC 4	-	-	-	-	-	GEN TOB	-	GEN-TOB
EC 5	-	-	-	-	-	-	-	-
EC 6	AMP	-	-	atm	-	gen tob	-	AMP
EC 7	AMP	-	-	-	-	gen tob	-	AMP
EC 8	AMC	-	-	-	-	-	-	AMC
EC 9	**AMP AMC**	**ctx caz**	**ert mem**	**ATM**	**-**	**GEN TOB**	**tig**	**AMP-AMC-ATM-GEN-TOB**
EC 10	**AMP AMC**	**caz**	**ERT mem**	**atm**	**nor**	**GEN TOB**	**tig**	**AMP-AMC-ERT-GEN-TOB**
EC 11	AMC	caz	-	atm	-	GEN TOB	-	AMC-GEN-TOB
EC 12	-	caz	ert	atm	-	GEN TOB	-	GEN-TOB
EC 13	-	caz	-	atm	-	GEN TOB	-	GEN-TOB
EC 14	-	-	ert	-	-	GEN TOB	-	GEN-TOB
EC 15	AMP AMC	caz	-	atm	-	gen TOB	-	AMP-AMC-TOB
EC 16	-	caz	-	atm	-	-	-	-
EC 17	-	caz	ert	-	-	gen TOB	-	TOB
EC 18	AMC	caz	-	atm	nor	GEN TOB	-	AMC-GEN-TOB
EC 19	-	caz	ert	-	-	GEN TOB	-	GEN-TOB
EC 20	**AMC**	**CAZ**	**ert mem**	**atm**	**cip**	**GEN TOB**	**-**	**AMC-CAZ-GEN-TOB**
EC 21	AMC	caz	-	atm	nor	gen TOB	tig	AMC-TOB
EC 22	AMP AMC	caz	ert	-	nor	GEN TOB	-	AMP-AMC-GEN-TOB
EC 23	-	caz	ert	-	-	gen TOB	-	TOB
EC 24	AMP AMC	caz	-	atm	-	GEN TOB	-	AMP-AMC-GEN-TOB
EC 25	**-**	**CAZ**	**-**	**atm**	**NOR**	**GEN TOB**	**-**	**CAZ-NOR-GEN-TOB**
EC 26	-	caz	ert	atm	nor	GEN TOB		GEN-TOB
EC 27	**AMP AMC**	**ctx CAZ**	**-**	**ATM**	**-**	**GEN TOB**	**tig**	**AMP-AMC-CAZ-ATM-GEN-TOB**
EC 28	-	caz	ert	-	-	GEN TOB	-	GEN-TOB
EC 29	AMP AMC	caz	ert	atm	-	GEN TOB	-	AMP-AMC-GEN-TOB
EC 30	-	caz	-	-	nor	GEN TOB	-	GEN-TOB
EC 31	-	caz	-	-	-	GEN TOB	-	GEN-TOB
EC 32	-	caz	-	atm	-	GEN TOB	-	GEN-TOB
EC 33	-	caz	-	-	-	GEN TOB	-	GEN-TOB
EC 34	AMC	-	-	-	-	GEN tob	-	AMC-GEN
EC 35	AMC	ctx caz	ert	atm	-	GEN TOB	-	AMC- GEN-TOB
EC 36	AMP AMC	caz	-	atm	nor	gen TOB	-	AMP-AMC-TOB
EC 37	AMC	ctx caz	-	-	-	gen TOB	-	AMC-TOB
EC 38	-	caz	-	-	-	GEN TOB	-	GEN-TOB
EC 39	-	caz	-	atm	nor	GEN TOB	-	GEN-TOB
EC 40	AMP AMC	caz	-	-	nor	GEN TOB	-	AMP-AMC-GEN-TOB
EC 41	**AMP AMC**	**ctx CAZ**	**-**	**ATM**	**-**	**GEN TOB**	**-**	**AMP-AMC-CAZ-ATM-GEN-TOB**
EC 42	AMC	caz	-	-	nor	GEN TOB	-	AMC-GEN-TOB
EC 43	AMP AMC	-	ert	atm	cip nor	gen TOB	-	AMP-AMC-TOB
EC 44	-	caz	-	-	-	GEN TOB	-	GEN-TOB
ATCC 25922	-	-	-	-	-	-	-	-

UPPERCASE = resistant, lowercase = intermediate resistance, – = no resistance.
AMP (ampicillin), AMC (amoxicillin-clavulanic acid), CTX (cefotaxime), CAZ (ceftazidime – 3rd generation cephalosporin), ERT (ertapenem), MEM (meropenem), ATM (aztreonam), CIP (ciprofloxacin), NOR (norfloxacin), GEN (gentamicin), TOB (tobramycin), TIG (tigecycline).
PC (Penicillins), CS (Cephalosporins), CP (Carbapenems), MB (Monobactams), FQ (Fluoroquinolones), AG (Aminoglycosides), TC (Tetracyclines).
Isolates highlighted in **bold** and with grey shading are multidrug-resistant.
ATCC 25922 = *E. coli* quality control strain.

Magiorakos et al. (2012). For the non-MDR isolates, resistance was limited to antibiotics representing the penicillins, and aminoglycosides (Table 1). The highest proportion of resistances among *E. coli* pit latrine fecal sludge isolates was detected for the two tested aminoglycosides tobramycin (TOB) (36/44) and gentamicin (GEN) (29/44), followed by the tested penicillins AMC (21/44) and AMP (15/44) (Fig. 1). Lower levels of resistances were established for the cephalosporin ceftazidime (CAZ, 4/44), the monobactam aztreonam (ATM, 3/44), the fluoroquinolone norfloxacin (NOR, 1/44) and the carbapenem ertapenem (ERT, 1/44), while no isolate showed resistance to either tigecycline (TIG), representing the tetracyclines, or cefotaxime (CTX), meropenem (MEM) and ciprofloxacin (CIP) (Table 1, Fig. 1). Intermediate resistance was observed in varying quantities for all antibiotics tested (Fig. 1, Table 1), with the exception of the penicillins AMP and AMC for both of which an intermediate category is not specified in the EUCAST breakpoint table (2016).

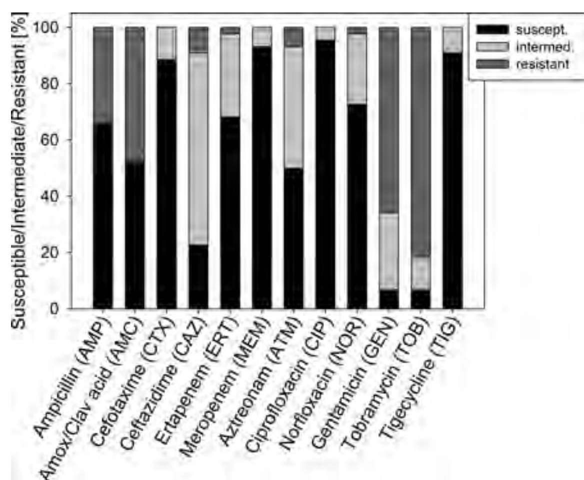

Fig. 1. Antibiotic resistance and susceptibility of *E. coli* pit latrine fecal sludge isolates (n = 44) to 12 selected antibiotics.

Among the *E. coli* pit latrine isolates, 34% were resistant to the β-lactam ampicillin (Fig. 1). Maré and Coetzee (1966) established in an early study that about 50% of fecal *E. coli* isolates collected from patients in Pretoria (South Africa) showed ampicillin resistance, while Grabow and Prozesky (1973) established that this was the case for about 38% of coliforms present in Edendale hospital sewage (Pietermaritzburg, South Africa). Similarly, Galane and Le Roux (2001) reported that out of 30 *E. coli* fecal isolates from healthy patients in South Africa, 50% exhibited ampicillin resistance. Barreto et al. (2009) established that about 40% of 92 *E. coli* strains isolated from the feces of healthy children in Portugal were ampicillin resistant, while most recently de Francesco et al. (2017) demonstrated that this was the case for 41 out of 87 *E. coli* isolates from the feces of children in rural Limpopo (South Africa), which is in line with our results.

For amoxicillin-clavulanic acid (AMC), the resistance level observed for fecal pit latrine *E. coli* isolates of about 48% is within the range (0–57%) reported for clinical *E. coli* isolates from private hospitals in South Africa (Brink et al., 2008). The elevated level of AMC resistance among the *E. coli* pit latrine isolates might be due to the frequent use of AMC to treat respiratory and urinary tract infections, as was shown in Spain where AMC resistances coincided with AMC consumption in communities (Oteo et al., 2008).

The majority (39 out of 44) of *E. coli* fecal pit latrine sludge isolates were susceptible to the cephalosporin cefotaxime (CTX) and no isolate was resistant. This is in line with a recent report, showing that only 2 out of 87 *E. coli* isolates from the feces of children from rural Limpopo (South Africa) were resistant to this cephalosporin (de Francesco et al., 2017). However, for ceftazidime (CAZ), four *E. coli* pit latrine isolates (all MDR) showed resistance (Table 1).

None of the fecal pit latrine sludge isolates was resistant to the carbapenem meropenem (MEM) and only one out of 44 isolates was resistant against ertapenem (ERT), the second carbapenem tested. This matches two studies from South Africa, with Brink et al. (2008) reporting that the resistance levels for these two antibiotics – even among clinical *E. coli* isolates – were only 1 and 2%, and McKay and Bamford (2015) showing that blood stream infection causing clinical *E. coli* isolates were highly susceptible to both MEM and ERT. Similarly, for non-clinical fecal *E. coli* isolates from South Africa, no resistance was detected for another carbapenem, imipenem (de Francesco et al., 2017).

The majority of pit latrine sludge *E. coli* isolates was susceptible to the tested fluoroquinolones ciprofloxacin (CIP) (42/44) and norfloxacin (NOR) (32/44). In a study conducted by Yang et al. (2014), *E. coli* isolates from feces of healthy volunteers showed a low level of resistance to ciprofloxacin at 8.7%, while de Francesco et al. (2017) reported that no fecal *E. coli* isolates from South Africa were resistant to this fluoroquinolone. This result is similar to the current pit latrine study as no resistance was detected for the fluoroquinolone ciprofloxacin (CIP) and only one *E. coli* isolate showed resistance to norfloxacin (NOR) (Fig. 1). The limited degree of resistance to fluoroquinolones among the pit latrine *E. coli* isolates might be a reflection of a possibly limited use of these antibiotics in rural or peri-urban communities. However, 95% of *E. coli* isolates obtained from water sources used by rural Venda communities in South Africa were resistant to the fluoroquinolone ciprofloxacin (Obi et al., 2004), indicating potential variation in the use of these antibiotics in peri-urban and rural settings in South Africa.

A high degree of resistance to the tested aminoglycoside antibiotics gentamicin (GEN) and tobramycin (TOB) was established for the fecal pit latrine sludge *E. coli* isolates in the current study (Fig. 1). For antibiotics considered important in human and veterinary medicine, resistance levels exceeding 50% are categorized as very high (EFSA, 2017). Similarly, both environmental *E. coli* isolates from sewage effluent as well as clinical *E. coli* isolates from outpatients with urinary tract infections showed elevated levels of gentamicin resistance (Korzeniewska et al., 2013; Mamani et al., 2015), thereby matching our results. However, *E. coli* isolates from human feces in Botswana (Pesapane et al., 2013) and in Limpopo, South Africa (de Francesco et al., 2017), exhibited a much lower degree of resistance to gentamicin (<10%), again possibly reflecting different usage patterns.

None of the pit latrine fecal sludge isolates displayed resistance to tigecycline (Table 1), the first of a new class of antibiotics controlling even MDR *E. coli* (Peterson, 2005), thereby matching a study showing that even 326 clinical *E. coli* isolates collected in South Africa were susceptible to tigecycline (Kanj et al., 2014). The fact that 30–40% of *E. coli* isolates from human feces in Botswana (Pesapane et al., 2013) exhibited resistance to tetracycline, which was not tested in our study, indicates that tigecycline is clearly more effective. However, four out of 44 isolates (three of which categorized as MDR) exhibited intermediate resistance to tigecycline.

In total, six pit latrine *E. coli* isolates were categorized as MDR, with two isolates (EC27, EC41) showing resistance toward six individual antibiotics representing four different classes (Table 1). All of these isolates exhibited resistance to the tested aminoglycosides gentamicin and tobramycin. In addition, more than 60% of these MDR isolates were resistant to both penicillins tested and the cephalosporin ceftazidime. In line with these results, van der Meeren et al. (2013) found high levels of resistance for gentamicin in 12 HRMO (highly resistant microorganisms) *E. coli* isolates from a malnutrition and pediatric ward in Mozambique. In contrast, Eshetie et al. (2015) reported lower levels of gentamicin and amoxicillin-clavulanic acid resistance but similar levels for ceftazidime and ampicillin in clinical MDR *E. coli* isolates from Ethiopia. Fifty percent of the pit latrine MDR *E. coli* isolates were resistant to aztreonam (ATM), which is concerning as this antibiotic is considered as a reserve group antibiotic to be used as last resort option (WHO, 2017).

However, only a single MDR *E. coli* pit latrine isolate (EC25) exhibited resistance towards the fluoroquinolone norfloxacin (NOR) and one isolate (EC10) towards the carbapenem ertapenem (ERT) (Table 1, Fig. 1). It is noteworthy that resistance to fluoroquinolones and carbapenems was only detected in *E. coli* pit latrine isolates categorized as MDR. Carbapenems are considered a last-line therapy for infections caused by multidrug-resistant Gram-negative bacteria (Cantón et al., 2012), and carbapenem-resistant *E. coli* strains are normally associated with invasive infections and high mortality rates (Eshetie et al., 2015). It is therefore concerning that pit latrine *E. coli* isolates might have already acquired resistances against at least one of these important antibiotics.

In addition, intermediate resistances observed for medically important antibiotics such as third generation cephalosporins, carbapenems, aztreonam or tigecycline (Table 1) in the present study are a reason for concern given recommendations to categorize certain intermediate

resistances as resistant to avoid potential treatment failure (Leclercq et al., 2013).

4. Conclusion

AMR as well as MDR *E. coli* strains were detected in pit latrine samples from a peri-urban community in KwaZulu-Natal (South Africa), which might be a reflection of the diverse fecal input entering these pit latrines. However, the current study was of an exploratory nature, as only a limited number of *E. coli* isolates from pit latrine fecal sludge samples was analyzed. Nevertheless, the results obtained indicate that additional work is needed to assess the degree of AMR and MDR in *E. coli* and other relevant bacterial species from pit latrines in South Africa. The presence of AMR and MDR bacteria in pit latrine fecal sludge can contribute to the spread of antibiotic resistances and cause bacterial contamination on and off site, especially when the pit emptying is done using manual procedures. If pathogenic AMR and MDR bacteria are present in pit latrines, these should be considered as a potential health risk in rural and peri-urban communities, given current pit latrine fecal sludge handling practices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijheh.2017.08.002.

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Supplemental Material
for Beukes, King and Schmidt
Assessment of pit latrines in a peri-urban community in KwaZulu-Natal (South Africa) as a source of antibiotic resistant *E. coli* strains

Figure S1. Pit latrine-emptying practices on site.

(PiD staff is acknowledged for providing pictures)

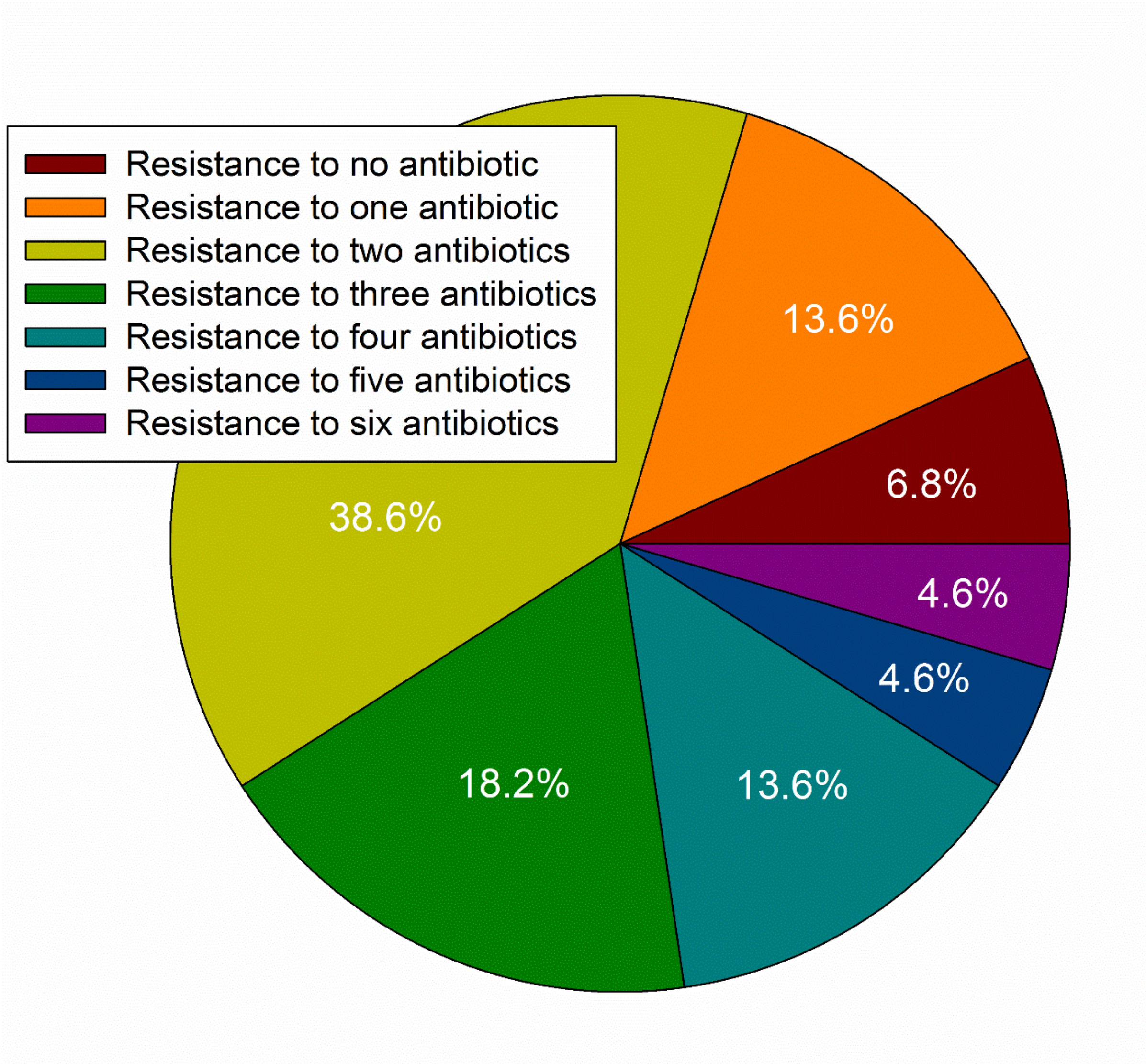

Figure S2. Incidence of antibiotic resistances in *E. coli* pit latrine isolates (n = 44).

Chapter 4: Appendix

Detection of β-lactamase activity in *E. coli* pit latrine isolates using the nitrocefin test

Abstract

The emergence of *Escherichia coli* strains with broad-spectrum resistance to β-lactams is a great concern as they present a challenge when treating infections caused by pathogenic *E. coli* strains. In the following study, β-lactamase activity in *E. coli* pit latrine isolates displaying resistance or intermediate resistance to β-lactam antibiotics was verified by a rapid procedure employing the chromogenic cephalosporin, nitrocefin. Single colonies of *E. coli* pit latrine isolates grown for 24h on nutrient agar plates were suspended in 20µl of nitrocefin solution (0.5mM) to determine the presence of β-lactamase activity, which was depicted by a red color change upon complete hydrolysis of nitrocefin. The results obtained from the nitrocefin test were compared to results obtained for β-lactam resistance using the European committee on antimicrobial susceptibility testing (EUCAST) disk diffusion assay. Of the 42 *E. coli* pit latrine isolates tested, 20 isolates were confirmed positive for β-lactamase activity, 2 isolates tested negative and the remaining 20 isolates displayed incomplete hydrolysis of nitrocefin. Of the 16/42 isolates that displayed only intermediate resistance to β-lactam antibiotics based on the EUCAST disk diffusion assay, 62% displayed incomplete hydrolysis of nitrocefin. However, 87% of the isolates that displayed resistance to ampicillin based on the EUCAST disk diffusion assay tested positive for β-lactamase activity using the nitrocefin test. The data in this study indicate that the nitrocefin test was useful for the detection of β-lactamase activity in *E. coli* pit latrine isolates with resistance to ampicillin. The incomplete hydrolysis of nitrocefin by approximately 48% of the *E. coli* pit latrine isolates is possibly due to a low level of β-lactamase activity in these isolates or the use of different mechanisms of resistance to β-lactam antibiotics. Thus, the nitrocefin test enables the detection of β-lactamase activity and also highlights that in the case of an isolate displaying resistance to β-lactam antibiotics using the EUCAST disk diffusion assay, more than one mechanism of resistance can be expected.

Introduction

The main mechanisms that render *Enterobacteriaceae* resistant to β-lactam antibiotics are alterations of the antibiotic target by possessing an altered or acquired penicillin binding protein

(PBP) with low affinity to β-lactams, secondly, the enzymatic inactivation of the antibiotic by the production of β-lactamases and finally, decreased antibiotic transport through the outer membrane by altered porins or efflux pumps (Allen et al. 2010; Vaidya 2011; Cag et al. 2016). In *E. coli*, outer membrane permeability for β-lactam antibiotics is due to the presence of porins, also known as outer membrane proteins. Porins like OmpF and OmpC form pores in the outer membrane that enables tiny molecules to diffuse into the periplasmic space. (Nikaido and Nakae 1979; Sawai et al. 1982; Yoshimura and Nikaido 1985). Multidrug efflux pumps, particularly AcrAB-TolC and Mex pumps, mediate intrinsic and acquired multidrug resistance (MDR) in *E. coli*. Efflux pumps can also synergistically interact with other resistance mechanisms like external membrane permeability to boost resistance levels (Li et al. 2015).

In a study by Hiraoka et al. (1989), a cephalosporinase produced by *Proteus vulgaris* displayed a broad spectrum of activity ranging from penicillins to cephalosporins including oxyimino-cephalosporins when expressed in *E. coli*, demonstrating that the substrate spectrum of β-lactamases is one of the major factors contributing to antibiotic resistance. In the early 1980s, the introduction of extended-spectrum cephalosporins and carbapenems was a milestone in antimicrobial chemotherapy for the treatment of infections caused by *Enterobacteriaceae* and other Gram negative pathogens (Paterson and Bonomo 2005; Pfeifer et al. 2010). However, due to the over and misuse of β-lactam antibiotics, new β-lactamases termed extended-spectrum β-lactamases (ESBLs) emerged that could hydrolytically inactivate and thus confer resistance to these compounds (Pfeifer et al. 2010). This problem is further exacerbated in strains that display low permeability to β-lactam antibiotics. While ESBLs confer bacterial resistance to penicillins and cephalosporins of 1st, 2nd, and 3rd generation, including aztreonam, ESBLs cannot hydrolyze cephamycins and carbapenems and they are inhibited by clavulanic acid (Rasmussen and Bush 1997). However, recent studies have shown that some combinations of β-lactam-inhibitors are no longer useful against common Gram negative pathogens producing ESBLs, carbapenemases or various β-lactamases in the same microorganism (Bush and Bradford 2019).

Since the mechanism of resistance to β-lactam antibiotics is frequently due to the production of β-lactamase enzymes, resistance in bacterial isolates can be rapidly determined by detecting the activity of such enzymes using the chromogenic cephalosporin nitrocefin, which changes from yellow to red upon complete hydrolysis (O'Callaghan et al. 1972). The nitrocefin test is usually used to confirm the outcome of antimicrobial susceptibility tests for isolates showing

resistance to β-lactam antibiotics. *E. coli* strains that are resistant to β-lactam antibiotics, based on sufficient β-lactamase activity will generally be nitrocefin positive. Those strains that are β-lactam resistant but are nitrocefin negative are possibly exhibiting low β-lactamase activity, using a different mechanism for resistance e.g. porins, efflux pumps or possess certain metallo-β-lactamases (MBLs) which cannot hydrolyze nitrocefin even though they usually have a broad substrate spectrum (Markowitz 1980; Livermore and Brown 2001). MBLs are metalloenzymes with Zn (II) ions that are linked to their active site and are vital to their activity (Walsh et al. 2005; Crowder et al. 2006). Chromogenic tests such as the nitrocefin test and the Cica-β-test, described by Livermore et al. (2007), may be useful for the early detection or confirmation of ESBLs, metallo-β-lactamases (MBLs) and AmpC β-lactamases in clinical isolates of *E. coli*. Early detection of such hydrolases is indeed critical in minimizing mortality in the case of severe infections caused by MDR pathotypes of *E. coli* frequently found in healthcare facilities (Kollef 2000). The aim of this study was therefore to assess the presence of β-lactamase activity in *E. coli* pit latrine isolates displaying resistance or intermediate resistance to β-lactam antibiotics based on the EUCAST disk diffusion assay, using the nitrocefin test.

Materials and methods

E. coli isolates used in this study were originally isolated and characterized according to Beukes et al. (2017). The chromogenic cephalosporin nitrocefin (SR0112, Oxoid® UK) was used to detect β-lactamase activity in *E. coli* pit latrine isolates. A lyophilized pellet of nitrocefin was resuspended per manufacturer's instructions in dimethylsulphoxide (DMSO) and 0.1M phosphate buffer solution (1:1 ratio) and stored in the dark at -20°C until further use. Single colonies of all *E. coli* pit latrine isolates showing resistance or intermediate resistance against at least one β-lactam antibiotic, were scraped from nutrient agar plates after incubation for 24 hours and suspended in 20µl of nitrocefin solution (0.5mM) in microtiter plates. In addition, 20µl of nitrocefin solution without inoculum was used as a blank and the *E. coli* reference strain ATCC 25922, which is susceptible to all tested β-lactam antibiotics, was used as a negative control. β-Lactamase activity was detected by a red color change within 10min of incubation at 37°C in the dark (Fig. 1). A yellow color after incubation indicated a negative result and an orange/brown color change indicated incomplete or partial hydrolysis of nitrocefin. Isolates, which did not show any β-lactamase activity or incomplete hydrolysis of nitrocefin, after this period, were further incubated for 20min under the same conditions.

Chemicals

Unless indicated otherwise, all media and chemicals used were of the best quality commercially accessible.

Results and discussion

Of the 42 tested *E. coli* pit latrine isolates that were resistant or showed intermediate resistance to at least one β-lactam antibiotic based on the EUCAST disk diffusion assay, 20 (48%) isolates were confirmed positive for β-lactamase activity using the nitrocefin test. While 2 isolates tested negative (EC25, EC16), the remaining 20 (48%) isolates displayed incomplete hydrolysis of nitrocefin (Table 1). In addition, of the 16 (38%) isolates that displayed only intermediate resistance to β-lactam antibiotics (EUCAST disk diffusion assay), 62% displayed incomplete hydrolysis of nitrocefin. However, of the 15 isolates that were resistant to ampicillin (EUCAST disk diffusion assay), 87% tested positive for β-lactamase activity using the nitrocefin test. Overall, 65% of the isolates that tested positive for β-lactamase activity using the nitrocefin test were resistant to ampicillin. Hiraoka et al. (1989) observed a decrease in susceptibility of an *E. coli* strain deficient of both OmpF and OmpC porins to 11 of 15 β-lactam antibiotics tested except for ampicillin, piperacillin and imipenem. However, in the presence of the type I penicillinase the activities of ampicillin, piperacillin and cefoperazone were significantly reduced. This could indicate that resistance to ampicillin in *E. coli* is generally mediated by the production of β-lactamases, which matches the current study as the majority of *E. coli* pit latrine isolates resistant to ampicillin tested positive for β-lactamase activity.

Although isolate EC25 displayed resistance to ceftazidime and intermediate resistance to aztreonam and isolate EC16 displayed intermediate resistance to both ceftazidime and aztreonam based on the EUCAST (2016) antimicrobial susceptibility test, both tested negative for β-lactamase activity using the nitrocefin test (Table 1, Fig. 1). Hiraoka et al. (1989) found that the activity of a type I β-lactamase was more reduced in ampicillin resistant *E. coli* strains for ceftazidime, cefepime, and ceftizoxime than for the other cephalosporins tested, possibly indicating an involvement of porins or efflux pumps in mediating resistance to ceftazidime in *E. coli* isolates. Furthermore, Jaffe et al. (1982) found that isolates deficient of both OmpF and OmpC porins, revealed a higher degree of resistance to the tested β-lactam antibiotics (cefoxitin, ampicillin, cefaloridine, cefazolin) than those deficient of only one porin. Only 4/6 MDR isolates were confirmed positive for β-lactamase activity, one was negative and one displayed incomplete hydrolysis of nitrocefin (Table 1, Fig. 1). The isolates in the current study

that displayed a negative result or incomplete hydrolysis of nitrocefin but showed resistance to β-lactam antibiotics based on the EUCAST (2016) antimicrobial susceptibility test, might use a different mechanism for β-lactam resistance such as porin deficiency or efflux pumps (Nikaido 1996).

Table 1 Detection of β-lactamase activity in *E. coli* pit latrine isolates using the nitrocefin test.

Pit Latrine Isolate	EUCAST β-lactam disk diffusion assay				Nitrocefin Test		
	PC	CS	CP	MB	Positive (Red)	Negative (Yellow)	Incomplete hydrolysis (Orange/Brown)
EC 1	AMP AMC	-	-	-	+		
EC 2	AMP	-	-	-	+		
EC 3	-	caz	-	-			
EC 6	AMP	-	-	atm	+		
EC 7	AMP	-	-	-	+		
EC 8	AMC	-	-	-			
EC 9	**AMP AMC**	**caz ctx**	**ert mem**	**ATM**	+		
EC 10	**AMP AMC**	**caz**	**ERT**	**atm**	+		
EC 11	AMC	caz	-	atm			
EC 12	-	caz	ert	atm			
EC 13	-	caz	-	atm			
EC 14	-	-	ert	-	+		
EC 15	AMP AMC	caz	-	atm	+		
EC 16	-	caz	-	atm		-	
EC 17	-	caz	ert	-			
EC 18	AMC	caz	-	atm	+		
EC 19	-	caz	ert	-	+		
EC 20	**AMC**	**CAZ**	**ert mem**	**atm**			
EC 21	AMC	caz	-	atm			
EC 22	AMP AMC	caz	ert	-			
EC 23	-	caz	ert	-			
EC 24	AMP AMC	caz	-	atm	+		
EC 25	-	**CAZ**	-	**atm**		**-**	

Pit Latrine Isolate	EUCAST β-lactam disk diffusion assay				Nitrocefin Test		
	PC	CS	CP	MB	Positive (Red)	Negative (Yellow)	Incomplete hydrolysis (Orange/Brown)
EC 26	-	caz	ert	atm	+		
EC 27	**AMP AMC**	**ctx CAZ**	**-**	**ATM**	+		
EC 28	-	caz	ert	-	+		
EC 29	AMP AMC	caz	ert	atm	+		
EC 30	-	caz	-	-			
EC 31	-	caz	-	-			
EC 32	-	caz	-	atm	+		
EC 33	-	caz	-	-			
EC 34	AMC	-	-	-			
EC 35	AMC	ctx caz	ert	atm			
EC 36	AMP AMC	caz	-	atm	+		
EC 37	AMC	ctx caz	-	-			
EC 38	-	caz	-	-			
EC 39	-	caz	-	atm	+		
EC 40	AMP AMC	caz	-	-	+		
EC 41	**AMP AMC**	**ctx CAZ**	**-**	**ATM**	+		
EC 42	AMC	caz	-	-			
EC 43	AMP AMC	-	ert	atm			
EC 44	-	caz	-	-			
ATCC 25922	-	-	-	-		-	
Blank						-	

UPPERCASE - resistant, lowercase - intermediate resistance, - susceptible

EC (*E. coli*), AMP (ampicillin), AMC (amoxicillin-clavulanic acid), CTX (cefotaxime - 3rd generation cephalosporin), CAZ (ceftazidime - 3rd generation cephalosporin), ETP (ertapenem), MEM (meropenem), ATM (aztreonam), - Yellow fill (susceptible/negative for nitrocefin), + Red fill (positive for nitrocefin), orange/brown fill (incomplete hydrolysis of nitrocefin), blank (nitrocefin only), PC (Penicillins), CS (Cephalosporins), CP (Carbapenems), MB (Monobactams). Isolates highlighted in bold are multi-drug resistant.

In addition, these isolates could be exhibiting low β-lactamase activity or producing certain MBLs not capable of hydrolyzing cephalosporins (Matthew et al. 1975; Queenan and Bush

2007). These MBLs are called subclass B2 β-lactamases that vary from subclasses B1 and B3 because they contain one zinc ion in their active site and have a limited substratum range that almost solely hydrolyzes the carbapenems (Wommer et al. 2002). CphA (Carbapenem-hydrolyzing MBL) and Sfh-I (*Serratia fonticola*) are subclass B2 MBLs found in *Enterobacteriaceae* such as *E. coli* and have a single zinc ion at the Zn^2 site (Garau et al. 2005; Fonseca et al. 2011). Isolates showing resistance to β-lactams based on the EUCAST disk diffusion assay and a positive result for the nitrocefin test might also use a synergistic effect between porin deficiency and the production of β-lactamases, as Hiraoka et al. (1989) demonstrated an enhanced resistance to β-lactam antibiotics in *E. coli* isolates showing β-lactamase activity and porin deficiency.

Livermore et al. (2007) used the chromogenic cephalosporins nitrocefin and the oxyimino-cephalosporin HMRZ-86 (i.e. Cica-β-test), to confirm β-lactamase activity in clinical isolates of *E. coli* - among other bacteria - with known β-lactamase activity. The results obtained by these authors indicated that the nitrocefin test was successful in detecting β-lactamase activity in all tested isolates with known β-lactamase activity.

In a study by Shannon and Phillips (1980), the nitrocefin test along with other tests such as an acidimetric paper-strip test and a tube acidimetric test could not reliably detect β-lactamase activity in various tested *Enterobacteriaceae*. Similarly, Smith et al. (2014) found that out of 41 *E. coli* isolates resistant to penicillin and oxacillin from Herring gulls and 51 from Hybrid deer, less than 25% of *E. coli* isolates tested positive for β-lactamase activity using the nitrocefin test. However, Uri (1985) found the nitrocefin test useful in separating β-lactamase producing *E. coli* from non-producers before performing further experimental analysis. Similarly, Chah and Oboegbulem (2007) confirmed β-lactamase activity in the majority (99%) of ampicillin resistant *E. coli* strains using a nitrocefin solution. The results in the current study are therefore similar to the results reported by Uri (1985) and Chah and Oboegbulem (2007) as the nitrocefin test reliably detected β-lactamase activity in the majority (>85%) of *E. coli* pit latrine isolates that displayed resistance to ampicillin.

Figure 1 Detection of β-lactamase activity in *E. coli* pit latrine isolates EC11-20 using the nitrocefin test.

A potential disadvantage of color-based phenotypic tests employing substrates such as nitrocefin is that enzymatic reactions can be incomplete, therefore only leading to partial color (orange/brown) reactions within a specified time (Voladri and Kernodle 1998). These partial color reactions in the nitrocefin test may be due to the difference in substrate specificities of various β-lactamase enzymes or varying expression levels rendering isolates intermediate resistant (Voladri and Kernodle 1998). Additionally, these partial color reactions could indicate an alternate mechanism for β-lactam resistance among *E. coli* pit latrine isolates, other than the production of a β-lactamase, as previously highlighted.

Conclusion

The nitrocefin test was useful in confirming β-lactamase activity in *E. coli* pit latrine isolates displaying resistance to the β-lactam, ampicillin. In addition, the nitrocefin test in the current study could potentially detect intermediate resistance against β-lactam antibiotics in isolates based on a high level of agreement (62%) between the nitrocefin test for isolates showing partial color reactions (incomplete hydrolysis) and the EUCAST disk diffusion assay for the same isolates showing intermediate resistance to at least one β-lactam antibiotic. Furthermore, the results even indicate a possible involvement of alternative β-lactam resistance mechanisms such as porins or efflux pumps in mediating antibiotic resistance in some isolates. This was demonstrated in isolates EC25 and EC16, which both displayed resistance or intermediate resistance to β-lactam antibiotics in the EUCAST disk diffusion assay but tested negative for β-lactamase activity using the nitrocefin test. Overall, both the nitrocefin test and the EUCAST disk diffusion assay confirm the high level of resistance of *E. coli* pit latrine isolates to β-lactam antibiotics and indicate that the nitrocefin test is a useful addition to confirm or improve

surveillance for the emergence of resistance to clinically relevant β-lactam antibiotics in bacterial isolates from South African pit latrines.

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Chapter 5

Antibiotic resistance profiles of coagulase-positive and coagulase-negative staphylococci from pit latrine fecal sludge in a peri-urban South African community

The majority of this chapter dealing with the isolation and characterization of multi-drug resistant Staphylococcus spp. in pit latrine samples, has been published under the title: Antibiotic resistance profiles of coagulase-positive and coagulase-negative staphylococci from pit latrine fecal sludge in a peri-urban South African community in Folia Microbiologica 63:645-651 and the published manuscript is reproduced on the following pages.

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ORIGINAL ARTICLE

Antibiotic resistance profiles of coagulase-positive and coagulase-negative staphylococci from pit latrine fecal sludge in a peri-urban South African community

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Abstract

The aim of this study was to assess pit latrine samples from a peri-urban community in KwaZulu-Natal (South Africa) for the presence of multidrug-resistant (MDR) *Staphylococcus* spp. Standard procedures were used to isolate *Staphylococcus* spp. from pit latrine fecal sludge samples, with confirmation at genus level by polymerase chain reaction (PCR). Sixty-eight randomly selected pit latrine *Staphylococcus* spp. isolates were further characterized by using established disk diffusion procedures. An average *Staphylococcus* spp. count of 2.1×10^5 CFU per g fecal material was established using two randomly selected pit latrine samples. Of the 68-selected *Staphylococcus* spp. pit latrine isolates, 49% were identified as coagulase positive, 51% as coagulase negative and 65% (12 coagulase positive, 32 coagulase negative isolates) were categorized as MDR. The majority (66/68) of *Staphylococcus* spp. isolates displayed resistance to fusidic acid while only 5/68 isolates displayed resistance to chloramphenicol. The pit latrine samples analyzed in this study are a source of MDR *Staphylococcus* spp., highlighting the need for proper hygiene and sanitation regimes in rural communities using these facilities.

Abbreviations

BSAC	British Society for Antimicrobial Chemotherapy
CFU	Colony-forming unit
CNS	Coagulase-negative staphylococcus
CPS	Coagulase-positive staphylococcus
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ISO	International Organization for Standardization
MDR	Multidrug-resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
ORSA	Oxacillin-resistant *Staphylococcus aureus*
PCR	Polymerase chain reaction

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Introduction

The World Health Organization (WHO (World Health Organization) 2001) highlighted a lack of laboratory-based surveillance for antimicrobial resistance on the African continent. Furthermore, the absence of comprehensive policies and plans to address antimicrobial resistance as well as limited regulatory capacity and the circulation of low-grade antimicrobials were identified as major challenges (Ndihokubwayo et al. 2013). Dissemination of multidrug-resistant (MDR) bacteria is aggravated by factors such as poverty and improper sanitation practices (Visser et al. 2011), which are common in peri-urban and rural areas where communities are forced to use inferior sanitation systems such as pit latrines (WHO (World Health Organization) 2017a). Although antibiotics have been used for decades to treat infections, their limited efficacy and lifespan due to antibiotic-resistant bacterial pathogens is becoming a serious problem (Wright and Poinar 2012). This is further exacerbated as antibiotic-resistant bacteria and resistance genes can be scattered in the environment by abiotic and biotic factors such as wind, water, waste, humans, or animals (Allen et al. 2010).

Staphylococcus spp. are Gram-positive microorganisms found in humans (von Eiff et al. 2002), animals (Schmidt et al. 2014), and the environment (Schulz et al. 2012). While coagulase-positive staphylococci (CPS) such as *Staphylococcus*

aureus are well-established pathogens, coagulase-negative staphylococci (CNS) have increasingly been recognized as important pathogens as well (Becker et al. 2014). *Staphylococcus aureus* (coagulase-positive) and *Staphylococcus epidermidis* (coagulase-negative) are two prominent human commensal species, which can cause morbidity and mortality in susceptible individuals (Otto 2009). The coagulase-positive *S. aureus* is the most common opportunistic pathogen found in the nostrils, on the skin, and in mucous membranes of humans and animals (Gorwitz et al. 2008; Mainous et al. 2006) and has also been isolated from human stool (Campillo et al. 2001; Lindberg et al. 2011). *Staphylococcus aureus* can cause diverse infections and is particularly detrimental to burn patients as they are more prone to bacterial colonization and infection due to the nature of their wounds and the resulting immune-suppression (Cook 1998).

Methicillin, now substituted by oxacillin, was first introduced as celbenin in 1959 to treat infections caused by penicillin-resistant *S. aureus* strains. Not long after its introduction into clinical use, the first methicillin-resistant *S. aureus* (MRSA) strain was reported (Jevons 1961). Both MRSA and oxacillin-resistant *S. aureus* (ORSA) strains express the *mecA* gene encoding a penicillin-binding protein (PBP2A) with very low affinity for β-lactams, thus rendering penicillins ineffective (Pinho et al. 2001). As ORSA strains are usually multidrug-resistant (MDR), they are difficult to treat in case of infections (Hiramatsu et al. 2001). At the same time, MDR ORSA strains are ubiquitous in nature, are not confined to hospital settings (hospital-acquired ORSA), and have emerged in community settings where they can infect healthy individuals (community-acquired ORSA) (Chambers 2001). These strains combine oxacillin resistance with enhanced virulence and fitness (Otto 2013).

As recent work has demonstrated the presence of multidrug-resistant *Escherichia coli* in pit latrine fecal sludge sampled in a peri-urban South African community (Beukes et al. 2017), we screened such samples for the presence of antibiotic-resistant *Staphylococcus* spp., as such data are lacking for pit latrines in South Africa.

Materials and methods

Sample collection

As reported previously (Beukes et al. 2017), fecal sludge samples (one each) were collected in autumn 2014 from peri-urban pit latrines, when ten of these were due for manual emptying based on the eThekwini municipality's (KwaZulu-Natal, South Africa) pit latrine emptying schedule. Samples were transported on ice from the sampling sites to the laboratory and analyzed within 12–18 h upon arrival in the laboratory.

Enumeration and isolation of *Staphylococcus* spp.

The enumeration of *Staphylococcus* spp. at the time of sampling essentially followed the ISO 6888–1 guideline procedure (ISO 6888–1, 1999). One gram fresh pit latrine fecal sludge was serially diluted with sterile saline (0.85% NaCl) up to 10^{-8} and 100 μL aliquots of each dilution were spread plated in duplicate onto pre-dried Baird-Parker agar plates (Merck). A coagulase test (Bactident Coagulase, Merck) was used together with PCR employing the genus-specific primers TstaG422/Tstag765 (Martineau et al. 2001) as described previously (Gemmell and Schmidt 2013), to confirm isolates at genus level as coagulase-positive (CPS) and coagulase-negative (CNS) staphylococci.

Antibiotic disk diffusion assay

To determine the antibiotic resistance profiles of *Staphylococcus* spp. from pit latrine fecal sludge samples, a random selection of 33 coagulase-positive and 35 coagulase-negative fecal sludge isolates (in total 68) was further characterized using the established EUCAST disk diffusion procedure (EUCAST (European Committee on Antimicrobial Susceptibility Testing), 2015) and, for oxacillin, the BSAC procedure (BSAC (British Society for Antimicrobial Chemotherapy) 2015a). Antibiotic test disks (Oxoid) with the following concentrations and inhibition zone breakpoints—using or adapting established breakpoints (EUCAST (European Committee on Antimicrobial Susceptibility Testing) 2016; BSAC (British Society for Antimicrobial Chemotherapy) 2015b)—were used: oxacillin (OX; 1 μg, S ≥ 15 mm/*R* ≤ 14 mm), cefoxitin (CFX; 30 μg, CPS S ≥ 22 mm/*R* < 22 mm, CNS S ≥ 25 mm/*R* < 25 mm), cefalexin (CFL; 30 μg, CPS S ≥ 22 mm/*R* < 22 mm, CNS S ≥ 25 mm/*R* < 25 mm), doripenem (DOR; 10 μg, CPS S ≥ 22 mm/*R* < 22 mm, CNS S ≥ 25 mm/*R* < 25 mm), imipenem (IMP; 10 μg, CPS S ≥ 22 mm/R < 22 mm, CNS S ≥ 25 mm/R < 25 mm), ciprofloxacin (CIP; 5 μg, S ≥ 20 mm/*R* < 20 mm), moxifloxacin (MXF; 5 μg, S ≥ 24 mm/*R* < 21 mm), gentamicin (GEN; 10 μg, CPS S ≥ 18 mm/*R* < 18 mm, CNS S ≥ 22 mm/*R* < 22 mm), tobramycin (TOB; 10 μg, CPS S ≥ 18 mm/R < 18 mm, CNS S ≥ 22 mm/R < 22 mm), erythromycin (E; 15 μg, S ≥ 21 mm/*R* < 18 mm), clindamycin (CLM; 2 μg, S ≥ 22 mm/*R* < 19 mm), quinupristin-dalfopristin (QD; 15 μg, S ≥ 21 mm/*R* < 18 mm), tigecycline (TGC; 15 μg, S ≥ 18 mm/R < 18 mm), chloramphenicol (C; 30 μg, S ≥ 18 mm/R < 18 mm), fusidic acid (FD; 10 μg, S ≥ 24 mm/*R* < 24 mm), and rifampicin (R; 5 μg, S ≥ 26 mm/*R* < 23 mm). All isolates were tested in duplicate, inhibition zones were measured to the next millimeter using a digital vernier caliper (Marshal Tools, India), and average inhibition zones were established and compared to the breakpoints specified above. Resistance levels for tested antibiotics in CPS and CNS isolates were statistically compared using Fisher's exact test using Graph Pad Prism (v.7.03).

Results and discussion

Enumeration of *Staphylococcus* spp.

An average presumptive *Staphylococcus* spp. count of 2.1 × 10^5 CFU/g was established at the time of sampling for fresh fecal material from two randomly selected pit latrine samples. This is similar to counts (ranging from $\log_{10}$ 4.9 to $\log_{10}$ 5.4 CFU/g) established for *Staphylococcus aureus* in feces from Italian and Swedish infants (Lindberg et al. 2011) and the counts reported for coagulase-positive staphylococci in raw (10^2–10^5 CFU per g dry matter; Dumontet et al. 2001) and *Staphylococcus* spp. in lagooned sewage sludge (1.2 × 10^6 CFU per g of total suspended solids; Dudley et al. 1980).

Antibiotic resistance profiles of coagulase-positive *Staphylococcus* spp. pit latrine isolates

While the majority of the 33 coagulase-positive *Staphylococcus* spp. isolates was resistant to fusidic acid (31/33), all CPS isolates were susceptible to chloramphenicol (Fig. 1). This matches a recent study by Kumburu et al. (2018), reporting that no chloramphenicol-resistant phenotypes were detected among 30 methicillin-resistant *Staphylococcus aureus* (MRSA) hospital isolates from Tanzania. In addition, all CPS pit latrine isolates were resistant to at least one antibiotic (Table 1) and intermediate resistance was observed for oxacillin (8/33), moxifloxacin (11/33), erythromycin (2/33), clindamycin (14/33), quinupristin-dalfopristin (12/33), and rifampicin (11/33) (Fig. 1, Table S1). Resistance levels exceeding 20% were detected for oxacillin (16/33), clindamycin (11/33), and rifampicin (8/33), while resistance levels for tigecycline, tobramycin, erythromycin, quinupristin-dalfopristin, gentamicin, cefalexin, cefoxitin, doripenem, moxifloxacin, ciprofloxacin, and imipenem were lower than 20% (Fig. 1). Tsuji et al. (2007) reported that 8% and 12% of community-associated MRSA isolates were resistant to clindamycin and the fluoroquinolone levofloxacin respectively, which is lower than the value for clindamycin in the current study but similar for the fluoroquinolones tested. Notably, approximately 33% of CPS isolates in the current study displayed intermediate resistance to moxifloxacin (Fig. 1). While no resistance against tigecycline was detected among 350 clinical isolates of *S. aureus* (255 MSSA and 95 MRSA) collected in South Africa (Kanj et al. 2014), about 18% of coagulase-positive *Staphylococcus* spp. pit latrine isolates—including oxacillin-resistant and oxacillin-susceptible isolates—were resistant to tigecycline in the current study. This indicates that tigecycline-resistant phenotypes of *Staphylococcus* spp. might be emerging as was reported recently for Nigeria (Ayepola et al. 2014).

Flemming and Ackermann (2007) reported that less than 5% of *S. aureus* stool isolates were resistant to tested carbapenems, tetracycline, and macrolides. In the current study, resistance to antibiotics representing these categories was higher (Fig. 1). A study done in Cape Town (South Africa) reported that about 48% of healthcare-acquired staphylococcus isolates were resistant to cloxacillin and about 44% to clindamycin (McKay and Bamford 2015), which is similar to the CPS resistance patterns for oxacillin (48%) and clindamycin (33%) in the current study (Fig. 1). While all clinical *S. aureus* isolates from 14 health institutions in KwaZulu-Natal (South Africa) were susceptible to fusidic acid, less than 10% of these isolates were resistant to chloramphenicol and ciprofloxacin, about 30% were resistant to erythromycin, clindamycin, and gentamicin, and one in five was resistant to rifampicin (Shittu and Lin 2006). These resistance patterns match those of CPS pit latrine isolates in the current study except for fusidic acid, for which 94% of CPS isolates displayed resistance, and the antibiotics erythromycin and gentamicin, for which resistance levels were clearly lower. Of the 33 CPS pit latrine isolates analyzed in the current study, 12 (i.e., 36%) were categorized as MDR based on resistance to at least one antibiotic from three or more antibiotic categories (Table 1, Table S1).

Among coagulase-positive *S. aureus* and *S. pseudintermedius* isolates from companion animals and the environment in Zambia, only 10% of the *S. pseudintermedius* isolates were categorized as MDR (Youn et al. 2014). In addition, a very low level of resistance to chloramphenicol was detected for both *S. aureus* and *S. pseudintermedius* isolates, which is similar to the CPS pit latrine isolates as all of these were susceptible to chloramphenicol. A recent study on staphylococcal isolates from diabetic patients with foot ulcers identified 36% of these isolates as MDR (Mottola et al. 2016), thus matching the percentage of MDR CPS pit latrine isolates in the current study. It is therefore evident that antibiotic resistance is not only present in nosocomial but also in community-related and environmental CPS, demonstrating the potential for transfer of resistance genes between humans, animals, and the environment.

Antibiotic resistance profiles of coagulase-negative *Staphylococcus* spp. pit latrine isolates

About 97% of the coagulase-negative *Staphylococcus* spp. pit latrine isolates analyzed were resistant to three or more antibiotics (Table 1). All 35 pit latrine CNS isolates were resistant to fusidic acid and more than 85% to oxacillin (Fig. 2). Intermediate resistance was observed for moxifloxacin (11/35), erythromycin (5/35), clindamycin (5/35), quinupristin-dalfopristin (11/35), and rifampicin (2/35). Resistance levels below 15% were detected for chloramphenicol (5/35) and tigecycline (4/35), while resistance levels to tobramycin, erythromycin, quinupristin-dalfopristin, gentamicin, cefalexin, cefoxitin, and doripenem exceeded 50% and those for moxifloxacin, ciprofloxacin, and imipenem 30% (Fig. 2,

Fig. 1 Antibiotic resistance and susceptibility of coagulase-positive *Staphylococcus* spp. pit latrine fecal sludge isolates (n = 33) to 16 selected antibiotics

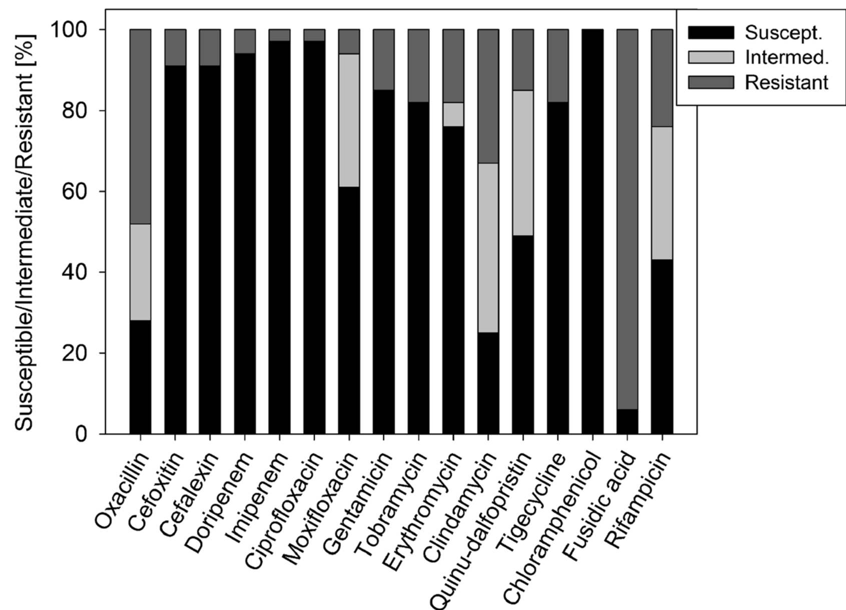

Table S2). Ntirenganya et al. (2015) reported that all coagulase-negative staphylococci isolated in a healthcare facility in Rwanda were oxacillin-resistant and 50% showed resistance to erythromycin, which is similar to the coagulase-negative *Staphylococcus* spp. pit latrine isolates in the current study. In contrast to these pit latrine CNS isolates, a study in the Democratic Republic of the Congo (DRC) showed that only 50% of coagulase-negative *Staphylococcus* spp. (CNS) isolates were resistant to gentamicin and all isolates were susceptible to erythromycin (Irenge et al. 2015). Ayepola et al. (2014) reported that out of 100 clinical CNS isolates from Nigeria, 41% were categorized as methicillin-resistant phenotypes based on their resistance to oxacillin, which is lower than the proportion of oxacillin-resistant (89%) phenotypes among the CNS pit latrine isolates from South Africa in the current study (Fig. 2). However, both the study by Ayepola et al. (2014) and the current study analyzing pit latrine fecal sludge CNS isolates show that a higher degree of antibiotic resistance is evident among coagulase-negative *Staphylococcus* spp. isolates exhibiting an oxacillin-resistant phenotype than among oxacillin-susceptible isolates. Similar to the South African CNS pit latrine isolates, the majority of CNS strains isolated from blood cultures of septicemic patients in Turkey showed oxacillin resistance, and these isolates exhibited a higher degree of resistance to antibiotics from other classes than oxacillin-susceptible CNS isolates (Koksal et al. 2009). Among 135 oxacillin-resistant CNS strains from Turkey, more than 55% were resistant to gentamicin, erythromycin, clindamycin, ciprofloxacin, and chloramphenicol while only 25% showed resistance to fusidic acid (Koksal et al. 2009). Among the oxacillin-resistant coagulase-negative *Staphylococcus* spp. pit latrine isolates from South Africa, more than 80% showed resistance to gentamycin, clindamycin, and fusidic acid, more than 40% for erythromycin and ciprofloxacin, and above 10% for tigecycline and chloramphenicol. The high degree of resistance to clindamycin and gentamycin therefore matches the report by Koksal et al. (2009).

Table 1 Distribution of resistances against single or multiple antibiotics among coagulase-positive and coagulase-negative *Staphylococcus* spp. pit latrine fecal sludge isolates

	CPS isolates (n = 33) with		CNS isolates (n = 35) with	
	ABR	MDR	ABR	MDR
No resistance	0	0	0	0
1 antibiotic	10	0	1	0
2 antibiotics	6	0	0	0
3 antibiotics	9	5	3	1
4 antibiotics	2	1	0	0
5 antibiotics	2	2	0	0
6 antibiotics	1	1	0	0
7 antibiotics	1	1	0	0
8 antibiotics	0	0	3	3
9 antibiotics	0	0	6	6
10 antibiotics	0	0	3	3
11 antibiotics	1	1	6	6
12 antibiotics	0	0	5	5
13 antibiotics	1	1	2	2
14 antibiotics	0	0	3	3
15 antibiotics	0	0	2	2
16 antibiotics	0	0	1	1
∑	33	12 (36%)	35	32 (91%)

ABR antibiotic resistance, *MDR* multidrug resistance

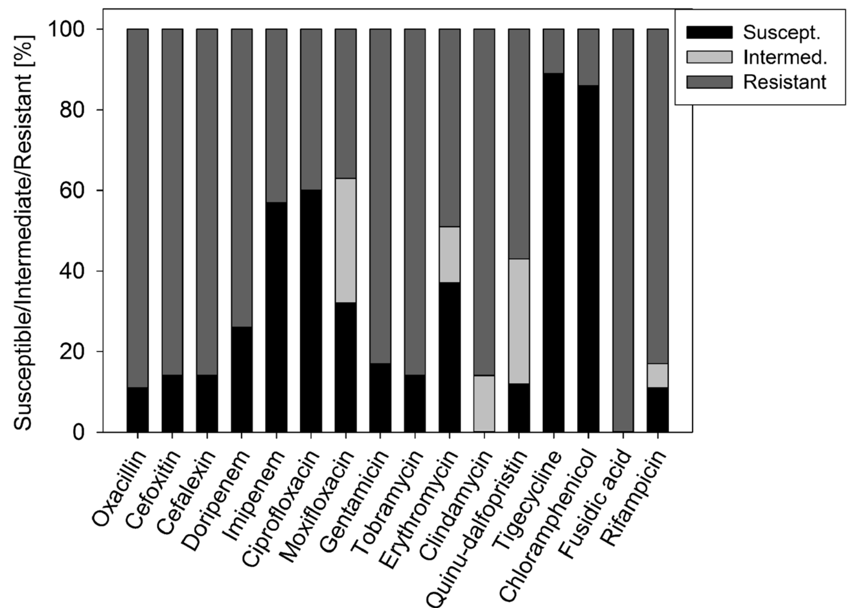

Fig. 2 Antibiotic resistance and susceptibility of coagulase-negative *Staphylococcus* spp. pit latrine fecal sludge isolates (n = 35) to 16 selected antibiotics

More recently, Szczuka et al. (2016) reported that 93% of methicillin-resistant coagulase-negative *S. hominis* isolates from clinical specimens were resistant to erythromycin and 77% were resistant to clindamycin. However, less than 10% of clinical methicillin-resistant coagulase-negative isolates of the same species were found to be resistant to quinupristin-dalfopristin (Petinaki et al. 2005). Among the CNS pit latrine isolates of the current study, resistance to erythromycin was lower at 49%, but higher for clindamycin at 93% and for quinupristin-dalfopristin at 58% of oxacillin-resistant CNS isolates, which contrasts with the findings of Szczuka et al. (2016) and Petinaki et al. (2005). Faria et al. (2009) showed that 34% of CNS isolates from wastewater—which would have received fecal input—were resistant or exhibited an intermediary phenotype to two or more antibiotics. The antibiotic resistance level among pit latrine CNS isolates—with more than 90% categorized as MDR (Table 1, Table S2)—is therefore much higher than that among wastewater isolates. Hence, the resistance patterns of coagulase-negative *Staphylococcus* spp. pit latrine isolates more closely resemble those of clinical coagulase-negative *Staphylococcus* spp. isolates reported by Koksal et al. (2009), Ayepola et al. (2014), and Ntirenganya et al. (2015), although the antibiotic resistance levels appear to be much higher in the pit latrine isolates.

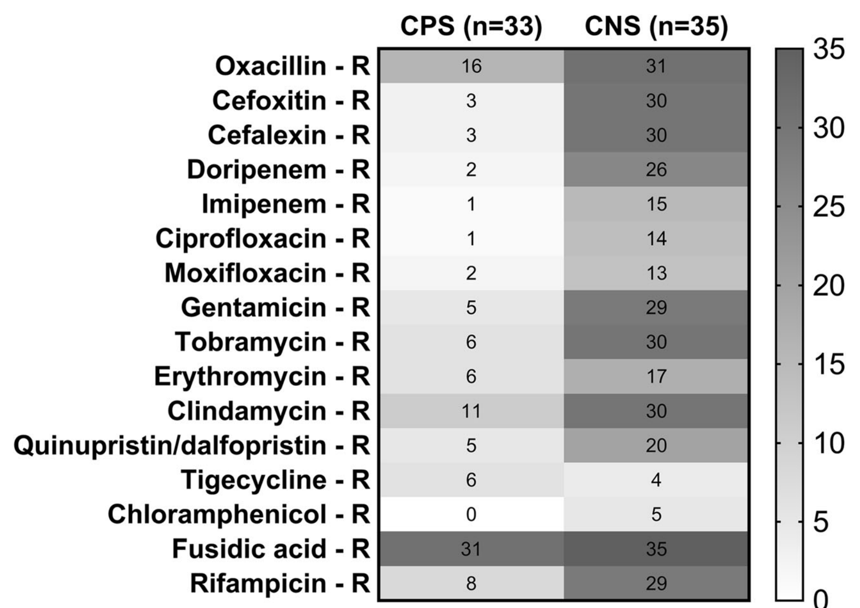

Fig. 3 Resistance heat map for coagulase-positive (CPS) and coagulase-negative (CNS) *Staphylococcus* spp. pit latrine isolates

Comparison of coagulase-positive and coagulase-negative *Staphylococcus* spp. pit latrine isolates

In the current study, coagulase-negative *Staphylococcus* spp. pit latrine isolates generally displayed a higher level of resistance to individual antibiotics (more than 80% of these isolates were resistant to more than 7 antibiotics) and a higher level of multidrug resistance (more than 90% of these isolates were categorized as MDR), than the coagulase-positive isolates (Table 1, Fig. 3), which in most cases was statistically significant (Table S3). Although the reliability of the disk diffusion method for detecting oxacillin resistance has been critically discussed (EUCAST (European Committee on Antimicrobial Susceptibility Testing), 2015; York et al. 1996), the data obtained nevertheless indicate the presence of a higher proportion of oxacillin-resistant coagulase-negative than of oxacillin-resistant coagulase-positive *Staphylococcus* spp. isolates in pit latrine samples. This is in agreement with the results for the other antibiotics tested, with resistance levels for antibiotics representing the clinically important aminoglycosides, cephalosporins, or macrolides being evidently higher among coagulase-negative than in coagulase-positive *Staphylococcus* spp. pit latrine isolates (Fig. 3).

Conclusions

The majority of *Staphylococcus* spp. isolates from pit latrines—particularly the coagulase-negative isolates—possessed antibiotic resistances against numerous clinically relevant compounds. The high resistance levels detected for clinically relevant antibiotics among these isolates are concerning, as it even included resistances against the reserve group antibiotic tigecycline (WHO (World Health Organization) 2017b), thus potentially rendering infections caused by such antibiotic-resistant strains untreatable. In addition, these results highlight the need for microbiological surveillance of those pit latrine facilities in peri-urban and rural communities in South Africa that require manual pit emptying, as this practice can cause contamination potentially putting workers and communities at risk.

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Compliance with ethical standards

Ethical clearance for this study was granted by the Humanities and Social Science Research Ethics Committee of the University of KwaZulu-Natal (ref. no. HSS/0653/013).

Conflict of interest The authors declare that they have no conflict of interest.

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Supplemental material for
Beukes and Schmidt

Table S1

Pit latrine isolate	Antibiotic resistance of coagulase positive *Staphylococcus* spp.								Antibiotic resistance profile
	PC	CS	CP	FQ	AG	MLS	TC	MISC	
CPS1	ox	-	-	mxf	-	-	-	FD	FD
CPS2	-	-	-	-	-	clm	-	FD·r	FD
CPS3	-	-	-	-	-	CLM·qd	-	FD·R	CLM·FD·R
CPS4	-	-	-	-	-	-	-	FD·R	FD·R
CPS5	OX	-	-	-	-	clm	-	FD·r	OX·FD
CPS6	ox	-	-	-	-	e·CLM	-	FD·R	CLM·FD·R
CPS7	-	-	-	-	-	e·CLM·QD	-	FD·R	CLM·QD·FD·R
CPS8	ox	-	-	-	-	clm·qd	-	FD·r	FD
CPS9	OX	-	-	-	-	clm	-	FD·R	OX·FD·R
CPS10	-	-	-	-	-	-	-	FD·r	FD
CPS11	ox	-	-	mxf	-	clm	-	FD·r	FD
CPS12	**OX**	**CFX·CFL**	**IPM**	**MXF**	**GEN·TOB**	**E·CLM·QD**	**TIG**	**FD·R**	**OX·CFX·CFL·IPM·MXF·GEN·TOB·E·CLM·QD·TIG·FD·R**
CPS13	-	-	-	-	-	E	-	FD	E·FD
CPS14	-	-	-	-	-	clm·qd	-	FD·r	FD
CPS15	-	-	-	mxf	-	qd	-	FD	FD
CPS16	**OX**	**-**	**-**	**-**	**-**	**clm**	**TIG**	**FD·r**	**OX·TIG·FD**

Pit latrine isolate	Antibiotic resistance of coagulase positive *Staphylococcus* spp.								Antibiotic resistance profile
	PC	CS	CP	FQ	AG	MLS	TC	MISC	
CPS17	ox	-	-	-	-	clm	-	FD·r	FD
CPS18	**OX**	**-**	**-**	**-**	**-**	**E·CLM·QD**	**TIG**	**FD·R**	**OX·E·CLM·QD·TIG·FD·R**
CPS19	OX	-	-	-	-	clm·qd	-	FD	OX·FD
CPS20	**OX**	**-**	**-**	**-**	**-**	**clm·qd**	**TIG**	**FD**	**OX·TIG·FD**
CPS21	ox	-	-	-	-	clm	-	FD	FD
CPS22	**OX**	**-**	**-**	**mxf**	**GEN·TOB**	**clm·qd**	**TIG**	**FD·r**	**OX·GEN·TOB·TIG·FD**
CPS23	ox	-	-	mxf	-	E·CLM·qd	-	FD·r	E·CLM·FD
CPS24	OX	-	-	-	-	-	-	FD	OX·FD
CPS25	**ox**	**-**	**-**	**-**	**-**	**E**	**TIG**	**FD**	**E·TIG·FD**
CPS26	**OX**	**-**	**-**	**mxf**	**GEN·TOB**	**clm**	**-**	**FD**	**OX·GEN·TOB·FD**
CPS27	OX	-	-	mxf	GEN·TOB	clm·qd	-	-	OX·GEN·TOB
CPS28	-	-	-	-	-	E	-	R	E·R
CPS29	**OX**	**-**	**-**	**mxf**	**-**	**CLM·qd**	**-**	**FD**	**OX·CLM·FD**
CPS30	**OX**	**CFX·CFL**	**DOR**	**mxf**	**-**	**CLM·qd**	**-**	**FD·r**	**OX·CFX·CFL·DOR·CLM·FD**
CPS31	**OX**	**CFX·CFL**	**DOR**	**CIP·MXF**	**GEN·TOB**	**CLM·QD**	**-**	**FD**	**OX·CFX·CFL·DOR·CIP·MXF·GEN·TOB·CLM·QD·FD**
CPS32	**OX**	**-**	**-**	**mxf**	**TOB**	**CLM·QD**	**-**	**FD**	**OX·TOB·CLM·QD·FD**
CPS33	**OX**	**-**	**-**	**mxf**	**-**	**CLM·qd**	**-**	**FD**	**OX·CLM·FD**

UPPERCASE - resistant, lowercase - intermediate resistance.

CPS (Coagulase positive *Staphylococcus* spp. isolate), OX (oxacillin), CFX (cefoxitin), CFL (cefalexin), DOR (doripenem), IMP (imipenem), CIP (ciprofloxacin), MXF (moxifloxacin), GEN (gentamicin), TOB (tobramycin), E (erythromycin), CLM (clindamycin), QD (quinupristin-dalfopristin), TIG (tigecycline), C (chloramphenicol), FD (fusidic acid), R (rifampicin), - (no resistance), PC (Penicillins), CS (Cephalosporins), CP (Carbapenems), FQ (Fluoroquinolones), AG (Aminoglycosides), MLS (Macrolides, Lincosamides, Streptogramins), TC (Tetracyclines), MISC. (Miscellaneous).

Isolates highlighted in bold and with grey shading are multidrug-resistant.

Table S2.

Pit latrine isolate	Antibiotic resistance of coagulase negative *Staphylococcus* spp.								Antibiotic resistance profile
	PC	CS	CP	FQ	AG	MLS	TC	MISC	
CNS1	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·MXF**	**GEN·TOB**	**e·CLM·QD**	**TIG**	**FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·MXF·GEN·TOB·CLM·QD·TIG·FD·R**
CNS2	**OX**	**CFX·CFL**	**DOR·IPM**	**MXF**	**GEN·TOB**	**e·CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·MXF·GEN·TOB·CLM·QD·FD·R**
CNS3	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·MXF**	**GEN·TOB**	**E·CLM·qd**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·MXF·GEN·TOB·E·CLM·FD·R**
CNS4	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·MXF**	**GEN·TOB**	**E·CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·MXF·GEN·TOB·E·CLM·QD·FD·R**
CNS5	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·MXF**	**GEN·TOB**	**E·clm**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·MXF·GEN·TOB·E·FD·R**
CNS6	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·MXF**	**GEN·TOB**	**E·CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·MXF·GEN·TOB·E·CLM·QD·FD·R**
CNS7	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·mxf**	**GEN·TOB**	**E·CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·GEN·TOB·E·CLM·QD·FD·R**
CNS8	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·MXF**	**GEN·TOB**	**E·CLM·QD**	**TIG**	**C·FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·MXF·GEN·TOB·E·CLM·QD·TIG·C·FD·R**
CNS9	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·MXF**	**GEN·TOB**	**E·CLM·QD**	**-**	**C·FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·MXF·GEN·TOB·E·CLM·QD·C·FD·R**
CNS10	**OX**	**CFX·CFL**	**DOR**	**mxf**	**GEN·TOB**	**CLM·qd**	**TIG**	**FD·R**	**OX·CFX·CFL·DOR·GEN·TOB·CLM·TIG·FD·R**
CNS11	**OX**	**CFX·CFL**	**DOR·IPM**	**CIP·MXF**	**GEN·TOB**	**E·CLM·QD**	**-**	**C·FD·R**	**OX·CFX·CFL·DOR·IPM·CIP·MXF·GEN·TOB·E·CLM·QD·C·FD·R**
CNS12	**OX**	**CFX·CFL**	**DOR·IPM**	**MXF**	**GEN·TOB**	**CLM·qd**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·MXF·GEN·TOB·CLM·FD·R**
CNS13	**OX**	**-**	**-**	**mxf**	**GEN·TOB**	**CLM·QD**	**TIG**	**FD·R**	**OX·GEN·TOB·CLM·QD·TIG·FD·R**
CNS14	**OX**	**CFX·CFL**	**DOR**	**-**	**GEN·TOB**	**CLM·qd**	**-**	**FD·R**	**OX·CFX·CFL·DOR·GEN·TOB·CLM·FD·R**
CNS15	**OX**	**CFX·CFL**	**-**	**-**	**TOB**	**E·CLM·qd**	**-**	**C·FD**	**OX·CFX·CFL·TOB·E·CLM·C·FD**

Pit latrine isolate	Antibiotic resistance of coagulase negative *Staphylococcus* spp.								Antibiotic resistance profile
	PC	CS	CP	FQ	AG	MLS	TC	MISC	
CNS16	**OX**	**CFX·CFL**	**-**	**-**	**TOB**	**E·CLM·qd**	**-**	**FD·R**	**OX·CFX·CFL·TOB·E·CLM·FD·R**
CNS17	**OX**	**CFX·CFL**	**DOR**	**CIP·MXF**	**GEN·TOB**	**e·CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·CIP·MXF·GEN·TOB·CLM·QD·FD·R**
CNS18	**OX**	**CFX·CFL**	**DOR**	**CIP·MXF**	**GEN·TOB**	**e·CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·CIP·MXF·GEN·TOB·CLM·QD·FD·R**
CNS19	**OX**	**CFX·CFL**	**DOR**	**-**	**GEN·TOB**	**CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·GEN·TOB·CLM·QD·FD·R**
CNS20	**OX**	**CFX·CFL**	**DOR·IPM**	**mxf**	**GEN·TOB**	**CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·GEN·TOB·CLM·QD·FD·R**
CNS21	**OX**	**CFX·CFL**	**DOR**	**-**	**GEN·TOB**	**CLM·qd**	**-**	**FD·R**	**OX·CFX·CFL·DOR·GEN·TOB·CLM·FD·R**
CNS22	**OX**	**CFX·CFL**	**DOR·IPM**	**mxf**	**GEN·TOB**	**CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·GEN·TOB·CLM·QD·FD·R**
CNS23	**OX**	**CFX·CFL**	**DOR**	**-**	**GEN·TOB**	**CLM·qd**	**-**	**FD·R**	**OX·CFX·CFL·DOR·GEN·TOB·CLM·FD·R**
CNS24	**OX**	**CFX·CFL**	**DOR**	**CIP·mxf**	**GEN·TOB**	**E·CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·CIP·GEN·TOB·E·CLM·QD·FD·R**
CNS25	**-**	**CFX·CFL**	**DOR**	**CIP·mxf**	**GEN·TOB**	**E·CLM·QD**	**-**	**FD·R**	**CFX·CFL·DOR·CIP·GEN·TOB·E·CLM·QD·FD·R**
CNS26	**OX**	**CFL**	**-**	**CIP·MXF**	**GEN·TOB**	**e·CLM·QD**	**-**	**FD·r**	**OX·CFL·CIP·MXF·GEN·TOB·CLM·QD·FD**
CNS27	**OX**	**CFX·CFL**	**DOR·IPM**	**-**	**GEN**	**E·CLM·qd**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·GEN·E·CLM·FD·R**
CNS28	**OX**	**CFX·CFL**	**-**	**-**	**GEN·TOB**	**E·CLM·qd**	**-**	**C·FD**	**OX·CFX·CFL·GEN·TOB·E·CLM·C·FD**
CNS29	**OX**	**CFX·CFL**	**DOR**	**-**	**GEN·TOB**	**E·CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·GEN·TOB·E·CLM·QD·FD·R**
CNS30	**OX**	**CFX·CFL**	**DOR**	**mxf**	**GEN·TOB**	**CLM·qd**	**-**	**FD·R**	**OX·CFX·CFL·DOR·GEN·TOB·CLM·FD·R**

Pit latrine isolate	Antibiotic resistance of coagulase negative *Staphylococcus* spp.								Antibiotic resistance profile
	PC	CS	CP	FQ	AG	MLS	TC	MISC	
CNS31	**OX**	**CFX·CFL**	**DOR·IPM**	**mxf**	**GEN·TOB**	**CLM·QD**	**-**	**FD·R**	**OX·CFX·CFL·DOR·IPM·GEN·TOB·CLM·QD·FD·R**
CNS32	-	-	-	mxf	-	clm	-	FD	FD
CNS33	**OX**	**CFX**	**-**	**-**	**-**	**clm**	**-**	**FD·r**	**OX·CFX·FD**
CNS34	-	-	-	-	-	E·clm·QD	-	FD	E·QD·FD
CNS35	**-**	**-**	**-**	mxf	**-**	E·clm	**-**	FD·R	E·FD·R

UPPERCASE - resistant, lowercase - intermediate resistance.

CNS (Coagulase negative *Staphylococcus* spp. isolate), OX (oxacillin), CFX (cefoxitin), CFL (cefalexin), DOR (doripenem), IMP (imipenem), CIP (ciprofloxacin), MXF (moxifloxacin), GEN (gentamicin), TOB (tobramycin), E (erythromycin), CLM (clindamycin), QD (quinupristin-dalfopristin), TIG (tigecycline), C (chloramphenicol), FD (fusidic acid), R (rifampicin), - (no resistance), PC (Penicillins), CS (Cephalosporins), CP (Carbapenems), FQ (Fluoroquinolones), AG (Aminoglycosides), MLS (Macrolides, Lincosamides, Streptogramins), TC (Tetracyclines), MISC. (Miscellaneous).

Isolates highlighted in bold and with grey shading are multidrug-resistant.

Table S3.

Antibiotic phenotype	CPS isolates (33/68)	CNS isolates (35/68)	*P* value*
Oxacillin - R	16	31	0.0005
Oxacillin - I/S	17	4	
Cefoxitin - R	3	30	<0.0001
Cefoxitin - S	30	5	
Cefalexin - R	3	30	<0.0001
Cefalexin - S	30	5	
Doripenem - R	2	26	<0.0001
Doripenem - S	31	9	
Imipenem - R	1	15	<0.0001
Imipenem - S	32	20	
Ciprofloxacin - R	1	14	0.0002
Ciprofloxacin - S	32	21	
Moxifloxacin - R	2	13	0.0028
Moxifloxacin - I/S	31	22	
Gentamicin - R	5	29	<0.0001
Gentamicin - S	28	6	
Tobramycin - R	6	30	<0.0001
Tobramycin - S	27	5	
Erythromycin - R	6	17	0.0107
Erythromycin - I/S	27	18	
Clindamycin - R	11	30	<0.0001
Clindamycin - I/S	22	5	
Quinupristin/dalfopristin - R	5	20	0.0004
Quinupristin/dalfopristin - I/S	28	15	
Tigecycline - R	6	4	0.5066
Tigecycline - S	27	31	
Chloramphenicol - R	0	5	0.0539
Chloramphenicol - S	33	30	
Fusidic acid - R	31	35	0.2318
Fusidic acid - S	2	0	
Rifampicin - R	8	29	<0.0001
Rifampicin - I/S	25	6	
MDR	12	32	<0.0001
Non MDR	21	3	

* *P* value for the null hypothesis that the groups are not significantly different

Chapter 6

Pit latrine antibiotic resistant *Staphylococcus* spp. and *E. coli* isolates from a peri-urban community in South Africa - biofilm formation on household surfaces and the impact of a reference biocide and two commercial household cleaners

Abstract

Current pit latrine emptying practices enable the transfer of potential bacterial pathogens onto household surfaces. Subsequent biofilm formation can cause the cross contamination of household items and food products. Therefore, the ability of selected *Staphylococcus* spp. and *Escherichia coli* pit latrine fecal sludge isolates to form biofilms was determined using the crystal violet (CV) assay and confirmed by microscopy. Thereafter, the ability of a reference biocide and two commercially available household cleaning formulations to tackle such biofilms was assessed via confocal laser scanning microscopy (CLSM). A multi-drug resistant (MDR), coagulase positive (CP) strain of the genus staphylococcus was identified as the most prolific biofilm former among the tested pit latrine isolates. Using 48 hour biofilms formed on substrate coupons, CLSM analysis showed that a 5 minute treatment with 3% formaldehyde was most effective in reducing the metabolic activity of cells within *Staphylococcus* spp. and *Escherichia coli* biofilms. While a commercial household bleach was able to reduce the metabolic activity within *Staphylococcus* spp. and *E. coli* biofilms; a biocide-free commercial, household dishwashing liquid was least effective in reducing the metabolic activity within biofilms formed. As potentially pathogenic antibiotic resistant bacterial pit latrine isolates can form biofilms on household surfaces, proper hygiene measures are essential to avoid health risks due to pit emptying practices.

Keywords: Pit latrines, peri-urban communities, South Africa, *Staphylococcus* spp., *E. coli*, biofilm formation, biocides, detergents

Introduction

Biofilms are the common mode of bacterial colonization on biotic and abiotic substrates, including typical household materials (Bellamy et al. 1998; Rusin et al. 1998). In fact,

Ojima et al. (2002) discovered the most bacterial contamination in kitchens, followed by bath rooms. Not surprisingly, biofilms play an important role in disease transmission and are therefore a serious problem in food-processing environments (Butz et al. 1993; Srey et al. 2013; Cappitelli et al. 2014). In addition to household surfaces, biofilms can persist on surfaces in clinical settings and on various public surfaces (Reynolds et al. 2005) for extended periods.

Strains of coagulase negative (CN) *Staphylococcus epidermidis* and coagulase positive (CP) *Staphylococcus aureus* are both known to form biofilms (Geoghegan et al. 2013; Büttner et al. 2015). In the case of *S. aureus*, these biofilms have been linked to persistent hospital infections (Costerton et al. 1999). Similarly, *E. coli* is known to form biofilms on diverse abiotic (Marti et al. 2017) and biotic surfaces (Amrutha et al. 2017). The successful inactivation and removal of biofilms is, in addition to factors such as environmental conditions and bacterial strain type, dependent on the types of surfaces they adhere to. Plastic, a cheap and durable polymer and glass, albeit costlier than plastic, are both common food contact surfaces in kitchens (Schmidt and Ericksson 2005; Careli et al. 2009). Despite cleaning efforts, biofilms can persist for months or even years on surfaces (Hingston et al. 2013). A potential strategy for tackling persistent biofilms is the use of an appropriate biocide. However, while some biocides do not sufficiently penetrate the biofilm, others can inactivate cells within the biofilm without destroying it (Simões et al. 2010). In addition, if biocides are not administered at the recommended dosage, bacteria within biofilm communities can become resistant (Ortega et al. 2013). It is also known that bacteria which are resistant to biocides also display resistance to antibiotics (Akimitsu et al. 1999).

Bacterial resistance to biocides can be either intrinsic, which is defined as an inherent property of the microorganism rendering it resistant to a particular biocide; or acquired, resulting from genetic changes in the microorganism by either mutation or the acquisition of plasmids or transposons (Russell 1995). However, unlike antibiotics typically sporting only one specific cellular target site thereby facilitating the development of resistance (Fraise 2002), bacteria less frequently acquire resistance to biocides due to their mostly broad spectrum of activity and action at several cellular target sites (Denyer and Maillard 2002). As bacteria within biofilms are less sensitive to biocides than their planktonic counterparts, partly due to the limited access of the biocide to biofilm embedded cells (Carpentier and Cerf 1993), the formation of biofilms can provide resistance against various antimicrobial agents (Mah and O'Toole 2001). This resilience has been attributed to the complex nature of extracellular polymeric substances

(EPS) enclosing the cells within the biofilm (Flemming and Wingender 2010) and the release of extracellular DNA (eDNA) (Olwal et al. 2018). A large proportion of South African households make use of pit latrines (Graham and Polizzotto 2013), which can serve as a potential breeding ground for MDR bacteria (Beukes et al. 2017; Beukes and Schmidt 2018). As manual pit emptying and a lack of hygiene can cause contamination of household surfaces in peri-urban communities, the aim of this study was to assess the potential of selected antibiotic resistant pit latrine fecal sludge bacterial isolates to form biofilms on typical household surfaces and thereafter to determine the impact of selected locally used commercial household cleaning formulations on the inactivation of these biofilms.

Materials and methods

Bacterial strains used in the study

All strains used in this research were initially obtained from fecal pit latrine sludge gathered in a peri-urban community in the eThekwini municipality in KwaZulu-Natal (South Africa), as previously reported (Beukes et al. 2017; Beukes and Schmidt 2018).

Biofilm formation assay and biofilm development

A selection of *Staphylococcus* spp. and *E. coli* pit latrine isolates representing different antibiotic resistance profiles (Beukes et al. 2017; Beukes and Schmidt 2018) - including two MDR isolates in each group (CPS12, CNS17, EC9 and EC10) - were used for the biofilm assay. The biofilm assay employing crystal violet was essentially carried out over 96h according to a procedure described by Beukes and Schmidt (2012). However, nutrient broth and glass Petri dishes were used instead of Leucoberbelin blue medium and plastic Petri dishes. In addition, staphylococcus strain CPS7 was used as a representative to demonstrate biofilm development on plastic coupons over a 96h period and to determine the biocide effect on cells, using scanning electron microscopy (SEM) analysis.

Biofilm inhibition testing using CLSM

CLSM analysis was carried out using a Zeiss LSM 710 laser scanning confocal microscope. 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) (Sigma Life Sciences) reduction by metabolically active cells was detected via the red fluorescence of the formazan formed after excitation at 568nm and measurement at 590nm using a long pass filter (566-670nm). 4',6-Diamidino-2-phenylindole (DAPI) (Sigma Life Sciences) bound to double stranded DNA was detected as blue fluorescence after excitation at 405nm and measurement at 460nm using a

long pass filter (410-507nm). The commercial dishwashing liquid, in the absence of DAPI, CTC and bacterial cells, showed some background fluorescence when excited at 405nm (Fig. 1). However, no background fluorescence was detected when the commercial dishwashing liquid was excited at 568nm (Fig. 1). For confocal analysis, biofilms of strain CPS12 and *E. coli* EC10 were grown on sterile glass slides (26×76mm, average thickness of 1-1.2mm) for 48h. For this purpose, glass slides were individually added to sterile glass Petri dishes (90mm). Thereafter, 25ml of the test strain cultures pre-grown in nutrient broth at 25°C to about 2×10^8 cells per ml were aseptically added to the Petri dishes, followed by incubation for 48h at 25°C. After incubation, glass slides with biofilms were aseptically removed and gently rinsed with sterile distilled water and left to air dry in a clean bench cupboard and thereafter exposed for 5 min to water (control), the reference biocide (3% formaldehyde) and two cleaning formulations (dishwashing liquid at 0.1% (v/v) and 1.25% (v/v) bleach with a 0.07% concentration of NaOCl in solution). Following exposure, the biofilm was again gently rinsed with sterile distilled water to remove any dislodged cells and left to air dry.

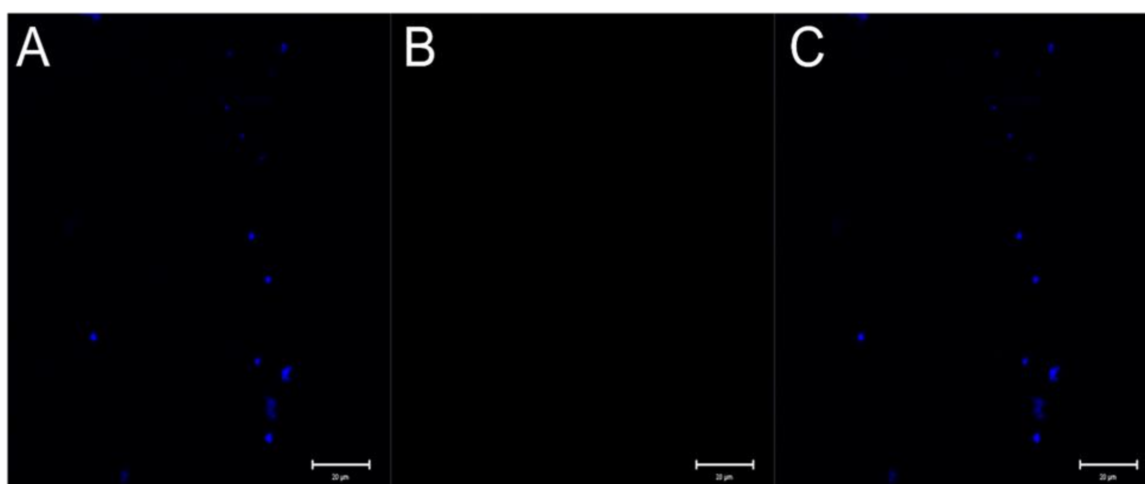

Fig. 1 CLSM micrographs of an unstained sample of 0.1% Dishwashing liquid, excited with a (A) 405nm and (B) 568nm laser line and (C) shows combined laser line signals.

These slides were then stained with CTC and DAPI to determine the presence and metabolic activity of cells within biofilms after treatment using CLSM. The staining procedure was carried out according to Tashyreva et al. (2013) with minor modifications. The biofilms were stained with a 1mM CTC solution in sterile distilled water and incubated in the dark for 45min at room temperature. After staining, slides were rinsed with sterile distilled water to remove

any unbound stain. Thereafter, slides were stained with a 5µg/ml DAPI solution in sterile distilled water and incubated in the dark for 15min at room temperature. After staining, slides were rinsed with sterile distilled water to remove any unbound stain and stored in the dark until qualitative CLSM analysis was performed. To quantify the effect of biocide treatment on the metabolic activity of cells present within the biofilm of pit latrine isolate CPS12 (a prolific biofilm former), mean pixel intensity values at 590nm, as a measure of metabolic activity, were determined in at least two selected regions of interest with biofilm present (134.89×134.89µm, pixel size 0.13µm, DAPI fluorescence used as an indicator for the presence of biofilm), using ZEN 2010 software and a Zeiss LCI Plan Neofluar 63x lens. The scan speed was set at 3 and a pixel scanning average of 4, which is equal to a pixel dwell time of 25.21µsec.

Chemicals

Two formulations for household cleaning were bought from a local supermarket and unless otherwise mentioned, other chemicals used in this study were of the best quality commercially available.

Results and discussion

Identification of the most prolific biofilm former

Among the *E. coli* pit latrine isolates, MDR *E. coli* isolate EC10 was identified as the most prolific biofilm former up to 72h incubation (Fig. 2A). Overall, among the twenty two tested pit latrine isolates, the MDR staphylococcus isolate CPS12 was identified as the most prolific biofilm former based on the CV assay (Fig. 2B). *Staphylococcus* spp. are well known as prolific biofilm formers (Stepanović et al. 2000), and the strong biofilm forming abilities of CP staphylococci such as *S. aureus* have been accredited to cell wall proteins, the expression of which varies between strains and is dependent on growth conditions (Mack et al. 1996; Foster et al. 2014). SEM analysis confirmed the strong biofilm forming abilities of antibiotic resistant staphylococcus pit latrine isolates. Sterile plastic coupons were as expected free of detectable biofilm at 0h incubation, while a single layer of staphylococcal (CPS7) cells had typically developed after 24h incubation (Fig. 3A), increasing to two cell layers after 48h (Fig. 3B), more than three layers of cells after 72h (Fig. 3C) and up to 5-7 layers of clustered cells after 96h incubation (Fig. 3D).

Biofilm treatment effects

Formaldehyde

According to CLSM analysis, a 5min treatment with the reference biocide formaldehyde (3%) caused a visible reduction in metabolically active cells within the staphylococcus strain CPS12 biofilm (Fig. 4D; Fig. 9), when compared to the untreated control (Fig. 4A), based on at least two selected regions of interest with biofilm present.

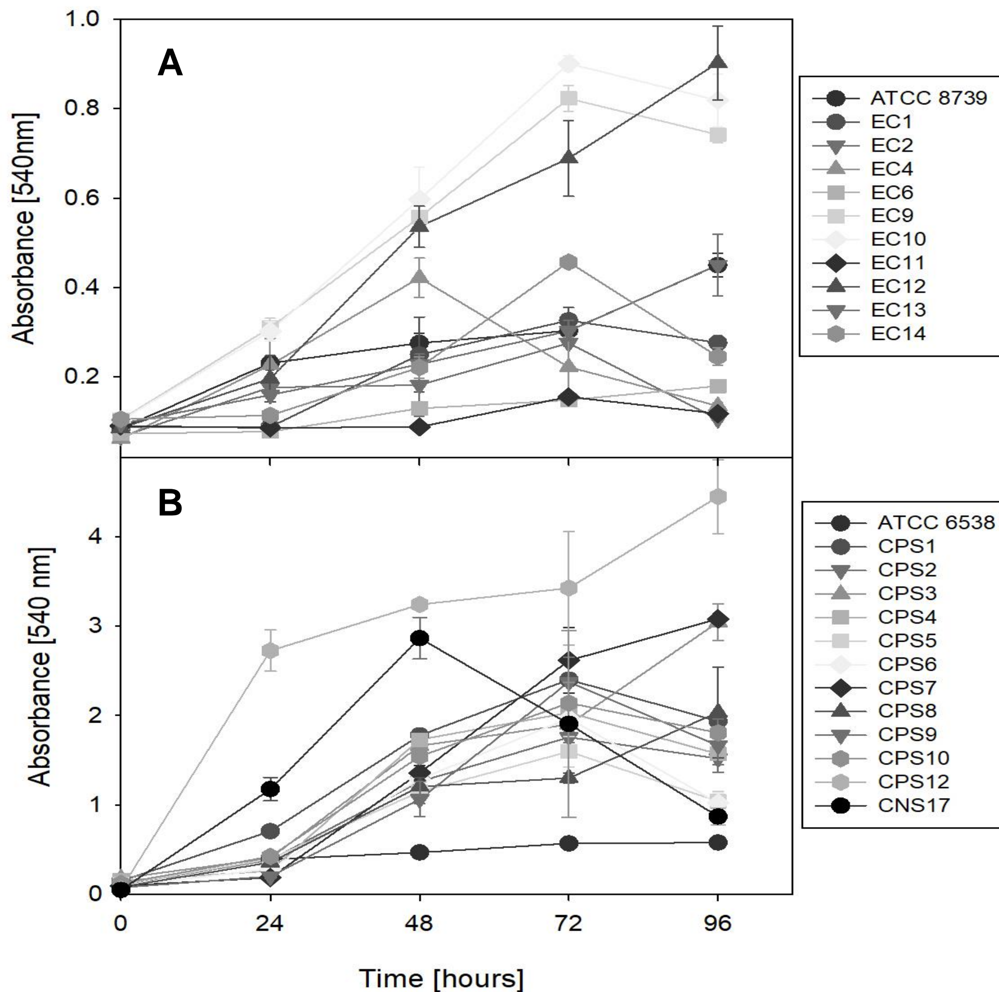

Fig. 2 CV biofilm assay to evaluate the most prolific biofilm former among the 10 selected *E. coli* pit latrine isolates (A) in comparison to the 12 selected *Staphylococcus* spp. pit latrine isolates (CP and CN) (B). *E. coli* (ATCC 8739) and *Staphylococcus aureus* (ATCC 6538) were employed for comparison. The data shown are the means obtained from measurements done in duplicate experiments.

In addition, 3% formaldehyde caused a notable difference in cell morphology within the staphylococcus biofilm (CPS7) after treatment (Fig. 5B), in comparison to the control (Fig. 5A). Based on the qualitative assessment of the MDR *E. coli* isolate EC10 biofilm, CLSM analysis revealed that a 5min treatment with 3% formaldehyde (Fig. 6E) clearly reduced the number of respiring cells in comparison to the control (Fig. 6B). Formaldehyde has long been used as sterilizing agent in hospitals for heat-labile and electrical equipment (Nyström 1991). It is a mutagenic agent, alkylating carboxyl, hydroxyl and sulfhydryl groups in the target organism (Loveless 1951; Phillips 1952), particularly in the cytoplasm of bacterial cells. In a study by Penna et al. (2001) on planktonic cells, a 0.5-1.0% aqueous solution of formaldehyde provided a viable cell reduction of 6-9 $\log_{10}$, with an average minimum inhibitory concentration (MIC) of 156mg/L reported for *S. aureus* and *E. coli* for overnight decontamination of items. According to Spicher and Peters (1976), Gram positive bacteria such as *S. aureus* and *Enterococcus faecalis* are more resistant to formaldehyde than Gram negative *Proteobacteria* such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

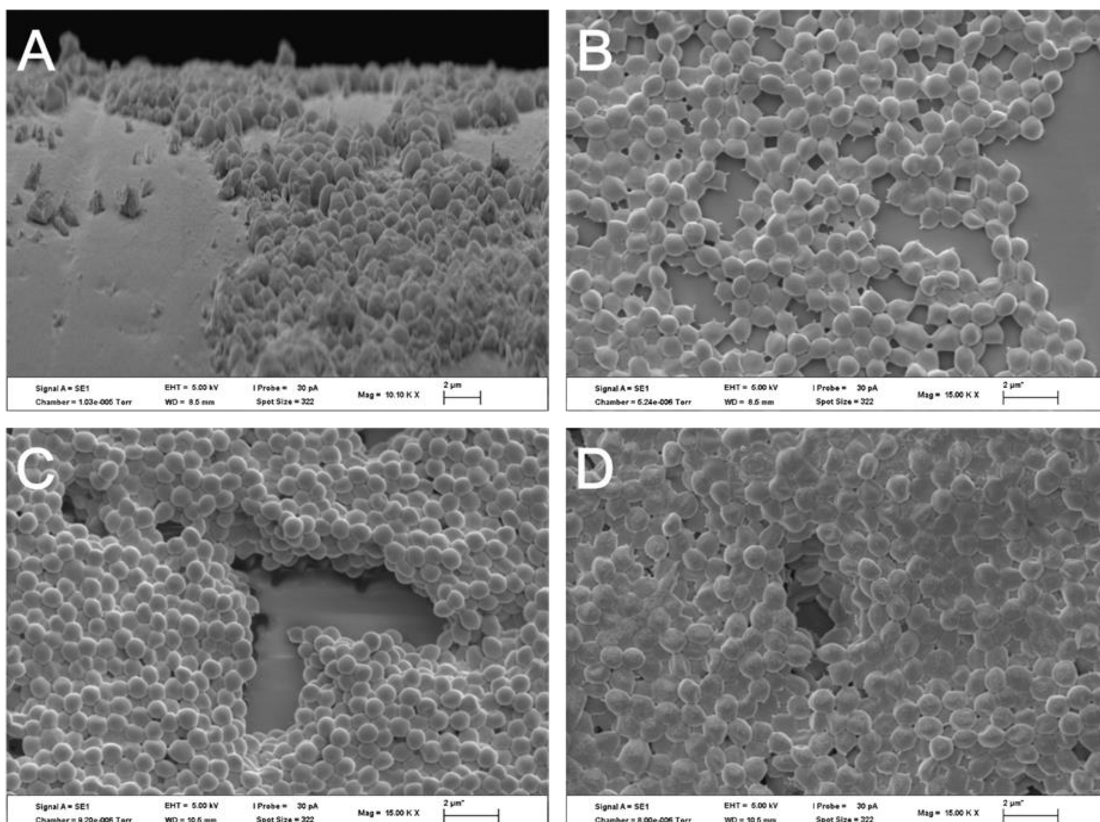

Fig. 3 SEM micrographs of staphylococcus isolate CPS7 biofilms at 24 (A), 48 (B), 72 (C) and 96 (D) hours incubation.

After 2h exposure, formaldehyde concentrations of 0.9% and 3.2% were effective against *S. aureus* and *E. faecalis* respectively (Spicher and Peters 1976). In the current study, based on the biofilm regions with formazan present due to actively respiring cells (Fig. 4D and Fig. 6E), the staphylococcus biofilm was more resistant to formaldehyde than the *E. coli* biofilm, matching the biocide susceptibility results obtained by Spicher and Peters (1976). While formaldehyde was effective in reducing cell metabolic activity within both staphylococcus and *E. coli* biofilms, it was not effective in the removal of cells. This could be due to the formaldehyde triggering cross linking reactions in the EPS layer of the biofilms, thereby rendering them resistant to removal (Simões et al. 2005).

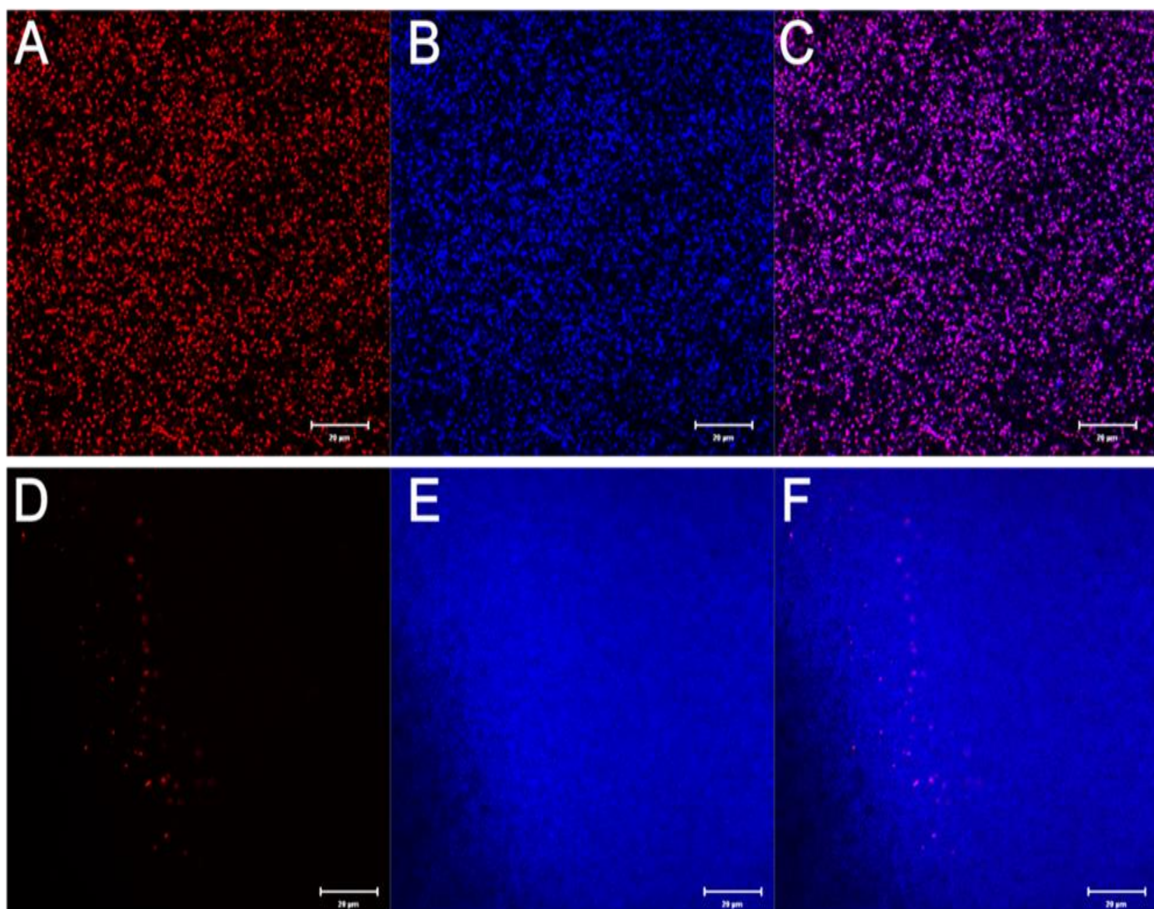

Fig. 4 CLSM micrographs of staphylococcus isolate CPS12 biofilms after 48h incubation without biocide and detergent treatment (A-CTC, B-DAPI and C-combined DAPI and CTC signals) and after 48h incubation and a 5min treatment with 3% formaldehyde (D-CTC, E-DAPI and F-combined DAPI and CTC signals).

Household bleach (Sodium hypochlorite)

A 5min treatment with 1.25% bleach (0.07% NaOCl) caused a reduction in cell metabolic activity (Fig. 7A; Fig. 9) and an apparent reduction in regions with formazan formation within the staphylococcus biofilm, in comparison to the control (Fig. 4A). For the *E. coli* biofilm, a 5min treatment with 1.25% bleach also caused a reduction in cell viability and regions with formazan formation within the biofilm (Fig. 8A).

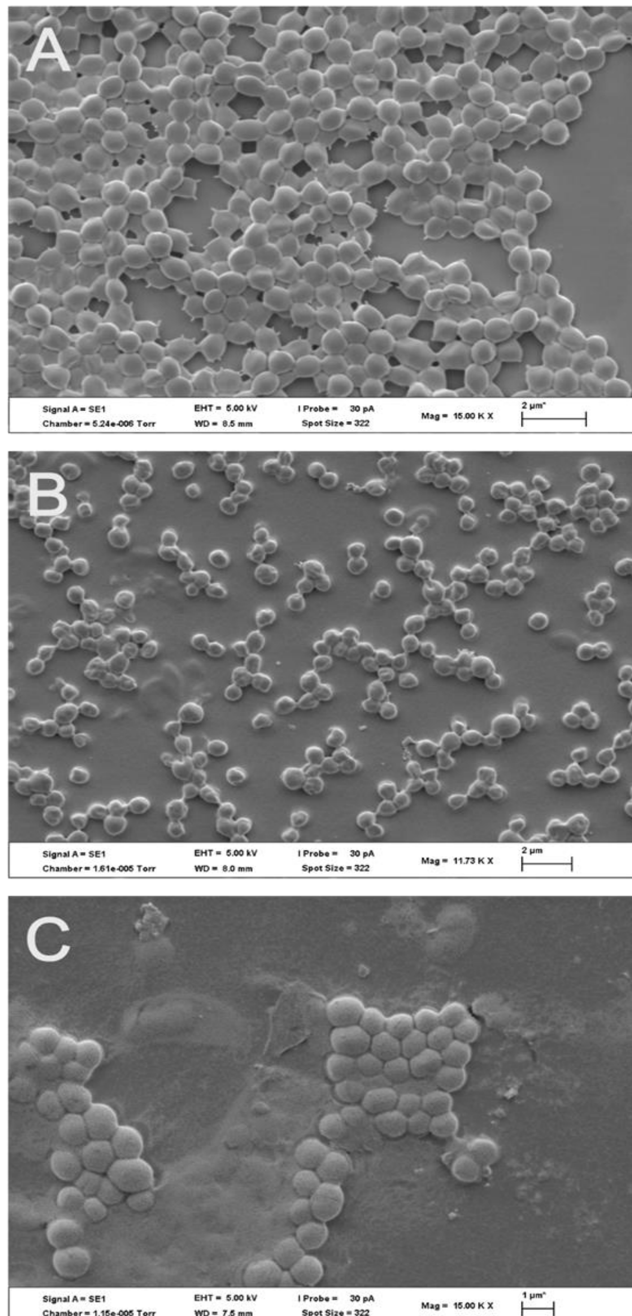

Fig. 5 SEM micrograph of staphylococcus isolate CPS7 biofilm after 48h incubation without any biocide or detergent treatment (A), after a 5min treatment with 3% formaldehyde (B) and after a 5min treatment with 0.1% Dishwashing liquid (C).

However, based on a qualitative assessment, this was more pronounced than in the staphylococcus biofilm. Sodium hypochlorite (NaOCl) is an oxidizing agent, which has long been used as a disinfectant (McDonnell and Russell 1999; Estrela et al. 2002). As an antimicrobial oxidizing agent, NaOCl interferes with bacterial cellular metabolism, causes phospholipid destruction and formation of chloramines and irreversibly inactivates enzymes involved in lipid and fatty acid metabolism (Estrela et al. 2002). Jimenez et al. (2010) found that a hard surface sanitizing spray containing 0.095% of sodium hypochlorite as active ingredient was the best disinfectant among three other tested disinfectants for the reduction of *S. aureus* and *S.* Typhimurium cells present on surfaces. This disinfectant resulted in a reduction of 3.92 $\log_{10}$ for *S. aureus* and 3.22 $\log_{10}$ for *S.* Typhimurium on an artificially contaminated non-porous plastic high chair in a child care center (Jimenez et al. 2010).

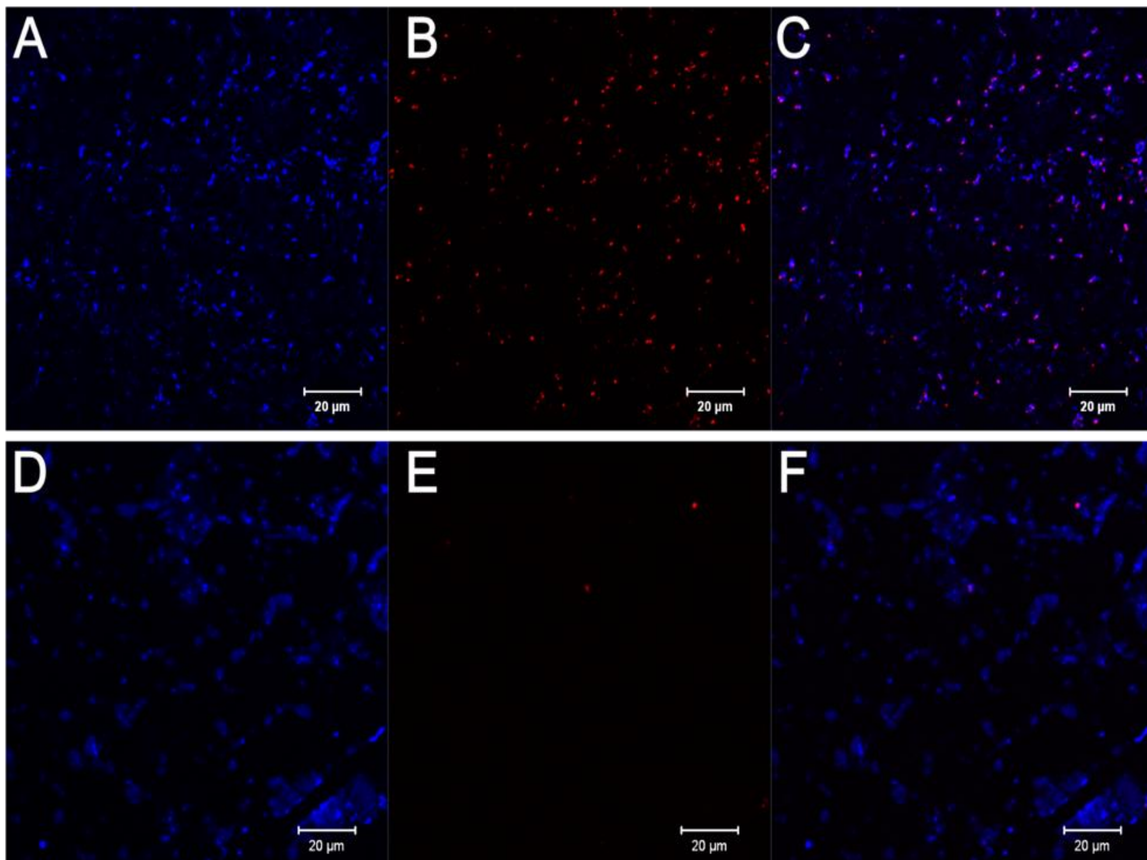

Fig. 6 CLSM micrographs of *E. coli* isolate EC10 biofilms after 48h incubation without biocide and detergent treatment (A-DAPI, B-CTC and C-combined DAPI and CTC signals) and after 48h incubation and a 5min treatment with 3% formaldehyde (D-DAPI, E-CTC and F-combined DAPI and CTC signals).

Parnes (1997) evaluated the capacity of sodium hypochlorite-based bleach at dilutions suggested for non-porous surface disinfection and dishwashing liquid detergent at levels above standard guidelines to kill and/or remove bacteria like *S. aureus*, *Salmonella* Typhi and *E. coli* from surfaces. The results of the Parnes (1997) study showed that only bleach was effective against *S. aureus* and *E. coli* and prevented the transfer of these bacteria to other surfaces. However, alternatives such as undiluted ammonia and vinegar while effective against *E. coli*, were ineffective against *S. aureus* (Parnes 1997). The antimicrobial and antibiofilm impacts of NaOCl on *S. aureus* isolates obtained from patients with atopic dermatitis were explored by Eriksson et al. (2017). NaOCl showed antibacterial effects against planktonic cells at levels of 0.01-0.08% after 1h treatment and the eradication of *S. aureus* biofilms in vitro at levels of 0.01-0.16% was observed (Eriksson et al. 2017).

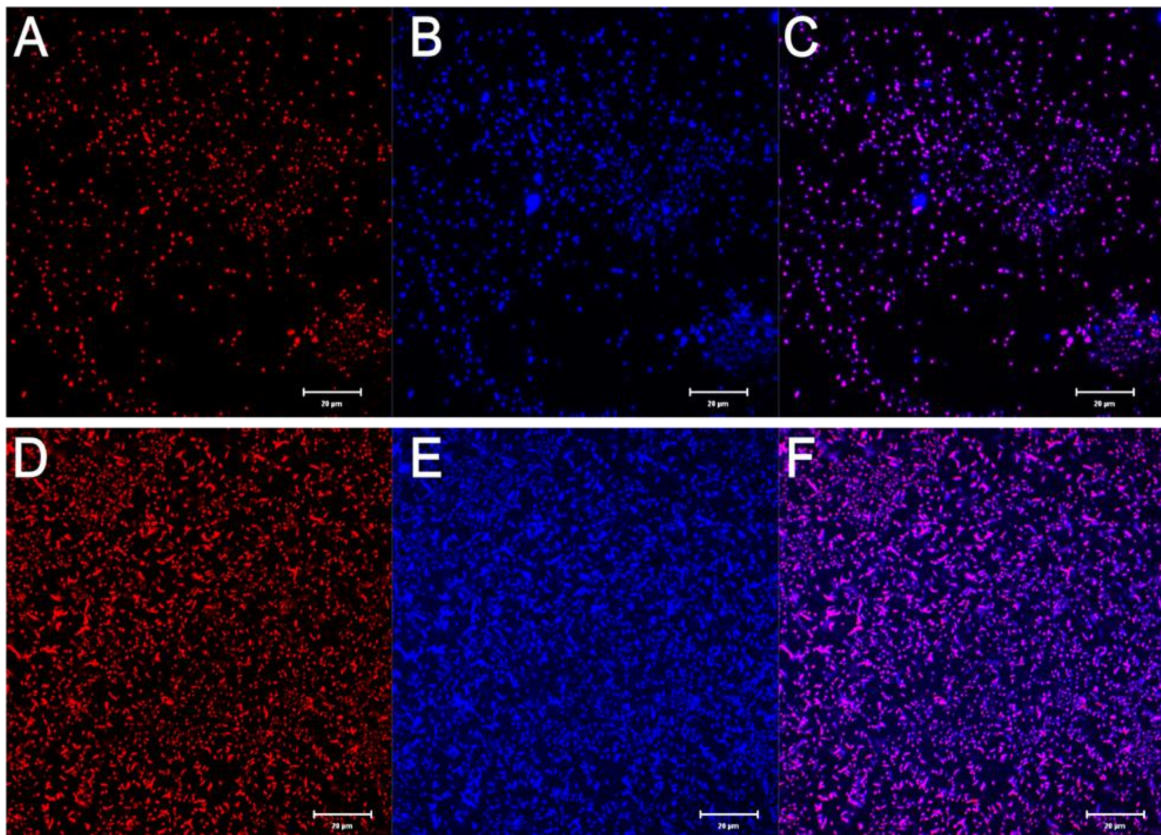

Fig. 7 CLSM micrographs of staphylococcus isolate CPS12 biofilms after 48h incubation and a 5min treatment with 1.25% Bleach (sodium hypochlorite) (A-CTC, B-DAPI and C-combined DAPI and CTC signals) and after 48h incubation and a 5min treatment with 0.1% Dishwashing liquid (D-CTC, E-DAPI and F-combined DAPI and CTC signals).

Olwal et al. (2018) found that treatment with a solution containing 150µl of waterguard (0.178M NaOCl) in 1L of water, caused a greater log reduction of CFU's/mL for *Staphylococcus epidermidis* planktonic cells than biofilm cells for both 30min and 60min treatments. In addition, *S. epidermidis* biofilms treated with 5mM NaOCl exhibited significantly increased extracellular DNA (eDNA) release than the corresponding planktonic cells (Olwal et al. 2018). However, this was not evident for the CPS12 biofilm but might have been the case for the formaldehyde treated CPS12 biofilms (Fig. 4E) as a large smear-like area of DNA was present after treatment. Lastly, Almatroudi et al. (2016) quantitatively tested the efficacy of NaOCl against *S. aureus* dry-surface biofilms using the crystal violet assay and viable plate counts and qualitatively using a live/dead stain with CLSM. Almatroudi et al. (2016) found that plate counts reduced by a factor of 7 $\log_{10}$ and biofilm biomass by a factor of 100 after a 10min exposure to 0.1-2 percent NaOCl. However, staining of the remaining biofilm showed that viable *S. aureus* cells remained after treatment, which regrew and formed biofilms after prolonged incubation (Almatroudi et al. 2016).

Notwithstanding the different exposure times employed, the results in the current study were similar to Eriksson et al. (2017), as similar concentrations of sodium hypochlorite were effective in reducing areas with formazan formation within the staphylococcus CPS12 biofilm (Fig. 7A), in comparison to the control (Fig. 4A), and similar to the results obtained by Almatroudi et al. (2016) as metabolically active cells of strain CPS12 were also present after treatment judging by the remaining areas with formazan formation. It is also worth noting that 1.25% bleach (household biocide) in the current study was less effective than formaldehyde in reducing the metabolic activity of cells within the staphylococcus biofilm but was more effective in reducing the apparent biofilm coverage based on the few regions of biofilm present after treatment (Fig. 7A-C).

Household dishwashing liquid (detergent)

After treatment with 0.1% dishwashing liquid, the staphylococcus biofilm remained mostly intact and there was a large portion of metabolically active cells present (Fig. 7D-F; Fig. 9), similar to the control (Fig. 4A). In addition, after treatment with 0.1% dishwashing liquid, cells within the staphylococcus biofilm (CPS7) appeared to be more plump (Fig. 5C) in comparison to the control (Fig. 5A) and the formaldehyde treatment (Fig. 5B). However, qualitative CLSM analysis revealed that dishwashing liquid was more effective on the *E. coli* biofilm, causing a

reduction in regions with formazan formation within the biofilm after treatment (Fig. 8E) when compared to the control (Fig. 8B).

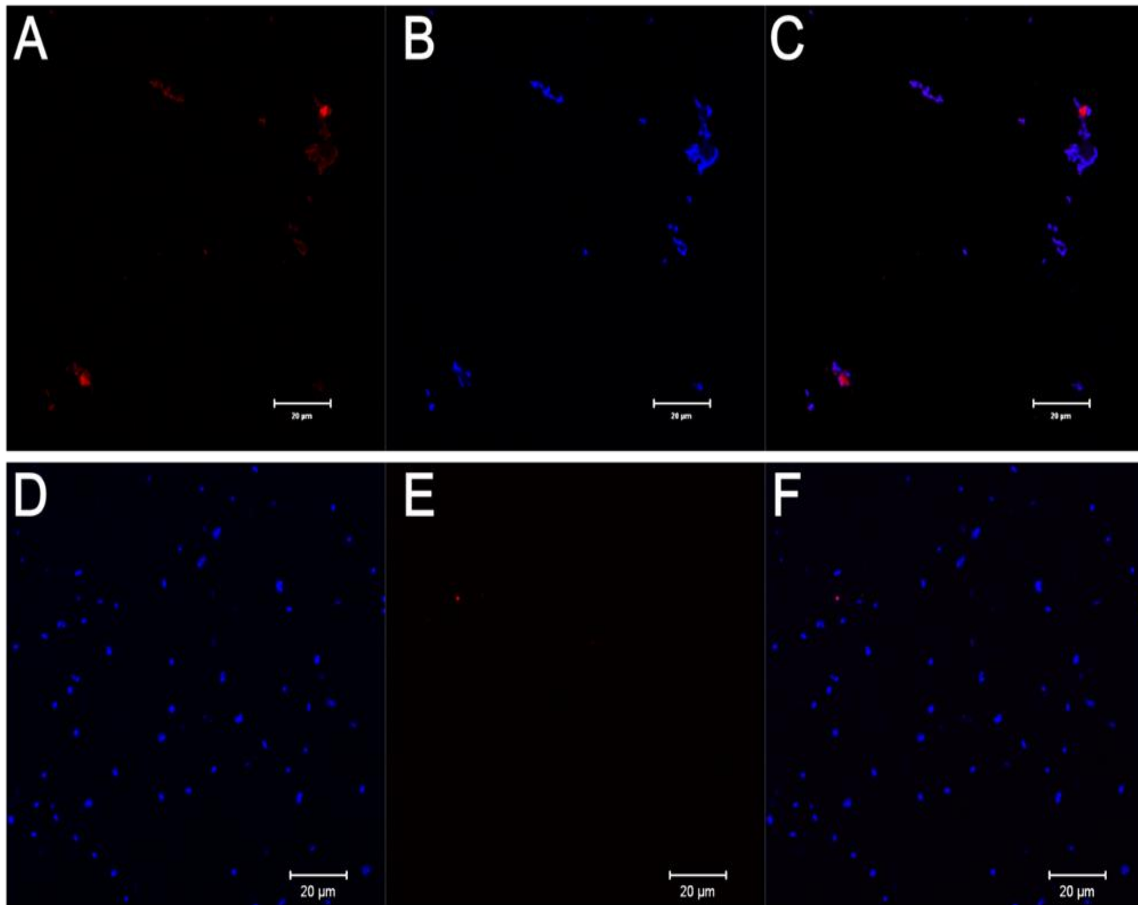

Fig. 8 CLSM micrographs of *E. coli* isolate EC10 biofilms after 48h incubation and a 5min treatment with 1.25% Bleach (sodium hypochlorite) (A-CTC, B-DAPI and C-combined DAPI and CTC signals) and after 48h incubation and a 5min treatment with 0.1% Dishwashing liquid (D-DAPI, E-CTC and F-combined DAPI and CTC signals).

Baker et al. (1941) evaluated the effects of a range of anionic and cationic detergents, at a concentration of 0.03%, on Gram positive and Gram negative bacteria. Their results indicated that Gram positive and Gram negative bacteria were equally sensitive to the action of cationic detergents e.g. Zephiran and Triton K-12 (Baker et al. 1941). All the anionic detergents tested (e.g. Cetyl sulfate, Duponol LS, etc.) selectively inhibited the metabolism of Gram positive bacteria (Baker et al. 1941). Shafa and Salton (1960) discovered that 0.2% sodium dodecyl sulfate (SDS) (anionic surfactant) at pH values between 2 and 9 resulted in the full

disaggregation of cell walls isolated from six Gram negative bacteria. However, this effect was different in intact cells of Gram negative bacteria as actively metabolizing cells can exclude SDS from its target site in the cell wall (Bolle and Kellenberger 1958). The antimicrobial activity of surfactants is influenced not only by the chemical properties of the surfactants but also by the growth conditions of the target microorganism before and during surfactant treatment (Russell 1992).

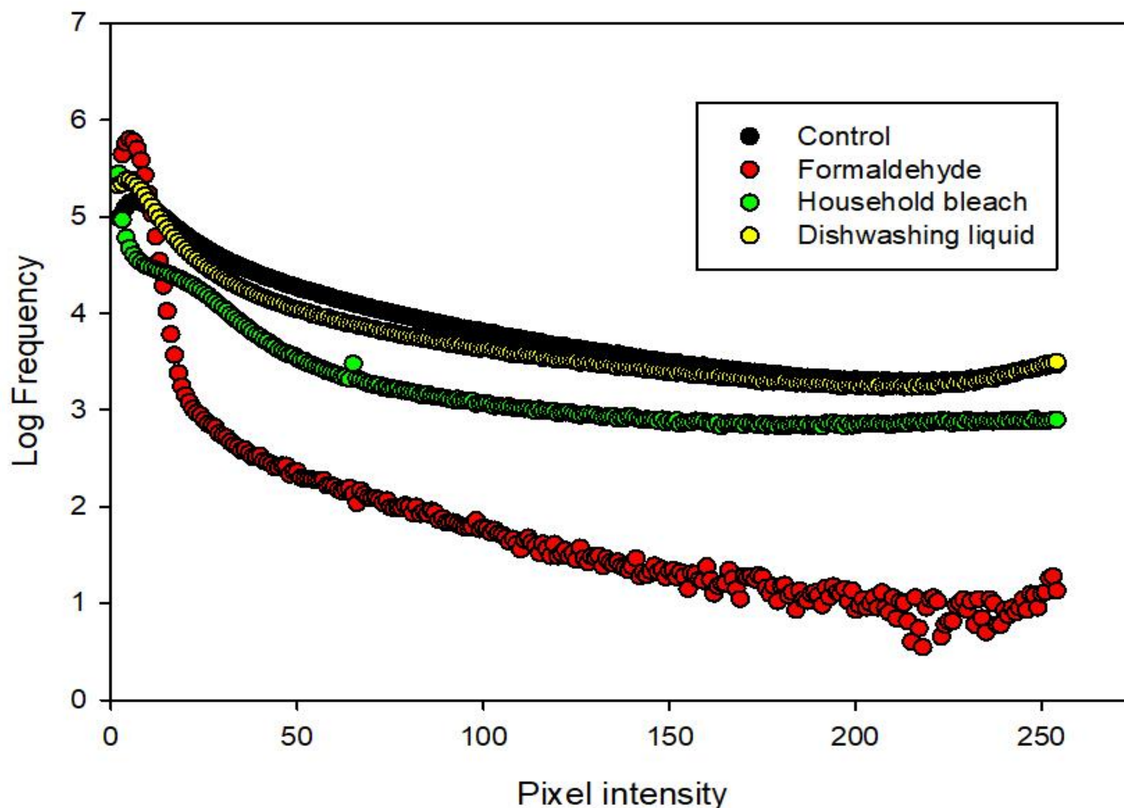

Fig. 9 Log normalized frequency of detection of metabolic activity within the staphylococcus strain CPS12 biofilm before and after treatment with 3% formaldehyde, 1.25% bleach and 0.1% dishwashing liquid. Mean pixel intensity values were determined in at least two selected regions of interest with biofilm present (134.89 x 134.89µm, pixel size 0.13µm) at 590nm.

Kim et al. (2015) exposed *S. aureus* and *E. coli* planktonic cells to plain and antibacterial soaps for 20s at 22°C (room temperature) and 40°C. These temperatures and exposure times were selected to simulate hand washing conditions. Both plain and antibacterial soaps had the same formulation containing an anionic detergent except that the antibacterial soap additionally contained 0.3% triclosan. At either of the temperatures tested, there was no notable difference

in the bactericidal activity of plain and antibacterial soaps, indicating that temperature has no impact on surfactant antibacterial activity. In addition, both soaps were more effective against Gram positive than Gram negative bacteria. The results obtained by Kim et al. (2015) do not match the current study as 0.1% dishwashing liquid (containing anionic detergents) was least effective against the staphylococcus biofilm. However, this could be due to Kim et al. (2015) using planktonic cells, while the strong biofilm forming ability of pit latrine isolate CPS12 in the present study probably rendered cells embedded therein less susceptible to the detergent. Voss (1963) found that in the presence of low concentrations of divalent cations, the bactericidal effectiveness of two alkyl benzene sulfonates and three other types of anionic surfactants against *S. aureus* increased. After a 10min exposure to 25ppm (0.0025%) of C12ABS surfactant alone, on average 85% of *S. aureus* survived and after the addition of Ca^{2+} (cations), even though insufficient to convert all of the C12ABS to the calcium salt, only 28% of the *S. aureus* cells survived (Voss 1963). Voss (1963) also observed that this effect varied between cations and that low concentrations of C12ABS had little or no killing action on *E. coli*. In the current study, dishwashing liquid was found to be one of the weaker biocides as it caused the lowest visible reduction in cell metabolic activity after treatment of the staphylococcus biofilm (Fig. 7D). However, it was useful in reducing regions with formazan formation thus inactivating cells within the *E. coli* biofilm (Fig. 8E) in comparison to the control (Fig. 6B) and the other treatments.

Gram positive versus Gram negative bacteria

Gram negative bacteria are frequently more resistant to antimicrobial agents than many Gram positive bacteria (Russell and Gould 1988; Russell et al. 1997). However, this depends on the antimicrobial agent used, the bacterial species tested and the growth conditions of the bacterial strain. The manner in which the bacterial cells are grown and their state - e.g. planktonic versus biofilm cells - affects their susceptibility to biocides (Brown and Williams 1985). Tachikawa et al. (2005) found that biofilm cells were more resistant to biocides such as hypochlorite (HOCl) than planktonic cells and the extent of resistance varied with the structure of biofilms formed and the biocide tested. Cellular impermeability is one of the resistance mechanisms of bacterial biofilms, which reduces the concentration of a chemical as it interacts with, and penetrates the various layers of the biofilm. (Anderson and O'Tool 2008; Tart and Wozniak 2008). Olwal et al. (2018) further proposed that the enhanced release of eDNA in reaction to sub-lethal heat and oxidative stress exposure can enhance the resistance of biofilms to physico-chemical stress. This eDNA release was also evident in the staphylococcus biofilm in the

current study, which displayed a large smear-like area of DNA (Fig. 4E) after treatment with 3% formaldehyde. The stronger biofilm forming ability of staphylococcus strain CPS12 might explain why this Gram positive pit latrine isolate was more resistant to the tested biocides and detergents than the Gram negative *E. coli* strain EC10 based on the regions of formazan present after treatment.

Previous studies demonstrated that confocal microscopy and fluorescent probes are useful tools for the examination of biofilms both qualitatively and quantitatively (Korber et al. 1994; Jun et al. 2010). A study by Huang et al. (1995) determined respiratory activity within biofilms during disinfection with monochloramine using CTC as an indicator for the presence of metabolically active cells, which matches the current study where CTC was successfully employed to determine the metabolic activity within biofilms after biocide treatment by means of confocal microscopy. The reliability of CTC was also verified using an inactivated (via autoclaving) CPS12 biofilm (Fig. 10). A lack of detectable fluorescence signal for CTC after excitation indicated that the reduction of CTC only takes place in the presence of metabolically active bacterial cells.

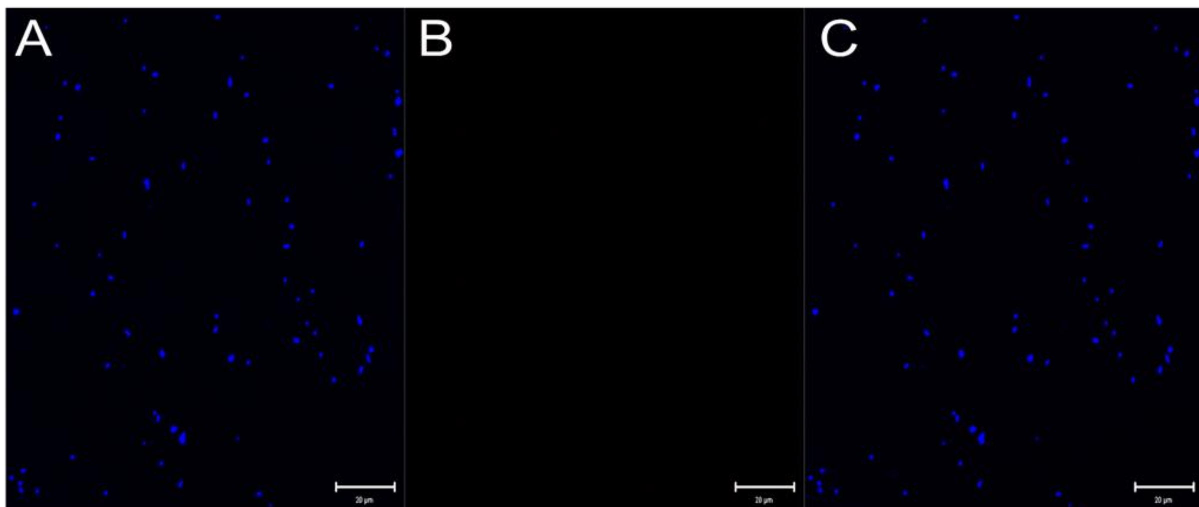

Fig. 10 CLSM micrographs of an autoclaved isolate CPS12 48h biofilm stained with DAPI (A) and CTC (B). Micrograph C shows combined DAPI and CTC signals.

Conclusion

Overall, the results in the current study indicate that generally *Staphylococcus* spp. pit latrine isolates were more prolific biofilm formers than *E. coli* pit latrine isolates. In addition, 3% formaldehyde, while not feasible for use in households due to its toxicity, was clearly more

effective than household bleach and dishwashing liquid in reducing the proportion of active cells within MDR CPS and *E. coli* biofilms. Although *E. coli* isolates did not display a strong biofilm forming capacity based on the CV assay, these isolates still present a hygiene challenge if they make contact with food items and they can pose an even greater risk if they become incorporated into established biofilms. This is a major concern since biofilms have the ability to promote the horizontal transfer of genes, including those of antimicrobial resistance and virulence.

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Chapter 6: Appendix

Antimicrobial activity of a sporicidal bedscreen curtain on selected MDR and non-MDR *Staphylococcus* spp. and *E. coli* pit latrine isolates

Abstract

Infection control procedures play an important role in clinical environments to prevent and reduce the spread of disease. Patients in hospitals can be exposed to potential pathogens on contaminated healthcare garments, uniforms, bedscreen curtains and other fabrics through direct or indirect contact. The aim of this study was to qualitatively assess the bacteriostatic activity of a commercially available sporicidal bedscreen curtain against multidrug resistant (MDR) and non-MDR coagulase positive and negative *Staphylococcus* spp. (CPS, CNS) and *Escherichia coli* pit latrine isolates. Zone of inhibition (ZOI) testing was done to evaluate whether the curtain can inhibit and prevent the growth and reproduction of bacteria on its surface. Zone diameters for *E. coli* and *Staphylococcus* spp. (CPS and CNS) pit latrine isolates were compared to zones of inhibition stipulated by the manufacturer of the bedscreen curtain, against *E. coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) respectively. The average zone diameter established for both the CPS and CNS isolates tested in the current study was 14mm and 11mm for the tested *E. coli* pit latrine isolates while *E. coli* isolate EC26 was the only isolate displaying a smaller zone of inhibition than that stipulated by the manufacturer. The remainder of the *E. coli* and staphylococcus pit latrine isolates had larger zones of inhibition than the zones stipulated for these two microorganisms by the manufacturer. In addition, the sporicidal bedscreen curtain was more effective against *Staphylococcus* spp. isolates as indicated by the larger zones of inhibition for these isolates. Overall, the results indicate that the tested bedscreen curtain can inhibit the growth of MDR and non-MDR CPS, CNS and *E. coli* pit latrine isolates and can thus provide an effective infection control barrier in peri-urban healthcare facilities.

Introduction

Patients, healthcare workers, and visitors in healthcare facilities can be exposed to potential pathogens via hand-touch contact (Cheng et al. 2015), contaminated healthcare equipment, garments, bedscreen curtains, and other fabrics through direct or indirect contact (Whyte and Bailey 1985; Gaspard et al. 2009). Despite infection control measures such as scrupulous hand

washing by healthcare staff before and after contact with patients and medical procedures (Corcoran and Kirkwood 1999), items such as clinical surfaces and bedscreen curtains that are frequently touched can be contaminated with potential pathogens, making them important sites of bacterial contamination and thus reservoirs for the transmission of infectious diseases (Das et al. 2002; Ohl et al. 2012). Due to cross-contamination of bedscreen curtains, cross-infection can occur (Old 1998) as some bacteria are able to survive on clinical fabrics for extended periods of time (Neely and Maley 2000). In addition, Kramer et al. (2006) found that *Clostridium difficile* spores and MRSA were able to survive on non-biocidal inanimate surfaces for up to 5 and 7 months, respectively.

Disposable antimicrobial and sporicidal bedscreen curtains can contain a variety of antimicrobial agents and are used as a passive infection prevention strategy in healthcare facilities (Schweizer et al. 2012). Sporicidal bedscreen curtains consisting of 100% polypropylene, impregnated with antibacterial and antimildew chemicals and nanometer silver particles demonstrated excellent antimicrobial and sporicidal activity for up to 6 months in an intensive care unit (Kotsanas et al. 2014). However, recent formulations have extended the antimicrobial and sporicidal activity of these curtains for up to 2 years, with not only benefits of extended protection but also a reduction in costs related to laundering and labor (Kotsanas and Gillespie 2016). The curtains are typically impregnated with a biocidal formulation, which inhibits bacteria, fungi and spores that make contact with the fabric thus preventing pathogen transmission. As community members who make use of pit latrines can become hospitalized, potentially carrying MDR *E. coli* and *Staphylococcus* spp., the aim of this study was to test the bacteriostatic activity of a commercially available sporicidal bedscreen curtain used in healthcare facilities against selected MDR and non-MDR CPS, CNS and *E. coli* pit latrine isolates.

Materials and methods

Isolation and characterization of *Staphylococcus* spp. and *E. coli* pit latrine isolates

Staphylococcus spp. and *E. coli* pit latrine isolates were previously isolated and characterized as described by Beukes and Schmidt (2018) and Beukes et al. (2017) respectively. A total of 10 randomly selected isolates (MDR and non-MDR) from each group of microorganisms was used to test the bacteriostatic activity of the bedscreen curtain including an *E. coli* (ATCC 8739) and a *Staphylococcus aureus* type strain (ATCC 6538).

Zone of inhibition (ZOI) test

ZOI testing was done to evaluate the bacteriostatic properties of the curtain fabric. For this purpose, 100µl cell suspensions of overnight cultures (Nutrient broth (Merck), 120rpm, 37°C) of selected *Staphylococcus* spp. and *E. coli* pit latrine isolates adjusted to 2×10^8 cells/ml (equivalent to 0.5 McFarland standard) using a Helber-type bacterial counting chamber (Marienfeld, Germany), were spread plated in duplicate onto 25ml Mueller-Hinton agar (Oxoid) in 90mm Petri dishes, similar to the EUCAST (2015) antibiotic susceptibility testing method. Thereafter, test disks of approximately 0.8-1mm in diameter were cut using a sterile disk cutter from the bedscreen curtain, placed on the agar plates (Fig. 1) and incubated at 35°C for 24h. After incubation, zones of growth inhibition were measured with a digital Vernier caliper (Marshal Tools, India) and all mean values obtained were rounded to the nearest millimeter. Zone diameters for *E. coli* and *Staphylococcus* spp. (CPS and CNS) pit latrine isolates were compared to zones of inhibition stipulated by the manufacturer for the bedscreen curtain biocidal efficacy against *E. coli* and MRSA respectively.

Chemicals

All chemicals used were of the best grade commercially available.

Results and discussion

The zone diameters for the CPS isolates tested ranged from 11mm (CPS31) to 18mm (CPS29) with an average of 14mm, from 12mm (CNS25, CNS18) to 16mm (CNS22) with an average of 14mm for CNS isolates (Table 1) and from 9mm (EC26) to 11mm (EC5, EC10, EC12, EC22, EC31, EC43) with an average of 11mm for the *E. coli* pit latrine isolates tested (Table 2). The tested curtain fabric was apparently more effective against CPS and CNS isolates followed by *E. coli* pit latrine isolates based on the average zones of inhibition. *E. coli* isolate EC26 was the only isolate that had an average ZOI, which was lower than the stipulated control ZOI according to the manufacturer. None of the MDR CPS, CNS, or *E. coli* isolates had an average ZOI smaller than that stipulated by the manufacturer.

Ohl et al. (2012) performed a study to determine the frequency and persistence of bacterial contamination with *Staphylococcus aureus*, MRSA, *Enterococcus* spp., vancomycin-resistant enterococcus (VRE) or aerobic Gram negative rods on standard hospital privacy curtains over a 3 week period. These authors showed that over 90% of curtains placed during this study were contaminated within a week, while others showed persistent and frequent contamination with

VRE, suggesting that hospital bedscreen curtains are rapidly contaminated with potentially pathogenic bacteria and could possibly be involved in pathogen transmission.

Table 1 Zone of inhibition test to determine the bacteriostatic effect of a sporicidal bedscreen curtain on selected *Staphylococcus* spp. pit latrine isolates.

Pit Latrine Isolate *S. aureus** (MRSA)	Zones of Inhibition Mean (mm)** **11mm**
CPS2	14
CPS9	14
CPS12	14
CPS19	14
CPS20	13
CPS22	17
CPS26	16
CPS29	18
CPS30	13
CPS31	11
S. aureus ATCC 6538	14
Coagulase negative *Staphylococcus* spp.	
CNS1	13
CNS4	13
CNS8	13
CNS12	14
CNS18	12
CNS22	16
CNS25	12
CNS27	15
CNS30	14
CNS34	14

Grey fill – MDR

*Zone of inhibition stipulated as an efficacy indicator by the manufacturer.

**All values reported are the means of two independently performed measurements rounded to the next mm value.

Mahida et al. (2014) confirmed this by demonstrating that hospital bedscreen curtains were linked to an outbreak of group A *Streptococcus* (GAS) in an ENT (ear, nose and throat) ward,

with poor hand hygiene and a lack of laundering implicated as the source of contamination of these curtains.

Table 2 Zone of inhibition test to determine the bacteriostatic effect of a sporicidal bedscreen curtain on selected *E. coli* pit latrine isolates.

Pit Latrine Isolate *E. coli**	Zones of Inhibition Mean (mm)** **10mm**
EC4	10
EC5	11
EC10	11
EC12	11
EC20	10
EC22	11
EC26	**9**
EC31	11
EC36	10
EC43	11
E. coli ATCC 8739	10

Grey fill - MDR, Isolate highlighted in bold displayed a smaller average ZOI than the ZOI stipulated by the manufacturer.

*Zone of inhibition stipulated as an efficacy indicator by the manufacturer.

**All values reported are the means of two independently performed measurements rounded to the next mm value.

However, Woodland et al. (2010) found that laundering was not completely effective in removing bacteria such as CNS present on bedscreen curtains in a podiatry clinic as there was no significant change in colony counts following laundry. In addition, McIntyre (2013) found that 93% of tested laundered fabric contained bacteria that could result in healthcare-associated infections. These studies suggest that laundering procedures are not reliable in removing all microorganisms found on hospital bedscreen curtains, making them a reservoir for potential pathogens and a source for cross-contamination in healthcare facilities.

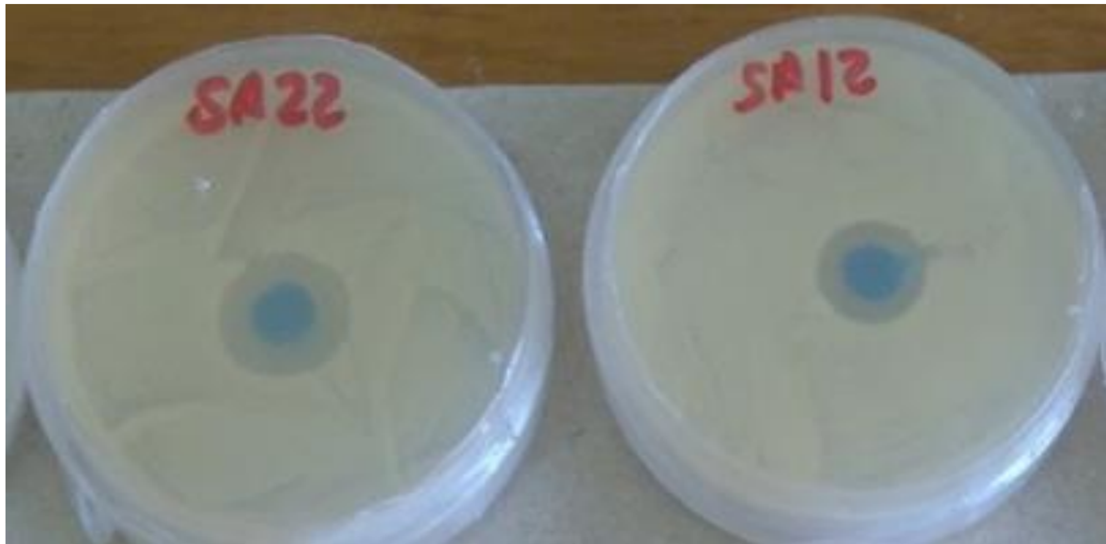

Figure 1 Zone of inhibition test showing *Staphylococcus* spp. strain CPS22 and CPS12 grown in the presence of the biocidal curtain disks after 24 hours incubation.

Thus, antimicrobial and sporicidal hospital bedscreen curtains appear more beneficial than standard hospital curtains in terms of cost and antimicrobial efficacy. Using a similar procedure (curtain fabric samples placed directly on Mueller Hinton agar inoculated with clinical isolates), Rinck (2010) compared curtains with antimicrobial properties and standard hospital bedscreen curtains in terms of cost and antimicrobial efficacy. The curtains with antimicrobial properties displayed broad-spectrum activity even against antibiotic resistant clinical isolates such as MRSA, VRE, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, *Enterobacter cloacae* and *Acinetobacter baumannii* (Rinck 2010). The standard bedscreen curtain without biocide showed no antimicrobial activity and was more expensive to maintain, due to costs associated with laundering and curtain changes. Similarly, Kotsanas and Gillespie (2016) tested the bacteriostatic properties of a commercial hospital bedscreen curtain, with both antimicrobial and sporicidal activity, against a range of antibiotic resistant microorganisms over a 24 month period. Testing followed a procedure matching the current study wherein zones of inhibition and contact inhibition were determined against a range of clinical isolates. Again, the tested curtain was found to be more beneficial than the standard bedscreen curtain with benefits of extended protection and a reduction in costs related to laundering and labor by more than 50% (Kotsanas and Gillespie 2016).

Conclusion

The results obtained in the current screening study are similar to results reported by Rinck (2010) and Kotsanas and Gillespie (2016), as the tested sporicidal bedscreen curtain displayed bacteriostatic activity against both MDR and non-MDR *Staphylococcus* spp. and *E. coli* pit latrine isolates. Sporicidal and germicidal PPE can also be recommended for use by municipal workers emptying pit latrines to prevent the spread of pathogens within communities. Additionally, the germicidal agents in the curtains can also be incorporated into surface cleaning agents as they demonstrated broad spectrum activity. The data in this study also indicate that the use of these bedscreen curtains in hospitals situated in communities who make use of pit latrines will be able to reduce or even prevent the spread of illnesses caused by MDR and non-MDR pit latrine bacteria within these healthcare facilities.

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Concluding remarks

The detection of *Escherichia coli* and *Staphylococcus* spp. on certain surfaces surrounding households and on municipal workers' skin after pit emptying indicates that the presence of pit latrines can present a health risk to both municipal workers and the individuals living in communities were manual pit latrine emptying takes place. Overall, coagulase negative *Staphylococcus* spp. (CNS) pit latrine isolates were found to be more resistant to individual antibiotics in comparison to coagulase positive *Staphylococcus* spp. (CPS) and *E. coli* isolates from pit latrine fecal sludge samples and the nitrocefin test was useful in confirming β-lactamase activity in *E. coli* pit latrine isolates displaying resistance to ampicillin. In addition, it was established that generally pit latrine isolates from the genus *Staphylococcus* are more prone to colonize typical household surfaces than *E. coli* isolates from the same environment due to their excellent biofilm-forming abilities. Formaldehyde, albeit not suitable for household use, was more effective in reducing cell viability within *Staphylococcus* spp. and *E. coli* biofilms than commercial household biocides and detergents. Additionally, the results obtained indicate that the tested bedscreen curtain was effective against multi-drug resistant (MDR) and non-MDR CPS, CNS and *E. coli* pit latrine isolates. The detection of MDR *E. coli* and *Staphylococcus* spp. in pit latrine fecal sludge samples indicates the presence of antibiotic resistant pathogens. As a result of poor hygiene and manual pit latrine emptying, the MDR bacteria found in pit latrine fecal sludge can contaminate household surfaces giving rise to the spread of antibiotic resistance genes within resilient biofilms. These biofilms may contaminate food products, which can ultimately affect the health of individuals at these households, unless proper hygiene measures are implemented. In light of the above findings, the manual emptying of pit latrines in this study should be considered a potential health risk to peri-urban communities.

Despite the effort to increase basic sanitation facilities, approximately 15% of the world's population still practice open defecation (WHO sanitation fact sheet 2018). An increase in urbanization has also caused many sanitation and hygiene problems, one of them being that of a full pit latrine. Due to insufficient space at households to dig new pits and a lack of pit emptying services, household members may be forced to resort to unsafe sanitation practices such as open defecation (Thye et al. 2011). In addition, peri-urban community members, in particular women and children, practice geophagia (Meel 2012; Mathee et al. 2014). Given that

manual pit latrine emptying and open defecation are still taking place in peri-urban communities in South Africa, geophagia poses a major health risk as pit latrine fecal sludge containing MDR *E. coli* and *Staphylococcus* spp. as well as other pathogens can be sprayed onto soil and thus be consumed. Only recently, a 5-year-old child from the Eastern Cape in South Africa died from falling into a pit latrine, further emphasizing the danger of pit latrines in peri-urban communities (Mail and Guardian 2018).

Combatting issues related to improper sanitation and poor hygiene in peri-urban communities requires a combination of finances, training, knowledge, and proper attitudes towards these practices. According to Skhosana (2001), in addition to the provision of safe drinking water and adequate sanitation facilities, there is also a need for education on proper hygiene practices. In a study based on knowledge, attitude, and practices on water, sanitation and hygiene in selected schools in Limpopo, South Africa, it was found that although learners had sufficient knowledge on safe hygienic practices, there were gaps in knowledge related to disease transmission routes (Sibiya and Gumbo 2013). In addition, there was a lack of maintenance of sanitation facilities at these schools, reflecting a poor attitude of school authorities towards these practices (Sibiya and Gumbo 2013). In order to combat antibiotic resistance, previous studies have suggested the use of alternate drug therapies to treat infections in humans due to antibiotic resistance and stewardship programs to encourage proper hygiene and sanitation regimes in peri-urban communities. Guiteras et al. (2015) found that a stewardship program combining motivation, health education and subsidies for the purchase of hygienic latrines reduced open defecation in peri-urban Bangladesh. However, stewardship programs can be very costly and not all municipalities can afford them. Dik et al. (2015) highlighted the numerous information gaps regarding the financial evaluation of antibiotic stewardship programs. The high costs of some of these programs were related to the cost of antimicrobials, medical staff, proper sanitation facilities, education, and alternate antibiotic therapies (Dik et al. 2015). Although costly, these stewardship programs can be beneficial from both a health and financial perspective in the future.

According to the integrated global action plan for the prevention and control of pneumonia and diarrhea (GAPPD) (WHO/UNICEF 2013), one of the millennium development goals for 2025 is to reduce diarrhea related deaths in children under the age of 5 to less than 1 in 1000 live births. Improving access to adequate sanitation and proper hygiene all play a pivotal role in attaining this goal. However, even though sanitation delivery in South Africa has increased

since early 2000 (South African National Sanitation Policy 2016), research does indicate that diarrheal diseases are still one of the top ten leading causes of child deaths in South Africa, where the rates of infection with human immunodeficiency virus (HIV) and tuberculosis (TB) are high (Stats SA 2019). The results obtained in the current study indicate that there is a clear lack of knowledge and/or poor attitude toward proper hygiene among municipal workers and peri-urban household members and despite the improvement of sanitation facilities, there is no guarantee that these systems will be used correctly. Therefore, there is an urgent need for education, for both municipal workers and household members, on personal hygiene and efficient handling of pit latrine fecal sludge in order to reduce the risk of contamination and the spread of disease within peri-urban communities, given that manual pit latrine emptying is taking place. Overall, this study highlights the complexity of identifying the source of bacterial contamination on municipal workers' skin and PPE and on household surfaces within peri-urban communities.

The current study only forms a first baseline hygiene survey of peri-urban households using pit latrines and therefore has limitations. Additional studies are required to identify sources and exposure pathways of enteric pathogens not targeted in the present study such as *Salmonella* spp. from pit latrine fecal sludge and to identify effective interventions. In addition, the identification of *E. coli* pathotypes and of *Staphylococcus* species present in pit latrine fecal sludge is required. Furthermore, confirmation of resistance genes in bacterial antibiotic resistant isolates from pit latrine fecal sludge should be carried out along with chemical analysis of sludge samples to identify antibiotics present therein.

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