PREVALENCE OF SOME VIRULENCE FACTORS AMONG BACTERIA ISOLATED FROM LAUNDRY WASTEWATER

BY

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CERTIFICATION

I hereby declare that this project titled **PREVALENCE OF SOME VIRULENCE FACTORS ISOLATED FROM LAUNDRY WASTEWATER** was carried out by Miracle Chisom EZEKWEMBA with (Matriculation no: LSC2009964), from the department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Edo State, Nigeria in partial fulfillment for the award of B.sc Degree in Microbiology.

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(Head of Department)

DEDICATION

This report is dedicated to God for His mercies, grace and love and also to my beloved family for their support, love and care especially my mother Mrs. Mary Agbokhie for her endless cheers and unfailing support all through the years.

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ABSTRACT

Laundry wastewater, a category of greywater, contains various chemical constituents such as soap, suspended solids, oils, perfumes, and other pollutants at high concentrations. This study investigated the prevalence of virulence factors in bacterial isolates obtained from laundry wastewater to highlight their potential health risks.

Samples were collected from residential and industrial laundry facilities, and the microbial isolates were analyzed using biochemical, molecular, and phenotypic methods. The total heterotrophic bacterial counts were recorded as 82.25 ± 4.7 CFU/mL × 10^7 in Laundry Wastewater V and 94.50 ± 6.1 CFU/mL × 10^7 in Laundry Wastewater W.

Twenty-five bacterial isolates which include *Staphylococcus aureus*, *Staphylococcus* spp., *Escherichia coli*, and *Pseudomonas* spp. were characterized. The most frequently isolated bacterium was *Staphylococcus aureus* [11/25 (44.0%)], followed by *Escherichia coli* [7/25 (28.0%)], *Pseudomonas* spp. [4/25 (16.0%)], and *Staphylococcus* spp. [3/25 (12.0%)]. Virulence factors such as DNase activity, lipase activity, and hemolytic activity were assessed. DNase activity was observed in 40% of the isolates, lipase activity in 52%, and hemolytic activity in 92%. Among *Staphylococcus aureus* isolates, DNase activity was 45.5%, lipase activity was 54.5%, and hemolytic activity was 100%. Similarly, hemolytic activity was prevalent in all *Escherichia coli* isolates (100%) and 75% of *Pseudomonas* spp. isolates.

This study highlights the high prevalence of virulence factors in bacterial isolates from laundry wastewater and underscores its potential as a reservoir of pathogenic microorganisms. Untreated discharge of such wastewater poses serious public health and environmental risks. Therefore, it emphasizes the need for enhanced wastewater treatment systems and further research on the ecological and epidemiological implications of virulence factors in laundry effluents.

CHAPTER ONE

INTRODUCTION

1.1 Background of study

Laundry wastewater is a type of grey water that contains soap, suspended particles, oil, fragrances, and other high levels of chemicals (Braga and Maria, 2014). Bacteria enter the washing machine through worn clothing, household linen, and influent water, and the laundry process is supposed to produce cosmetically and hygienically clean laundry (O'Toole *et al.,* 2009; Teufel *et al.,* 2010). Many bacteria organisms are transferred to textiles through skin contact with laundry products. Direct body contact, for example, can usually bring members of the human skin and mucosal biota to garments and towels (Smith *et al.,* 1987). Notably, large volume of water is used for laundry activities on daily basis (Massoumeh and Kargari, 2016). However, improper disposal of laundry wastewater could result into unquantifiable environmental menace especially the contamination of natural water sources such as rivers and streams. Water contamination has multiple adverse effects on living organisms (Cidu *et al.,* 2011; Frichot *et al.,* 2021; Maharjan *et al.,* 2021; Ng and Elshikh, 2021).

Water contamination can result from various sources, including metals, pesticides, fertilizers, surfactants, disinfectants, phenolic compounds, dyes, pharmaceuticals, hormones, endocrine disruptors, and microorganisms (Aonghusa and Grey, 2002; Wang et al., 2015; Peña-Guzmán et al., 2019; Patel et al., 2020; Georgin et al., 2023; Warren-Vega et al., 2023). Additionally, hydrocarbons, trace organic pollutants, pesticides, nanoparticles, microplastics, and other contaminants pose significant risks to human health, ecosystem services, and sustainable socioeconomic development (Li, 2020; Li and Wu, 2019). However, biological contaminants which include pathogenic microorganisms such as viruses, bacteria, parasites, and protistans

have been noticeably implicated in water contamination over the years (Behnam *et al.,* 2013). Bacteria are the most abundant microbes in nature and they are found in every conceivable habitat, from the soil beneath our feet to the depths of the Earth's crust, and even in extreme environments such as acidic hot springs and radioactive waste-contaminated areas (Bardgett and van der Putten, 2014; Thakur *et al.,* 2022; Rappaport and Oliverio, 2023). Water can be contaminated by wide variety of bacteria, such as *Vibrio cholerae*, *Escherichia coli*, *Salmonella* typhi etc. which can lead to diverse waterborne illnesses, including cholera, typhoid, and diarrhoea (Cabral, 2010; Al-Abdan *et al.,* 2021; Ali *et al.,* 2021).

Nevertheless, the manifestation and control of these infections induced by bacteria varies from one person to another and this is determined by host factors such as genetics, lifestyle, age, previous infections, nutrition, environment and several virulence factors (Ogunrinola *et al.,* 2020; Hou *et al.,* 2022). Environmental contaminants, chemicals, and air pollution all reduce the body's ability to combat bacterial infections (Kraemer *et al.,* 2019). Water pathogens are able to trigger infection, survive, and stay in their host due to a variety of virulence factors. Virulence factors are defined as cellular structures or specialized techniques used by pathogenic microorganisms to colonies, escape or suppress the immune response, get nutrients from the host, and detect environmental changes (Casadevall and Pirofski, 2009). These include the production of a wide range of molecules that act singly or in combination to enhance their ability to evade host defenses and cause disease (Leitão, 2020), phenotypic heterogeneity that includes antigenic variation (van der Woude and Bäumler, 2004), and dormancy capacity that allows the establishment of chronic infections (Lewis, 2010). These elements are crucial to the pathogen's ability to infect host cells, influence the host's defense systems, and affect the host-pathogen relationship. Numerous bacterial virulence factors have been identified by advances in

transcriptomics, proteomics, and genome studies; nevertheless, these factors can only be considered in relation to host-pathogen interactions (Wu *et al.,* 2008). Some important factors that contribute to the acquisition of virulence elements that result in the formation of pathogenic forms by horizontal gene transfer are toxins, adhesions, and aggressions that encode and provide the pathogenic host with virulence-associated traits (Saunders *et al.,* 2001; Keen, 2012). The bacterium's virulence is a relative indicator of the damage it can do when these adaptations cause host damage because of its pathogenic nature (Khan *et al.,* 2010). The incidence and distribution of these virulence factors in laundry wastewater reveals a gap in understanding the full scope of the public health dangers associated with improper wastewater disposal and reuse.

Aim

The aim of this study was to evaluate the presence of some virulence factors in bacteria associated with laundry wastewater in Benin City, Edo state.

Objectives of the study

The specific objectives were to:

i. determine the total heterotrophic bacterial count of laundry wastewater;
ii. characterize and identify bacteria isolates obtained from laundry wastewater;
iii. evaluate the diversity of the bacteria isolates and their level of occurrence;
iv. determine the prevalence of selected virulent factors in the bacteria isolates.

CHAPTERTWO

LITERATURE REVIEW

2.1 BACTERIAL CONTAMINATION IN WASTEWATER

In wastewater environments, both pathogenic and nonpathogenic bacteria can thrive (Abdel-Raouf et al., 2012). Harmful pathogens present in these settings include bacteria, viruses, protozoa, parasitic worms, and their eggs (Abdel-Raouf et al., 2012). Research indicates that a significant portion of the harmful bacteria contaminating aquatic ecosystems originates from human and animal waste, as well as water contaminated with feces used for domestic activities such as laundry and bathing (Gerardi and Zimmerman, 2004). While many bacteria are beneficial or harmless to their hosts, certain enteric bacterial pathogens dominate the pathogenic bacterial population in wastewater (Varela and Manaia, 2013). Common human bacterial pathogens found in wastewater include Salmonella spp., Escherichia spp., Shigella spp., Yersinia spp., Klebsiella spp., Leptospira spp. Vibrio cholerae, Aeromonas hydrophila, Legionella pneumophila, Mycobacterium spp., and Pseudomonas (Kristian Stevik et al., 2004; Maynard et al., 2005; Cai and Zhang, 2013).

Enteric bacterial pathogens such as Salmonella spp., Escherichia spp. Shigella spp., Yersinia spp., and V. cholerae are often responsible for gastrointestinal illnesses like diarrhea, dysentery, and gastroenteritis (Okoh et al., 2007; Anastasi et al., 2010; Varela and Manaia, 2013). Additionally, Helicobacter pylori, which is linked to gastrointestinal ulcers and certain cancers, may also be transmitted through water, although its exact transmission pathways remain unconfirmed (Anastasi et al., 2010). Other diseases associated with wastewater exposure include

leptospirosis (caused by Leptospira), lung infections (caused by mmL. pneumophila and Mycobacterium avium), and wound infections (caused by Pseudomonas aeruginosa (Gerardi and Zimmerman, 2004; Levy et al., 2010). Some bacteria, such as L. pneumophila M. avium, P. aeruginosa and A. hydrophila, are opportunistic pathogens that typically cause disease in individuals with compromised immunity or physical vulnerabilities, such as burns or wounds (Gerardi and Zimmerman, 2004).

Escherichia coli, a genetically diverse bacterium, is generally harmless and part of the normal gut flora in humans and other warm-blooded animals. It is commonly used as an indicator of fecal contamination, with concentrations in raw sewage ranging from 10^5 to 10^10 colony-forming units per liter. However, certain strains of E. coli possess virulence genes that enable them to cause intestinal or extra-intestinal diseases, including gastroenteritis, diarrhea, urinary tract infections, hemolytic uremic syndrome, and meningitis (Anastasi et al., 2010).

VIRULENCE FACTORS

Virulence factors are loosely defined as products that contribute to a bacterium's pathogenicity (Emtenas *et al.,* 2003). Researchers have used virulence components to conduct rigorous experiments on bacterial pathogenicity (Falkow, 2004). Toxins, adhesions, and biofilm contribute to the formation of pathogenic forms by horizontal gene transfer, resulting in virulence-associated characteristics (Saunders *et al.,* 2001; Keen, 2012).

2.2.1 Biofilm formation

Biofilms are dense aggregates of bacteria embedded within an exopolysaccharide matrix (Cvitkovitch et al., 2003). These structures are prevalent in nature and are critically involved in the development of certain infectious diseases (Donlan, 2002; Parsek and Singh, 2003). The cells within a biofilm produce extracellular polymeric substances (EPS), which consist of a complex mixture of polysaccharides, proteins, lipids, and DNA (Hall et al., 2004). Biofilms exhibit a three-dimensional architecture and represent a communal lifestyle for microorganisms, often referred to metaphorically as "cities for microbes" (Watnick and Kolter, 2000). They can develop on both living (biotic) and non-living (abiotic) surfaces and are commonly found in natural, industrial, and clinical environments. Biofilms may constitute the entire microbiome or only a portion of it. The microbial cells within a biofilm exhibit physiological differences compared to their planktonic counterparts, which are free-floating or swimming single cells in liquid media (O'Toole and Kolter, 1998).

Microorganisms form biofilms in response to various factors, such as the recognition of specific or non-specific attachment sites on surfaces, nutritional signals, and, in some cases, exposure to subinhibitory concentrations of antibiotics (O'Toole and Kolter, 1998; Karaten and Watnick, 2009). When a cell transitions to the biofilm mode of growth, it undergoes a phenotypic shift, leading to the differential regulation of numerous genes (An and Parsek, 2007). Virtually any bacterial species can form biofilms under suitable conditions, and it is estimated that more than 90% of microorganisms exist in biofilm form (Costerton et al., 1999). Due to their physiological differences from planktonic cells, biofilm-forming bacteria are highly resilient, enabling them to survive harsh environments and resist antibacterial treatments, including antibiotics (Roy et al., 2018; Hawas et al., 2022).

Biofilms are associated with a range of infections, including catheter-related infections, middle ear infections, dental plaque, gingivitis, contact lens contamination, and cystic fibrosis-related infections. They are also implicated in more severe conditions, such as endocarditis, infections of permanent indwelling devices like heart valves, joint prostheses, and intervertebral discs (Lewis, 2001).

2.2.2 Toxin production

Organic chemist Ludwig Brieger (1849–1919) coined the term “toxins”, which comes from the word toxic (Brade, 1999). As a specific byproduct of a live organism's metabolic processes, toxins are colloidal, proteinaceous, poisonous substances that are generally very unstable, extremely toxic when injected into tissues, and usually capable of causing the production of antibodies (Merriam-Webster, 2008). The two types of toxins include exotoxins, which are polypeptides generated by both Gram-positive and Gram-negative bacteria, and endotoxins, which include LPS produced by Gram-negative bacteria (Wu *et al.,* 2008; Levinson, 2010; Szentirmai *et al.,* 2021).

Lipopolysaccharides (LPS) are big molecules made up of three covalently bound components: Lipid A (which is primarily responsible for toxicity), an inner core oligosaccharide, and an outer core polysaccharide called the O-antigen (Lehmann *et al.,* 1987). Lipopolysaccharide, which is now more often used as endotoxin (Rietschel *et al.,* 1994), refers to a group of structurally similar elements found in the outermost membrane of the cell envelope of Gram-negative bacteria, including *Salmonella* and *E. coli* (Avila *et al.,* 2021).

2.2.3 O antigen

The structure and composition of the O chain vary greatly between strains, dictating the serological specificity of the parent bacterial strain (approximately 160 distinct O antigen structures are produced by various E. coli strains (Galanos and Freudenberg, 1993). The presence or absence of O chains influences whether an LPS is "rough" or "smooth". Full-length O-chains would smooth the LPS, but the absence or reduction of O-chains would roughen the LPS (Rittig *et al.,* 2003). Bacteria with rough LPS usually have more permeable cell membranes to hydrophobic antibiotics because rough LPS is more hydrophobic (Tsujimoto *et al.,* 1991). O antigen is exposed on the extreme outer surface of the bacterial cell and, as a result, is a target of recognition by host antibodies (Reatz and Whitfield, 2002).

2.2.4 Core

Typically, the core domain comprises sugars like heptose and 3-Deoxy-D-manno-oct-2-ulosonic acid (also called KDO, keto-deoxyoctulosonate), which are oligosaccharides that connect directly to lipid A (Galanos and Freudenberg, 1993). Large groups of bacteria share a common core structure, making the shape and composition of the core oligosaccharide less variable (Hershberher and Brinkley, 1968).

2.2.5 Lipid A

Lipid A is made up of two glucosamine (amino sugar) units in a β (1→6) linkage, with connected acyl chains ("fatty acids") and typically one phosphate group on each carbohydrate (Reatz *et al.,* 2009). The Lipid A moiety is a well conserved LPS component (Tyzeng *et al*., 2002). However, the structure of Lipid A varies among bacterial species. Lipid A structure has a significant impact on the degree and character of overall host immune activation (Khan *et al.,* 2018). When macrophages encounter LPS molecules, they release cytokines that cause inflammation and attract more immune cells to the infection site (Raetz and Whitfield 2002).

2.2.6 Exotoxin

Exotoxins are a class of soluble proteins produced by bacteria that enter host cells and catalyze the covalent alteration of a host cell component(s) to modify host cell function (Joseph, 2009). Exotoxins are produced by both Gram negative and Gram positive bacteria (Joseph 2009). It is found that these substances are produced in modest levels and do not excite the immune system, thus neutralizing immunological responses (Boquet and Ricci, 2014). Bacteria emit exotoxins into the extracellular space. Exotoxins are classified based on their mechanism of action, such as cytotoxins, superantigens, or membrane-damaging toxins. Toxins that damage membranes include haemolysin, which destroys red blood cells, and leukocidin, which destroys white blood cells. Superantigen toxins can trigger systemic inflammation and shock by stimulating an excessive immune response. Cytoxins can disrupt or weaken host cells' metabolic systems, leading to cell death (Jinyoung *et al.,* 2021). The cholera toxin generated by *Vibrio cholerae* is a cytotoxin that causes diarrhoea by disrupting ion transport in the stomach (Kaper *et al.,* 1995).

2.2.7 Antibiotic resistance

The phenomenon known as antibiotic resistance arises when an antibiotic loses its effectiveness in preventing bacterial growth and is characterized by microorganisms' capacity to withstand the effects of antimicrobial drugs (Beceiro *et al.,* 2013; Nadeem *et al.,* 2020). Antibiotic resistance can arise through mutations or the acquisition of resistance genes, as noted by Davies (1997) and Martinez and Baquero (2000). These resistance mechanisms are commonly observed in natural environments (Davies, 2006; Yim et al., 2007; Fajardo and Martinez, 2008). Even low levels of antibiotics can induce transcriptional changes in microorganisms that provide them with advantages (Tsui et al., 2004; Yim et al., 2006; Fajardo and Martinez, 2008). These changes are

distinct from the microbial networks associated with the general stress response (Goh et al., 2002).

Antibiotic resistance is an inherent characteristic of bacteria in environments with little human influence, such as wildlife or remote regions of the Earth (Allen et al., 2010; D'Costa et al., 2006, 2011; Dantas et al., 2008; Riesenfeld et al., 2004; Segawa et al., 2013). The production of antibiotics has a long history, spanning over 106-109 years (D'Costa et al., 2011). Naturally occurring antibiotics have been associated with various functions, including molecular signaling, transcription activation, suppression of virulence genes, bacterial adhesion stimulation, and modulation of mutation frequency (Dantas et al., 2008; Davies et al., 2006; Sengupta et al., 2013; Wright, 2007). The roles of bacteria depend on their genetic makeup and physiological context. Natural antibiotic resistance pathways are essential for maintaining normal cellular functions (Sengupta et al., 2013; Wright, 2007).

2.3 LAUNDRY WASTEWATER CHARACTERISTICS

Industrial washing is one of the most water-intensive businesses, generating significant amounts of wastewater (WW) daily, putting a strain on public sewage systems (Thiem, 2022). Industrial laundry wastewater contains large levels of organic matter and heavy metals from soiled textiles and detergents (Thiem, 2022). Wastewater is the principal pollutant in the laundry sector. Untreated effluent from laundry facilities can damage public sewage systems and natural water resources (Thiem, 2022). Industrial laundries' wastewater management relies heavily on wastewater analysis (Thiem, 2022).

The two primary criteria for analysing the pollutant level and other features of wastewater from industrial laundry are quantity and quality (Thiem, 2022). Laundry procedure, washed textiles, and washing formulas are the main elements influencing the characteristics of effluent from laundry (Thiem, 2022).

Water washing and dual-phase washing are thought to be the two operations in industrial laundries that produce the most wastewater (EPA 2000). After passing through several washing steps, supplied water enters the wastewater system. Water is introduced during the dry cleaning phase and subsequently separated during the solvent recovery process, making the dry cleaning procedure another source of wastewater (Thiem, 2022). The separated dry cleaning water is released by certain laundry facilities into the same effluent stream as other processes (Thiem, 2022). The volume of textiles, washing formulas, laundering procedures, facility size, and recycling level all affect wastewater flow (Thiem, 2022).

Laundry wastewater contains a variety of suspended particles, salts, minerals, organic debris, and pathogens originating from clothing, laundry detergents, and fabric softener residues (Howard et al., 2005). This wastewater also includes additional components such as heat, lint, soil, dyes, finishing agents, and other chemical compounds from detergents (Josef and Frederike, 2011). The inclusion of detergents raises the alkalinity and pH of the effluent, which is characterized by high levels of salt, nitrogen, phosphorus, and surfactants but has a relatively low biological oxygen demand (BOD) (Shi et al., 2018). Typical values for chemical oxygen demand (COD), BOD, and turbidity in laundry wastewater range from 375–4155 mg/L, 48–1200 mg/L, and 14–400 NTU, respectively (Manouchehri and Kargari, 2017).

Laundry wastewater is typically alkaline, with a pH ranging from 9.5 to 11, and often has a high temperature, as noted in the Finnish Industrial Wastewater Guide 2018. Common pollutant parameters in laundry wastewater include total suspended solids (TSS) at around 1000 ppm, COD at 5000 ppm, BOD at 1300 ppm, and fats, oils, and grease (FOG) at 1100 ppm (Janpoor et al., 2011). These effluents are highly complex, containing elevated levels of COD, BOD, microbial load, and toxicity, along with significant concentrations of particulate matter, proteins, starch, fats, oils, detergents, disinfectants, and pharmaceutical residues (Kern et al., 2015;

Zotesso et al., 2017). The COD and BOD in laundry wastewater arise from the use of diverse commercial formulations that include organic and mineral substances such as surfactants, builders, disinfectants, stain removers, alkalis, oxidants, bleaching agents, enzymes, softeners, optical brighteners, preservatives, corrosion inhibitors, perfumes, and waterproofing agents (Nicolaidis and Vyrides, 2014; Sheth et al., 2017; Swartz et al., 2017).

The chemical oxygen demand (COD) is a key parameter for assessing water pollution, measuring the amount of oxygen required to oxidize the contaminants in the water (Sibil et al., 2014; Morin-Crini and Crini, 2017; Tomšič et al., 2023). COD is determined by oxidizing the reducing substances in the sample using a potassium dichromate solution in an acidic medium and then measuring the results colorimetrically (Lacalamita et al., 2023). In contrast, BOD measures the aerobic decomposition of biodegradable organic matter by bacteria in the sample (Morin-Crini and Crini, 2017; Lacalamita et al., 2023). The high proportion of solids in laundry wastewater is primarily due to chemicals and soil residues from textiles (Thiem, 2022).

Table 2.1: Conventional and non-conventional pollutant concentrations of sewage generated from domestic and industrial laundries

Pollutant	Domestic Concentration Range (mg/L)	Industrial Laundry Average concentration (*) (mg/L)
BOD	100 -300	1300
COD	250 -1000	5000
TOC	100 -300	1400
TSS	100 -350	1000
Oil & Grease	50 -150	1000

(Source: EPA 1989)

The flow of wastewater affects treatment system efficiency, yet contaminants in effluent can cause contamination. Washing chemicals and soil in textiles determines the type and concentration of impurities present. Pollutants in laundry wastewater vary among industrial launderettes. Laundry wastewater contamination levels are often analysed for pH, TSS, BOD5, COD, phosphorus, and surfactants (both anionic and non-ionic) (Thiem, 2022). Analytical tests for priority and non-convention pollutants, including pesticides and herbicides, heavy metals, and contaminants including cyanide, pathogens, and nutrients, may be undertaken based on testing purposes and authority criteria (Thiem, 2022).The treatment procedure is determined based on the wastewater quality. (EPA, 1989)

Laundry wastewater quality is mostly determined by detergents, which are commonly used in industrial settings (Thiem, 2022).Washing chemicals play a crucial role in determining the quality of wastewater due to their high consumption rate (Thiem, 2022). Washing chemicals primarily remove grime from fabrics and majority of added chemicals are not left behind (Thiem, 2022). As a result, washing chemicals can enter the wastewater stream (Thiem, 2022). Laundry wastewater contains these chemicals, either in their natural form or altered through chemical processes (Thiem, 2022). The quality of wastewater is determined by the requirement to clean textiles, which are the second primary source (Thiem, 2022). Textile soil is a substantial contributor of solids to wastewater streams (Thiem 2022). The type of customer's business impacts the volume of laundered textiles and soil level, making it a factor in determining laundry wastewater quality (Thiem, 2022). Laundry effluent from a petrol station typically contains oil and grease, whereas hospital textiles may contain bacteria, germs, viruses, or blood (Thiem, 2022).

Table 2.2: Pollution Load in Wastewater from Washing Work Uniforms and Shop Towels

Pollutant	**Average Raw Wastewater Shop Towels (A)**	**Average Raw Wastewater Uniforms (B)**	**The ratio of A: B (Pounds/ 1000 pounds towels) (Pounds/ 1000 pounds uniforms)**
Total Volatile Organic Compounds	1.49	0.014	106
Total Semi-Volatile Organic Compounds	1.11	0.032	35
Total Pesticides and Herbicides			
Total Priority Pollutant Metals	0.718	0.154	4.7
Total Common Metals	16.6	3.55	4.7
Total Other Metals	0.420	0.115	3.7
BOD	46.3	8.29	5.6
COD	188	47.3	4.0
TSS	78.6	9.14	8.6
Oil and Grease	113	3.58	32
Flow (gal/lb production)	2.1	1.7	1.2

Shop towels, wipers, and rags, which are used to clean up solvents, grime, and spilt liquids at repair and machinery stations, contain high levels of volatile organic compounds (Thiem, 2022).Water from washing towels has 106 times more VOCs than water from washing uniforms (Thiem, 2022). Laundry shop towels produce five times more BOD5 in their wastewater than uniform laundry (Thiem, 2022).

2.4. HEALTH IMPLICATIONS OF CONTAMINATED LAUNDRY WASTEWATER

Laundry facilities produce large amounts of wastewater with a complex mixture of organic and inorganic contaminants, causing ecological and human health hazards (Kumar *et al.,* 2023). Textiles contaminated with pathogens (viruses, bacteria, and fungi) have been linked to outbreaks of illness, with health care workers and institutions being the most commonly affected (Bloomfield *et al.,* 2011). Untreated or insufficiently treated laundry wastewater (LWW) discharged into aquatic systems, in particular, causes environmental contamination (Akarsu and Deniz, 2021). Research indicates that typical washing products include endocrine-disrupting and asthma-related chemicals (Dodson, 2012). EDCs, which mimic oestrogen, can affect reproductive, neurological, and metabolic systems, as well as cause cancer and harmful developmental effects in humans (Colborn, 1993; Parlett, 2013; Jurewicz, 2011; Chen, 2014; Ventrice 2014).

2.4.1 Microfibers

The terms "microfiber" and "microplastic fibre" are frequently used interchangeably in the textile business in the course of discussing microplastic pollution (Xu *et al.,* 2021). In order to symbolise micro-denier products, the Japanese fibre manufacturing company Toray first proposed the idea of microfiber in the 1970s. In the 1980s and 1990s, major production of microfiber took place in Europe and America (Song, 2011). "Staple fibres or filaments of linear

density with no more than one denier and above 0.3 deniers" are the formal definition of microfibers in textile engineering (Liu *et al.,* 2019). The mass of grammes per 9000 meters of fibre is known as the denier, or simply "D," which is a unit used to characterize the linear mass density of fibres (Jianli *et al.,* 2022). A 9000-meter silk strand weighing roughly one gramme serves as the natural standard for a denier (Amutha, 2016). From the perspective of professional textile engineers, the definition of microfiber in textile engineering is a well-established agreement that has gained widespread acceptance. Nevertheless, there is still no universally accepted definition that is broad enough to cover all the elements required to characterize microfibre as a worldwide environmental contaminant with a wide dispersion.

"Microfibers are any natural or artificial fibrous materials of threadlike structure with a diameter less than 50 μm, length ranging from 1 μm to 5 mm, and length to diameter ratio greater than 100" is the broad and comprehensive definition of microfiber pollutants that we proposed in 2019 (Liu *et al.,* 2019). Although we all try to agree on a definition of microfiber from an ecological and environmental standpoint, it is a methodological challenge and a topic of ongoing debate because microfiber is one type of emerging pollutants and the research associated with it is still in its early stages (Jianli *et al.,* 2022). At first, it was more probable to characterise microfibers as synthetic fibres that were removed from garments while they were being laundered (Napper and Thompson, 2016). Approximately 700,000 microfibers, weighing around 0.5 grammes, have been reported to be released with laundry sewage with each rotation of the washing machine drum (Karkkainen and Sillanpaa, 2021; Napper and Thompson, 2016). The primary source of microfibers was thus found to be household and commercial washing (Cai *et al.,* 2020a). Each cycle, up to 700,000 can be released into the wastewater, totalling around 0.5 g (Jianli *et al.,* 2022).

Domestic washing is predicted to emit 500,000 tonnes of microfibers into the ocean globally each year (Boucher and Friot, 2017). Textiles account for approximately 14% of plastic waste output per sector and are the second leading cause of plastic pollution after packaging (Smith and Vignieri, 2021). As a result, domestic washing was first considered a major potential source of microfibers (Cai *et al.,* 2020). During laundry, synthetic fabrics emit microfibers into the environment, which can have a major and permanent effect (Henry *et al.,* 2019). Laundry operations and wastewater discharge from the textile sector are intimately associated with the release of MFs into the environment (Henry *et al.,* 2019).

2.4.2 Organic load

High levels of surfactant and Chemical Oxygen Demand (COD) in laundry wastewater reduced the LC50, indicating that the wastewater is becoming more toxic and raising the risk of wastewater pollution for the environment and biota (Esmiralda *et al*., 2012). The high biological oxygen demand in wastewater could cause a methane explosion, according to the Finnish Industrial Wastewater Guide.

2.4.3 Chemicals

Surfactants are the most active component of laundry detergents (Braga and Varesche, 2014). Water bodies become eutrophic due to phosphates found in laundry effluent. Numerous surfactants have been used and discharged as a result of industrial expansion and population growth, creating a major environmental issue (Tripathi *et al.,* 2013). They build up in the environment because they are not biodegradable and produce foaming in receiving rivers and other water sources (Nguyen *et al.,* 2015).

2.4.4 Toxin

Kohler discovered that the phosphorus in sodium tripolyphosphate of wastewater from washing causes water hyacinth, algae, and cyanobacteria to grow more (Kohler, 2006). As a result, the water's oxygen concentration decreases, hastening the eutrophication process. Using eutrophic waters or laundry effluent directly to water a vegetable garden can put human health at greater risk. Toxins generated by cyanobacteria that proliferate in the water are the source of the danger (Sivonen, 1999).

2.4.5 Microorganisms

There are several pathogenic bacteria in wastewater (Wéry *et al.,* 2008; Zheng *et al.,* 2020). *Escherichia coli*, *Enterococcus,* and *Pseudomonas*, among bacteria, *Aspergillus fumigatus*, and *Candida*, among fungi, and norovirus, sapovirus, and enteric adenovirus, among viruses, are common pathogenic microorganisms in wastewater that can cause diarrhoea, respiratory system diseases, upper respiratory tract infections, and gastrointestinal diseases in humans under certain conditions (Millner *et al.,* 1977; Greer *et al.,* 2009; Levantesi *et al.,* 2012).

2.4.6 Agriculture

Wastewater is used for irrigation in both treated and untreated forms, with its usage varying based on geographic and economic conditions. In many developing nations, the majority of wastewater is used untreated due to limited treatment infrastructure, often leading to its direct discharge into water bodies (Scott et al., 2010; Qadir et al., 2010). Untreated wastewater is particularly common in urban and peri-urban agriculture, accounting for approximately 11% of irrigated croplands globally (Thebo et al., 2014). However, untreated wastewater can contain various pollutants from municipal, agricultural, and industrial sources, posing significant health risks (Sarah et al., 2016). These risks affect farmers, agricultural workers, their families, nearby communities, and consumers of irrigated produce, including exposure to excreta-related pathogens, skin irritants, and toxic chemicals (Qadir et al., 2007).

Exposure to untreated wastewater has been linked to numerous diarrheal diseases, such as salmonellosis, shigellosis, cholera, giardiasis, amoebiasis, hepatitis A, and viral enteritis (WHO, 2006). Additionally, helminth infections like ascariasis, associated with anemia and impaired physical and cognitive development, have been connected to wastewater exposure (Bos et al., 2010). Agricultural workers frequently suffer from skin conditions, including dermatitis and rashes, due to prolonged contact with untreated wastewater (Sarah et al., 2016).

Chronic health issues are also associated with exposure to heavy metals such as arsenic, cadmium, lead, and mercury, which can accumulate through the consumption of contaminated food or occupational exposure to polluted soil (Sarah et al., 2016). For instance, cadmium buildup can lead to osteoporosis and kidney damage, a condition known as itai-itai disease in Japan, which was first linked to the use of heavily contaminated water for irrigating rice paddies (Järup, 2003). Emerging contaminants in wastewater, such as polycyclic aromatic hydrocarbons (PAHs), personal care products, and endocrine-disrupting chemicals, also pose potential health risks, though their pathways into the food chain are not yet fully understood (Dodgen et al., 2013; Wang et al., 2012).

To address these widespread health risks, organizations like the WHO have established guidelines to ensure that pollutant levels in wastewater remain below harmful thresholds (WHO, 2006). However, these guidelines recognize the challenges of achieving full treatment and provide progressive targets for different scenarios (Sarah et al., 2016). Despite the health risks, the use of untreated or inadequately treated wastewater remains critical for smallholder farmers, particularly in water-scarce or economically disadvantaged regions, as it supports livelihoods and food production (Raschid-Sally and Jayakody, 2008). In many developing cities, farmers in urban and peri-urban areas rely heavily on wastewater for irrigation, even though it contributes to a high prevalence of water-related diseases (Qadir et al., 2010). Furthermore, wastewater use is increasingly seen as a strategy to adapt to climate change, providing a reliable water source in unpredictable or arid conditions (Trinh et al., 2013).

Balancing the need for improved food security, nutrition, and livelihoods with efforts to reduce health risks remains a significant challenge (Drechsel et al., 2002). Given these concerns, further research is needed to fully understand the extent of exposure to wastewater contaminants and their health impacts (Sarah et al., 2016). This review aims to critically analyze research published since 1995 on the health risks and exposure pathways associated with agricultural wastewater use (Sarah et al., 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in in Benin City, Edo State, Nigeria using samples obtained from two commercial laundry shops. Benin City is the Capital City of Edo State and it lies between latitude 6.5^0 North and longitude 5.8^0 East of the Greenwich Meridian.

3.2 Sample Collection

A total of eight (8) laundry wastewater samples were collected from two commercial laundry shops (Laundry shop V and Laundry shop W). The samples include initial-wash effluent V1 (IW-EV1), initial-wash effluent V2 (IW-EV2), initial-wash effluent W1 (IW-EW1), initial-wash effluent W2 (IW-EW2), rinse-water effluent V1 (RW-EV1), rinse-water effluent V2 (RW-EV2), rinse-water effluent W1 (RW-EW1) and rinse-water effluent W2 (RW-EW2). The samples were properly labeled and immediately transported to the laboratory for analysis.

3.3 Enumeration of Total Heterotrophic Bacteria

The enumeration of total heterotrophic bacteria was carried out using nutrient agar culture media. Nutrient agar (NA) (Lab M, Lancashire, United Kingdom) was prepared by dissolving 28 g of the agar powder in 1000 mL of distilled water and sterilized by autoclaving at 121^{o}C for 15 minutes. The wastewater samples were diluted serially using ten-fold dilution techniques in test tubes to get eight diluents by transferring one mL of the wastewater samples into 9 mL sterile distilled water. Total heterotrophic bacterial count was determined using the spread plate method by inoculating 200 µL of diluent 10^7 from the samples in duplicates into sterile nutrient agar plates which were then incubated at 37^{o}C for 18-24 h. Enumeration of the bacterial isolates was

carried out and the mean counts were expressed as colony-forming units per millilitre (CFU/mL). Discrete colonies were purified by sub-culturing on nutrient agar plate for characterization.

3.4 Morphological and Biochemical Characterization of Bacteria Isolates

The bacterial isolates were characterized via the following morphological and biochemical assays.

3.4.1 Gram reaction

The morphological characteristics of the bacteria isolates were determined by Gram reaction test. The presumptive bacteria colony was smeared on a clean grease free slide and heat fixed over flame. The smear was flooded with crystal violet (primary stain) for 1 min then rinsed with water, flooded with Lugol's iodine solution for 30 sec and then rinsed off with water, flooded with 95% alcohol (decolorizer) and immediately rinsed off with water. Finally, the smear was counter stained with safranin for 1 min and rinsed off with water. The slides were allowed to air dry before observing under the microscope using an oil immersion objective lens of ×100 magnifications.

3.4.2 Coagulase test

Coagulase test was conducted by placing drops of saline on a grease free glass slide and a discrete colony of presumptive isolate was emulsified in the saline drops using a sterile inoculating loop to make bacteria suspension. Using a Pasteur pipette, a drop of plasma was placed on the bacteria suspension and saline drop then mixed gently. Formation of clumps in the bacteria suspension within 10-15 seconds indicates coagulase positive result.

3.4.3 Oxidase test

This was carried out by wet filter paper method using Kovacs oxidase reagent (1% tetra-methyl-p-phenylenediamine dihydrochloride. The filter paper was soaked with freshly prepared reagent

and a loopful of the bacteria isolate was smeared on the piece filter paper. A positive reaction was indicated by purple to blue coloration appearing with 5-30 seconds while absence of purple coloration was considered as a negative result.

3.4.4 Indole test

Spot indole test was conducted using a fresh culture of the test organism. Several drops of 1% p-dimethylaminocinnamaldehyde reagent were moistened on a filter paper. A loopful culture of the presumptive bacteria isolate was smeared on the reagent saturated area of the filter paper. Positive result is shown by the presence of a blue to blue-green colour change within 2-3 minutes while negative results remain colourless or appears light pink.

3.4.5 Catalase test

The catalase test was conducted by preparing a suspension of the fresh test culture using sterile distilled water on a clean glass microscope slide. A few drops of hydrogen peroxide (H_2O_2) were added to the suspension using a dropper. The formation of bubbles indicated a positive result, while the absence of bubbles suggested a negative result.

3.4.6 For the starch hydrolysis test, starch agar medium was used. The test organism was streaked in a single line onto the center of the starch agar plate, which was then incubated at 37°C for 48 hours. After incubation, the surface of the agar plate was flooded with iodine solution and left for 30 seconds. Excess iodine was then poured off. A clear zone around the growth line after adding iodine indicated starch hydrolysis (positive result), while a blue-black coloration around the streak line indicated no starch hydrolysis (negative result).

3.4.7 Hydrogen sulphide test

This test was carried out using triple sugar iron (TSI). The test organisms were inoculated by stab inoculation and the test tubes were incubated at 37^{o}C for 18-24 hours. The formation of black coloration in the medium was considered to be hydrogen sulphide positive while the absence of black coloration was recorded as hydrogen sulphide negative.

3.4.8 Motility test

The motility test was conducted using sulphide indole motility (SIM) medium. The SIM was needle-stabbed deeply with bacteria isolate of the test culture and incubated at 37^{o}C for 24-48 hours. Motility is positive when zone of growth diffuses out of inoculation line. Confinement of growth to the stab line and leaving the medium clearly transparent indicate negative result.

3.4.9 Methyl-red Voges-Proskauer (MRVP) test

The methyl red test was performed using MRVP broth. The test culture was introduced into the broth medium and incubated at 37°C for 24 hours. Following incubation, 2 mL of the broth was transferred into two separate test tubes labeled MR and VP, which were then incubated again for an additional 24 hours. After this period, methyl red reagent was added to the MR-labeled broth**,** Statistical analysis was carried out on the data using Microsoft Excel 2013. Mean values were expressed using descriptive statistics.

CHAPTER FOUR

4.0 RESULTS

4.1 Total Heterotrophic Bacterial Counts of Laundry Wastewater

The mean total heterotrophic bacterial counts of laundry wastewater were shown in Table 4.1. The distribution as observed in laundry shop V was 137±3.7 CFU/mL × 10^7 [initial-wash effluent V1 (IW-EV1)], 91±3.4 CFU/mL × 10^7 [initial-wash effluent V2 (IW-EV2)], 102±5.9 CFU/mL × 10^7 [initial-wash effluent W1 (IW-EW1)] and 154±1.4 CFU/mL × 10^7 [initial-wash effluent W2 (IW-EW2)]. The distribution as observed in laundry shop W was 43±2.9 CFU/mL × 10^7 [rinse-water effluent V1 (RW-EV1)], 58±2.5 CFU/mL × 10^7 [rinse-water effluent V2 (RW-EV2)], 91±3.3 CFU/mL × 10^7 [rinse-water effluent W1 (RW-EW1)] and 31±2.8 CFU/mL × 10^7 [rinse-water effluent W2 (RW-EW2)].

The total heterotrophic bacterial counts based on location were shown in Figure 4.2. It was observed to be 82.25±4.7 CFU/mL × 10^7 [Laundry Wastewater V] and 94.50±6.1 CFU/mL × 10^7 [Laundry Wastewater W].

Table 4.1: Total Heterotrophic Bacterial Counts in Laundry Wastewater

Sample Code	Mean Counts of Heterotrophic Bacteria CFU/mL $\times 10^7$
IW-EV1	137±3.7
IW-EV2	91±3.4
IW-EW1	102±5.9
IW-EW2	154±1.4
RW-EV1	43±2.9
RW-EV2	58±2.5
RW-EW1	91±3.3
RW-EW2	31±2.8

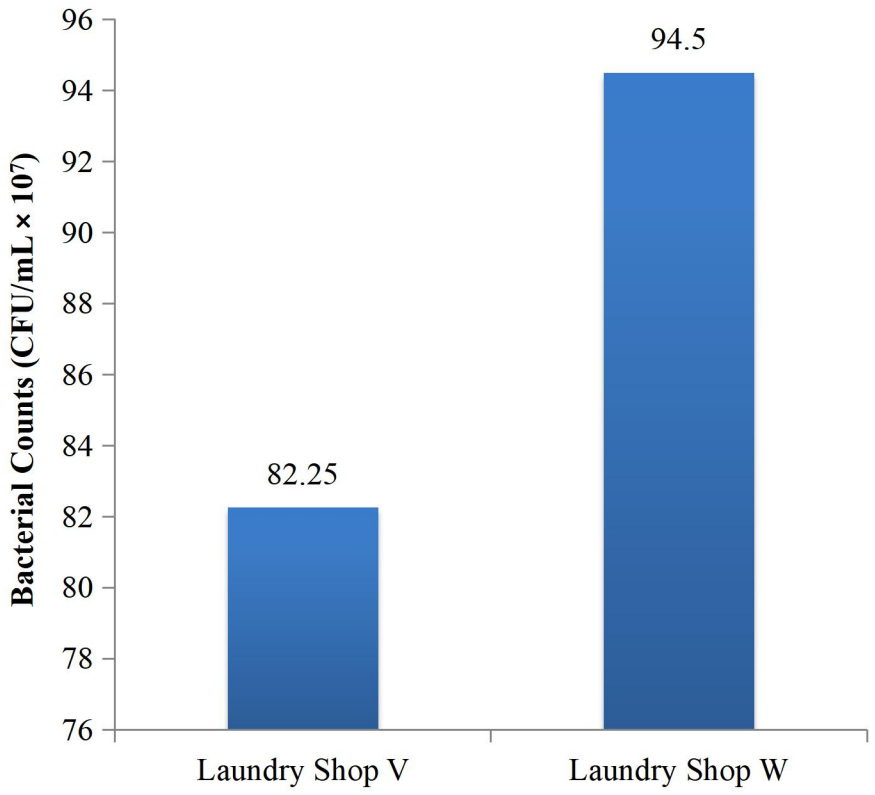

Figure 4.1: Mean Heterotrophic Bacterial Counts Based on Laundry Shop Location

4.2 Bacterial Diversity and their Level of Occurrence in Laundry Wastewater

The diversity of the bacterial population associated with laundry wastewater based on their morphological and biochemical characterization was shown in Table 4.2. In total, twenty five (25) bacterial isolates were characterized and the bacterial identified include *Staphylococcus aureus*, *Staphylococcus* spp., *Escherichia coli* and *Pseudomonas* spp. The frequency of occurrence revealed that the highest occurring bacteria were *Staphylococcus aureus* [11/25 (44.0%)]. Other bacterial isolates as characterized was *Pseudomonas* spp. [4/25 (16.0%)], *Escherichia coli* [7/25 (28.0%)] and *Staphylococcus* spp. [3/25 (12.0%)] as shown in Figure 4.2.

4.3 Evaluation of Selected Virulence Factors in the Bacterial Isolates

In this study, the prevalence of selected virulence factors as observed in the bacterial isolates was DNase activity [10/25(40.0%)], lipase activity [13/25(52.0%)] and hemolytic activity [23/25(92.0%)] as shown in Figure 4.3. The distribution as observed in *Staphylococcus aureus* was DNase activity [5/11(45.5%)], lipase activity [6/11(54.5%)] and hemolytic activity [11/11(100.0%)]. The distribution as observed in *Pseudomonas* spp. was DNase activity [1/4(25.0%)], lipase activity [2/4(50.0%)] and hemolytic activity [3/4(75.0%)]. The distribution as observed in *Escherichia coli* was DNase activity [3/7(42.9%)], lipase activity [3/7(42.9%)] and hemolytic activity [7/7(100.0%)]. The distribution as observed in *Staphylococcus* spp. was DNase activity [1/3(33.3%)], lipase activity [2/3(66.7%)] and hemolytic activity [2/3(66.7%)].

Table 4.2: Biochemical characterization of the bacterial isolates

Isolate groups	Growth on Nutrient agar	Gram staining	Shape	Catalase	Oxidase	Coagulase	Urease	Motility	Indole	Citrate	Hydrogen sulphide	Starch hydrolysis	Methyl red	Voges proskauer	Sugar fermentation					Presumptive Bacterial
															Glucose	Mannitol	Lactose	Sucrose	Maltose	
A	Yellow	+ve	Cocci	+ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	A-	A-	A-	A-	A-	*Staphylococcus aureus*
B	Greenish	-ve	Rod	+ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-G	AG	-G	-G	-G	*Pseudomonas* spp.
C	White	-ve	Rod	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	AG	AG	AG	AG	AG	*Escherichia coli*
D	White	+ve	Cocci	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	AG	-G	AG	AG	AG	*Staphylococcus* spp.

Keys: Positive (+ve); Negative (-ve); Acid and Gas Production (AG); Acid Production Only (A-); Gas Production Only (-G); Absence of both Acid and Gas Production (- -).

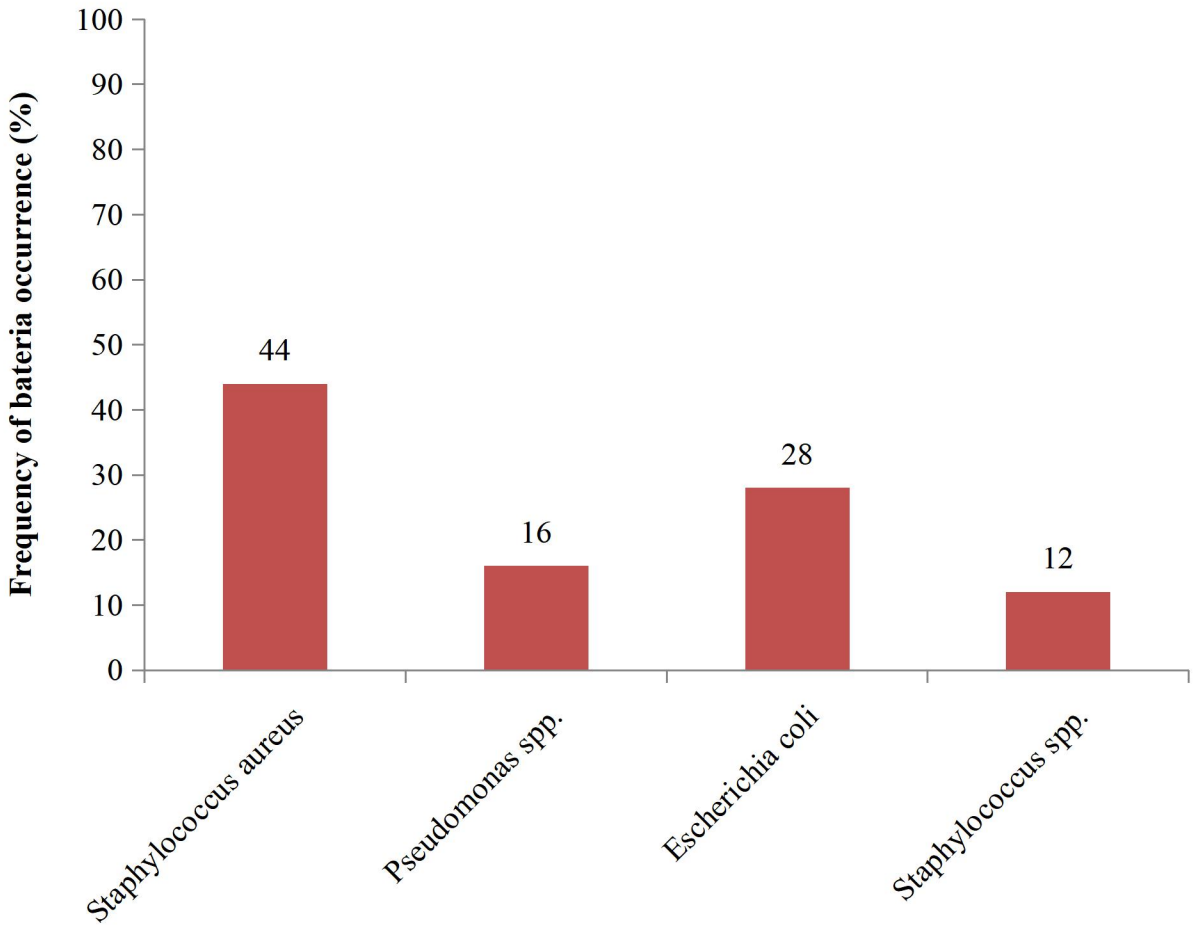

Figure 4.2: Occurrence of Level of Diverse Bacteria in Laundry Wastewater

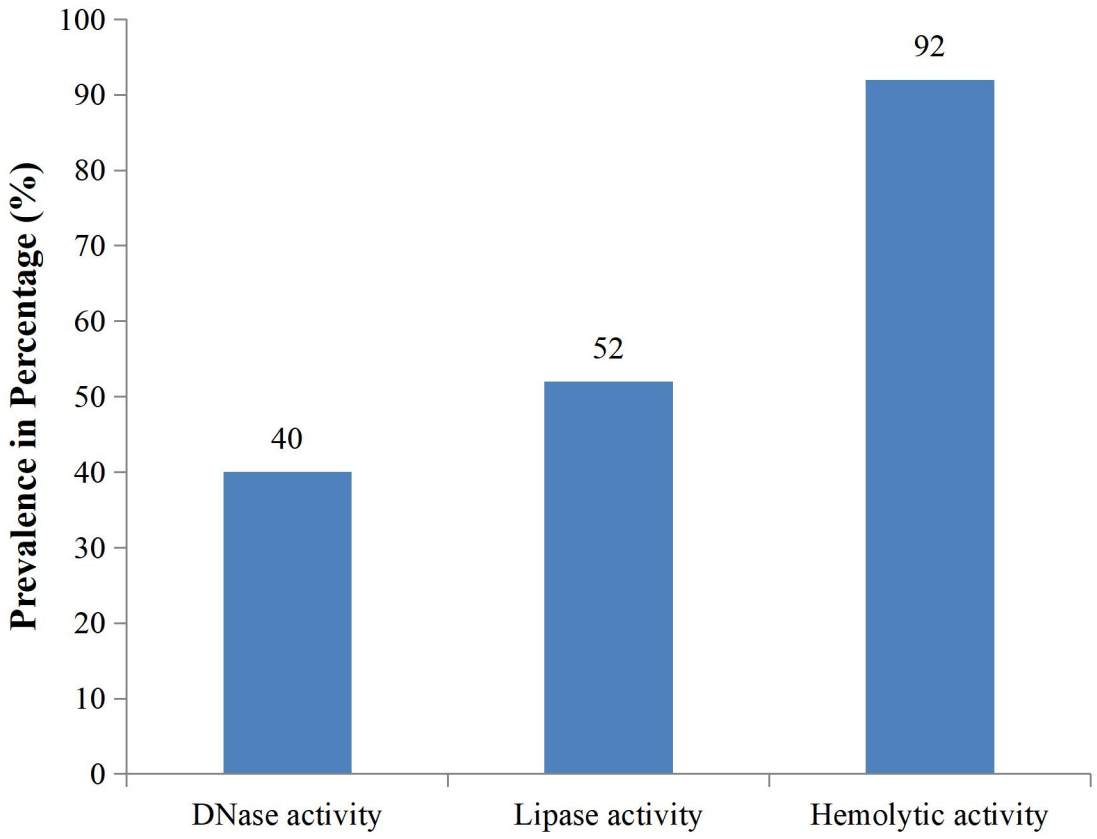

Figure 4.3: Prevalence of Virulence Factors in the Bacterial Isolates

CHAPTER FIVE

5.1 Discussion

Laundry wastewater and several other anthropogenic wastes have been a prominent source of bacterial contamination, encompassing both pathogenic and non-pathogenic species. The bacteria population in these effluents varies and they are notably influenced by diverse environmental factors (Callewaert *et al.,* 2015). Low washing temperatures and the development of biofilm in washing machines allow a variety of bacteria, including *Pseudomonas aeruginosa* and *Staphylococcus aureus* to thrive in laundry settings (Abney *et al.,* 2021). These bacteria could be harmful due to their association with some virulence factors including adhesion and toxin synthesis (Rasko and sperandio, 2010). Among the many pathogens that can be found in wastewater, especially laundry effluent, are enteric bacteria that can cause serious health problems, such as *Salmonella* spp. and *Escherichia coli* (Chahal *et al.,* 2016). The existence of these pathogens emphasizes the necessity of efficient care and good personal cleanliness to stop the spread of illness.

Developing measures to reduce bacterial contamination in laundry settings requires an understanding of the presence of virulence factors in laundry wastewater. To lessen the release of harmful bacteria into the environment, this involves employing higher washing temperatures, performing routine maintenance on washing machines, and putting modern wastewater treatment technology into place (Callewaert *et al.,* 2015). From the result, it was observed that the mean heterotrophic bacterial counts between two laundry shops, labeled Shop V and Shop W. Shop W exhibits a considerably higher bacterial count compared to Shop V. This aligns with Zige *et al.* (2019) suggesting that disparity likely stems from variations in hygiene practices and equipment maintenance between the two shops. Shop W might have inadequate cleaning procedures, poor

handwashing practices among staff, or use of contaminated water, all of which could contribute to elevated bacterial levels. According to result, the frequency of various bacterial species detected in wastewater from laundry facilities. *Staphylococcus aureus*, a common skin bacterium, is the most prevalent, found in 44% of samples. Escherichia coli, an indicator of fecal contamination, is present in 28% of samples, raising concerns about hygiene practices. *Pseudomonas* spp., known for their environmental adaptability, is detected in 16% of samples. This is in line with Callewaert *et al.* (2015) which suggests that washing machines facilitate microbial exchange between influent water, skin-related bacteria, and biofilm-related bacteria, leading to the presence of various bacterial species in the effluent water. The detection of *Staphylococcus aureus*, *Pseudomonas* spp., and *Escherichia coli* in laundry wastewater is consistent with earlier findings which highlighted the survival and potential growth of bacteria like *Staphylococcus aureus* even at moderate washing temperatures. This underscores the importance of effective wastewater treatment to mitigate environmental and health risks.

From the results, the percentage of bacterial isolates exhibiting three different virulence factors: DNase activity (40%), lipase activity (52%), and hemolytic activity (92%). Virulence factors are traits that enhance a bacterium's ability to cause disease. Hemolytic activity, the ability to lyse red blood cells, is the most prevalent virulence factor among the isolates, indicating a high potential for these bacteria to cause infections. Lipase activity, the ability to break down lipids, is also prevalent, suggesting that these bacteria may be capable of colonizing and surviving in environments rich in lipids. DNase activity, the ability to degrade DNA, is present in a lower percentage of isolates but still indicates potential for tissue damage. This aligns with Whitehead *et al.* (2022) by considering the microbial exchange and potential pathogenicity in washing machines indicating that washing machines can harbor a diverse range of bacteria, including

potential pathogens like *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, which are often found in laundry wastewater. It also aligns with Callewaert *et al.* (2015) suggesting that the presence of virulence factors such as hemolytic and lipase activities in bacterial isolates suggests that these pathogens could be transferred through laundry processes, similar to how microbial communities are exchanged in household washing machines. This highlights the importance of managing laundry wastewater to prevent the spread of pathogens.

5.2 Conclusion

The prevalence of virulence factors among bacteria isolated from laundry wastewater is a significant public health concern. This study demonstrated that laundry wastewater could harbor a diverse range of bacteria, including those possessing virulence factors such as hemolysis. The high prevalence of these virulence factors among isolated bacteria highlights the potential risks associated with exposure to laundry wastewater, particularly for individuals with compromised immune systems. These findings emphasize the need for proper treatment and disposal of laundry wastewater to prevent the dissemination of virulent bacteria into the environment. By addressing these critical issues, we can mitigate the risks associated with laundry wastewater and promote a safer environment for public health.

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