Risk assessment of pathogens in vegetables irrigated with contaminated water and wetland treated water

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By

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Dedicated To

Prophet Muhammad (SAWW)

My Beloved Father

My Sweet Mother

&

My Brother and Sisters

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Sanam Islam Khan

CERTIFICATE

This thesis entitled as "**Risk assessment of pathogens in vegetables irrigated with contaminated waste water and wetland treated water**" submitted by ***Sanam Islam Khan*** to the Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan is accepted in its present form as satisfying the thesis requirement for the Degree of Master in Philosophy in Microbiology.

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Abstract

This research work was design to evaluate the microbiological safety of vegetables irrigated with untreated, tap and wetland treated sewage waste water. A significant difference in aerobic plate count was observed between vegetables irrigated with sewage water and wet land treated water. Significant difference in microbial load was observed in summer season and winter season both groups of vegetables viz irrigated with treated and untreated water. However, overall microbial count was higher for summer season vegetables as compare to winter season vegetables. Microbial load was also differing significantly with type of vegetable in both seasons. Washing of vegetables with sterile distilled water significantly reduced microbial count, after washing microbial count came down to permissible limits in all vegetables irrigated with treated water however, this was not observed in case of untreated sewage water. Pathogenic isolates includes *Bacillus cereus, Escherichia Coli, Alcaligenes faecalis, Corynebacterium xerosis, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Enterobacter aerogenes, Salmonella* Typhimurium, *Klebsiella pneumonia, Micrococcus luteus, Shigella dysentriae, Streptococcus lactis* were isolated from vegetables and were preliminary identified based on biochemical parameters. Non-significant difference was found between the growth of vegetables irrigated with sewage water and treated water, after 60th days of sowing. Therefore, it could be inferred from this study that wet land treatment significantly reduced the risk of pathogen spread in vegetables irrigated with sewage water. Moreover, this study helps in better understanding of the transfer of pathogens to growing vegetables. It would serve as guideline for local vegetable growers using sewage water for irrigation.

Introduction

The use of vegetables as a part of nutrition and daily consumption is a very important part of human life. Fresh vegetables are not only a source of essential minerals, vitamins and food nutrients but also help in maintenance of immunity. Five to nine servings of fruits and vegetables are recommendations per day by FAO and WHO because it is reported that 31% of the cardiovascular disease cases can be caused by deficiency of fruits and vegetables intake (Johnston *et al.*, 2006). The use of vegetables has been expressively increased in recent years due to increasing health hazards in human society.

Pakistan being an agriculture country produces a variety of vegetables in different seasons. The seasonal vegetables of Pakistan are not only consumed within different parts of country but also export to many countries of the world. Potato, tomato, chilies, cucumber, okra and gourds are popular vegetables of summer and spring. In winter season cauliflower, cabbage, spinach, lettuce, potato, carrot, onion, radish, turnip, coriander, fenugreek and peas are grown. It is estimated in an economic survey that 7612.4 thousand tones vegetables were grown in Pakistan during 2012 (Wasim, 2013).

Most of the vegetable growing is done traditionally in agriculture field where soil is nutritional and supporting medium. There is a problem with soil medium it may contain pathogens which can be transferred to human and plants through contact with soil or pathogens can become air borne. Before any plantation risk of pathogen transmission should be minimize, it can be decrease by covering soil with fine mesh or weed mat, it can be reduce by covering soil with gravel or crushed stone. Soil contains large number of microorganisms so these attempts do not completely eliminate the risk of microbial transmission. Other approach could be the use of soil from healthy environment. Source of soil contamination can be the use of untreated sewage (Alex *et al.*, 2009).

The demand of safe irrigation water for agriculture especially horticulture crops is increasing day by day (Shiklomanov, 1998). In a survey by FAO in 2010 it is estimated that 2000-5000 liters of water is utilized to produce per day food per person. Fresh water used for irrigation purposes is only 1% of the total earth water

(Zia *et al.*, 2013). The water reservoirs for agricultural purposes contain ground water, surface water, rain water and sewage water. In many countries rain fed water is also a source for irrigation (Li *et al.*, 2000). Rain water is stored in synthetic reservoir then it is use for irrigation (Makoto, 1999; Prinz, 1999). The surface water of rivers, lakes, streams and ponds has always been a common source of irrigation (Winter *et al.*, 1999; Gil *et.al.*, 2013).

In recent few decades the urbanization has increased and utilization of fresh water for domestic purposes caused shortage of water for agricultural lands. This urbanization generates large amount of waste water (lazarova and Bahria, 2005; Qadir *et* al., 2007a; Aasano *et al.*, 2007). This waste water released into natural water bodies like streams, lakes and ponds without any treatment, farmers in urban areas uses this waste water for irrigation of their crops. This is considered as cheaper source of water for irrigation and also contains large amount of nutrients which enhances the growth of plants (Keraita and Drechsel, 2004; Scott *et al.*, 2004). But long term application of wastewater to soil can lead to accumulation of nutrients into soil, this shows toxic effect on plant growth. (Esmailiyan et al., 2008). The sewage water that comes from domestic utilizations contains a variety of human pathogenic microbes. The species of *Salmonella spp., E.coli, Campylobacter spp. Shigella, Listeria monocytogenes Streptococcus spp., Staphylococcus auras Yersinia, Noroviruses, Rota viruses, Protozoan* and *Helminthes* were reported in sewage water by many researchers (USEPA 1992; Calci *et al.*, 1998; Steele and odumeru, 2004; Nwachuku and gerba., 2006). These microbes reproduce in water and resume their cells for a longer period of time as *E. coli* can persist in ground water up to 58 days while in sewage water more than 12 weeks at 8 °C (Wang and Doyle., 1998). The survival of microbes especially the pathogens in water bodies depends on many factors like temperature, particle matter; soluble organic matter and sunlight contribute to the survival of pathogens in water (Gerba, 2009). Microorganisms may remain on plant surfaces or enter into plant tissues and appear as micro colonies in tissues (Szabo and Conventry, 2001). Colonization of pathogens on vegetables depends on type of pathogens, vegetables, plant physiology and environmental conditions (Critzer and Doyle, 2010; harris *et al.*, 2003). Stomata allows pathogens to enter into leaves of plants

(Staskawicz *et al.*, 2001; Barker-Reid *et al* 2009; Gomes *et al.*, 2009; Arascavage *et al.*, 2008) while pathogens spreads to the fruit by leaves, damage branch and flower (Mcmahon *et al.*, 2002). Plants does not allow the proliferation of plant pathogens on their surfaces by releasing hormone like ethylene but cannot inhibit the attachment and proliferation of human pathogens because they do not recognize human pathogens (Berger *et al*, 2010). Plants provide favorable conditions for growth of pathogens such as protect from UV light, disinfectants and have large amount of nutrients (Heaton and Jones, 2008).

In 2014 Mathur *et al.* reported infectious bacteria genera like corny bacterium, streptococcus and staphylococcus in vegetables in India. Human consumption of these contaminated vegetables causes outbreaks of illnesses (EFSA, 2013). Centre of disease control and prevention reported large number of cases in United kingdom 76 million people become ill with foodborne infections each year and 5000 people die, 4% due to intake of fresh vegetables (Little and Gillespie, 2008). Another survey of CDC reported, the number of epidemics related to vegetables in the US doubled from 1973-1987 and 1988-1992 (Olsen *et al.*, 2000). From 1998 to 2008, produce-related foodborne illnesses accounted for 46% of all reported foodborne illnesses (Painter *et al.*, 2013). Batz *et al.* (2012) estimated the disease load linked with several food products and foodborne pathogens. Among twelve food categories, vegetables were ranked fourth in cost of illness with approximately 1.4 billion dollars in estimated annual cost of infection.

Sewage water can be treated through constructed wetlands by different plants. Vegetables like tuber crops and leafy greens are not suggested to grow in constructed wetlands or in soil which is irrigated with the treated waste because normally the constructed wetlands do not include a disinfection step. But plants of bananas, papaya, rice, dwarf coconuts can be grown in constructed wetlands for treated of water (Nelson, 1999). Treated water form constructed wetlands can be utilized for the irrigation of food crops and other plants, plants can utilize the remaining nutrients for their growth. In those areas, root crops should be avoided unless a decontamination step is included, but choice is not limited to wetland-tolerant species. Other advantage of constructed wetland is that it offers a low-energy solution for reutilizing wastewater and keeping nutrients in forms usable

by plants. UV light sterilizer can be used for further treatment of water from constructed wetlands and mixed into the irrigation stream for the agricultural system. So all the nutrients were returned to the farm soils paying to their sustainable long-term use (Nelson, 1997).

Objectives

The main objectives of the present study are followings:

1. To study the difference in microbial load of selected vegetable irrigated with sewage, wetland treated and tap water.

2. To check the effect of washing on microbial load of selected vegetables.

3. To evaluate the impact of soil microbiology and seasonal variation on microbial load in vegetables.

4. To determine the impact irrigation water type on vegetable growth.

REVIEW OF LITERATURE

2.1. Back Ground of Research:

All vegetables can be contaminated during growth, harvesting, transporting and storage, so they become a source of disease transmission. Contamination leads to spoilage of vegetables during storage. Decay is more expected if the vegetable is broken as this enables pathogen to enter the tissues. A cracked skin of vegetable discharges nutrients which helps the microorganism to grow on vegetables and make biofilms. Microbial damage results in release of watery contents form vegetables which leads to spoilage of vegetables. pH influence the growth of microorganisms and vegetables have a greater pH than fruit, though they are still slightly acidic, bacteria and fungus grow well at this pH level. 33% of the vegetable spoilage is caused by bacterial growth. These bacteria not only spoil vegetables but also transmitted to human and causes different diseases. Contamination of vegetables can be avoided by careful growth, harvesting, handling, transport and storage. Refrigeration decelerates microbial growth; control of humidity is also important as condensation will encourage bacterial growth and too dry atmosphere will lead to wilting of the crop. Packaging can control the amount of oxygen and water reaching the vegetables and the microbes growing on it.

There is increasing evidence that consumption of raw fresh vegetable is a major factor contributing to human foodborne outbreaks, due to the potential for contamination with pathogenic microorganisms. Foodborne epidemics can be defined as a series of infections spread because of intake of infectious agent's through food (WHO). It becomes epidemic when more than one person is affected with the same symptoms by intake of same infectious food. Disease severity depends on the agent involved in cause of that disease, symptoms like nausea, vomiting, abdominal cramps, diarrhea, fever, headache and weakness can appear (Besser *et al.*, 2003). According to the Center for Disease Control (CDC) foodborne diseases affect 76 million persons, cause 325,000 people to be hospitalized, and 5,000 deaths per year (Klonsky, 2006).). Fresh vegetables are progressively recognized as possible sources of diseases (Beuchat, 2002;

Ibenyassine *et al.*, 2006; NACMCF, 1999). Occurrences of food borne illnesses related with consumption of fresh crop have also increased -- from 1% to 12% from 1970s to 1990s. From 1990 to 2004, fresh produce was responsible for the largest number of foodborne illness, accounting for 21% stated by the Center for Science in the Public Interest Database (Ilic *et al.*, 2008). Since the first recognized outbreaks of foodborne disease linked with *E. coli O157:H7*, the pathogen have become increasingly prominent as a cause of serious illness in many countries around the world (Mclure, 2000). Multiple surveys have been performed to determine the local prevalence of pathogenic microorganisms on fruit and vegetables. The list of pathogens of interest includes bacteria *enterohemorrhagic Escherichia coli* (e.g., *E. coli O157: H7*), *enterotoxigenic E. coli, Staphylococcus aureus, Campylobacter spp., Bacillus cereus, Listeria monocytogenes, Salmonella spp., Shigella spp., Yersinia enterocolitica, protozoa Cryptosporidium spp., Cyclospora cayetanensis, Giardia spp., Entamoeba histolytica, helminths* such as *Ascaris spp.*, and viruses, in particular, adenoviruses, enteroviruses, *noroviruses*, and *rotaviruses*. Incidence of foodborne pathogens on fruits and vegetables varies by region and can be extremely high in some developing countries.

2.2 Importance of vegetables:

Vegetables are the edible portions of plants. They contain valuable food components which can be fruitfully used to build up and repair the body. Different portions of vegetables like tuber, stem, leave, fruit and seed are eatable. Each part playing his part to nutrition in its own mode. They are important food and helpful in the maintenance of health and avoidance of diseases. They also paly key role in maintaining alkaline reserve of the body. They contain carbohydrates, vitamins and minerals. (Robinson, 1990). Minerals are utilized by human body to continue the metabolic activities of body tissues. Minerals are the integral parts of bones, teeth, muscles, hair and nerve cells. Correct balance of minerals is required to assimilate Vitamins. (Sonni Alvarez, 2002).

Large amount of vitamins are present in vegetables. Vitamins are involved in repairing of epidermis, mucous membranes, skeletons, teeth, hair, and visualization, also plays role in reproduction. They are also involve in absorption of phosphorous and calcium; necessary for bones, also help in clotting of blood, nervous system also require vitamins for proper functioning (Chatterjea and Shinde, 1998). Vegetables are considered as natural reserves of nutrients e.g. carrot is a good source of vitamin A, vitamin C can be obtain from spinach and tomato which is used to prevent and cure scurvy. Potato is a good source of carbohydrates. Sponge gourd and bottle gourd are rich dietary fibers, these help to prevent constipation. World Health Organization (WHO) reported that four of five main disease burdens in the Western world like high blood pressure, high cholesterol, and fatness are closely related with low fruit and vegetable consumption. According to previous studies, low consumption of Fruits and Vegetables is projected to cause about 19 % of digestive tract cancer and 31 % of heart illness (Guilbert 2003). Increased fruits and vegetables consumption prevents the chances of cancer but their prevention rate varies between 19 and 32 % in different populations (Grundgaard *et al.* 2003; Hoffmann *et al.* 2003). Although recent Meta analyses recommend more modest estimates of the protective role of fruits & vegetables in cancer (Key 2011; Soerjomataram *et al.* 2010). Prevention of atherosclerosis is also associated with a food rich in fruits and berries especially in elderly men (Ellingsen *et al.*, 2008). Intake of Green leafy vegetables decreases the threat of type 2 diabetes (Carter *et al.*, 2010), body also perform good physical functions by intake of fruits and vegetables (Myint *et al.*, 2007).

2.3. Irrigation water:

According to FAO (2011) food demand is increasing and food production is expected to increase 70% universally and almost 100% in developing countries up to 2050. This increased requirement will put extraordinary burden on many agricultural production systems around the world; this will also increase the water requirement for agriculture. Water used to substitute or increase precipitation in the production of produce is called irrigation water (Hargreaves & Merkley,

1998). Agricultural and horticultural production is increased by irrigation of crops. According to the Food and Agriculture Organization (FAO), one person used to drink 2-4 L of water per day, but to yield one person's daily food requires 2000-5000 L of water. 1000-3000 L of water is require to produce 1 kg of rice and 13,000-15,000 L to produce 1 kg of grain-fed beef (FAO, 2010; Pimentel *et al.*, 1997). Requirement for irrigation water is increasing (Shiklomanov, 1998) nearly 274 million hectares of cultivated land were irrigated world-wide in 2000, which is almost 16% of the total cultivated area on world (Sieberta *et al.*, 2006). In 2003 53,000 ha area was irrigated in Sweden (Wriedt *et al.*, 2008).

2.4. Sources of Irrigation Water:

Before use irrigation water is stored in natural or synthetic sources. There are different sources of irrigation water, including rainwater, groundwater, surface water and untreated or treated wastewater. Rainwater is thought to be the easiest source of irrigation (Li *et al.*, 2000). The term 'Rainwater harvesting' is used for gathering and storing rainwater in artificial reservoirs (Makoto, 1999; Prinz, 1999). Groundwater can be obtained through wells and springs this is called fresh water. Water from wells and spring is hygienically harmless than surface water for crop production (Ayers & Westcot, 1985). Fresh water that can be used for crop production and which is available to humans comprises less than 1% of the Earth's total water resources (Zia *et al.*, 2013). Irrigation can be done through several surface water sources (Winter *et al.*, 1999). Surface water comes from groundwater sources, but it can become polluted with the addition of wastewater, storm water and farming run-off, which contains large amount of pathogens (Winter *et al.*, 1999). Deficiency of freshwater for irrigation has forced growers to use wastewater, and around 20 million hectares were irrigated with wastewater around the world each year (Scott *et al.*, 2004). Wastewater contains a wide range of contaminants: salts, metals, microorganisms, residual medicines, organic complexes and remains of personal care stuff (Qadir *et al.*, 2007). These products can damage human health and the environment. Wastewater usage in the developing countries has increased because it contains large amounts of nutrients which are required for growth of plants and is also a dependable source of water

(Hussain et al., 2001). Sewage water is utilized for the irrigation of 26% of the vegetables in Pakistan (Ensink *et al.*, 2004). About 80% of vegetables are irrigated with sewage water in Hanoi (Lai, 2000). People in Ghana irrigates 11,500 ha, area each year with waste water (Keraita and Drechsel, 2004). Untreated waste water is used in Mexico to irrigate about 260,000 ha of land (Mexico CAN, 2004). Farmers are facing the problem of soil productivity, presence of nutrients in sewage water increases soil productivity so sewage water application increases production of leafy vegetables in the short run, however, non-stop use badly effects production (Field, 2001).

2.5. Irrigation Methods:

Different methods can be used to apply water for irrigation. Surface irrigation and localized irrigation are two main methods of irrigation (Cuenca, 1989). Surface irrigation is the easiest method of irrigation, in which water runs under gravity without pushing. Surface irrigation can be achieved as channel, flood or frame strip irrigation and the water is not applied directly to the plant surface, so the plant surface cannot be directly contaminated if waste water is used (Solomon *et al.*, 2002). In localized irrigation water is applied through connected pipes (Vermeiren & Jobling, 1983). In localized irrigation method, water can be supplied through trickle irrigation (water is applied to the root zone of each plant), spray or micro-sprinkler irrigation (water is supplied directly to the plant covering) or bubbler irrigation (water is applied in low quantities to the soil nearby plants) (Frenken, 2005).Surface irrigation systems leads to larger water losses due to lack of control (mainly evaporation and technical faults in the distribution system) (Rivas *et al.*, 2007). In Zimbabwe 50% of water is lost through dehydration during surface irrigation of gardens (Batchelor *et al.*, 1996). Hence, connection of a water circuit is vital for improving the efficacy and 10-50% water can be saved (Postel, 1992).

The Irrigation water distribution system can contain large amount of microorganisms, microbiological load of the water sources depends on the complex environment, nutrient availability, microbial interactions and accumulation of sediments (Pachepsky *et al.*, 2012). When untreated wastewater

is used for irrigation as waste water contains large amount of microbes, these microorganisms can persist in the water circuit within biofilms (Yan *et al.*, 2009; LeChevallier *et al.*, 1987). Their persistence is affected by various environmental factors as well as nutrient availability, microbial interactions, pipe material, system hydraulics, use of sanitizers and sediment accumulation (Pachepsky *et al.*, 2011; USEPA, 2002). Regrowth of pathogens, like *Legionella* and *Aeromonas*, has been reported in the USA on membrane bioreactor treatment plants (Jjemba *et al.*, 2010). These microbes mix with irrigation water passing through irrigation systems and may reach the plant surface. Washing of the irrigation system decreases the microbial persistence in pipes (Pachepsky *et al.*, 2012).

Aquatic plants and sediments can help pathogen survival in the open irrigation system, whereas in case of pipe-based irrigation systems pathogens can survive through biofilms. It has been shown that E. coli can survive for up to 300 days in autoclaved, filtered river water at 4 °C (Flint, 1987). Bottom sediment could be one of the major reservoirs of pathogenic microorganisms as it provides nutrient availability and protection from UV sunlight (Burton *et al.*, 1987; Lewis *et al.*, 1986).

Pachepsky *et al.* (2011) reported that fecal coliforms are multiple-fold greater in sediments than in the water column. Thus, it is suggested that the total suspended solids (TSS) content must be reduced before water treatment (Rose *et al.*, 1996). Use of contaminated water for irrigation of crops is considered to be responsible for several outbreaks of disease following consumption of such crops (Beuchat and Ryu, 1997).

2.6. Concentrations of Microorganisms in Irrigation Waters:

A complete investigation of pathogens level in irrigation water has not been assembled for the USA or for any other country yet (Stoeckel, 2009). Reliable reporting on the microbial quality of irrigation waters is not done regularly anywhere in the world, due to different factors; the charge of broad sampling and also producers/growers who have started to gather information on microbial quality of irrigation water are unwilling to share them (Suslow, 2010). However accessible data shows that pathogens contaminate the irrigation water.

Thurston- Enriquez *et al.* (2002) reported the presence of human pathogens in water used for irrigation of crops in the United States and several Central American countries. Their findings conclude that 28% of water samples contained *microsporidia*, 60% contained Giardia cysts, and 36% samples contained *Cryptosporidium* oocysts.

Duffy et al. (2005) detected *Salmonella* in 9% of waters samples used for irrigation in Texas. A large survey of USA groundwater found that 11% of sites were positive for *Cryptosporidium, Giardia*, or both (Moulton-Hancock *et al.*, 2000). Close *et al.* (2008) demonstrated the leaching of *E. coli* and *Campylobacter* to groundwater; *E. coli* and Campylobacter were detected in 75% and 12% of samples, respectively.

In Nigeria large-scale irrigation is done through river water Chigor *et al.* (2010) found *E. coli O157* from 2% of water samples from river. Johnson *et al.* (2003) reported the occurrence of *E. coli O157:H7* in 1% of the samples and *Salmonella* in 6% of the surface water samples in Southern Alberta. Gannon *et al.* (2004) observed *E. coli O157:H7* in 2% of 1608 water samples supplied in period of 2-year period. Eight percent of the irrigation water samples collected from six districts in Alberta, Canada contained >100 fecal coliform/100 ml (Cross, 1997). Salmonella were noted in 6% of water samples in Greece (Arvanitidou *et al.*, 1997). In an investigation of private wells in the Netherlands, Schets *et al.* (2005) found that 11% of the samples contained fecal indicators, while *E. coli O157:H7* was isolated from 3% of the samples.

2.7. Human Pathogens in vegetables:

Different sources can contaminate the vegetables but a large number of pathogens can be transmitted to plants via irrigation water, then these persist on external and internal parts of the plant for many days (Islam *et al.*, 2004). Wound surfaces and stomatal cavities allow these pathogens to enter into the plant tissues (Barker-Reid *et al.*, 2009; Gomes *et al.*, 2009; Aruscavage *et al.*, 2008). Plant tissues provides favorable living condition to pathogens, provide protection from sterilization treatment, UV light and also have large amount of nutrients for pathogen proliferation (Heaton & Jones, 2008).

Different studies shows different results on the population and survival of human pathogens, applied with irrigation water either in the plant surface or by the root system, so it is hard to make broad statements on pathogen survival and their populations on plants (Berger *et al.*, 2010).

Waste water is used for irrigation can be responsible for large number of foodborne outbreaks especially consumption of leafy vegetables are responsible for outbreaks because leafy vegetables are normally irrigated near harvest to increase their market price (EFSA, 2013; Harris *et al.*, 2003). Irrigation results in humid environment and pathogens easily persist in humid environment on plant surfaces (Dreux *et al.*, 2007). Different studies shows that intake of raw vegetables irrigated with poor quality of water results in high numbers of foodborne infections, especially diarrhea (Harris *et al.*, 2003).

2.8. Important Pathogens in vegetables through Irrigation water:

Many studies documented that transmission of pathogens occurs from contaminated irrigation water to vegetables (Chigor *et al.*, 2010; Islam *et al.*, 2004; Solomon *et al.*, 2002; Steele and Odumeru, 2004). Once transmission of bacteria occurs, survival and overall persistence of pathogens on crop depends upon environmental condition, crop type and strain of bacteria. In addition, time elapsed between the latest irrigation and time of harvest can determine the degree of crop pollution for different pathogens (Soon *et al.*, 2012). Some pathogens have been shown to persist on plant surfaces for the whole growing period (Solomon *et al.*, 2002). Studies also reported that introduction of pesticide with irrigation water promote growth of certain bacteria like *Salmonella*, it can persist up to 15 days on tomato surfaces (*73*). To correctly evaluate the risks of using irrigation water of poor microbiological quality, more studies need to be designed to enumerate pathogens on produce surface Foodborne infections mostly occur through the ingestion of *E. coli O157:H7* and *L. monocytogenes* with fresh fruits and vegetables (EFSA, 2013; Brackett, 2001).

The infectious dosage of both bacteria is low (Ramaswamy *et al.*, 2007; Ackers *et al.*, 1998). Both have been present in surface water (Wilkes *et al.*, 2009) and

can be transferred to the plant surface via irrigation water (Steele & Odumeru, 2004). Both *E. coli O157:H7* and L. monocytogenes have been reported in several outbreaks related to fruits and vegetables world-wide (EFSA, 2013). Intake of lettuce results in an *E. coli O157* outbreak in Sweden and this lettuce was irrigated with waste water (Söderström *et al.*, 2008). Some studies reported that concentration of pathogens in water is important for transfer of pathogens to vegetables, so food contamination is dependent on pathogen concentration, but Pachepsky *et al.* (2011) reported that internalization and colonization of pathogens in plants does not depends upon concentration of pathogens in irrigation water.

The plant phyllosphere possess a threatening environment for enteric pathogens. This environment is typically categorized by unstable temperatures, unreliable nutrient availability, opposition of local microbiota, UV-light and water activity (Heaton & Jones, 2008; Cooley *et al.*, 2006). So, human pathogens (outside their host) are considered not to be part of the phyllosphere. Nevertheless, foodborne outbreaks show that these microorganisms are able to adjust the unstable environment of plant's phylloshere (Berger et al., 2010).

Studies shows that there are some factors which help the pathogens to persist and colonize in the plants, these factors includes gene expression, motility and extracellular compound production (Aruscavage *et al.*, 2006; Solomon & Matthews, 2006). Plants shows immunity against plant pathogens and does not allow the persistence and proliferation of plant pathogens on their surfaces, plants shows different immune mechanisms e.g. plants releases hormone ethylene which inhibits certain plant pathogens, but plants does not show any defense against human pathogens. As all immune mechanism depends on recognition of foreign particles so this can be the reason that plants do not recognize human pathogen as a foreign particle, that's why plants cannot inhibit the colonization of human pathogens on their surfaces. Therefore, human pathogens can be present as a part of the plant phyllosphere (Berger *et al.*, 2010).

Both *E. coli O157:H7* and *L. monocytogenes* colonizes plant surfaces and persist for long period of time on plants but it depends upon environmental factors and nutrient accessibility (Islam *et al.*, 2004; Beuchat, 1996a).

Takeuchi *et al.* (2000) observed that *E. coli O157:H7* can attach better to surfaces and the cut edge of lettuce leaves than *L. monocytogenes*. Active motility and simple diffusion is required by pathogens to colonize themselves on the surfaces of plants (Cooley *et al.*, 2003). Colonization also depends upon the formation of pathogens aggregates, especially near stomatal cavities and intercellular junctions, therefore pathogens can defend themselves from hostile environmental conditions, as well as post-harvest decontamination treatments (Heaton & Jones, 2008). Both aggregate formation and internalization of pathogens in the plants helps the pathogens to survive for longer period of time (Heaton & Jones, 2008).

Survival of pathogens on leafy vegetables also affected during pre-harvest. Studies have shown that environmental conditions, particularly temperature increases and precipitation pattern changes, can affect the survival of human pathogens on leafy vegetables (Liu *et al.*, 2013). Plant characteristics, e.g. leaf water content, nutrient content, antioxidants and leaf morphology, may affect the phyllosphere microbiota. Leaf physiology and morphology may also affect the development of microbial populations in the phyllosphere and it is possible that certain spots on the leaf surfaces that are suitable for microbial growth can develop. Pre-harvest cultural practices that can alter leaf morphology and physiology, and consequently the prevalence of human pathogens on the leaf surfaces, could be exploited in order to prevent proliferation of human pathogens.

2.9. Pathogen pathways into plants:

Different mechanisms are adapted by pathogens to attach themselves on surfaces, these include: fimbriae (pilli), fibrils and flagella interaction with surface by means of electrostatic, hydrogen bonding and hydrophobic forces followed by release of extracellular binding polymers (Frank, 2001). Attachment is the first step in the establishment of pathogens on plants usually attachment occur at stomata, broken trachoma or cracks in the cuticle (Critzer and Dolye 2010). SEM has been used to examine the plant surfaces and pathogens attachment to surfaces.

Critzer and Dolye (2010) reported that pathogens form biofilms on plants surfaces. They forms biofilms on cotyledons, hypocotyls and roots of cabbage, they examined microbial attachment by SEM. If a pathogen succeeds to survive on the surfaces of the plants a chance exist that the organism may remain on the plants at an infectious dose at time of consumption (Heaton and Jones, 2008). Roots of plants can transport E. coli to the leaves of the lettuce from soil (Solomon *et al.*, 2002b), similarly aerial parts of Romaine lettuce seedlings can become contaminated with *Salmonella* through the roots of the plant (Bernstein *et al.*, 2007b). Conversely, some studies have not confirmed these results. *E. coli* was found in root tissue but not in shoot tissue of spinach plants grown on contaminated soil (Sharma *et al.*, 2009).

Jablasone *et al.* (2004), Miles *et al.* (2009), Zhang *et al.* (2009), Erickson *et al.* (2010) observed that internalization of *E. coli* and *Salmonella* via the root system does not occur or is a very rare event. *E. coli* enters into the lettuce tissues through damage surfaces (Barker-Reid *et al.*, 2009) and promotes its survival (Aruscavage *et al.*, 2008; Brandl *et al.*, 2004). Stomatal cavity was the preferential route of entry of *E. coli* to the vegetable leaf was observed by Gomes *et al.* (2009) with four different varieties of lettuce.

2.10. Adherence to plants:

Adherence of pathogen to the surface of plants depends on strain of pathogen and type of plant. For example, Lapidot and Yaron (2009) reported that strain-specific properties of Salmonella (curli and cellulose) affected its ability to enter parsley plants from irrigation water. In 2011 Guo *et al.* observed Significant differences in survival on and in tomato plants for various serotypes of *Salmonella* and Plants also vary in their susceptibility to become contaminated with pathogens when irrigated with contaminated water. Quantitative risk assessment models showed that risk differs between crops type, leafy vegetable lettuce showed higher risk than cucumber, but similar to broccoli and cabbage (Hamilton *et al.*, 2006). In an experiment *E. coli* and *Clostridium perfringens* were added to irrigation water then cantaloupe, lettuce and pepper were irrigated with that water, pathogens were detected on the surfaces of cantaloupe and lettuce, but were never recovered

on the bell peppers (Song *et al.*, 2006). Irrigation with contaminated water resulted in concentrations of total coliforms in amaranthus much higher than in other vegetables in the study of Okafo *et al.* (2003). Melloul *et al.* (2001) showed that lettuce and parsley were more contaminated with Salmonella as compared to tomatoes and pimento because eatable parts of lettuce and parsley grows on ground surfaces.

There are indications that differences between cultivars may influence the extent of contamination from irrigation water to different levels (Barak *et al.*, 2008 for tomatoes, and Mitra *et al.*, 2009 for spinach), although reasons for that are currently unknown.

2.11 Previous studies on vegetables contamination:

Cordelia *et al.* in 2003 compared the contamination level, the seasonal distribution and the toxigenicity of pathogens in waste water and vegetables irrigated with waste water. They took 196 samples of water from Kawo drain, Sabon Gari drain and River Galma used for irrigation and 326 samples of vegetables grown in two seasons from January–April and from July–October and irrigated with water from these sites. They also compared the presence of foodborne pathogens like *E. coli, Salmonella, Vibrio* and microbial count in dry and rainy seasons in water and vegetable. Kawo drain water equally contaminated with coliform in both season but microbial count was higher than other two sites, but overall dry season showed higher microbial count than wet season. 133 Vegetable samples were observed in dry season 21 of them were contaminated and 47 samples were examined in rainy season only 6 of them were contaminated so the frequency for contamination in vegetables in dry season was 15.8% and 6.4% in wet season. All vegetables showed higher coliform counts range from 3.40–6.38 log cfu/ml. No contamination of *Salmonella* and *vibrio* was found in any vegetable sample during wet season. 39 isolates of enteropathogenic *E. coli* was found, 15 (38.5%) were toxigenic.

In 2006 Minhas *et al.* reported the prevalence of pathogenic contamination in different vegetables, animal food and grain crops irrigated with waste water. They also studied faecal coliform in waste water before irrigation of crops averaged count was 1.5×10^8/100 ml was detected in water. Then they applied this water

for irrigation, contamination transferred from water to produce. The total microbial counts for vegetables fluctuated between 2 · 106 and 3.5×10^7, fodder 6×10^6 and 3×10^7 and grain crops 2×10^8 and 3.8×10^8. Whereas the average Faecal coliform observed between <2 and 9×10^5 for vegetables, 9×10^2 for fodder and 2×10^5 for grain crops. Fungal contamination was high in shoots of clover but the shoots of clover was free of Salmonella and Shigella but cabbage sample exhibited Shigella population up to 9×10^4 cfu/g. Okra fruits showed highest bacterial count about 7.2×10^7. Riged gourd showed highest fecal coliform about 9×10^5. Some practices was done to decrease microbial load they harvested crops and kept in sunlight and sheds. Pathogenic load was decrease to acceptable level in sunlight dried produced while parts of produce that were in direct contact with sewage water were highly polluted. The coliform number in fresh produce was decreased by washing with water and removing of outer leaves of cabbage.

In 2010 Forslunda *et al.*, performed an experiment in Belgrade, Serbia and in Bologna, Italy in order to check the microbial contamination in potatoes by using subsurface drip irrigation. They irrigated potatoes with treated sewage water, stream water and tap water. All samples including soil, water and potato samples were collected from March 2007 to September 2008 were analyzed for the presence of fecal indicator Escherichia coli. Water and potatoes samples were analyzed for the existence of helminthes eggs, which is also key indicator of fecal pollution. In order to check the contamination level in irrigation water whether it lies within the permissible limits set by WHO they used quantitative microbial risk assessment (QMRA) model with Monte Carlo simulations. They reported that levels of *E. coli* contamination was low in irrigation water and soil as well as in potatoes. They also reported that contamination level (1.0×10^3 disease risk) in treated waste was above the recommended guideline of World Health Organization (WHO) for the accidental ingestion of soil by farmers (Serbia: 0.22 and Italy: 5.7×10^2). This suggest that subsurface drip irrigation practice is safer for consumer of vegetables as well as for farmers.

Forslund *et al.*, in 2012 assessed the fecal contamination in tomatoes. They irrigated tomatoes by surface and subsurface drip irrigation with treated domestic wastewater. They done their study in two cropping seasons in 2007 and 2008 at fields in Crete and Italy. Sewage water was treated through Membrane Bio Reactor (MBR) technology, gravel purification or UV light. They studied the amount of fecal indicator bacterium from soil, irrigation water and tomatoes samples. They reported high contamination of *E. coli* about 1753 CFU/100ml at Italy 488 CFU/100ml at Crete from irrigation water, but low amount of *E. coil* was found in soil samples ranged from 33 to 95 CFU/ g from both sides. Only two tomato sample were contaminated with *E.coli* out of 84 in Crete but no *E. coli* were found from any sample in Italy. All samples of irrigation water were devoid of *helminth* eggs in Crete. 36 samples of tomatoes were collected from Italy which were irrigated with treated water and tap water only two of them were contaminated by *helminth* eggs.

In another study Tiimub *et al.* (2012) assessed microbial contamination in waste water irrigated vegetable. They took lettuce and grown in Kumasi Metropolis in Ghana at three different locations i.e. Karikari farms, Gyinyaase and Atonsu they irrigated lettuce with waste water. The bacteriological quality of lettuce on three farms was studied for their Total colifoms, faecal coliforms, *enterococci, E. coli* and *Salmonella* CFU. Total coliforms was estimated by most probable number their count ranged from 4.93×10^4 CFU to 6.17×10^4 CFU highest count was observed at Atonsu and lowest at Karikari. Faecal coliforms ranged from 3.48×10^3 CFU to 4.66×10^4 CFU and E. coli 2.98×10^3 CFU to 3.86×10^4 CFU, again highest count was observed at Atonsu and lowest at Karikari. *Salmonella* varied from 2.50×10^2 CFU to 2.72×10^2 CFU, similar result in case of sites Atonsu showed highest contamination and Karkari showed lowest. The differences in total colifom counts at the two locations were substantial ($P < 0.001$). Faecal colifoms ($P < 0.000$) and *E. coli* ($P < 0.000$) counts were higher than other bacterial counts in all samples. Microbial counts of all samples were higher than the suggested values of World Health Organization (WHO). Their study presented that irrigation water is the main source of contamination in lettuce

leaves. Effective washing of vegetables before use decreases the microbial counts reduces the risk associated with vegetables.

In 2012 Javier Castro-Rosas *et al.*, evaluated the quality and occurrence of *E. coli* in ready to eat salads vegetables. They purchased vegetables from different restaurants of Mexico, where mostly vegetables were irrigated with waste water. They collected 130 vegetable samples from three kinds of restaurants: local restaurant, national chain restaurant both known for high levels of hygiene and local inexpensive restaurant. Total 6 restaurants were taken 2 from each group. Each sample was analyzed for occurrence of faecal coliforms and *E. coli*. *E. coli* were further studied for the presence of DEPs loci by using polymerase chain reactions. 99% of the samples were contaminated with feacal coliforms, 85% contained *E. coli* and 7% sowed the presence of DEPs. There was no difference found between number of positive samples between restaurants and categories. The Fecal coliforms were significantly higher ($p \leq 0.001$) than the E. coli in all samples. They concluded that untreated wastewater is unsafe for irrigation it should be avoided.

Avazpour *et al.*, (2013) studied the microbial contamination of salad used in Ilam's restaurants. 42 samples were collected from all restaurants and transported to the research laboratory. They used different Medias for identification of *E. coli* like Brilliant Green Medium, Trypton water and Coax reagent. For *salmonella* identification they used Selenit systein, Tetrationat, Salmonella-shigella agar and Briliant green and for detection of *Enterococcus,* KF agar medium was used. Yeast and Mold were detected by using Sabro dextrose agar medium (SDA). They found that 66% of samples were contaminated with *Enterococcus*, 69% with *E. coli* and 83% of samples were contaminated with yeast. *Salmonella* and mold were not found form any sample. Their results showed that all samples were contaminated and proper disinfectants and washing decreases microbial count.

In 2013 Osamwonyi *et al.* reported microbial count and different pathogens in vegetable samples. Eighteen vegetable samples were collected from three different restaurants located at Okada town, Edo State. They investigated

bacteriological attributes in samples. The mean heterotrophic and coliform counts from restaurant A were recorded ranged from 1.46×10^4 to 2.80×10^4 CFU/g and 1.46×10^4 to 2.84×10^4 CFU/g respectively. From restaurant B aerobic plate and total coliform count ranged from 1.74×10^4 to 2.36×10^4 CFU/g and 1.36×10^4 to 2.10×10^4 CFU/g were recorded. Microbial counts for vegetables obtained from restaurant C varied from 2.08×10^4 to 2.60×10^4 CFU/g and 1.12×10^4 to 2.90×10^4 CFU/g for total heterotrophic count and total coliform count respectively. They showed non-significant differences between the mean bacterial counts ($P>0.01$). They isolated; *Bacillus* sp., *Pseudomonas* sp., *Proteus* sp., *Staphylococcus* sp., *Micrococcus* sp., *Acinetobacter* sp., *Enterobacter, and Klebsiella* sp. The high microbial load indicates that vegetables provide favorable conditions for survival and proliferation of microorganisms inside vegetables. They recommended that vegetables should be properly decontaminated before salad preparation.

2.12 Irrigation water hygiene:

Sterility of crops can be guaranteed either by providing pathogen-free water or by disinfecting water before it reaches the plants. Hygienic quality of water can be improve by different methods like heat treatment or pasteurization, filtration, UV irradiation, chlorination, ozonation (Newman, 2004), waste stabilization, use of sedimentation ponds, waste storage or filtration through sand and soil (Keraita *et al.*, 2010; Mara & Silva, 1986). All these have been shown to decrease the levels of microorganisms in irrigation water.

Water treatment methods includes: chemical methods, physical methods and biological methods. Every treatment system has its own benefits and drawbacks. Water treatment can be done through chlorination it is quite old and economical method, chlorine can be used in different forms, e.g. chlorine gas, hypochlorite and chlorine dioxide (Newman, 2004). Chlorine dioxide is very effective in killing bacteria and viruses, but it is very unstable and needs to be produced at the site of use. Chlorine can become carcinogenic as it exist in the form of hypochlorous acid and hypochlorite in water and these can react with organic matter in water (Nieuwenhuijsen *et al.*, 2000). Chlorine treatment also fails to deactivate oocysts of Cryptosporidium parvum (Korich *et al.*, 1990; Peeters *et al.*, 1989).

Water treatment can be done through Ozone. Ozone can make pores in microbial cell membranes due to its highly oxidative properties. But, ozone releases some byproducts that may be lethal to humans (Glaze & Weinberg, 1993; Haag & Hoigne, 1983). Hydrogen peroxide is an unstable, strong oxidizer that can lyse the cell membrane of microorganisms. It has been found to be useful against fungi, bacteria and algae, and can therefore be used for sterilization of irrigation water (Glaze *et al.*, 1987).

Filtration is another method to remove microbes, especially protozoan oocysts and *helminth* eggs (Landa *et al.*, 1997). In filtration water passes through a permeable rough medium, which removes microbes. Filtration is a simple and relatively safe method, as there is no hazard of chemicals forming. But filtration have disadvantage it involves large land areas and environmental issues can affect the efficacy of the system (Huisman & Wood, 1974).

Wetlands are a suitable low-cost technology for removal of water microbes (Greenway, 2005). Constructed wetlands are planned wetlands that have substrates, plants and different microbial communities that are deliberately used for water treatment. Constructed wetlands are environment friendly systems (Cooper, 2009; Dan *et al.*, 2011). Water tolerant plants, soil and sand are used in wetlands for treatment of water (Kadlec and Knight, 1996). Environment friendly and low cost technology makes constructed wetlands a very important technology of water treatment (Sim *et al.*, 2008; Contreras *et al.*, 2010; Lee *et al.*, 2010; Bruch *et al.*, 2011). These are suitable for pathogen removal through physical, chemical and biological processes (Greenway, 2005; Zdragas *et al.*, 2002; Davies & Bavor, 2000). For example, wetlands are able to eliminate faecal coliforms, *Enterococci* and the total bacterial load from water (Greenway, 2005; Bolton & Greenway, 1999).

High operational costs and capital investments are major drawbacks of the conventional waste water systems therefore their applicability in villages and rural communities is not suitable. An alternative to this is constructed wetlands which is not only cost and operation wise economical but also independent of mechanical gears or external energy supply. An extensive study is carried on

constructed wetlands but still many mechanisms are unknown such as the interactions between soils, plants, water and micro-organisms are not well documented (Toscano*et al.*, 2009). Constructed wetlands are designed to remove contaminants from polluted waters its use has been increasing in developing countries for water treatment (Brix, 1999; Gopal, 1999; Vymazal, 2005).

Projected costs for domestic wastewater treatment via constructed wetland are 4 times less than conventional approaches (Rousseau *et al.*, 2004). CWs are easy to control, harness robust natural treatment routes, as well as they provide esthetic value (Tanner *et al.*, 2012). Also, they produce valuable biomass through different kinds of vegetation grown on it and support wildlife habitat (Wetzet, 2001). Wetlands epitomize the transition zone between terrestrial and aquatic surroundings. Several case studies predicted that wetlands deliver effective nutrient sinks and buffering locations for organic and inorganic contaminants (Hammer and Bastian, 1989).

2.13 Regional and local differences of Pathogens in vegetables:

Developing countries usually report much higher levels of pathogens in irrigation water than developed countries (Thurston-Enriquez *et al.*, 2002).In developing countries; untreated raw wastewater is frequently used for irrigation. In Pakistan quarter of vegetables are irrigated with waste water. In most parts of Sub-Saharan Africa, about 60–100% of the perishable vegetables sold in most cities are irrigated with waste water in urban and peri-urban areas (Scott *et al.*, 2004). Fecal indicator concentrations can reach to high level. Singh *et al.* (2010) found concentrations of fecal coliforms from 105 to 109 MPN/100 mL in waters of Indo-Gangetic riverine system used for irrigation of leafy greens. Sewage or improperly treated effluents from sewage treatment plants can mix with water it may contains hepatitis A, Norwalk viruses, or enter viruses along with bacterial pathogens (Beuchat, 1998). Local differences in developed countries have also been seen (Kavka *et al.*, 2006).

Chaturvedi *et al.*, (2013) investigated the microbiological quality of fresh vegetables, collected from several regions of Ropar, Punjab, India. They collected 36 vegetable samples from low economic area and high economic area. Their study showed that Contamination was mainly found in cauliflower, peas, cabbage, and potato. The microbial load in vegetables obtained from low economic area was significantly higher in comparison to vegetables from high economic areas. In low economic area maximum total plate count was observed in onion followed by carrot and radish while in high economic area it was recorded in peas followed by potato. Equally, in low economic area maximum yeast and mold count was recorded in radish, cauliflower while in high economic area maximum yeast and molds count were recorded in radish, onion and cauliflower, followed by cabbage. Maximum coliform count was observed in low economic areas in cauliflower, followed by onion. Considerable numbers of microbes were also detected in carrot, peas, cabbage and potato in the area. Maximum coliform count in high economic area was recorded in radish followed by carrot. *Eshcerichia coli* were detected only in onion obtained from shops of low economic area.

Intraregional variations in microbial quality of surface waters are significant. For example, in about 3500 surface-water samples from Ohio, 35% samples contained *E. coli* about 126 CFU/100 ml, 13% samples contaminated with 235 CFU/100 mL, 20% contaminated with 576 CFU/100 mL, and 32% samples were contaminated with 576 CFU/100 mL (Stoeckel, 2009).

Microbial quality of well water can be affected by the design of wells, nature of the substrata, depth to groundwater and rainfall (Gerba, 2009).In the USA, the majority of drinking water disease outbreaks documented are caused by fecal contamination of wells (Reynolds *et al.*, 2008). Close *et al.* (2008) reported that fine soil filter pathogens well than stony soil but macropores of stony soil allow quick movement of pathogens (Guber *et al.*, 2005). Deeper soils filter more microorganisms and reduces the contamination of ground water.

Water leaching and microbial transport depends on hydrological system. Travel time of water also depends on water contents, structure of soil, geochemical properties of soil and the deepness of ground water table. If groundwater table is deeper than microorganisms require more time to reach to the ground water. Clay and sandy soil increases the distance of pathogens so they may become die before reaching to ground water (Gerba, 2009). A study by Johnson et al. (2010) found high occurrence of viral contamination (averaging _50 MPN/100 L) in karts aquifers of East Tennessee, and further suggested that co-occurrence rates of viruses and bacterial indicators were higher for karts aquifers than for other aquifer types. Although size of viruses makes them better suited to travel in pore spaces, their interactions with surfaces of the solid matrix can make their transport comparable with the transport of bacteria and parasite oocysts. Unprotected wells routinely have lower microbial water quality than protected wells (e.g., Shortt *et al.*, 2003).

2.14 Incidence of diseases in areas where waste water is used for irrigation:

As long as 60 years ago, Norman and Kabler (1953) observed that poor microbiological quality of irrigation water was associated with the incidence of human pathogens in leafy vegetables. Connections between contaminated irrigation water and clinical studies are typically reported in areas where irrigation water may have unsatisfactory microbial quality, most often having waste origin. Katzenelson *et al.* (1976) compared the incidence of gastrointestinal diseases in 77 kibbutz uses waste water for irrigation, incidence of diseases were two to four times higher in population uses sewage water for irrigation. A study in Mexico compared the incidence of diarrheal disease and microbial quality of the irrigation water in households irrigating vegetables with either untreated wastewater or natural rainfall (Cifuentes, 1998). Rates of diarrhea were significantly higher in households irrigating with untreated wastewater than in households irrigating with rainfall alone. In Morocco, vegetables irrigation with sewage water results in high rate of enteric fever in people working in fields (Ait Melloul and Hassani, 1999).

Populations near microbiologically contaminated surface water sources can be affected via transmission pathways other than irrigation such as aerosols from the surface micro layer as demonstrated in marine environments (Aller *et al.*, 2005), or transfer from domestic animals, insects, etc. Animal and composting services are distinguished sources of airborne particulates and dust as well as insects that vector enteric pathogens, however, emission rates, transport, survival, and deposition of particulates and insects carrying *E. coli*, *Salmonella*, and other fecal bacteria from these sources currently are not counted (Duan *et al.*, 2008; Millner, 2009). Insect vectors may harbor and successively spread Enterobacteriaceae and plant pathogens to plants and animals by direct physical contact (Mitchell and Hanks, 2009). Information about such transport is very limited, but potential vectors for contamination of leafy greens have been recognized and studied (Talley *et al.*, 2009).

Creation of irrigation water storages affects local ecological systems, and can modify pathogen transmission. Ecosystem changes concomitant with irrigation development in Sri Lanka, for example, resulted in long-term changes in the composition of the mosquito fauna, which was characterized by the increasing dominance of species with the potential to transmit human pathogens (Amerasinghe and Indrajith, 1994). Low microbial quality of water can be translated in higher disease incidence not only via agricultural production but also via household uses, including drinking unboiled water (Cifuentes, 1998; Van der Hoek *et al.*, 2001).

In summary, transmission of microorganisms to produce and their consequent survival are evident by incidence studies and multiple recent outbreaks (Mandrell, 2011). However, details of the potential mechanisms of transport have been documented mostly in laboratory studies. More field data are needed to establish reservoirs and patterns of transmission occurring in farm operation environments, and to evaluate the relative importance of various factors such as pathogen concentration, pathogen strain, plant state, irrigation regime, weather patterns, etc. Results of studies of the incidence of E. coli O157:H7 and Salmonella in watersheds and other environments in a major produce production

environment of California emphasize the need for more specific data about these factors (Cooley *et al.*, 2007; Gorski *et al.*, 2011).

Materials and Methods

3.1. Study site

This study was conducted in Islamabad. Islamabad is the capital city of Pakistan. Its population has increased to 2 million conferring by National Census Department in 2012 (Wikipedia). It is situated in north-eastern side of Pakistan. Islamabad experience five different seasons: Winter starts from November and ends in February, March and April comes in spring, summer from May to June, July and August makes rainy season and September and October comes in autumn. June is the warmest month, average temperature increase to 38 °C in June.

3.2. Experimental Design:

Selected v

egetables were grown in two seasons: summer season (from 14th April to 30th June) and winter season (from 10th October to 15th December). Tomatoes, coriander, spinach, radish and okra were grown in summer while cabbage, turnip, lettuce, fenugreek and carrot grown in winter. Temperature was recorded during experiment. For planting of vegetables in summer three tillage were prepared each with five rows of 12 feet long. Shovels and hoes were used for digging before digging these was dipped into ethanol for 2 hours in order to decontaminate them. After digging vegetation and stones were removed and prepared for sowing.

Sterilized seeds for both summer and winter vegetables were purchased from Horticulture department of National Agricultural Research Centre (NARC), Islamabad. Seeds were sowed by trough method. Very shallow trenches were made and seeds were sprinkled lightly and evenly along the trenches and then soil was gently pushed over the seeds for close up. Same method was used for sowing of vegetables in winter.

3.3. Water types and irrigation practice:

Irrigation was done with three different kinds of water quality.

i) Sewage water ii) Wetland treated water iii) Tap water

Irrigation was done by direct application of water over vegetables by water cans. Sewage water was taken from settling tank of constructed wetland; this constructed wetland is running to treat sewage water of residential colony of Quaid-i-Azam University Islamabad. Treated water of this wetland was also used for irrigation and tap water was taken from a house of that colony. Vegetables were irrigated after every two days.

3.4. Sample collection:

3.4.1. Soil sampling:

Random soil sampling was done. Five gram of soil was collected in sterile polythene bags from three different points before vegetables planting. All sampling was done on the day of experiment; collected samples were kept in cooling boxes and transferred to laboratory for analysis.

3.4.2. Water sampling:

All samples were collected in sterilised bottles of 500ml. Sampling was done from sewage water, wetland treated water and tap water. Samples were transferred to laboratory in cooling box for analysis within 2 hours of collection for aerobic plate count. Physico-chemical analysis of water was also done in order to check the nutrient level in water.

3.4.3. Vegetable sampling:

Half kilogram of each vegetable was collected in a sterile polythene zip bags, names of vegetables and tillage were noted on bags. Vegetables were collected randomly from each row. Extensive care was taken to avoid the risk of accidental contamination. The polythene bags with vegetables were kept in an ice box maintained at 5–10°C to keep the normal conditions of the microflora of vegetables and transported to the laboratory for microbial analysis. Study was carried out within 2-3 hours of collection.

3.5. Sample analysis:

Before analysis each sample was coded in laboratory in order to avoid any problem. All samples including soil, water and vegetables were analysed for total microbial count and pathogens present in these. Each vegetable sample was examined as unwashed and washed. Washing of vegetables was done with sterile distilled water. Physico-chemical analysis of water was also done.

3.5.1. Sterilization of Apparatus:

Detergent and tap water was used for washing of apparatus. After washing apparatus was kept for drying and then sterilized in autoclave at 121°C and 15 PSI for 15 to 20 minutes.

3.5.2. Culture Media and Chemicals:

In present study prepared culture media and chemicals were used, obtained from DIFCO Laboratories (Detroite, Michigan, USA), BDH Laboratory Chemical Division (Poole, Dorset, England), ICI America 9211 North Harborgate street Portland, Sigma Chemicals Co., St. Louis, E. Merk (Dernstadt, Germany) and Oxoid chemical company UK.

3.5.3. Media preparation:

Leco 250 analytical balance was used for weighing of media. Required amount of synthetic media was added in distilled water according to recipe mentioned on bottle. Magnetic stirrer was used for mixing of media after mixing it was sterilized in autoclaved by above mention method.

3.5.4. Microbial Analysis of Soil:

Fresh soil sample was sieved by using the sterile sieve, beaker and pestle. For serial dilutions 9ml of autoclaved water was taken in 10 tests tubes then 0.1g of sieved soil was used to made serial dilutions up to 10^{-9}. After that 0.1 ml of diluted sample was taken through micropipette from 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} and spread on petri plates containing nutrient agar. These petri plates were incubated 24 hours at 37°C temperature. After incubation period different bacterial colonies appeared on nutrient agar plates then these colonies were counted using colony counter. Colony forming unit of each colony was calculated with the help of formula:

CFU/ ml = number of colonies x dilution factor/Volume inoculated

Morphologically differentiated colonies from these plates were sub cultured on nutrient agar for further analysis. Pure culture was obtained by sub-culturing on different agars. Pseudomonas Cetrimide agar was used for isolation of *Pseudomonas* sp., Salmonella-Shigella agar was used for isolation of *Salmonella – Shigella*, Eosine methylene blue agar and MacConkey's agar were used for isolation of all Gram$^-$ bacteria. After sub-culturing on different agars, identification of isolated bacteria was done on the basis of morphological depictions, microscopic observations and biochemical examinations.

3.5.5. Microbial analysis of water:

Microbial count was done by plate count method, for sewage, treated and tap water samples, samples were diluted through serial dilutions. One ml of water sample was added to the tube containing 9 ml of sterilized water and dilutions were made up to 10^{-10}. Then 0.1 ml of diluted sample was taken by the help of micropipette from 10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} dilutions and spreading of samples

on plates of nutrient agar was done through spreader made up of glass rod. 24 hours of incubation period was given to these plates at 37°C temperature. Further analysis was done by above mentioned method

3.6. Physico-chemical analysis of water:

3.6.1. Dissolved Oxygen:

"Digital DO Meter" was used for determination of dissolved oxygen in water samples. "Digital DO Meter" contains sensory probe for measurement of dissolve oxygen, sensory probe was washed thoroughly before measurement and de-ionized water was water used for washing of probe. Sensory probe was dipped into sample and reading was noted.

3.6.2. Biochemical oxygen demand (BOD_5)

BOD_5 is the measurement of oxygen rate consumed by microorganisms for degradation of organic material in water under five day period. Standard Method 5210B (APHA, 21st Edition) was used for measurement of BOD_5 of the waste water, treated water and tap water.

Reagents:

- **Calcium Chloride Solution:** In reagent water 27.5 g of $CaCl_2$ was mixed and then water was added to make 1 L final volume.
- **Magnesium sulphate solution:** 22.5 g of magnesium sulphate was dissolved in reagent water, to make final volume of 1 L more water was added in solution.
- **Ferrous Chloride Solution:** 1 L solution of $FeCl_2$ was made, 0.25 g ferrous chloride hexahydrate was dissolved in reagent water and final volume was made by adding more water.
- **Phosphate Buffer Solution:** Phosphate buffer solution contained 1.7 g of ammonium chloride, 21.7 g of dipotassium hydrogen phosphate, 33.4 g sodium hydrogen phosphate and 8.5 g potassium dihydrogen phosphate. All chemicals were mixed in 500 ml reagent water and 1 litre final volume solution was made adding water. pH of the solution was kept 7.2.

Procedure:

- **Preparation of dilution water:** Dilution water was flooded with organic free air. 1 ml of each chemical solution was added in 1000 ml of dilution water.
- **Dilution Technique:** 295 ml of dilution water was taken in aspirator bottle which contained long rubber tubing attached to lower end and 5 ml of sample was dissolved in this water and air entrapment was avoided. Two BOD bottles were taken and filled with mixed dilution water, one bottle was used to measure the initial dissolved oxygen and other bottle was tightly air locked and incubated at 20°C for 5 days. Following formula was used to determine the BOD_5 of water;

Calculations:

$$BOD_5 \text{ (mg/l)} = (DO_1 - DO_2)/\text{volume of the dilution sample} \times 1000$$

Where,

DO_1 = DO of diluted sample taken after preparation

DO_2 = DO of dilution sample taken after 5 days incubation period

3.6.3. Chemical oxygen demand (COD):

Closed Reflux method was used to determine chemical oxygen demand of the water. Titrimetric method 5220 C (APHA, 21st edition).

Reagents:

- **Sulphuric Acid Reagent:** Conc. Sulphuric acid was taken and 5.5 g of silver sulphate was mixed in sulphuric acid at rate of per kg of sulphuric acid. Then it was kept for dissolution of silver sulphate for two days.
- **Digestion Solution:** Digestion solution contained 10.2 g of potassium dichromate (dried for two hours at 37°C), 33.3 g of mercuric sulphate and 167 ml concentrated Sulphuric acid, all these were dissolved in distilled water and diluted to 1000 ml.
- **Potassium Hydrogen Phthalate Solution**: small quantity of distilled water was taken and 425 mg of KHP was dissolved in it, KHP was dried

at 120° C to achieve a constant volume before its use. Final volume of 1 L solution was achieved by adding water.

Procedure:

COD of sample was determined in 16× 100mm culture tubes. 3.5 ml sulphuric acid reagent, 2.5 ml standard solution and 1.5 ml digestion solution was supplemented in each tube. All tubes were tightly closed and heated in oven for two hours at 150° C. then tubes were allowed to become cool and solids were removed after settled down. Spectrophotometer was used for measurement of COD at wavelength of 600nm. Recorded absorbance was equated with calibration curve.

3.6.4. Orthophosphate:

- **Ammonium Molybdate Solution:** 175 ml distilled water was taken in a reagent bottle and 25 g ammonium molybdate was mixed in it and in other reagent bottle 400 ml distilled water was taken and conc. sulphuric acid of 80 ml was mixed to water, allowed to cool. Solution of ammonium molybdate and sulphuric acid solution was mixed and this solution was diluted to obtain the final volume of the 1 litre.
- **Phenolphthalein indicator Solution:** 100 ml 60 % ethyl alcohol was taken in reagent bottle and 0.5 g of phenolphthalein powder was added in it to make phenolphthalein indicator solution.
- **Stannous Chloride Solution:** 100 ml of glycerol was used to dissolve 2.5 g of stannous chloride, dissolution was speed up by heating.
- **Strong Acid Solution:** 600 ml distilled water was taken in flask and 300 ml conc. sulphuric acid was slowly added in it and kept for cooling. After cooling 4 ml of conc. HNO3 was mixed and diluted to get final volume of the 1 litre solution.

Procedure:

25 ml water sample was taken in a beaker and added 4-5 drops of phenolphthalein indicator solution and 3 to 4 drops of strong acid solution. Allowed to develop ponk colour pink colour. After the appearance of pink colour 1 ml of ammonium

molybdate solution was mixed in it, after that stannous chloride solution about 25 microliter mixed. Sample was left unshaken for 10 minutes to develop the colour. Spectroquant was used to measure the concentration of phosphate.

3.6.5. Sulfates:

Hardness of water is caused by sulphates. Concentration of sulphates was measured by EPA method 0375 Barium chrometery method.

Reagents:

- **Barium Chloride Crystals**
- **Buffer Solution A**: initially 500 ml of distilled water was taken in reagent bottle and 5g of sodium acetate, 30g of magnesium sulphatehexa hydrate, 1g potassium nitrate, and 20ml of 99% acetic acid were mixed in it, after dissolution more water was added in solution to get the final volume of 1 litre.

Procedure:

25ml sample was taken in 100 ml flask and 5ml of buffer solution was mixed to it. Then, crystals of $BaCl_2$ was mixed and dissolved by stirrer. Spectroquant was used for measurement of sulphates.

3.6.6. Nitrate-nitrogen (NO_3-N):

Concentration of nitrates in water was measured by EPA method 4500 (APHA, 21st edition).

Reagents:

1 Normal HCl

Procedure:

Spectroquant was used to determine the level of nitrate-nitrogen, 0.5ml 0f 1N HCl was mixed to 25 ml of sample.

3.6.7. Nitrite-nitrogen (NO_2-N):

Nitrites concentration was determined by EPA method 4500 (AHPA 21st edition)

Reagents:

- **Buffer coloured Reagent:** 800ml of distilled water was used for dissolution of 10g of sulphanilamide, 100 ml 85% phosphoric acid and 1g of N (1-naphthyl) ethylene diamine dihydrogen chloride. After dissolution more distilled water was added to make the solution of 1 litre.

Procedure:

Water sample was filtered and 25 ml sample was taken in a 100 ml flask and added 1ml of buffer reagent, it was mixed till to appearance of colour. Spectroquant was used to determine the amount of nitrite-nitrogen.

3.6. Vegetables Analysis:

3.6.1. Preparation of Buffer:

Vegetables were grinded for serial dilutions. Butterfield's Phosphate Buffer was used for blending of vegetables and serial dilutions. Stock solution of buffer was made. For preparation of buffer 500 ml distilled water was taken in a reagent bottle and 34grams of potassium dihydrogen phosphate was dissolved in it. More distilled water was added to solution to obtain the final volume of 1 L. pH of buffer was kept to 7.2. Stock solution of butterfield's phosphate buffer was stored at 4° C. working solution was made by taking 1.25 ml of stock buffer through micropipette (100-1000 µl) in a graduated cylinder of 1000 ml and further diluted up to 1 L. This solution was used for blending and serial dilution of vegetable samples. For serial dilutions 9 ml of working buffer solution was taken in test tubes and autoclaved for sterilization.

3.6.2. Preparation of Sample:

Preparation of sample and serial dilutions was done according to the method defined in manual of FAO (1992). Sterilized knife was used to cut the vegetables into small parts. For blending of vegetables 225 ml sterile Butterfield's phosphate buffer ml was taken in Warring blender and 25 grams of vegetable sample was added in buffer and blended for two minutes at 12000 rpm. During blending volume of buffer was decreased to half and this aliquot was considered as 10^{-1} dilution, then serial dilutions were made from the blended samples in sterile butter field's phosphate buffer. All dilutions were vortex for about 5 seconds.

3.6.4. Total Microbial Count:

For total bacterial count 0.1 ml diluted sample from 10^{-1}, 10^{-3} and 10^{-5} was put on nutrient agar plates and spreaded with the help of spreader. Then, these nutrient agar plates were allowed to incubate for 24 hours at 37° C. Various colonies were appeared on incubated plates, these were counted in colony counter. While morphologically differentiated colonies were picked through sterile wire loop and cultured on nutrient agar plates. Later on colonies from these plates were used for further analysis. These colonies were sub-cultured on Eosine methylene blue agar, MacConkey's agar media to get the pure cultures of Gram⁻ bacteria. Salmonella-Shigella agar media was used for isolation of *Salmonella* and *Shigella* sp., Pseudomonas Cetrimide agar was used for *Pseudomonas* sp. Cultures were streaked on plates containing above media and incubated for 24 hours at 37°C. Isolated bacterial strains were identified on the basis of morphology, microscopic depiction and biochemical analysis.

3.7. Identification of Bacterial isolates:

3.7.1. Morphological Description:

Morphology of Colonies:

Microorganisms can be distinguished on the basis of their morphological features because each microorganism has its own morphology. On the basis of opacity, colour, mass, forms, boundary and elevation isolated strains were sorted out.

Microscopy:

Smear was prepared by taking a bacterial colonies with the help of wire loop and attached on glass slides, slides were dried out and cultures were fixed by heat on slides. Smear was flooded with Gram's Crystal violet dye and rinsed after 1 minute. After that smear was flooded with Gram's iodine and allowed to stand for one minute before washing, then rapid decolourization was done with 95% C_2H_3OH, after decolourization slides were rinsed with tap water. After decolourization smear were flooded with Safranin and was allowed to stand for 45 seconds again washing was done. Then these slides were dried out and observed under microscope with oil emulsion objective (100x). Under microscope Gram negative bacteria looked pink and Gram positive bacteria appeared purple as they obtained the colour of secondary and primary dye respectively.

3.7.2 Biochemical Representation:

Biochemical reactions of isolated strains were observed and identification of strains was done by consulting the Bergey's Manual of Determinative Bacteriology (9^{th} Edition). Following biochemical tests were conducted:

- Triple sugar iron for lactose/Glucose fermentation
- Indol and H_2S test
- Citrate utilization test
- Nitrate reduction test
- Catalase test
- Carbohydrate fermentation
- Urease test
- Methyl red Vogas-Proskauer test
- Carbohydrate fermentation test
- Oxidase test

3.7.2.a. Triple sugar iron test:

Procedure:

Triple sugar iron was conducted for fermentation of Glucose or lactose. Slants of triple sugar iron agar were prepared. Stab-streak method was used for inoculation

of bacterial colonies into slants, inoculation was done with the help of sterile needle. These slants were incubated at 37° C for 24 hours. After incubation slants were observed for colour change and acid production, Pink red colour of slants indicated alkaline reaction while acid production was indicated by yellow colour of butt. Lactose fermentation was indicated by slant colour while glucose fermentation was indicated by butt colour.

3.7.2.b. Indole and H_2S test:

Some bacterial strains utilizes amino acids and produces indole and H_2S and addition of kovac's reagents confirm the presence of indole.

Kovac's Reagents:

Composition:

150 ml Amyl alcohol, 50ml hydrochloric acid, and 10g p-dimethylaminebenzaldehyde.

Procedure:

SIM agar deep tubes were made under sterile condition and inoculation was done by stab inoculation method. Inoculated slants were incubated at 37° C for 24 hours. Slants were observed for presences of H_2S and indole production was observed by appearance of cherry red colour layer on agar after the addition of 2 to 3 drops of kovac's reagent and absence was indicated by no colour change. Appearance of insoluble black ferrous sulphate precipitate along the line of stab inoculation showed the presence of H_2S gas.

3.7.2.c. Citrate Utilization Test:

Procedure:

Some bacterial strains utilizes citrate as their carbon source and this test was conducted to identify citrate utilizing bacterial strains. Simmon's citrate agar slants were prepared. Inoculation of bacterial strains was done by inoculating needle and left for incubation for 24 hours at 37° C. Citrate utilizing strains were identified by colour change of agar from green to blue while in case of negative strains colour of agar remained green.

3.7.2.d. Nitrate Reduction Test:

Two reagents were used for identification of nitrate reduction.

Reagent A:

Composition:

8grams Sulfuric acid and1000 ml 30% acetic acid (5M).

Reagent B:

Composition:

5 g Alpha-naphthylamine and 1000 ml acetic acid (5M).

Procedure:

This test was done to identify the bacterial strains which reduces nitrate to nitrite. Tubes of nitrate reduction broth was prepared and inoculation was done with the help of sterile wire loop and inoculating tubes were incubated at 37° C for 24 hours. After incubation period reagent A and reagent B was added in the tubes and positive reaction was indicated by appearance of cherry red colour immediately after addition of reagents. Those tubes which did not show the colour change, small amount of Zinc dust was added to them. Those showed colour change after addition of Zinc dust was considered as positive test.

3.7.2.e. Catalase Test:

Procedure:

Clean glass slide was taken and single bacterial colony was placed on it by wire loop. Bubble formation indicated positive reaction after the addition of one drop of hydrogen peroxide while negative test was confirmed by no bubble formation.

3.7.2.f. Urease Test:

Procedure:

Urea broth was prepared according to recipe cited at chemical bottle, then this media was autoclaved for sterilization. After autoclaving it was left for cooling and then 5ml urea per 95 ml urea broth was added through syringe filter. Urea

was prepared by dissolving 0.2 g of urea in 5 ml distilled water. Tubes of this urea broth was prepared and inoculated with strains. These tubes were incubated at 37° C. Positive test was indicated by appearance of light pink colour.

3.7.2.g. Methyl Red and Vogas-Proskauer Test:

MR-VP broth was prepared and 5 ml broth was transferred in each test tube. Inoculation of bacterial strains was done and these tubes were incubated for 24 hours at 37° C.

Reagents used for identification includes:

Methyl Red Indicator:

Composition:

300 ml of 95 % ethyl alcohol, 0.1 g Methyl Red and 2000 ml distilled water.

Barrit's Reagent:

Barrit's reagent was used for Vp test, it includes solution A and solution B.

Solution A:

Composition:

5g Alpha-naphthol and 100 ml Ethanol.

Solution B:

Composition:

40 NaoH and100 ml deionized water.

Procedure:

Isolated bacterial colonies were grown in MR-VP broth in test tubes at 37° C. then cultures were divided into two parts by shifting half of culture to sterile test tubes. For methyl red test two drops of methyl red indicator was added and observed the colour change. Bright red colour was appeared in case of positive test and negative test did not show any colour change.

3 ml of Alpha-naphthol and 1 ml of NaoH was added in other tubes and tubes were shaken continually to maintain reaction. Pink colour was appeared in case of positive test. The appearance of pink colour within two to three minutes accompanied a positive test. During this time interval test tubes were shaken constantly to maintain reaction.

3.7.2.h. Carbohydrate fermentation test:

Medium Composition:

Peptone.....5 g

Phenol red.....0.018 mg

Glucose, lactose, sucrose.....5 g

Beef extract.....3 g

Distilled water.....1000 ml

Procedure:

Anaerobic conditions were given for fermentation test. Durham tubes were used for observation of gas production. After inoculation, tubes containing fermentative medium were kept for incubation at 37° C for 24-48 hours and observed for colour change from red to yellow and gas production which was indicated by bubble in inverted Durham tubes. Cultures that were not capable of fermenting a carbohydrate substrate did not change the indicator and the tubes observed red, there was no affiliated evolution of gas. That was considered as negative reaction.

3.7.2. i. Oxidase test:

Procedure:

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. Took a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride. Moistened the paper with a sterile distilled water. Colony was picked with platinum loop and

smear in the filter paper. Then observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

Measurement of Plants Height:

Heights of plants was measured by measuring tape. Height of plants was measured from the base of the plant to the top of the plant. It was measured two times first at forty days after sowing and second time after 60 days.

4.1.a. Statistical analysis:

All experiments were performed in triplicate. Statistical analysis of difference between mean values obtained for aerobic plate count of vegetables grown in sewage water, treated water and tap water was depicted by Analysis of Variance (ANOVA), Tukey's test was used for all pairwise comparison to calculate LSD values. APC values were converted into log 10 prior to statistical analysis. P-values of 0.05 or less were considered as significantly different. Paired 'T' test was applied to assess the significance level between unwashed and washed vegetables. Analysis of Variance (ANOVA) and Tukey's test was applied to calculate the significance level between growths of vegetables.

RESULTS

This study was conducted to assess the risk related to vegetables that are grown in different qualities of water and also the effect on the growth of vegetables irrigated with these waters. During this research work ten vegetables were grown in sewage water, wetland treated water and tap water in two different seasons; okra, radish, spinach, tomato and coriander grown in summer season (from 15th April to 30th June) while lettuce, turnip, fenugreek, carrot and cabbage grown in winter (from 10th October to 15th December). These were grown in two seasons in order to check the microbial count in different seasons. Average temperature in summer growing season was recorded 29°C and in winter growing season it was recorded 16°C. Before planting of vegetables Microbial counts of soil, sewage water, tap water and wetland treated water were analyzed. Physic-chemical analysis of water was also done to check the level of nutrients for plant growth. Random sampling of vegetables was done from all tillage. All these vegetables are grouped into two sub samples and analyzed as unwashed and washed. After collection all samples were coded and transported to laboratory. In laboratory these samples were analyzed for aerobic plate count, presences of pathogenic bacteria. Growth of vegetables in terms of height was also measured in all vegetables; measurement was done two times during growth season.

4.1. Sample Analysis:

4.1.b. Soil analysis:

Soil type : Non agricultural

Location : D-type colony West of Quaid I Azam University Islamabad

Seasons : Winter and Summer

Crops yielded : Okra, Radish, tomato, spinach, coriander (April to June) and Turnip, Cabbage, Lettuce, Carrot and Fenugreek (Oct to Dec)

Bacterial count from soil samples shown in Table (1)

Table. 1. APC of soil samples

Sample #	Aerobic plate count media	Colony #	CFU/ml
01	Nutrient agar	75	750
02	Nutrient agar	62	620
03	Nutrient agar	64	640

The bacterial isolates obtained from soil were characterized according to Bergey's Manual of Determinative Bacteriology (9th edition). Six different strains were isolated form soil, 'a' and 'd' were G^+ rods, 'b', 'e' and 'f' were G^- rods and 'c' was G^- coccobacilli (Table 7). These were then sub-cultured on selective media as well as nutrient agar and after that, they were sorted on the basis of their unique morphology found on these media (Table 6). Further identification of these isolates was done by performing biochemical test shown in Table (8).

Based on microscopic observation, morphology depiction and biochemical analysis, bacteria identified from soil are *B. cereus, E. coli, Alcaligenes faecalis, Corynebacterium xerosis, Pseudomonas aeruginosa and Proteus mirabilis.*

4.1.c. Microbial analysis of water:

Microbial quality of sewage water, treated water and tap water was assessed before they were used for irrigation. Treated water used in this study were treated through constructed wetland. In constructed wetland water were exposed to anaerobic digestion, sand filter and plants. Plants utilizes different compounds from water for their growth and also extract microorganisms from water by attaching them to their roots. Mean CFU/ml for sewage water was recorded to 7 x 10^7, for treated water it was 8.1 x 10^2 and 1.8 x 10^2 was recorded for tap water in summer season. Twelve different strains were isolated from sewage water, strains 'a' and 'd' showed G^+ reaction both strains were rods, strain b, e, f, h, j, k, l were pink rods, strain g, I, m were purple cocci and strain 'c' showed G^- reaction it was coccobacilli (Table 7).

After microscopic observation, these isolates were sub-cultured on nutrient agar and selective media so that they could be morphologically distinguished from each other (Table 6). The isolates were then biochemically examined for identification (Table 8). On the basis of above tests, bacteria isolated from sewage water are, *B. cereus, E. coli, Corynebacterium xerosis, Pseudomonas aeruginosa, Proteus mirabilis, Shigella dysentriae Staphylococcus aureus, Streptococcus lactis, Enterobacter aerogenes, Klebsiella pneumoniae, Micrococcus luteus and Salmonella* Typhimurium.

Eight strains a, b, d, e, f, g, h and I were isolated from treated water. Strain b, e, f, h were pink rods, strain 'g' and 'I' were purple cocci, strain 'a' and'd' were purple rod (Table 7). These strains were morphologically distinguished on selective media (Table 6) and then subjected to biochemical tests (Table 8).

Isolated strains include *E. coli, Salmonella* typhimurium, *Staphylococcus aureus, Corynebacterium xerosis, Enterobacter aerogenes, Micrococcus luteus, Pseudomonas aeruginosa* and *B. cereus.* Seven strains were isolated from tap water, these were identified through microscope shown in Table (7), selective media (Table 6) and biochemical tests (Table 8). Isolated strains are *B. cereus, E. coli, Proteus mirabilis, Salmonella* typhimurium, *Streptococcous lactis, Staphylococcus aureus and Pseudomonas aeruginosa.*

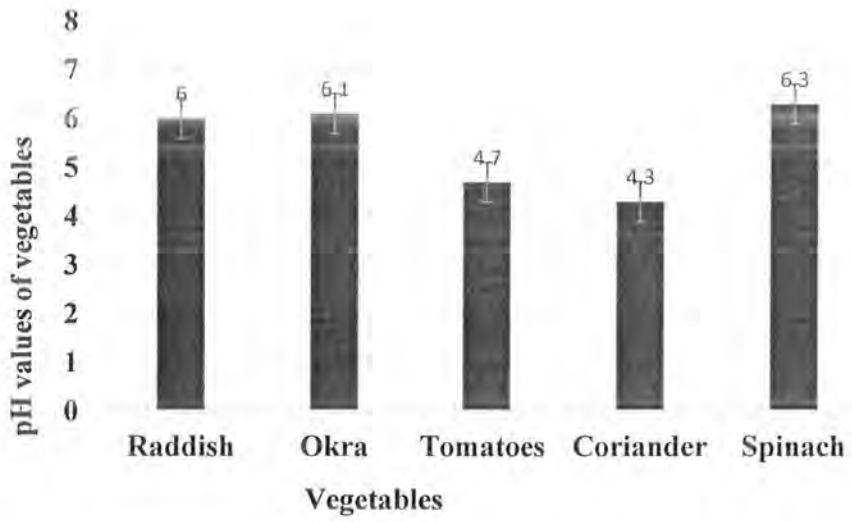
In winters mean number of bacteria from sewage water was 2.1×10^4, treated was 1.3×10^2 and tap water was 7×10^1. Morphologically ten different strains were isolated from sewage water in winter season. On the basis of microscopy (Table 7), morphology (Table 6) and biochemical tests (Table 8) these were identified as *Corynebacterium xerosis, Pseudomonas aeruginosa, E. coli, Proteus mirabilis, B. cereus, Staphylococcus aureus, Streptococcus lactis, Klebsiella pneumoniae, Micrococcus luteus* and *Salmonella* Typhimurium.

From treated water *Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, B. cereus, Streptococcus lactis* were isolated, their morphology, microscopy and biochemical tests are given in Tables (6, 7 and 8)

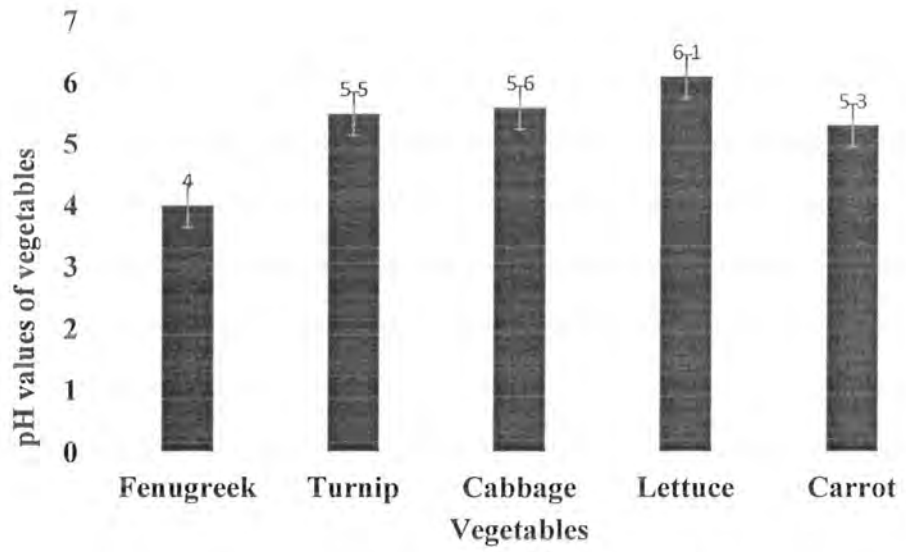
Proteus mirabilis, Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus luteus were isolated from tap water in winter season, their morphological depiction, microscopy and biochemical tests are given in Tables.

4.1.d. Determination of pH of vegetables samples:

Each vegetable sample was blended in sterile distilled water and its pH was measured with the help of digital pH meter. Lowest pH values were recorded for fenugreek and spinach showed highest pH in overall samples. Vegetables grown in summer varied for their average pH values from 4.3 to 6.3 (Graph 1). Highest pH was recorded for spinach followed by okra, radish, tomato and coriander. While vegetables grown in winter varied for their average pH values from 4 to 6.1 (Graph 2). While lettuce exhibited highest pH and followed by cabbage, turnip, carrot and fenugreek in winter season. pH of spinach, lettuce and okra were found to be close to one another.



Graph. 1. Comparison of pH values of vegetables grown in summer season



Graph. 2. Comparison of pH values of vegetables grown in winter season

4.2. Comparison between Aerobic Plate Count of Vegetables Grown in Sewage, Treated and Tap Water in Summer Season:

All the samples of vegetables grown in sewage water showed higher microbial count as compared to vegetables of treated and tap water. Highest microbial count was noted in okra (1×10^7) followed by spinach (8.9×10^6), radish (1.89×10^5), tomatoes (1.36×10^5) and coriander (8.4×10^4) was least contaminated in all sewage water vegetable samples. While in treated water highest microbial count was found in spinach (1×10^4) followed by okra (4.3×10^3), tomatoes (2.75×10^3), radish (1.84×10^3) and coriander (1.83×10^3), whereas vegetables grown in tap water showed highest microbial count in okra (3.6×10^4) followed by spinach (2.9×10^3), tomatoes (1.57×10^3), radish (1.11×10^3) and coriander (1.01×10^3).

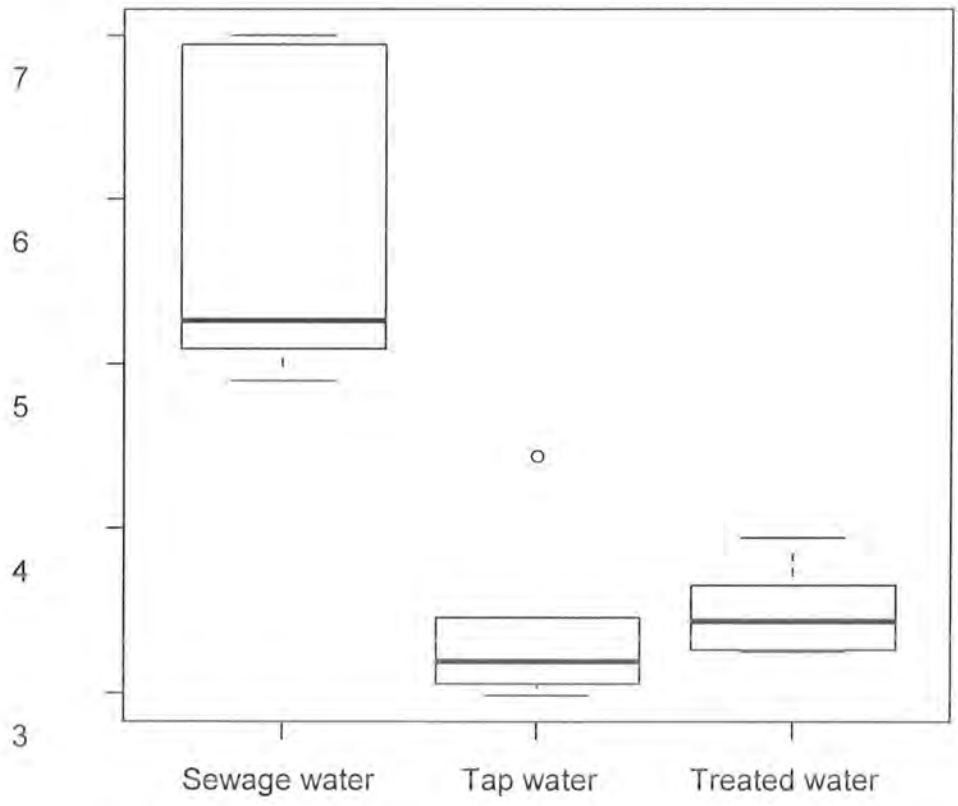
Spinach was highly contaminated in sewage water but in treated water and tap water okra was highly contaminated and coriander was least contaminated in all samples of vegetables in all waters. Analysis of variance (ANOVA) indicated highly significant difference at $p \leq 0.05$ between sewage water and treated water irrigated vegetables and highly significant difference was also found between sewage water and tap water irrigated vegetables but treated water and tap water irrigated vegetables differed non-significantly with each other (Graph 3) Although all treated water vegetables samples showed high microbial count except for okra, which showed high microbial count in tap water vegetables as compared to treated water but difference was non-significant.

Table. 2. Analysis of Variance for APC for vegetables grown in sewage water, treated water and tap water

Source of variation	DF	Sum of Squares	Mean Square	F	P
Water	2	18.702	9.351	18.51	0.00021
Residual	12	6.063	0.505		

Table. 3. All pairwise significant difference between sewage water, treated water and tap water irrigated vegetables

Comparison	Difference	Lower	Upper	P value
Tap water-sewage water	-2.4083	-3.6076	-1.2090	0.0004
Treated water-sewage water	-2.3269	-3.5262	-1.1275	0.00062
Treated water-Tap water	0.0814	-1.1178	1.2807	0.9820



Graph. 3. Comparison between APC of Vegetables Grown in Sewage, Treated and Tap water

4.3. Comparison between Aerobic Plate Count of Vegetables Grown in Sewage, Treated and Tap Water in Winter Season:

Sewage water, treated water and tap water presented highest influence on number of colonies on vegetables in winters just like summer season. In sewage water lettuce showed highest count (1.6×10^4) followed by cabbage (1×10^4), turnip (3.99×10^3), carrot (3.3×10^3), fenugreek (2.8×10^3).

Lettuce also presented highest count in treated water up to 1.14×10^3 followed by cabbage and carrot with similar microbial count up to 1.9×10^3, turnip revealed 1×10^3 count and fenugreek presented minimum microbial count 3.2×10^2.

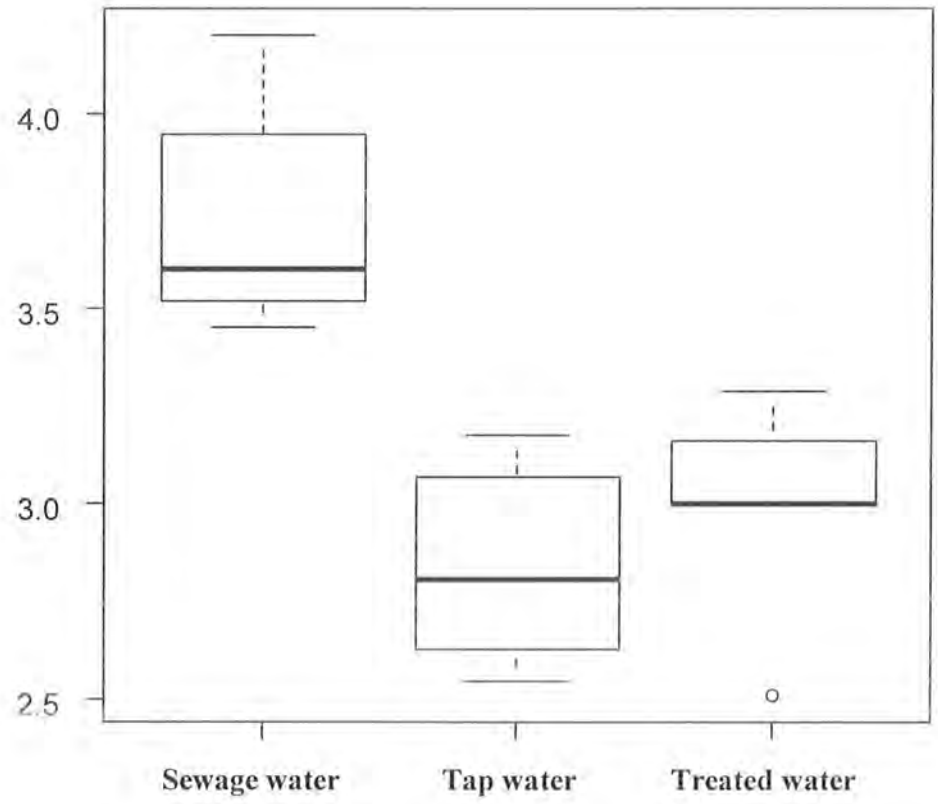
In tap water lettuce exhibited 1.16×10^3 count, turnip showed 1.4×10^3 count followed by cabbage with 6.3×10^2 microbial count and fenugreek with 3.5×10^2 showed lowest microbial count in tap water irrigated vegetables. Analysis of variance conducted for APC of winters vegetables grown in sewage water, treated water and tap water revealed that a highly significant difference was ($p \leq 0.05$) lied between vegetables grown in sewage water and tap water and highly significant difference was also found between vegetables grown in sewage water and treated water but vegetables grown in treated water and tap water varied non significantly shown in graph (4).

Table. 4. Analysis of Variance for Aerobic Plate Count for vegetables grown in sewage water, treated water and tap water in winter season

Source of variation	DF	Sum of Squares	Mean Square	F	P
Water	2	2.331	1.1653	13.33	0.0008
Residual	12	1.049	0.0874		

Table. 5. All pairwise significant difference between APC of sewage water, treated water and tap water irrigated vegetables in winter season

Comparison	Difference	Lower	Upper	p adj
Tap water-Sewage water	-0.9002	-1.3990	-0.4013	0.0011
Treated water-Sewage water	-0.7524	-1.2513	-0.2535	0.0044
Treated water-Tap water	0.1477	-0.3510	0.6466	0.7158



Graph. 4. Comparison between APC of vegetables grown in different qualities of water in winter season

4.4. Comparison between APC of Unwashed and Washed Vegetables:

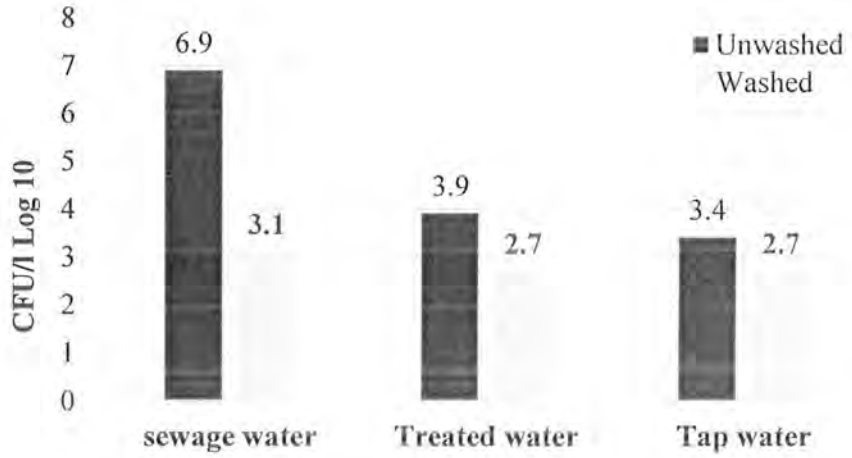
Vegetables grown in sewage water, treated water and tap water were analyzed for APC in unwashed and washed samples. Vegetables grown in sewage water contained highest number of mesophilic heterotrophic bacteria in unwashed spinach samples followed by okra, radish, tomato and coriander and same trend was noted for vegetables grown in treated water but in tap water grown vegetables highest count was noted in okra followed by spinach, tomato, radish and coriander. Washing prominently reduced bacterial count in all samples. Highly significant difference was found by paired T test analysis (Appendix A.2)

Significant difference was noted at $p \leq 0.5$ between unwashed and washed samples of spinach (Graph 5). Highly significant difference was observed between unwashed and washed samples of okra grown in sewage water and treated water while significant difference was noted for tap water irrigated okra samples (Graph 6). Significant difference was found at $p \leq 0.5$ between unwashed and washed samples of tomatoes irrigated with sewage water but highly significant difference was noted for other two samples (Graph 7). Minimum bacterial count was observed on tap water irrigated coriander and highly significant difference was observed for unwashed and washed coriander samples (Graph 8) and all unwashed and washed radish samples showed highly significant difference at $p \leq 0.5$ (Graph 9).

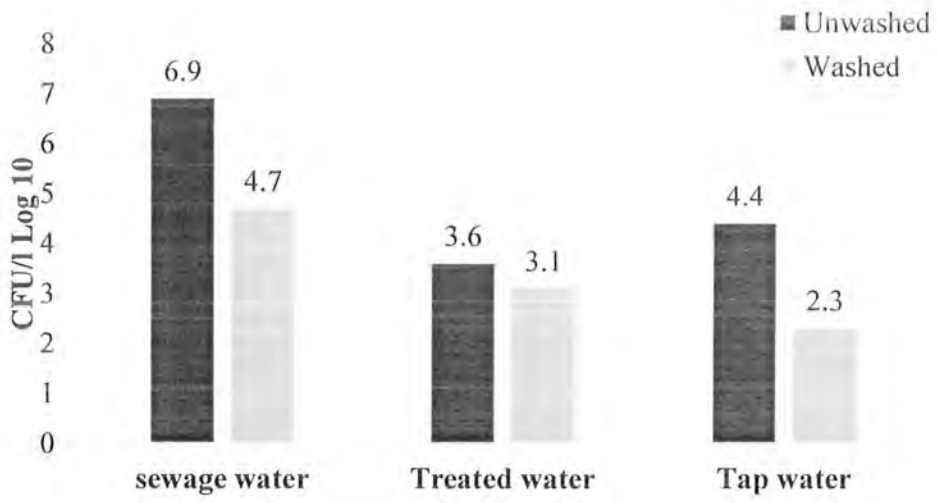
Winters vegetables revealed results that lettuce from sewage water was highly contaminated followed by cabbage, turnip, carrot and fenugreek. Washing significantly reduced the count of bacteria on all lettuce samples (Graph 10). Significant difference was found between unwashed and washed cabbage samples of sewage water and treated water, while highly significant difference was found in cabbage sample of tap water and microbial count was reduced up to permissible limits in tap water samples (Graph 11).

Fenugreek harbored minimum microbial count in all samples, highly significant difference was found between unwashed and washed samples of sewage water fenugreek but non-significant difference was noted for unwashed and washed samples of fenugreek grown in treated water and tap water but CFU in tap water

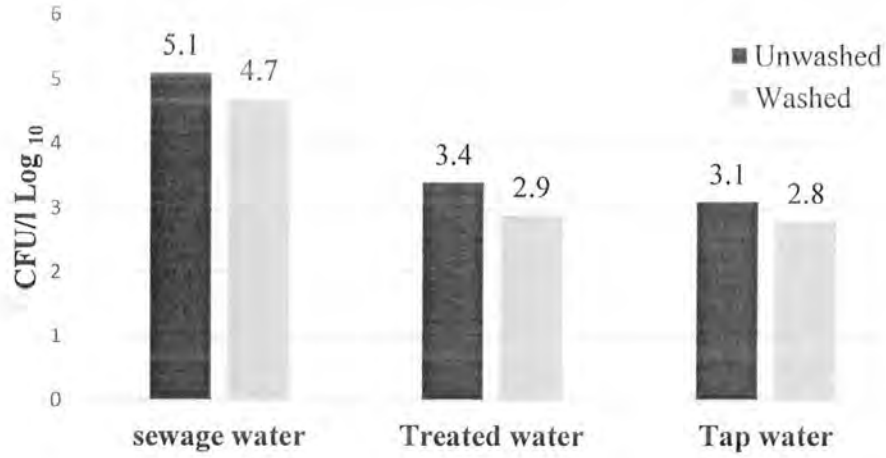
irrigated fenugreek came down to permissible limits. (Graph 12). Highly significant difference was also noted for turnip samples (Graph 13). Paired T test values showed highly significant difference between unwashed and washed samples of carrot, microbial count reduced up to permissible limits in washed samples of carrot grown in tap water (Graph 14)



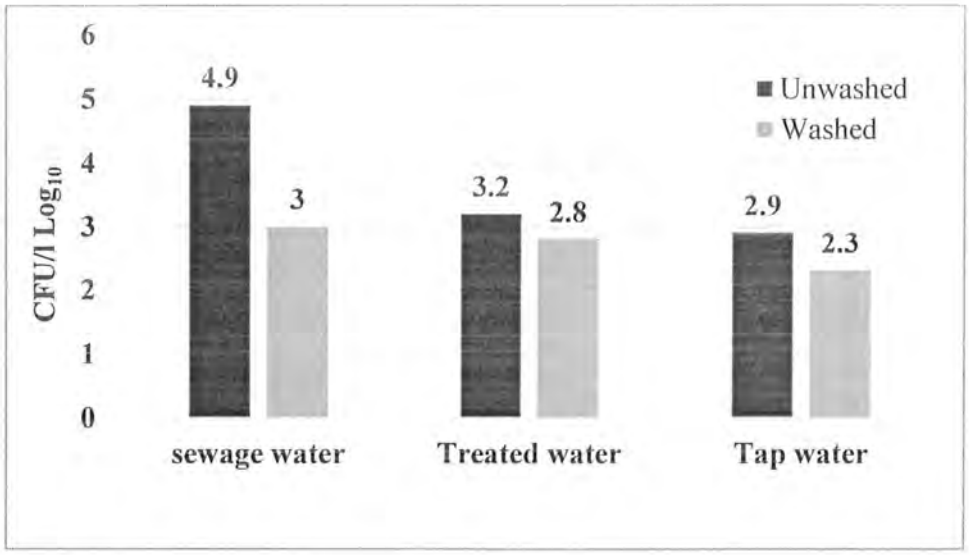
Graph. 5. Comparison between CFU of unwashed and washed samples of spinach grown in sewage, treated and tap water



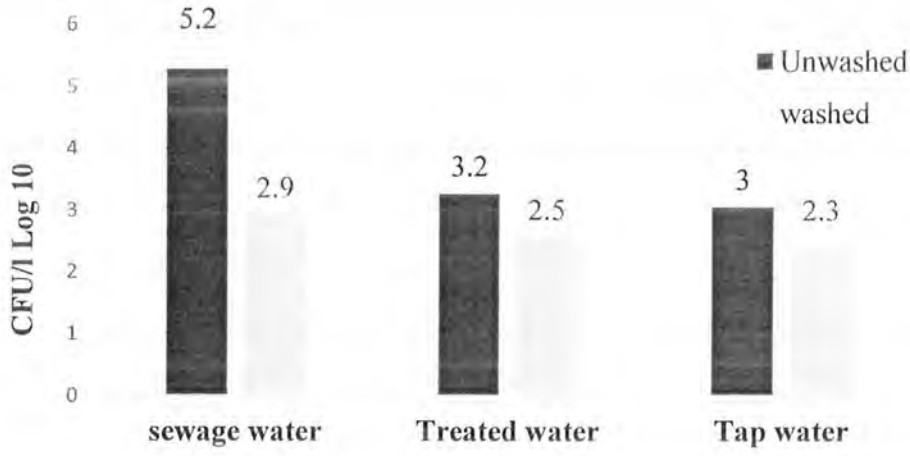
Graph. 6. Comparison between CFU of unwashed and washed samples of okra grown in sewage, treated and tap water



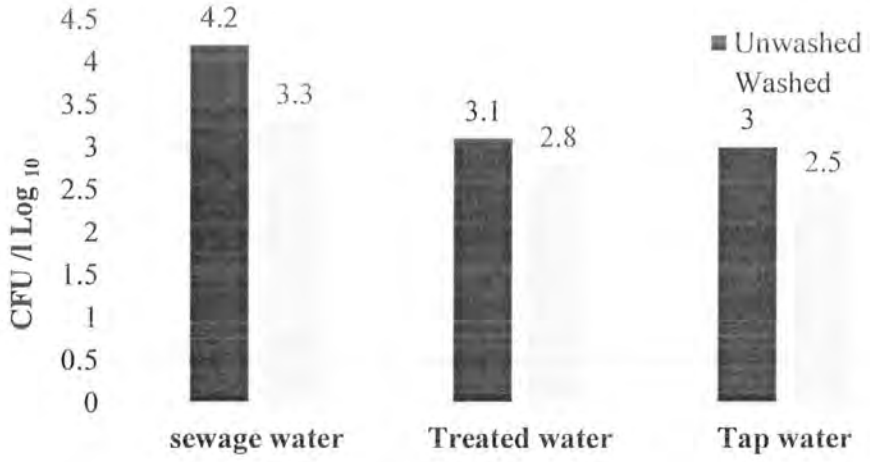
Graph. 7. Comparison between CFU of unwashed and washed samples of tomatoes grown in sewage, treated and tap water



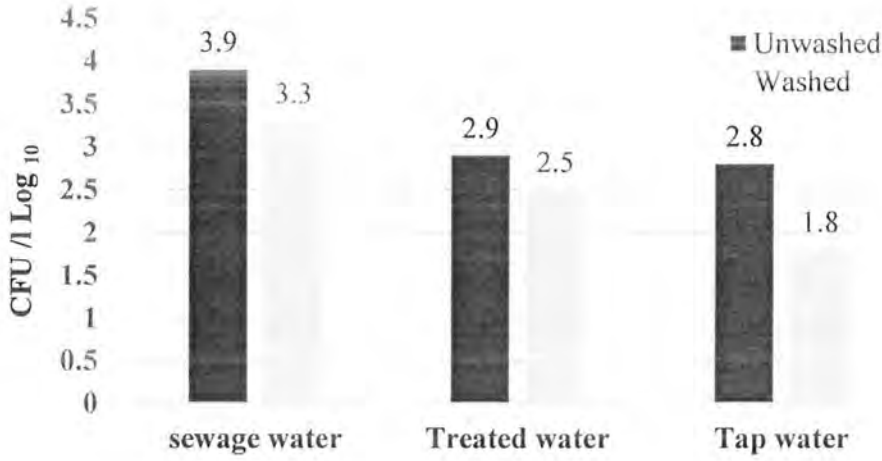
Graph. 8. Comparison between CFU of unwashed and washed samples of coriander grown in sewage, treated and tap water



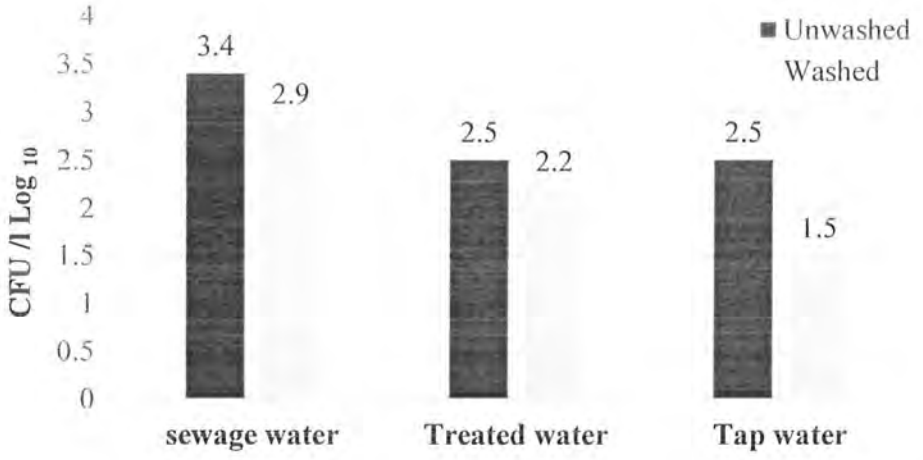
Graph. 9. Comparison between CFU of unwashed and washed samples of radish grown in sewage, treated and tap water



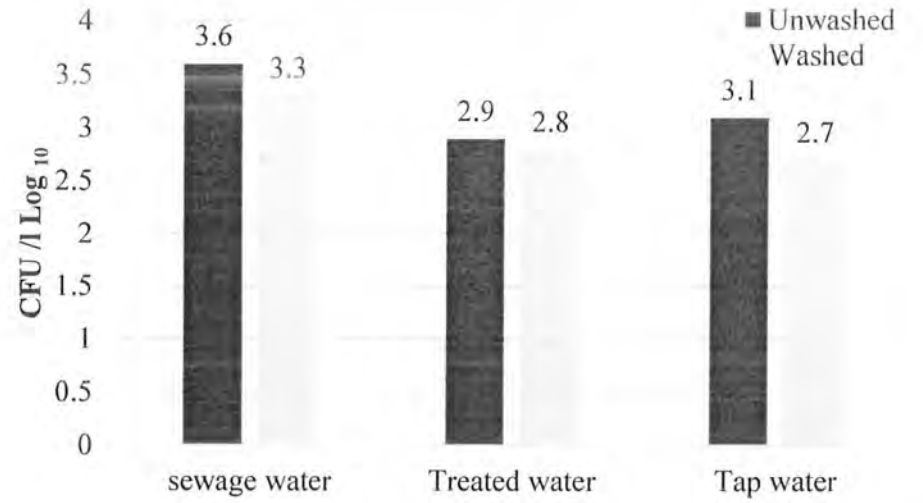
Graph. 10. Comparison between CFU of unwashed and washed samples of lettuce grown in sewage, treated and tap water



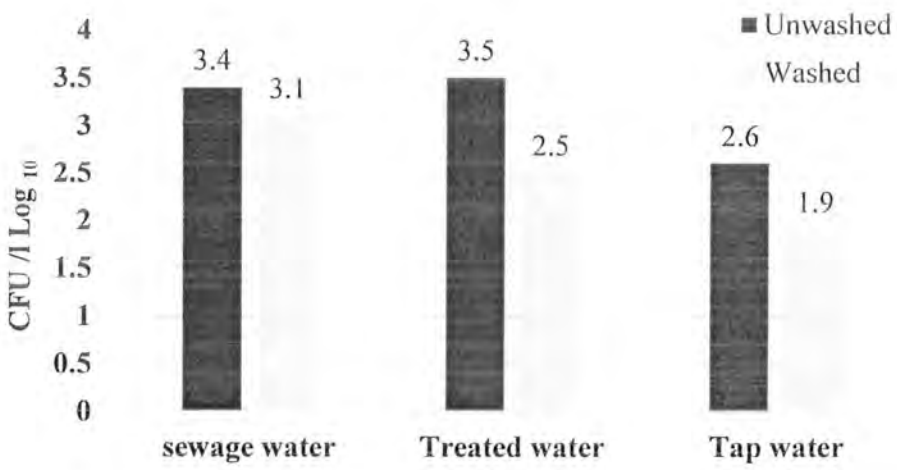
Graph. 11. Comparison between CFU of unwashed and washed samples of cabbage grown in sewage, treated and tap water



Graph. 12. Comparison between CFU of unwashed and washed samples of fenugreek grown in sewage, treated and tap water



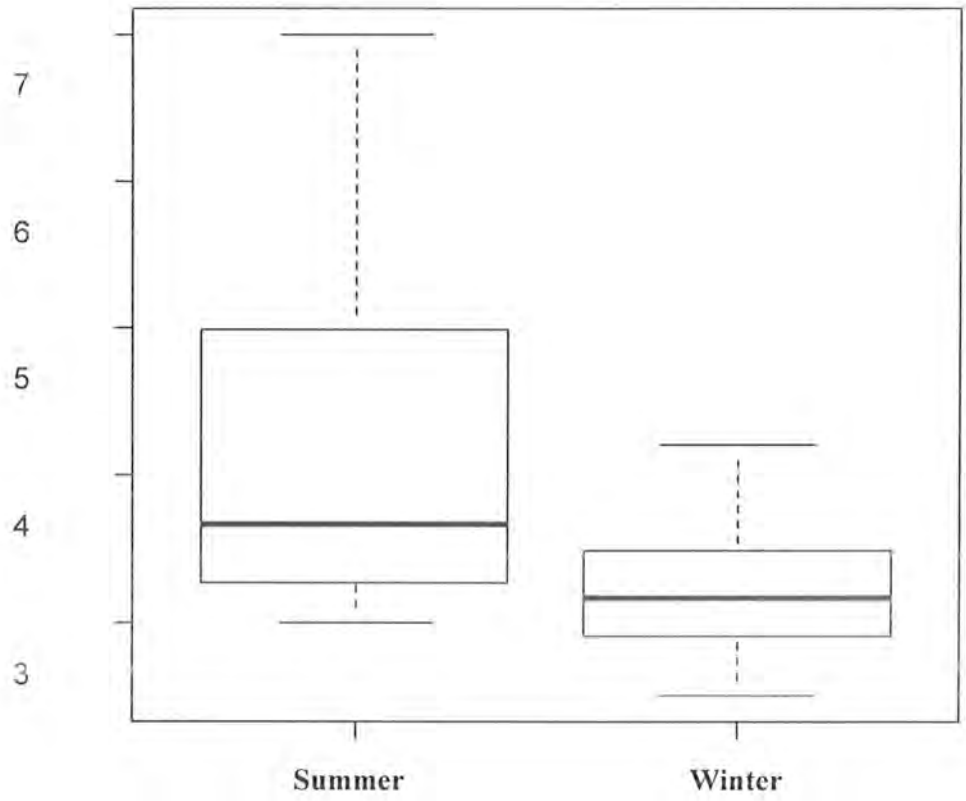
Graph. 13. Comparison between CFU of unwashed and washed samples of turnip grown in sewage, treated and tap water



Graph. 14. Comparison between CFU of unwashed and washed samples of carrot grown in sewage, treated and tap water

4.5. Comparison of Microbial Count between Summer and Winters Vegetables:

Vegetables were grown in summer and winter in order to compare the microbial count in vegetables in both seasons. Leafy, fruity and tuber vegetables were grown in both seasons. T test was applied to check the significant difference between bacterial counts in both seasons. Summer vegetables showed higher microbial count in all water qualities (sewage water, treated water and tap water) irrigated vegetables then winters vegetables. Leafy vegetables like spinach grown in summer showed higher APC then leafy vegetable lettuce grown in winter season. Tuber vegetables radish grown in summer showed higher aerobic plate count then turnip which was grown in winters. T test revealed highly significant difference between summer and winter vegetables (Graph 15)



Graph. 15. Comparison between CFU of summer and winter vegetables

4.5. Characterization of Micrbial Community from Vegetables:

Samples were prepared and serially diluted according to method described in FAO Manual (1992). These diluted samples were spreaded on nutrient agar and incubated at 37° C for 24 hours. After incubation various colonies that appeared on nutrient agar plated were differenciated from each other on the basis of their morphological characteristics. In order to get the pure cultures of bacteria, different colonies were further sub-cultured on Salmonella-Shigella Agar (SS), Pseudomonas Cetrimide Agar (PCA), MacConkey's agar (MaC), Eosin methylene blue agar (EMB) and plates were incubated at 37°C for 24 hours. After incubation, indentification of sub-cultured microbes was done according to morphology, microscopy and biochemical tests. Comprehensive discription of morphology, microscopy abd biochemical tests is given in Table (6), Table (7) and in Table (8).

Table. 6. Morphological depiction of isolated strains

Isolates	NA	MaC	EMB	PCA	SS
a.	Dense, white waxy growth	--	--	--	--
b.	White, moist growth	Pink lactose fermenters	Metallic green sheen	--	Pink lactose fermenters
c.	Light creamy, viscous growth	Pale yellow non lactose fermenters	--	--	--
d.	Grayish granular growth	--	--	--	--
e.	Abundant, thin white growth	Colorless non-lactose fermenters	Pink non-lactose fermenters	White growth turning media green	--
f.	Large, irregular translucent growth	Colorless non-lactose fermenters	Pink non-lactose fermenters	--	Pale pink
g.	Abundant, opaque, golden colony	--	--	--	--
h.	White, slimy growth	Pink lactose fermenters	Dark brown colony	--	--
I.	Thin, gray growth	Colorless, non-lactose fermenters	Gray, mucoid colony	--	White slimy colony

J.	White mucoid, slimy, translucent	--	Purple dark centered colony	--	White slimy colony
k.	Smooth, yellowish growth	--	--	--	--
l.	Thin, grayish colony	Colorless, non-lactose fermenters	Small white growth	--	Brown colored
m.	Abundant, opaque, golden colony	--	--	--	--

Key: NA = Nutrient Agar; -- = Not streaked; MaC = MacConkey Agar; EMB = Eoisen Methylene Blue Agar; SS = *Salmonella-shigella* Agar; PCA = Pseudomonas Cetrimide Agar

Isolates	Colour	Form	Organization
a.	Purple	Rods	Single
b.	Pink	Rods	Single
c.	Pink	Cocco-bacilli	Single
d.	Purple	Rods	Palisade
e.	Pink	Rods	Single
f.	Pink	Rods	Single
g.	Purple	Cocci	Bunches
h.	Pink	Rods	Single
i.	Purple	Cocci	Tetrads
j.	Pink	Short rods	Single
k.	Pink	Rods	Single
l.	Pink	Short rods	Single
m.	Purple	Cocci	Short chains

Tables. 7. Microscopic Depiction of bacterial isolated strains

Table. 8. Biochemical analysis of bacterial isolates

Isolates	Lactose	Dextrose	Sucrose	MR	VP	H2S	Indole	NO3	Catalase	Oxidase	Urease	Citrate	TSI
a.	-	AC	AC	-	V	-	-	+	+	-	-	-	AC/NC
b.	AG	AG	A, V	+	-	-	+	+	+	-	-	-	AC/NC
c.	-	-	-	-	-	-	-	-	+	-	-	V	-
d.	-	AC	AC	-	-	-	-	+	+	+	-	-	-

e.	-	-	-	-	-	-	-	+	+	+	-	+	-
f.	-	AG	AC	+	-	+	-	+	+	-	+	-	Y/Y
g.	AC	AC	AC	+	V	-	-	+	+	-	-	-	Y/Y
h.	AG	AG	AG	-	+	-	-	+	+	-	-	+	AC
i.	-	AG	AC	+	-	+	-	+	+	-	-	+	
j.	AG	AG	AG	V	V	-	-	+	+	-	+	+	-
k.	-	-	-	-	-	-	-	V	+	+	-	-	RC
l.	-	AC	AC	+	-	-	V	+	+	-	-	-	R/Y H_2S
m.	AC	AC	AC	+	-	-	-	-	-	-	-	-	-

Key; - + = Positive; reaction; = Negative; V = Variable A = Acid production; AG = Acid and Gas; AC/NC = Acid/ No change; R/Y = Red/ Yellow; Y/Y = Yellow/Yellow; R/NC = Red/ No color change

Table. 9. Distinguished Bacterial Strains from Soil, Water and Vegetables

Isolates	Distinguished Bacteria
a.	*Bacillus cereus*
b.	*Escherichia coli*
c.	*Alcaligenes faecalis*
d.	*Corynebacterium xerosis*
e.	*Pseudomonas aeruginosa*
f.	*Proteus mirabilis*
g.	*Staphylococcus aureus*
h.	*Enterobacter aerogenes*
i.	*Salmonella* typhimurium
j.	*Klebsiella pneumoniae*
k.	*Micrococcus luteus*
l.	*Shigella dysentriae*
m.	*Streptococcus lactis*

4.6. Pathogens Isolated from Vegetables:

Bacterial isolates obtained from vegetables were characterized according to Bergey's Manual of Determinative Bacteriology (9th edition). Interpreting the results of microscopy, morphological appearance on different media, and biochemical tests different strain were isolated from vegetables. A comprehensive description of microscopic observation, morphological and biochemical analysis of the identified strains are given in tables. Identified strains from radish given in table # 10, okra in table # 11, spinach in table # 12, coriander in table # 13, tomatoes in table # 14, cabbage in table # 15, turnip in table # 16, lettuce in table # 17, fenugreek in table # 18 and from carrot given in table # 19.

Table. 10. Pathogens isolated from radish samples

No. of isolates	Sewage water unwashed radish	Sewage water washed radish	Treated water unwashed radish	Treated water washed radish	Tap water unwashed radish	Tap water washed radish
a	*S. dysentriae*	*E. coli*	*S.* typhimurium	*M. luteus*	*P. mirabilis*	*P. mirabilis*
b	*B. cereus*	*B. cereus*	*P. aeruginosa*	*B. cereus*	*S. aureus*	*B. cereus*
c	*E. coli*	*S.lactis*	*B. cereus*	*P. aeruginosa*	*E. coli*	*S. aureus*
d	*S. aureus*	*S. aureus*	*E. coli*		*B. cereus*	*E. coli*
e	*P. aeruginosa*	*E. aerogenes*	*M. luteus*		*M. luteus*	
f	*S. lactis*					
g	*E. aerogenes*					
h	*M. luteus*					

Table. 11. Pathogens Isolated From Okra Samples

No. of isolates	Sewage water unwashed okra	Sewage water washed okra	Treated water unwashed okra	Treated water washed okra	Tap water unwashed okra	Tap water washed okra
a	*E. aerogenes*	*E. coli*	*S.* typhimurium	*S. aureus*	*P. mirabilis*	*E. coli*
b	*E. coli*	*B. cereus*	*P. aeruginosa*	*B. cereus*	*E. coli*	*B. cereus*
c	*B. cereus*	*E. aerogenes*	*B. cereus*	*P. aeruginosa*	*S. aureus*	*S. aureus*
d	*S. aureus*	*S. aureus*	*S. lactis*	*S. lactis*	*B. cereus*	
e	*P. aeruginosa*		*S. aureus*		C. *xerosis*	
f	*S.* typhimurium					
g	C. *xerosis*					

Table. 12. Pathogens Isolated From Spinach Samples

No. of isolates	Sewage water unwashed spinach	Sewage water washed spinach	Treated water unwashed spinach	Treated water washed spinach	Tap water unwashed spinach	Tap water washed spinach
a	*P. aeruginosa*	*E. coli*	*S.* typhimurium	*S. aureus*	*P. aeruginosa*	*P. mirabilis*
b	*E. coli*	*B. cereus*	*P. aeruginosa*	*B. cereus*	*E. coli*	*B. cereus*
c	*S.* typhimurium	*M. luteus*	*B. cereus*	*P. aeruginosa*	*P. mirabilis*	*E. coli*
d	*S. aureus*	*S. lactis*	*A. faecalis*		*B. cereus*	
e	*B. cereus*	*S. aureus*	*S. aureus*		*C. xerosis*	
f	*S. dysentriae*		*E. coli*		*S. aureus*	
g	*M. luteus*					
h	*S. lactis*					
i	*K. pneumoniae*					

Table. 13. Pathogens Isolated From Coriander Samples

No. of isolates	Sewage water unwashed coriander	Sewage water washed coriander	Treated water unwashed coriander	Treated water washed coriander	Tap water unwashed coriander	Tap water washed coriander
a	*P. aeruginosa*	*E. coli*	*E. coli*	*E. coli*	*P. aeruginosa*	*E. coli*
b	*E. coli*	*B. cereus*	*P. aeruginosa*	*B. cereus*	*E. coli*	C. *xerosis*
c	*M. luteus*	*M. luteus*	*B. cereus*	*P. aeruginosa*	*P. mirabilis*	*P. mirabilis*
d	C. *xerosis*	*S. lactis*	*S. aureus*		C. *xerosis*	
e	*B. cereus*				*S. aureus*	
f	*S. lactis*					
g	*K. pneumoniae*					
h	*A. faecalis*					

Table. 14. Pathogens Isolated From Tomato Samples

No. of isolates	Sewage water unwashed tomato	Sewage water washed tomato	Treated water unwashed tomato	Treated water washed tomato	Tap water unwashed tomato	Tap water washed tomato
a	*S. typhimurium*	*E. coli*	*S. typhimurium*	*P.aeruginosa*	*E. coli*	*E. coli*
b	*E. coli*	*S.dysentriae*	*P. aeruginosa*	*B. cereus*	*S. aureus*	*B. cereus*
c	*P. aeruginosa*	*E. aerogenes*	*S. lactis*	*S. aureus*	*P. mirabilis*	*P. mirabilis*
d	*E. aerogenes*	*S. lactis*	*B. cereus*	*E. coli*	*B. cereus*	*S. aureus*
e	*S. dysentriae*	*B. cereus*	*S. aureus*			
f	*M. luteus*		*E. coli*			
g	*A.faecalis*					
h	*S. lactis*					

Table. 15. Pathogens Isolated From Turnip Samples

No. of isolates	Sewage water unwashed turnip	Sewage water washed turnip	Treated water unwashed turnip	Treated water washed turnip	Tap water unwashed turnip	Tap water washed turnip
a	*B. cereus*	*M. luteus*	*E. coli*	*E. coli*	*P. aeruginosa*	*S. aureus*
b	*K. pneumoniae*	*B. cereus*	*E. aeruginosa*	*B. cereus*	*E. coli*	*C. xerosis*
c	*M. luteus*	*E. coli*	*B. cereus*	*E. aeruginosa*	*C. xerosis*	*P. mirabilis*
d	*C. xerosis*	*S. lactis*	*S. aureus*		*P. mirabilis*	
e	*A.faecalis*				*S. aureus*	
f	*S. lactis*					
g	*E. coli*					

Table. 16. Pathogens Isolated From Cabbage Samples

No. of isolates	Sewage water unwashed cabbage	Sewage water washed cabbage	Treated water unwashed cabbage	Treated water washed cabbage	Tap water unwashed cabbage	Tap water washed cabbage
a	*S.* typhimurium	*E. coli*	*B. cereus*	*S. lactis*	*M. luteus*	*E. coli*
b	*E. coli*	*B. cereus*	*C. xerosis*	*B. cereus*	*E. coli*	*S. aureus*
c	*B. cereus*	*P. aeruginosa*	*S.* typhimurium	*M. luteus*	*P. mirabilis*	*P. mirabilis*
d	*A. faecalis*	*C. xerosis*	*S. lactis*		*S. aureus*	*M. luteus*
e	*P. aeruginosa*	*S. lactis*	*M. luteus*			
f	*S. lactis*					
g	*C. xerosis*					
h	*P. mirabilis*					

Table. 17. Pathogens Isolated From fenugreek Samples

No. of isolates	Sewage water unwashed fenugreek	Sewage water washed fenugreek	Treated water unwashed fenugreek	Treated water washed fenugreek	Tap water unwashed fenugreek	Tap water washed fenugreek
a	*E. coli*	*E. aerogenes*	*S. aureus*	*M. luteus*	*B. cereus*	*M. luteus*
b	*B. cereus*	*S. aureus*	*P. aeruginosa*	*B. cereus*	*M. luteus*	*B. cereus*
c	*S.* typhimurium	*E. coli*	*B. cereus*	*P. aeruginosa*	*C. xerosis*	
d	*S. aureus*	*B. cereus*	*C. xerosis*			
e	*E. aerogenes*		*M. luteus*			

Table. 18. Pathogens Isolated From Lettuce Samples

No. of isolates	Sewage water unwashed lettuce	Sewage water washed lettuce	Treated water unwashed lettuce	Treated water washed lettuce	Tap water unwashed lettuce	Tap water washed lettuce
a	*S.* typhimurium	*B. cereus*	*P. aeruginosa*	*P. aeruginosa*	*P. mirabilis*	*C. xerosis*
b	*S. dysentriae*	*S. lactis*	*S. lactis*	*B. cereus*	*S. aureus*	*S. aureus*
c	*S. lactis*	*E. aerogenes*	*S. aureus*	*S. aureus*	*E. coli*	
d	*E. aerogenes*		*B. cereus*		*C. xerosis*	
e	*E. coli*					
f	*A. faecalis*					
g	*M. luteus*					

Table. 19. Pathogens Isolated From Carrot Samples

No. of isolates	Sewage water unwashed carrot	Sewage water washed Carrot	Treated water unwashed Carrot	Treated water washed Carrot	Tap water unwashed Carrot	Tap water washed Carrot
a	*E. aerogenes*	*E. coli*	*E. coli*	*S. aureus*	*P. mirabilis*	*E. coli*
b	*E. coli*	*S. aureus*	*P. aeruginosa*	*B. cereus*	*E. coli*	*C. xerosis*
c	*S. aureus*	*E. aerogenes*	*P. mirabilis*		*S. aureus*	*S. aureus*
d	*S. typhimurium*	*C. xerosis*	*S. aureus*		*C. xerosis*	
e	*P. aeruginosa*		*C. xerosis*			
f	*C. xerosis*					

4.7. Physicochemical Characterization of Water Used for Irrigation:

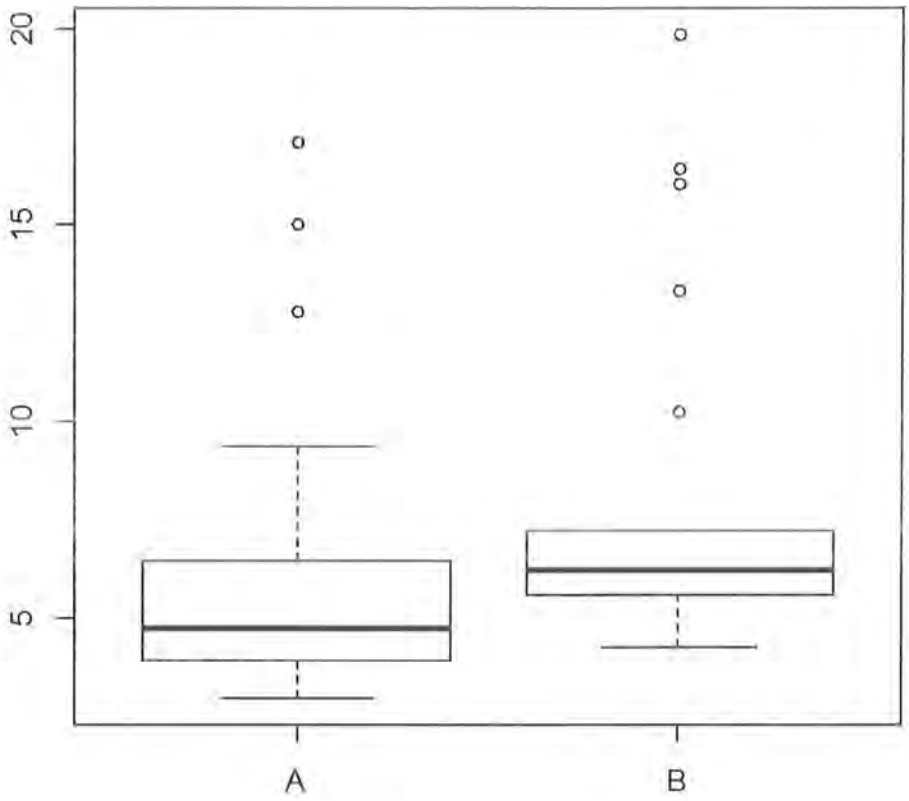
Plants require particular amount of organic nutrients for their growth like nitrites, nitrates, sulphates and phosphates. Amount of these nutrients were determined in sewage water, wetland treated water and tap water. Amount of COD, BOD, DO. Phosphates. Sulphates, nitrite-N and nitrate- N were higher in sewage water than treated and tap water (Table).

Table. 20. Quality assessment of water used for irrigation

Factors	Sewage water	Treated water	Tap water
COD	196.2 mg/l	40 mg/l	6 mg/l
BOD	131.57 mg/l	26.9 mg/l	4 mg/l
DO	2.75 mg/l	6.83 mg/l	2.1 mg/l
Nitrate-N	70.46 mg/l	9.06 mg/l	5.11 mg/l
Nitrite-N	1.99 mg/l	0.22 mg/l	0.01 mg/l
Sulphates	94.91 mg/l	23 mg/l	4.01 mg/l
Phosphates	28.83 mg/l	10.55 mg/l	2.33 mg/l

4.8. Comparison between Growth of Vegetables in Sewage Water, Treated Water and Tap Water:

Highest of vegetable plants were measured in inches, measurement were done two times one at 40 days after sowing and second time at 60 days after sowing. Welch Two Sample t-test conducted to check the significant difference between growths of vegetables in sewage water and wetland treated water. Statistical analysis revealed that a non-significant difference ($p \leq 0.1$) lied between growth of vegetables grown in sewage water and wetland treated water (Graph) .



Graph. 16 comparison between growths of vegetable plants in sewage water and wetland treated water

Discussion

Vegetables are important part of human nutrition and plays role in maintenance of human body by providing lot of essential nutrition. Due to this nutritive value consumption of fresh vegetables has increased significantly over the last decade. Foodborne illness has recently gained much attention worldwide due to its deleterious effects on human health and consequentially on national economy. Increased consumption of fresh vegetables in the form of raw and minimally processed salads has resulted in increase in foodborne outbreaks which some time may be fatal. Most of diseases linked with vegetables are primarily those transferred by the fecal-oral route, and therefore, contamination occurs during growth and handling of vegetables (De Roever, 1998). Different sources can contaminate the vegetables but a large number of pathogens can be transmitted to plants via irrigation water, then these persist on external and internal parts of the plant for many days (Islam *et al.*, 2004).

In present study characterization of the amount of contamination occurring from contaminated irrigation water and isolation of pathogenic bacteria from vegetables those transmitted from irrigation water and soil.

The normal pH range of the vegetables was 4.0 to 6.3. Tomatoes pH range from 4.6 to 4.8. Dobricevic *et al.* (2005) reported same results for tomatoes pH with slight differences may be different due to different assortment of tomatoes used in this study. Tomatoes pH was lower than pH of lettuce and cabbage. The cabbage studied for pH values presented that it ranged from 5.5 to 5.6. This result could not be linked with other results as pH values related to blended cabbage sample in sterile distilled water was lacking in literature. Lettuce presented the pH range from 5.9 to 6.1. These results coincide with the results of Chutichudet *et al.* (2011). pH of other vegetables used in this study could not be compared as literature for their pH also lacking. It was noted from literature that pH of vegetables has strong influence on the presence of microbial count in vegetables. Vegetables with higher pH harbored more microorganism as compared to vegetables with low pH values. Highest microbial count was recorded for spinach (6.9 log CFU/g) and okra (6.9 log CFU/g) with pH of 6.3 and 6.1 respectively followed by radish (5.2) with pH of 6, tomatoes (5 log CFU/g) with 4.7 pH, in

winter season highest microbial count was observed for lettuce (4.2 log CFU/g) with pH of 6.1 followed by cabbage (3.9 log CFU/g) with 5.6 pH, turnip (3.6 log CFU/g) with 5.5 pH, carrot (3.5 log CFU/g) with 5.3 pH and fenugreek (3.4 log CFU/log) with 4 pH. Weissinger *et al.* (2000) reported similar results. James and Ngarmsak (2011) also reported that pH influence the growth of food-borne pathogens related with vegetables.

Vegetables were grown in two season summer and winter season, these vegetables were irrigated with sewage water, wetland treated water and tap water. Aerobic plate counts (APC) results for vegetables grown in sewage water indicated higher counts for aerobic microorganisms. Spinach and okra have 6.9 log cfu/g, radish 5.2 log cfu/g, tomato and coriander 5.1 and 4.9 logcfu/g respectively. Ofosu *et al.* (1999) also observed high viable count in vegetables irrigated with sewage water. Itohan *et al.* 2011 reported similar results in sewage water irrigated vegetables. Benti *et al.* (2014) reported high microbial count for spinach than present study; this contrast may be due to highly polluted water used for irrigation. Study of Thunberg *et al.* (2004) presented similar results for spinach sample. Minhas *et al.* 2006 showed same results of APC for vegetables grown in sewage water. In fruit vegetables okra showed higher microbial count then tomato, Tasado *et al.* (2013) founded higher APC for okra then tomato which is similar to present study, they also reported same microbial count for tomato.

In winter season highest count was noted for lettuce (4.2 log CFU/g) followed by cabbage, turnip, carrot, fenugreek with 3.9, 3.6, 3.47 and 3.44 respectively. Abdullahi and Abdulkareem (2010) observed high microbial count for lettuce then present study, this variation may be due to different condition of the study. Microbial count of vegetables grown in winter season was lower than the summer season. Similar trend was reported by Caponigro *et al.* in 2010. Rao et al. (2012) also observed higher microbial count in summer season then winter season. Results of present study also supported by the findings of Odeyemi (1990) who detected higher aerobic bacterial population in vegetables during the summer season.

The reason for high microbial count in summer is that the water which is used for irrigation contained high amount of microorganism in summer than winter, microorganisms cannot survive at low temperature as temperature in winter

growing season decreased up to average 16 °C. It was noted that leafy green harbored more bacteria and the count associated with such vegetables was high in both season. This result is in accordance with the findings of Valentin-Bon *et al.* (2008) where they recorded increased count of microorganisms with spinach and lettuce. Abdullahi and Abdulkareem (2010) and Aliyu *et al.* (2005). reported similar results for leafy vegetables. The high microbial counts in spinach could be due to the wide surface area of vegetable leaves which is appropriate for water contact and microbial contamination (Anonymus, 2002). Lettuce showed high microbial count than cabbage similar finding was reported by Viswanatha and Kaur (2001) although they reported higher microbial count for both vegetables. Leafy vegetables have more unprotected area on which pathogens from neighboring soil get attach, enter and proliferate in leaf tissue (Rosas *et al.*, 1984). More risks were associated with leafy vegetables, therefore WHO/FAO (2008) has kept them on top priority for food safety measures. Spinach and okra in sewage water were considered in spoilage food according to WHO guidelines for aerobic plate count for vegetables and other vegetables in sewage water showed microbial count near to unsatisfactory level. Aerobic plate count was analyzed at the time of harvest in present study, if vegetables have such amount of contamination at their growth level then it will become hazardous to human health as processing and storage of vegetables increases microbial count, so many other factors contaminating vegetables in markets so these become heavily contaminated before reaching to consumer.

Washing significantly decreased the amount of microorganisms in all vegetables. This result coincide with the findings of Rajkowski and Fan (2003) and Park *et al.* (2008) but Rosas *et al.* (1984) reported that washing of vegetables reduced the pathogens significantly but not to the permissible limits contradiction results was also reported by Saper *et al.* (2007). This contradiction may be due to use of non-sterile water for washing of vegetables by authors. Minhas et al. (2006) also suggested that thoroughly washing of vegetables with tap water reduces microbial count to permissible limits.

In present study members of *Bacillus, Corynebacterium, Streptococcus, Staphylococcus, Lactobacillus, Micrococcus, Pseudomonas* and *Enterobacteriacea* were isolated from vegetables. Majority of samples contained

E. coli, Bacillus cereus and *Staphylococcus aureus*. Itohan *et al.* (2011) confirmed the findings of *Escherichia coli, Klebsiella* and *Enterobacter, Proteus, Pseudomonas aeruginosa, Salmonella* and *Shigella* from vegetables, they also isolated *Staphylococcus aureus* from majority of the samples. Mathur *et al.* (2014) also reported the presence *Corynebacterium, Staphylococcus and Streptococcus* from vegetables. Avazpour *et al.* (2013) observed the presence of *E. coli, Salmonella* and *Shigella* in vegetables. In present study a number of bacteria was also observed in vegetables irrigated with tap water, these bacteria were also present in tap water and soil. Abaidoo *et al.* 2010; Gerba and Choi, 2006; Kirby *et al.* 2003; Steele and Odumeru, 2004 also demonstrated the role of soil and irrigation water in pathogens transfer to vegetables. The mean heterotrophic count detected in vegetables are revealing the fact that the microenvironment with in these foods provided favorable condition for growth and proliferation of pathogens.

In present study growth of vegetables observed in sewage water and treated water was non-significantly different from each other. Although there as a significant difference was found between growths after forty days of seedling but after sixty days non-significant difference was observed. Water used for irrigation was highly alkaline with high BOD, COD, DO, nitrates, phosphates and sulphates contents these results coincide with the findings of many workers (Al-Fredan, 2006 ; Nazif *et al.*, 2006 ; Mahmood & Maqbool, 2006) for waste water. Long use of this alkaline water for irrigation effects the growth of vegetables as observed in present study, alkaline contents blocks the pores of soil then it blocks the circulation of water and other nutrients in soil and inhibits the availability of these for plant roots. Tamoutsidis *et al.* (2002) reported the long term application of municipal wastewater on vegetables for edible leaves (lettuce, spinach,) and roots (radish, carrots and beets), reduced the overall yield of plants. Iqbal *et al.* 1991; Jana & Harjee, 1996; Singh & Mishra, 1997; Wahid *et al.* 2000; Farid, 2006 reported similar results. Chen & Chia (2002) also observed similar impact of municipal wastewater vegetables including cabbage, carrot and sweet peas. Vegetables irrigated with treated waste water showed good growth with long term used of this water. Darvishi *et al.* (2010) reported similar results for plant growth in treated waste water.

Conclusion

Present study concludes that:

- High microbial load was found in vegetables irrigated with sewage water irrigation of vegetables with sewage water transmit pathogens to vegetables which results in food borne illnesses so contaminated water and soil contaminates the vegetables during growth.
- Microbial number sewage water irrigated vegetables recorded in present study range from 8.4×10^4 to 1×10^7 CFU/g, in some vegetables microbial number is above the ICMS (International Commission on Microbiological Specification for Foods 1998) level and in some vegetables it is near to permissible limits.
- Although these microorganisms can be a part of epiphytic flora but their high number and isolation of pathogenic bacteria like *Salmonella typhimurium, Klebsiella pneumonia, Shigella dysentriae, Streptococcus lactis, Proteus mirabilis* and *Staphylococcus aureus* reflects the poor hygiene condition, their load further increases after harvesting during handling and storage of vegetables which poses threat to human health.
- In treated water vegetables microbial count range from 3.2×10^2 to 1×10^4 CFU/g, clearly less amount of microorganisms were isolated from vegetables irrigated with wetland treated water, and although pathogens were found in treated water irrigated vegetables but their number and variation was less than sewage water irrigated vegetables.
- Sewage water effects the growth of plants if it is used for long period of time for irrigation, as in this study a significant difference was found between heights of plants after forty days of seedlings but after sixty days heights of vegetables showed non-significant difference in sewage water and treated water.
- Hence, great attention must be paid using polluted water for production of vegetables.

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Appendix. A. 1.

Cultural media composition

Nutrient Agar

Chemicals	g/l
Peptone	5g
Beef extract/yeast extract	3g
NaCl	5g
Agar	15g

Final pH: 6.8 at 25 °C.

Pseudomonas Cetrimide Agar

Chemicals	g/l
Pancreatic digest of gelatine	20g
Cetrimide	0.3g
Magnesium chloride	1.4g
Potassium sulfate	10g
Agar	15g

Final pH: 7.2 ± 0.2 at 25°C

Eosine Methylene Blue Agar

Chemicals	g/l
Enzymatic Digest of Gelatin	10g
Lactose	10g
Dipotassium Phosphate	02g
Eosin Y	0.4g
Methylene Blue	0.065g
Agar	15g

Final pH: 7.1 ± .2 at 25

Salmonella-shigella agar

Chemicals	g/l
Beef Extract	5g
Enzymatic Digest of Casein	2.5g
Enzymatic Digest of Animal Tissue	2.5g
Lactose	10g
Bile Salts	8.5g
Sodium Citrate	8.5 g
Sodium Thiosulfate	8.5g
Ferric Citrate	1g
Brilliant Green	0.00033 g
Neutral Red	0.025 g
Agar	13.5g

Final pH: 7.0 ± 0.2 at 25° C

MacConkey Agar

Chemicals	g/l
Pancreatic Digest of Gelatin	17g
Lactose monohydrate	10g
Sodium chloride	5g
Peptone (meat and casein)	3g
Bile Salts	1.5g
Neutral Red	0.03 g
Crystal violet	0.001g
Agar	1.5g

Final pH: 6.8 ± 0.2 at 25° C

MR-VP Medium

Chemicals	g/l
Buffered peptone	7
Dextrose	5
Dipotassium Phosphate	5

Final pH: 6.9 ± 0.2 at 25° C

Nitrate reduction broth

Chemicals	g/l
Peptic digest of animal tissue	5g
Meet extract	3g
Potassium nitrate	1g
Sodium chloride	30g

Final pH: 7.0±0.2 at 25°C

Urease broth

Chemicals	g/l
Yeast extract	0.1g
Dipotassium hydrogen phosphate	9.5g
Potassium dihydrogen phosphate	9.1
Urea	20g
Phenol red	0.01g

Final pH: 6.8 ± 0.2 at 25°C

SIM Medium

Chemicals	g/l
Enzymatic Digest of Casein	20g
Enzymatic Digest of Animal Tissue	6.1g
Ferric Ammonium Citrate	0.2g
Sodium Thiosulfate	0.2g
Agar	3.5g

Final pH: 7.3 ± 0.2 at 25°C

Appendix. A.2: Paired T test and p values of comparison between APC of unwashed and washed vegetables irrigated with sewage water, treated water and Tap water.

S. No.	Vegetables	T value	P value	Irrigation water
1	Radish	20.86456	0.001144	Sewage water
2	Okra	12.95914	0.002950	Sewage water
3	Tomatoes	2.75016	0.055345	Sewage water
4	Coriander	34.60854	0.000416	Sewage water
5	Spinach	16.93909	0.001730	Sewage water
6	Fenugreek	30.76989	0.000527	Sewage water
7	Turnip	4.074527	0.027643	Sewage water
8	Cabbage	3.128512	0.044387	Sewage water
9	Lettuce	4.165564	0.026541	Sewage water
10	Carrot	8.487824	0.006799	Sewage water
11	Radish	6.998103	0.009907	Treated water
12	Okra	3.543944	0.03561	Treated water
13	Tomatoes	6.810634	0.010443	Treated water
14	Coriander	4.212855	0.025995	Treated water
15	Spinach	5.671082	0.014857	Treated water
16	Fenugreek	2.565245	0.062132	Treated water
17	Turnip	3.797911	0.031431	Treated water
18	Cabbage	3.127277	0.044418	Treated water
19	Lettuce	4.113604	0.027162	Treated water
20	Carrot	5.137156	0.017933	Treated water
22	Radish	7.008671	0.009878	Tap water
23	Okra	5.174622	0.017688	Tap water
24	Tomatoes	6.777837	0.010541	Tap water
25	Coriander	9.272065	0.005716	Tap water
26	Spinach	5.728089	0.014576	Tap water
27	Fenugreek	1.797036	0.107081	Tap water
28	Turnip	3.990177	0.028725	Tap water
29	Cabbage	4.511842	0.022889	Tap water
30	Lettuce	3.652839	0.033725	Tap water
31	Carrot	2.907464	0.050369	Tap water