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Physicochemical and Microbiological Evaluation of Irrigated Vegetables with Wastewater "Yemen"

[1,2,3]*Muamar AL-Jaboobi,* [2]*M'hamed Tijane,* [3]*Shawqi EL-Ariqi and* [1]*Mohammed Bouksaim*

[1]INRA, RCAR-Rabat. P.O.Box 6570,Rabat Institute, 10101 Rabat Morocco
[2]Laboratory of Biochemistry-Immunology, Faculty of Sciences,
University Mohammed V-Agdal Rabat, Morocco
[3]Faculty of Agriculture, Sana'a University, Sana'a Yemen

Abstract: Ninety samples of fresh vegetables from Bani-Alharth area in Yemen were examined to assess the quality of vegetables irrigated using wastewater. So, physico-chemical and microbial characteristics of randomized vegetables samples were carried out. The analyses showed that the physicochemical characters were observed ordinary with averages 5.5, 4.6, 0.18 and 89.1, respectively, for pH, electrical conductivity EC, acidity and moisture content. Microbial flora in all tested samples showed relative unsatisfactory quality with an average of $4.1x10^6$, $7.9x10^3$, $2.2x10^3$, $4.5x10^3$, $2.7x10^4$, $1.1x10^4$ and $8.5x10^3$CFU/g, respectively, for total aerobic plate counts (APC), total coliform, fecal coliform, Sterptococci spp, *Staphylococcus aureus*, yeast and mould counts, while, Salmonella, Shigella and Vibiro were not detected in all samples. In contraste, *Clostridium prefergeces* was detected in low count in some samples. And when API identification was applied, a presence of *Escherichia coli* (35 percent), Klebsiella genus (21.7 percent), Enterobacter genus (17.4 percent), Citerobacter gens (13 percent) and Serratia genus (13 percent) was observed. On the other hand, this research showed that Antibiotic susceptility of Enterobacteriaceae isolates recoded high prevalence of resistance to Ampicillin, Amoxicillin, Amoxicillin+Ac clavulanic, Tetracycline and Erythromycin. The results emphasize that using of wastewater in vegetables irrigating have considerable influence on the microbiological quality of produced vegetables. Therefore, these products are of unacceptable quality.

Key words: Vegetables · Wastewater · Enterobacteriaceae · Antibiotic Susceptility

INTRODUCTION

Consumers perceive fresh vegetables as an important source for a healthy diet [1] because of the plethora of the health and nutritional benefits associated with the consumption of fresh fruits and vegetables [2]. Vegetables nutrient content, carbohydrate and amount of water, are sufficient to support growth of microorganisms. [3, 4]. Microorganisms usually produce multiple degradative enzymes, such as cellulases, pectinases, amylases and proteases that release more nutrients from the injured plant's tissue, assisting development of other populations that feed on these nutrients [5].

Microorganisms that are responsible to cause human illness can be transferred by raw vegetables and a number of Fresh products [3, 4]. Salmonella [6] and *Escherichia coli* [3] have been isolated from raw vegetables. So, risks associated with contaminated fresh fruits and vegetables has increased in recent years [3, 7-9]. Irrigation water led to real increases in diseases associated with produced vegetables even if there are no sources of sewage discharge. Water is essential in the growth of crops, removal of adhere soil, cooling of produce, decontamination, washing of equipment and personal hygiene. Hence, pathogens that might have been introduced at any point of the production chain may still be present when the product is consumed [10-12].

Corresponding Author: Mohammed Bouksaim, INRA, RCAR-Rabat.
P.O. Box 6570, Rabat institut, 10101 Rabat Morocco.
Tel: +212-661 138803.

An irrigated vegetable with wastewater is a common practice in arid and semi-arid regions which are confronting increasing water shortages. Therefore, over 85000 hectares are irrigated with wastewater in Mexico [13]. In Pakistan 26% of national vegetable production is irrigated with wastewater [14]. It is clear that using contaminated wastewater in vegetable irrigation sustained the infection in the field [15].

In zone of Bani Al-Harth in Yemen shortage of water led to use untreated or partly treated wastewater as source for agriculture irrigation. So, vegetables were selected in this area due to the export to several markets because of their distinguished quality. In addition these products are wastewater irrigated and they were eaten without further treatment. Meanwhile, consumers suffer of swelling and Diarrhea that attracts attention to triggered doubts of those products. Thus, the absence of standardization guideline to use wastewater and evaluate the healthy risk pinpoints could be dangerous for human and animal health.

The aim of this study is to concentrate on a better understanding of the source of pathogens when wastewater is used in vegetables production, their behavior in the field environment: plant, soil and water.

MATERIALS AND METHODS

Selection of Samples: A total of 90 samples were collected from Bani Al-Harth area between August and December 2012. Collected including Tomato, leek, Parsley, Mint, Onion, Carrot, Lettuce and Cabbage were chosen since they represent uncooked vegetables as well as those commonly available to consumers in Yemen.

Twenty five gram of each sample was homogenized in 225 mL of peptone water mother solution. Then, serial dilutions of slain solution (8.5 g NaCl, 1000 ml distilled water, pH 7.0) were made and 1ml of mother solution was transferred into 9ml serial dilutions up to 105 of slain solution. 1ml from each dilution was cultured for presence of potential pathogen using the selective media.

Physicochemical Characterization: Physicochemical determination concerning moisture content, acidity, pH and electrical conductivity were evaluated according to AOAC [16].

Microbiological Characterization: Microorganisms including total aerobic plate counts and pathogens germs were examined by using Standard Microbiological Methods Health Protection HPA 2004 a, b, c, d [17-20], HPA 2005 [21] and Difco Manual [22]. While, yeasts and moulds were determined by using NM08.0.123-2005 [23].

Isolation and Identification of Enterobacteriaceae: In this step of research, red colonies were transferred from Violet Red Bile Agar and purified on nutrient agar. All strains were tested by Gram stain and oxidase activity and isolates identification was performed by the biochemical reaction profile using commercial API 20E kit (Biomerieux, Marcyl'Etoile, France).

Bacteria Antibiotic Sensitivity: Bacteria isolates were tested for their sensitivity to certain known antibiotics. The antimicrobial sensitivity was tested using *E. coli,* Enterobacter, Klebsiella and Citerobacter isolates and was performed using agar disk diffusion method according to (CLSI) [24] and the following antibiotic disks (Oxoid Ltd., England): AMP: Ampicillin 10µg, AMX: Amoxicillin 10µg, AMC: Amoxicillin+Ac clavulanic 20µg, CTX: Cefotaxime 30µg, GEN: Gentamicin 500 µg, CIP: Ciprofloxacin 5µg, C: Clorophenecole 30µg, TET: Tetracycline 30 µg and E: Erthromycine 15µg were used to monitor and evaluate these bacteria's sensitivity.

Statistical Analysis: The data were statistically analyzed using statistical package, SAS copy right © 2002.

RESULTS AND DISCUSSION

Physicochemical Characterization: Physicochemical results of analyzed vegetables samples produced from different Places and irrigated using wastewater are presented in Table 1. Results showed that the pH values varied between 4.4 to 6.3. Mint had the highest value and the low one was obtained with tomato. In all, these values could support microbial growth and considered slightly normal because the pH of vegetable is ordinarily near neutrality [25, 26]. The concentration found in the present study is similar to those detected by Rico *et al*. [27] who reported that pH of the vegetables is often between 5 and 6.5.

On the other hand, acidity values were observed in the range of 0.1 - 0.3, these values are considered normal because of vegetables organics acids content according to Wiley [25] and Beuchat [10] who reported that, vegetables contain many organics acids so as Acetic, citric, succinic, malic, tartaric, when benzoic and sorbic acids are the mostly.

Table 1: Physicochemical characterization of the tested fresh vegetables samples

Varieties	N.S.	pH	EC (mS /cm)	AC	M%
Tomato	10	4.4	3.8	0.31	92.8
leek	10	6.0	6.2	0.09	87.7
Parsley	10	5.8	7.3	0.26	85.7
Mint	10	6.3	6.4	0.24	88.6
Onion	10	5.6	3.5	0.14	86.3
Carrot	10	5.6	4.6	0.16	88.2
Lettuce	10	5.7	3.8	0.17	88.4
Radish	10	5.9	3.4	0.12	91.3
Cabbage	10	6.1	2.8	0.10	92.5
Total /Average	90	5.7	4.6	0.18	89.1

N.S: Number of samples, EC: Electrical Conductivity, AC: Acidity, M: Moisture

Table 2:Results of microbiological analysis of the investigated fresh vegetable samples

Varieties	N.S	APC UFC/g	TC UFC/g	FC UFC/g	Sal/SH/V	SF	STF	CL	Y UFC/g	M UFC/g
Tomato	10	$8.0x10^5$	$3.8x10^3$	$1.1x10^2$	abs	$2.3x10^2$	$1.7x10^2$	-	$2.1x10^4$	$6.7x10^3$
Leek	10	$1.1x10^6$	$4.3x10^3$	$5.9x10^2$	abs	$1.2x10^4$	$3.1x10^4$	+	$2.5x10^3$	$1.6x10^3$
Parsley	10	$7.2x10^6$	$1.3x10^4$	$2.3x10^3$	abs	$5.5x10^3$	$7.8x10^4$	+	$4.4x10^3$	$4.0x10^3$
Mint	10	$1.4x10^7$	$9.7x10^3$	$4.7x10^3$	abs	abs	$1.1x10^4$	+	$8.0x10^2$	$5.3x10^3$
Onion	10	$3.5x10^6$	$3.8x10^3$	$4.1x10^2$	abs	$3.7x10^3$	$4.7x10^2$	+	$5.4x10^4$	$3.0x10^4$
Carrot	10	$1.4x10^6$	$9.4x10^3$	$5.3x10^3$	abs	$3.2x10^3$	$1.7x10^4$	-	$4.4x10^3$	$6.1x10^3$
Lettuce	10	$4.3x10^6$	$1.7x10^4$	$3.1x10^3$	abs	$1.5x10^4$	$8.7x10^4$	-	$1.3x10^3$	$1.2x10^4$
Radish	10	$5.5x10^5$	$6.7x10^3$	$2.5x10^3$	abs	$1.1x10^3$	$6.3x10^3$	+	$3.4x10^3$	$1.1x10^4$
Cabbage	10	$6.9x10^6$	$3.2x10^3$	$1.0x10^3$	abs	abs	$9.7x10^3$	-	$3.0x10^3$	$2.7x10^2$
Total /Average	90	$4.1x10^6$	$7.9x10^3$	$2.2x10^3$	abs	$4.5x10^3$	$2.7x10^4$		$1.1x10^4$	$8.5x10^3$
Significance		P<.0001	P=0.0026	P=0.0001	-	P=0.1087	P=0.0082		P=0.0407	P=0.0563

APC: Aerobic Plate Count, TC: total coliforms, FC: fecal coliforms, STF: *Staphylococcus aureus*., SF: *Streptococcus fecalis*, Sal: Salmonella, SH: Shigella, V. Vibiro CL: Clostridium, Y: Yeasts, M: Molds and abs: Absent

Concerning electrical conductivity (EC) results obtained varied from 7.3 to 2.8 mS /cm with an average of 5.5 mS/cm, the highest value was obtained in parsley, while the lowest value was determined with Lettuce samples. The EC explains the presence of ions and organic acids in solution [28]. Then it was also reported that Electric conductivity and bacterial contamination are interrelated parameters [29].

Moisture results were ranged between 92.8 and 85.7 with an average of 89.1. These values are considered normal because fresh vegetables are rich with water [16]. These values agreed with the results observed by Jay [30] who showed that vegetable water content is about 88%.

Microbiological Characterization: The analyzed samples as seen in Table 2, showed that studied microbial type counts were found high in the most vegetables varieties when compared to different guidelines microbial limits as well as other recommendations [3, 4, 31, 32]. A total aerobic plate counts (APC) incidence level was observed from $1.4x10^7$ to $5.5x10^5$ with the main of $4.1x10^6$. And, Mint had the highest contamination among all vegetables. The levels of APC bacteria agree with other studies where the bacteriological quality of vegetables has been assessed [33]. It is reported that the APC contamination can be considered as an efficient indicator of food pollution [28].

A rise in total coliform and fecal coliform numbers was also seen, in all samples, exceeded level for the maximum rang allowable. This was because of a poor quality of vegetables irrigation water source, which exposed vegetables in different production stages [34]. The average of the selected microorganism found in the present research are observed similar to those detected in vegetables by Silva *et al.* [35] and Abadias *et al.* [36]. Many research reports from different parts of the world have revealed that production factors condition could pose a risk to human health caused by microbial spoilage, so as the water, soil, air, equipment and workers handling [1, 7, 37, 38]. It is noticed that coliform bacteria were also reported in a wide variety of vegetables as part of their normal flora [36]. But, total levels of fecal coliform organisms are considered a danger contamination index [3, 36, 39]. However, tomato contamination was only in low significance numbers. We expected the lesser may be because of its high acidity content [25]. On the other hand theoretically, it is not close to the ground [40].

More Sterptococci incidence in the samples, more than we expected varied in Onion and in Tomato, respectively, between $1.2x10^4$ and $2.3x10^2$ CFU/g with an average of $4.5x10^3$ CFU/g. The present result is in concordance with that obtained by Turantas [41]. Also Ibenyassine *et al*. [42] who showed high enterococci levels in vegetable produced using wastewater irrigation. Streptococcus species are considered responsible for many diseases. Furthermore, they can be reservoirs of antimicrobial resistance. As noted in northern Georgia that isolated Enterococcus from fruits and vegetables recorded high resistant to lincomycin and bacitracin, resistant relatively low were to salinomycin, penicillin, or nitrofurantoin. Except for vancomycin all isolates showed 100% sensitivity [43].

When Staphylococcus bacteria were studied, highest *Staphylococcus aureus* count value of $1.4x10^5$ CFU/g was observed in parsley but the $1.7x10^2$ CFU/g as lowest value was found in tomato. Indeed, vegetables contamination with staphylococci has long been recognized as a result of human contact [12, 33, 44]. And, *S. aureus* is a major cause of food intoxications, also it is the most frequently occurring bacterial pathogen among clinical isolates from hospital inpatients and is the second most prevalent bacterial pathogen among clinical isolates from outpatients [34, 45]. In Clostridium case, results showed a very low incidence. But, *Clostridium prefergeces* has been isolated from leek, parsley and onion. It was reported before, that this bacteria genus is often present when the same vegetables were analyzed in Canada by Metcalf *et al*. [46].

In parallel, yeasts incidence when treated found between $5.4x10^4$ (Onion) and $8.0x10^2$ CFU/g. (Mint). Investigations in several countries had found that yeasts were prevalent organisms found in vegetables at different levels in USA by Tournas [47], in Italy by Corato [48] and in Singapore by Seow *et al*. [38]. Yeasts were the predominant organisms that can grow in a wide variety of vegetables [47], it has also ability to grow in a broad range of pH values and temperatures [15, 28, 49].

Concerning mould counts were ranged from $2.7x10^2$ CFU/g (Cabbage) to $3.0x10^4$ CFU/g (Onion). Some genera of moulds are common plant pathogenic fungi and could be one of potential inoculate for vegetables that could be carried by different vectors. So, many lost production has been reported [48, 50], also it feel important to declare that moulds were found in all salad types [48, 51]. Therefore, good attention could be taken to preserve quality and discard mycotoxins production. Because these substances are very dangerous and are considered carcinogenic components [52, 53].

Identification of Pathogenic Enterobacteriaceae: In the present work, isolates obtained showed that all bacteria isolates samples were Gram negative, short rods and oxidase negative. The identified isolates of Enterobacteriaceae are presented in Fig. (1).

The most common bacteria were identified as *Escherichia coli* (35%). All samples were *E.coli* positive. This constitutes an excellent indicator organism of fecal contamination of fresh products which can be implicated in food pollution outbreaks. [4, 7, 35, 54]. In the same idea it is reported that infection by *E. coli* O157:H7 can be caused only by a few cells and often resulted in an extremely dangerousillness [55, 56].

In parallel, the Klebsiella genus is the second one presented by two species *K. pneumoniae* (60%) and *K. oxytoca* (21%). Klebsiella genus were already present in a wide range of vegetables [32, 33, 57]. Also it is known that Klebsiella genus is considered the main reason to many problems to human including nosocomial infection, urinary tract infection, diarrhea and other diseases [58].

In Enterobacter case results show the presence of *E. aerogenes* (40%), *E. cloacae* (20%) and *E. intenrmedius* (40%). Enterobacter spp were isolated from vegetables by Osterblad *et al*. [59] and Viswanathan and Kaur [33]. *E. aerogenes* has been reported to cause bloodstream infections [60]. However, Citrobacter genus (13 percent) was represented by two species: *Citerobacter freundii* with percentage (66%) and *Citerbacter diversus* (33%). *Citrobacter freundii* can constitute an additional food safety concern as implicated in food spoilage and food poisoning outbreaks [61, 42]. Several works have been reported that *Citerobacter freundii* is considered as one of causes of urinary tract infection, [62, 63]. But, also *Citerbacter diversus* was known as the cause of sepsis and meningitis [64].

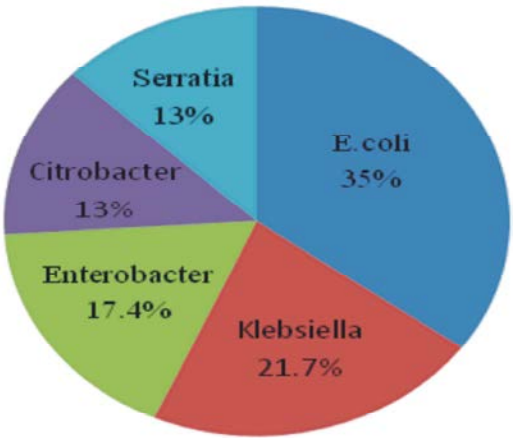

Fig 1: Identified Enterobacteriaceae isolates from the studied fresh vegetable samples

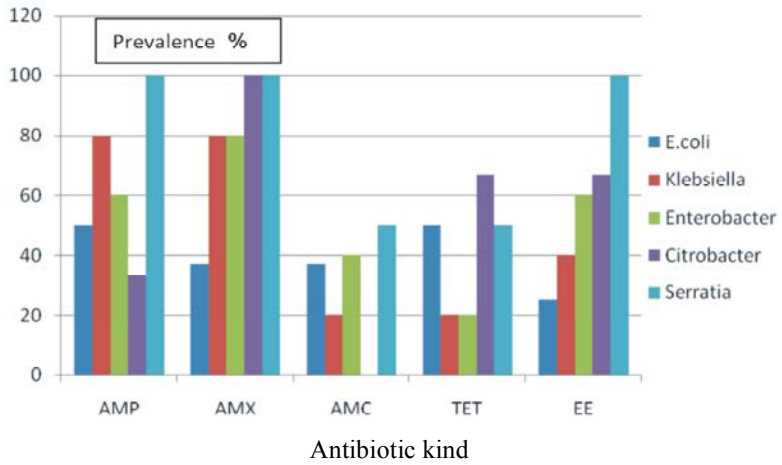

Fig. 2: Antibiotic resistance of some geneses of Enterobacteriaceae

In the present study, the vegetables products when analyzed have shown a presence of other Enterobacteriaceae genus such as Serratia (13 percent), this genus was identified as *Serratia marcescens* with percentage (66%) and *Serratia liquetacience* (33%). It is known that Serratia. are often present in high numbers in vegetables [3, 36, 42, 65]. As reported previously, *Serratia marcescens* is generally an opportunistic pathogen causing infections for immunocompromised patients. Therefore these bacteria can cause certain illness such as respiratory tract and a lethal septicemia tract infection [66].

Bacteria Antibiotic Sensitivity Assay: To evaluate the incidence of antibiotic resistance between the Enterobacteriaceae isolates revealed in Figure 2, that there is a clear difference concerning antibiotic resistance among genus. All isolates have showed the same trend 100% sensibility to Ciprofloxacin, Cefotaxime, Gentamicin and Chloromphenecole.

The isolates were particularly resistant versus Ampicillin, Tetracycline, Amoxicillin, Erythromycin and Amoxicillin+Ac clavulanic-which are confirmed to be the most commonly used antibiotics in the studied area. And then, Serratia isolates have the highest resistance rates against the used antibiotics*:* Ampicillin (100%), Amoxicillin (100%), Amoxicillin+Ac clavulanic (50%) Tetracycline (50%) and Erythromycin (100%). Previous studies revealed that *Serratia marcescens* is found resistant to Ampicillin and other antibiotics Ball *et al*. [67] AlAskari *et al.,* [28]. The observed rate of antibiotic resistant Klebsiella were as follow: Ampicillin (80%), Amoxicillin (80%), Amoxicillin+Ac clavulanic (20%), Tetracycline (20%) and Erythromycin (40%). It is noticed that Ajayi and Egbebi [68] have confirmed before Klebsiella resistance to ampicillin, Amoxicillin+Ac clavulanic and Tetracycline. Indeed, resistance to antimicrobial components is a well recognized problem among Enterobacteriaceae [69, 70]. As example, *K. pneumoniae* resistance to antibiotic is caused by the gaining of plasmids containing genes that encode for extended-spectrum beta-lactamases (ESBLs) and these plasmids often carry other resistance genes as well as ESBL-producing [71]. Hence, infections by these bacteria have limited treatment options and have been associated with high mortality rates [72].

Enterobacter isolates were presented resistance to Ampicillin (60%), Amoxicillin (80%) and Amoxicillin+Ac clavulanic (40%) Tetracycline (20%) and Erythromycin (60%). Resistant in Enterobacter spp. has been reported by Charrel *et al*.[73]. Result obtained relatively to *E. coli* showed resistance percentage to Ampicillin, Amoxicillin, Amoxicillin+Ac clavulanic, Tetracycline and Erythromycin evaluated to, respectively, 50, 37, 37, 50 and 25%. The *Enterobacter cloacae* and *Escherichia coli* resistance is predominant especially by the acquisition of ESBL production mechanism [69, 71, 74].

Genus Citobacter was studied, the following percentage antibiotic resistance values, Ampicillin (33%), Amoxicillin (100%) and Tetracycline (67%) and Erythromycin (67%), were recorded on genus Citerobacter. Citerobacter resistance to antimicrobial therapy was documented by Samonis *et al*. [75]. The direct use of antibiotic during cultivation, or the use of contaminated fertilizers or irrigation water are reasons of colonize vegetables by resistant bacteria [59]. Consequently, the danger posed by growing resistance to antibiotics should be ranked along with pathogen on a list of threats to the consumer. Overall resistance to antimicrobials in the present work was relatively high.

This may lead to an additional food safety concern. It will be a challenge to environment, so pathogens could be public health problem.

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