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ORIGINAL ARTICLE

Sanitary impact evaluation of drinking water in storage reservoirs in Moroccan rural area

Faissal Aziz [a,b,*], Juan Parrado Rubio [c], Naaila Ouazzani [a,b], Mohammed Dary [d], Hamid Manyani [d], Bruno Rodríguez Morgado [c], Laila Mandi [a,b]

[a] National Center for Research and Studies on Water and Energy, University Cadi Ayyad, Marrakech, Morocco
[b] Laboratory of Hydrobiology, Ecotoxicology & Sanitation (LHEA, URAC 33), Faculty of Sciences Semlalia, Marrakech, Morocco
[c] Department of Biochemical and Molecular Biology, Faculty of Pharmacy, University of Seville, Spain
[d] Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Spain

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Abstract In Morocco, storage reservoirs are particular systems of water supply in rural areas. These reservoirs are fed with rainwater and/or directly from the river, which are very contaminated by several pathogenic bacteria. They are used without any treatment as a drinking water by the surrounding population. In this context, the aim of this study is to evaluate the impact of consuming contaminated water stored in reservoirs on health status for six rural communities located in Assif El Mal, Southern East of Marrakech. This was investigated using a classical methodology based on population survey and by molecular approach using PCR–DGGE technique to determine the intestinal bacterial diversity of consumers. The survey showed that, the residents of the studied area suffered from numerous health problems (diarrheal diseases, vomiting or hepatitis A) due to the lack of waste management infrastructures. The consumer's stool analysis by molecular approach revealed that numbers of *Escherichia coli*, *Aeromonas hydrophila* and *Clostridia*, were significantly higher in the diarrheal feces. In addition, PCR–DGGE study of the prevalence and distribution of bacteria causing human diseases, confirmed that, there is a relationship between water bacterial contaminations of storage reservoirs and microbial disease related health status. Therefore, water reservoir consumption is assumed to be the mean way of exposure for this population.

It's clear that this approach gives a very helpful tool to confirm without any doubt the relationship between water bacterial contamination and health status.

* Corresponding author at: National Center for Research and Studies on Water and Energy, University Cadi Ayyad, Marrakech, Morocco. Tel.: +212 641639142.
E-mail address: faissalaziz@gmail.com (F. Aziz).
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1. Introduction

One third of the world's populations live in countries with some level of water stress. Due to the increase of human population and the resulting impact of human activity on the environment, water scarcity will increase in the future (Asano et al., 2007). Water resource contamination has harmful effects on the environment and human health (Emmanuel et al., 2009; Muhammad et al., 2011). Irregular water supply and insufficient treatment seem to be associated with self-reported diseases (Abu-Amr and Yassin, 2008). It is therefore important to understand the potential indicating that the natural and drinking water can contribute to the transmission of pathogenic microorganisms.

In 2006, Maged et al. (2006) reported a correlation between drinking water contaminated with bacteria and waterborne diseases such as diarrheal and hepatitis A. Moreover, an estimation game for 2 million children dies each year because of diarrheal disease (WHO, 2002). Almost all of them are living in developing countries and are less than 5 years of age. Children younger than 1 year account for more than 50 percent of these deaths, and the risk can be 2–3 times higher among children who are not exclusively breastfed (Arifeen et al., 2001; Bhandari et al., 2003). Many of these deaths are attributed to the use of unsafe drinking water.

Consequently, waterborne diseases are important public health issues, and many of them are derived from contact with contaminated water by human fecal material (Balarajan et al., 1991; Zahra and Jamil, 2001; Scott et al., 2002). Diarrhea can be classified as acute inflammatory disease of the intestinal tract. Its composition change has been related to different metabolic disorders and infections (Brigidia et al., 2001). Diarrhea induced by pathogens can cause dysbacteriosis which leads to changes in the intestinal microbiota and the destruction of protective microbial barrier (Chaofeng et al., 2011).

The bacteriological study by the isolation of bacteria on culture medium demonstrates that a very small proportion of these bacterial species (Nocker et al., 2007). For example, from 1012 bacteria in one gram of feces only 20–40% can be grown (Macfarlane et al., 2004; Suchodolski et al., 2004). Indeed, to identify one species by this technique, the biochemical or serological procedure used for that needs at least one week according to the standards of microbial analysis. To ensure a good public water quality, we must develop improved methods, more precise to identify human fecal pollution. Consequently, scientists have been searching for other rapid methods, that are very sensible to detect all bacterial diversity in environmental samples. Therefore, a molecular detection method is required, since such methods are highly specific and sensitive. The molecular approach is typically based on the detection and quantification of specific segments of the pathogen's genome (DNA or RNA).

These techniques allow researchers to speedily and exclusively detect microorganisms of public health concern. Additionally, recent methods have allowed immediate detection of numerous microorganisms in a simple test (Marcelino et al., 2006). They are the new techniques of multiplex PCR, real-time PCR, nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), oligonucleotide DNA microarray (Law et al., 2014), and magneto-DNA nanoparticle system (Chung et al., 2013).

The detection of bacteria in clinical microbiological research and diagnosis using molecular techniques has increased significantly (Tannock et al., 2004; Murray et al., 2005). Recently, the Polymerase Chain Reaction (PCR) technique has allowed fast and effective diagnosis of microbial infections due to its specificity and sensitivity (Sibleya et al., 2012). PCR–DGGE also has been performed for rapid changes tracking and diagnosing of bacterial diversity in healthy human neonates' intestinal tract (Favier et al., 2002) and in patients suffering from combined infections (Muyzer et al., 1993; Ariefdjohan et al., 2010). It is therefore apparent that this approach will contribute to the understanding of the genetic diversity of complex microbial communities.

Up to now, the nature and magnitude of endemic waterborne disease are not well characterized in Morocco. Epidemiologic studies can give an estimate of the waterborne risk along with other types of information. Endemic gastrointestinal illnesses are rarely seen by the medical authorities in Morocco, for the simple reason that the majority of gastrointestinal illnesses are not declared through the medical care system. In evaluating the drinking water risks, investigators must also study the factors and exposure risks.

Poor rural communities in Morocco, like those in other developing countries that do not have access to piped water, have mainly been reliant on other water resource harvesting systems as part of low cost strategies for improving water supply and sanitation. Typically, rainwater and surface water are collected and stored in traditional reservoirs and then conserved for drinking and cooking. It's the case of Assif El Mal valley (Marrakech region); our study site in which its water is very contaminated with many pathogenesis bacteria (Aziz et al., 2013). This bacterial pollution exposes the user population to many gastrointestinal illnesses.

Global studies have identified and analyzed the pathogenic bacteria in water that cause diarrhea, but there have been a few studies in Morocco, and none of them has evaluate the human impacts using a molecular technique.

The aim of this study is to investigate the human health impact due to contaminated drinking water stored in reservoirs, via an epidemiological study in the consuming population. This was done by (i) a survey questionnaire and (ii) by studying the 16S-rDNA diversity in children feces, using Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis (PCR–DGGE) technique.

2. Methods

2.1. Study area

The basin of Assif El Mal is located on the north side of the High Atlas, one hundred kilometers southwest of Marrakech (Fig. 1). In the valley Assif El Mal, the population living in the plain suffers from drinking water shortage and lack of minimum hygiene conditions. The poor socioeconomic status of the local population does not enable them to dig wells. As a consequence, they are using an archaic method as the only source of water for any kind of use (consumption, watering of livestock, etc), water is stored in a kind of traditional cistern buried in the ground, called "*Matfya*" with no prior treatment. They are supplied by river and/or rain water through channels called "*Seguia*".

The studied population in this work used the water of six traditional reservoirs distributed in the study area from upstream to downstream (*R*1–*R*6) (Fig. 1). According to Aziz et al. (2013), these reservoirs are contaminated with several pathogenic bacteria. This situation indicates a fecal contamination of these water resources (Table 1).

2.2. Data collection for the survey

Data collection was accomplished through a questionnaire which involved a representative sample of 300 households in six rural communities located in Assif El Mal. The local population used to store water in reservoirs without any treatment.

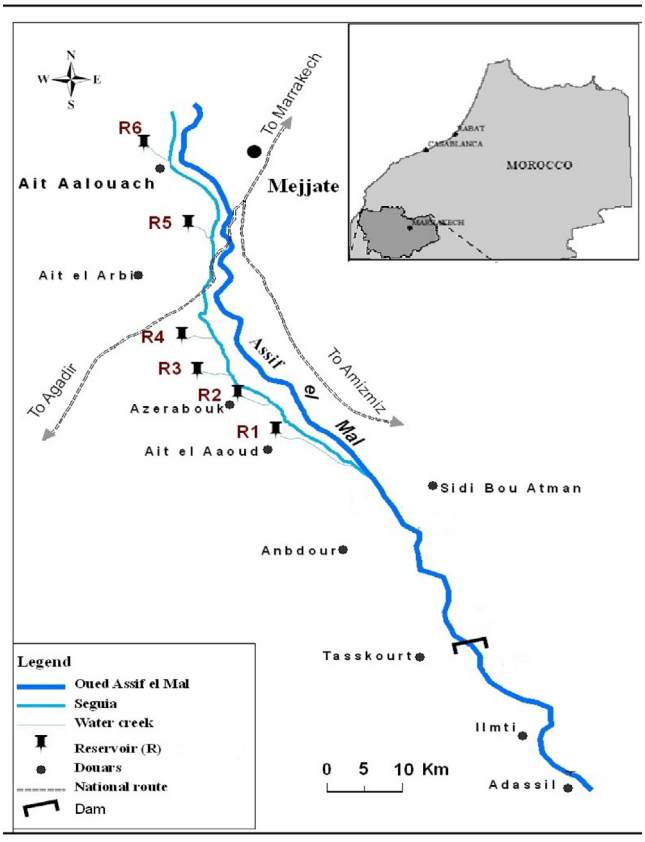

Figure 1 Valley Assif El Mal; map of situation and location of sampling sites (R: reservoir of sampling).

The control area is chosen in Mejjat village, where a municipal drinking water network is available (Fig. 1). The questionnaire ascertained information on demographic factors (age, sex, and number of residents in house), other factors were taken into account such as possibly predictive of Acute Gastrointestinal illness (the presence of pets and livestock, type and frequencies of diseases), and the water reservoirs characteristics.

The Acute Gastrointestinal illness (AGII) was defined by the following grouping of symptoms signaled (occurred as a maximum in the 28 days ago): (1) vomiting or liquid diarrhea or (2) nausea or soft, loose diarrhea combined with abdominal cramps (Payment et al., 1991).

2.3. Samples collection and DNA extraction

A coprolite analysis study was carried out among children living in the studied area with those in the nearest area as a control (Mejjat village). During our visits to both the studied areas, the children (male and female) were randomly selected. 126 children were investigated, including 108 children from the exposed area (Assif El Mal).

To collect human fecal samples, children were requested to place approximately 10 g of fresh feces into sterile vials, using a sterile spatula. The samples were kept on ice for transport to the lab, and stored at −20 °C. To determine fecal moisture content, frozen specimens were thawed at 4 °C. Then, around 0.5 g of each fecal sample was put in a vacuum dryer for 3 days and reweighed for the measurement of dry weight percentage.

To ensure sample homogeneity, the fecal aliquots were diluted 1:4 in sterile water then put in separate sterile bags and mixed. DNA was extracted with the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol and then immediately stored at −20 °C.

2.4. PCR amplification

For PCR purposes, the DNA concentration was measured by NanoDrop (RND-1000 spectrophotometer, NanoDrop Technologies Inc.) and adjusted to a concentration of 10 ng/µl.

The variable region V3–V5 of the 16S rDNA was amplified using the universal primers 341F-GC and 907R as shown in Table 2. This set of primers was designed to be specific for most bacteria (Muyzer et al., 1997). The use of universal primers in 16S rRNA gene-DGGE population fingerprinting ensure the evaluation of microbial diversity, the reason for that the fecal microbiota is host specific and relatively stable

Table 1 Bacterial load (CFU. 100 ml^{-1}) in the studied water reservoirs (R) (Aziz et al., 2013).

	Fecal coliforms	*E. Coli*	Fecal streptococci	*Staphylococcus aureus*	*Clostridia*	*Salmonella* sp	*Pseudomonas aeruginosa*
*R*1	2.20*E*+04	1.55*E*+04	7.43*E*+14	850 ± 6	1.47*E*+03	+	+
*R*2	2.92*E*+04	2.46*E*+04	2.31*E*+03	9.71*E*+02	1.66*E*+03	+	–
*R*3	2.03*E*+04	1.53*E*+04	1.63*E*+03	1.27*E*+03	1.91*E*+03	+	+
*R*4	2.77*E*+04	1.52*E*+04	9.04*E*+03	2.22*E*+03	2.12*E*+03	+	+
*R*5	1.13*E*+04	1.03*E*+04	5.20*E*+03	6.55*E*+02	1.83*E*+03	+	+
*R*6	3.12*E*+04	2.48*E*+04	1.38*E*+04	2.38*E*+03	2.87*E*+03	+	+

(+): Present.
(−): Absent.
CFU: (Colony forming units).

Table 2 Oligonucleotide primers used for PCR.

Primer	Primer sequence (5′ → 3′)
341F	5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3′
907R	5′-CCG TCAATT CCT TTG AGT TT-3′

F: Forward.
R: reverse.
*GC clamp: CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG.

temporally (Vanhoutte et al., 2004; Sánchez et al., 2007). PCRs were performed in a Thermal Cycler (TECHNE TC-5000).

Each PCR mixture contained 100 ng of DNA, 1x reaction buffer implemented with 2.5 mM $MgCl_2$, 50 pmol of each primer, 0.2 mM of each dNTP, and 3 units Taq-polymerase in a final volume of 50 ml. The PCR protocol had an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 30 s at 92 °C, 30 s at 55 °C, and 30 s at 72 °C and a final extension step at 72 °C for 30 min was carried out. The quality of the amplicon products was checked by electrophoresis in 1.5% agarose gels using a molecular weight marker (1 kb DNA ladder), on Ethidium bromide-stained.

2.5. Denaturing Gradient Gel Electrophoresis (DGGE)

After the optimization of experiments, PCR products (25 ml) were analyzed through DGGE, in 8% polyacrylamide gel (in 1 mm vertical), using a parallel gradient of 45% urea formamide on the top and 65% at the bottom of the gel (100% denaturing gradient is 7 M urea and 40% deionized formamide). The vertical electrophoresis was carried out using the Bio-Rad Dcode system using 0.5 × TAE buffer (20 mM Tris, 10 mM acetic acid, and 0.5 mM EDTA) during 16 h at 75 V, with an initial step at 120 V for 15 min. The gel was then stained in an ethidium bromide solution and then photographed in a Molecular Imager system (Bio-Rad), after an agitation in distilled water. The most intense bands from DGGE profiles were excised from the gel and used as templates in a new amplification using the same primers.

2.6. Sequencing and nucleotide sequence accession numbers

The bands of DGGE were excised and their nucleotide sequence was amplified with the similar primers under the same PCR conditions described above and then sequenced. The sequences determined were compared with 16S rRNA sequences available in the GenBank database. The BLAST program (Basic Local Alignment Search Tool) was used to explore similarity against sequences deposited in the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The species with 97% similarity (< 3% sequence differences) to the isolate sequences were considered as the same species.

2.7. Statistical analysis

For the data survey exploitation, odds ratios at 95% confidence intervals were used to quantify the relationship between AGII and contamination of water supplies. Multivariable models were used to estimate the odds ratio controlling for important covariates.

Concerning the DGGE fingerprints analysis, basing on the number of bands and their relative intensities, the total biodiversity of the concentration of the Dominance or Simpson index (D or S) and the Shannon index (H') were calculated. The universal model used in statistic is the Shannon–Weiner index: $H' = -\sum \text{pi ln pi}$, where pi is the frequency of the ith species.

For DGGE results, the presence or absence of migrate bands was converted to a binary matrix (0/1), taking into account each band present in at least one sample as a single descriptor.

Using the Pearson correlation coefficient (95% probability) we calculated the similarities between the banding patterns then a dendrogram was constructed using the average linkage between group method (UPGMA) (Fromin et al., 2002).

3. Results and discussion

3.1. Epidemiological survey

3.1.1. Population study

Six rural communities located in Assif El Mal, Southern East of Marrakech were selected for this study, representing a cross section of rural populations in this area. The hamlets were largely composed of Amazigh speaking residents (95%) averaging 3 individuals per household. The mean age of the residents was between 33 and 38 years and the mean household income ranged between 100 and 300 Euro for month (CDRT, 2008).

According to the response of the studied population to our questionnaire, various characters of water reservoirs are summarized in Table 3. In addition, we noted that in the exposed area, only 17% of people drink municipal water and 60% of them reported that these waters may have an interruption of two days per week on average. However, 83% claimed that they depended on water storage reservoirs for drinking, washing and bathing purposes. About half of the people (n = 379; 56.5%) said that they never added chlorine or any disinfectant product in drinking water. Also, most people (n = 640; 95.3%) are conscious that water could transmit diseases. But, 508 (75.7%) of people, especially the most elderly, think that the water in their reservoirs is a healthy water and better than other resources according to their local beliefs. Moreover, some householders take benefit from potable water network, but they keep storing rainwater and surface water in reservoirs because of the interruption of the piped water supply.

About 466 (69.5%) of persons interviewed regarding exposed area (n = 671) reported that the stored water is turbid

Table 3 Water resource characterization (R: reservoir).

Reservoir	Age (years)	Distance from agglomeration (m)	Capacity (m^3)	Consumer (person)	Disinfection (Chlorination)
*R*1	45	25.5	120	92	Some time
*R*2	38	14.5	90	89	Rarely
*R*3	64	19	150	168	Never
*R*4	29	6.5	72	136	Never
*R*5	35	22	96	84	Rarely
*R*6	63	5	180	154	Never

and has the presence of insects and settlements, but only 10.8% of them used to clean their reservoirs. In addition, 636 (94.7%) people claimed that they have never closed water reservoirs properly. For All people (n = 671) homes aren't connected to the sewage networks system, 120 (17.9%) disposed wastewater in cesspools and 82.1% drained wastewater in an open area. From the six storage reservoirs sampled, two of them (*R*1, *R*2) were less than 15 year old, three were between 15 and 50 years (*R*3, *R*4, *R*5) and the lats one (*R*6) is greater than 50 year old.

Demographic characteristics of the studied population are depicted in Table 4. The average age of participants was 34 ± 1.5 years old. 33.77% of the interviewees haven't had any school degree, reflecting a low educated community. A total of 254 (37.8%) were unemployed.

3.1.2. Gastrointestinal illness and health related quality of the population

In our study, self-reported diseases were claimed by 107 (10%, 2%) of the interviewees. A number of 45 (42%), 36 (33.6%), 6 (5%, 6%) and 5 (4%, 6%) reported diarrheal diseases and vomiting, diarrheal diseases, vomiting or hepatitis A, respectively. Such diseases were more prevalent among people who used water reservoirs (OR = 1, 15) than people living in the control area (Table 4). In addition, children of 10 years or younger were more exposed to acute gastrointestinal symptoms (OR = 1, 81) and individuals who had used these resources for 10 or more years were less to report acute gastrointestinal symptoms. Such diseases were more prevalent among analphabet people (OR = 4, 03). Moreover, for all studied factors the statistical analysis ($P < 0.05$) showed that the most affected populations are those who use water from storage reservoirs *R*4 and *R*6.

Most exposed people (n = 427%; 55.5%) received treatment; 276 (64.7%) of them received traditional self treatment at home, 102 (24%) were treated in the governmental hospital and only 1% in special clinics. According to this survey, there is evidence that the Assif El Mal population suffer from water-related diseases due to contaminated water supply which affects their health status.

This impact is accentuated by the low socioeconomic and intellectual levels. These results are similar to those reported by other studies in rural areas over the world. It was shown that in rural areas in India the provision of private inputs to children's health depends on socioeconomic characteristics of the child's family (Jalan and Ravallion, 2003). It is estimated that 81% of the poorest quintile (in terms of a composite wealth index) of families did not use oral re-hydration therapy when a child had diarrhea, as compared to 50% in the richest quintile (Gwatkin et al., 2000; Bellani, 2012). According to Jalan and Ravallion (2003), 48% of those in the poorest quintile did not seek medical treatment, as compared to 22% in the richest. There is also evidence suggesting that parental education, notably of the mother, matters to child health outcomes.

Table 4 Incidence of Acute Gastrointestinal illness (AGII) according to demographic characteristics of the studied population (n = 1049) in each station.

	Population characterization (%)								Incidence of AGII (%)								Odds ratio
	Control (n = 378)	Exposed (n = 671)							Control (n = 9)	Exposed (n = 98)							
		Tot.	*R*1	*R*2	*R*3	*R*4	*R*5	*R*6		Tot.	*R*1	*R*2	*R*3	*R*4	*R*5	*R*6	
Sex																	
Female	51.1	53.6	17	16	15	20	15	18	33.3	57	10.3	9.9	10.8	20.9	20.8	27.7	1.15
Male	48.9	46.3	17	16.4	15.4	17.4	16.7	17	66.6	43	7	9	15.2	23.1	18.1	28	
Age																	
40 + years	15.2	26.2	19	15.3	21.6	16.5	13.4	14.2	0	17	13.9	12	15.9	18.8	16.8	22.6	0.54
11–39 years	57	48.7	16.5	17.1	17.7	15	16.2	17.4	0	46	11.9	11.6	17.2	19.3	17.6	22.4	0.91
0–10 years	27.8	25	13.7	15.5	17.3	19	14.9	19.6	100	37	9.7	10.3	15	19.7	15.8	29.5	1.81
Education completed																	
Analphabet	29.7	27.3	13.7	15.3	16.4	18	15.8	20.8	0	60	14.3	17.1	15.7	17.1	16.9	19.1	4.03
Grade school	46	56	16.8	17.0	16.0	17.3	16.8	16.2	100	37	14.3	15.0	16.4	18.2	17.1	20.7	0.45
High school	22	15.6	16.2	18.1	18.1	16.2	17.1	14.3	0	3	0	0	0	33	0	66	0.17
University	2.3	1	42.9	28.6	28.6	0	0	0	0	0	0	0	0	0	0	0	0.03

This finding has also been reported in Kenya by Bhargava (1999) when he found a very strong correlation between children morbidity and the parental scores on cognitive tests. He also pointed out that the maternal score was a much stronger predictor than the paternal score.

3.2. Bacterial prevalence in studied population

3.2.1. Diversity and Dominance

Using PCR–DGGE to examine feces from the consumers of contaminated water stored in traditional reservoirs, we aimed to mention which taxa might be exist as commensal flora and which might have a causal role.

A band similarity coefficient of >95% in the DGGE profile of total bacteria was observed for the studied fecal samples (Fig. 2). We note a significant difference ($P < 0.001$) in the banding pattern between the exposed population (predominant number of bands: 16.9 ± 3.8 and 7.24 ± 1.8 for non-diarrhea and diarrhea subjects respectively) and control subjects (9.9 ± 3.5). This significant difference was also confirmed by a higher intensity of the bands (54%) observed for the exposed non-diarrhea subjects than the control.

The similarities between the studied groups were calculated based on the DGGE profiles (Fig. 2). The similarity indices for total bacteria are presented in Table 5; which revealed that the biodiversity of dominant taxa was higher in the non-diarrhea exposed subjects (51.3 ± 2.4%) than in the control samples (46.3 ± 1.5%). But these indices are lower for diarrhea exposed subjects (39.5 ± 2.5) (Table 5).

An important bacterial diversity ($H' = 3.7$, $P < 0.05$) and Dominance ($D = 0.92$, $P < 0.05$) were noted in the exposed group samples. In the opposition, the band numbers and H' index of the exposed diarrhea groups ($H' = 2.5$, $P < 0.05$) were significantly lower as compared with the exposed non-diarrhea group ($P < 0.05$) and more significantly ($P < 0.001$) lower as compared with the control ($H' = 3.1$). This reflects the reduction in the diversity of intestinal microbiota for the diarrhea group.

The dendrogram based on Jaccard similarity index of DGGE banding patterns, was discriminated between 7 groups of samples that shared only 75% of the detected bands (Fig. 3) and were significantly different ($P < 0.01$). Using the DGGE profile, the seven main clusters were marked as the following (C, *R*1–*R*6), where the groups clustered together according a very recognized gradient.

3.2.2. Bacterial group's community

On 126 samples studied, a total of 28 representative DGGE bands were excised from the gels (Fig. 2) and sequenced, but only eighteen bands gave clear results in the sequencing; that are shown in Table 6.

DGGE band patterns showed a gastrointestinal bacterial variability of human population along the Assif El Mal valley. The majority of the dominant bands sequences (72%) displayed more than 97% similarity with the known sequences in database. The most abundant bacterial groups detected were members of the Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes, but the diarrhea groups were mainly composed by phylum Firmicutes and Proteobacteria. Among the dominant bands, 94% (16/17) had more than 98% of similarity with the known sequences in the database and were found in both the studied groups (exposed and control).

Fingerprinting analysis showed that the dominant diarrheagenic organisms isolated from the stool samples were *Escherichia coli*, *Streptococcus* and *Aeromonas*. However, *Aeromonas hydrophila* was present exclusively in diarrhea of subjects who have the lowest band number (the lowest biodiversity).

So, we can state that, the dysbacteriosis is dependent on the type of opportunistic pathogen responsible of the infection. This result might indicate also that pathogen infection caused intestinal damages and resulted in changes of the structure and composition of intestinal microbiota that might aggravate the illness.

According to Bravo et al. (2003), in fecal samples from diarrheal children, many microbial pathogens have been identified in the intestinal tract, of which *E. coli* is the dominant. Many authors in recent works reported that the *Aeromonas* cause diarrhea (Albert et al., 2000; Aslani and Alikhani, 2004; Al-Mayahie et al., 2011; Subashkumar et al., 2012; Tomás, 2012). In addition Mühldorfer et al. (1996), noted that *E. coli* was the most prominent bacteria among the total germs isolated from diarrhea according to their pathogenicity factors.

The largest proportion of microbes which resides in human intestine falls into two groups, the Bacteroidetes and the Firmicutes (Xu et al., 2007; Chaofeng et al., 2011). *Bacteroides vulgatus* is the numerically predominant bacteroides species in the human colonic microbiota which are beneficial for intestinal colonization (Wexler, 2007). Lactobacilli and Bifidobacteria are also very important groups of intestinal microbiota as they have many beneficial effects on the host (Boesten and De Vos, 2008).

Another predominant bacterial genus is *Clostridium* which has been proven to be a major cause of epidemic diarrhea (Susan et al., 2004; Harrison et al., 2005). The genus *Clostridium* consists of a heterogeneous group of micro-organisms that can adapt to diverse habitats (Woodmansey et al., 2004). According to Codling et al. (2010), there are no differences within control individuals and a similar instability of *Clostridium* was noted.

3.3. Water-GI tracts bacterial incidence

Regarding the correlation approach, cluster analysis, attempts to differentiate between cohorts that contain large differences and within the cohort itself. Cluster analysis, based on band patterns, showed a clear gradient upstream–downstream in fecal samples in parallel to the gradient that was shown for water reservoirs with reference to bacterial contamination level (Aziz et al., 2013). An obvious grouping of all subjects of each station is also viewed (Fig. 3). Therefore, this clear grouping of fecal samples is related to bacterial diversity in water reservoirs. So, this spatial gradient from the station *R*1–*R*6, due to an accumulation of microbiological contaminants from upstream to downstream in water (Aziz et al., 2013), is proportionally reflected on GI tract of the user population. This is due mainly to the increasing degree of the pollution impact caused by the activity of neighboring populations along the studied area.

Generally, the dendrogram has grouped together populations presenting the same characteristics of GI tracts and that

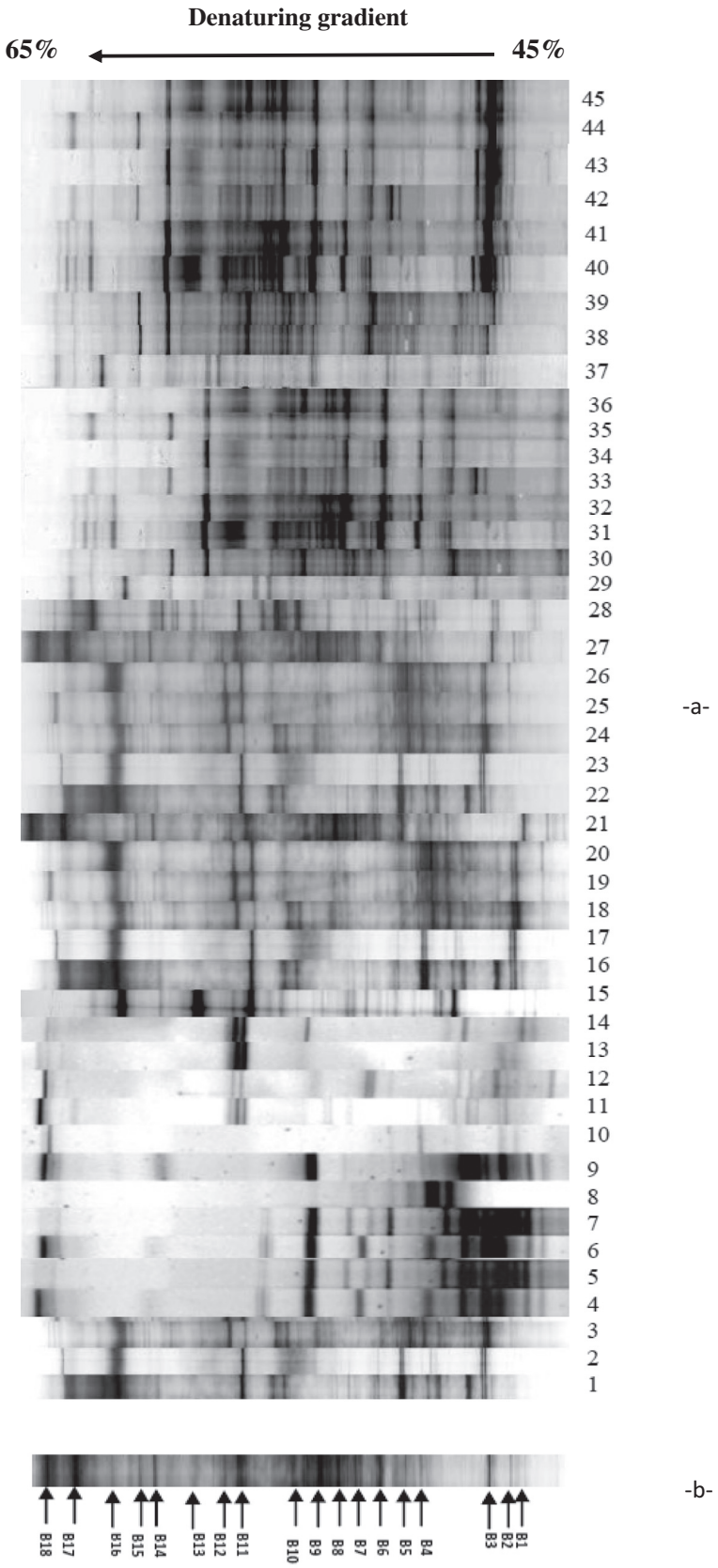

Figure 2 DGGE banding profiles of V3–V5 regions produced from the community DNA extracted from stool samples of the studied population (a. DGGE profile, b. bands legend [B1–B18]).

Table 5 Dice similarity coefficient (Dsc), Simpson index of Dominance (*D* or *S*), Shannon index of general diversity (*H'*) of total bacteria in the AGII versus non AGII fecal community, $^{*}P < 0.05$.

	AGII		Non-AGII
	Diarrhea	Non-diarrhea	
Dsc (%)*	39.5 ± 2.5	51.3 ± 2.4	46.3 ± 1.5
Dominance*	0.76	0.87	0.92
Shanon H'	2.5 ± 0.12	3.7 ± 0.45	3.1 ± 0.23

using the same drinking water reservoirs (Fig. 3). This analysis showed that bacterial communities in these water reservoirs and GI tracts had a stepwise ascending shape, suggesting that they had similar gene diversity. This similarity confirms the influence of high selective forces in these environments, even on the GI tract bacterial community.

The proportion of the bacterial genera incidence was predominantly higher in the reservoirs *R*4 and *R*6, which are respectively about $6.5 \cdot 10^5$ and $8.3 \cdot 10^5$ CFU/100 ml for total coliform (Aziz et al., 2013). The individualization of these two stations (*R*4 and *R*6) is related to their exposition to intensive local pollution sources according to the data obtained in the current study.

In addition, as reported during the questionnaire survey, due to ingestion of contaminated water from storage reservoirs in Assif El Mal valley, most residents of this areas especially children suffer from waterborne diseases such as gastroenteritis, dysentery, diarrhea and viral hepatitis (A, B and C).

It's clear that the obtained results using the molecular approach in this study give a very helpful tool to confirm without any doubt the relationship between water quality and health status of the Assif El Mal population. This study can be viewed as a model approach for: (i) applying molecular techniques to be used as routine monitoring methods for many emerging pathogens and (ii) collecting quantitative data necessary for assessing potential health threats due to a wide range of pathogens in storage waters. Even though some bands obtained by 16S rDNA PCR amplification are short and cannot be used to distinguish the exact taxonomic groups, we can use them to understand the distribution of bacterial population. In addition, the use of this molecular technique helps to give accurate information on the most dominant bacteria groups in the human tract. This could be seen as a preventive evaluation of the sanitary risk, for vulnerable population, before causing the diarrhea or other water borne diseases.

In conclusion, the data collected during the survey in the Assif El Mal rural area, indicated that the residents of the studied area suffer from numerous health problems due to the lack of sewage and solid waste disposal systems which are the major

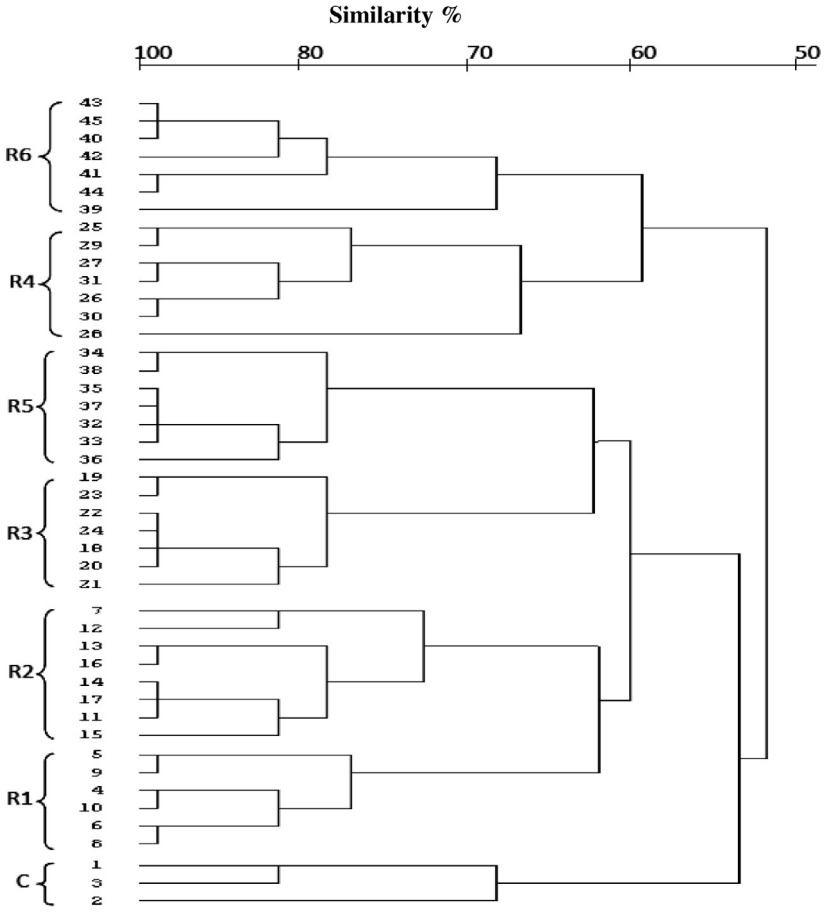

Figure 3 Clustering tree from the analysis of DGGE profiles of fecal samples based on the 16S rRNA gene.

Table 6 Phylogenetic affiliation of eighteen representative 16S rRNA gene sequences obtained from DGGE bands of the fecal DGGE profiles.

Band number	Nearest species	Taxon	Band number	Nearest species	Taxon
1	*Bacteroides faecis* JCM 16477	Bacteroidetes	10	*Bacteroidetes uniformis* S13	Bacteroidetes
2	*Bifidobacterium bifidum* strain LMG 11041	Actinobacteria	11	*Lactobacillus salivarius* CH-9	Firmicutes › Lactobacillales
3	Uncultured bacteroidales bacterium clone MS146A1 B01	Bacteroidetes	12	*Streptococcus mitis* bv2	Firmicutes › Lactobacillales
4	Uncultured *Bacteroides* sp. clone Sew1-210	Bacteroidetes	13	*Clostridium sp.* N8, N6	Firmicutes › Clostridia
5	Uncultured bacterium clone L243	Firmicutes	14	*Clostridium sordellii* JCM 3814	Firmicutes › Clostridia
6	*Escherichia coli* JM109	Proteobacteria gammaproteo-bacteria	15	*Enterococcus sp.* SF-1	Firmicutes › Lactobacillales
7	*Lactobacillus casei* FHHMB206-KNM12	Firmicutes › Lactobacillales	16	*Bacillus thuringiensis* CCM15B	Firmicutes › Bacillales
8	*Bacteroides eggerthii* DSM 20697T	Bacteroidetes	17	*Aeromonas hydrophila* LMG 19562	Proteobacteria (Gammaproteobacteria)
9	*Clostridium colinum* DSM 6011T	Firmicutes clostridia	18	*Bacteroidetes vulgatus* BCRC12903	Bacteroidetes

threats for water resources. This threat is accentuated by the low socio-economic and intellectual levels of the population.

The analysis of the microbial community in intestinal tract of the user population demonstrated a clear discrepancy between the fecal microbial diversity and richness measured as the presence of 16S rRNA gene signatures of bacteria. Key bacterial communities were shown to be significantly different, in diversity and Dominance, between control and the exposed populations. Fingerprinting analysis showed that the dominant diarrheagenic organisms isolated from the stool samples were *E. Coli*, *Clostridia* and *Aeromonas*. Pathogen opportunist infection caused intestinal damage and resulted in changes of the structure and composition of intestinal microbiota that might worsen the diarrheal sickness.

Comparative Cluster analyses of the DNA based fingerprints revealed seven major types of communities based on microbial richness of fecal samples, reflecting a strong correlation between them. The accumulations of microbiological contaminants from upstream to downstream in water was proportionally reflected on GI tract of the user population. The PCR–DGGE study to evaluate the prevalence and distribution of bacteria causing human diseases, show that water reservoir consumption is assumed to be the primary route of exposure for this population, especially the oldest and the most polluted ones.

It's clear that using the molecular approach in this study gives a very helpful tool to confirm without any doubt the relationship between water bacterial contamination and health status of the Assif El Mal population.

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