Iranian Journal of Microbiology
Volume 18 Number 2 (April 2026) 204-216
DOI: http://doi.org/10.18502/ijm.v18i2.21295

ORIGINAL ARTICLE

Impact pattern of heavy metals on gut microbiota in the polluted city of Tehran

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Received: November 2025**, Accepted:** March 2026

ABSTRACT

Background and Objectives: This article focuses on the effects of six heavy metals on gut microbiota, which plays a key role in human health. Gut microbiota plays a key role in metabolism, immunity, and maintaining homeostasis. Heavy metals can affect microbiota composition and function, with health consequences. Consuming large amounts of heavy metals may have harmful impacts, including alteration in microbial composition and bacterial population changes.
Materials and Methods: Six heavy metals—cadmium, chromium (toxic metals), copper, zinc, iron, and selenium (beneficial trace elements)—were detected in peripheral blood, serum, or urine, while feces were used for 16S rRNA sequencing. Serum samples from 100 volunteers from Tehran (polluted area) and Firoozkooh (clean city) were collected. Subjects were analyzed for the presence of *Escherichia coli*, *Bacteroides fragilis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Clostridium clostridioforme*, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* to evaluate correlations between metals and microbial composition using biochemical, microbial, and molecular methods.
Results: *Escherichia coli* and *Bifidobacterium longum* levels in polluted areas were not significantly different from those in unpolluted areas. *Bacteroides fragilis* in polluted areas was significantly higher compared to non-polluted locations. *Clostridium*, *Akkermansia*, *Faecalibacterium*, and *Lactobacillus acidophilus* were significantly lower in polluted areas, amounting to less than half the levels in clean areas. Heavy metal concentrations showed no gender differences in either location.
Conclusion: Some heavy metals change intestinal microbiota composition and metabolic profiles, potentially resulting in metabolic diseases and environmental risks.

Keywords: Gastrointestinal microbiome; Heavy metals; Environmental pollution; Bacteria; Iran

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INTRODUCTION

The industrial revolution has significantly increased chemical release, including heavy metals, into the environment, causing health alterations, including disruption of the food cycle (1). Heavy metals may originate from mining, metal processing, or chemical production and enter the human body via food, skin, or inhalation (2).

Human exposure to both essential and toxic heavy metals is widespread (3). The main mechanism of heavy metal toxicity involves free radical production causing oxidative stress, damage to biological molecules including enzymes, proteins, lipids, nucleic acids, and DNA damage (4). Increased oxidative stress, altered gut microbial composition, and inflammation may result from exposure to heavy metals such as arsenic (As), lead (Pb), mercury (Hg), cadmium (Cd), and chromium (Cr), potentially leading to intestinal permeability and barrier dysfunction (5). The intestine is where continuous immune system activation through direct microbiota contact occurs (6). Heavy metals transfer to cells and tissues through protein and nucleic acid binding, potentially destroying macromolecules and disrupting cellular functions (4). The human body interacts with many heavy metals including copper, iron, cadmium, chromium, selenium, and zinc through food, water, air, and industrial products (7). The relationship between heavy metals and gut microbiota may create various problems including mental disorders and damage to blood components, lungs, liver, kidneys, and other vital organs by affecting central nervous system functioning (8). Long-term heavy metal accumulation may slow muscle and nerve cell development (9). Unfavorable metals exert destructive effects by directly impacting human cells and causing physiological disturbances in digestive system microflora (10).

Some trace elements are categorized between toxic heavy metals and essential trace elements, such as chromium (Cr) as an essential trace element for livestock and poultry growth, where their addition to feed can improve animal productivity and efficiency (11). Conversely, hexavalent chromium (Cr^{6+}) is toxic, leading to liver and kidney damage and respiratory diseases (12). Cadmium is another problematic heavy metal (13). Cd, as a pollutant, is found in drinking water and soil, produced in human activities such as mining and agricultural fertilizers through air deposition or industrial divisions such as pigments and plastics. Cd exposure alters abundance, diversity, and composition of gut microbiota in freshwater crayfish (14). Cd exposure affects short-chain fatty acid (SCFA) concentrations by altering bacteria capable of biosynthesizing SCFAs, such as *Bacteroides fragilis* (11).

Some heavy metals, including zinc (Zn), iron (Fe), copper (Cu), and selenium (Se) participate in various biochemical and physiological functions at low concentrations and are considered as necessary trace elements (11). Zinc (Zn) is essential for almost all organisms. Even mild deficiencies can reduce cellular differentiation, influence the immune system, and deeply affect growth (15). Zinc may have negligible effects on gut microbiota (16). Iron deficiency is associated with slight T cell decreases and reduced neutrophil bacterial killing capacity (17). Iron alters intestinal microbiota by regulating microbial energy acquisition from host nutrients (18). Bacterial survival is regulated by iron through several key metabolic pathways, including butyrate production. Iron is tightly regulated in the gut and plays a crucial role in maintaining healthy microbial communities (11).

Copper deficiency may lead to decreased cellular antibody responses and increased sensitivity to microorganisms (11). Cu serves as a cofactor for various enzymes involved in antioxidant reactions (e.g., superoxide dismutase), biological membrane and DNA integrity, and ATP production. Selenium has important functions in biological growth and development due to antioxidant, anti-inflammatory, and immune roles. It interacts with gut microbiota, influencing absorption through the strong thyroid-gut axis, and selenium intake affects gut microbiota through hormone release (19).

Gut microbiota refers to the vast community of microorganisms inhabiting the gastrointestinal tract, primarily composed of bacteria, archaea, viruses, and eukaryotes (20). The bacterial component includes members from phyla Firmicutes, Bacteroidetes, and Actinobacteria, and less abundant Proteobacteria members (21). This community plays important roles in host intestinal epithelial expansion and differentiation, immune regulation, pathogen defense, and intestinal homeostasis maintenance (22). Human gastrointestinal tract microbiota is a dynamic ecosystem including 400 to 1000 bacterial species (23). Microflora interact with humans symbiotically (commensal) (24), where the gastrointestinal tract is the first organ sensitive to heavy metals, consist-

ing of different bacterial populations in bidirectional relationships with intestinal epithelial cells (25). Imbalance leads to gastrointestinal tract dysfunction (26). Industrialization has resulted in global environmental pollution (27), and heavy metal toxicity effects on mammalian intestinal microbiota have caused severe digestive tract wall damage and decreased total intestinal bacterial numbers (28). The bacterial genera studied were *Escherichia*, *Bacteroides*, *Lactobacillus acidophilus*, *Bifidobacterium*, *Clostridium*, *Akkermansia*, and *Faecalibacterium*. *Escherichia* species are usually found in lower intestinal regions of warm-blooded organisms and may cause intestinal infections (29). Studies show high prevalence of heavy metal resistance genes (HMRGs) in *E. coli* across arsenic, cadmium, copper, and mercury (30). Bacteroides fragilis species are found in the human intestinal tract and can establish symbiotic relationships with humans. *Bacteroides fragilis* susceptibility and resistance to heavy metals has been studied (31, 32). Studies show that *Lactobacillus acidophilus* an important probiotic member, can maintain intestinal flora balance, relieve constipation, improve intestinal motility, and promote nutrient absorption. *Lactobacillus acidophilus* can adsorb heavy metals such as cadmium to reduce toxicity (33, 34). *Bifidobacterium longum* species represent probiotics playing significant roles in digestive disease treatment (35), such as inflammatory bowel disease (36). Probiotics can bind to numerous targets and eliminate them with feces, including heavy metals such as cadmium, which are detoxified by probiotics (37). *Clostridium* is a genus of gram-positive bacteria causing diseases through exotoxin production (38). Its susceptibility to heavy metals such as cadmium and copper has been determined (39). Studies have shown that increasing cadmium concentrations decrease culture growth rates. *Clostridium* has significant potential in bioremediation of heavy metals, particularly lead (40). *Akkermansia muciniphila* colonizes most animal intestinal tracts and abundantly exists in humans since infancy (41), interacting with human body cells (42) and can be exposed to heavy metals (43) including chromium, zinc, and selenium (44). *Faecalibacterium prausnitzii* produces short-chain fatty acids such as butyrate through dietary fiber fermentation, making it an important gut microbiota component fighting inflammation. Butyrate is the preferred energy source with anti-inflammatory properties generally considered beneficial to intestinal health (45). While previous research has largely focused on the effects of individual heavy metals in controlled animal models, this study addresses a critical knowledge gap, and for the first time in a human cohort quantitatively investigates the association between real-world, chronic exposure to a mixture of toxic and essential heavy metals and the abundance of seven functionally distinct gut bacterial species in a highly polluted urban environment.

Therefore, this article aims to develop the current understanding of how heavy metal exposure disrupts the composition and function of key gut microbial populations.

MATERIALS AND METHODS

Experimental sampling. Tehran records more than 17 million vehicle trips daily, and many vehicles have outdated technology. Thus, Tehran air is among the most polluted worldwide. Tehran, the capital of Iran, is consistently ranked as one of the most polluted cities in the world, with annual mean PM2.5 concentrations frequently exceeding 3-4 times the World Health Organization's (WHO) air quality guideline limit, a situation primarily attributed to high-density traffic, industrial emissions, and geographical factors conducive to pollutant trapping (WHO, 2021; Iran Department of Environment, 2024). We based our research in this city, considering Tehran as an environmentally polluted city from May 2022 to May 2023. For comparison, the suburban city of Firoozkooh, accessible to our team, was chosen as a clean area and the negative control. Environmental pollution levels for both locations were reconfirmed by official sources (46).

Fifty samples were collected from each location in Tehran province (Iran) as the polluted area and from Firoozkooh as the non-polluted area. The health questionnaire collected personal information including job, weight, age, years of residence, sex, blood pressure, dietary habits, physical exercise, infectious or hereditary illness signs, digestive disease, current medication, smoking, or addictive drugs. Selected samples were from both sexes, similar in age and years of regional presence. Subjects with heavy metal concentrations within normal ranges represented the polluted group. Subjects had taken no medication, especially antibiotics, nor supplementary heavy metals in the last 3 months. This study is designed to investigate the effects of chronic, low-dose environmen-

tal exposure to heavy metals, as opposed to acute, high-dose toxicity, by recruiting long-term residents from a highly polluted urban area, thereby reflecting real-world human exposure scenarios. Samples were collected from sterile peripheral blood (for iron, selenium, zinc, and copper), urine (for cadmium and chromium), and feces (for all heavy metals) under microbiological standard sampling conditions. Samples were transferred to the laboratory. Sera were immediately separated from blood via centrifugation at 1500 rpm for 5 minutes and stored frozen. Samples were assayed by atomic absorption. Whole blood was taken according to medical lab standards and serum was prepared by centrifuging blood at 2500 rpm for 10 minutes. Collected sera were stored at -20°C until analysis. For heavy metal tests, serum samples were diluted with distilled and deionized water in 1:10 proportion and tested at the Biochemistry Department of Pasteur Institute of Iran.

Heavy metal assay. This article focuses on six heavy metals: chromium (Cr), cadmium (Cd), iron (Fe), zinc (Zn), selenium (Se), and copper (Cu). This specific set of six heavy metals was chosen to encompass both priority toxic pollutants (cadmium and chromium) and essential micronutrients (copper, zinc, iron, and selenium), enabling a comprehensive investigation into how both harmful and beneficial metals, common in urban environments, differentially correlate with gut microbial populations.

Heavy metal concentrations were determined by flame atomic absorption spectrophotometry (Thermo Jarrel Ash Corporation Model: UNICAM 929 with deuterium background correction, Germany). The flame accessory atomizes samples, converting them into free atom clouds. Sample atoms absorb light at unique wavelengths, and absorption degree is directly proportional to element concentration. Metal elements were measured at ppb range (parts per billion-µg/l) by GBC Scientific Equipment Ltd.-SensAA, China (47). Fe, Zn, and Cu were measured in ppm using flame accessory instruments; Cr, Cd, and Se were assayed in ppb by GBC accessory. Different trace element concentrations were prepared for standard figure calibration. To ensure accuracy, standard solutions were analyzed after every 10 test samples. Serum samples were run in triplicate, and individual values were averaged (48, 49).

Although, the authors acknowledge the potential value in screening for a wider array of heavy metal contaminants, the study's focus was strategically confined to chromium (Cr), cadmium (Cd), iron (Fe), zinc (Zn), selenium (Se), and copper (Cu) strictly on the basis of budgetary limitations, which governed the extent of the analytical work and restricted the number of elements that could be reliably quantified within the available financial framework (Table 1).

Age range. Subject age range was 20-50 years, averaging 39.34 for Tehran and 39.96 for Firoozkooh. The frequency of people aged 40-50 years was the highest, equal to 24 people for Tehran and 31 people for Firoozkooh. Selected samples from both genders were similar in BMI (body mass index), age, smoking status, and years of regional presence. Age and body mass index (BMI) measurements were converted to standard deviation scores (SDS).

Gender of subjects. The potential gender effect on 6 metal elements (Se, Fe, Cu, Zn, Cd, Cr) individually and in dual combinations (iron/copper, iron/selenium, copper/zinc, copper/selenium, zinc/selenium, iron/zinc) on gut microbiota was assessed. For this, 23 men and 27 women for Tehran and 24 men and 26 women for Firoozkooh were studied. To avoid extending the length of the manuscript due to the journal’s standard, these results are not presented in a separate table or figure in this article.

Fecal sampling and DNA extraction. Stool samples were rapidly carried to the laboratory in cold chain storage and stored frozen until processing. DNA was extracted using QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Extracted DNA quality and quantity were analyzed by agarose gel electrophoresis and NanoDrop ND-8000 (Thermo Scientific, USA), respectively (2).

qPCR and bacterial load determination. The 16S rDNA gene, representing all bacteria, was used as the reference gene to normalize the data. The complete list of primer sequences for both the reference gene and the target bacterial groups is provided in Table 2. To determine the relative fold change of the target bacterial populations, we employed the 2^-ΔΔCt method, using the 'all bacteria' 16S rDNA gene (with primers listed in Table 2) as the normalizer.

Bacterial abundance was quantified by SYBR Green qPCR (LightCycler® 96 SW 1.1; Roche, Ger-

Table 1. Device reading parameters according to manufacturer's guidelines

Element/Device Conditions	Iron	Copper	Zinc	Selenium	Chromium	Cadmium
Wavelength (nm)	248.3	324.7	213.9	196	357.9	228.8
Bandwidth	0.3	1	1	2	0.5	1
Emission source	Halo cathode	Halo cathode	Halo cathode	Halo cathode	Halo cathode	Halo cathode
Measurement	flame	flame	Flame	graphite furnace	graphite furnace	graphite furnace
Halo-cathode current (mA)	8	5	3	5	6	3

Table 2. Nucleotide sequence of primers used to determine intestinal microbiota population

Bacterial target	Sequence (5'–3')	Amplicon	Size (bp)	Ref
All bacteria	F: TCCTACGGGAGGCAGCAGT	466	132	
	R: GGACTACCAGGGTATCTATCCTGTT			
Escherichia coli	F: TGAAACTAAAGGAATTGACG	155	136	(73)
	R: ACCATGCACCACCTGTC			
Bacteroidetes	F: CRAACAGGATTAGATACCCT	204	136	(72)
	R: GGTAAGGTTCCTCGCGTAT			
Lactobacillus acidophilus	F: TGGATGCCTTGGCACTAGGA	92	133	(70)
	R: AAATCTCCGGATCAAAGCTTACTTAT			
Bifidobacterium	F: GGGTGGTAATGCCGGATG	278	132	
	R: TAAGCCATGGACTTTCACACC			
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC	195	132	(70, 73)
	R: CTCTACGAGACTCAAGCTTGC			
Enterococcus faecalis	F: AACCTACCCATCAGAGGG	360	132	(71)
	R: GACGTTCAGTTACTAACG			
Clostridium clostridioforme	F: AATCTTGATTGACTGAGTGGCGGAC	148	132	(70)
	R: CCATCTCACACTACCGGAGTTTTTC			
	R: CGGCACCGAAGAGCAAT			
Faecalibacterium prausnitzii	F: AGAGTTTGATCATGGCTCAG	191	135	(72)
	R: GGTTACCTTGTTACGACTT			
Akkermansia muciniphila	F: CAGCACGTGAAGGTGGGGAC	327	74	(74)
	R: CCTTGCGGTTGGCTTCAGAT			

many). All reactions were performed in triplicate in 25 µL volumes. Primers targeting 16S rDNA for individual bacterial species and total bacterial load were used in a final concentration of 20 µL (50).

Standard curves were generated using dilutions of DNA extracted from a standard *E. coli* strain. The resulting Cq values were converted to DNA concentration based on the genome size and 16S rRNA gene copy number of the target bacterium, enabling calculation of colony-forming units (CFU) (33, 36).

Total bacterial load was quantified by real-time PCR following standard procedures (e.g., Nadkarni et al.) (29). Microbiota composition at the order, family, and genus levels was analyzed using the primers listed in Table 2. Unpolluted individuals served as the control group, and the universal "all bacteria" gene was used as the reference. Each 20 µL PCR reaction contained 12 µL Master Mix, 0.5 µL primer, 3 µL DNA, and 4 µL molecular grade water. The cycling conditions were: initial denaturation at 95°C for 15 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for one minute. Data were analyzed using the 2^-ΔΔCt method, with the universal bacterial gene as the normalizer.

Regarding water, molecular grade water (nuclease-free) was employed for all reaction preparations. This is standard practice in qPCR to prevent nucleic acid degradation and ensure the reliability and reproducibility of the amplification results.

Total viable counts and fecal coliform enumeration

were not performed in this study because our primary objective was to achieve species-specific absolute quantification of seven functionally distinct bacterial taxa using targeted qPCR, which provides higher resolution and specificity for detecting subtle changes in key microbial populations than conventional culture-based methods.

This method was calculated from the formula RQ= 2^-ΔΔCt and Ct = (Target Ct- Ref Ct (all bacteria)) IBD - (Target Ct- Ref Ct (all bacteria)) control ΔΔ. The RQ=2^-ΔΔCt number was used as the fold change number for group comparisons. The control group fold change in expression was one, and fold change in expression was calculated for other groups compared to the control group.

As expected, we targeted bacterial DNA rather than RNA. Our methodology employed quantitative PCR (qPCR) directly on extracted bacterial genomic DNA to quantify 16S rDNA gene copies, which serves as a reliable marker for bacterial abundance and eliminates the need for reverse transcription steps typically required for gene expression analyses.

In our study, a targeted quantitative PCR (qPCR) approach was deliberately employed to achieve high-resolution, absolute quantification of seven specific, functionally critical bacterial taxa (*Escherichia coli*, *Bacteroides fragilis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Clostridium clostridioforme*, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*), selected a priori based on their well-documented and contrasting roles in gut barrier function, immune modulation, and specific susceptibility or resistance to heavy metal toxicity (33, 34, 37, 39, 40, 43, 45). Consequently, while we acknowledge that this hypothesis-driven method does not provide the broad, untargeted diversity assessment offered by 16S rRNA amplicon sequencing, it provides a robust and statistically powerful test of our predefined hypotheses regarding specific microbial biomarkers, and we have therefore carefully framed our conclusions to reflect these specific quantitative changes in key bacterial populations rather than making unsupported claims about overall community structure.

Standard curve. Bacterial abundance was calculated as previously described. The standard curve was prepared using serial dilutions of DNA from standard strain *E. coli*. This curve allows DNA concentration calculation for each bacterium from fecal samples. The standard curve is graphically represented as a semi-log regression line plot of CT value vs. log of DNA concentration.

Statistical analysis. SPSS software version 26, t-tests, and Pearson's correlation coefficients were utilized. Characteristics that were descriptively analyzed included age and sex. T-tests were used for six heavy metal elements in two independent groups living in polluted and non-polluted areas. If significance is $p < 0.05$, each element's correlation in people in these two regions would be reported as significant.

In our study, the application of t-tests and Pearson correlation was strategically confined to testing a limited set of pre-specified hypotheses derived directly from our primary objective—specifically, to compare predefined bacterial targets and heavy metal concentrations between two distinct geographical locations (polluted vs. non-polluted areas)—thereby reducing the risk of Type I error inflation that typically necessitates corrections for multiple comparisons in exploratory, hypothesis-generating research.

Ethical statement. All experiments containing human materials were performed according to relevant guidelines and regulations of the granting institute, Pasteur Institute of Iran, which issued ethical code IR.PII.REC.1399.097. All human subjects were fully informed of questionnaires and willingly signed written informed consent forms.

RESULTS

Heavy metal determination results. The result presented in Table 3 explicitly states that chromium in the non-polluted area (Firoozkooh) was "extremely lower" than in the polluted area (Tehran). For Firoozkooh (as the Control) the finding is 0.15 ± 0.060.15 ± 0.06 µg/dl, whereas, the amount for Tehran (as the Test) is : 1.03 ± 0.841.03 ± 0.84 µg/dl, This represents an almost 7-fold increase in the polluted region. This is a drastic environmental and biological difference.

Iron levels in serum samples from Firoozkooh were slightly lower than in Tehran. A slight difference was observed for cadmium between polluted and non-polluted regions. Zinc concentration was not different between study regions. Copper and selenium were slightly higher in Firoozkooh sera compared to Tehran. Based on the negative correlation coefficient, we concluded that with iron increases, copper amounts

Table 3. Average and standard deviation of heavy metal concentration in study areas

Variables	Firoozkooh (control)	Tehran (test)
Zinc µg⁄ml	14/0 ± 90/0	16/0 ± 89/0
Copper µg⁄ml	19/0 ±04/1	17/0 ± 99/0
Selenium ng⁄ml	23/9 ±80/129	56/10 ± 70/127
Iron µg⁄dl	36/22 ± 78/80	62/29 ± 70/95
Chromium µg⁄dl	06/0 ± 15/0	84/0 ± 03/1
Cadmium µg⁄dl	03/0 ± 09/0	05/0 ± 13/0

decrease, and the opposite was also observed, meaning iron decreases resulted in copper increases. Results are reflected in Table 3.

According to Table 4, average chromium is significant at 95% confidence interval with significance level sig= 0.00≤0.05. The average chromium in Tehran and Firoozkooh is different. Considering the positive t sign, chromium amount in Tehran is higher than Firoozkooh. Average cadmium is significantly different at 95% confidence interval with significance level sig= 0.002≤0.05. Therefore, average cadmium in Tehran and Firoozkooh is different, and considering the positive t sign, cadmium amount in Tehran is higher than Firoozkooh. For mean iron levels, there was a significant difference at 95% confidence interval with significance level 0.05≤0.005=sig. This means mean iron in Tehran and Firoozkooh is different, and considering the positive t sign, iron amount in Tehran is higher than Firoozkooh.

The last row of the table clearly confirms the statistically significant finding for cadmium, showing a calculated t-value of 3.259 and a significance level of p = 0.002, which unequivocally supports the text's conclusion that cadmium levels are higher in Tehran.

Gender and age of the subjects. The concentration levels of six metal elements (Se, Fe, Cu, Zn, Cd, Cr) in 100 subjects of male and female with age groups between 20 and 50 were analyzed. However, there is a considerable relationship between chromium amount in Tehran people's sera and their age, whereby chromium amount decreases when age increases. Regarding gender, there were no significant differences in individual levels of cadmium, chromium, iron, zinc, copper, or selenium. Similarly, there was no significant relationship between dual assessment of iron/copper, iron/selenium, copper/zinc, copper/selenium, zinc/selenium, iron/zinc elements in Tehran or Firoozkooh people's sera. According to statistical analysis, there is no correlation between various element levels in samples received and individuals' gender. Based on Pearson correlation analysis, a statistically significant correlation was found between serum iron and copper in Firoozkooh individuals (P<0.05, r=0.45, N=50) and between chromium in Tehran individuals and their age (P<0.05, r=0.003, N=25). This means that with increasing iron, copper levels in Firoozkooh individuals decrease, and chromium levels in Tehran individuals decrease with increasing age. Pearson's correlation coefficient analysis showed that chromium levels decrease with increasing age. Also, the reverse was observed, meaning chromium amount increases if age lowers. Additionally, selenium, iron, zinc, and copper concentration levels in Firoozkooh people's sera do not show significant relationships with age. Chromium amount decreases with age, and reversely, increased copper is shown in lower age subjects, regardless of region. Unlike chromium, there was no significant relationship between age and serum levels of cadmium, iron, copper, zinc, and selenium in Tehran tested as the polluted area. Unlike chromium, there is no significant relationship between age and serum levels of cadmium, iron, zinc, copper, and selenium in Tehran and Firoozkooh. Table 5 confirms that se-

Table 4. T-test in two independent groups from Tehran and Firoozkooh

Standard error difference	Mean difference	df	T	Sig (p-value)	Samples	Metals
0/03154	-0/0036	98	-0/114	0/90	50	zinc
0/03670	-0/5020	98	-1/368	0/175	50	copper
1/98448	-2/10	98	-1/058	0/293	50	selenium
5/24957	14/92	98	2/842	0/005	50	iron
0/17027	0/87784	24/263	5/156	0/00	25	chromium
0/012	0/40	48	3/259	0/002	25	cadmium

lenium, iron, zinc, and copper concentration levels in Tehran people's sera have little or no correlation with age.

Table 6, indicates selenium, iron, zinc, and copper concentration levels in Firoozkooh people's sera. According to the Table 6, the correlation coefficients indeed show: Selenium (r = -0.020, p = 0.890) and iron (r = -0.097, p = 0.505): negative but non-significant relationships with age. Also, Zinc (r = 0.105, p = 0.469) and copper (r = 0.102, p = 0.480): positive but non-significant relationships with age. Therefore, all p-values exceed the conventional significance threshold (p > 0.05), confirming that none of these metals demonstrate statistically significant associations with age in our study population: therefore, these metals were not affected by age.

Microbiome pattern in polluted and unpolluted regions. Based on our results, *Escherichia coli* amounts in polluted area intestines are not significantly different from those living in non-polluted areas (Fig. 1). Similar results occurred with *Bifidobacterium longum* (Fig. 4). Whereas, *Bacteroides fragilis*, amounts (Fig. 3) in polluted area intestines are significantly higher than those living in non-polluted areas. Expectedly, yet interestingly, amounts of four other bacteria—*Lactobacillus acidophilus* (Fig. 2), *Clostridium clostridioforme* (Fig. 5), *Akkermansia muciniphila* (Fig. 6), *Faecalibacterium prausnitzii* (Fig. 7)—existing in polluted area intestines compared to those living in non-polluted areas have significantly decreased and in some cases reached half.

Overall, the results (Fig. 8) demonstrated a correlation pattern between heavy metals and some of humans' normal flora bacteria.

DISCUSSION

Heavy metals play various roles in biological systems, including immune homeostasis and intestinal epithelial barrier (51, 52). The interaction between gut microbiota and heavy metals can be either toxic or beneficial to the body according to concentrations, different valence states, and forms of the same heavy metal (53). Microflora helps maintain human health by detoxifying and removing toxic compounds from the intestine (54). *Lactobacillus acidophilus* and *Bifidobacterium longum* were the most important species studied, followed by *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* (11). Nevertheless, all effects of heavy metal toxicity on mammalian intestinal microbiota remain unclear (55). Intestinal microbiota may function as a physical barrier to heavy metal absorption and by changing

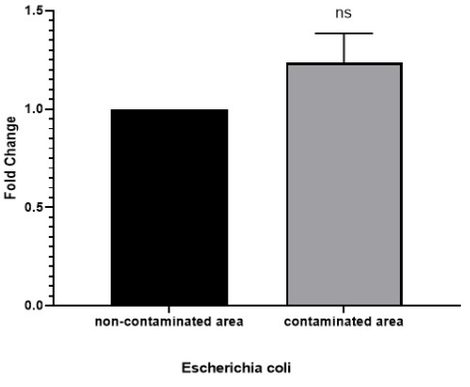

Fig. 1. *Escherichia coli* * Data are presented as mean ± SD for each group (*p-values < 0.05, ** p-values < 0.01, *** p-values < 0.001, and **** p-values < 0.0001); ns: not significant.

Table 5. Iron, zinc, copper, and selenium concentration and age in Tehran

Iron, Zinc, Copper & Selenium vs age		Age	Fe	Zn	Cu	Se
age	Pearson Correlation	1	.075	-.195	-.069	-.007
	Sig. (2-tailed)		.605	.174	.635	.960
	N	50	50	50	50	50

Table 6. Selenium, iron, zinc and copper concentrations and age in Firoozkooh

Selenium, Iron, Zinc & Copper vs age		Age	Se	Fe	Zn	Cu
age	Pearson Correlation	1	-.020	-.097	.105	.102
	Sig. (2-tailed)		.890	.505	.469	.480
	N	50	50	50	50	50

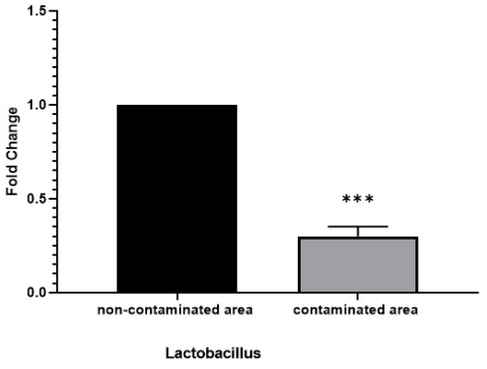

Fig. 2. *Lactobacillus acidophilus*

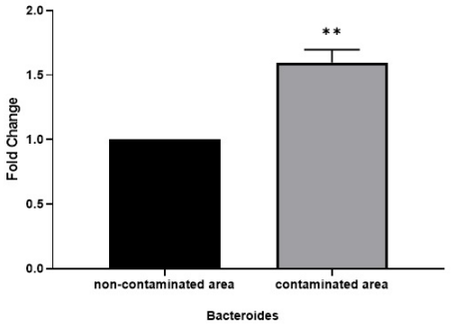

Fig. 3. *Bacteroides fragilis*

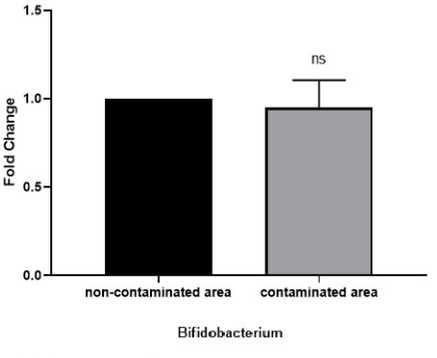

Fig. 4. *Bifidobacterium longum*

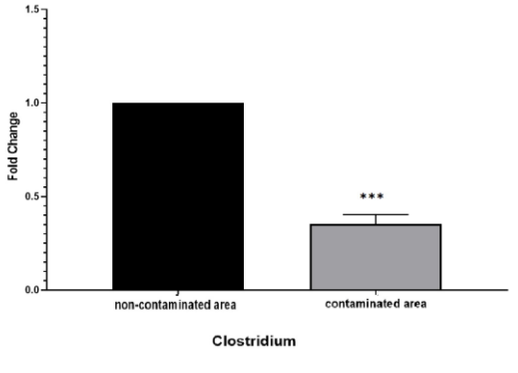

Fig. 5. *Clostridium clostridioforme*

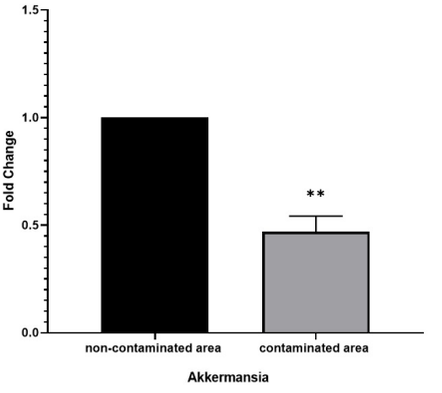

Fig. 6. *Akkermansia muciniphila*

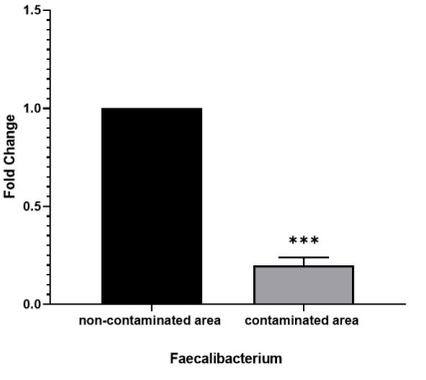

Fig. 7. *Faecalibacterium prausnitzii*

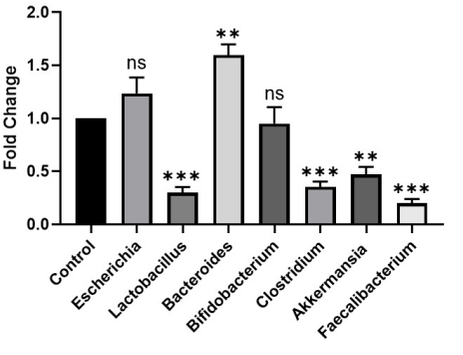

Fig. 8. Collective results

pH, oxidative balance, and detoxification concentration changes heavy metal absorption and metabolism (56). This may occur through modulation of enzymes or proteins involved in heavy metal metabolism (3). Some bacteria including probiotics have been shown to reduce heavy metal absorption in the intestine through heavy metal detoxification, changing transporter protein expression, and maintaining intestinal barrier function (57). Thus, probiotic bacteria have

been used as medicinal supplements to reduce heavy metal harmful effects (43).

The first bacteria in our research was *Escherichia coli*, showing that its amount in polluted area intestines is not significantly different from that of people living in non-polluted areas. Our finding was almost in compliance with results obtained by Ashley S. Tseng et al. (60). *Bacteroides fragilis* is one of the important bacterial species in the human gut microbiome, playing roles in food digestion and providing part of the body's needed energy (61). Our research, probably for the first time, has shown that *Bacteroides fragilis* in polluted area intestines are significantly higher compared to people in non-polluted locations. Therefore, it might be concluded that the presence of heavy metals in polluted areas correlates with *Bacteroides fragilis* levels with the same species in people's normal flora. *Bifidobacterium longum* is a gram-positive bacterium making up mammalian flora, known as probiotic playing significant roles in digestive disease treatment such as inflammatory bowel disease (62). We showed that *Bifidobacterium longum* amounts in polluted area intestines are not significantly different from people living in unpolluted areas. However, it has previously been shown that cadmium increases and *Bifidobacterium longum* increase intestinal inflammation (43). Based on our findings, it was expected to witness that *Clostridium, Akkermansia, Faecalibacterium,* and *Lactobacillus acidophilus* were less dominant in polluted area intestines compared to non-polluted area people, accounting for even less than half. This comparative finding was also very likely the first to be reported. According to our research, Heavy metal element presence in polluted areas was associated with marked differences in *Clostridium* amounts in normal people's intestinal flora compared to those living in non-polluted areas, resulting in markedly reduced *Clostridium clostridioforme* levels in polluted area intestines. Our finding regarding *Clostridium clostridioforme* was slightly similar to results obtained years ago in another study (63). In other studies regarding five heavy metals, *Akkermansia muciniphila* was significantly reduced regardless of nutritional regime. *Akkermansia muciniphila* abundance was opposite to cadmium in feces (25). Some heavy metals such as cadmium were associated with reduced *Akkermansia muciniphila* abundance in gut microbiomes. These changes may be correlated with heavy metal toxicity effects on this bacterium and in intestinal environments, as well as disrupting balance and abundance of *Akkermansia muciniphila* on intestinal microbiome health and immune systems (64). On the other hand, its reduction can probably indicate an association with disease risks caused by heavy metal exposure (65). Expectedly, our research showed that *Akkermansia muciniphila* amounts in polluted area intestines significantly decreased compared to those living in non-polluted areas. For *Faecalibacterium,* some heavy metals such as cadmium may correlate with changes in this type of bacteria in human gut microbiomes (66). Our results showed that *Faecalibacterium prausnitzii* in polluted area intestines significantly decreased compared to people living in unpolluted areas and reached less than half the bacterial population compared to unpolluted areas. *Lactobacillus acidophilus* has been shown to bind to heavy metals, which may contribute to reduced toxic effects by binding to some heavy metals such as cadmium (67), which is near to our finding. Based on our results, *Lactobacillus acidophilus* amounts in polluted area intestines significantly decreased compared to people living in unpolluted areas and reached less than half, which is consistent with another study (68). Consequently, we observed decreases in beneficial bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium*, which may have implications for the various benefits these bacteria provide in normal conditions (69). According to the current study, average heavy metal concentrations did not differ significantly between men and women living in Tehran and Firoozkooh. This observed correlation between specific bacterial populations and heavy metal levels raises the intriguing possibility that shifts the abundance of sentinel taxa like *Lactobacillus, Faecalibacterium,* or *Akkermansia.* This could potentially serve as a novel, non-invasive biomarker for assessing chronic, low-level environmental heavy metal exposure in human populations. Also, exposure to heavy metal mixtures may be needed as they might have additive, synergistic, or antagonistic effects on gut microbiota, which is a matter of importance for future research. As future recommendations, it is highly significant to raise awareness against heavy metal-induced toxicity and provide concerned people with guidelines for the benefit of dual human-environment relationships. Consequently, other toxic heavy metals (THMs) not tested in this research, such as arsenic (As), mercury (Hg), and lead (Pb) can cause damage to multiple organs even

at low exposure levels. Additionally, in future it is essential to decrease environmental pollutant levels and their effects on microbiota with several strategies including green belts in cities, promotion of public transportation, reduction of industrial emissions, and most importantly raising public awareness.

ACKNOWLEDGEMENTS

The authors would like to thank the laboratory staff of Microbiology Research Center (MRC), Department of Biochemistry of Pasteur Institute of Iran, Department of Bacteriology, Pasteur Institute of Iran, and finally Department of Mycobacteriology and Pulmonary Research of Pasteur Institute of Iran. This study was funded by Pasteur Institute of Iran (Grant No.: 1809).

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