

Gut microbiota shift of spangled emperor under pollution stress

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Abstract

Hepatic antioxidant enzymes as oxidative stress biomarkers were investigated and correlated with the identified dominant gut microbial phyla. The results showed that while the antioxidant enzymes, Superoxide Dismutase (SOD), and Catalase (CAT) lev-

els were reduced in the polluted PO site, significant elevation (* $P \geq 0.05$) was observed at the clean reference CR site indicating negative correlation to pollution stress. On the other hand, among five significant bacterial genera, *Lactobacillus* and *Vagococcus* showed a positive relationship to the oxidative pollution stress between PO and CR sites. Diversity and bacterial richness had been observed in the PO site compared to the CR site. As a result, 429,346 sequences were obtained from the pooling of 20 samples identified into 10 phyla and 79 genera in which *Firmicutes* was dominant in both PO and CR sites. The number of common OTUs was 221 for both CR and PO samples. The results revealed that under the stressed environmental state, the homo-lactic *Vagococcus* genus is dominant over the hetero-lactic *Lactobacillus*, which uses less energy in the derived process.

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Introduction

Industrial and urban pollution stress influence gut microbial community which could affect the immunity of the species, and the change in the gut microbial community depends on the surrounding environment and its equilibrium with the species.¹ Compared to other mammals, the gut microbial composition of fish could shift quickly in response to changes in the aquatic environment.² This change in the surrounding environment leads to a physiological response, which increases the biological production of reactive oxygen species (ROS) leading to the development of antioxidant defense systems to overwhelm the encountered ecological stress.³ The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity of these agents. Ecological factors such as pollution stress would affect the activities of significant biological biomarkers, the antioxidant enzymes.⁴ These enzymes include Catalase (CAT) (EC1.11.1.16) reducing the H_2O_2 to H_2O , Superoxide Dismutase (SOD) (EC 1.15.1.1) converting O_2^- to H_2O_2 , Glutathione reductase (GSH) (EC 1.8.1.7) catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH) and Malondialdehyde (MDA) as oxidative stress markers.⁵

The host's dietary factors and the surrounding environment influences the activities of the antioxidant enzymes and the gut microbial community.⁶ Several studies about the responses of antioxidant fish enzymes to pollutants enhancing ROS production have been reported, although often they have been inconclusive and shown vast individual differences.⁷ Significant changes have been studied in marine fish by workers studying several molecular biomarkers, such as the glutathione redox status, the concentration of thiobarbituric acid, the appearance of new GST isoenzymes or the presence of new SOD forms of higher electrophoretic mobility.⁸

Numerous industries are in and around the city of Jeddah coast

due to its strategic location as one of the shipping industry and urbanization centers of Saudi Arabia. Still, at the same time these industries are a source of toxic chemicals and effluents.⁹ The southern corniche area of Jeddah receives through Al-Kumra effluent the equivalent of 300,000 m³ of semi-treated sewage. Since 2001, the same volume of municipal wastewater is disposed from underwater diffuser situated at about 3 km south of the old effluent.¹⁰ The dumping of pollutants from point and diffuse source, and in cities where there is not a system of efficient sewage treatment has affected aquatic habitat. The domestic sewage is a major source of pollution, stimulating growth of bacteria and other micro-organisms, including those of fecal origin. As the sewage of domestic origin are characterized by the presence of fecal material,¹¹ can be considered that this source of pollution most influenced the microbiota of aquatic habitat.

Many species of marine benthic fish are shown to reflect environmental status. Fish could, therefore, be possibly used as indicators in areas affected by human activities to describe the state of the environment. One such habitat transition is the migration of wild fish from clean to a polluted marine aqua system. Very little is known about the adaptation and structure of gut microbiota among fish species from oxidatively stressed environments. In this study, we characterized the composition of the gut microbiome of the spangled emperor (*Lethrinus nebulosus*), which is abundant in the Red Sea coastal of Jeddah City in Saudi Arabia. We investigated microbiome correlation with liver antioxidant enzymes as oxidative stress biomarkers in pollution stress conditions.

Materials and Methods

Sample collection and preparation

Twenty fish of the spangled emperor (*Lethrinus nebulosus*) were sampled during the summer of 2018 from a Polluted (PO) area near the location 21°16'14.2"N, 39°07'22.4"E, where an abundance of sewage was released, and a Clean Reference (CR) area near the site 21°12'24.3"N, 39°09'57.9"E in the Red Sea coastal region of Saudi Arabia (Figure 1). Jeddah city seawater annual temperature ranged from 25 to 29°C. Only fish over 20 cm in length were sampled. Experimental fish were randomly harvested, placed in clean, sealable plastic bags, transferred to the laboratory on ice, and stored at 4°C for processing. Fishing was conducted according to the guidelines (Decree No. 21911, 7 November 1988) of the Ministry of Agriculture, Kingdom of Saudi Arabia. The Royal Coast Guard of the Kingdom of Saudi Arabia fish sampling authorized fish sampling by (Decree No. 2, 3 February 1990). All procedures performed at King Abdulaziz University abided by Royal Decree No. M/59, August 24, 2010, entitled "Research Ethics for Handling of Living Animals."

The fish were dissected on the same day, and the intestines were removed aseptically from the abdominal cavity of each fish. The contents were carefully collected and labeled according to the area of collection. Seawater was collected from three different sites using sterile glass vials. To avoid surface contamination, water samples were collected from 15–20 cm below the surface of the water. Water quality for both sites was assessed. The pH and Electrical Conductivity (EC) were determined in-situ electronically with appropriate meters. Standard laboratory methods were employed for the examination of water samples for the analysis of Total Solids (TS) and Total Dissolved Solids (TDS). Chemical Oxygen Demand (COD) was estimated by convectional reflux method, followed by colorimetric measurement at wavelength

435nm. Biochemical Oxygen Demand (BOD) was measured based on oxygen consumed in a 5 days test period (BOD₅) at 20°C after arrival of sample to the laboratory, and pH. Standard protocols were used to estimate all other parameters.¹² Statistical analysis such as mean, standard deviation and test of statistical significance, t-test were performed using MS Excel version 2013.

Biochemical analysis of fish liver

Aqueous liver extract in ice-cold saline was used for the estimation of antioxidant enzymes. Total proteins were determined by the method of Lowry *et al.*¹³ The assay of Glutathione Reductase (GSH) activity was determined by the method of Foyer and Halliwell,¹⁴ modified by Rao.¹⁵ In vitro activity of Catalase (CAT) was determined by the method of Aebi,¹⁶ Malondialdehyde (MDA) level was measured by the modified method of Kei¹⁷ and *in vitro* assay of Superoxide Dismutase (SOD) activity was estimated by the method of Beyer Jr and Fridovich.¹⁸ Activities differences in detoxifying enzymes between the control and the stressed groups were analyzed by Student's *t*-test (one-tailed) using Excel program, and the significant differences were indicated as **P* ≥ 0.05.

DNA extraction

The contents of the posterior portion of the intestine were transferred to a sterile 10 mL Falcon tube and kept at -20 for isolation of DNA. For 16S rRNA gene fragments, 50 mg of pellet per sample was used. The extraction of the genomic DNA from the fecal contents was performed using QIAamp DNA Stool Mini Kit (QIAGEN, Germany) conferring to the producer's instructions, and the Picogreen method was used to detect the DNA quantity using Victor 3 fluorometry (Invitrogen, cat. #P7589). The enriched fragments size of the PCR was checked using an Agilent Technologies 2100



Figure 1. Sampling sites of Polluted (PO) at Al-Badac area near the location 21°16'14.2"N, 39°07'22.4"E, where an abundance of sewage was released, and a clean reference (CR) at Al-Goza'in area near the site 21°12'24.3"N, 39°09'57.9"E on the Red Sea, southern coast of Jeddah, Saudi Arabia.

Bioanalyzer. The 16S rRNA gene sequence of PO and CR samples was achieved using by MacroGen Inc. (Seoul, Korea).

Amplicon library preparation

The PCR products were progressively diluted and quantified. Amplicon library construction was targeted using 16S rRNA primers (V3-V4), 341F: CCTACGGGCGWCA and 805R: GAC-TACHVGGGTATCTAATCC. For cluster generation, samples were sequenced on the MiSeq sequencing stage (Illumina, USA), ensuring the producer's procedure. Automated paired-end sequencing and cluster generation with dual index reads were performed. The spans of short reads were prolonged by finding the join between paired-end reads using the FLASH program. Sample's microbiota raw sequence reads have been registered to the database: Sequence Read Archive (SRA) of GenBank of NCBI. The accession number of SRA is SRP158594.

Quality control was performed for each sample sequence, and the non-specific reads, low-value data, and illusions were removed using QIIME program. Sequences were grouped to Operational Taxonomic Units (OTUs) using UCLUST program. OTUs were clustered with cutoff values of 97% similarity. To minimize error, Alpha diversity investigation was practiced examining species variety in a single sample by species coverage (Coverage), species richness (Chao), and species assortment (Shannon and Simpson's diversity indexes) using MOTHUR program. The number of the OTUs in bacterial groups was evaluated by the Chao richness index. Shannon's and Simpson indexes were practiced attaining the variety of the OTUs. Phylum and genus taxonomy levels were used to evaluate the community structure.

Results and Discussion

Disposing of pollutants into the seawater in cities where there is an oxidative pollution system of efficient sewage treatment has affected aquatic habitat. The growth of microorganisms is stimulated by domestic sewage. Water quality data from PO versus CR areas were compared. Both samples were assessed for pH, EC, TS, TDS, BOD, and COD, as summarized in Table 1. The samples from PO areas showed greater pollution than samples from CR areas owing to the higher COD of 65.70 ± 0.53 mg/L (* $P \geq 0.05$). The BOD of polluted water was not very high compared to the clean reference one, suggesting that the amount of biodegradable organic pollutant matter was low. The concentration of salt was similar between the two sample areas, indicating that pollution did not affect the salinity of the water. The TS content in samples from PO areas (58.06 ± 0.26 mg/L) was higher than that in samples from

CR area (41.8 ± 0.10 mg/L) owing to the presence of additional pollutants in the water (* $P \geq 0.05$). In PO samples, most of the pollutants were in the dissolved form, and the TS content the fraction of TDS was quite high (56.67 ± 0.06 mg/L), whereas the TSS was exceedingly low (1.49 ± 0.007 mg/L). Pollution did not appear to affect the pH of the water.

The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity of these agents. Pollution oxidative stress is toxic for fish, stimulated by the release of Reactive Oxygen Species (ROS). Collaborative action of antioxidant defense mechanisms equilibrates the levels of ROS. The removal of excessive harmful ROS induces the activities of antioxidant enzymes as potential indicators of oxidative stress. In the present study, the mean CAT and SOD activities of fish from CR site were appeared to be significantly higher (* $P \geq 0.05$) when compared to the mean activities in the fish from the PO site. GSH and MDA indicated non-significant ($P < 0.05$) activities between the PO and CR areas, as shown in Figure 2. The habitat modifications force the fish to develop many morphological and physiological adaptations to survive in stressed conditions. From the waste sites of several chemical industries in the river of Bernesga in Spain, the liver antioxidant enzymes of two fish species, *Rutilus arcasii* and *Gobio gobio* were studied by Almar *et al.*¹⁹ Animals from polluted sites exhibited diminished glutathione concentration, which is consistent with the current study. Comparative variations of glutathione and glutathione-dependent enzymes in both fish species indicate a diverse exposure to toxins.²⁰ The antioxidant enzymes activities of shorthorn sculpin (*Myoxocephalus scorpius*) were compared between polluted and clean ports²¹ and revealed that fish captured in the polluted wharf had amplified activity of CAT and GSH. Due to contamination by oils, phenols, ammonia, and Polycyclic Aromatic Hydrocarbons (PAHs) in the Yellow River in China, pollution-induced oxidative impairment in carp.²² While GSH and SOD were disturbed in all examined tissues, both intestinal and kidney tissues showed diminished CAT and GSH, and the same tissues indicated advanced MDA levels. This signifies that the insufficiency of antioxidant response could lead to oxidative damage. Assessments of water quality in a river in Amsterdam have been confined by traditional carp. Samples captured from the polluted area had amplified activity of GSH but not CAT and SOD.²³ In the present study, the imbalance of antioxidant enzyme activities among different organ tissues and their influence by certain toxicants makes them ineffective biomarkers in representing ecological pollution toxicity. Moreover, the diminished activities of hepatic antioxidant enzymes from CR and PO samples may indicate that when the antioxidant system was overwhelmed, an alternative biological mechanism takes place hindering antioxidant enzyme activity in the PO site. This mechanism

Table 1. Physico-chemical characteristics of sludge Polluted (PO) and Clean Reference (CR) seawater.

Parameters	PO	CR
COD (mg/L)	$65.70 \pm 0.53^*$	30.6 ± 0.69
BOD (mg/L)	14.38 ± 0.20	6.72 ± 0.30
Electrical conductivity (salinity; μ S)	428.6 ± 0.74	445.7 ± 0.20
Total solids (TS; mg/L)	$58.06 \pm 0.26^*$	41.8 ± 0.10
Total suspended solids (TSS; mg/L)	$1.49 \pm 0.007^*$	1.23 ± 0.016
Total dissolved solids (TDS; mg/L)	56.67 ± 0.06	40.6 ± 0.04
pH	5.39 ± 0.008	5.49 ± 0.008

Values are presented as means $\pm$ SE (n = 5). (* $P > 0.05$).

could be the change in the microbial community to overwhelm the pollution stress.

Metagenomic analysis advocates mutable microbiomes in PO vs CR fish gut. The fish intestine is the most organ that interrelates with the surrounding environment and is intricate in adaptations and stress replies. The various and immense population of microorganisms make the community of the intestinal microbiota.²⁴ Pooled fecal samples revealed a total of 429,346 sequences with the number of sequences 199,900 for clean reference area and 229,446 for the polluted area after filtering quality. Then all the sequences were collected sequences binned into 221 Operational Taxonomic Units (OTUs) for the CR and PO areas at the 97% sequence similarity value, respectively. The OTUs clustering read counts of the fecal samples

were 94,820 and 110,048 for CR and PO groups, respectively. The microbial community richness and diversity indicated 180 and 115 OTUs for the CR and PO groups, respectively. This indicated higher microbial diversity in the CR site than in the PO site.

Complexities of fish gut microbes from stress area PO compared with clean referenced CR site were assessed based on the indexes of alpha-diversity, Chao1, and Shannon. Species richness was assessed by the Chao1 index, and the species diversity was assessed by Shannon's index. The results indicated that PO samples had the major alpha-diversity indexes compared to the CR sample, as indicated in Table 2. Good's coverage results for the OTUs for the CR and PO groups were $99.9 \pm 0.09\%$ and $99.9 \pm 0.07\%$, respectively. These results indicated a nearly complete selection of the group in

Table 2. Alpha diversity indices of bacterial Community richness & diversity in fish fecal samples (n=2).

Sample	OTUs	Chao1	Shannon	Simpson	Goods Coverage
CR	180	185	1.800531	0.498269	0.999947269
PO	115	116	2.682847	0.664035	0.999981826

* OTUs: Operational Taxonomic Unit is an operational definition of a species or group of species often used when only DNA sequence data is available
 * Chao1: returns the Chao1 richness estimate for an OTU definition
 * Shannon: The Shannon index takes into account the number and evenness of species.
 * Simpson: The Simpson index represents the probability that two randomly selected individuals in the habitat will belong to the same species.
 * Goods Coverage: Coverage is calculated as $C=1-(s/n)$, where s is the number of unique OTUs and n is the number of individuals in the sample. This index gives a relative measure of how well the sample represents the larger environment.

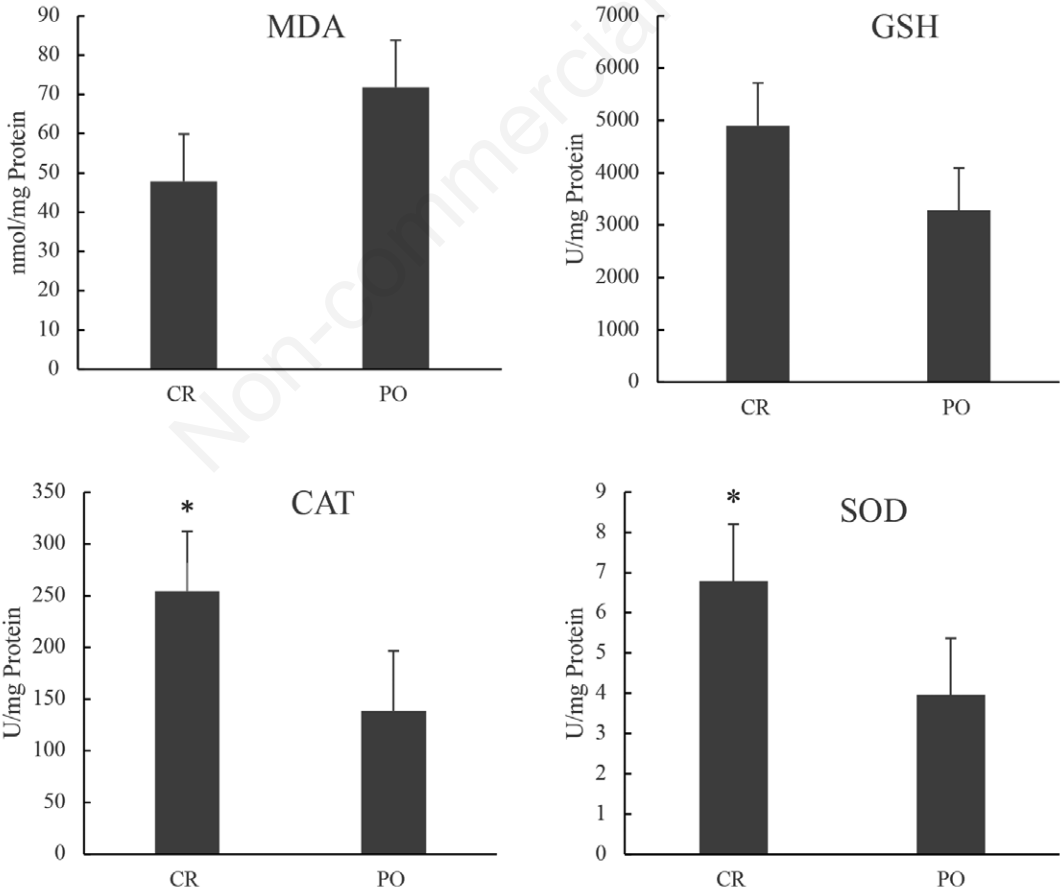


Figure 2. Comparison of antioxidant enzymes activities of fish between the polluted area (PO) and clean reference area (CR). Malondialdehyde (MDA), Catalase (CAT), Superoxide Dismutase (SOD), and Glutathione Reductase (GSH). Significant statistical differences between two groups (* $P \leq 0.05$) are indicated with asterisks.

either of the two sites. Also, the Chao1 was utmost in the PO group, Shannon and Coverage were highest in the PO group, and Simpson was last in the CR group. The analysis of OTUs indicates the sharing and variations of OTUs by the random fish species of polluted versus clean reference sites, which specifies the existence of some similar microbiota viability in the intestine of both samples because of the same living circumstances. At the same time, different organisms are dependent on the gut for different life customs and species.

In the current experiment, the metagenomic analysis indicated that 100% of the metagenomes were attributed to bacteria. The libraries of both samples were sufficient primarily to evaluate the phylotype richness and microbial community variety at the 97% similarity edge. The results identified 10 phyla among all sequences, and unspecified microbial phyla of the total sequences were only 0.1%. At the phylum level, the most dominant groups were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. The phyla abundance ratio (CR: PO) was assessed between the clean reference and polluted sites. The abundant ratios of phyla were ordered as *Firmicutes* (95:90) < *Bacteroidetes* (4:5) < *Proteobacteria* (1:4) < *Actinobacteria* (0.2:0.5). The most dominant phyla were *Firmicutes*, followed by *Bacteroidetes* showing almost even distribution between the PO and CR areas. The phyla *Proteobacteria* and *Actinobacteria* showed a higher abundance ratio in the polluted area than in the clean reference area; however, their overall abundance ratios are low and therefore, would not be represented as significant pollution biomarkers. Ye *et al.*²⁵ found that *Firmicutes* phylum was a dominant group in their study, which is consistent with the current experiment. *Bacteroidetes* and *Firmicutes* obtain nutrients from the diet as a part of the proteins and carbohydrates fermentation of the host's gut.²⁶ Other studies observed similar results for the intestine of other fish species. In grass carp, *Firmicutes* phylum was primary in the gut bacteria, and in adult zebrafish as a mutual adherent of the intestinal microbiota.²⁷ Therefore, the change in bacterial communi-

ties between PO and CR areas may be arisen in by progressed alterations that have caused the context of stressed settings.

Little is known about the effect of sewage pollution on fish gut microbiota in Saudi Arabia aquatic habitat. There is an obvious deficiency in the knowledge of the load and composition of bacteria present in fish culture ponds in Saudi Arabia. In every microbial habitat the nutritional competition between organisms plays an important role in influencing the composition of microflora. Some common bacterial microflora in water, such as *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Vibrio* spp., and *Myxobacteria* can cause major fish epizootics under stressful conditions.²⁸

At the species level, taxonomic abundance revealed significant count of homo-lactic *Vagococcus fessus* in the PO area versus hetero-lactic *Lactobacillus plantarum* in the clean reference area (Table 3). This significant count of lactic acid bacteria species explains the shift from hetero-lactic to homo-lactic as a consequence of pollution-induced stress. In total, 79 genera were identified from the sequence reads. Figure 3 indicated the highest abundance ratios of *Firmicutes* genera ordered as *Lactobacillus* (68:0) <

Table 3. Dominant lactic acid bacteria species abundance count of clean reference (CR) versus polluted (PO) areas.

Species	CR	PO
<i>Vagococcus fessus</i>	30	60,630
<i>Lactobacillus plantarum</i>	64,632	0
<i>Lactobacillus reuteri</i>	3	0
<i>Lactobacillus ruminis</i>	23	81
<i>Lactobacillus sake</i>	0	6

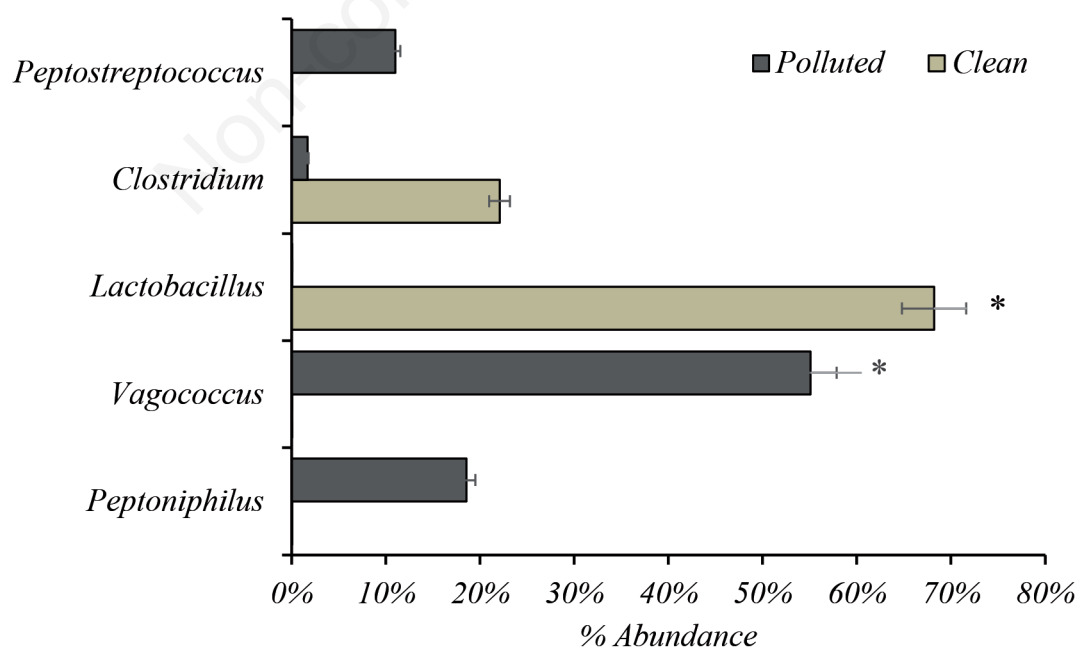


Figure 3. Metagenomic analysis of microbial genera isolated from polluted and clean reference areas in the Red Sea costal of Saudi Arabia. Asterisks indicated the highest % abundances.

Vagococcus (0:55) < *Clostridium* (22:2) < *Peptoniphilus* (0:19) < *Peptostreptococcus* (0:11). These genera were pollution sensitive for which *Lactobacillus* and *Clostridium* were representatives of the clean reference area, and *Vagococcus*, *Peptoniphilus*, and *Peptostreptococcus* were selective for the polluted area. Dominant Lactic Acid Bacteria (LAB) *Vagococcus* sp. takes over in the PO instead of the *Lactobacillus*, which was dominant in the CR sample. According to the hexose sugar metabolic pathway, LAB are conventionally categorized in two metabolic sub-groups included hetero- and homo-fermentatives.²⁹ Throughout the glycolysis metabolic pathway, homo-fermentative lactic acid bacteria metabolize hexoses to produce 2 mol of lactic acid and 2 mol of ATP out of 1 mol of hexose. On the other hand, the hetero-fermentative lactic acid bacteria use another metabolic pathway where 1 mol of hexose produced 1 mol lactic acid and 1 mol of ATP.³⁰ Homofermentative produces more lactic acid and energy from glucose than heterofermentatives where other waste products are produced instead of lactic acid as well as less energy is derived from the process.

A research study showed that the genera *Pseudomonas*, *Paracoccus*, *Flavobacterium*, *Providencia*, *Vagococcus*, *Bacillus*, and *Alcaligenes* were strongly related to the methomyl and carbofuran degrading strains using partial 16S rDNA sequence analysis.³¹ The biodegradation capability of isolated *Vagococcus* strains makes them aspirants for tender in bioremediation trials in pesticide polluted soils.³¹ Another study indicated categorized the bioflocculant from *Vagococcus* sp. The bioflocculant properties comprised thermostable and have robust flocculating activity in a varied array of pH with moderately low quantity prerequisites. It is expected that the *Vagococcus* genus would be exploited not only in the area of wastewater treatment, but also in drinking water dispensation, and food and fermentation industry because of its efficient flocculation and naivety towards humans and the environment.³² Another study indicated efficient budding for the bioremediation of chromium polluted sites by the *Vagococcus* sp.³³ These facts together reveal the observed results in the current study for the *Vagococcus* genus for taking over in harsh conditions. This indicated that the homofermentative *Vagococcus* dominance shift is a sensitive ecological pollution biomarker.

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