Test of polystyrene toxicity on Aeromonas-*sobria* and staphylococcus-*homini* bacteria

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Abstract. The spread of nano-plastic particles arising from the decomposition of plastic has become a global problem because it poses a threat to primary products in food chains, and thus threatens the entire system. We tested the apparent effects on the metabolism of Gram-negative and Gram-positive bacteria after exposure to nano-plastics (nano-cpolystyrene) by using carbon as an energy source. After diagnosis bacteria samples and then exposed to nanopolystyrene solution with different concentrations (200, 500 and 1000) ppm. Biology Eco Microplate (BEMP) was used to determine changes in bacteria after uptake of a carbon source. Average well-color development (AWCD) was used for data analysis, the result showed there are increase in size of bacteria and in absorption of carbon sources.

1 Introduction

Plastic is used in various fields of life because of the properties it contains. It may turn into microplastic as a result of many natural environmental processes, such as heat from sunlight and the movement of water waves. Microplastics are divided into primary and secondary depending on the bumps on their surface. These materials are found in water and sediment, as mentioned. Many studies have shown its inhibitory role for organisms that live in most environments because of its random entry with food into the body of living organisms, and sometimes it is considered a carrier of other pollutants such as some organic materials and antibiotics, so its effect related to the size, concentration, and the amount of substances attached to its surface, so it may move through the food chain and accumulate in members of living organisms [1]. The interaction of pollutants with microplastics increases their toxicity and makes their treatment more difficult because they can travel by land and sea and may also be transmitted by air. When living organisms are exposed to them, they may cause physiological, biological and molecular damage [2]. The presence of microplastics in the soil leads to its deterioration as a result of the impact on the organisms that live in the soil and for which fertility and aeration are provided [3,4]. Recent studies have shown have reached to the Tibetan Plateau in the third pole Where studies showed high concentrations of these pollutants in the living and non-living components, so that they were more when compared to the concentrations of the ocean. The reason for this increase was attributed to the tourists in that region, in addition to the air transport of these pollutants [5] Numerous studies indicate

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the arrival of microplastics to agricultural lands, where their estimates reached (8.2 × 1010 to 1.29 × 1015 microplastics/year) through biosolids and wastewater, more than 60 studies showed the huge quantities of these materials that are present in sewage sludge in variations across countries of the world [6]. Microplastics are available in fresh water, lakes and ponds, and their abundance increases in rivers, especially in locations that are close to residential areas. Therefore, the effect of agricultural lands increases on increasing the concentrations of microplastics 7 Due to its small size, which does not exceed 5 micrometers, it enters through filters in wastewater treatment A diversity of some types of pathogenic bacteria was found, forming biofilms on the surfaces of microplastic particles, after examining many wastewater treatment works, especially during the incubation period of the treated water [8, 9] These pollutants may escape with the bacteria attached to their surfaces to healthy drinking water and cause many negative effects on the environment in general and on humans in particular, such as pneumonia, meningitis and skin infections [10]. Many plants can filter microplastic particles, especially polystyrene (Zostera marina), and thus reduce their intensity in the marine environment [11]. Microplastics include many types, including (polypropylene, polyethylene, polyethylene terephthalate,polyvinyl chloride, and polystyrene) [12] and polystyrene is one of the common types in most studies, which has clear negative effects on biological communities , due to its relationship with climate change, as it had a negative impact combined with carbon dioxide on the metabolic activities of bacteria, especially in the metabolism of amino acids in the soil [13]. And showed its effect on microalgal-bacterial granular sludge (MBGS) in wastewater with a size (5 μm, and 10 μm), where it reduced the diversity of all species [14]. Types of bacteria, such as Proteobacteria and Bacteroidetes, showed toxic effects due to the presence of polystyrene in concentrations (10 and 200 μm), as it affected (carbon fixation, glycolysis, tricarboxylic acid cycle, and glycan biosynthesis and metabolism) [15]. The protein concentration and esterase activity decreased in Bacillus cereus CH6 bacteria due to exposure to polystyrene microplastics, although the bacteria were considered polystyrene decomposers, but it caused adverse results on their metabolism [16]. Also, polystyrene was toxic to Gram-negative Escherichia coli and Gram-positive Bacillus cereus [17]. Therefore, the aim of the study was to evaluate the change that gram-negative and gram-positive bacteria will undergo after exposure to microplastics, and what is the effect of this change on carbon consumption?

2 Material and methods

Collect the samples:

Colonies of pure cultures were identified by Morphological and Cultural Characteristic method, Vitek 2GN System Method (using GM-ID cards of VITEK2) by Al-Amin Laboratory in Najaf.

Preparation Stuck polystyrene (PS)

1- 0.1 g of PS pellets (99.99 purity) and 4 g of ethyl acetate were weighed and placed in a capped vial with a magnetic stir bar.

2- This vial was left on a magnetic stir plate set at 40 °C and 500 rpm for 2 h until the PS was completely solubilized.

4- Using a glass Pasteur pipette, the ethyl acetate mixture was then slowly dripped into a 100 mL sized beaker with 50 mL of ethanol being stirred at 400 rpm.

5- The mixture was transferred into a Falcon tube and centrifuged at 4000 rpm for 10 min.

6- The effluent was placed in a hazardous waste container and the 40 mL of dissolved water was poured into the Falcon tube and vortexes to wash the particles before being centrifuged again. This washing step was repeated two more times. Because some PS is lost in the washing process, 7- the final concentration was determined by drying 1 mL of the

mixture in three pre-weighed vials in a vacuum oven at 60 °C. The average of the difference before and after drying was used to adjust the concentration of PS in the dissolved water to 1 mg/ml.

8- Then (0.036 g) was weighed from the banned polystyrene and placed in a beaker, and distilled water was added to it to complete the volume to 50 ml.

9- Then, the sample was placed in an ultrasound device for two hours until the sample was completely dissolved, after which five dilutions through Take (1.2.4.8) ml and complete the volume for each one to 100 ml to measure the absorbance to make the standard curve and the straight line equation to measure the unknown concentrations of the water samples [18].

2.1 Test of polystyrene toxicity on bacteria

After diagnosing the bacteria with Vitek 2GN, they were cultivated for the two types (*Aeromonas sobria* and *staphylococcus homini*) in petri dishes Fig 1, then 6 holes were placed in each dish, then polystyrene was added, which was diluted in concentrations (0,5ppm, 1ppm, 2ppm, 4ppm, 200 ppm, 400ppm, 600ppm, 800ppm, 1000ppm, 2000ppm), and the volume of each concentration was completed to 10 ml, finally placed in incubator with temperature 121°C. The experiment was repeated by placing concentrations of polystyrene (200ppm, 500ppm, 1000ppm) in tubes of 10 ml capacity, and completing the volume to 10 ml with distilled water, then bacteria were added to each tube and for both types to form a suspension. It was shaken well, then placed in the incubator for an hour at a temperature of 37C to activate and stabilize the bacteria, then samples were taken from each tube and cultured on a plate containing a nutrient medium and incubated at a temperature of 37Cc to make sure whether the polystyrene affects the bacteria or not.

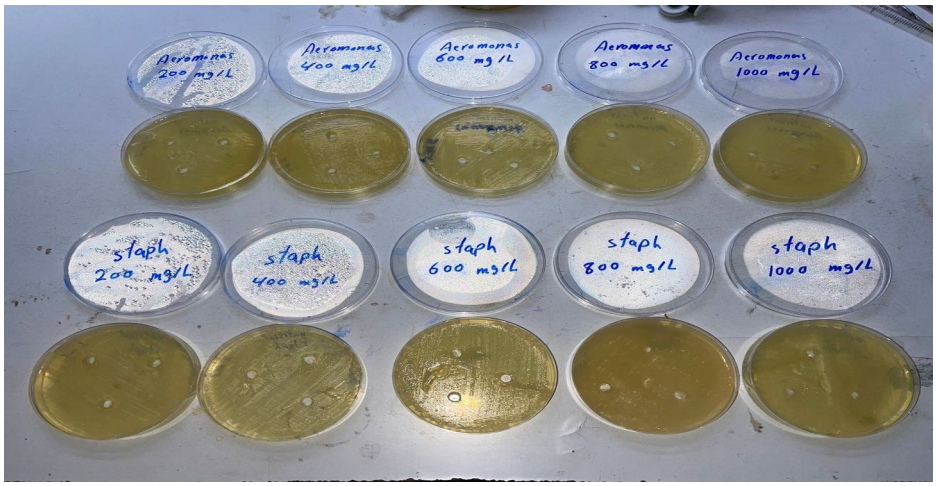

Fig. 1. Exposure *Aeromonas sobria* and *staphylococcous homini* to micro polystyrene.

2.2 Carbon source consumption test by Ecomicroplate

After making a suspension of bacteria with different concentrations of polystyrene (200ppm, 500ppm, 1000ppm) and one to distilled water, 75 microns were taken from each concentration by a micropipette and added to each hole in ecomicroplate Fig 2 and incubated in the incubator at 37°C and 121 lbs pressure.

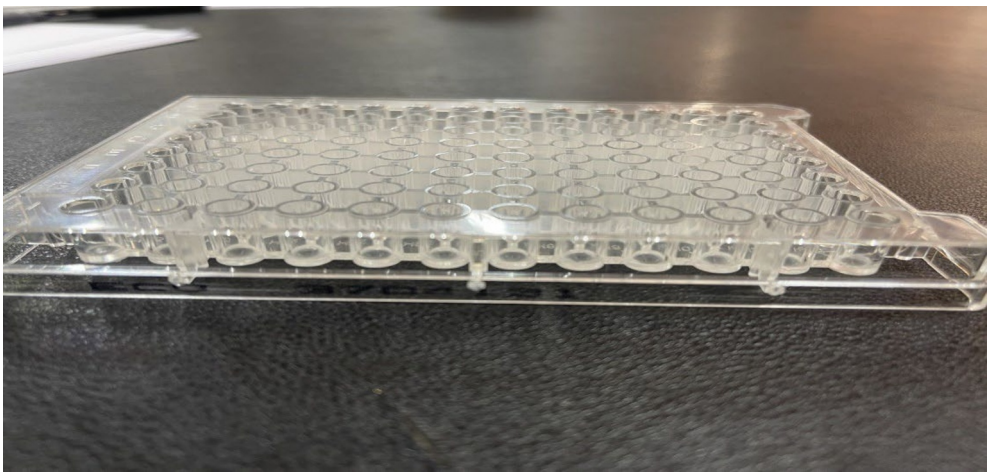

Fig. 2. Ecomicroplate.

2.3 Preparation samples to scan electron microscopy (SEM) technique

Samples were prepared for examination by (SEM), where the highest concentration of polystyrene (1000 ppm) and distilled water were prepared as a control in a 10 ml glass tube then bacteria were added to them, after that incubated for 24 hours with temperature 37^{o}C, finally planted in Petri dishes containing a nutrient agar and return it to incubation for 24 hours under the same pressure and temperature conditions before sending it for examination.

2.4 Statistical Analysis

For statistical analysis SPSS-24 software was used to test our hypothesis by applied ANOVA-Test, Pearson's correlation coefficient, and T-test. P values ≤ 0.05 significant, ≤ 0.01 high significant

3 Result and discussion

3.1 Diagnosis of *Aeromonas* and *Staphylococcus*

The bacteria were diagnosed using Vitek 2GN system, and the results showed the following species (*Klebsiella pneumonia, Acinetobacter baumannii, Aeromonas sobria, staphylococcus hominis, E-coli*). Only two species were selected, one of which was (G+ ve) including (*staphylococcus hominis*) (and the other was (G- ve) including (*Aeromonas sobria*) to be treated with micro-polystyrene.

3.2 Toxicology test of polystyrene on isolation bacteria

After treating the bacteria for two type ***staphylococcus hominis*** (G+ve bacteria), and ***Aeromonas sobria*** (G-ve bacteria) with different concentrations of polystyrene, the result showed that there was no any effected of polystyrene any effect on the bacteria after treatment (Fig 3).

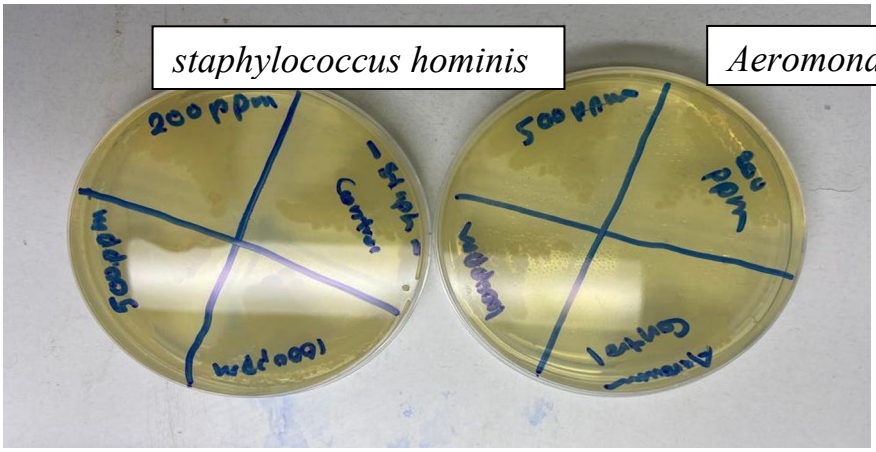

Fig. 3. *Aeromonas* and *staphylococcus* after treatment with PS.

The reason is due to composition of the surface of nano-polystyrene: its surface may contain substances that may make it unable to adhere to or interact with bacteria, and this reduces the effect on bacterial growth [19]. Perhaps increasing the concentration of nano-polystyrene, which reached 1000 ppm, led to a large aggregation of its particles, which leads to a reduction in its effectiveness in interacting with bacteria [20], moreover, environmental conditions, temperature and concentration of other chemicals have directed effect on this results.

3.3 Carbon source consumption test by biology Eco microplate

The results showed a variation in the carbon source consumption for both species of bacteria.Our result was identical to the study of [[2]1], where the growth of studied species were enhanced when the Shannon diversity indicator gave an increase in the average numbers of Bacteria from 4.3% to 38.6%.

This indicates that MP provided a benefit to the microorganisms of the intestine. This is similar to the results of our study when the presence of polystyrene enhanced the feeding of both species of bacteria inside the microplate, also the variation in the response to these pollutants depending on the species of bacterial, some of which may inhibit its growth after exposure, as stated in a study of [17] , who exposed *E.coli* bacteria to the micro-polystyrene, where they adhered to the surface of micro plastics of size 5mm and formed plastispher, where micro plastics provided a source of nutrients for microorganisms. On the other hand, gram-positive bacteria (*B.cereus*) promoted growth, and here micro plastics provided a source of food for the bacteria [22-27]. The results showed a difference in the absorption of sugars for both studied of bacteria (*Aeromonas sobria* and *staphylococcus hominis*) Which were classified as gram negative and gram positive respectively , with an increase in absorption after treatment with nanopolystyrene.

Bacterial species are differ in their response to nanopolystyrene, in addition to their differences between G - and G +, some of which may inhibit their growth after exposure to nanopolystyrene such as *E.coli* and *Bacillus cereus* due to the weakness of their cell wall [17]. However, in our study growth was enhanced for both Gram-positive and Gram-negative bacteria, this indicates the ability of bacteria to form EPS (Extracellular polymeric substances) on the surfaces of micropolystyrene, which consists of sugars, proteins, fats, and sometimes DNA.

The bacteria form EPS in a solid or colloidal form that protects it from external toxic substances when they live in a medium called plastisphere Which is considered a suitable

place to incorporate polystyrene and microorganisms[24]. The EPS charge may differ between (G -ve and G +ve) and this leads to the difference in electrostatic attraction and repulsion between EPS and polystyrene [25].

The amount of EPS produced may affect the accumulation of bacteria around the polystyrene [26]. Therefore plastic additives can be considered a carbon source for some types of microorganisms that have the ability to decompose plastic. Thus, polystyrene plays an additional surface for bacterial growth [28]. It is worth noting that the concentrations of polystyrene that we used were higher than concentrations in the natural river environment, i.e. the equivalent of (20-70 mg\l to 1000 mg\l) respectively. This gives them a suitable place for reproduction and growth. As aresult, their metabolism of carbon sources will increas, then will cause flux more CO_2 from equatic ecosystems and this is what we have seen in the ecomicroplate. It is inferred from this result that by feeding on polystyrene, the bacteria increased their requirements for nutrients represented by sugars. Which, when withdrawn from the aquatic ecosystem and excreting carbon dioxide, leads to an increase in its quantity in the aquatic environment then transfer to the atmosphere, and that consedared as one of the green house gases, this indicates that the presence of polystyrene has increased the percentage of carbon dioxide in the aquatic ecosystem and atmospheric atmosphere [29]. Exposure to polystyrene or other microplastics can induce various metabolic changes in both (G -ve and G +ve) bacteria. However, the exact nature of these changes can vary depending on several factors, including the specific bacterial species, the properties of the microplastics, and the environmental conditions [30]. There are some general metabolic changes that can occur that which bacteria can experience stress when exposed to microplastics. This stress can lead to the activation of stress response pathways, including the production of stress-related proteins and changes in gene expression. These responses can divert metabolic resources away from other processes. It was observed that consumption of carbohydrates represented by (Tweens 80 , Tweens 40, α-Cyclodextrin, Glycogen, D-Cellobiose, α-D-Lactose, i-Erythritol, D-Mannitol, N-Acetyl-D-Glucosamine, D-Glucosaminic acid, Glucose-1-phosphate and D-Xylose), which are carbon sources with high energy inputs by both species of bacteria, increased the concentration of micro polystyrene for studied bacteria, was consumption increased with increasing concentration of micropolystyrene. This indicates that sites containing high concentrations of micro plastics exert an unstable energy on the microorganisms [31] there were clear significant differences between the absorbance of bacteria to different concentrations of micropolystyrene, (p-value< 0.05), (Table 1). Also Bacteria may adjust their nutrient uptake strategies in response to the presence of microplastics. This could involve changes in the expression of transporters and receptors for various nutrients, including carbon sources like sugars. Where Tween 40 and 4-Hydroxybenzoic acid of microorganisms were consumed in a study that used some pollutant stress which may have acquired new mechanisms with micro polystyrene [32]. Morover it could changes in energy metabolism the bacteria may modify their energy metabolism to adapt to the new conditions. For example, they may switch between different metabolic pathways (e.g., glycolysis, TCA cycle) depending on the availability of substrates and the need for energy [33]. D-Galactonic acid-γ-Lactone and γ-Hydroxybutyric acid consedered the most commonly used carbon sources to induce resistance to pollutants stress in microbial communities, which explains the consumption of these carbon sources by the bacteria in our study). From the other hand it could be formation of Biofilm that bacteria exposed to microplastics may form biofilms on the plastic surface. Biofilm formation involves the production of extracellular polymeric substances (EPS), which can be rich in carbohydrates. This can change the overall metabolic profile of the bacterial community within the biofilm [34], This is what our study proved when examining bacteria exposed to micropolystyrene (SEM). Some bacteria produce secondary metabolites with antimicrobial properties. Exposure to microplastics could influence the production of such compounds as part of the

bacterial response [35]. Bacteria may experience changes in their redox balance when interacting with microplastics, which could lead to the production of reactive oxygen species (ROS). ROS can affect various metabolic processes and cellular structures [36]. It's essential to recognize that the metabolic responses of bacteria to microplastics are complex and can vary widely among different bacterial species and environmental conditions [37]. Bacteria sometimes secrete exopolysaccharides when they form a biofilm, which is also one of the protective mechanisms [38]. This was demonstrated by the results in our study, where there were significant differences (p-value<0.05, Table: 2, 3 and 4) between the different concentrations of micropolystyrene for the amino acids represented by L-Asparagine, L-Phenylalanine, L-Serine, L-Threonine, Glycyl-L-Glutamic Acid, Phenylethylamine and Putrescine and L-Arginine) of and the organic acids represented by (Pyruvic Acid Methyl Ester, D-Galacturonic Acid, 2-Hydroxy Benzoic Acid, 4-Hydroxy Benzoic Acid, Itaconic Acid, α-Keto Butyric Acid and D-Malic Acid), and others like: D,L-α-Glycerol Phosphate, γ-Amino Butyric Acid, c-Methyl-D- Glucoside and D-Galactonic Acid γ-Lactone)

Table 1. Mean ±SD for some carbohydrates of two species of bacteria (*Aeromonas sobria and staphylococcus hominis*) after treatment with (D.W) as control and (200, 1000) ppm micro polystyrene.

Parameters	Control		200ppm		1000ppm		Sig	LSD
	Aeromonas	*Staph*	*Aeromonas*	*Staph*	*Aeromonas*	*Staph*		
Water	0.298±0.033	0.305±0.084	0.279±0.022	0.280±0.030	0.409±0.068	0.552±0.024	0:001*	0.011
Tween 80	0.686±0.159	1.032±0.053	2.186±0.063	1.91±0.052	1.460±0.098	2.051±0.034	<0:001*	0.031
α-Cyclodextrin	0.335±0.051	0.763±0.106	0.328±0.029	2.038±0.165	0.611±0.164	1.709±0.188	<0:001*	0.07
Glycogen	1.739±0.026	0.692±0.235	0.420±0.017	2.169±0.104	1.603±0.338	2.199±0.024	<0:001*	0.122
D-Cellobiose	0.344±0.040	0.283±0.066	0.381±0.042	0.313±0.039	0.686±0.053	0.704±0.076	<0:001*	0.012
α-D-Lactose	0.324±0.018	0.269±0.070	0.321±0.008	0.421±0.034	0.485±0.052	1.294±0.577	0.009*	0.230
Tween 40	0.692±0.062	0.223±0.066	1.625±0.300	0.395±0.057	1.726±0.079	1.248±0.681	0.001*	0.385
i-Erythritol	0.298±0.058	0.265±0.080	0.296±0.084	0.257±0.037	0.967±0.049	0.625±0.042	<0:001*	0.015
D-Mannitol	1.080±0.463	0.926±0.079	1.975±0.028	1.892±0.028	1.825±0.098	2.127±0.027	<0:001*	0.157
N-Acetyl-D-Glucosamine	1.478±0.238	1.007±0.194	2.001±0.032	1.915±0.050	1.334±0.435	2.165±0.092	0.001*	0.199
D-Glucosaminic Acid	0.256±0.042	0.820±0.101	2.054±0.037	1.978±0.013	1.567±0.279	2.220±0.005	<0:001*	0.062
Glucose-1-Phosphate	0.823±0.701	0.258±0.051	0.371±0.030	0.403±0.026	0.621±0.088	0.855±0.034	0.266	0.339
D-Xylose	0.444±0.037	0.380±0.031	0.433±0.034	0.405±0.030	1.046±0.262	1.733±0.721	0.005*	0.399
*Significant at 0:05								

Table 2. Mean ±SD for some Amino Acids and Related of two type of bacteria (*Aeromonas sobria and staphylococcus hominis*) after treatment with (D.W) as control and (200, 1000) ppm micro polystyrene**.**

Parameters	Control		200ppm		1000ppm		Sig	LSD
	Aeromonas	*Staph*	*Aeromonas*	*Staph*	*Aeromonas*	*Staph*		

Water	0.298±0.033	0.305±0.084	0.279±0.022	0.280±0.030	0.409±0.068	0.552±0.024	0:001*	0.011
L-Asparagine	0.892±0.471	0.962±0.338	1.867±0.002	1.844±0.053	1.744±0.236	2.032±0.046	0.02*	0.267
Glycyl-L-Glutamic Acid	0.306±0.028	0.225±0.028	0.993±0.382	1.031±0.352	0.965±0.134	2.136±0.045	<0:001*	0.196
Phenylethyl-amine	0.595±0.222	0.664±0.080	1.940±0.062	1.819±0.035	1.267±0.532	2.196±0.056	<0:001*	0.234
L-Phenylalanine	0.713±0.586	0.829±0.074	1.823±0.091	1.596±0.023	1.441±0.223	2.162±0.020	0:001*	0.274
L-Serine	0.244±0.021	0.248±0.054	0.694±0.058	0.598±0.046	1.289±0.579	1.520±0.199	0.001*	0.258
L-Threonine	0.878±0.364	0.379±0.047	2.036±0.029	1.981±0.012	1.544±0.280	2.255±0.039	< 0.001*	0.145
Putrescine	0.611±0.028	0.553±0.093	1.944±0.040	2.081±0.116	1.127±0.609	2.276±0.027	<0:001*	0.267
*Significant at 0:05								

Table 3. Mean ±SD for some Organic Acids of two type of bacteria (*Aeromonas sobria and staphylococcus hominis*) after treatment with (D.W) as control and (200, 1000) ppm micro polystyrene.

Parameters	**Control**		**200ppm**		**1000ppm**		**Sig**	**LSD**
	Aeromonas	***Staph***	***Aeromonas***	***Staph***	***Aeromonas***	***Staph***		
Water	0.298±0.033	0.305±0.084	0.279±0.022	0.280±0.030	0.409±0.068	0.552±0.024	0:001*	0.011
D-Galacturonic Acid	0.294±0.030	0.212±0.015	1.846±0.055	1.720±0.025	1.781±0.169	2.001±0.020	<0:001*	0.0226
2-Hydroxy Benzoic Acid	0.294±0.044	0.227±0.057	1.679±0.220	1.646±0.105	0.701±0.243	2.148±0.029	<0:001*	0.0839
4-Hydroxy Benzoic Acid	0.336±0.136	0.836±0.097	1.983±0.020	1.842±0.080	1.557±0.151	2.158±0.016	<0:001*	0.039
Itaconic Acid	0.335±0.026	0.227±0.003	0.375±0.044	0.315±0.008	0.592±0.079	0.835±0.026	<0:001*	0.006
α-Keto Butyric Acid	0.308±0.017	0.255±0.049	0.339±0.046	0.359±0.049	0.585±0.079	0.810±0.033	<0:001*	0.010
D-Malic Acid	0.431±0.040	0.406±0.078	1.636±0.052	1.703±0.269	0.927±0.360	1.959±0.122	< 0.001*	0.153
Pyruvic Acid Methyl Ester	1.399±0.159	0.192±0.016	1.929±0.021	0.371±0.039	1.938±0.168	0.791±0.024	<0:001*	0.038
*Significant at 0:05								

Table 4. Mean ±SD for some Organic Acids of two type of bacteria (*Aeromonas sobria and staphylococcus hominis*) after treatment with (D.W) as control and (200, 1000) ppm micro polystyrene**.**

Parameters	**Control**	**200ppm**	**1000ppm**	**Sig**	**LSD**

	Aeromonas	*Staph*	*Aeromonas*	*Staph*	*Aeromonas*	*Staph*		
Water	0.298±0.033	0.305±0.084	0.279±0.022	0.280±0.030	0.409±0.068	0.552±0.024	0:001*	0.011
D,L-α-Glycerol Phosphate	0.282±0.007	0.236±0.033	0.409±0.028	1.212±0.587	0.640±0.064	0.735±0.044	0.015*	0.238
γ-Amino Butyric Acid	1.624±0.113	1.328±0.227	1.889±0.043	1.883±0.045	1.731±0.275	2.209±0.005	0.002*	0.097
c-Methyl-D-Glucoside	0.247±0.057	0.268±0.047	0.272±0.027	0.262±0.026	1.364±0.533	0.504±0.030	0.002 *	0.196
D-Galactonic Acid γ-Lactone	1.036±0.551	0.767±0.256	1.994±0.035	1.920±0.037	2.153±0.077	2.086±0.046	<0:001*	0.255
L-Arginine	0.573±0.273	0.473±0.157	1.822±0.048	1.722±0.079	0.674±0.041	2.184±0.023	<0:001*	0.074
*Significant at 0:05								

3.4 Scan electron microscopy (SEM) analysis

The results of treatment with polystyrene at a concentration of 1000 ppm showed an increase in the size of bacteria, as the (Fig 4), (Table 5).

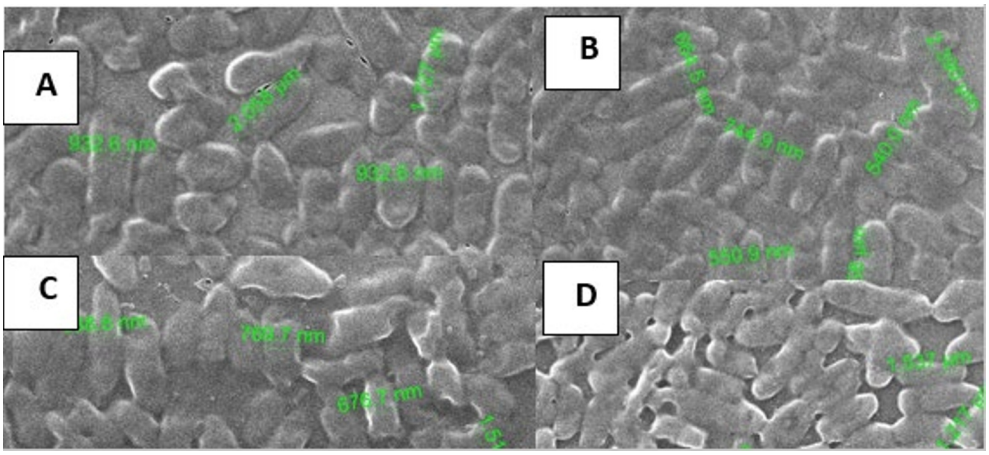

Fig. 4. A (*A.sobria* with (1000 ppm) polystyrene), B (*A.sobria* with D.W), C (*Staph* with 1000 ppm), D (*Staph* with D.W) under (SEM) microscope.

Table 5. Mean ±SD for two type of bacteria (*Aeromonas sobria and staphylococcus hominis*) after treatment with (1000) ppm micro polystyrene.

Parameters	**Bacterial Volume**	**Mean ±SD**		**df**	**Sig**
		Control	**Treatment**		
Polystyrene mg/L	*Aeromonas*	673.57±178	1231±171	7	0.008*
	Staph	246±19.62	357±50.81	7	0.011*
* significant at 0.05					

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