Toxic Effect of Ammonium Nitrate and *Bacillus Subtilis* on the Wild Rat, *Rattus Norvegicus*

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Abstract: The effect of ammonium nitrate at different tested concentrations and *Bacillussubtilis* (*B. subtilis)* spores on the mortality of the wild rat, *Rattus norvegicus* was examined. The antibacterial activity of ammonium nitrate and *B. subtilis* at the sublethal concentration against the population levels of intestinal bacteria of rats on the Nutrient agar and Mac Conkey agar media was also investigated. The susceptibility of the isolates to ammonium nitrate and *B. subtilis* was assayed by disc diffusion method. Results indicated that the mortality percents which recorded by ammonium nitrate at the tested concentrations 0.5, 1.0 and 1.5% were 46.66, 63.33 and 80% after 28 days of oral administration, respectively. But it wasn't gave any mortality at the lowest concentration 0.1% till the end of experiment. *B. subtilis* recorded only 30% mortality at its highesttested concentration 10.4×10^4spores/mlafter 28 days of administration. In concerning to the effect of ammonium nitrate at the sublethal concentration 0.1% on the intestinal bacteria, it was completely preventing the growth of *Klebsiella granulomatis* on both tested media. While, it was showed observed increasing in the number of *Klebsiella pneumonia* and *Klebsiella oxytoca* colonies in comparison with the control.On the contrary, this concentration causing a high decreasing in the count of *Staphylococcus epidermidis* on the MacConkey agar media.*B. subtilis* at the sublethal concentration 1.3×10^4spores/ml was markedly prevent the growth of all bacterial flora in the intestine. The highest antimicrobial effect of ammonium nitrate was recorded against *K. granulomatis* with inhibition zone diameter 23 mm. On the other hand, *B. subtilis* was more susceptible to *E. coli* with inhibition zone 21.6 mm. The latter draws a conclusion that ammonium nitrate considered a promising effective rodenticide and *B. subtilis* was a successful natural biocontrol agent against the intestinal bacteria of rat which strongly controls all metabolic processes and therefore the rate of nutrition and crops damage in the fields and may be also causing the rat future death.
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1. Introduction

Wild rat (*Rattus norvegicus*) is considered a dangerous agricultural pest which wide spread in the different fields in Egypt **(Abdel-Azeem, 2013)**. It is a strong pest living on the supplies or harvested cereals, root crops and also on livestock feeds that can be found in farm buildings lead to untold economic loss to farmers **(Cowan *etal*., 2003)**. On the other hand, it is considered one important vector of human and livestock diseases acts as carriers of parasites and other infectious organisms that affect humans and animals makes them unwanted **(Webster and Macdonald, 1995; Stojcevic *et al*., 2004)**. A wide range of control measures have been used from time to time for the control of rodents as chemical, biological and other means. Application of chemical substances is widely used method for rodents control **(Olumide, 2008)**. Chemical pesticides were used to control rodents but it has large adverse effects on human and environmental health **(Beshay, 2005)**. Ammonium nitrate is one of the most commercially important ammonium compounds in terms of usage. It has extensive use in the area of nitrogen fertilizers lead to an increase in agricultural out puts. At the same time, it has a successful effect in the rodents control and it is safely used when applied at low rates (recommended concentrations) **(Testud, 2004; Koller, 2009)**. The toxic effect of ammonium nitrate against rats is due to raised ammonium levels in blood and it is responsible for the hyper ammonemic condition and hyper ammonemic is mainly responsible for the neurological alterations. It cause also oxidative stress and damage of the rat organs lead to death **(Siva *etal*., 2017)**. In the direction of biological control, *Bacillussubtilis* represent a novel natural probiotic which is preferably used in the rats control. Moreover, it is safe and cost-effective mean for using **(Selvam *et al*., 2009)**. The pathogenic effect of this bacterium is due to releasing of peptidoglycan and lipopeptide cause formation of TNF-alpha, Shock, organ injury and also necrosis hypatocyte lead to the rat mortality at the end **(Rihab *et al*., 2014)**. At other trend, the intestinal bacteria in rats as *Enterobacteriaceae*, *Peptostreptococcus*, sp., *Eubacterium* sp., *Lactobacillus* sp. and *Colstridium* sp. plays a very important role in the development and activation of the immune system and also has a major effect on the in vivo physiology and the digestion

process by activation of the digestive enzymes **(Rance *et al*., 1998; Geraldine and Beth, 2008)**. Furthermore, this bacteria considered one of the essential factors of in vivo response, expression of genes, a major energy source for enterocytes and also contributes significantly in the rats resistance to the different infectious diseases **(Masami *etal*., 1975; Itoh and Narushima, 2005)**. The sublethal concentrations of ammonium nitrate does not cause mortality of rats but keep it under ammonia stress **(Priyadarshini and Neeraja, 2015)**. Nitrates, in case of their oral absorption, are reabsorbed rather quickly in intestines affected directly on the intestinal bacteria **(L'Hirondel, 1998)**. Ammonium nitrate has a strong antibacterial activity against large number of intestinal bacteria in rats including *Escherichiacoli* and *Staphylococcus aureus* **(Ira *etal*., 2010)**. At the same trend, *B. subtilis* also has an antibacterial potential against variety of intestinal bacteria. It is found to secrete the surfactins (cyclic lipopeptides) which reduced the growth of bacteria **(Selvam *et al*., 2009)**. Reduction in the number of intestinal bacteria as *Lactobacillus* causes active colitis in rat **(Fabia *et al*., 1993)**.

This study carried out to assess the toxic effect of ammonium nitrate and *B. subtilis* sporeson the wild rat, *R. norvegicus* and also evaluate the inhibition activity of each of them at the sublethal concentration against the intestinal bacteria which plays a very important role in keeping the rat in a healthy state.

2. Materials and Methods

Rats

Adult males of the wild rat, *Rattus norvegicus* were trapped from near drainage canal and plantation area at Meniet El-Kamh district, Sharkia Governorate, Egypt. The rats were carefully transported to the laboratory by using traps and then maintained in metallic cages, supplied with enough food (crush maize) and water for acclimatization. Rats were observed daily for about two weeks before any experiments **(Abdel-Azeem, 2013)**.

Preparation of ammonium nitrate solution

Pure ammonium nitrate (33% N) produced in form of pills was obtained from El-Gomhouria Company, Egypt. Four concentrations; 0.1, 0.5, 1 and 1.5% from the active ingredient of this fertilizer were prepared by dissolving the required amount in distilled water to obtain the appropriate concentration **(Samira *etal*., 2006)**.

Preparation of bacterial inocula

Bacillus subtilis strain was obtained from the Insect Pathogen Unit (IPU), Plant Protection Research Institute, Agricultural Research Center, Egypt. It was identified according to Bergeys Manualof Systematic Bacteriology, (**Holt *etal*., 1984**). The strain was grown with Nutrient broth medium (5 g / l bactopeptone, 5 g / l beef extract, 5 g / l NaCl) (pH 7.2) at 37ºC. The bacterial spores were collected and the spores concentration was determined using a hemocytometer and adjusted to 1.3×10^4, 2.6×10^4, $5.2\times10^4$and 10.4×10^4 spores / ml by adding distilled water **(Kosaka*etal*., 1998)**.

Treatment of rats

The active and healthy adult rats were chosen and separated into three experimental batches. The first batch was orally administered with ammonium nitrate, this batch divided into four groups for the four concentrations of this fertilizer. Three replicates, each containing 10 rats were used for each concentration. The second batch of rats was orally administered with *B. subtilis* bacterium, divided into four groups for the four tested concentrations of this bacterium. Three replicates were prepared for each concentration, each replicate containing 10 rats. Third batch was considered as control comprising of three replicates each one containing 10 rats orally administered with only distilled water. Before the administration with each of ammonium nitrate and bacteria, rats were fasted for 6 hrs. After two weeks of administration, the experimental rats were retreated with the same tested concentrations of each agent by the same manner as mentioned previously. During the experiment period 28 days the mortality percents were recorded **(Siva *etal*., 2017)**.

*The obtained data were statistically analyzed and the difference between means was tested using the method of **Costat (2005)** statical program analysis, computer program software. The least significant difference (L.S.D.) was also calculated.

Rats which treated by the sublethal concentration of each of ammonium nitrate and bacteria were anesthetized by ether and sacrificed by decapitation for remove the small intestines to explore the inhibition effect of these sublethal concentrations on the intestinal bacteria of these individuals **(Moussa, 2005)**.

Isolation and identification of intestinal bacteria

5cm segment of the small intestine of 10 rats which randomly selected from the three replicates of the sublethal concentration of ammonium nitrate and *B. subtilis* were removed. These intestines were transferred into five Petri-dishes (12 ml diameter), each one containing two intestines provided with 20 ml distilled water. Intestines were cut by using fine scissors and flat pointed dissecting needle. Each suspension of the five plates was distributed into 20 Petri-dishes each one containing 1 ml of this suspension. These plates were divided into two groups each one comprising of ten plates. Nutrient agar media was poured aseptically into the first group plates and the plates of second group were provided

with Mac Conkey agar media. The same isolation was occurred also with the intestines of control replicates. The culture plates were incubated at 37ºC for 24 hours. Total mean count of bacterial colonies was calculated. These colonies were purified by sub-culturing on appropriate media and incubating at 37ºC for another 24 hours before identification **(Shokry, 2007)**. Cultures were Gram stained and characterized based on the cultural morphological and sugar fermentation reactions as biochemical reactions such as catalase, oxidase, coagulase, citrate utilization, urease, methyl red, indole, Voges Proskaeur tests according to Bergeys Manual of Determinative Bacteriology **(Holt *etal*., 1984)**.

*Nutrient agar and Mac Conkey agar media were obtained from the Bacteriology Laboratory, Plant Department, Faculty of Science, Zagazig University, Egypt.

Determination of ammonium nitrate and *B. subtilis* antibacterial activity

The antibacterial activity of sublethal concentration of both ammonium nitrate and *B. subtilis* against the intestinal bacteria was assayed using the disc diffusion method (DDM). In this method, six (6) millimeter diameter discs cut out from No. 1 What man filter paper, sterilized by autoclaving at 121ºC for 15 minutes. The sterilized discs were soaked in each sublethal concentration of ammonium nitrate and *B. subtilis*. The impregnated discs were then placed in triplicate on plates containing Nutrient agar medium and seeded with 1 ml of 24 hour old broth culture of intestinal isolates. The zones of inhibition were measured in millimeter as degree of susceptibility of the intestinal isolates to ammonium nitrate and *B. subtilis* and means of the inhibition zones were noted **(Lawrence *etal*., 2016)**.

* Simple means of data and standard error were computed as appropriate by using Microsoft Excel Program.

3. Results and Discussion

Effect of ammonium nitrate on the wild rat

As shown in Table (1) and Figure (1) no mortality was found at the concentration 0.1%. After 14 days of administration 46.66, 56.66 and 70% mortality observed at 0.5, 1 and 1.5% concentrations, respectively. At the end of experiment (28 days), the mortality reached to its maximum value 46.66, 63.33 and 80% at the concentration 0.5, 1 and 1.5%, respectively.

Table (1): Mortality of wild rat at different concentrations of ammoniumnitrate

Concentrations (%)	Mortality percent after indicated days						General mean
	1	4	7	14	21	28	
0.1	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{d}	0.00^{d}	0.00^{d}
0.5	0.00^{c}	20.00^{b}	30.00^{b}	46.66^{b}	46.66^{c}	46.66^{c}	31.66^{c}
1.0	36.66^{b}	40.0^{a}	56.66^{a}	56.66^{b}	63.33^{b}	63.33^{b}	52.77^{b}
1.5	53.33^{a}	53.33^{a}	66.60^{a}	70.00^{a}	80.00^{a}	80.00^{a}	67.21^{a}
Control	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{d}	0.00^{d}	0.00^{d}
P	.0000***	.0001***	.0000***	.0000***	.0000***	.0000***	.0000***
L.S.D. $_{0.05}$	1.56	1.69	1.05	1.05	0.66	0.66	13.48

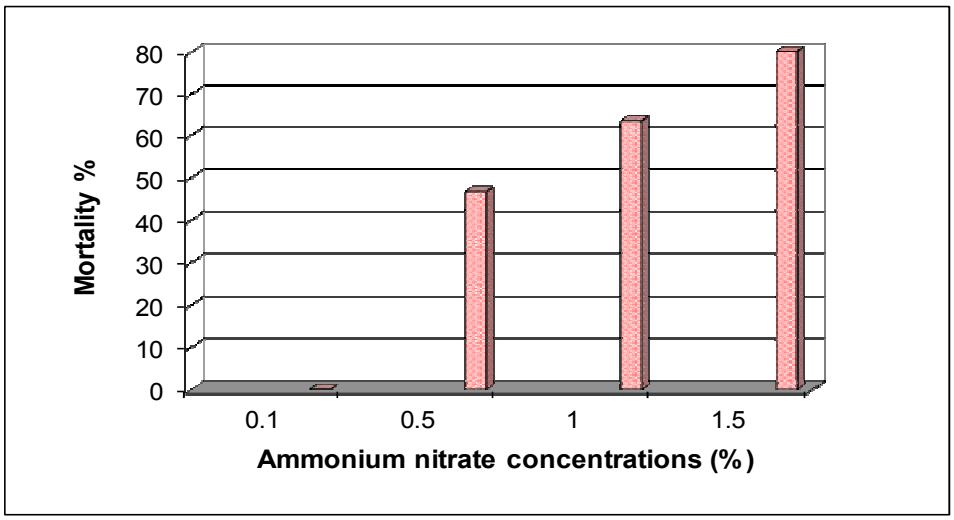

Fig. (1):Effect of ammonium nitrateon the wild rat after 28 days ofadministration.

The mortality rate increased with an increase in the concentration of ammonium nitrate. These results are confirmed by **Priyadarshini and Neeraja (2015)** reported that ammonia is toxic, leading to functional disturbances of the central nervous system of albino rat that can lead to coma and death. At the same trend, ammonium nitrate considered a dangerous explosive killed rats by causing extremely high internal pressure buildup. The oral administration of sodium nitrate has also lethal effect on rats caused tumors in the liver, skin and testes **(Pradyot, 2007). Siva *etal.* (2017)** showed that the mortality rate of albino rats was increased with an increasing of the dose concentration of ammonium sulfate. The concentration 10 mg / kg b.w. of this fertilizer not caused any mortality of rats but the other concentrations 30, 50 and 70 mg / kg b.w. recorded 20, 30 and 40% mortality, respectively. The highest mortality values 60, 70, 80 and 90% were exhibited by this fertilizer at the concentration 110, 130, 150 and 170 mg / kg b.w., respectively. On the other hand, zinc phosphide at the low concentration 0.06% not recorded any mortality of *Rattus norvegicus* after four days of the orally administration but the same concentration exhibited 20% mortality of the house mouse, *Musmusculus* after only one day of administration **(Abdel- Azeem, 2013)**.

Effect of *Bacillus subtilis* on the wild rat

Pathogenic effect of the tested concentrations 1.3×10^4, 2.6×10^4, 5.2×10^4 and 10.4×10^4 spores / ml of *B. subtilis* was investigated against the wild rat, *R. norvegicus*. Data in Table (2) indicated that the lowest concentration 1.3×10^4spores / ml did not achieve any mortality of rats till the end of experiment. On the other hand, the tested concentration 2.6×10^4 spores / ml was recorded its first effect after 14 days of administration with mortality 16.66%. After 21 days this percent increased reached to 20% and still stable till the end of experiment.The highest concentrations 5.2×10^4 and 10.4×10^4spores / ml gave the same mortality 23.33% after 14 days of administration then the mortality increased to 26.66 and 30% at the end of experiment for the two concentrations, respectively. In general, *B. subtilis* did not achieve acute toxicity against rats at the all tested concentrations during the experiment period. These findings were agree with those obtained by **Nakamura *etal.* (1999)** showed that *B. subtillis* not recorded any death of rats which feeding on diet containing 0.13%, 0.55%, 1.66% and 5% of it. In addition, it was not exert serious toxicity of rats even the highest dose. The same results were obtained also by **Edward and Elaina (1991)** cleared that *B. subtilis* not exhibited any mortality at the concentration 100.000 ppm in rats and 25.000 ppm in mice.

Although it has not achieved toxic effect against rats, it is reduced the creatinine and urea level and also caused a potent inhibition of ACE activity **(Raida*etal.*, 2017)**. At the same direction, **Youn – Hwan *etal.* (2009)** illustrated that surfactin C, produced by *B. subtilis* at dose of 500, 1000 and 2000 mg / kg not causes toxicities in survival of rats. But it showed decrease in body weight and increase the liver weight and also has an adverse effect on the different enzymes at the concentration 1000 or 2000 mg / kg. Zonal necrosis of hepatocyte around the hepatic vein was observed after oral administration of the same doses.

Table (2): Mortality of wild rat at different concentrations of *B.subtilis* spores

Concentrations (Spores / ml)	Mortality percent after indicated days						General mean
	3	6	9	14	21	28	
1.3×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.6×10^4	0.00	0.00	0.00	16.66	20.00	20.00	9.44
5.2×10^4	0.00	0.00	13.33	23.33	26.66	26.66	14.99
10.4×10^4	0.00	0.00	20.00	23.33	30.00	30.00	17.22
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Bacterial flora in the small intestine of rats administered with ammonium nitrate

Bacterial flora in the small intestine of rats which orally administered with ammonium nitrate at the sublethal concentration 0.1% were counted on the Nutrient agar and Mac Conkey agar media in comparison with the other count in the untreated rats (control). As cleared in Table (3) and Figure (2) *Klebsiellap neumoniae* was the most predominant bacterium in the small intestine of the untreated rats appeared by mean 61.2 colonies on the Nutrient agar media. It was followed by *Klebsiella oxytoca* by mean number 44.2 colonies on the Mac Conkey agar media. With regards to the effect of ammonium nitrate on the bacterial flora in the intestine, it was completely reduced the growth of *Klebsiella granulomatis* on both Nutrient and Mac Conkey agar media in comparison with the control. It was also highly reduced the growth or existence of *Staphylococcus epidermidis* to 1.6 and 9.4 colonies on the Nutrient and Mac Conkey agar media, respectively compared with 6.8 and 21 colonies in the control at each culture media, respectively. At the same trend, ammonium nitrate showed a little reduction against

Escherichiacoli and *Staphylococcus aureus* with mean count 9.6 and 2.4 colonies on Nutrient agar media, respectively. On the other hand, it was markedly increased the mean number of *K. pneumoniae* from 61.2 colonies in the control to 105.8 colonies on the Nutrient agar media and also causing highly increase of the number of *K. oxytoca* to 97 colonies in comparison with 44.2 colonies in the control on Mac Conkey agar media. These results are supported by **Itoh and Mitsuoka (1985)** reported that the intestinal flora of rats has a very complex structure as the other animal species and it consists of more than 90% strict anaerobes. These authors compared viable bacterial numbers in different types of media using the plate – in-bottle method, the highest count (5.6×10^{10} / g) was obtained with the SM10 medium and about 73% of the bacteria observed in feces by Gram staining could be cultured. The common intestinal flora in rats are obligate anaerobes and the most abundant bacteria are follow the genus Bacteroides, anaerobic Gram – positive cocci **(Geraldine and Beth, 2008)**.*S. aureus* colonies were detected in the rat gut **(Yoshida, 1999)**.

Table (3): Number of bacterial flora in the intestine of wild rats administered with 0.1% of ammonium nitrate

Bacterial flora	Treated with ammonium nitrate on culture media		Untreated (control) on culture media	
	Nutrient agar	Mac Conkey agar	Nutrient agar	Mac Conkey agar
Escherichia coli	9.6± 1.03	3.2 ± 0.86	10.0 ± 1.18	4.0 ± 0.70
Klebsiella granulomatis	-	-	3.0 ± 0.54	3.4 ± 0.92
Klebsiella oxytoca	-	97.0 ± 1.76	-	44.2 ± 1.77
Klebsiella pneumoniae	105.8 ± 2.78	-	61.2 ± 1.35	-
Staphylococcus aureus	2.4± 0.74	-	3.2 ± 1.15	-
Staphylococcus epidermidis	1.6 ± 0.60	9.4 ± 0.81	6.8 ± 1.74	21.0 ± 2.79

On the other hand, **Masami *etal.* (1975)** showedthat *E. coli* was the most predominant species in the small intestine of rats isolated on the Mac Conkey agar medium. At the same trend, **Umesaki *et al.* (1997)** indicated that *E. coli*, *Streptococcus faecalis* and *Colstridium paraputrificum* were the predominant intestinal bacteria in rats have an important role in normalization of immune function in the small intestine. Ammonium nitrate has an antimicrobial activity against some bacterial species as *E. coli* and *S. aureus* **(Wanxue*etal.*, 2010)**. The antimicrobial treatment of rat increased the number of *K. pneumoniae* in the intestine of mice up to 10^9cfu/g feces. This level decreased in rat without antimicrobial treatment due to competitive exclusion by the indigenous rat flora **(Karen, 2008)**. The intestinal bacterial overgrowth plays an important role in bacterial translocation in the experimental model of cirrhosis in rats **(Carlos *et al.*, 1997)**. Changes in the composition of intestinal flora of rats are often associated with diseases and possibly may be their cause **(Masami *et al.*, 1975)**.

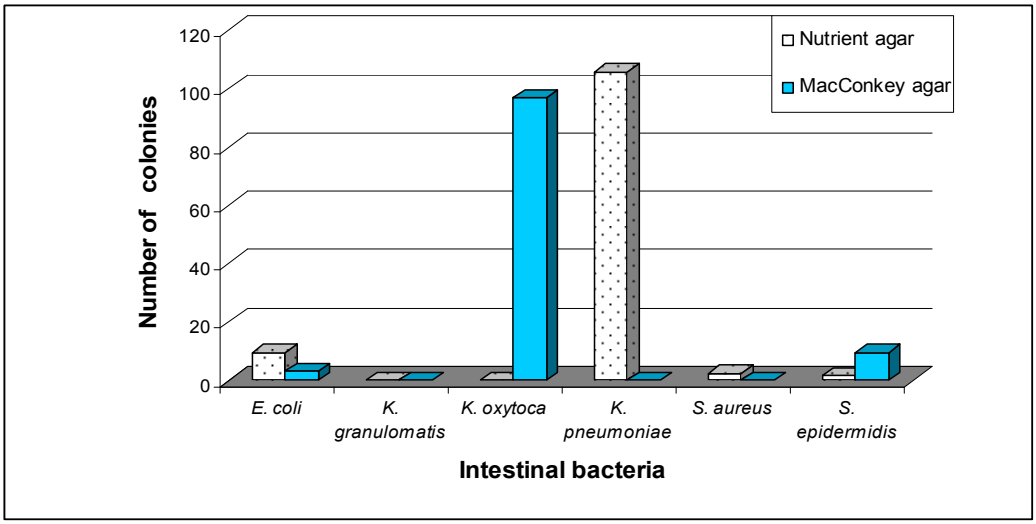

Fig. (2): Count of bacterial flora in the intestine of rats administeredwith 0.1% of ammonium nitrate on tested media.

Bacterial flora in the small intestine of rats administered with *Bacillus subtilis*

The Nutrient agar and Mac Conkey agar plates which inoculated with the small intestine inoculum of rats administered with *B. subtilis* at the sublethal concentration 1.3×10^4spores / ml were examined. Results showed that *B. subtilis*at this concentration was completely prevent the growth of bacteria on the all plates of both culture media. This finding explained clearly that *B. subtilis* has a supernatural antibacterial activity against the intestinal bacteria. These results are agree with those obtained by **Nalisa *et al.* (2015)** indicated that *B.subtilis* has a high inhibitory effects on the intestinally flora specially the Gram – positive species. This antibacterial effect is due to secrete a surfactins (cyclic lipopeptides) which have a high inhibition potential against bacteria **(Selvam *et al*., 2009)**. In the same trend, **Vander Pool *etal*. (2008)** published that *B. subtilis* produced a bactericidal substances which inhibit the growth of enteric bacteria. Reduction of the intestinal bacteria was negatively affected on the immune system activation causing dangerous diseases to rats which may lead to future death **(Fabia *et al*., 1993)**.

Antibacterial activity of ammonium nitrate against the intestinal bacteria

This test is performed to explain and justify why many bacteria did not appear in the intestines of rats administered with ammonium nitrate at the sublethal concentration 0.1% in comparison with the control in the previous count. As illustrated in Table (4) ammonium nitrate exert the highest inhibition activity against *K. granulomatis* isolate (Photo 1) with inhibition zone diameter 23 mm. The other bacterial species *K. oxytoca*, *S. epidermidis* and *E. coli*were inhibited by ammonium nitrate with inhibition zones 11.6, 9.0 and 4.3 mm, respectively. On the other hand, there is no inhibition was recorded by this fertilizer against *K. pneumoniae* (Photo 2) and *S. aureus* species. Thus confirming and explained the disappearing, increasing or decreasing the number of intestinal bacteria in rats administered with this fertilizer in comparison with control in the previous count. These results are in general agree with **Joe *etal*. (2008)** reported that ammonium has broad spectrum of antimicrobial activity. It was exert an bactericidal activity against many bacterial species as *Pseudomonas aeruginosa*, *E. coli*, *S. aureus* and *Salmonella enterica*. **Lei*etal*. (2008)** demonstrated that ammonium nitrate has significant inhibition effect on the growth of microorganisms, and the bactericidal rates in 15 min for *E. coli* and *S. aureus* were 99.99%. The same finding was obtained also by **Wanxue *et al*. (2010)** confirmed that ammonium nitrate has a strong antibacterial activity against the same bacterial species, *E. coli* and *S. aureus*. Nitrate has an antibacterial effect against *Clostridium botulinum* and other bacteria including enteric pathogens such as *Salmonella* and *E. coli* by retarding bacterial development. The antibacterial effect of nitrate was disputed for a long time **(Jean, 2002)**. The antibacterial properties of ammonium depend on the size of the dendrimer, the length of hydrophobic chains in the ammonium groups and the concentration **(Chris *etal*., 2000)**.

Antibacterial activity of *B. subtilis* against the intestinal bacteria

The presented data in Table (5) recorded that *B. subtilis* at the sublethal concentration 1.3×10^4 spores/ml was more susceptible to*E. coli* with inhibition zone diameter 21.6 mm, followed by *K. granulomatis*, *S. aureus* and *K. pneumoniae* with diameter of inhibition zones 19, 18 and 15.6 mm, respectively. The lowest antibacterial effect of *B. subtilis* was showed against *K. oxytoca* and *S. epidermidis* with inhibition zones 14.3 and 12 mm, respectively. This finding is similar to that reported by **Ramya *et al*. (2014)** stated that *B. subtilis* has a strong and broad – spectrum antimicrobial activity against the Gram negative bacteria *E. coli*, *Pseudomonas aeruginosa*, *K. pneumoniae*, *Proteus vulgaris* and *Yersiniaaldovae*. The antimicrobial activity was observed against the Gram positive bacteria such as *S. aureus*, *Streptococcus pyogenes* and *Enterococcus faecalis*.

Table (4): Antibacterial activity of ammonium nitrate at 0.1% against the intestinal bacteria

Bacterial flora	Inhibition zone (mm)
Escherichia coli	4.3 ± 1.20
Klebsiella granulomatis	23.0 ± 1.73
Klebsiella oxytoca	11.6 ± 0.32
Klebsiella pneumoniae	No inhibition
Staphylococcus aureus	No inhibition
Staphylococcus epidermidis	9.0 ± 1.50

Photo (1):Very weak growth of *K. granulomatis* surround the disc of filter paper submerged in 0.1% of ammonium nitrate.

Photo (2): Overgrowth of *K. pneumonia* surround the disc of filter paper submerged in 0.1% of ammonium nitrate.

Table (5): Antibacterial activity of *B. subtilis* at 1.3 × 10^4spores / ml against the intestinal bacteria

Bacterial flora	Inhibition zone (mm)
Escherichia coli	21.6 ± 0.87
Klebsiella granulomatis	19.0 ± 1.15
Klebsiella oxytoca	14.3 ± 1.32
Klebsiella pneumoniae	15.6 ± 1.45
Staphylococcus aureus	18.0 ± 2.31
Staphylococcus epidermidis	12.0 ± 1.73

It was showed preferentially more active against the Gram negative especially *E. coli* and Gram positive *S. aureus* **(Huda, 2010)**. At the same direction, **Mirac*etal*. (2006)** recorded that *B. subtilis* is effective against Gram – positive and Gram – negative bacteria whereas its extensive inhibition activity is particularly against Gram – posiotive species. *Pseudomonas syringae* and *Xanthomonascampestris* were the most sensitive bacterial species to *B. subtilis* with sterile zones 48 and 50 mm in diameter, respectively **(Todorova and Kozhuharova, 2010)**. The diameters of the inhibition zones which recorded also by *B. subtilis* against *Vibrioharveyi*, *V. anguilarum*, *V. vulnificus* and *V. damsela* were 7, 3, 12 and 4 mm, respectively **(Vaseeharan and Ramasamy, 2003)**. **Abriouel *et al.* (2011)** cleared that *B. subtilis*, the model system for Gram – positive organisms are able to produce more than two dozen antibiotics with different structures and functions depending on the ecological niche. In addition, it is produce a vast array of structurally unrelated antimicrobial compounds which include lipopeptides as iturin, surfactin, fengycins, bacteriocins and bacteriocin like inhibitory substances (BLTS) **(Stein, 2005)**. *B. subtilis* also produce subtilosin which showed a high bactericidal activity specially against the Gram – negative bacteria **(Marx *etal*., 2001)**.

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