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Researches on the Identification of the Intestinal Bacterial Flora of *Locusta migratoria migratoriodies* R and F. (Orthoptera: Acrididae) and the Pathogenicity of Some of these Bacteria for this Species

by

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Faculté des Sciences de l'Université d'Ankara
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Researches on the Identification of the Intestinal Bacterial Flora of *Locusta migratoria migratorioides* R. and F. (Orthoptera: Acrididae) and the Pathogenicity of Some of these Bacteria for this Species*

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The intestinal bacterial flora of *Locusta migratoria migratorioides* R. and F. was investigated qualitatively and quantitatively in all nymph stages and in adults obtained both from laboratory cultures and fresh material collected from the field, as well as from new cultures dating back to material collected from the same place, and comparisons were made of the results.

As many as 1565 basterial cultures were isolated from 400 nymphs and adults whose intestinal flora was investigated. These cultures were formed by 33 species of bacteria belonging to 6 families.

In addition, the pathogenic effects of certain bacteria on *L. migratoria* were investigated by injection into body fluids, and LD_{50} was calculated. Some of the bacteria tested were isolated in the course of determining the intestinal flora (*Serratia marcescens*, *P. vulgaris*, *P. mirabilis*, *P. rettgeri*, *B. cereus*). Others were those which were never observed in *L. migratoria* (*P. aeruginosa*, chromogenic strain of *S. marcescens*). As far as the latter species were concerned, collection cultures were used.

S. marcescens, *P. aeruginosa*, *P. vulgaris* and *P. mirabilis* were recognised as pathogenic, their LD_{50} being below 10 000 bacteria. However, our results being different from those obtained by other research workers, the conviction was reached that the LD_{50} may vary in great proportion, according to the different types of each bacterium species as well as to the insect species tested.

INTRODUCTION

Insect microbiology and pathology is a scientific subject which has been developing rapidly in recent years. E. A. STEINHAUS

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was the first research worker to conceive of this subject as a new branch of science and to use the above denominations in his works (1, 2, 3).

There are certain publications dealing with the identification of bacterial flora of several insect species (4, 5, 6, 7),

The attention of research workers was drawn to locust diseases for the first time in about 1910, when d'HERELLE observed an epizootic in the population of *Shistocerca paranensis*. He gave the name *Cocobacillus acridiorum* d'Hérelle to the bacterium he isolated from grasshoppers which had died of the epizootic, and he considered this bacteria the cause of the epizootic. In the years immidiately following, much field work was done on this bacterium, and its importance was investigated with respect to its biological control (2).

DU PORTE and VANDERLECK (8) conducted experiments on the pathogenicity of this bacterium on laboratory cultures. Recently, BUCHER (9) identifed the *Cocobacillus acridiorum* d'Hérelle (Culture Nr. 2163) of the Lister Institute as *Cloacae type A* and asserted that this bacterium was not really pathogenic for grasshoppers.

Epizootics similar to those observed in nature among grasshoppers have also been observed in permanent laboratory cultures by a number of research workers (14, 15, 16, 17). *S. marcescens* and *P. aeruginosa* were given as their agents. Laboratory experiments of artificial infection were also carried out with the same bacteria (14, 15, 16, 17).

BUCHER (18), in an article, classified the insect pathogens and described their characteristics. DUTKY (19), dealth with the microorganisms pathogenic for insects from the angle of biological control.

WEISER (20), critisized the recent advances achieved by researches on insect pathology and microbiology and pointed out the special subjects on which research generally concentrates.

The researches carried out on the bacterial flora of acrididae are rather few. BUCHER and STEPHENS (21, 22) described the cha-

racteristics of the bacteria they had isolated from the grasshoppers of western Canada (*M. bilituratus*, *M. bivittatus*, *M. packardii*, and *Camnula pellucida*). BUCHER (23) supplied information concerning the pathogenicity of these bacteria and the percentage of their occurance in grasshoppers.

In the present study the intestinal bacterial flora in *L. migratoria*, which species had never been investigated heretofore, was examined and the pathogenicity of some bacteria for this species was investigated.

The import of this study may be summed up in three points: a) it gives a suggestion as to whether the cultural and the biochemical characteristics of the bacteria isolated are different from those given in the literature on the subject (bacteriological aspect); b) it gives a further suggestion as to whether some of the bacteria can be used in the biological control of *L. migratoria* (biological control aspect); c) it supplies information on the bacteriological ecology.

MATERIAL AND METHOD

The material used for this research was taken from the permanent cultures of nymphs and adults collected in the surroundings of Diyarbakır and reared in the Zoology Department of the Sciences Faculty of Ankara.

One hundred eggs from 20 different coccoons were examined as to sterility. For the purpose of isolating and identifying the intestinal bacterial flora from the laboratory cultures, 100 adult grasshoppers and 150 nymphs were examined.

Field experiments were immediately carried out on 100 adult grasshoppers collected from several rice fields in the surrounding of Diyarbakır, as well as on the intestinal flora of adult individuals of a new culture dating back to material collected from the same place.

Twenty-five adults and 100 nymphs were used for the determination of the total number of bacteria in the intestine.

While the intestinal bacterial flora was investigated, the bacteria described as pathogenic for grasshoppers in literature were

isolated. For the purpose of investigating the pathogenicity of these bacteria for *L. migratoria*, they were injected into the body fluid of the insects, and the median lethal dose for each strain was calculated. In the pathogenicity tests, adult individuals not having reached puberty were used. For each dilution of bacteria between 1 /10 and 1 /1 000 000, 10 individuals were used for injection. These injections were given with an Agla (Beckman, Kent, England) micro injector between the first and second abdominal sternits.

External surface disinfection: the external surface of the grasshopper was disinfected with a 1 /100 solution of mercuric chloride prepared with 70 % alcohol.

To prevent the disinfectant from penetrating into the intestine, the mouth and anus of the grasshopper were closed by immersion into paraffin.

The intestine dissected out of the body under sterile conditions was placed in a small sterile porcelain mortar and triturated with 2 ml of nutrient broth.

Inoculation: A 1 ml sample was taken from the mortar with a sterile pipette and inoculated into nutrient broth and serum broth. As the insects are cold blooded creatures, the tubes, after the incubation of 24 hours at 37° were further incubated at room temperature for two days (1). There upon the broth was inoculated with nutrient agar and blood agar and identification was proceeded to.

In identification, normal bacteriological methods were used.

Effect on carbohyrates and similar compounds: in investigating carbohyrate fermentation, the sugars indicated in Table I were used.

TABLE I.

Monosacc.	Disacc.	Trisacc.	Polisacc.	Sugar alc.	Glycoside
Arabinose	Sucrose	Raffinose	Inuline	Gliserole	Salicine
Ksilose	Maltose		Dextrine	Adonitole	Esculine
Rhamnose	Lactose		Starch	Mannitole	
Glucose	Trehalose				
Fructose	Cellobiose				
Mannose					
Galactose					

The indol, methyl red, Voges-Proscauer, gelatin liquefaction, hidrogen sulphide, Catalase, and Phenylalanine Deaminase tests were carried out according to the methods described in CRUICKSHANK's work (24). For the urease test; the Coleman-Wilson medium, and for the citrare test; Simmon's citrate agar were used. The anaerobic breakdown of amino acids by bacteria was shown by means of a medium to which an indicator was added (25). Of the amino acids, the following were used: L-lysine hydrochloride, L-ornithine monohydrochloride, L-arginine hydrochloride and glutamic acid. For the Potassium cyanide test, the medium described by MΦLLER (26) was employed.

Living bacterium count by the drop plate method: For this purpose, the method described by REED and REED (27) was applied, modified as follows: Tenfold dilutions between 1/10 and 1/1 000 000 were made from a bacterial suspension with saline. The blood agar in Petri dishes was evaporated at 37° for two hours. In this way the spreading as wide as possible of the bacterial suspension dropped, and its absorption by the medium was insured. The bacterial suspension from a micro-injector was dropped as 0.01 ml drops from a height of 2.5 cm. It takes 15 minutes for the medium to absorb the drops completely. After the plates were incubated for 24 hours, the colony produced by each living bacterium was counted, and the total number of the living bacteria in the suspension was calculated.

EXPERIMENTS AND RESULTS

I- THE IDENTIFICATION OF THE BACTERIAL FLORA

A- Investigation of the sterilitiy of egg:

BUCHER (23) states that grasshoppers are sterile at the time of hatching, but soon acquire a bacterial flora; he did not investigate the egg. PRINSLOO (28), in a baceriological investigation of *Locustana pardalina*) (Walk), isolated *Pseudomonas aeruginosa* (Shroeter) Migula and *Flowobacterium deodorans* (Zimmerman) from the bad eggs, but found the normal eggs to be sterile.

As for us, we wished to form an idea concerning the sterility

of eggs by investigating one hundred eggs from the bacteriological standpoints.

After performing surface sterilization, the eggs were triturated in a mortar with 2 ml nutrient broth and inoculated.

One hundred eggs examined by this method were found sterile.

B- Investigation of the bacterial flora of nymphs at different stages:

Nymphs at different stages were examined from the point of view of bacterial flora in order to find out at which stage the acquisition of bacterial flora begins, as well as the difference between the stages with respect to the microorganisms and the percentages thereof they contain.

1– Bacterial flora of nymphs in the first stage:

Of the 25 nymphs examined within the first 24 hours after hatching, 15 were found sterile, whereas from the remaining 10, soil microorganisms belonging to the Bacillaceae family were isolated. The bacteria are distributed in male and female individuals as shown in Table II.

TABLE II.

Distribution of bacteria isolated from male and female nymphs within 24 hours of hatching

Bacteria isolated	Number of nymphs examined			Number of nymphs infected		
	Male	Female	Total	Male	Female	%
Bacillaceae family						
Bacillus subtilis	13	12	25	5	5	40
Bacillus cereus	13	12	25	2	4	24

Twenty five nymphs up to the begining of the second stage after the first 24 hours examined; five of them being found sterile. From the remaining 20, some bacteria belonging to the Bacillaceae family were isolated.

These bacteria are distributed in male and female individuals as shown in Table III.

TABLE III.

Distribution of bacteria isolated from nymphs aged 24 hours after hatching up to second stage

Bacteria isolated	Number of nymphs examined			Number of nymphs infected		
	Male	Female	Total	Male	Female	%
Bacillaceae family						
Bacillus subtilis	13	12	25	8	7	60
Bacillus cereus	13	12	25	4	6	40
Bacillus mycoides	13	12	25	3	2	20

2– Bacterial flora of nymphs in the second stage:

The numbers of bacteria isolated from male and female nymphs in the second stage are given blow (Table IV).

TABLE IV.

Distribution of bacteria isolated from male and female nymphs in the second stage

Bacteria isolated	Number of nymphs examined			Number of nymphs infected		
	Male	Female	Total	Male	Female	%
Enterobacteriaceae family						
Aerobacter aerogenes	12	13	25	10	9	76
Aerobacter cloacae	12	13	25	1	4	20
Escherichia coli	12	13	25	—	1	4
Bacillaceae family						
Bacillus cereus	12	13	25	3	—	12
Bacillus mycoides	12	13	25	2	3	20
Lactobacillaceae family						
Streptococcus faecalis	12	13	25	9	7	64
Strep. liquefaciens	12	13	25	1	3	16
Micrococcaceae family						
Staphylococcus albus	12	13	25	—	2	8

3– Intestinal bacterial flora of nymphs in the third stage:

The distribution of the bacteria forming the intestinal flora in male and female nymphs in this stage is shown in Table V.

TABLE V.

Distribution of bacteria in males and females isolated from nymphs in the third stage

Bacteria isolated	Number of nymphs examined			Number of nymphs infected		
	Male	Female	Total	Male	Female	%
Enterobacteriaceae family						
Aerobacter aerogenes	13	12	25	8	7	60
Aerobacter cloaceae	13	12	25	—	4	16
Bacillaceae family						
Bacillus cereus	13	12	25	5	3	32
Bacillus subtilis	13	12	25	3	—	12
Lactobacillaceae family						
Streptococcus faecalis	13	12	25	8	9	68
Strep. liquefaciens	13	12	25	1	2	12
Micrococcaceae family						
Staphylococcus aureus	13	12	25	2	—	8

As seen in the Table, the bacteria most frequently met are *A. aerogenes* (60 %), and *S. faecalis* (68 %). The Bacillaceae family, which is predominant in nymphs in the first stage, is superseded by the Enterobacteriaceae and Lactobacillaceae families in the second and third stages.

4– Intestinal bacterial flora of nymphs in the fourth stage:

The distribution of the bacteria isolated in male and female nymphs is shown in Table VI.

TABLE VI.

Distribution of bacteria isolated from male and female nymphs in the fourth stage

Bacteria isolated	Number of nymphs examined			Number of nymphs infected		
	Male	Female	Total	Male	Female	%
Enterobacteriaceae family						
Aerobacter aerogenes	13	12	25	10	9	76
Aerobacter cloacae	13	12	25	5	5	40
Escherichia coli	13	12	25	—	1	4
Lactobacillaceae family						
Streptococcus faecalis	13	12	25	10	10	80
Strep. liquefaciens	13	12	25	3	2	20
Strep. salivarius	13	12	25	1	2	12
Bacillaceae family						
Bacillus sphaericus	13	12	25	—	1	4
Micrococcaceae family						
Staphylococcus albus	13	12	25	3	1	16
Micrococcus candidus	13	12	25	2	1	12

5– Intestinal bacterial flora of nymphs in the fifth stage:

The distribution of bacteria forming the intestinal flora in male and female nymphs is shown in Table VII.

TABLE VII.

Distribution of bacteria in males and females isolated from nymphs in the fifth stage

Bacteria isolated	Number of nymphs examined			Number of nymphs infected		
	Male	Female	Total	Male	Female	%
Enterobacteriaceae family						
Aerobacter aerogenes	13	12	25	12	10	88
Aerobater cloacae	13	12	25	9	5	56
Proteus rettgeri	13	12	25	—	2	8
Lactobacillaceae family						
Streptococcus faecalis	13	12	25	10	8	72
Strep. liquefaciens	13	12	25	1	3	16
Bacillaceae family						
Bacillus subtilis	13	12	25	1	1	8
Achromobacteraceae family						
Alcaligenes faecalis	13	12	25	1	1	8
Micrococcaceae family						
Staphylococcus albus	13	12	25	2	1	12

The Tables relating to the intestinal bacterial flora of nymphs in 5 different stages may be summarized as follows: The majority of first stage nymphs were found sterile, from the others only members of the Bacillaceae family were isolated. Beginning from the second stage, the flora becomes qualitatively enriched; in the third stage, there is no substantial change with respect to the second; but in the fourth and fifth stages bacteria absent from the other stages appear.

No significant difference was noticed between the intestinal bacteria of male and female nymphs.

C- Intestinal bacterial flora of adults obtained from laboratory cultures:

The distribution of these bacteria in male and female is shown in Table VIII.

TABLE VIII.

Distribution of bacteria in males and females isolated from adults (LABORATORY CULTURE)

Bacteria isolated	Number of adults examined			Number of adults infected		
	Male	Female	Total	Male	Female	%
Enterobacteriaceae family						
Aerobacter aerogenes	50	50	100	45	42	87
Aerobacter cloacae	50	50	100	20	15	35
Serratia marcescens	50	50	100	1	1	2
Proteus vulgaris	50	50	100	12	9	21
Proteus rettgerii	50	50	100	4	3	7
Klebsiella pneumonia	50	50	100	3	4	7
Escherichia intermedia	50	50	100	1	1	2
Achromobacteraceae family						
Alcaligenes faecalis	50	50	100	11	7	18
Corinebacteriaceae family						
C. pseudodiphtheriticum	50	50	100	3	2	5
Lactobacillaceae family						
Streptococcus faecalis	50	50	100	42	48	90
Streptococcus salivarius	50	50	100	10	4	14
Streptococcus liquefaciens	50	50	100	16	13	29
Streptococcus sp.	50	50	100	1	4	5
Bacillaceae family						
Bacillus mycoides	50	50	100	3	—	3
Bacillus sphaericus	50	50	100	6	3	9
Bacillus vulgatus	50	50	100	—	4	4
Bacillus circulans	50	50	100	4	1	5
Bacillus cereus	50	50	100	11	8	19
Bacillus subtilis	50	50	100	10	7	17
Micrococcaceae family						
Sarcina flava	50	50	100	2	5	7
Staphylococcus albus	50	50	100	10	8	18
Staphylococcus aureus	50	50	100	1	3	4
Micrococcus conglomeratus	50	50	100	7	6	13
Micrococcus candidus	50	50	100	8	9	17
Micrococcus sp.	50	50	100	5	7	12

As shown in this table: With respect to species a significant difference exists to between the bacteria isolated from adults and nymphs. In spite of qualitative wealth observed in the flora of adult grasshoppers as compared to that of nymphs, the percentages of *A. aerogenes*, *A. cloacae* of the Enterobacteriaceae family and *S. faecalis* of the Lactobacillaceae family are approximately the same in adults and nymphs. These families are followed in descending percentage by the Bacillaceae, Micrococcaceae, Achromobacteraceae, Corynobacteriaceae families.

D- Intestinal bacterial flora in adults collected in the fields:

It was thought probable that the grasshoppers reared as laboratory cultures, fed on wheat blades and in touch with only the earth in their cages, would be confronted by bacteria species less abundant and varied than those in nature; therefore, field experiments were carried out in an attempt to discover the difference in intestinal flora between individuals in cultures and in nature. For this purpose, in August 1968, a large number of *L. migratoria* were collected from the rice fields round Diyarbakır, where they abound and examined without getting other food than that which they got in the fields.

The bacteria isolated from 50 male and 50 female adult individuals are shown in Table IX.

As seen in Table IX, *Serratia marcencens*, found in 2 % of laboratory culture adults (Table VIII), was isolated from field individuals in the proportion of 27 %. In addition, bacteria of the *Erwinia* genus, wholely absent from laboratory cultures, were isolated from field individuals in the proportion of 22 %. Furthermore, *Klebsiella ozeanea*, *Escherichia freundii*, *Streptococcus lactis*, *Micrococcus varians*, *Micrococcus caseolyticus* were also found in the intestinal flora of field individuals.

E- Intestinal bacterial flora in adults of a new culture aged 6 months:

Part of the grasshoppers collected from the surrounding of Diyarbakır were taken to the laboratory and fed in a cage for six months until a new generation was reared. In order to make a comparison among 1) the flora of individuals from a new culture, 2) that of field individuals and 3) that of individuals from cultures several years old, intestinal bacteria in 50 adults of this new culture were isolated and classified.

The distribution of these bacteria in male and female individuals is shown in Table X.

As seen in that Table, 24 species of bacteria belonging to 5 families were isolated from that Six-month group. While the *Serratia*

genus of bacteria was found in 27 % of the field individuals, it could not be isolated from individuals collected from the same area and reared for six months. As to the percentage of bacteria of *Erwinia* genus, this fell to 4 %.

TABLE IX.

Distribution of bacteria in males and females isolated from adults (FIELD MATERIAL)

Bacteria isolated	Number of adults examined			Number of adults infected		
	Male	Female	Total	Male	Female	%
Enterobacteriaceae family						
Aerobacter aerogenes	50	50	100	40	45	85
Aerobacter cloacae	50	50	100	27	31	58
Serratia marcescens	50	50	100	10	17	27
Proteus vulgaris	50	50	100	10	15	25
Proteus mirabilis	50	50	100	5	9	14
Proteus rettgeri	50	50	100	8	2	10
Escherichia freundii	50	50	100	4	7	11
Klebsiella ozaenae	50	50	100	3	1	4
Klebsiella pneumoniae	50	50	100	4	6	10
Escherichia coli	50	50	100	3	7	10
Erwinia sp.	50	50	100	10	12	22
Achromobacteraceae family						
Alcaligenes faecalis	50	50	100	10	15	25
Corynebacteriaceae family						
Arthrobacter sp.	50	50	100	3	5	8
Lactobacillaceae family						
Streptococcus faecalis	50	50	100	40	43	83
Streptococcus salivarius	50	50	100	10	8	18
Streptococcus liquefaciens	50	50	100	9	12	21
Streptococcus lactis	50	50	100	4	7	11
Streptococcus sp.	50	50	100	3	1	4
Bacillaceae family						
Bacillus mycoides	50	50	100	4	6	10
Bacillus sphaericus	50	50	100	5	8	13
Bacillus vulgatus	0	50	100	6	3	9
Bacillus circulans	50	50	100	5	5	10
Bacillus cereus	50	50	100	6	5	11
Bacillus subtilis	50	50	100	4	7	11
Micrococcaceae family						
Sarcina flava	50	50	100	2	1	3
Staphylococcus albus	50	50	100	9	10	19
Micrococcus caseolyticus	50	50	100	10	5	15
Micrococcus conglomeratus	50	50	100	7	7	14
Micrococcus varians	50	50	100	3	6	9
Micrococcus candidus	50	50	100	4	5	9

TABLE X.

Distribution of bacteria in adult males and females isolated from SIX-MONTH CULTURE

Bacteria isolated	Number of adults examined			Number of adults infected		
	Male	Female	Total	Male	Female	%
Enterobacteriaceae family						
Aerobacter aerogenes	25	25	50	19	24	86
Aerobacter cloacae	25	25	50	15	14	58
Serratia marcencens	25	25	50	—	—	—
Proteus vulgaris	25	25	50	2	2	8
Proteus mirabilis	25	25	50	3	2	10
Proteus rettgeri	25	25	50	2	2	8
Escherichia freundii	25	25	50	3	4	14
Escherichia coli	25	25	50	2	—	4
Erwinia sp.	25	25	50	1	1	4
Achromobacteraceae family						
Alcaligenes faecalis	25	25	50	4	4	16
Lactobacillaceae family						
Streptococcus faecalis	25	25	50	21	20	82
Streptococcus salivarius	25	25	50	3	5	16
Streptococcus liquefaciens	25	25	50	3	4	14
Streptococcus lactis	25	25	50	3	4	14
Bacillaceae family						
Bacillus sphaericus	25	25	50	2	2	4
Bacillus vulgatus	25	25	50	5	7	24
Bacillus cereus	25	25	50	4	3	14
Bacillus subtilis	25	25	50	3	5	16
Micrococcaceae family						
Sarcina flava	25	25	50	—	1	2
Staphylococcus albus	25	25	50	3	3	12
Staphylococcus aureus	25	25	50	1	—	2
Micrococcus caseolyticus	25	25	50	3	2	10
Micrococcus varians	25	25	50	5	2	14
Micrococcus conglomeratus	25	25	50	7	8	30
Micrococcus candidus	25	25	50	6	—	12

II- IDENTIFICATION AND CLASSIFICATION OF THE BACTERIA

The bacteria isolated in the course of this study have been classified according to their morphological, cultural and biochemical characteristics. Their serologies have not been taken into consideration. The classification was carried out according to the plan adopted in Bergey's Manual of Determinative Bacteriology

TABLE XI

ENTEROBACTERIACEAE FAMILY

Biochemical Reactions of the Isolated Types Belonging to *Aerobacter* and *Klebsiella* Genera

	Aerobacter Genus						*Klebsiella* Genus	
	A. aerogenes			*A. cloacae*			*K. pneumoniae*	*K. ozaena*
	Type I	Type II	Type III	Type I	Type II	Type III		
Glucose	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
Lactose	⊗	⊗	⊗	⊗	⊗	(+)	+	—
Maltose	⊗	⊗	⊗	⊗	⊗	⊗	+	⊗
Mannnitol	⊗	⊗	⊗	⊗	—	⊗	+	⊗
Sucrose	⊗	⊗	⊗	⊗	—	—	+	⊗
Arabinose	⊗	⊗	⊗	⊗	⊗	⊗	+	⊗
Trehalose	⊗	⊗	⊗	⊗	⊗	⊗	⊗	—
Raffinose	⊗	⊗	⊗	⊗	⊗	⊗	⊗	⊗
Dulticol	—	⊗	—	—	—	⊗	—	—
Sorbitol	⊗	⊗	⊗	⊗	—	⊗	+	—
Salicin	⊗	⊗	⊗	⊗	⊗	⊗	+	—
Dextrin	⊗	⊗	—	⊗	—	⊗	—	—
Galactose	⊗	⊗	⊗	⊗	⊗	⊗	⊗	+
Rhamnose	⊗	⊗	⊗	⊗	—	⊗	+	+
İnositol	—	⊗	⊗	⊗	—	⊗	+	—
Starch	⊗	⊗	⊗	—	+	—	+	⊗
Glycerol	⊗	⊗	⊗	+	+	(+)	—	—
Mannose	⊗	⊗	⊗	⊗	⊗	⊗	+	—
Inulin	—	⊗	—	—	—	⊗	—	—
Esculin	⊗	⊗	⊗	⊗	—	—	+	+
Cellobiose	⊗	⊗	⊗	⊗	⊗	⊗	+	+
Lysine	—	—	—	—	—	—	+	+
Arginine	+	+	+	+	+	+	—	—
Ornithine	+	+	+	—	+	+	—	—
G. acid	—	—	—	+	—	—	—	—
Gelatin	—	—	—	+	+	(+)	—	—
Citrate	+	+	+	+	+	+	+	—
Urease	—	—	+	+	—	+	—	—
H_2S	—	—	—	—	—	—	—	—
Indole	—	—	—	—	—	—	—	—
V. P	+	+	+	+	+	(+)	+	—
M. R	—	—	—	—	—	—	—	—

KEY: ⊗, acid and gas from carbohydrates; +, reaction positive, acid only; (+), reaction weak or delayed; —, reaction negative

ENTEROBACTERIACEAE FAMILY

Biochemical Reactions of the Isolated Types Belonging to *Escherichia* and *Serratia* Genera

Medium	*Escherichia* Genus				*Serratia* Genus	
	E. coli				*Serratia marcescens*	
	Type I	Type II	*E. freundii*	*E. intermedia*	Type I	Type II
Glucose	⊗	⊗	⊗	⊗	⊗	⊗
Lactose	⊗	⊗	(+)	+	—	—
Maltose	⊗	⊗	⊗	⊗	⊗	+
Mannitol	⊗	⊗	⊗	⊗	⊗	+
Sucrose	⊗	—	—	+	⊗	⊗
Arabinose	⊗	⊗	⊗	⊗	—	—
Trehalose	⊗	—	⊗	⊗	⊗	⊗
Raffinose	—	⊗	⊗	+	—	—
Dulcitol	⊗	—	—	—	—	—
Sorbitol	⊗	—	—	+	+	+
Salicin	—	+	⊗	⊗	+	+
Dextrin	—	—	—	⊗	—	+
Galactose	⊗	⊗	—	⊗	+	+
Rhamnose	⊗	—	⊗	⊗	—	—
Inositol	—	—	—	—	+	—
Starch	+	—	+	+	+	—
Glycerol	⊗	⊗	⊗	—	+	+
Mannose	⊗	+	—	+	+	+
Inulin	—	—	—	+	+	—
Esculin	—	⊗	⊗	—	—	—
Cellobiose	⊗	—	+	—	+	—
Lysine	+	+	+	+	+	+
Arginine	+	+	+	+	—	—
Orhitnine	+	+	+	+	+	+
G. acid	+	—	+	—	—	—
Gelatin	—	—	—	—	+	+
Citrate	—	—	+	+	+	+
Urease	—	—	(+)	+	+	+
H_1S	—	—	+	—	—	—
Indole	+	+	—	—	—	—
V. P	—	—	—	—	+	+
M. R	+	+	+	—	—	—
KCN	—	—	—	—	(+)	(+)
P. A. D.	—	—	—	—	—	—

The key is the same as the key in table XI

(29). Furthermore, in the identification of the Enterobacteriaceae family, the following publications were used: EDWARDS and EWING (30); EWING et alii (31).

The biochemical characteristics of all the bacteria isolated in the preceding parts of this study (B. C. D. E) have been given in the Tables in this part.

TABLE XIII.

ENTEROBACTERIACEAE AND ACHROMOBACTERACEAE FAMILIES

Biochemical Reactions of the Isolated Types Belonging to *Proteus*, *Erwinia* and *Alcaligenes* Genera

	Proteus Genus			*Erwinia* Genus	*Alcaligenes* Genus
Medium	*P. vulgaris*	*P. mirabilis*	*P. rettgeri*	*Eriwinia sp*	A. *facealis*
Glucose	⊗	+	+	+	—
Lactose	—	—	—	—	—
Maltose	⊗	—	—	+	—
Mannitol	—	—	+	+	—
Sucrose	—	—	+	+	—
Arabinose	—	—	—	+	—
Trehalose	—	—	—	+	—
Raffinose	—	—	—	—	—
Dulcitol	—	—	—	—	—
Sorbitol	—	—	—	—	—
Salicin	—	—	+	+	—
Dextrin	—	—	—	—	—
Galactose	⊗	⊗	+	+	—
Rhamnose	—	—	+	+	—
Inositol	—	—	+	—	—
Starch	—	—	—	—	—
Glycerol	+	+	+	—	—
Mannose	—	—	+	+	—
Inulin	—	—	—	—	—
Esculin	—	—	+	—	—
Cellobiose	+	—	—	—	—
Lysine	—	—	—	—	—
Arginine	—	—	—	—	—
Ornithine	—	+	—	—	—
G. asid	—	(+)	+	—	—
Gelatin	+	+	—	—	—
Citrate	—	—	+	+	+
Urease	+	+	+	—	—
H_2S	+	+	—	—	—
Indole	+	—	+	—	—
V.P	—	—	—	+	—
M.R	+	+	—	+	—
KCN	+	+	+	+	—
P.A.D	+	+	+	—	—

The key is the same as the key in table XI.

TABLE XIV

LACTOBACILLACEAE FAMILY

Biochemical Reactions and Growth Characteristics of the Isolated Types Belonging to *Streptococcus* Genus

Medium	*Streptococcus* Genus								
	Enterococ group					*Viridans* group		*Lactic* group	
	S. faecalis			*S. liquefaciens*		*S. salivarius*		*S. lactis*	*S. sp*
	Type I	Type II	Type III	Type I	Type II	Type I	II		
Salt broth 6,5 %	+	—	+	+	—	—	—	—	—
Broth pH 9.6	+	+	+	+	+	—	—	—	—
+10°C	—	+	+	+	+	—	—	+	—
+45°C	+	+	—	+	—	+	—	—	—
Methylene blue milk	+	+	+	+	—	+	+	+	—
Litmus milk (reduction)	+	+	+	+	+	+	+	+	—
Litmus milk (peptonis)	+	+	—	+	+	+	+	+	—
Hemolysis	A	—	A	—	A	—	A	A	A
Gelatin	—	—	—	+	+	—	—	—	—
Trehalose	—	+	+	+	—	+	+	—	—
Sorbitol	—	—	+	—	—	—	—	—	—
Glycerol	—	+	—	+	+	—	—	—	—
Mannitol	+	+	+	+	+	—	+	+	+
Lactose	+	+	—	+	+	+	+	+	—
Sucrose	—	+	+	+	+	+	+	—	—
Raffinose	—	—	+	—	+	+	+	—	—
Salicin	+	+	+	+	+	+	—	+	+
Inulin	—	—	—	—	—	+	+	—	—
Esculin	+	+	—	—	—	+	+	+	—

Key: +, Growth positive, acid from carbonhydrates; —, growth negative; A, alpha hemolysis.

TABLE XV

MICROCOCCACEAE FAMILY

Biochemical Reactions of the Isolated Types Belonging to *Micrococcus*, *Staphylococcus* and *Sarcina* Genera

Medium	*Micrococcus*					*Staphylococcus*		*Sarcina*
	M. candidus	*M. Conglomeratus*	*M. varians*	*M. caseolyticus*	*M. sp*	*S. aureus*	*S. albus*	*S. flava*
Glucose	+	+	+	+	—	+	+	—
Lactose	+	+	+	+	—	+	—	—
Maltose	+	+	+	+	—	+	+	—
Mannitol	+	—	+	+	—	+	—	—
Sucrose	+	+	+	+	—	+	+	—
Arabinose	+	+	+	+	—	—	—	—
Trehalose	+	+	+	+	—	—	—	—
Raffinose	+	+	+	—	—	—	—	—
Dulcitol	—	—	—	—	—	—	—	—
Sorbitol	—	—	—	—	—	+	+	—
Salicin	+	—	—	—	—	—	—	—
Dextrin	—	—	—	—	—	—	—	—
Galactose	+	+	—	+	—	+	+	—
Rhamnose	—	—	—	—	—	—	—	—
Inositol	—	—	—	—	—	—	—	—
Starch	—	—	+	—	—	—	—	—
Glycerol	+	—	—	+	—	+	+	—
Mannose	+	+	+	+	—	—	+	—
Inulin	+	+	—	—	—	—	—	—
Esculin	+	+	—	—	—	—	—	—
Cellobiose	+	—	—	—	—	—	—	—
Urease	—	—	—	+	—	—	—	—
Citrate	+	+	+	+	—	—	—	—
V.P	(+)	—	—	—	—	—	—	—
M.R	—	—	—	—	—	—	—	—
Indol	—	—	—	—	—	—	—	—
Lysine	—	—	—	—	—	—	—	—
Arginine	—	—	—	—	—	—	—	—
G. acid	—	—	—	—	—	—	—	—
P.A.D	—	—	—	—	—	—	—	—
Catalase	+	+	+	+	—	+	+	—
Gelatin	+	(+)	—	+	—			
Litmus milk	—	+	+	+	+	+	+	—

Key: +, reaction positive, acid only; (+), reaction weak or delayed; —, reaction negative.

TABLE XVI

BACILLACEAE FAMILY

Biochemical Reactions of the Isolated Types Belonging to *Bacillus* Genus

Medium	*Bacillus Genus*						
		B. cereus					
	B. subtilis	Type I	Type II	*B. circulans*	*B. vulgatus*	*B. sphaericus*	*B. mycoides*
Glucose	+	+	+	+	+	—	+
Lactose	—	—	—	—	—	—	—
Maltose	+	+	+	+	—	—	+
Mannitol	+	—	—	+	—	—	—
Sucrose	—	—	+	+	—	—	+
Arabinose	+	—	—	+	—	—	—
Trehalose	+	+	+	+	—	—	+
Raffinose	—	—	—	+	—	—	—
Dulcitol	—	—	—	—	—	—	—
Sorbitol	—	—	—	—	—	—	—
Salicin	—	+	+	+	—	—	+
Dextrin	—	—	—	+	—	—	—
Galactose	—	—	+	+	—	—	—
Rhamnose	—	—	—	—	—	—	—
Inositol	—	+	—	—	—	—	—
Starch	+	+	+	+	—	—	+
Glyserol	—	+	+	+	—	—	+
Mannose	—	—	—	+	—	—	+
Inulin	—	—	—	+	—	—	—
Esculin	+	+	+	+	—	—	+
Cellobiose	—	—	+	+	—	—	—
Lysine	—	—	—	—	—	—	—
Arginine	+	+	+	—	—	—	+
Ornithine	—	—	—	—	—	—	—
G. acid	—	—	—	+	—	—	—
Gelatin	+	+	+	+	—	—	+
Citrate	+	+	+	—	+	—	+
Urease	—	—	—	—	+	+	—
H_2S	—	—	—	—	—	—	—
Indole	—	—	—	—	—	—	—
V.P	+	+	+	+	+	—	+
M.K.	—	—	+	—	—	—	—
Catalase	+	+	+	+	+	+	+

KEY: +, reaction positive, acid only; —, reaction negative.

III- THE COUNTING OF THE INTESTINAL BACTERIA OF L.MIGRATORIA

BUCHER showed, the grasshoppers carry bacteria only in their bowels, in other words, under normal conditions, blood and all organs are sterile (23).

According to STEINHAUS; bacteria taken with food into the bowels and which can best adapt to intestinal conditions and find there facilities for proliferation, form the bacterial flora of insects. Doubtless the proliferation of the bacteria in the bowels is limited. Though the limiting factors are not known precisely, the following factors are likely to play an important part: feeding under non-optimal conditions, acidity, the existence of anaerobic conditions, certain characteristics of intestinal liquids and the dilution coused by feeding. The presence of too many bacteria in the bowels is a sign that the phsiological state of the insect is abnormal. Such abnormalities stop the mechanism of control of bacterial proliferation, and this leads to the death of the insect (2).

In this division of our study, the intestinal bacteria were counted in 25 adults and 20 nymphs in each stage in order to find out between what limits the total of intestinal bacteria varies.

For the counting of bacteria, the drop plate method described in "Material and Method" was used.

A- Number of intestinal bacteria in nymphs in different stages:

As seen in table XVII, both the minima and the maxima of bacteria are directly proportional to the size (stage) of the nymphs. A large number of nymphs in the first stage were found sterile. We may suppose that in those nymphs the number of bacteria is too small for counting by drop plate method. In the first-stage nymphs the bacteria numbered: 0–13 000, The successive stage showing the following numbers: 3000–900 000; 9000–2 100 000; 200 000–11 000 000; 300 000–21 000 000.

B- Number of intestinal bacteria in adults:

As shown in table XVII, the number of intestinal bacteria in

TABLE XVII.

Number of intestinal Bacteria in Adults and Nymphs of *L. migratoria*

First stage	Second stage	Third stage	Fourth stage	Fifth stage	Adult (male)	Adult (female)
Sterile	8 000	1 000 000	1 300 000	5 500 000	10 000 000	21 000 000
2 000	20 000	240 000	3 700 000	3 320 000	320 000	2 300 000
13 000	15 000	800 000	11 000 000	10 000 000	50 000 000	1 500 000
Sterile	370 000	1 200 000	1 500 000	800 000	28 000 000	38 000 000
Sterile	48 000	300 000	890 000	12 000 000	920 000	3 700 000
1 800	260 000	580 000	4 300 000	19 000 000	2 000 000	12 300 000
1 000	16 000	630 000	340 000	2 000 000	1 600 000	90 000 000
Sterile	215 000	580 000	2 000 000	2 100 000	560 000	8 200 000
2 500	900 000	1 500 000	1 200 000	1 000 000	13 000 000	320 000
5 000	165 000	310 000	1 600 000	4 300 000	3 300 000	400 000
Sterile	510 000	270 000	400 000	300 000	4 300 000	42 000 000
Sterile	3 000	1 230 000	690 000	5 600 000	5 600 000	67 000 000
5 000	12 000	3 120 000	1 100 000	8 200 000	22 000 000	
10 000	50 000	700 000	520 000	920 000		
Sterile	66 000	2 100 000	380 000	5 200 000		
1 900	7 500	1 000 000	420 000	21 000 000		
32 200	11 000	900 000	200 000	16 000 000		
Sterile	25 000	30 000	1 000 000	4 200 000		
Sterile	13 000	9 000	800 000	7 800 000		
1 100	4 000	50 000	1 300 000	2 200 000		

adults varies between 320 000 and 90 000 000. STEINHAUS (2) and BUCHER (23) asserted and our study confirms that among healthy individuals there are great differences in respect to bacteria numbers.

IV PATHOGENICITY TESTS

In this part of our study, the pathogenic effect of certain bacteria on *L. migratoria* was investigated and the median lethal dose (LD_{50}) was calculated for each bacterium. The experiments were carried out by injecting varying dosages of bacteria into the body fluid. Some of the bacteria experimented on were those isolated from intestinal flora (*S. marcescens*, *P. vulgaris*, *P. mirabilis*, *P. rettgeri*, and *Bacillus cereus*). Others were those bacteria (*P. aeruginosa*, chromogenic strain of *S. marcescens*) which were never observed in *L. migratoria*. These were taken from the culture collections of the Central Institute of Hygiene - Ankara.

For the pathogenicity tests young adults below puberty were used. Fifth-stage nymphs were segregated in small cages and subjected to the test as soon as they had reached adult stage.

The number of bacteria present in 0.01 ml of bacterial suspension were counted by Drop plate method, 10 grasshoppers being used for each bacteria dilution between 1 /10 and 1 /1 000 000, and observed for fifteen days. On the other hand, 0.01 ml saline was injected into 10 grasshoppers kept in a different cage as a control group.

From the individuals found dead during the inspection carried out once every 12 hours, bacterial cultures were made to find out the cause of death.

The median lethal dose was calculated according to the Reed and Muench method (32, 33).

a- **Tests with** *Serratia marcescens:*

The two types were isolated from either the laboratory cultures or the field material (Table XII).

Type I: 1 /10 - 1 /1 000 000 dilutions were prepared from suspension containing 220 000 000 bacteria per ml and injected in a dose of 0.01 ml into the body fluids of young adults.

After injection, the following facts were observed: The individuals that received high doses ceased to take food 5–6 hours after the injection and stayed motionless on same spot for a long time. After death, the abdomen of the insect assumed a darker colour, its tissues became soft and sticky and it was observed that when the grasshopper was grasped by the head with a pincer, the abdomen fell apart. Similar facts were observed also by STEVENSON (12).

220 000 - 2 bacteria were injected into grasshopper groups of 10 each.

The numbers of individuals found dead or alive within 15 days, are shown in Figure I.

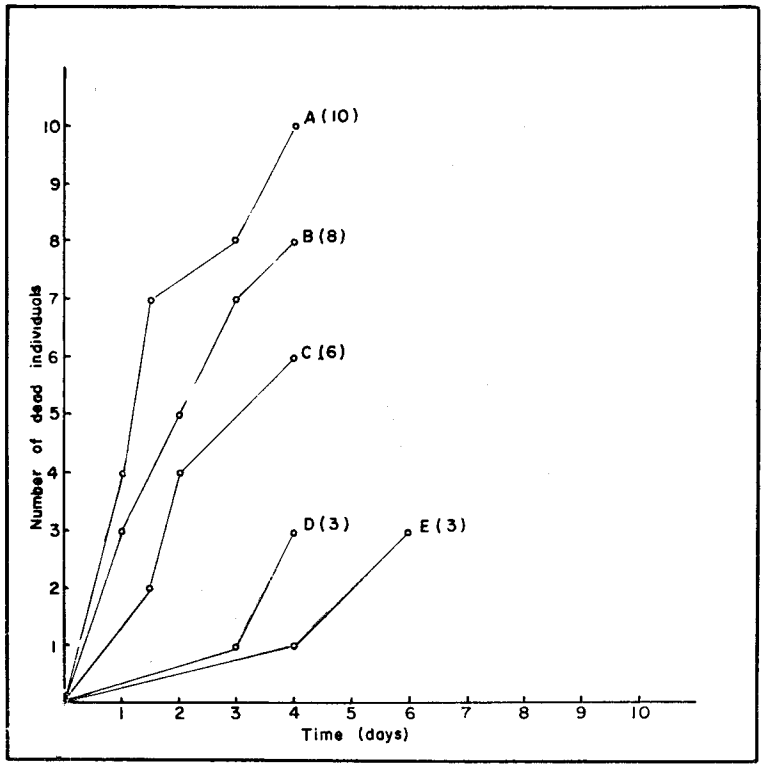

Figure I- Distribution over the days of the number of dead individuals in *L. migratoria* groups infected with *S. marcescens*

A: 1/10 dilution, B: 1/100 dilution, C: 1/1000 dilution D: 1/10 000 dilution, E: 1/100, 000 dilution

(In each test series 10 adults were used. The figures in parentheses indicate the number of dead individuals)

TABLE XVIII.

Death Percentages of Adult Grasshoppers Infected with Different Dilutions of S. *marcescens* (Type I)

Bacteria dilution	Mortality Ratio	Died	Survived	Accumulated Values			
				Died (D)	Survived (S)	Mortality Ratio	per. cent. $\frac{(D)}{(D+S)} \times 100$
10^{-1}	10/10	10	0	30	0	30/30	100
10^{-2}	8/10	8	2	20	2	20/22	90
10^{-3}	6/10	6	4	12	6	12/18	66
10^{-4}	3/10	3	7	6	13	6/19	31
10^{-5}	3/10	3	7	3	20	3/23	13
10^{-6}	0/10	0	10	0	30	0/30	0

From total death percentages corresponding to the dilutions shown in Table XVII the LD_{50} was calculated.

The LD_{50} found for nonchromogenic *S. marcescens* (Type I) is 1909 bacteria.

Type II- Though the two types of *S. marcescens* isolated in this study are biochemically very similar, the supposition that there might be a difference in virulence between them led us to investigate the pathogenic effect of Type II also. The LD_{50} of this Type was found to be 2016 bacteria, a figure near that found for Type I.

Tests carried out with the *S. marcescens* collection culture: The results of the tests carried out with the two types of nonchromogenic *S. marcescens* isolated in the course of our study being very different from those found by BUCHER, we felt the necessity of also testing a typical collection culture. For this purpose *S. marcescens* Nr. A 173 from the collection of the R. S. Central Institute of Hygiene was used. This culture was obtained by the Institute of Hygiene from the Pasteur Institute in Paris.

Of this bacterium, the initial suspension containing 240 000 000 bacteria per ml was used. Tenfold dilutions were made of this suspension, and doses containing 240 000 - 2 bacteria in 0.01 ml were injected into the grasshoppers. The distribution of the number of individuals which died in each group over the days is shown as follows (Fig. 2).

From the total death percentages corresponding to the different dilutions shown in Table XIX, the LD_{50} was calculated. This was found to be 2274 bacteria This result being close to that found for Type I and Type II, they confirm each other.

b- **Tests carried out with *Pseudomonas aeruginosa* culture Nr. 649.**

No member of Pseudomonas family was found among the bacteria forming the intestinal flora of *L. migratoria.*

P. aeruginosa is foremost among the bacteria described in literature as having a pathogenic effect on grasshoppers (13, 16,

17, 23). That is why we found it interesting to investigate its pathogenicity for *L. migratoria*. For this purpose we used for our tests the typical *P. aeruginosa* culture Nr. 649 obtained from the R. S. Central Institute of Hygiene. This culture was obtained from the Staten Serum Institute in Copenhagen.

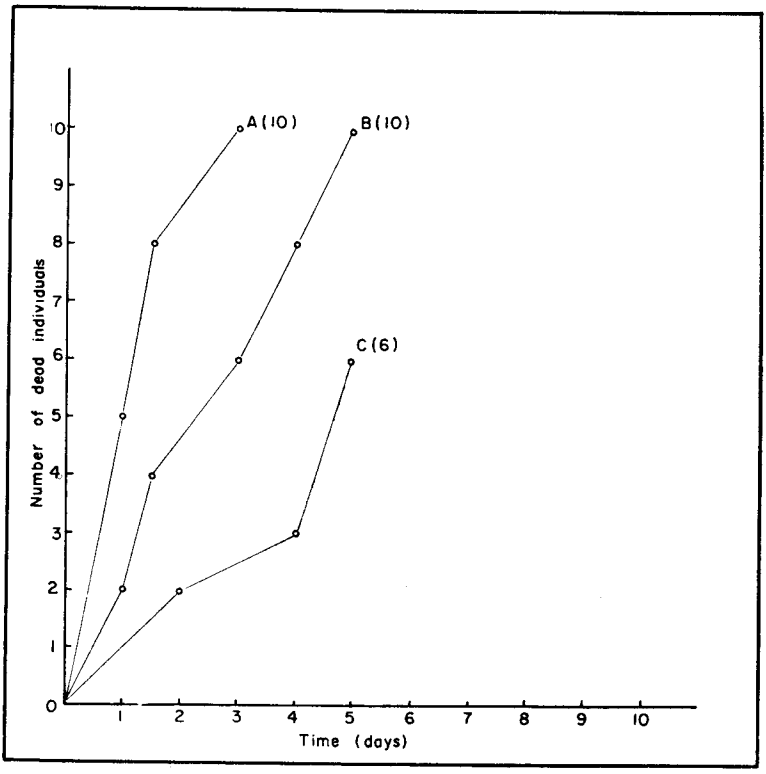

Figure 2– Distribution over the days of the number of dead individuals in *L. migratoria* groups infected with *S. marcescens* (A.173)

The grasshoppers infected with this bacterium do not show any sign of infection until a short time before death. In the dead grasshoppers the abdomen assumes a slightly reddish colour, the body loses its normal consistency, the tissues rot in a short time and the parts of the body are transformed into a sticky liquid. The dead grasshoppers acquire a typical smell of *P. aeruginosa*.

TABLE XIX.

Death Percentages of Adult Grasshoppers Infected with Different Dilutions of Chromogenic *S. marcescens* (A. 173)

Bacteria dilution	Mortality Ratio	Died	Survived	Accumulated Values			
				Died (D)	Survived (S)	Mortality Ratio	Per. cent. $\frac{(D)}{(D+S)} \times 100$
10^{-1}	10/10	10	0	26	0	26/26	100
10^{-2}	10/10	10	0	16	0	16/16	100
10^{-3}	6/10	6	4	6	4	6/10	60
10^{-4}	0/10	0	10	0	14	0/14	0
10^{-5}	0/10	0	10	0	24	0/24	0
10^{-6}	0/10	0	10	0	34	0/34	0

Of this bacterium the initial suspension containing 250 000 000 per ml was prepared and dilutions 1/10 - 1/1 000 000 were made. In a 0.01 ml each solution, 250 000 - 2 bacteria are to be found.

The number of individuals living and dead within 15 days were shown in Fig. 3.

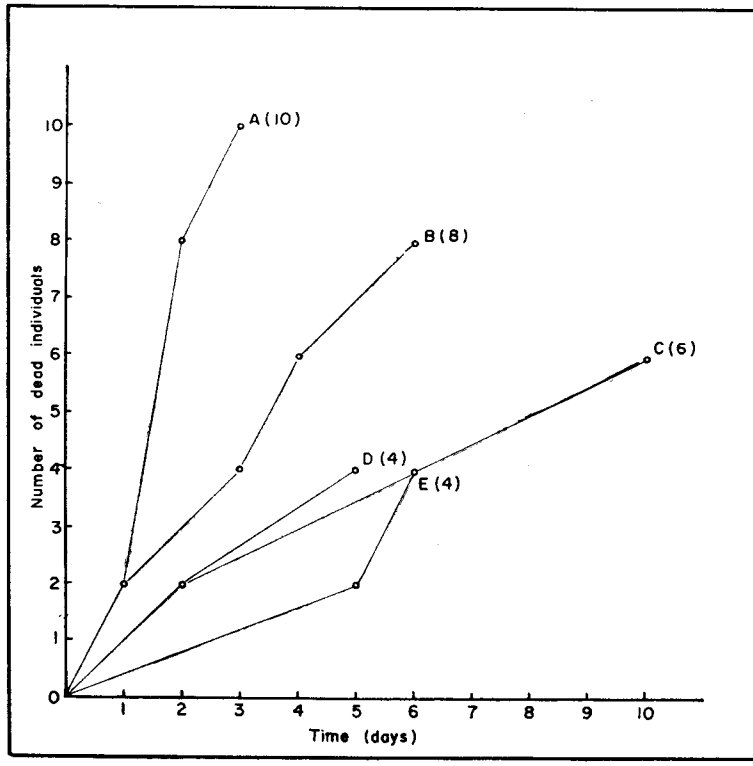

Figure 3- Distribution over the days of the number of dead individuals in *L. migratoria* groups infected with *P. aeruginosa*

From the total death percentages corresponding to the different dilutions shown in Table XX, the LD_{50} was calculated.

The LD_{50} of *P. aeruginosa* was found to be 2046 bacteria.

c- **Tests with** *Proteus vulgaris*:

The types isolated from *L. migratoria* were used in the pathogenicity tests carried out with the genus Proteus. Also in grasshop-

TABLE XX.

Death Percentages of Adult Grashoppers Infected with Different Dilutions of *P. aeruginosa*

Bacteria Dilution	Mortality Ratio	Died	Survived	Accumulated Values			
				Died (D)	Survived (S)	Mortality Ratio	Per. cent. $\frac{(D)}{(D+S)} \times 100$
10^{-1}	10/10	10	0	32	0	32/32	100
10^{-2}	8/10	8	2	22	2	22/24	91
10^{-3}	6/10	6	4	14	6	14/20	70
10^{-4}	4/10	4	6	8	12	8/20	40
10^{-5}	4/10	4	6	4	18	4/22	18
10^{-6}	0/10	0	10	0	28	0/28	0

pers infected with this bacterium, no sign of infection was appeared until a short time before death. In the last moments, a slowing down in the movements of the insect was noticed, and it was seen that it had stopped feeding. Dead grasshoppers soon rot, and the characteristic smell of the proteus genus is produced.

Of this bacterium, the initial suspension 100 000 000 bacteria per ml was used. Tenfold dilutions were made of this suspension, and doses containing 100 000 - 1 bacteria in 0.01 ml were injected into grasshoppers. The distribution of the number of individuals which died in each group over the days is shown as follows (Fig. 4).

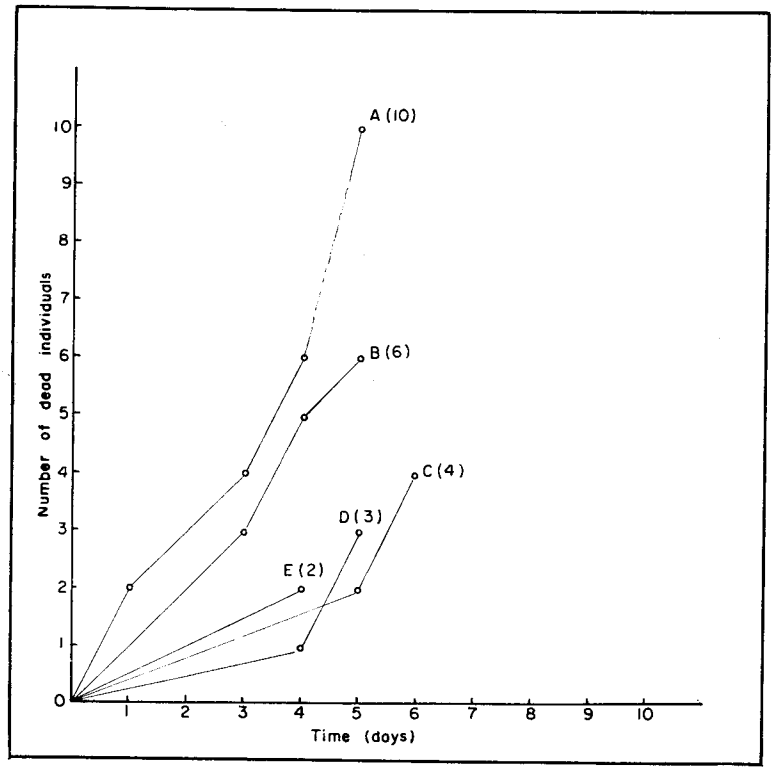

Figure 4– Distribution over the days of the number of dead individuals in *L. migratoria* groups infected with *P. vulgaris*

From the total death percentages corresponding to the different dilutions shown in Table XXI, the LD_{50} was calculated. This was found to be 6888 bacteria.

TABLE XXI.

Death Percentages of Adult Grasshoppers Infected with Different Dilutions of *P. vulgaris*

Bacteria Dilution	Mortality Ratio	Died	Survived	Accumulated Values			
				Died (D)	Survived (S)	Mortality Ratio	Per. cent. $\frac{(D)}{(D+S)} \times 100$
10^{-1}	10/10	10	0	25	0	25/25	100
10^{-2}	6/10	6	4	15	4	15/19	78
10^{-3}	4/10	4	6	9	10	9/19	47
10^{-4}	3/10	3	7	5	17	5/22	22
10^{-5}	2/10	2	8	2	25	2/27	7
10^{-6}	0/10	0	10	0	35	0/35	0

d- Tests with Proteus mirabilis:

In the test the initial suspension containing 260 000 000 bacteria per ml was used, and tenfold dilutions were prepared. 260 000 - 2 bacteria were injected into grasshopper groups of 10 each. The numbers of individuals found dead or alive within 15 days are shown in Figure 5.

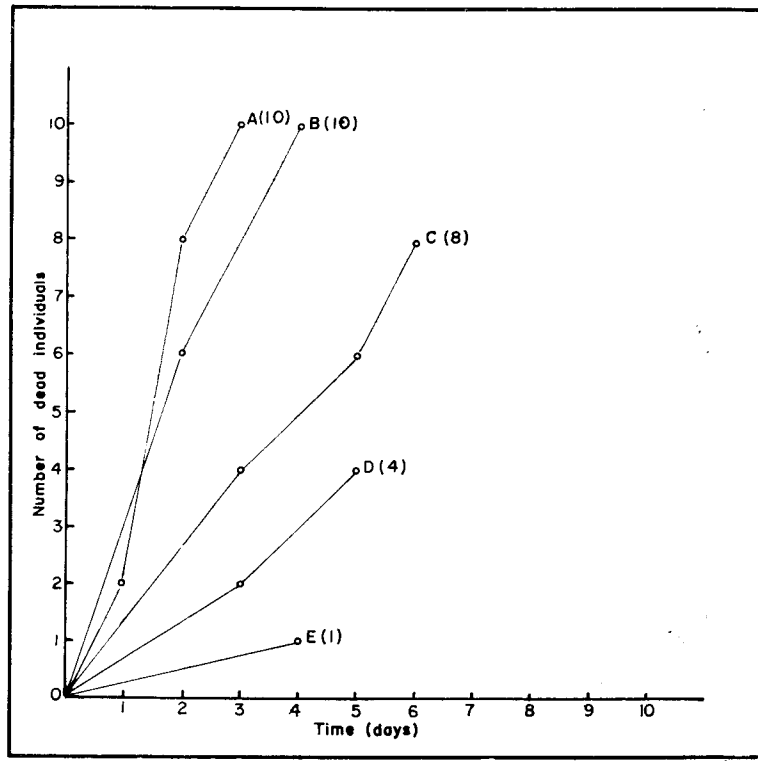

Figure 5– Distribution over the days of the number of dead individuals in *L. migratoria* groups infected with *P. mirabilis*

From the total death percentages corresponding to the different dilutions shown in Table XXII, the LD_{50} was calculated. This was found to be 2099 bacteria.

TABLE XXII.

Death Percentages of Adult Grasshoppers Infected with Different Dilutions of *P. mirabilis*

Bacteria Dilution	Mortality Ratio	Died	Survived	Accumulated Values			
				Died (D)	Survived (S)	Mortality Ratio	Per. cent. $\frac{(D)}{(D+S)} \times 100$
10^{-1}	10/10	10	0	33	0	33/33	100
10^{-2}	10/10	10	0	23	0	23/23	100
10^{-3}	8/10	8	2	13	2	13/15	80
10^{-4}	4/10	4	6	5	8	5/13	38
10^{-5}	1/10	1	9	1	17	1/18	5
10^{-6}	0/10	0	10	0	27	0/27	0

e- **Tests with** *Proteus rettgeri*:

Of this bacterium, the initial suspension containing 140 000 000 bacteria per ml was used. Tenfold dilutions were made of this suspension, and doses containing 140 000 - 1 bacteria in 0.01 ml were injected into grasshoppers. The distribution of the number of indivuduals which died in each group over the days is shown as follows (Fig. 6).

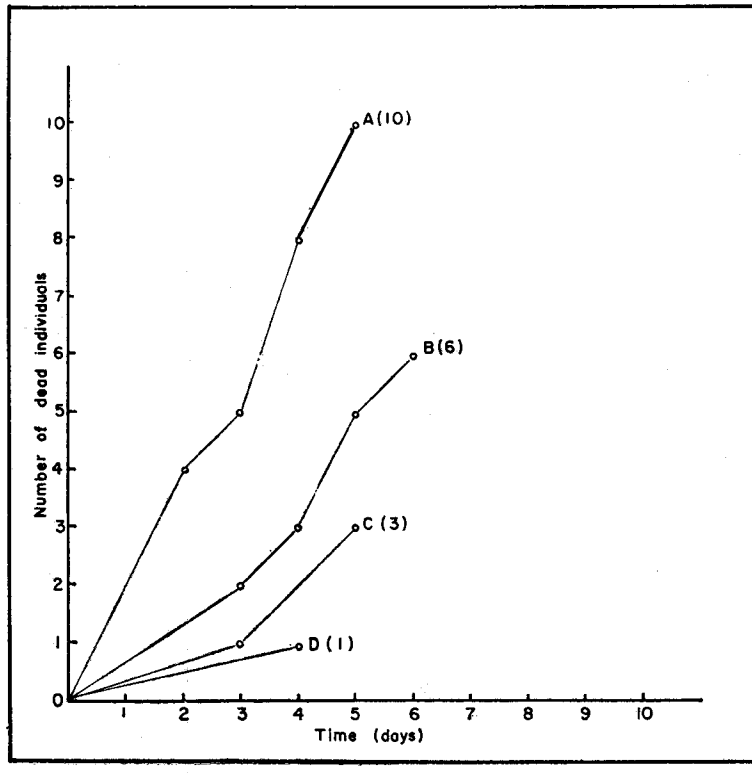

Figure 6– Distribution over the days of the number of dead individuals in *L. migratoria* groups infeted with *P. rettgeri*

From the total death percentages corresponding to the different dilutions shown in Table XXIII, the LD_{50} was calculated. This was found to be 11 341 bacteria.

TABLE XXIII.

Death Percentages of Adult Grasshoppers Infected with Different Dilutions of *P. rettgeri*

Bacteria Dilutions	Mortality Ratio	Died	Survived	Accumulted Values			
				Died (D)	Survived (S)	Mortality Ratio	Per. cent. $\frac{(D)}{(D+S)} \times 100$
10^{-1}	10/10	10	0	20	0	20/20	100
10^{-2}	6/10	6	4	10	4	10/14	71
10^{-3}	3/10	3	7	4	11	4/15	26
10^{-4}	1/10	1	9	1	20	1/20	5
10^{-5}	0/10	0	10	0	30	0/30	0
10^{-6}	0/10	0	10	0	40	0/40	0

DISCUSSION

According to our knowledge, until BUCHER and STEPHENS (21, 22) published their research concerning West Canada's grasshoppers (*M. bilituratus*, *M. bivittatus*, *M. packardii* and *Camnula pellucida*), no study existed on the bacterial flora of the Acrididae.

For the first time the intestinal bacterial flora of *L. migratoria* in all nymph stages and in adults, both in laboratory cultures and individuals collected from the field, has been studied by us in detail.

A total of 400 nymphs and adults was used as material in this research and 1565 bacterial cultures were isolated. These cultures from 33 various bacterial species belonged to 6 families.

One hundred eggs taken from 20 coccoons have been examined and all found sterile.

The eggs being sterile, the nymphs from them must also be sterile at the moment of hatching. Sixty per. cent of the 24-hour-old nymphs were actually found sterile. These data confirm BUCHER's (23) In 40 % of these nymphs, only members of the Bacillaceae family (*B. subtilis*, *B. cereus*) were isolated. Twenty per cent of the nymphs examined within the period from the age of 24 hours to the second stage were found sterile, whereas, bacteria belonging to the Bacillaceae family were isolated from 80 % of them.

In the second stage, the flora comprises species of greater variety. In this stage, the place occupied by the Bacillaceae family in the first stage was taken by *A. aerogenes* of the Enterobacteriaceae family (76 %) and *S. faecalis* of the Lactobacillaceae family (64 %).

The flora in passing to third stage does not exhibit a great change, but new species appear in the intestinal bacterial flora of the fourth and fifth stages. Here, also, *A. aerogenes* and *S. faecalis* continue to predominate (Table V, VI, VII).

Despite the fact that immediately after hatching the nymphs had been first infected with individuals of the Bacillaceae family, it was interesting to note that, in the further stages, these bacteria

gradually decreased in number, whereas the proportion of nymphs infected with *S. faecalis* and *A. aerogenes* of the Enterobacteriaceae and Lactobacillaceae families increased by 60 – 88 %. This fact, in our opinion may be explained as follows: The nymphs, when hatching are infected by earth microorganisms present on the surface of the egg. But only those bacteria taken into the bowels with the food in the later stages that are best adapted to intestinal conditions and find their facilities for proliferation come to constitute the bacterial flora of the insects.

A significant difference exists, in respect to variety of species, between bacteria isolated from adult grasshoppers and nymphs, but this may be due to the shortness of the time spent by the nymphs in each stage and the smaller number of bacterial species with which they are confronted during this limited period. *A. aerogenes*, *A. cloacae* of the enterobacteriaceae family and *S. faecalis* of the Lactobacillaceae were found to predominate in the adults and all the nymphs from the second stage on. The Micrococcaceae, Bacillaceae, and Achromobacteraceae families ranked next. These data of ours coincide with those found by BUCHER and STEPHENS (21, 22) and BUCHER (23) in the other species of the Acrididae. SHREWSBURY and BARSON (6) stated that the most widespread group among the intestinal bacteria of *Blattella germanica* are Gram negative bacilli. STEINHAUS (2) generalizes this fact as follows: "Perhaps the most generally distributed group in insects is that composed of Gram-negative small rods. In this respect the bacterial flora is similar to that of higher animals. Next frequently seen are the micrococci and sporeforming bacilli".

In order to find out the effect of the natural environment on the intestinal bacterial flora of the insect, the grasshoppers collected from the field were examined. This flora comprises more various species than those of laboratory cultures; e.g., bacteria of *Erwinia* group which are never found in laboratory cultures have been isolated in 22 % of the field material. Similarly, *K. ozaenae*, *E. freundii* of the same family have entered the intestinal flora of these individuals. The bacteria forming the intestinal flora of grasshoppers collected in the field exhibit a greater variety of species than those of laboratory cultures, probably because these insects

feed in greater areas on a greater number of plants. However, in these insects also, *A. aerogenes* and *S. faecalis* are among the bacteria always found in their intestinal flora.

To compare the flora of the individuals in a new culture formed from the material collected from the field with the individuals in nature and the permanent laboratory cultures used in our study, the intestinal bacterial flora of a sixmonth - old laboratory culture was investigated. While the *Serratia* genus of bacteria was found in 27 % of the individuals collected from the field, this genus could not be isolated at all from the six-month-culture of grasshoppers collected from the same area. In addition *K. pneumonia*, *K. ozaenae* and *Arthrobacter sp.* disappeared from the flora. This again is proof that the intestinal bacteria are closely related with the environment of the insects. The facility of introducing more bacteria into the bowels through different plants increases the variety of the flora. One of the studies leading to this conclusion is that of WEDBERG et alii (34). The researchers fed *Blaberus cranifer*, maintained motionless, on food reasonably free from bacteria and found out that the flora of droppings changed after some time. The number of bacterial species in each began to decrease. The cause of this change is doubtless the decrease in the chances to introduce bacteria into the bowels through the limitation in the kind of food. STEINHAUS (2, 35) points out that in the formation of the intestinal flora, the insect's feeding habits and its vital environment play a great part. Accordingly, the intestinal bacteria of differrent insect species are qualitatively widely different. This difference can also be found in different individuals of the same species. The data obtained from our research confirm this supposition. While a bacterial species was found in one of the individuals of the same stage, it could not be isolated from another (Table II–X).

As striking as the qualitative difference observed in the intestinal bacteria between individuals of the same species, and indeed more so, it is the quantitative difference. The number of intestinal bacteria in a healthy adult *L. migratoria*, varies from 320 000 to 90 000 000. BUCHER (23), also, wrote that the number of bac-

teria fluctuates between 500 000 and 50 000 000 in some other grasshopper species.

As to the bacteria counts carried out in each nymphal stage. they show that the age of the insect influences the intestinal bacteria quantitatively. Besides, the number of bacteria in nymphs in the same stage varies considerably.

The bacteria isolated in the course of our investigation have been classified according to cultural, morphological and biochemical characteristics, and these have been shown in the tables. Part of the bacteria we had isolated showed reactions different from those of well known species and types, which fact in turn of bacteriological importance.

The third part of our study, the pathogenic effect of certain bacteria on *L. migratoria* was investigated. The question of pathogenicity was taken up from two standpoints: 1) The study of the pathogenic effects of bacteria we have isolated from *L. migratoria;* 2) The testing of species which we have not met in the course of our investigation, but the pathogenic effect of which on grasshoppers was asserted.

The epizootics observed in both the field populations and the laboratory cultures of grasshoppers have induced researchers to identify and investigate the pathogenicity of the bacteria cousing them (8, 14, 15, 16, 17, 23, 36).

BUCHER (18) divides the bacteria pathogenic for incests into three groups; 1) Obligate pathogens; 2) Potential pathogens; 3) Facultative pathogens.

It was found that *S. marcescens*, which we isolated generally from individuals in nature, causes epizootics in laboratory cultures (10, 12). BUCHER (18) classified this bacterium with the group of facultative pathogens. Several researches state that when this bacterium is injected directly into the body fluids of the insect, a general septicemia results and the insects dies within 1–3 days. In the case of adult individuals (*M. bivittatus*, *Camnula pellucida*) when this bacterium was injected into their body fluids, LD_{50} was found to be 10–50 (17, 23). STEINHAUS (14) found that when

he injected about 300 000 bacteria into various species of insects, the mortality rate rose to 90 %.

S. marcescens Type I, and Type II, which we used in our tests on pathogenicity, were isolated from *L. migratoria*. In addition, tests were also carried out with a typical collection culture Number: A 173 from the Hygiene Institute. For the *S. marcescens* (Type I) the LD_{50} is 1909 bacteria. The mortality in group A was found to be 100 %, in group B, 80 % (Fig. 1). For Type II the LD_{50} is 2016 bacteria. The mortality in group A and B is 100 % – % 90 (Fig. 2). In the culture Nr. A. 173 the LD_{50} is 2274 bacteria. The mortality in groups A and B is 100 %. These data are similar to the results obtained by STEINHAUS (14) with other insect species, and *S. marcescens*, injected into the body cavity of *L. migratoria*, shows that the latter is even more sensitive than the other species tested by STEINHAUS. However, our findings are much higher those of BUCHER (23) for *M. bivittatus*. This difference may be accounted for as follows: as STEINHAUS also remarks, just as there are differences in virulence between the *S. marcescens* types, there can well be a difference in the resistance to *S. marcescens* of the insect species infected.

No members of the *Pseudomonas* group were found the intestinal bacteria of *L. migratoria*. BUCHER and STEPHENS (13,21) state that they could not trace *P. aeruginosa* in healthy grasshoppers, but were able to isolate this bacterium only from diseased or dead individuals. The research workers point out that the bacterium is eliminated from the intestine in a very short time and that the infection is caused by the bacteria which succeeded in passing into the blood under particular circumstances. The fact that we did not find *P. aeruginosa* in the intestine of healthy grasshoppers tends to confirm this supposition.

P. aeruginosa comes first among the bacteria pointed out as pathogenic to grasshoppers. BUCHER (18) gives this bacterium as an example of potential pathogens. For this reason, though this bacterium was not isolated in our study, its pathogenic effect on *L. migratoria* was tested with collection culture Number: 649.

BUCHER and STEPHENS (13) state that when a sufficient dose of bacteria is injected into the body fluid, the number of bacteria, after a latent period of six hours, increases logarithmically, but that in some individuals this number does not increase at all and is eliminated from the blood. In our tests all grasshoppers, infected with *P. aeruginosa*, died within 3 days when the dose was 250 000 bacteria and the nearer to the LD_{50}, the greater their chances of survival (Fig. 4).

For a bacterium to be considered pathogenic, the LD_{50} of the bacterium injected into the body fluid must be 10 000 or below (18). Our tests have shown that the LD_{50} for *P. aeruginosa* is 2046 bacteria. BUCHER (18) found an LD_{50} of 10 – 20 bacteria for a species of this bacterium isolated from grasshoppers. The mortality rate found in injecting the above amounts was only 18 % in our tests. This difference may be accounted for by the lower virulence of the species of bacteria we used or in the greater resistance of the insect species.

P. vulgaris, *P. mirabilis*, *P. rettgeri* of the *Proteus* group of bacteria are potential pathogens. BUCHER (18, 23) states that their LD_{50} varies respectively from (50–100, 20–500, 300–1000).

The LD_{50} of *P. vulgaris*, *P. mirabilis*, and *P, rettgeri* isolated from *L. migrotoria* are respectively 6888, 2099, and 11341 bacteria. According to these figures, P. rettgeri should not be considered pathogenic for *L. migratoria*.

Our tests carried out with *B. cereus* showed that this bacterium was not lethal even in doses above 100 000 bacteria.

As it appears from all these comparisons, the LD_{50} may vary greatly according to the various types of bacteria species used and the insect species tested. The cause of this differences may be, as indicated above, either the differences in virulence of the bacteria types used or in the resistance of the insect species to the bacteria types in question.

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ÖZET

Locusta migratoria migratorioides R. ve F. in barsak bakteri florası bütün nimf evrelerinde ve erginlerde, gerek laboratuvar kültürlerinde gerekse araziden toplanan taze materyalde ve bu materyalden itibaren hazırlanan yeni kültürde kalitatif ve kantitatif yönden incelenmiş ve bunlar arasında karşılaştırma yapılmıştır.

Barsak bakteri florası araştırılan 400 nimf ve erginden 1565 adet bakteri kültürü izole edilmiştir. Bu kültürler 6 familyaya giren 33 bakteri türüne aittir.

Ayrıca *L. migrotoria* üzerinde bazı bakterilerin patojenik etkisi vücut sıvısı içine bakteri enjekte edilerek araştırılmış ve LD_{50} (Ortalama öldürücü doz) hesaplanmıştır. Denenen bakterilerin bir kısmı barsak florasının tesbiti sırasında izole edilen bakterilerdir (*S. marcescens*, *P. vulgaris*, *P. mirabilis*, *P. retgerii*, *B. cereus*). Bir kısmı da *L. migratoria* da rastlanmayan bakterilerdir (*P. aeruginosa*, *S. marcescens* "pigmentli"). Sonuncu türlerin kolleksiyon kültürleri kullanılmıştır.

S. marcescens, *P. aeruginosa*, *P. vulgaris*, *P. mirabilis*'in ortalama öldürücü dozu 10 000 bakterinin altında olduğu için patojen kabul edilmişlerdir. Bununla beraber bulunan dozların diğer araştırıcıların verdikleri miktarlardan farklı olması nedeniyle LD_{50} nin her bir bakteri türünün çeşitli tiplerine ve denenen böcek türüne göre büyük oranda değişebileceği kanısına varılmıştır. Elde edilen veriler diğer araştırıcıların sonuçları ile karşılaştırılarak tartışılmıştır.

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