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TABLE OF CONTENTS

On The Threshold and Residual Numbers of *Salmonella schottmuelleri* In *Phormia regina* [1]

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Since 1940, the role of nonbiting flies as potential vectors of enteric pathogens has been reconsidered. Much of the extant literature has been cited in (1, 2) and other papers. Therefore, the findings of only a few pertinent studies will be mentioned.

Survival of enteric pathogens in axenically and nonaxenically reared immature stages (3) and in adult *Musca domestica* (1) and in pupal and adult *Phormia regina* (2) has been demonstrated. Decided multiplication of *Shigella* and *Salmonella* in houseflies fed bacteria (1) was questioned by Lindsay and Scudder (4) who felt that the technique used in handling fly excreta permitted significant multiplication in the receiving saline prior to platin. Knuckles (2) showed that *Salmonella typhimurium* and *Salmonella schottmuelleri* multiplied extensively in *Phormia regina* fed these species. The point in question relative to (1) was void in (2) because a more restricted fecal collection period was observed and non-nutrient agar was the collecting medium. The existence of infectivity thresholds for pathogens in houseflies (1), the transmission of *Salmonella* sp. from fly to fly via infectious feces (5), and the established correlation between fly population and shigellosis (6, 7) suggest a need for more basic studies on fly-bacteria relationships. Original bacterial threshold and residual studies are presented in this paper.

METHODS AND MATERIAL

Eggs oviposited on stale fish by 6 day-old or older stock flies were transferred to a vial and washed in 1.5 ml. of saline solution for from 3-5 min. The saline solution was decanted and the ova were then washed from 3-5 min. in 70% ethanol. Subsequently, the disinfectant was replaced with a second volume of itself and the eggs remained in it for from 5-10 min. They were then thoroughly washed in saline solution. The saline was discarded and the ova were then transferred to a larval medium (8) in the feeding flask of an apparatus for rearing larvae and paupae, aseptically (9). This apparatus consisted of a 1000 ml. (feeding) and a 250 ml. (pupation) Erlenmeyer flask. An inverted L-shaped piece of glass tube was affixed near the junction of the body and neck of the large flask and its distal end inserted into the mouth of the smaller flask which contained an inch layer

[1] A portion of a dissertation in the Department of Zoology and Entomology, and Bacteriology, University of Connecticut, Storrs, Connecticut.

of sawdust. Each flask was plugged with cotton and the apparatus was then incubated at 26 C.

Aseptically reared pupae were transferred from a pupation flask to vials which were then plugged with cotton. Emerging adult flies were lightly anaesthetized, individually mounted on paraffin blocks, and suspended over individual excrement collecting dishes on a rotable rack (10).

Mounted flies were fed measured numbers of *Salmonella schottmuelleri* with a potometer (1) graduated in thousandths of a milliliter, 24 hours after being restrained. Each insect was then fed *ad lib.* on 0.5 m sucrose solution twice daily as a maintenance diet. A tube of the maintenance solution was used for only a single feeding to prevent contamination. Stock flies were sustained on 0.5 mucrose and a 2% beef extract solution.

In one threshold experiment (Table 1), a small dish of 2% non-nutrient agar was placed under each fly on each of 9 days immediately after their morning meal and fecal collections were made for 20 min. thereafter. Separate fecal samples were made daily for each insect by transferring excrement to 2 ml. of distilled water. Similiarly, individual gut samples were made on the tenth day or day of fly deaths with the teased gut of each fly. Gut and fecal samples were used in the study of residual bacteria (Table 3), but a 7 hour fecal collection period was observed.

Bacteria were enumerated by the dilution method. One tenth milliliter of a well agitated fecal or gut sample was serially diluted out in 9 tubes, each of which contained 1.5 ml. of distilled water. One milliliter from each dilution was then transferred to separate tubes of selenite-F-broth (SFB). All inoculated media were incubated at 37 C for 24 hours.

Streaks were made on SS agar from the two highest dilutions showing growth in SFB. Growth from colonies typical of *Salmonella schottmuelleri* was fished to triple sugar iron agar (TSIA) slants, phenol red tartrate agar (PRTA) shakes, and urea (UB) broth. The identity of *Salmonellaa schottmuelleri* was based on negative PRTA and UB tests, and positive TSIA and macroscopic slide agglutionation tests.

Bacterial suspensions were prepared by washing growth from a 24-hour old slant culture with 0.5 m sucrose solution and adjusting a quantity of it turbidometrically to match a McFarland nephelometer stanard No. 0.5 (= 15x10 7 cells/ml). This suspension was diluted further with distilled water to regulate the number of bacteria to be ingested by the flies. A reasonable approximation of the numbers of *schottmuelleri* ingested by the flies was determined by recording the volume of suspension fed each fly.

All bacteriological media were sterilized as prescribed on their original containers. Flies were disinfected in a 1% aerosol - 1% sodium hydroxide - 5% formaldehyde solution (v:v:v:) for 15-20 min. The intact larval rearing apparatus and the various solutions were sterilized at 15 pounds of steam pressure for 15-20 min. Forceps, swab sticks, pipettes and glassware were sterilized in a hot air oven.

RESULTS AND DISCUSSION

Sixty determinations performed on the excreta and gut of 6 flies fed from 8,000 - 13,000 *Salmonella schottmuelleri* showed an average of less than a single bacterium per fly per day (Table 1). Only one of these flies was positive for the test microbe during a 10 day period. Four hundred bacteria were found in this specimen. Seventy determinations made on the feces and gut of 7 flies fed from 15,000 - 18,000 *schottmuelleri* showed an average of 2.8 billion bacteria per fly per day (Table 1). These flies were positive for *schottmuelleri* throughout the 10 day period. In a seond experiment (Table 2), determinations made on gut samples during a 10 day period from specimens fed from 5,000 - 13,000 bacteria gave similiar results as obtained in the first experiment. Three and two-tenth million bacteria were detected on the seventh day in a single insect fed 14,000 bacteria.

These data show that *Salmonella schottmuelleri* not only survives in but undergoes extensive multiplication in the gut of aseptically reared *Phormia regina* when they are fed a single meal of 14,000 or more bacteria. *Salmonella schottmuelleri* when fed to blowflies in smaller numbers than 14,000 undergoes a rapid decrement during their attempt to adjust to an *in vivo* environment and is destroyed, possibly by an enzyme mechanism or another chemical complex, therein. This bacterium may survive and multiply indefinitely not only in aseptically reared flies but also in nonaseptically reared specimens (2) once a microflora is established, therein. Results of the only other experiment of a similiar nature (1) support the existence of a bacterial threshold for infectivity in nonbiting flies. These authors found that 12,000 *Eschericha coli,* 12,000 *Shigella dysenteriae,* and 18,000 *Salmonella schottmuelleri* were required for the establishement of the respective species in the housefly.

Individual gut samples made during a 13 day period from 39 flies fed a single bacterial meal of 1.4 million *Salmonella schottmuelleri* and subsequent to a post-meal defecation period of 20 min. showed an average of 1.5 billion bacteria (Table 3). A defecation period of 20 min. following the morning meal was allowed prior to a fly dissection because most bacteria passed by *Phormia regina* are eliminated during this period. Like the threshold number, no difference was found in the number of residual *Salmonella schottmuelleri* in male and female insects. The residual bacteria alone show that *Salmonella schottmuelleri* underwent extensive multiplication in these flies and that they remain numerically sufficient to sustain infectivity and multiplication indefinitely, therein.

TABLE 1

On the threshold number of *Salmonella schottmuelleri* required for its establishment and multiplication in aseptically reared blowflies.

Fly No. and Sex	Calc. No. Bacteria Ingested	No. Bacteria Detected in Feces	No. Bacteria Detected in Gut	Total Bacteria Detected
1M	6×10^3	0	0	0
2M	8×10^3	0	0	0
3F	8×10^3	0	0	0
4M	8×10^3	0	0	0
5M	8×10^3	0	4×10^2	4×10^2
6F	13×10^3	0	0	0
7F	15×10^3	32×10^5	64×10^6	67×10^6
8M	27×10^3	67×10^6	26×10^9	27×10^9
9M	15×10^3	31×10^9	26×10^9	57×10^9
10M	15×10^3	33×10^9	26×10^9	59×10^9
11F	15×10^3	40×10^9	26×10^9	30×10^9
12F	15×10^3	39×10^9	26×10^9	69×10^9
13M	18×10^3	80×10^8	26×10^9	12×10^9

TABLE 2

On the threshold number of Salmonella schottmuelleri required for its establishment and multiplication in aseptically reared blowflies.

Fly No. Sex	Calc. No. of Bacteria	Day of Examination	No. of Bacteria Detected in Fly
1M	13×10^3	1	0
2F	9×10^3	2	0
3M	9×10^3	3	4×10^2
4F	13×10^3	4	0
5M	13×10^3	5	0
6F	9×10^3	6	0
7M	13×10^3	7	0
8F	11×10^3	8	0
9M	11×10^3	9	0
10F	9×10^3	10	0
11M	13×10^3	1	8×10^3
12F	13×10^3	2	20
13M	5×10^3	3	0
14F	13×10^3	4	0
15F	10×10^3	5	0
16M	9×10^3	6	0
17F	14×10^3	7	32×10^5
18M	13×10^3	8	4×10^2
19F	13×10^3	9	0
20F	13×10^3	10	0

TABLE 3

Residual *Salmonella schottmuelleri* in aseptically reared blowflies fed the test microorganism.

Fly No. and Sex	Calc. No. of Bacteria	Day of Examination	No. of Bacteria Detected in Fly
1F	14×10^5	1	16×10^4
2F	14×10^5	1	32×10^5
3M	14×10^5	1	8×10^3
4F	14×10^5	2	-------
5F	14×10^5	2	8×10^3
6M	14×10^5	2	16×10^4
7F	14×10^5	3	13×10^8
8F	14×10^5	3	8×10^3
9F	14×10^5	3	8×10^4
10M	14×10^5	4	16×10^4
11F	14×10^5	4	16×10^4
12M	14×10^5	4	-------
13F	14×10^5	5	8×10^4
14F	14×10^5	5	16×10^4
15M	14×10^5	5	8×10^4
16F	14×10^5	6	8×10^4
17F	14×10^5	6	64×10^6
18M	14×10^5	6	16×10^5
19F	14×10^5	7	4×10^2
20F	14×10^5	7	26×10^9
21M	14×10^5	7	16×10^4
22F	14×10^5	8	8×10^4
23F	14×10^5	8	4×10^2
24M	14×10^5	8	32×10^5
25F	14×10^5	9	64×10^7
26F	14×10^5	9	8×10^3
27M	14×10^5	9	16×10^4
28F	14×10^5	10	13×10^8
29F	14×10^5	10	32×10^5
30M	14×10^5	10	26×10^9
31F	14×10^5	11	8×10^3
32F	14×10^5	11	13×10^8
33M	14×10^5	11	64×10^6
34F	14×10^5	12	4×10^2
35F	14×10^5	12	8×10^3
36M	14×10^5	12	8×10^3
37F	14×10^5	12	13×10^2
38F	14×10^5	13	16×10^4
39M	14×10^5	13	64×10^6

SUMMARY

1. A threshold of from 13,750 - 15,000 *Salmonella schottmuelleri* was found necessary for its establishment and survival in the digestive tract of the black blowfly.
2. *Salmonella schottmuelleri* underwent a several-fold multiplication in the gut of aseptically reared blowflies which had ingested a threshold quantity of more of this species.
3. *Salmonella schottmuelleri* when ingested by blowflies in subminimal quantities underwent a rapid decrement and disappearance from their digestive tract and/or associated tissues.
4. Once an infection was established in *Phormia regina,* the number of residual *Salmonella schottmuelleri* therein, following the most proliferate period of defecation was found to be considerably above the infectivity threshold.

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