Naturally occurring antibodies against Bacillus pumilus and Streptococcus faecalis isolated from rabbits feces and the effect of increased antibody titers on these organisms
by Chester Armstrong Brown

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Bacteriology
Montana State University
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Abstract:
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1. To determine whether organisms normally found in rabbit feces had stimulated production of circulating antibodies in their hosts.

2. To determine what effect, if any, an increase in specific circulating antibody would have on the intestinal population.

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An increased agglutinin concentration showed two effects on the fecal organisms studied.

1. As the titer against Bacillus pumilus increased the numbers of Bacillus pumilus decreased. After the peak titer was reached and sustained for a short length of time a new organism, Lactobacillus sp., was found in the feces of the immune rabbits. This organism had not been demonstrated previously.

2. No decrease in the numbers of Streptococcus faecalis was found. However, a shift in antigenic type occurred. The original sero-type disappeared from the immune rabbits. The controls, however, still contained only the original sero-type.

Although efforts were made to demonstrate the presence of coproantibody in the feces of the immune animals it was not possible with the methods used in the experiments.

NATURALLY OCCURRING ANTIBODIES AGAINST *BACILLUS PUMILUS* AND *STREPTOCOCCUS FAECALIS* ISOLATED FROM RABBITS' FECES AND THE EFFECT OF INCREASED ANTIBODY TITERS ON THESE ORGANISMS

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CHESTER A. BROWN

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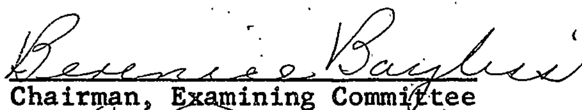
Approved:

Head, Major Department



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ABSTRACT

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CHAPTER I

INTRODUCTION

Early interest in the natural antibodies was prompted by the observation of the blood group agglutinins. The early hypothesis stated that these were genetically controlled. Later information pointed to outside stimuli as the causative agents responsible for the production of the natural antibodies.

Interest in coproantibody was prompted by the observation that it was demonstrable before serum antibody could be found. Thus coproantibody could be of diagnostic value in the early determination of the causative agent of disease.

The purpose of this study was to obtain information relating to the production of circulating antibodies in rabbits by some native intestinal organisms and to determine the effects an increased antibody titer would have on the intestinal organisms.

CHAPTER II

REVIEW OF LITERATURE

Naturally occurring antibodies

The occurrence of the so-called natural antibodies in the blood has been noted from the earliest days of immunology. Landsteiner (1936) described isohemagglutinins, as reported in his paper in 1900. However, no completely reasonable explanation of the natural antibodies has been proposed until recently.

Smith and Bryant reported in 1927 that certain strains of *Escherichia coli* from the ileum of calves suffering from diarrhea or scours mutated and gave rise to forms which had lost their capsular substance and were of decreased virulence. However, the mutant cells showed great increases in ability to be agglutinated and in ease of phagocytosis.

Bailey (1928) observed that experimental intranasal snuffles produced in rabbits by a strain of *Bacterium lepisepticum*, now known as *Pasteurella multocida*, containing a heterophile antigen caused production of a potent anti-sheep hemolysin. His results indicated that under natural conditions heterophile antibody is produced in rabbits by infection with similar strains of *Bacterium lepisepticum*, thus accounting for some of the variability in natural hemolysin content of sera from these animals.

Gibson (1930) studied natural agglutination as exemplified by serum reactions in 9 animal species with a variety of bacteria. This work revealed that the sera of different animal species showed an order of agglutination activity almost constant for all organisms used. He found that the natural

agglutinating substance differed from the immune agglutinating substance in that the natural substance was present in greater degree in the carbonic acid-insoluble fraction of the sera than in the carbonic acid-soluble fraction. The immune substance was chiefly located in the carbonic acid-soluble fraction.

Gibson also showed in this report that agglutination depends on non-specific and specific factors. For normal serum, the agglutination of bacteria is a 2-fold mechanism; (a) a non-specific effect reacting in varying degree with all organisms, and (b) a series of specific effects reacting as true "natural antibodies".

In 1931, Gibson reported on natural agglutinins and their relationship to somatic and flagellar antigens of bacteria. He found that normal sera from various mammalian animals contained agglutinins which reacted with the "H" and "O" antigenic constituents of many bacteria. Agglutinin-absorption experiments were used to show that the specificity of natural agglutinins was dependent chiefly on the "H" type antigen.

Curnen and Finland found in 1938 that when horses were immunized against type XIV pneumococci, their serum contained agglutinins in high titer for human erythrocytes of all four major blood groups. Only low titers of these agglutinins were found in non-immunized horses. Normal rabbit sera were found to contain agglutinins in low titer against the AB and A blood groups.

Emslie-Smith (1948) found at least seven distinct agglutinins for coliform and paracolon bacilli in the sera of two normal rabbits.

McCullough, Eisele, and Beal (1949) tried prolonged feedings of killed _Brucella_ organisms composed of equal portions of _Brucella abortus_ and _Brucella suis_ to healthy humans and failed to produce significant agglutination titers or dermal sensitivity. When using a maximum dosage of 49 billion organisms, they were able to stimulate demonstrable opsonic activity in some of the individuals, but no high agglutination titers.

Gillespie, Steber, Scott and Christ (1950) isolated aerobic and facultatively anaerobic bacteria from fecal specimens of two healthy humans and ran agglutination titers against their sera. They found in most cases that the organisms from a subject were agglutinated by his serum.

Wiener stated in 1951 that: "Evidence relating to the problem of the origin of natural antibodies, especially hemagglutinins and hemolysins, has been critically reviewed. Based on this analysis it is concluded that natural antibodies are, with possible rare exceptions, of immune origin." Wiener also stated: "Natural immunity to bacteria and viruses is generally conceded to be due to undiagnosed or symptomless infections, since such immunity is most frequent among individuals who have been exposed to the disease or who carry the microorganism in their bodies." It has been shown that polysaccharides isolated from the capsules of pneumococci possess chemical structures similar to the blood groups antigens. He also states: "Natural antibodies for red cells, such as the blood group antibodies and cold hemagglutinins, are shown to be of heterogenetic immune origin and are attributable to the presence of related antigens in bacteria and animal parasites."

Wilson and Miles (1955) state that the more commonly accepted view as to natural antibody formation is that these antibodies arise as the result of the response of the antibody-forming apparatus to external environmental stimuli. Such stimuli usually consist of overt or latent infections.

Springer, Horton, and Forbes (1959) have shown that germ-free chicks treated with live smooth Escherichia coli 086 develop agglutinins for the human blood group B. These agglutinins were not present in the chicks until after the infestation. However they did find antihuman B agglutinins present in normal chicks.

Osawa and Muschel (1960) found that some substance was necessary for the bactericidal action of normal serum, even when the properdin system was present. This necessary substance was antibody.

Cohen, Cowart, and Cherry (1961) reported finding antibodies against Staphylococcus aureus in the serum from non-immunized rabbits. The rabbits used were free from the specific pathogen at the time of testing. Tests were also run on the sera of rabbits obtained from a commercial source. These were found to contain other antibodies for staphylococci in addition to the two found in the specific-pathogen-free rabbits.

Michael, Whitby, and Landy (1962) made studies on natural antibodies to Gram-negative bacteria in the normal serum of several species of animals. They found antibody for several genera of the family Enterobacteriaceae in normal human serum. Serum from germ-free rats and chickens did not have demonstrable antibody titers against Escherichia coli or Salmonella typhosa, while no appreciable difference in titer was found between germ-free mice and

conventional mice with regard to the same two organisms.

Coproantibody

Sherman (1919) described an experiment in which the large intestine of a dog was severed from the small intestine and tied off. The free end of the small intestine was sutured to an artificial anus. The secretions from the large intestine were collected and found to contain hemolysins quantitatively the same as those in the blood.

Davies (1922) was the first to describe coproantibodies in the feces. He found these while investigating the serological properties of stools from cases of dysentery.

Burrows and Havens (1947) found that immune globulin, immunologically indistinguishable from serum globulin, is excreted in the feces and urine of actively immunized guinea pigs and humans. These fecal and urinary antibodies are independent of serum antibody and are not derived from it. This excretion is a normal process and the antibody titer is due to the partial substitution of immune globulin for normal globulin. This copro-antibody is excreted in the feces and urine of guinea pigs passively immunized with homologous or heterologous antiserum.

Burrows, Elliott, and Havens (1947) found antibody activity in the feces of guinea pigs with enteric cholera. This was found both in the case of immunized and infected animals. This activity was shown to be due to the presence of immune globulin. They found that the coproantibody did not persist long in the feces. It appeared early, peak titers slightly preceded peak serum antibody titers, but disappeared in 3 to 4 weeks, while the serum

antibody titer persisted for much longer. They stated that the pattern of vibrio excretion in relation to the total fecal flora was associated with the presence of coproantibody at the time of infection.

In 1947, Harrison and Barvard reported on coproantibody excretion during enteric infections. They examined patients with enteric infections and found that coproantibodies were excreted in 96.7 per cent of the cases studied. This they concluded made coproantibody a significant diagnostic factor in the determination of the causative organisms of infection. This is especially true since the coproantibody was excreted during the active phase of the infection, or in the case of chronic infection, during periods of clinical activity of the disease.

Koshland and Burrows (1950) studied methods of quantitative measurement of antibody response to the whole bacterial antigen. They used complement fixation tests modified for use with a particulate antigen. In this study the rate of coproantibody production was taken as a measure of the rate of antibody production. It was found that the agglutinating and complement-fixing antibodies to the cholera vibrio O antigen were closely similar if not identical. Also that fecal antibody behave independently of serum antibody formation.

Barksdale and Ghoda (1950a) observed reactions of fecal extracts and sera from a number of cases of Flexner and Sonne types of dysentery tested against the phase antigens from wild type Shigella paradysenteriae W, Shigella sonnei and certain of their serologically "non-specific" mutants. The serum and fecal extracts differed in the titer and specificity of their

agglutinins.

Barksdale and Ghoda (1950b) observed a low titer of coproantibody and serum agglutinins against strains of *Escherichia coli* in humans suffering with salmonellosis and/or shigellosis. The *E. coli* (97-C-K) was not agglutinated by standard salmonella or shigella antisera. In cases of bacillary dysentery the rise and fall in coproagglutinins directly against *E. coli* (97-C-K) roughly paralleled that of the agglutinins directed against the causal shigella.

Barksdale, Ghoda, and Okabe (1950) found, in a case of enteric disorder falling into the general category of chronic ulcerative colitis, that coproantibodies were present which gave agglutination of numerous antigens common to the shigella group of pathogens. Also a coliform organism was isolated which was capable of completely absorbing these various coproantibodies.

Gordon, Bennett, and Barnes (1950) used vaccine against *Shigella flexneri*. Coproantibody increase was found in only one group and only when the samples were collected one to two days after completion of a course of shigella vaccine. They showed that in a significant percentage of subjects, the parenteral shigella vaccine caused the excretion of fecal agglutinins, the appearance of which was early, transitory, and preceded the period during which the serum antibody response was at a maximum.

Gonzalez and Koppisch (1951) operated on normal and immune rabbits to produce an isolated segment in their bowels. They tied off the vermiform appendix and injected a suspension of *Shigella paradysenteriae*, type I, into

the lumen. After different lengths of time, the animals were killed and the contents of the appendices were examined for viable bacilli and for antibody. Agglutinins were found in low titer only in 5 immune rabbits and one of the normal rabbits which had received, in addition to the Shigella suspension, 1.0 ml of immune rabbit's serum. A probable explanation for the low level of antibody titer in the immune rabbits, and the disappearance of the agglutinins in the normal rabbits was the absorption of agglutinins by the homologous bacteria in the intestines, or the disintegration of antibody caused by enzymic action.

Koshland (1952) found that when cholera vaccine and adjuvant were injected into the abdominal cavity of young guinea pigs no excretion of antibody was observed, even though a parenteral injection of cholera vaccine alone gave significant fecal antibody concentrations. High titers of antibody were found in the sera of animals which received the vaccine and adjuvant. Thus it was suggested that serum and fecal antibody do not have a common origin and that neither the bile nor the lymphocytes are responsible for the transportation of demonstrable amounts of immune globulin to the bowels. Fecal antibody is synthesized independently of serum antibody, probably in local sites along the intestinal tract.

Burrows and Ware (1953) found that prophylactic immunity, like immunity to infection with overwhelming doses, is associated with the active secretion of antibody into the bowel, and suggested that coproantibody plays an integral and significant part in prophylactic immunity to enteric infection.

CHAPTER III

METHODS AND MATERIALS

Method of cage preparation for fecal collection

The rabbits' feces were collected as aseptically as possible while the animals remained caged. The refuse trays were cleaned thoroughly with soap and water and disinfected with a 2% amphyl solution. Fresh clean newspapers were placed in the bottom of the trays and a layer of beet pulp one half inch in depth placed on the newspaper. More newspaper, three layers in depth, which had been sterilized in an autoclave at 17 lbs for 20 minutes was placed over the beet pulp. The trays were then inserted into the cages. Newspaper was used as the sterile cover because of its water absorbency and availability, and beet pulp was used because of its absorbency.

Isolation and classification of two fecal organisms

Once the cages were prepared, they were checked every half hour until fresh fecal pellets were dropped. At this time a few pellets from each rabbit were placed in tubes containing 10 ml of sterile buffered water (phosphate buffer, pH 7.0). This was done with the aid of forceps which were sterilized by flaming after immersion in 70% alcohol. The fecal pellets were broken up with a sterile applicator stick. Some of the resulting fecal suspensions were streaked with sterile cotton swabs onto the following types of media: (1) nutrient agar (Baltimore Biological Laboratory, 01-138), (2) _Streptococcus faecalis_ agar (BBL, 01-320), (3) Eosine Methylene Blue agar (Difco, 0076-01), (4) crystal violet agar (nutrient agar ≠ 1.0 ml of 1.0% aqueous crystal violet solution and

(5) azide dextrose agar (BBL, 01-314 plus 1.5% Bacto. agar, Difco, 0140-05). Some of the fecal suspensions were smeared on slides, stained with Gram's stain, and examined microscopically. This gave an idea of the types of organisms present in the feces.

The organisms were incubated at 35 C unless otherwise stated. After incubation for an appropriate length of time, 24 hours for the nutrient agar and 48 hours for the other media, some of the resulting colonies were picked, stained with Gram's stain and examined microscopically. These organisms were then restreaked upon the types of media on which they had originally appeared. The freshly inoculated plates were incubated for an appropriate length of time, after which some of the resulting colonies were transferred to tubes containing 10 ml of trypticase soy broth (BBL, 01-162). The tubes were incubated for 24 hours, samples of the cell suspensions were Gram stained and inoculated into differential media for identification purposes.

The two most prevalent organisms isolated from the feces by these methods were designated as the two test organisms, S_1 and B_1. The S_1 isolate was later identified as _Streptococcus faecalis_, and the B_1 isolate identified as _Bacillus pumilus_. These two organisms grew well with ordinary culture methods and were easily determined from the other organisms present in the feces by their reactions on SF and EMB agars.

The two chosen organisms were then identified according to their morphology and reactions on various differential media.

Determination of the total number of each test organism per gram of feces

The cages were prepared as before and checked every half hour until fresh fecal pellets were dropped. The fecal pellets were removed from the tray and weighed on sterile paper. The sample weight was kept as close to 1.0 gram as possible without necessitating the splitting of a pellet. The weighed fecal pellets were placed in sterile Waring blendors and enough sterile buffered water (phosphate buffer, pH 7.0) was added to each blendor to give a 1:100 dilution of pellets to water. The mixture was agitated on the Waring blendor at low speed until a uniform suspension was achieved. This usually required 10 minutes.

Serial dilutions were plated to determine the number of test organisms per gram of feces. EMB agar was used for the B_1 organism and SF agar for the S_1 organism. The plates were allowed to solidify and then inverted and incubated for 48 hours. Depending on the number of colonies present on the plates, the counts were either made by the unaided eye or with the aid of a Darkfield Quebec Colony Counter.

The SF agar indicated the presence of the test organism S_1 by the formation of a yellowish zone around the colony. The EMB agar plates indicated the presence of the test organism B_1 due to the typical colony form which this organism displayed on the medium.

Preparation of bacterins and cell suspensions

The preparation of the various bacterins and cell suspensions differed in the methods of growing the organisms, formalin treatment, and standardization.

The S_1 organism was grown in the following manner. Five S_1 colonies were

picked from a SF agar plate and inoculated into one tube containing 10 ml of trypticase soy broth. The tube was incubated for 24 hours, after which a Gram stain was made of the resulting cell suspension. A sample of the cell suspension was streaked on a SF agar plate and incubated for 48 hours. Following this time 6 colonies were picked from the plate and each was inoculated into a separate tube containing 10 ml of trypticase soy broth. The tubes were incubated for 24 hours. Samples were removed from each tube and inoculated into differential media to ascertain if each tube contained the same organism.

Samples were removed from each tube and inoculated into two 250 ml glass Serval centrifuge bottles containing 200 ml of trypticase soy broth. These bottles were then incubated for 24 hours. After this time, 4 samples were removed from each bottle, each was smeared on a slide, and Gram stained as a check against contamination.

The B_1 organism was grown in the following manner. Six colonies were picked from an EMB plate and Gram stained. Cells from the picked colonies were streaked onto 6 new EMB agar plates and incubated for 48 hours. At the end of the incubation period, one colony from each plate was picked at random and inoculated into a tube containing 10 ml of trypticase soy broth. The 6 tubes were incubated for 24 hours. Samples were taken from each tube and inoculated into differential media to ascertain if each tube contained the same organism.

Samples were then transferred from the 6 tubes into one tube containing 10 ml of trypticase soy broth. The tube was incubated for 24 hours after

which time, 1.0 ml of the cell suspension was removed and used to inoculate two slants. The slants were made by placing 13.0 ml of trypticase soy agar in milk dilution bottles, plugging the bottles, and autoclaving for 20 minutes at 17 lbs. The bottles were removed and placed on their sides until the agar solidified. One-half ml of cell suspension was added to each slant and swished around the agar surface. The bottles were inverted and the excess cell suspension poured off. The inoculated slants were incubated for 24 hours in the inverted position.

The S_1 cells were harvested by centrifuging the bottles for 20 minutes at 1000 rpm in a Serval refrigerated centrifuge. The supernatant was removed and 100 ml of sterile saline added to each bottle. The cells were resuspended and again centrifuged at 1000 rpm for 20 minutes. The cells were washed in this manner 3 times. After the third wash, the cells were resuspended in 100 ml of saline containing 0.5% formalin.

The B_1 cells were harvested by addition of 10 ml of saline to each slant and loosening the cells with sterile, curved glass rods. The resulting cell suspension was placed in sterile Serval centrifuge tubes and washed three times with sterile saline. This was the same for both the living cell suspension and the killed bacterin. The difference in the methods of preparation was that the cells for the killed bacterin were resuspended after the third washing with 10 ml of saline containing 0.5% formalin, while only saline was used in the living cell preparation.

The cell suspensions were standardized according to two methods. The method of Hopkins was used for the S_1 bacterin, and the nephelometric method

of McFarland was used for the B_1 preparations. Both these methods are given in Clinical Diagnosis by Laboratory Methods (Todd, Stanford and Wells, 1953).

The method of Hopkins consisted of filtering the bacterial suspension through cotton into a 250 ml Erlenmeyer flask. Ten ml of the filtered cell suspension were placed in each of 4 Hopkins centrifuge tubes and run at 2800 rpm for 30 minutes in a clinical centrifuge. At the end of this time, the supernatant fluid and all the bacterial sediment above the 0.05 ml mark on the Hopkins tubes were removed with sterile capillary pipets. Saline containing 0.5% formalin was added to the 5.0 ml mark in each tube and the cells resuspended. This gave a 1% cell suspension, which for the S_1 organism was equal to a concentration of 8 x 10^{10} cells per ml. The 1% suspension was then diluted with saline containing 0.5% formalin to two concentrations. The first contained 1 x 10^{10} cells per ml and was used for the injections. The second contained 2 x 10^{10} cells per ml and was used for the agglutination tests.

The B_1 cells were standardized by turbidity. The cell suspension was filtered through cotton, and one ml of the filtrate was added to a standard test tube. Saline-formalin mixture was used to dilute the killed bacterin to agree with the McFarland tube # 7. Saline was used to dilute the 1.0 ml of cell suspension for the living cell mixture to the McFarland tube # 7. The amount of fluid necessary to dilute the 1.0 ml of cell suspension to this concentration was noted and the rest of the cell suspension was diluted accordingly. This gave a cell concentration of 2.1 x 10^{10} cells per ml, which was used for the agglutination tests. Twenty ml of this suspension

was then diluted 1:2 to give 1 x 10^{10} cells per ml, which was used for injections.

The diluted cell suspensions were placed in sterile vaccine bottles, capped and labeled. Those which were formalin treated were incubated for 24 hours. Following this, 1.0 ml was removed from each bottle and inoculated into sterile fluid thioglycollate tubes and into brain heart infusion tubes. The tubes were incubated for 48 hours to test organism kill.

As a check for possible serologic changes in the fecal test organisms by the end of the experiment, cell suspensions were made of the organisms which appeared on the last set of plates for the fecal organism counts. Plates with low numbers of organisms were chosen, and every colony on the plates picked and inoculated into tubes containing 15 ml of trypticase soy broth. The tubes were incubated for 24 hours.

After incubation the S_1-like organism suspensions were transferred to sterile centrifuge tubes. The tubes were centrifuged, the supernatant fluid was poured off and the cells resuspended in 10 ml of saline. The cells were washed 3 times as before. After the last washing the cells were resuspended in 10 ml of saline containing 0.5% formalin. The resulting cell suspensions were standardized by the Hopkins method, and diluted to concentrations of 2 x 10^{10} cells per ml. These cell suspensions were used in agglutination tests against both the sera collected from the rabbits at the start of the injection period and the sera from the final bleedings.

The B_1-like organisms were inoculated from the tubes onto slants of trypticase soy agar and incubated for 24 hours. Following this, the organisms were washed off each slant with saline and curved glass rods and placed in

sterile Serval centrifuge tubes. The cells were washed three times and resuspended in saline. The resulting cell suspensions were standardized using McFarland tube # 7. The cell suspensions were used in agglutination tests against the original sera and sera from the final bleedings.

A bacterin of Escherichia coli (Montana State College stock culture # 4) was made to check for nonspecific agglutinins in the rabbits' sera.

The organism was inoculated into 10 ml of trypticase soy broth and incubated for 8 hours. At the end of this period the resulting cell suspension was streaked onto milk dilution bottle slants. The cells were grown, harvested, and treated as previously explained for the B_1 cell suspension. The suspension was standardized against McFarland tube # 7, which gave an approximate concentration of 2.1 x 10^{10} cells per ml. This was the concentration used for the agglutination tests.

Determination of circulating antibody titer against the test organisms

Blood was collected from the rabbits by the ear puncture and heart puncture techniques. Weekly bleedings were done during the test period, starting one week before the first injection. At the time of the ear bleedings 10 to 15 ml of blood was collected from each rabbit. The blood was allowed to clot at room temperature for three hours, then the clots were ringed with sterile applicator sticks and stored over night at 5 C. The next morning the expressed sera were poured into sterile glass centrifuge tubes and spun at 1000 rpm for 20 minutes. The supernatant fluid was poured into sterile metal capped tubes and used immediately. In the heart puncture technique 40 to 50 ml of blood were collected and treated

as mentioned above.

The sera collected by these two methods were used in the agglutination tests. The test method used was a modification of that described in Carpenter's Immunology and Serology (1956). The modification was in the dilutions used. The dilutions used in this experiment are shown in Table I. For the B_1 organism, it was necessary to use dilutions in the range of 1:4,096, and for the S_1 organism, it was necessary to use dilutions in the range of 1:131,072.

The agglutination tubes were shaken and incubated in a 37 C water bath for 24 hours. At the end of this time, the tubes were read. This was the only temperature used in the agglutination studies.

Agglutination tests were run on the serum from each rabbit every week during the injection period. Tests were made with each serum against both the B_1 and S_1 bacterins. At the end of the experiments agglutination tests were also run with the various sera using *E. coli* and the S_1-like and B_1-like organisms as the antigens.

TABLE I

Dilutions used in the agglutination tests

tubes	saline ml	serum ml	antigen ml	dilution
1	0	0.5	0.5	1:2
2	0.5	0.5	0.5	1:4
3	0.5	0.5 of 1:2	0.5	1:8
4	0.5	0.5 of 1:4	0.5	1:16
5	0.5	0.5 of 1:8	0.5	1:32
6	0.5	0.5 of 1:16	0.5	1:64
7	0.5	0.5 of 1:32	0.5	1:128
8	0.5	0.5 of 1:64	0.5	1:256
9	0.5	0.5 of 1:128	0.5	1:512
10	0.5	0.5 of 1:256	0.5	1:1024
11	0.5	0.5 of 1:512	0.5	1:2048
12	0.5	0.5 of 1:1024	0.5	1:4096
13	0.5	0.5 of 1:2048	0.5	1:8192
14	0.5	0.5 of 1:4096	0.5	1:16,384
15	0.5	0.5 of 1:8192	0.5	1:32,768
16	0.5	0.5 of 1:16,384	0.5	1:65,536
17	0.5	0.5 of 1:32,768*	0.5	1:131,072
18 control	0.5		0.5	

* Discard 0.5 ml from last tube

Method of increasing the circulating antibody titer

After the original circulating antibody titer had been determined against the test organisms, the rabbits received injections of the two test bacterins. Of the test animals, 2 were chosen to receive the B_1 suspension, 2 to receive the S_1 bacterin, and the remaining 2 were kept as controls. The route of injection was through the marginal ear vein.

The injection schedules used were as given in Carpenter's Immunology and Serology (1956). Two different schedules were used due to the two different types of bacterins. These are given in Table II.

TABLE II

Schedules followed to administer bacterins

Time after first injection Days	Amounts	
	S_1 bacterin*	B_1 cell suspension
0	0.1 ml	0.1 ml **
1		0.2 ml **
2		0.3 ml **
3	0.3 ml	0.1 ml *
4		0.2 ml *
5		0.3 ml *
7	0.5 ml	
10	1.0 ml	0.5 ml *
14	2.0 ml	

* Cell suspension containing 1 x 10^{10} cells per ml.

** Cell suspension containing 1 x 10^8 cells per ml.

Determination of coproantibody concentration in the feces

The feces were collected as before except the cover newspaper was not sterilized. The feces were weighed as close as possible to 4 grams without cutting a pellet and were diluted 1:4 with saline. At first this was done in 30 ml beakers. The beakers were shaken on a rotary shaker for 10 minutes. The supernatant fluid was poured into plastic Serval centrifuge tubes and spun at 7,000 rpm for 10 minutes. The supernatant was poured off into sterile

test tubes and agglutination tests were run on this fluid using the B_1 and S_1 organisms as the antigens.

Because the first dilution possible with this method was 1:8 and there was not agglutination recorded, the fluid was concentrated. All the supernatant fluid present after centrifugation was placed in dialysis bags and allowed to evaporate. When this was first done, the concentrate was one half of the original volume. Agglutination tests were run on this material but no agglutination was present.

Another method was used in which the fecal samples were ground up and placed in plastic centrifuge tubes with 4 ml saline added for each gram of feces. The tubes were capped, and shaken for 2 minutes. The tubes were placed in a Serval refrigerated centrifuge and spun at 7,000 rpm for 10 minutes. The supernatant was removed and placed in dialysis bags and again evaporated. The fluid was concentrated 8 fold and agglutination tests run with it as the antiserum.

CHAPTER IV

EXPERIMENTS AND RESULTS

Description of test organisms

The organisms isolated from the rabbits' feces were designated B_1 and S_1. Based on the various reactions and characteristics the isolates exhibited (Table III), they were identified according to Bergey's Manual of Determinative Bacteriology (Breed, Murry, and Smith, 1957). The B_1 isolate was identified as *Bacillus pumilus*. The S_1 isolate was identified as *Streptococcus faecalis*.

TABLE III

Characteristics of the B_1 and S_1 isolates

Test	*B. pumilus*	*S. faecalis*
Gram reaction	+	+
O_2 requirement	aerobic	facultatively anaerobic
Spore formation	+	-
Swelling due to spores	-	-
Size	0.7 by 2.0 u	0.7 u
Colonies on EMB	circular, entire convex. 2 mm in diameter, smooth and mucoid.	absent
Colonies on SF	absent	circular, entire convex. 1 mm in diameter, smooth and mucoid.
Capsule present	-	-
Cells found	singly	in chains or pairs
Growth in broth	aerobic pellicle formed	flocculent heavy sediment
Motility	+ in SIM medium	- in SIM and hanging drop

TABLE III cont'd.

Test	*B. pumilus*	*S. faecalis*
Temperature range		
10 C	absent	1 +
20 C	2 +	3 +
28 C	4 +	4 +
35 C	4 +	4 +
45 C	2 +	2 +
56 C	absent	absent
1 + equals slight growth, 2 + equals fair growth, 3 + equals good growth, 4 + equals abundant growth.		
Gelatin liquefaction	+	-
Starch hydrolysis	-	-
Litmus milk	peptonized	reduced in 24 hours, acid curd in 48 hours
6.5% NaCl broth	growth	growth
7.0% NaCl broth	growth	-
9.0% NaCl broth	-	-
pH 9.6 broth	-	+
glucose	acid	acid
lactose	acid	acid
sucrose	acid	acid
galactose	acid	acid
arabinose	acid	acid
mannose	acid	-
maltose	acid	-

Number of test organisms per gram of feces

These experiments were designed to show any change in the number of test organisms per gram of feces during a period of active immunity. Bacterins of the two test organisms were given to groups of rabbits. The number of each test organism per gram of feces was checked in rabbits immunized with the B_1 bacterin, in rabbits immunized with the S_1 bacterin, and in non-immunized rabbits.

The feces were collected as previously described. After being diluted

1:100 with phosphate buffered water the fecal mixtures were agitated for 10 minutes in Waring blendors. Serial dilutions were made and the material plated.

To determine the number of _Bacillus pumilus_ organisms per gram of feces, EMB agar was used as the plating medium. On EMB agar the organism produced a colony form similar to that produced by species of _Aerobacter_. The colonies were circular, entire and convex. Their average size was 2 mm in diameter. They contained a dark purple center with light purple edges.

To determine the number of _Streptococcus faecalis_ organisms per gram of feces, SF agar was used as the plating medium. On SF agar the organism produced a yellowish zone around the colony.

Plate counts were run weekly, starting one week before the first injection and proceeding through the injection period. The last counts were made 46 days after the first injection. The counts were tabulated according to which bacterin the animals received. Group 1 animals received the _Bacillus pumilus_ bacterin, group 2 animals received the _Streptococcus faecalis_ bacterin, and group 3 received no bacterin and was the control group. Each group consisted of two rabbits.

As the circulating antibody concentration against _B. pumilus_ increased in the group one rabbits which had received the _B. pumilus_ bacterin, there were no appreciable differences in the number of _B. pumilus_ organisms found per gram of feces as shown on the EMB agar plates. There were also no appreciable differences in the number of _S. faecalis_ organisms per gram of feces. The counts are shown in Table IV.

TABLE IV

Number of Bacillus pumilus and Streptococcus faecalis organisms per gram of feces from rabbits immunized with a B. pumilus bacterin

Time after first injection	Rabbit #1		Rabbit #4	
	B. pumilus	S. faecalis	B. pumilus	S. faecalis
Days	Counts x 10^4			
0*	41	15	60	18
7	4	13.5	30	13.7
14**	5	12	37	8.3
22	4.5	12	9	4
25***	13	16	50	10.2
29	7	23	19	9.5
36	65	30	16.5	34
39	120	55	38	45
44	57	20	41	31
45	120	80	500	180
46	50	30	600	137

* Time on which immunization with a formalin killed Bacillus pumilus bacterin began.
** Time on which immunization with a living cell suspension of Bacillus pumilus began.
*** Time on which the peak circulating antibody titer was reached.

To determine the effect upon the test organism during the period of immunization additional isolations were made. When the final plate counts were made 46 days after the start of the injection period, all the colonies from the EMB and SF agar plates with the lowest number colonies were picked and transferred to tubes containing 15 ml of trypticase soy broth. One EMB and one SF agar plate was used per rabbit.

Twenty-seven colonies were picked from the EMB agar plates containing the fecal samples from the B. pumilus immunized group of rabbits. Of the 27 tubes containing the isolates, two showed aerobic growth. The other 25 tubes showed growth only in the bottom 2/3 of the broth. Samples from all

27 tubes were transferred to tubes of fluid thioglycollate medium and to the same differential media used to identify the original B. pumilus test organism. The cell suspensions remaining in the trypticase soy tubes were then standardized according to the method of McFarland using standard #7.

All the colonies picked from the EMB agar plates containing the fecal samples from groups 2 and 3 grew aerobically in the trypticase soy broth. Eight tubes were randomly chosen from each group and samples from each tube were inoculated into thioglycollate medium and the differential media used to identify the original B. pumilus. The remaining suspensions in these tubes were standardized according to the method of McFarland using standard #7.

The 25 organisms from group 1 which grew in the lower 2/3 of the trypticase soy broth also grew that way in the thioglycollate medium. Because of their reaction and characteristics on the differential media, they were identified as members of the genus Lactobacillus. The 2 aerobic organisms were found to be members of the species B. pumilus. All the organisms from groups 2 and 3 which were tested were found to be members of the species B. pumilus.

Agglutination tests were run on the 27 organisms from group 1 and on the 16 organisms from groups 2 and 3. The tests were done using sera collected from the group 1 rabbits before immunication and sera collected after immunization. The immune sera contained agglutinins against B. pumilus in a titer of 1:2048. The sera collected from the rabbits before immunization contained agglutinins against B. pumilus in a titer of 1:8. Agglutination tests were also run against the 43 isolated organisms using sera from the group 2 and 3 rabbits. These sera contained agglutinins

against B. pumilus in a titer of 1:8.

The 25 Lactobacillus organisms were found to have no titer in the low titer sera from group 1. In the sera from this group containing agglutinins against B. pumilus in high titer, these 25 organisms showed titers of 1:16 as compared to titers of 1:2048 for the B. pumilus organism. The 2 isolates from the final plate count which were identified as B. pumilus showed agglutination titers of 1:8 in the low titer sera and 1:2048 in the immune sera. These 2 organisms were therefore considered to be the same as the original B. pumilus organism. The results are shown in Table V.

TABLE V

Circulating antibody titers present in rabbits immunized with Bacillus pumilus bacterin against the Lactobacillus sp. and B. pumilus organisms isolated during the final plate counts

Organism	Low titer sera	Immune sera
Original B. pumilus	1:8	1:2048
Final B. pumilus	1:8	1:2048
Lactobacillus	0	1:16

The 16 organisms isolated from the feces of groups 2 and 3 showed the same titers as the original B. pumilus organism in both the low titer and immune sera collected from the group 1 rabbits.

The 25 Lactobacillus organisms showed no titer in the sera from the groups 2 and 3 rabbits. The 2 B. pumilus isolated from the final plates containing the fecal samples from group 1 showed the same titer in the sera from the groups 2 and 3 rabbits as the original B. pumilus organism (Table VI).

TABLE VI

Circulating antibody titers against Bacillus pumilus and Lactobacillus sp. in sera from groups 2 and 3

Organism	Group 2	Group 3
Original B. pumilus	1:8	1:8
Final B. pumilus	1:8	1:8
Lactobacillus	0	0

The plate count methods did not show any effect of increased antibody concentrations on the number of colonies resembling those of B. pumilus and S. faecalis per gram of feces. However, the increased antibody concentrations did have effect on the organisms. When the antibody concentration against B. pumilus was increased, the number of B. pumilus organisms found per gram of feces decreased and a Lactobacillus producing a colony similar to that of B. pumilus was found in large numbers. This organism was only found in the feces of the B. pumilus immunized rabbits, not in the other two groups.

As the circulating antibody concentration against S. faecalis increased in the group 2 rabbits which had received the S. faecalis bacterin, there was no appreciable differences in the number of S. faecalis organisms found per gram of feces as shown by the SF agar. There were also no appreciable differences in the number of B. pumilus organisms (Table VII).

TABLE VII

Number of Streptococcus faecalis and Bacillus pumilus organisms found per gram of feces in rabbits immunized with a S. faecalis bacterin

Time after first injection	Rabbit #2		Rabbit #5	
	S. faecalis	B. pumilus	S. faecalis	B. pumilus
Days	Counts x 10^4			
0*	1.9	18	4.8	5.2
7	2.2	7.5	3.7	3
14	1.2	4	3.1	4.3
22**	.5	3.7	5.9	3.9
39	100	153	3	3
44	6	8.3	4	5.9
45	700	1000	30	85
46	5.5	31	4.9	10.1

* Time on which immunization with the Streptococcus faecalis bacterin began.
** Time on which the peak circulating antibody titer was reached.

Twenty-five colonies were picked from the SF agar plates containing the fecal samples from group 2. This was the group of rabbits which received the Streptococcus faecalis bacterin. All 25 tubes showed the same growth the original S. faecalis possessed in this medium. Samples from each tube were inoculated onto the same differential media as were used to identify the original S. faecalis isolate. The remaining cell suspensions in the tubes were standardized according to the method of Hopkins.

All of the colonies picked from the SF agar plates containing the fecal samples for groups 1 and 3 exhibited the same type of growth in the trypticase soy tubes as the original S. faecalis. Eight tubes were picked at random from each group and samples inoculated into the same type of differential media as were used to identify the original S. faecalis organism. The cell suspensions remaining in these tubes were then standardized according to the

method of Hopkins.

From their reactions and characteristics on the various differential media, all of the picked organisms from the three groups were identified as members of the species Streptococcus faecalis.

Agglutination tests were run on the 25 organisms isolated from the rabbits immunized with a S. faecalis bacterin and on the 16 organisms isolated from groups 1 and 3. These tests were done using sera collected from the group 2 rabbits before the immunization with S. faecalis began and with sera collected after immunization. The low titer sera contained agglutinins against S. faecalis capable of agglutination in a 1:8 dilution. The immune sera contained agglutinins against S. faecalis in a titer of 1:65,536. Agglutination tests were also run against the 41 isolated organisms using sera from groups 1 and 3. These sera contained agglutinins against S. faecalis in a titer of 1:8.

The 25 organisms isolated from the feces of the group 2 rabbits were found to have no agglutination titers in the serum from the non-immunized rabbits. In the immune sera these 25 organisms were found to have agglutinins in the titer of 1:16 as compared to titers of 1:65,536 for the original S. faecalis organism. The results are shown in Table VIII.

TABLE VIII

Circulating antibody titer present in rabbits immunized with Streptococcus faecalis against the S. faecalis organisms present in the final plate counts

Organism	Low titer sera	Immune sera
Original S. faecalis	1:8	1:65,536
Final S. faecalis	0	1:16

The 16 organisms from groups 1 and 3 showed the same titers as the original S. faecalis organism in both the immune and low titer sera from the group 2 rabbits.

The 25 organisms from group 2 did not show any titer with the sera from groups 1 and 3.

The plate count methods did not show any effect of increased antibody concentrations on the number of colonies resembling those of B. pumilus and S. faecalis per gram of feces. However, the increased antibody concentrations did have effect on the organisms. When the circulating antibody titer was increased against S. faecalis a complete sero-type shift was found in the S. faecalis organisms isolated from the feces. Because of the manner in which the bacterin was made, all major sero-types of the organism which were present in the feces would have been included.

During the course of the experiment, the control group showed only a normal fluctuation in the number of B. pumilus and S. faecalis organisms found per gram of feces. This fluctuation would be expected using plate count methods and differential plating media. The consistency and water content of the rabbits' feces varied from count to count. This would also give a fluctuation in the organism count (Table IX).