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The gut flora of the chick

3*. Differences in caecal flora between 'infected', 'uninfected' and penicillin-fed chicks

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The gut flora of domestic animals has been studied extensively in recent years, mainly in attempts to elucidate the mechanism of antibiotic stimulation of the growth of young animals. There is strong reason to believe that the effect is mediated through the gut flora (Jukes, 1955), but considerable confusion exists because of the contradictory results obtained by different groups of workers. For example, when they gave chlortetracycline to chicks Dixon & Thayer (1951) reported an increase in the numbers of lactobacilli, March & Biely (1952) reported a decrease, whereas Eisenstark & Sanford (1953) and Anderson, Cunningham & Slinger (1953) found no change.

Coates, Dickinson, Harrison, Kon, Porter, Cummins & Cuthbertson (1952) have shown that in old premises that have housed several generations of chicks a growth-depressing condition exists which is transmissible and is counteracted by penicillin in the diet. In this connexion, Elam, Jacobs, Fowler & Couch (1954) reported a decrease in 'clostridia' in penicillin-fed chicks, and that 'clostridia' fed to chicks in a clean environment depressed growth; however, according to Smyser, Cleverdon, Kulp & Materson (1952) the numbers of *Clostridium perfringens* increased in the presence of dietary penicillin after 4 weeks, but chlortetracycline was without effect, and Brown & Luther (1950), Romoser (1951) and Anderson, Cunningham & Slinger (1951) found no reduction in numbers of anaerobes, and sometimes an increase, with chlortetracycline.

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In the present work we studied the anaerobic flora of the caeca of 'infected', 'uninfected' and penicillin-fed birds (see below) during the first few days of life, since an 'adult' flora is established in chicks 2 days after they begin to feed (Lev & Briggs, 1956*b*). A note on our findings has been published elsewhere (Lev, Briggs & Coates, 1956).

EXPERIMENTAL

Methods

Chicks. Rhode Island Red × Light Sussex male day-old chicks were used in all experiments; they were housed in electrically heated brooders or cages with wire floors. Five birds constituted an experimental group; in addition, twenty birds of the same batch and brooder and treated in exactly the same way were weighed at 1 and 2 weeks of age. A *t* test was done on the weights of 'infected', 'uninfected' and penicillin-fed groups of chicks at 2 weeks to determine the significance of differences between them.

Diet. The chicks received *ad lib.* the mash used in previous experiments (Lev & Briggs, 1956*b*). For certain groups of chicks procaine penicillin was incorporated in the diet at the rate of 45·5 mg/kg.

Housing. Two types of housing were used: (1) 'infected' premises in which chicks had been kept for some time and in which an habitual growth response to penicillin had been obtained; (2) 'uninfected' premises—a small room in an animal house situated some distance from the infected premises. The uninfected premises were disinfected before each experiment with formaldehyde for about 2 days, remaining traces being removed with ammonia. Under our conditions little or no growth response is obtained by feeding antibiotics to chicks in such premises, and these chicks were used as controls. Cages, heaters and feeding troughs were sterilized by autoclaving them before each experiment. Air samples were examined for *Clostridium welchii* with a slit sampler and neomycin–egg-yolk plates (see below).

Bacteriological methods. The caeca were removed for bacterial counts immediately the chicks were killed. Their contents were expressed into 9 ml. freshly boiled and cooled quarter-strength Ringer's solution and vigorously shaken with glass beads. Decimal dilutions were made of this solution, and plates inoculated from the dilutions were incubated anaerobically as quickly as possible. Several granules of calcium chloride were placed in each anaerobic jar to prevent spreading of colonies on the plates. After the total counts, spore counts were also made on the original dilution, after it had been heated at 80° for 15 min to kill vegetative cells. Miles & Misra's (1938) method was used and counts were related to the dry weight of material. For dry-weight determinations 2 ml. of sample were heated on a boiling water-bath for about $\frac{1}{2}$ h and then dried to constant weight at 100° in an electric oven. Counts were made on blood agar; in some earlier experiments liver-infusion agar and the medium described by Wynne, Schmieding & Daye (1955) were also used. Soluble starch (0·1%) was added to the media used for the enumeration of spores (Olsen & Scott, 1950). Total counts of *Cl. welchii* were made by the method of Lowbury & Lilly (1955) modified by the addition of 100 μg/ml. neomycin (Upjohn) to a 10% egg-yolk serum

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nutrient agar. Duplicate plates were used throughout and all incubations were made at 39° for 2 days. *Cl. welchii* was identified by cultural and biochemical characteristics and by the Nagler reaction (Hayward, 1943). Several strains were kindly typed by Professor C. L. Oakley.

Design of experiments

Comparative bacteriology of infected and uninfected chicks

Total counts. In previous work Lev & Briggs (1956*b*) showed that a balanced gut flora is established in chicks 2 days after feeding. To investigate any differences in the establishment of the total bacterial flora in the gut of infected and uninfected chicks counts were made on material from six sites in the gut, namely, crop, gizzard, duodenum, first and second parts of the ileum, and the caeca, 1 and 2 days after feeding.

Differences in clostridial population. Following the demonstration of *Cl. paraputrificum* in the caeca of chicks not fed or watered (Lev & Briggs, 1956*a*), the quantitative and qualitative development of the clostridial spore population of infected and uninfected chicks was examined on each of the first 4 days after feeding.

Reinfection of cleaned premises. The infected chick premises were thoroughly cleaned and redecorated, and spontaneous reinfection was studied, with *Cl. welchii* as an index of infection, on three successive batches of chicks; chicks from the animal house served as controls.

Comparative bacteriology of infected and penicillin-fed chicks

These experiments were made on lines similar to those with infected and uninfected chicks, with the addition that neomycin–egg-yolk plates for *Cl. welchii* were used, as well as blood-agar plates for total counts. Both penicillin-fed and control groups of chicks were reared in the infected premises.

RESULTS

Comparative bacteriology of infected and uninfected chicks

Total counts. The results of replicate experiments in which total counts of material from six sites in the chick gut were made showed no consistent quantitative or qualitative differences between infected and uninfected groups of chicks. The results of Miles & Misra's (1938) method and tube dilution counts used in parallel agreed well.

Differences in clostridial population. Although the total number of spores was similar in the infected and uninfected groups of chicks 1, 2, 3 and 4 days after feeding (Table 1), a distinct qualitative difference was found on the 1st day only; *Cl. welchii* spores appeared in large numbers in the infected chicks and none in the uninfected (Pl. 1, 1). The clostridia isolated from the caeca of older chicks 2, 3 and 4 days after feeding were similar in both number and type.

These results were confirmed in two further experiments. In addition, total counts made in parallel with spore counts showed that on the 1st day *Cl. paraputrificum* was present in as large numbers as *Escherichia coli*. By the 2nd day many other types of organism had appeared and a greater variety of organisms was isolated from infected chicks than from uninfected controls.

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Reinfection of cleaned premises. In the studies of reinfection of cleaned premises, *Cl. welchii* appeared contrary to expectation in the caeca of the control chicks from the animal house 1 day after feeding, but not in the caeca of chicks in the cleaned premises. From the results of previous experiments, the chicks with *Cl. welchii* in their caeca were regarded as being infected, whereas the others were not. These forecasts were confirmed later by the growth response of the controls to penicillin (Exp. 1, Table 2); however, the chicks in the cleaned premises showed a small response.

When a second batch of chicks (Exp. 2, Table 2) was put in the infected cleaned premises 2 weeks after the first, *Cl. welchii* appeared in their caeca 1 day after feeding, and their weight at 14 days of age showed that they were infected. No accommodation was available for a control group in this experiment.

When the experiment in the study of reinfection of premises was repeated with a third batch of chicks *Cl. welchii* appeared in the caeca of chicks from the infected cleaned premises but not in those from the animal house. However, both groups showed a significant response to penicillin, i.e. were infected (Exp. 3, Table 2).

The infected state was thus related to the presence of *Cl. welchii* in the caeca of chicks 1 day after feeding, except in one instance. Moreover, it was possible to determine the recrudescence of the infection by the appearance of *Cl. welchii* in chicks placed in the newly cleaned premises.

Comparative bacteriology of infected and penicillin-fed chicks

In the first experiment of this series (Table 3) *Cl. welchii* was not isolated from either group 1 day after feeding. An almost pure culture of coliforms was isolated from the penicillin-fed group, whereas many clostridial types other than *Cl. welchii* occurred in the unsupplemented controls. However, *Cl. welchii* appeared in large numbers in the unsupplemented group 2 days after feeding, but not in the penicillin-fed group. In one replicate of this experiment *Cl. welchii* was not isolated from the caeca of chicks receiving penicillin in the diet, though in this and another replicate (Exps. 2 and 3, Table 3) it appeared in the control birds 1 and 2 days after feeding (Pl. 1, 2). It appears, therefore, that *Cl. welchii* and other clostridia were eliminated, or their establishment prevented, by the small amount of penicillin incorporated in the diet.

In one experiment when chicks from an alternative source (University of Reading Poultry Farm) were used (Exp. 4, Table 3), *Cl. welchii* occurred in both penicillin-fed and control groups in approximately equal numbers. However, Pl. 1, 3 shows that, in contrast with the activity of *Cl. welchii* strains isolated from the control group, lecithinase (α-toxin) production on neomycin–egg-yolk agar was markedly less in strains from the penicillin group. Strains of *Cl. welchii* from this group produced as much lecithinase when subcultured in the absence of penicillin (and in pure culture) as strains isolated from the control group.

In the second experiment with chicks from this source (Exp. 5, Table 3) penicillin caused a reduction in both numbers and toxigenicity. This result is of interest, being intermediate between those obtained with our own chicks and that of the first experiment with chicks from another source.

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Table 1. *Correlation of* Cl. welchii *in the caeca of infected chicks with the depressed growth condition; weights of uninfected chicks, whose caeca contained no* Cl. welchii *1 day after feeding*

Exp. no.	No. of birds/ group	Weight (g) at 2 weeks: On diet	Weight (g) at 2 weeks: On diet + penicillin	P*	No. of *Cl. welchii* spores (1 day after feeding)	Total no. of organisms (1 day after feeding): Spores	Total no. of organisms (1 day after feeding): Vegetative	No. of *Cl. welchii* spores (2 days after feeding)	Total no. of organisms (2 days after feeding): Spores	Total no. of organisms (2 days after feeding): Vegetative	Total no. of spores on: 3rd day	Total no. of spores on: 4th day
1: infected	20	108	137	$P<0.001$	*c.* 6.5×10^{6}	7.5×10^{6}	—	—	2.2×10^{7}	—	3.0×10^{8}	8.1×10^{6}
animal-house room, control	20	122	122	$P>0.9$	None	1.1×10^{6}	—	—	2.3×10^{7}	—	5.4×10^{8}	1.1×10^{7}
2: infected	20	115	134	$0.01>P>0.001$	*c.* 1.0×10^{6}	1.2×10^{6}	7.0×10^{10}	Present†	9.2×10^{6}	7.2×10^{10}	—	—
animal-house room, control	20	125	133	$0.3>P>0.2$	None	8.7×10^{7}	7.6×10^{10}	Present†	3.1×10^{6}	1.2×10^{11}	—	—
3: infected	40	115	143	$0.01>P>0.001$	*c.* 1.0×10^{6}	1.4×10^{6}	7.0×10^{10}	*c.* 1.4×10^{5}	1.3×10^{7}	7.0×10^{10}	—	—
animal-house room, control	20	134	127	$0.3>P>0.2$	None	1.1×10^{7}	2.0×10^{9}	*c.* 3.0×10^{6}	9.3×10^{6}	8.5×10^{10}	—	—

* Probability of the difference in the weights of the groups of chicks arising by chance in a homogeneous population.
† $>10^{3}$ and $<10^{5}$.

Table 2. *Correlation of* Cl. welchii *in the caeca of chicks in newly redecorated premises with the appearance of the depressed growth condition*

Exp. no.	No. of birds/ group	Weight (g) at 2 weeks: On diet	Weight (g) at 2 weeks: On diet + penicillin	P*	No. of *Cl. welchii* spores (1 day after feeding)	Total no. of organisms (1 day after feeding): Spores	Total no. of organisms (1 day after feeding): Vegetative	No. of *Cl. welchii* spores (2 days after feeding)	Total no. of organisms (2 days after feeding): Spores	Total no. of organisms (2 days after feeding): Vegetative
1: infected	60	117	120	$0.4>P>0.3$	None	4.1×10^{7}	2.8×10^{10}	Present†	1.1×10^{8}	1.4×10^{10}
animal-house room, control	40	118	126	$0.1>P>0.05$	3.6×10^{7}	4.2×10^{7}	6.2×10^{9}	None	1.1×10^{6}	4.9×10^{10}
2: infected	30	105	125	$P<0.001$	1.4×10^{7}	5.5×10^{8}	8.9×10^{10}	1.5×10^{5}	3.2×10^{7}	1.5×10^{10}
3: infected	40	104	119	$0.05>P>0.02$	4.9×10^{5}	2.4×10^{8}	1.1×10^{11}	4.4×10^{5}	1.2×10^{8}	2.0×10^{11}
animal-house room, control	20	109	123	$0.02>P>0.01$	None	1.9×10^{8}	6.9×10^{9}	2.5×10^{6}	2.5×10^{7}	5.9×10^{11}

* Probability of the difference in the weights of the groups of chicks arising by chance in a homogeneous population.
† $>10^{3}$ and $<10^{5}$.

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Table 3. *Correlation of* Cl. welchii *in the caeca of infected chicks with the depressed growth condition; weights of chicks given penicillin, whose caeca contained either no* Cl. welchii *or atoxigenic* Cl. welchii

Exp. no.	No. of birds/ group	Weight (g) at 2 weeks	P*	Vegetative count, 1 day after feeding		Vegetative count, 2 days after feeding	
				Cl. welchii	Total	*Cl. welchii*	Total
1: no addition	20	119	<0·001	$<10^3$	$2{\cdot}0 \times 10^9$	$1{\cdot}5 \times 10^8$	$1{\cdot}3 \times 10^{11}$
given penicillin	20	125		$<10^3$	$5{\cdot}2 \times 10^{10}$	$<10^3$	$1{\cdot}7 \times 10^{11}$
2: no addition	20	113	<0·001	$1{\cdot}4 \times 10^7$	$7{\cdot}0 \times 10^{10}$	$2{\cdot}3 \times 10^7$	$2{\cdot}2 \times 10^{11}$
given penicillin	20	137		$<10^3$	$1{\cdot}2 \times 10^{11}$	$<10^3$	$2{\cdot}5 \times 10^{11}$
3: no addition	16	111	<0·001	Not tested		$6{\cdot}1 \times 10^6$	$9{\cdot}0 \times 10^{10}$
given penicillin	16	136				$<10^3$	$1{\cdot}5 \times 10^{11}$
4: no addition	106	134	<0·001	$1{\cdot}5 \times 10^8$	$3{\cdot}8 \times 10^{11}$	$9{\cdot}2 \times 10^5$	$8{\cdot}1 \times 10^{10}$
given penicillin	107	145		$1{\cdot}5 \times 10^8$†	$7{\cdot}8 \times 10^{11}$	$<10^3$	$9{\cdot}0 \times 10^{10}$
5: no addition	70	117	<0·001	$4{\cdot}4 \times 10^8$	$2{\cdot}2 \times 10^{10}$	$1{\cdot}7 \times 10^8$	$1{\cdot}2 \times 10^{11}$
given penicillin	72	143		$7{\cdot}8 \times 10^4$	$4{\cdot}0 \times 10^{10}$	$2{\cdot}0 \times 10^7$†	$1{\cdot}6 \times 10^{11}$

* Probability of the difference in the weights of the groups of chicks arising by chance in a homogeneous population.
† Strains showing reduced toxigenicity.

Other experiments

Distribution of Cl. welchii *in the chick gut.* In one experiment in which spore counts were made, *Cl. welchii* was found only in the caeca and not in the duodenum or ileum of infected chicks 1 and 2 days after feeding.

Typing of Cl. welchii. All the strains examined were of type A. They produced α-, θ-, and κ-toxins and one strain also produced μ-toxin. They showed the Nagler reaction, did not sporulate in culture, fermented lactose and formed acid and clot or a 'stormy clot' in litmus milk.

Air examination. Cl. welchii was about six times more numerous in the air of the infected chick room than in that of the animal-house room which contained about one per cu. ft. The same batch of diet was fed to both infected and uninfected groups of chicks, and numbers of *Cl. welchii* in the diet were very low (about 50/g); the diet was probably not, therefore, the source of *Cl. welchii* in the chicks.

Presence of Cl. welchii *in chicks* 1 *week old.* Comparisons of the numbers of *Cl. welchii* present in infected, uninfected and penicillin-fed chicks 1 week old showed very few of these organisms in infected chicks and none in the other two groups.

DISCUSSION

In our experience the improvement in the growth of young animals in old (i.e. infected) premises brought about by antibiotics in the diet does not occur in new premises. The growth-depressing condition in the former is transmissible and has been regarded as a subclinical infection which is relieved by antibiotics in the diet (Coates *et al.* 1952). Chicks that are growing suboptimally, i.e. that are infected, have a relatively thicker gut wall than uninfected chicks (Pepper, Slinger & Motzok, 1953; Coates, Davies & Kon, 1955). Numerous workers have reported the sparing action of antibiotics on a variety of nutrients ranging from inorganic ions such as Ca^{2+} (Common, Keefe, Burgess & Maw, 1950) to biotin and folic acid (Coates, Dickinson, Harrison & Kon,

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1951). Thus it may be that antibiotics stimulate the growth of the host animal by eliminating organisms producing substances which irritate, thicken, and hence decrease the permeability of, the gut, with consequent impairment of the absorption of nutrients.

Because *Cl. welchii* produces a variety of toxins, several of which—the enzymes lecithinase, hyaluronidase and collagenase—may be expected to attack cell surfaces, this organism has been considered by a number of workers to be the causative organism of the growth depression. However, Elam *et al.* (1954) counted only spores, which do not produce toxins, and these were present in faeces in numbers of only 10^4/g. Williams, Taylor, Stockstad & Jukes (1951) fed toxins and cultures of clostridia, among them *Cl. welchii*, to chicks, but they did not produce growth depression. However, the toxins may have been decomposed by digestive processes and, since *Cl. welchii* does not form spores under ordinary cultural conditions, it is quite probable that the vegetative cells did not survive the acidity of the gizzard and so failed to become established. Elam *et al.* (1954) prepared their clostridia for feeding experiments by heating a suspension of faeces; by this method spores alone were fed and a growth depression resulted.

The presence of *Cl. welchii* in the gut of chicks appears to be related to the growth depression. There are distinct differences in the presence and activity of *Cl. welchii* in the first few days of life between chicks from clean and infected premises and between infected and penicillin-fed chicks.

The elimination of *Cl. welchii* by dietary penicillin in our experiments is not in agreement with the work of Smyser *et al.* (1952), who detected this organism by the production of a 'stormy clot' reaction in litmus milk. They observed an increase in numbers of *Cl. welchii* when penicillin was fed to chicks in an experiment lasting 6 weeks. In the first of our experiments in which chicks from the University of Reading Poultry Farm were used no reduction in numbers of *Cl. welchii* was observed, but a marked impairment of toxigenicity was noted, which is equivalent (for the host) to the elimination of the organism (Lev *et al.* 1956).

It seems, therefore, that in the mode of action of antibiotics in growth stimulation biochemical function of the gut flora is a more important factor than population trends, or the presence or absence of a particular organism. The great variety of contradictory numerical results obtained by different groups of workers may illustrate this point.

Although not strictly analogous to the subclinical infection in chicks, enterotoxaemia in lambs, which is caused by *Cl. welchii* type D, has been reported by Jordan & Bell (1951) and Jordan (1952) to be reduced or eliminated when chlortetracycline is incorporated in the diet.

There was evidently a relationship between the presence and physiological activity of *Cl. welchii* and the infection or growth depression in our chicks, although occasional anomalous results were obtained in some of the experiments. We hope to show in further experiments whether *Cl. welchii* is, in fact, the infector, or merely an indicator.

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SUMMARY

1. Comparative bacteriological studies were made of 'infected' chicks which show a growth response to penicillin in the diet, and of 'uninfected' chicks which do not, and also of penicillin-fed chicks.

2. No differences were found in total numbers of lactobacilli, streptococci or coliforms in infected and uninfected groups of chicks when six sites in the gut were sampled.

3. Distinct differences were found in the clostridial population between infected and uninfected groups of chicks. *Cl. welchii* appeared 1 day after feeding in the caeca of the infected but not of the uninfected groups.

4. The presence of *Cl. welchii* was used as an index of the reappearance of infection in newly cleaned premises.

5. When penicillin was fed to chicks *Cl. welchii* was eliminated.

6. In an experiment made with chicks from another source the numbers of *Cl. welchii* were similar whether the chicks received penicillin or not, but the toxigenicity of the strains from the penicillin-treated group was markedly impaired on first isolation. In a further experiment the numbers of *Cl. welchii* were reduced and their toxigenicity was impaired.

7. The significance of these results in relation to the mode of action of antibiotics in growth stimulation is discussed.

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EXPLANATION OF PLATE

1. Differences in spore population of the caeca of chicks 1 day after feeding. Haemolytic colonies of *Cl. welchii* present on left blood plate (infected chicks), absent on right plate (uninfected chicks).

2. Differences in spore population of the caeca of chicks 1 day after feeding. Haemolytic colonies of *Cl. welchii* present on left blood plate (infected chicks), absent on right plate (infected chicks receiving penicillin).

3. Differences in lecithinase-production on neomycin–egg-yolk agar between *Cl. welchii* from infected chicks (left) and infected chicks receiving penicillin (right).

Note added in proof. Several strains of Cl. *welchii* isolated from chicks in Exp. 5 (Table 3) have recently been examined for toxin production; they resemble the food-poisoning strains described by Hobbs, B. C., Smith, M. E., Oakley, C. L., Warrack, G. H. & Cruickshank, J. C. (1953) (*J. Hyg., Camb.*, **51**, 75).

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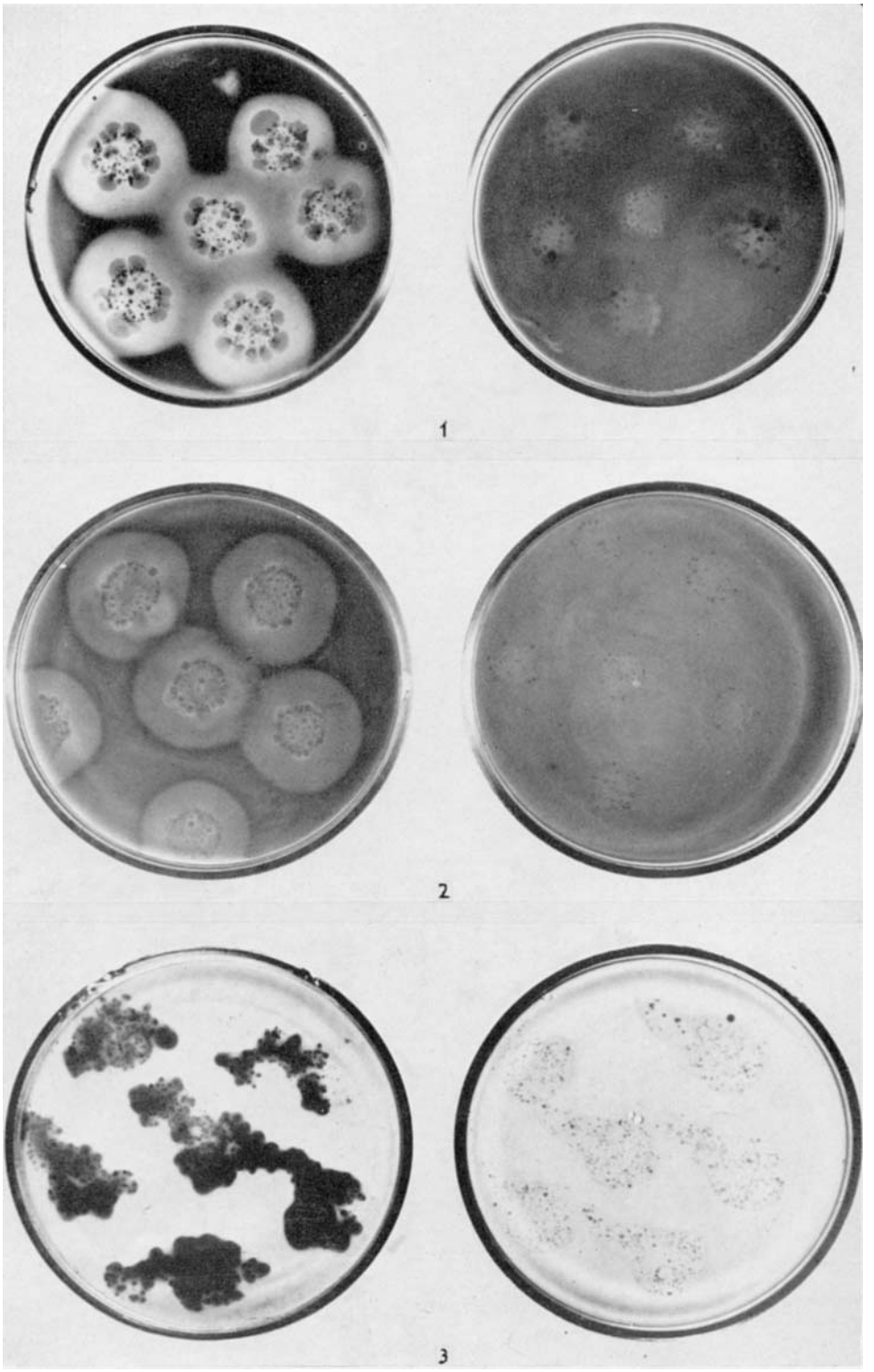

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