Growth, Morphogenesis, and Virulence of *Candida albicans* after Oral Inoculation in the Germ-Free and Conventional Chick¹

EDWARD BALISH² AND A. W. PHILLIPS

Department of Bacteriology and Botany, Biological Research Laboratories,
Syracuse University, Syracuse, New York

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ABSTRACT

BALISH, EDWARD (Syracuse University, Syracuse, N.Y.), AND A. W. PHILLIPS. Growth, morphogenesis, and virulence of *Candida albicans* after oral inoculation in the germ-free and conventional chick. J. Bacteriol. 91:1736-1743. 1966.—The effects of intestinal bacteria on the multiplication, morphogenesis, and infectivity of *Candida albicans* in the alimentary tract were investigated by comparing results obtained in germ-free and conventional chicks after oral inoculation. This challenge resulted in the establishment of large numbers of the pathogen in the alimentary tract of each group of chicks; these numbers were increased in crop contents from challenged bacteria-free chicks wherein hyphae predominated over the yeast form. These animals also had lesions of the crop epithelium containing numerous hyphae and few yeast-like forms. In contrast, challenged conventional chicks receiving an adequate diet displayed no evidence of infection. Their alimentary tract contained the yeast form of *C. albicans*; no hyphae were seen. Although we found bacterial inhibition of *C. albicans* multiplication in the alimentary tract, this in itself did not seem to explain the resistance to intestinal candidiasis in our conventional chicks. We argued that this resistance to infection was due chiefly to the prevention of hyphal development in *C. albicans* by intestinal bacteria. *C. albicans* in the gut of our conventional chicks resulted in some increase in numbers of enterococci in contents from the crop. Increased pH values in contents from the gut of germ-free chicks were not clearly related to infection after challenge. The E_h of the above crop contents were only slightly decreased in the germ-free crop. Thus the E_h did not appear to be involved in susceptibility to infection. Invasion of the blood stream and kidneys of conventional chicks by the yeast form of *C. albicans* occurred in challenged animals receiving a purified diet which had been radiation-sterilized and stored for 6 months at room temperature (25 C). Their growth rate decreased and they became moribund; no hyphae were observed in tissues or intestine of these animals. Challenged bacteria-free chicks receiving the same diet were resistant to the above invasion, although they had crop lesions containing hyphae as described. The resistance of these chicks to systemic invasion was attributed to absence of intestinal bacteria competing for low levels of vitamins in the stored diet. Germ-free chicks had decreased levels of serum γ -globulin which increased after challenge, whereas this value was unchanged in conventional birds after challenge.

The occurrence of *Candida albicans* as a commensal or as a pathogen in the alimentary

¹ Taken in part from a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree, Syracuse University, Syracuse, N.Y.

² Present address: Argonne National Laboratory, Argonne, Ill.

tract (and other sites) in man and other animals was reported by many workers (31). Others suggested that certain members of the intestinal flora may hinder infection of a host by *C. albicans*. For example, chicks and turkey poults were resistant to oral challenge (1, 2). Severe crop infections were described in orally challenged chicks receiving a diet containing antibiotics (25).

In the flora of the chick, unidentified species of lactobacilli were described as the most numerous group of bacteria in contents from all segments of the gut except the colon (24). In the ceca and colon, large numbers of coliforms and enterococci occurred. These findings were confirmed, and large numbers of unidentified lactobacilli in crop contents were described (12).

The growth of *C. albicans* as a yeast form or as hyphae and mycelia with blastospores may be related to its infectivity (23). The preferential growth of hyphae and mycelia during infection was described (4, 27, 33). Many in vitro studies on nutritional control of morphogenesis in *C. albicans* were performed by Nickerson and associates (5). The effects of phosphate (30) and carbon dioxide (Mardon and Phillips, *Bacteriol. Proc.*, p. 16, 1965) on morphogenesis were described.

The present investigation was undertaken to compare the responses of germ-free and conventional chicks to oral challenge with *C. albicans*. This study included: (i) enumeration of bacteria in the alimentary tract of challenged and unchallenged animals, (ii) enumeration of *C. albicans*, (iii) relative numbers of yeast and hyphal forms of *C. albicans*, (iv) pH and E_h in the gut, (v) macroscopic and histological examinations of tissues, and (vi) response to challenge in chicks receiving different diets. A preliminary report of this investigation has been presented (Balish and Phillips, *Bacteriol. Proc.*, p. 67, 1963).

MATERIALS AND METHODS

Organism. *C. albicans* ATCC 10231 was employed. The identity of the antigenic grouping of this strain is unknown to us (7).

Virulence. The virulence of *C. albicans* was tested in albino Swiss mice weighing 20 to 25 g. Animals were inoculated intraperitoneally with washed cells diluted in saline. The LD_{50} was calculated to be 10^6 cells (colony count). When killed and examined within 20 days of injection, surviving mice often manifested severe kidney infections.

Routine cultivation of *C. albicans*. *C. albicans* was transferred weekly on slants of Sabouraud dextrose agar (Difco). These cultures were incubated for 24 hr at 37 C and then stored at 4 C until subcultured at monthly intervals. Sabouraud dextrose broth (Difco) was utilized for growing inocula for chicks as described below. Corn meal agar (Difco) was employed to verify the formation of mycelia, blastospores, and chlamydospores by *C. albicans* isolated from animals; cultures were incubated at 37 C for 36 hr.

Preparation of inocula for oral challenge. Cultures were grown in 8 ml of broth in glass ampoules; these were incubated at 37 C for 24 hr. Colony counts were obtained after plating appropriate dilutions on Sabouraud dextrose agar and incubating for 48 hr at 37 C. After examination for contamination, duplicate

ampoules were sealed and immersed in a sulfuric acid-dichromate cleaning solution for 20 min, after which they were handled with clean rubber gloves.

Inoculation of animals. Duplicate ampoules containing inocula were immersed in the germicidal lock of an isolator for 30 min before they were transferred into the isolator containing the test animals. One ampoule was removed and the colony count was determined as described. The second sealed ampoule in the isolator was opened, and its contents were added to the diet.

Enumeration of *C. albicans*. A weighed sample of contents from the gut (0.2 to 1 g, wet weight) was mixed with 9 ml of sterile saline containing glass beads and was agitated on a Vortex Junior mixer (Scientific Industries, Inc., Queens Village, N.Y.). Appropriate dilutions were plated on Sabouraud dextrose agar containing penicillin and streptomycin (each at 58 μ g/ml). Three to five plates of each dilution were prepared and incubated for 48 hr at 37 C; colonies were counted.

Enumeration of bacteria. Weighed samples of contents from the intestine were suspended in sterile saline, as described above, then diluted and plated, in 1-ml amounts, on different media. Three to five plates of each dilution were prepared. Colonies were counted after incubation at 37 C for 48 hr. The media (Difco) included: (i) tryptone-glucose-yeast extract-agar for total aerobes; (ii) Brain Heart Infusion Agar containing 0.1% sodium thioglycolate for total anaerobes; (iii) EMB agar for coliforms; (iv) SF medium for enterococci; and (v) Tomato Juice Agar for lactobacilli.

Measurements of pH and E_h . To determine pH and E_h on contents from the alimentary tract, 0.2 to 1.0 g (wet weight) of sample was suspended in 5 ml of distilled water and was agitated as above. Samples were always held under nitrogen until after E_h values were taken with a Beckman Zeromatic meter equipped with platinum and calomel electrodes; appropriate corrections were made. The pH values were taken with the same instrument.

Maintenance of animals. Embryonated eggs were obtained from White Leghorn hens of the K-137 Kimber strain (Marshall Brothers Hatchery, Ithaca, N.Y.). Two different diets were employed in separate experiments. Diet A was a purified diet (C-IR) described previously (6) and purchased from Nutritional Biochemicals Co., Cleveland, Ohio. Diet B was a commercial chick-growing mash (Growena from Ralston Purina Co.) supplemented with vitamins (20). In earlier experiments, diets were sterilized by ionizing radiation (19). Later diets were autoclaved by a method similar to that described by others (10, 28).

Germ-free animals were housed in Plexiglas isolators and reared by gnotobiotic techniques (18, 19). Chicks of either sex were employed, and, unless noted otherwise, there were at least 10 animals in each experimental group. Conventional chicks were obtained, reared, and housed as described for germ-free chicks except that unsterilized ration (diet B) was fed only during the 1st day after hatching. Several reviews concerning germ-free vertebrates are available (13, 14, 15, 17).

Microbiological examinations of gnotobiotic animals. The microbiological status of gnotobiotic chicks was examined by routine procedures (29).

Examination of chicks for evidence of infection. During the period of challenge, all chicks were weighed weekly; amounts of diet and water consumed were recorded daily. Chicks were killed by decapitation. Blood samples were taken from the heart by sterile syringe and plated on Sabouraud agar to obtain colony counts of *C. albicans*. The blood was also examined microscopically. Internal viscera were grossly examined for evidence of infection. Material from suspected lesions was aseptically taken with sterile cotton swabs and streaked on Sabouraud agar plates. Some of this material was also Gram-stained and microscopically examined. Contents from different segments of the gut were removed and stored at 4°C for less than 2 hr. These samples were assayed, as described above, for viable microorganisms. The tissues taken for histological examination were: kidneys, liver, lungs, spleen, heart, pancreas, esophagus, crop, proventriculus, gizzard, small intestine, and ceca.

Histological procedures. Tissues were fixed in neutral buffered formalin and processed by routine methods. Duplicate tissue sections were stained with either hematoxylin and eosin (HE), or with periodic acid-Schiff stain.

Determination of serum proteins. Serum was recovered from clotted blood by centrifugation. Total protein was determined by the biuret method which was standardized with an assayed control serum (Hyland Laboratories, Los Angeles, Calif.). Serum proteins were separated by paper electrophoresis in a Durrum-type cell utilizing barbital buffer at pH 8.6 and an ionic strength of 0.075. A running time of 16 to 18 hr at 8 ma was employed. The paper strips containing separated proteins were stained with bromophenol blue (3) and analyzed with the Analytrol recording scanner and integrator (Beckman Instruments Co., Belmont, Calif.).

RESULTS

The pattern of our results is generally illustrated by the following experiment; an exception to this pattern will be described later in this paper. Germ-free and conventional chicks were fed the autoclaved crude ration (diet B); and at age 7 days, both groups of chicks were inoculated with *C. albicans*. During the period of challenge, all chicks appeared healthy with satisfactory growth rates; weight gains of germ-free birds were slightly larger than those of the conventional animals. The results are given below. In other experiments, similar results were obtained with chicks receiving the fresh, purified ration (diet A) sterilized either by ionizing radiation or by autoclaving. In Table 1, a comparison is given of the numbers of *C. albicans* in contents from the alimentary tracts of germ-free and conventional chicks after challenge. It is evident that *C. albicans* was established in the gut of each group of animals. However, with the

TABLE 1. Enumeration of *Candida albicans* in contents from the alimentary tract of germ-free and conventional chicks after oral inoculation with the pathogen

Contents from*	<i>C. albicans</i> colonies per g (wet wt) $\times 10^6$	
	Bacteria-free chicks	Conventional chicks
Crop.....	200†	3
Duodenum.....	3	1
Jejunum.....	5	2
Ileum.....	7	2
Cecum.....	80	10

* All chicks were 7 days of age when fed the challenge dose of 10^6 cells (colony count). The animal received an autoclaved crude ration (diet B in text), except that the conventional chicks were initially fed once with unsterilized diet to establish a flora. All animals were killed and examined at 38 days of age.

† The values given are averages taken from 10 chicks in each group.

exception of the small intestine, the numbers of *C. albicans* colonies obtained from bacteria-free chicks were considerably larger than those from the conventional animals. In the bacteria-free chicks, the number of *C. albicans* was larger in contents from the crop than from the ceca, whereas in conventional chicks, the ceca contained larger numbers than the crop. When numbers of *C. albicans* in contents from the small intestine were compared, no great differences were seen between bacteria-free and conventional animals. In conventional chicks, only the yeast form was observed in all specimens taken from the gut, whereas hyphae predominated in gut contents from bacteria-free chicks. In Table 2, the numbers of various bacteria in the intestine of challenged conventional chicks are compared with bacterial counts on samples from unchallenged animals. Coliforms, lactobacilli, and enterococci were established in the alimentary tract of both groups of chicks. In unchallenged birds, coliforms and lactobacilli appeared to be more numerous than enterococci in the crop, whereas the numbers of each of these bacteria appeared to be similar in the duodenum, ileum, and ceca. On the other hand, in challenged chicks no great differences were apparent in numbers of the above bacteria in comparable segments of the gut; enterococci in the crop were increased. Of those tissues that were examined, only the epithelium in the crop of each challenged bacteria-free chick displayed evidence of infection by *C. albicans*. The crops of these birds had white patches deposited on the surface of the inner lining. HE-stained sections manifested

TABLE 2. Enumeration of bacteria in contents from different segments of the alimentary tract of conventional chicks

Chicks	Contents from	Log ₁₀ no. of bacteria per g (wet wt)				
		Total aerobes	Total anaerobes	Coliforms	Lactobacilli	Enterococci
Fed <i>Candida albicans</i> *	Crop	9.2	7.2	9.2	8.3	6.2
	Duodenum	8.2	6.1	6.2	6.1	6.1
	Ileum	8.2	7.1	8.2	8.3	8.1
	Ceca	8.2	8.2	8.3	8.1	8.2
Not fed <i>C. albicans</i> †	Crop	9.1	7.1	8.3	8.5	8.5
	Duodenum	7.2	6.1	7.1	7.2	7.3
	Ileum	8.2	6.4	7.2	8.2	8.2
	Ceca	8.2	8.0	8.1	9.2	8.3

* Except as noted, these chicks were treated as described for conventional chicks in Table 1.

† These animals are the same ones described in a footnote to Table 1.

a partial denudation of the crop epithelium, and periodic acid-Schiff staining revealed a dense growth of hyphae with blastospores which was confined to the crop epithelium (Fig. 1b and c). Budding yeast forms were not found in any of the sections examined. Viable *C. albicans* was recovered from the crop lesions. In contrast, our conventional chicks were resistant to the challenge. No evidence of infection could be found in any of the specimens taken from these animals. The pH values on unchallenged germ-free and conventional chicks are compared in Table 3. The pH was higher in the germ-free animals than in the conventional group in all segments of the gut. The greatest difference was in the pH of contents from the crop, wherein the germ-free birds were at neutral pH and the conventional group at acid pH. We found a similar pattern of pH values in germ-free and conventional animals after challenge. In Table 4, the E_h of intestinal contents of unchallenged germ-free chicks are compared with the E_h values taken on conventional animals. The greatest differences between the two groups of animals were seen in contents from the crop and ceca. In the crop, E_h values were slightly lower in germ-free chicks than in conventional birds. In contents from the cecum, E_h values were decreased in conventional animals. Minor differences were seen in contents from the small intestine of the two animal groups. After challenge, no appreciable changes in the comparative E_h levels were seen. In unchallenged chicks, the γ -globulin concentration in germ-free animals was smaller than in conventional birds (Table 5). Although challenge did not alter the γ -globulin level in conventional animals, it increased this value in bacteria-free chicks.

FIG. 1. Photomicrographs of chick crop sections taken from animals killed at 38 days of age. (a) Germ-free chick not challenged with *Candida albicans* ATCC 10231. Periodic acid-Schiff (PAS) stain. Ca. $\times 100$. (b) Germ-free chick fed 10^6 cells of *C. albicans* at 7 days of age. The crop epithelium contains numerous hyphae of *C. albicans*. PAS stain. Ca. $\times 100$. (c) Same as (b). Blastospores are present on hyphae. Ca. $\times 400$.

TABLE 3. *pH of contents from the alimentary tract of germ-free and conventional chicks in the presence and absence of oral inoculation with Candida albicans*

Contents from	pH values			
	Unchallenged chicks		Challenged chicks	
	Germ-free (7)*	Conventional (10)	Bacteria-free (7)	Conventional (10)
Crop.....	7.0 ± 0.2†	5.2 ± 0.4	6.5 ± 0.2	4.7 ± 0.5
Duodenum.....	6.9 ± 0.2	6.3 ± 0.3	6.7 ± 0.2	6.1 ± 0.3
Jejunum.....	8.0 ± 0.2	7.2 ± 0.2	8.0 ± 0.2	6.7 ± 0.2
Ileum.....	8.1 ± 0.2	7.5 ± 0.3	8.0 ± 0.2	7.6 ± 0.3
Ceca.....	8.0 ± 0.1	7.4 ± 0.3	8.0 ± 0.2	6.7 ± 0.5

* Number of animals employed is given in parentheses. All chicks received the autoclaved crude ration (diet B); animals were challenged when 7 days old, then killed, and examined at 38 days, as described under Materials and Methods.

† Mean ± standard deviation.

TABLE 4. *E_h of contents from the alimentary tract of germ-free and conventional chicks in the presence and absence of oral inoculation with Candida albicans*

Contents from	E _h values (mv)			
	Unchallenged chicks		Challenged chicks	
	Germ-free (7)*	Conventional (10)	Bacteria-free (7)	Conventional (10)
Crop.....	+100 ± 5†	+160 ± 20	+120 ± 5	+150 ± 10
Duodenum.....	-60 ± 5	-40 ± 10	-80 ± 10	-60 ± 10
Jejunum.....	-70 ± 10	-50 ± 20	-100 ± 10	-80 ± 10
Ileum.....	-80 ± 5	-100 ± 5	-100 ± 10	-50 ± 20
Ceca.....	-50 ± 5	-140 ± 5	-80 ± 10	-150 ± 5

* Number of animals is given in parentheses. Values were taken on the chicks described in Table 3.

† Mean value ± standard deviation.

The pattern of the results described above became markedly different when chicks received a purified ration (diet A) which had been radiation-sterilized with 5 megarads of electron beams and stored at room temperature (25 C) for 6 months to test nutritional stability. This experiment was conducted as follows. Germ-free and conventional chicks were reared to 35 days of age prior to challenge. During this period, data were obtained on their growth rates and the amounts of diet consumed. When the chicks were 35 days old they were challenged once with the pathogen, each chick receiving about 10⁶ cells (colony count). These animals were observed for an additional 35 days at which time survivors were killed and examined; moribund chicks were killed before termination of the experiment. The following results were obtained. Prior to challenge, the conventional chicks had slightly decreased growth rates (Fig. 2). Also during this period, the germ-free chicks consumed about one-half the amount of diet for the same gain in

body weight as did the conventional chicks. With the exception of these differences, both groups of chicks appeared to be healthy. However, after challenge, only the conventional animals became moribund and were killed within 15 to 35 days. The illness was characterized by lethargy, losses in appetite and weight, drooping heads, and ruffled feathers. In contrast, challenged bacteria-free chicks maintained their healthy appearance (Fig. 2). The above conventional chicks succumbed to an invasion of the blood and kidneys by the yeast form of *C. albicans*, whereas the bacteria-free animals were resistant to this invasion. Blood from the conventional chicks contained approximately 10⁶ per milliliter of *C. albicans* colonies. The exterior surface of the kidneys was enveloped with purulent material which contained yeastlike cells; hyphae were not observed. *C. albicans* colonies were isolated from this purulent material (PAS-positive). Renal tubules were congested with leukocytes, and, in some instances, tubules were virtually blocked. On the other hand, all

TABLE 5. Serum proteins in germ-free and conventional chicks in the presence and absence of oral challenge with *Candida albicans*

Microflora of chicks*	Protein concn, g/100 g of serum				
	Albumin	α_1 -Globulin	α_2 -Globulin plus β -globulin	γ -Globulin	Total protein
Conventional	1.19 $\pm$ 0.25†	0.36 $\pm$ 0.05	0.54 $\pm$ 0.11	0.94 $\pm$ 0.06	3.0 $\pm$ 0.3
Conventional + <i>C. albicans</i>	1.36 $\pm$ 0.09	0.45 $\pm$ 0.08	0.54 $\pm$ 0.07	0.96 $\pm$ 0.05	3.3 $\pm$ 0.2
Germ-free	1.25 $\pm$ 0.14	0.38 $\pm$ 0.03	0.54 $\pm$ 0.08	0.81 $\pm$ 0.02	3.1 $\pm$ 0.1
<i>C. albicans</i>	1.35 $\pm$ 0.07	0.40 $\pm$ 0.05	0.52 $\pm$ 0.03	0.99 $\pm$ 0.04	3.3 $\pm$ 0.02

* All chicks received a crude ration (diet B in text) and were killed when 38 days of age.

† Mean of 3 animals $\pm$ standard deviation.

FIG. 2. Germ-free (GF) and conventional (CONV) chicks received a purified diet which was sterilized by high-energy electrons. This diet was stored at room temperature for 6 months prior to feeding. At 35 days of age, separate groups of germ-free and conventional animals were fed 10^6 cells of *Candida albicans* ATCC 10231 (YST). At 70 days, surviving chicks were killed and examined for infections. The curve showing the lowest growth rate is for the challenged conventional chicks.

bacteria-free chicks in this experiment continued to appear healthy and survived the challenge (Fig. 2). These animals were killed and examined when they were 70 days of age. Infections were present only in the crop of each animal. The crop epithelium contained large numbers of hyphae with blastospores, and *C. albicans* colonies were isolated. These lesions did not appear to be more severe than the crop lesions previously described in bacteria-free chicks receiving an adequate diet.

DISCUSSION

In the present experiments, oral challenge with *C. albicans* resulted in crop infections in all bacteria-free chicks and no such infections in conventional chicks. It is clear that the conventional intestinal flora provided protection against candidiasis of the gut. This interpretation of our

data is consistent with observations and suggestions of others concerning the protective influence of intestinal bacteria against candidiasis; references are given in the introduction to this report. On the other hand, our data give no evidence on the mechanism of this protection or the identification of a given bacterial species in the intestine responsible for the protection. Preferential growth of *C. albicans* hyphae occurred in all crop infections in our challenged germ-free chicks. These results are consistent with the observations of others that the conversion of the yeast form to hyphae is significant in candidiasis (4, 8, 11, 33).

Our pH data on intestinal contents from germ-free chicks indicated higher pH values than those reported by others (21); however, their birds were younger (12 to 13 days old) than our animals, and received a different diet. The contents of the digestive tract of the conventional chick is acid in all segments of the tract (12, 26). However, it appears that the large numbers of *C. albicans* found in the crop contents of our challenged germ-free chicks could not be due solely to the pH of this segment of the tract, since maximal growth of *C. albicans* occurs in the pH range 5.5 to 9.0 (9). The difference in E_h between crop contents from our germ-free and conventional chicks did not seem to be large enough to explain the relatively large numbers of *C. albicans* in the crop of challenged germ-free chicks. This interpretation is consistent with in vitro findings (16).

Our results essentially confirm those reported earlier with respect to decreased γ -globulin level in germ-free chicks compared with conventional birds (32). However, a considerably smaller difference was found in the present study. This discrepancy may have been due to a difference in the intestinal flora of the conventional chicks employed in the two laboratories. The susceptibility of our germ-free chicks to candidiasis of the crop, after challenge, may have been a result of their relatively low γ -globulin level.

Bacteria in the alimentary tract of conventional chicks appeared to inhibit the multiplication of *C. albicans*. This inhibition was maximal in the crop, but it is uncertain whether it was sufficient to prevent crop infection in conventional chicks. However, if the inhibition were the sole determinant of resistance, it is surprising that no infections were observed in the ceca of bacteria-free chicks where large numbers of *C. albicans* were found. In view of this and other evidence noted above, we suggest that the resistance of conventional chicks to intestinal candidiasis is due to inhibition by intestinal bacteria of morphogenesis in *C. albicans*, that is, unidentified bacterial products prevent hyphal development.

The challenge of conventional chicks appeared to alter somewhat the relative numbers of certain bacteria in the intestine (Table 2). One of the most notable shifts was the increase in numbers of enterococci in contents from the crop, which may account for the septicemia and kidney infection in our challenged conventional chicks receiving the stored diet, since several species of enterococci isolated from intestinal contents of the rat required amino acids which were also essential to the host (22). It was suggested that these enterococci may compete with the host for essential amino acids. It would be of interest to know the vitamin requirements of these enterococci, and whether a similar competition is possible with respect to certain vitamins.

Systemic candidiasis by the yeast form may be possible in conventional chicks under certain conditions, although our bacteria-free chicks were resistant to this form of infection. In the first instance, *C. albicans* invasion of the blood stream and kidneys of our conventional chicks may have been due to the presence of intestinal bacteria, combined with results of feeding a diet which was nutritionally deficient or contained toxic by-products due to radiation sterilization. We favor the following explanation for the above invasion. The purified diet was rendered deficient in vitamins during long storage at room temperature. This deficiency was further enhanced due to utilization of vitamins by intestinal bacteria in the conventional chicks; this competition was not present in bacteria-free chicks. Evidence for the above explanation was the observation that the conventional chicks consumed twice the amount of diet as did the germ-free chicks to achieve a given increase in body weight. There is no indication from this study as to the identity of specific dietary components which were involved in the postulated nutritional deficiency. Other explanations of the above results are not excluded. For example, there could have been an undetected virus infection in our conventional chicks which

may have enhanced their susceptibility to the above form of candidiasis, whereas the bacteria-free chicks could have been resistant because of the possible absence of such a virus infection.

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