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Nutritional Requirements of *Lactobacillus bifidus* Isolated from Poults and Chicks.* (20335)

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Romoser *et al.*(1) reported that large numbers of an unidentified branched gram positive organism were found in ceca of normal chicks. These organisms, later identified as *Lactobacillus bifidus*(2), were found in greatest numbers when 15% lactose was added to the basal diet and were decreased in number by the addition of 10 ppm of penicillin G. A high bifid count was correlated with poor chick growth. *L. bifidus* also constitutes 50% of the cecal population of turkeys(3). It is well known that under the usual growing conditions, poults grow faster when antibiotics are fed. This may be due to a reduction in numbers of bifid organisms. Since *L. bifidus* might utilize a known or unidentified substance(s) essential for rapid chick and poult growth, a knowledge of the nutritional requirements of avian bifids may contribute information concerning the mechanism of antibiotic action(4-6). Results of such a study are presented in this paper.

Experimental. Twenty-four cultures, tentatively classified as *L. bifidus,* have been isolated from the ceca of turkey poults. For the nutritional studies, 7 representative avian strains were selected, including 5 poult strains, one strain from adult turkeys(3) and one strain from chicks(2). The general procedures used in microbiological assays were employed for the determination of growth requirements. Stock cultures were transferred monthly on the basal medium containing 15 g per liter of Phytone (papaic digest of soybean, Baltimore Biological Laboratory) and 2% agar. The medium used for preparation of inoculum was of the same composition except for the omission of agar. Stock and inoculum cultures were incubated at 37°C, for 48 hours under anaerobic conditions using a Brewer anaerobic jar. The inoculum cultures were centrifuged, washed twice with physiological saline, resuspended and adjusted to a reading of 95 on the Evelyn photo-electric colorimeter, using

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a 660 mμ filter. One drop of this suspension was added to each of the assay tubes. Solutions were prepared so that the amount of supplement for each test was contained in 1 to 3 ml. In all cases the final volume in each tube was 6 ml. The basal medium was identical to that employed by Hassinen *et al.*(7) except for the following modifications: the lactose and sodium acetate were reduced to 7 and 5 g per liter of single strength medium, respectively. Cystine was replaced by cysteine hydrochloride and N-Z Case (enzymatic digest of casein) by Vitamin-Free Casamino Acids (Difco) or amino acid mix(8). Alanine and tryptophane were omitted when amino acid mix or Casamino Acids were used as the nitrogen source. Ascorbic acid was omitted except where indicated in the results. The final pH of the medium was adjusted to 6.8. The tubes were autoclaved at 15 lb pressure for 15 minutes, cooled, inoculated and incubated anaerobically at 37°C for 48 hours. The growth was measured turbidimetrically in the Evelyn colorimeter, using the 660 mμ filter.

Results and discussion. None of the 7 strains would grow in several partially chemically defined media, including the medium described by Hassinen *et al.*(7). Preliminary investigations using Eugon broth revealed that Phytone was necessary for growth; however, upon the addition of this supplement to Hassinen's medium, no growth was obtained. Thus, it was surmised that the concentration of ingredients was the factor responsible for the absence of bacterial growth. Under our experimental conditions, the avian bifids were inhibited by the concentration (25 mg per ml) of sodium acetate used by Hassinen *et al.*(7), Tomarelli *et al.*(9) and Kuhn(10) for the proliferation of *L. bifidus* of human origin. A low concentration (5 mg per ml) improved growth of avian bifids. The reduction of lactose from 35 to 7 mg per ml did not appreciably decrease growth. Cystine and cysteine hydrochloride were interchangeable, but cysteine hydrochloride was chosen for incorporation in the basal medium because of its non-toxic effect(11) and its reducing properties.

The 7 avian strains, when grown in the modified basal medium described above, had an absolute requirement for a growth-promoting substance(s) in Phytone. This growth response is shown in Table I. The substance was also present in yeast extract, beef extract and fish solubles. The latter crude material is well known to contain growth-promoting substances for chicks and turkeys.

TABLE I. Effect of Phytone and Ascorbic Acid on Growth of *Lactobacillus bifidus*. (Poult Strain 3.)

Phytone conc., mg/tube	Optical density*			
	No ascorbic acid		Ascorbic acid†	
	Aerobic	Anaerobic	Aerobic	Anaerobic
0 (Inoc.)	.00	.00	.00	.00
5	.04	.07	.13	.09
10	.09	.11	.14	.11
20	.13	.21	.21	.21
40	.12	.39	.40	.44
60	.14	.48	.62	.51
90	.32	.64	.75	.70

* $\text{Log} \frac{100}{\text{Galvanometer reading}}$.

† 12 mg/tube.

None of the following compounds singly or in combination replaced the Phytone requirement: vit. B_{12}, thymidine, desoxyribonucleic acid, choline, inositol, lyxoflavin, carnitine, Leucovorin (citrovorum factor), DL-6-thioctic acid (protogen), pantethine, pyridoxal hydrochloride, pyridoxal phosphate, pyridoxamine, orotic acid, oleic acid, polyoxyethylene sorbitan monooleate (Tween 80), blood group specific substances A and B, and galactose acetyl-glucosamine. It has been reported that the growth factor in human milk(12) required by variants of human bifids, could be replaced by galactose acetyl-glucosamine(13) or a nitrogen-containing polysaccharide from mucin(10) or partially replaced by blood group specific substances A and B(10). The variant human strains have not been made available for comparison in our tests. However, the unidentified factor required by the avian bifids appears to be different from that required by the variants of human bifids since galactose acetyl-glucosamine and blood group specific substances A and B failed to replace the Phytone activity when incorporated in the medium described by the above authors or

when incorporated in the basal medium used in our experiments.

The avian bifids also seem to be different from the human bifids, in that they exhibit a marked stability in oxygen requirement and bifid morphology. In contrast to the behavior of a number of bifids isolated from nursling stools of infants(9,14), they have not become aerobic nor lost their branched form upon continual subculture even after a period of one to 2 years. However, they can be grown in an aerobic atmosphere, provided ascorbic acid is added either aseptically or autoclaved in the medium.

In Table I the effect of ascorbic acid on the growth of one culture (poult strain No. 3) is shown. That some aerobic growth was obtained with high levels of Phytone in the absence of ascorbic acid was not too surprising, since a crude substance such as Phytone might contain a reducing compound. There was no apparent change in bifid morphology when the organism was grown in an aerobic atmosphere in the presence of ascorbic acid. From the results obtained, the bifids isolated from ceca of chicks and poults appear to be more fastidious in their nutritional requirements than the bifids from human origin.

Summary. The nutritional requirements of *Lactobacillus bifidus* isolated from the ceca of chicks and turkey poults have been studied. All strains were inhibited by high concentrations of sodium acetate. The organisms could be grown in an aerobic atmosphere, provided ascorbic acid was present, without any apparent change in bifid morphology. No growth was observed when a chemically defined medium was employed, but luxuriant growth was obtained in the basal medium when it was supplemented with Phytone or other crude substances. The activity of Phytone could not be replaced by the galactose acetyl-glucosamine or the incorporation of several other known growth-promoting substances into the basal medium. The nutritional fastidiousness of *L. bifidus* isolated from chicks and poults may suggest that these organisms compete with the host for mutually essential nutrients.

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