Growth-Promoting Activity of L-Lyxoflavin for *Lactobacillus lactis*.*† (19463)

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Lyxoflavin was reportedly isolated from human heart muscle by Pallares and Garza (1) and shown to be identical with synthetic L-lyxoflavin. They(2) considered it a "stereoisomer of vit. B_2." Emerson and Folkers(3,4) showed that synthetic L-lyxoflavin(5) possessed a growth-promoting activity distinct from riboflavin activity for rats. Wahlstrom and Johnson(6) reported significantly greater gains in weight of pigs when L-lyxoflavin was incorporated in a diet containing all known vitamins. Bruins *et al.*(7) obtained a 10% weight increase in chicks on a basal diet containing riboflavin, with L-lyxoflavin alone and in combination with dried whey, wheat bran and liver residue. Hendlin and Wall, quoted in Emerson and Folkers(4), found that L-lyxoflavin had less than 0.2% activity of riboflavin for this microorganism. Snell(7) states that lyxoflavin exerted a competitive inhibition against riboflavin for *L. casei* in high concentrations, although it was slightly stimulatory in the presence of suboptimal doses of riboflavin.

Studies reported in this paper show that L-lyxoflavin promotes the growth of *Lactobacillus lactis*.‡ The effect of this compound on the growth of 2 other bacterial species is also discussed.

Experimental. Three cultures have been used in these tests: *Lactobacillus lactis*, ATCC 8000, *Lactobacillus casei* ATCC 7469, both of which require riboflavin under the conditions of assay, and *Leuconostoc mesenteroides* ATCC 8293, which apparently synthesizes riboflavin under the conditions of assay. The riboflavin assays with *L. casei* were carried out by the modified method of Snell and Strong, as described by Snell(8). Turbidimetric measurements were made after 20 to 40 hours incubation using the Evelyn colorimeter fitted with a 620 mμ filter. The stock culture of *Leuc. mesenteroides* was maintained by monthly transfer in Eugon agar stabs (Baltimore Biological Laboratory). The stock culture of *L. lactis* was transferred

TABLE I. Composition of Double Strength Medium.*

	mg		mg
DL-α-Alanine		Adenine sulfate	
L-Arginine mono-HCl		Guanine HCl	2
DL-Aspartic acid		Uracil	
L-Glutamic acid		Xanthine	
Glycine		K_2HPO_4	100
DL-Histidine mono-HCl		KH_2PO_4	100
DL-Isoleucine	40	$MgSO_4 \cdot 7H_2O$	40
L-Leucine		$MnSO_4 \cdot 4H_2O$	
L-Lysine mono-HCl		NaCl	2
DL-Methionine		$FeSO_4 \cdot 7H_2O$	
DL-Phenylalanine		$CaCl_2$	20
DL-Serine		Thiamin HCl	2
DL-Threonine			μg
DL-Tryptophane	80	Ca. pantothenate	400
L-Tyrosine		Vit. B_{12}	.8
DL-Valine	40	Pyridoxal phosphate	200
L-Asparagine		*p*-Aminobenzoic acid	400
L-Cystine		Nicotinic acid	800
Sodium ethyl oxalacetate	100	Biotin	1
Tween 80	20	Pteroylglutamic acid	1
		KCN	50
		LBF factor†	10 units
	g		
Na acetate, anhyd.	1.2	Distilled water to	100 ml
Dextrose	2		

* Cystine should be dissolved in concentrated HCl and water before adding to dissolved amino acids, kept in stock as a dry mix. Pyridoxal phosphate and sodium ethyl oxalacetate are added after the pH is adjusted to 6.8.

† LBF factor, Lederle 8000 unit concentrate of pantethine (synthetic LBF) Merck.

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‡ *L. lactis* is synonymous with *Bacillus lactis acidi*, according to Bergey's Manual of Determinative Bacteriology, 6th Edition, 1948. Williams & Wilkins Co., Baltimore.

TABLE II. Growth Response of *Lactobacillus lactis*, *Lactobacillus casei* and *Leuconostoc mesenteroides* to Riboflavin and L-Lyxoflavin.

Substance added	μg/10 ml	*Lactobacillus lactis*		*Lactobacillus casei*		*Leuconostoc mesenteroides*	
		Incubation period, hr					
		22	40	20	40	22	40
		Galvanometer reading*					
0	.0	92	91	94	94	77	44
Riboflavin	.025	76	69				
	.05	67	57	78	81	71	41
	.1	58	41	66	67	65	42
	.25			49	47		
	.3	53	36				
	.5	50	35	47	38	60	40
L-Lyxoflavin	.05	91	87			93	95
	.1	83	73	92	96	94	97
	.3	65	45				
	.6	55	39			95	98
	1	54	36	92	93	96	96
	5	61	37	91	94	96	97
	10			94	94	96	89
	50	62	32	94	94	96	69
	100	62	37				

* Galvanometer reading of 100 represents no growth.

biweekly on a stock medium consisting of 10% clarified tomato juice, 0.5% Bacto-yeast extract, and 10% Bacto-dehydrated skim milk (adjusted to pH 6.8 and sterilized at 121°C for 15 minutes). Both organisms were grown in 10 ml of Difco Micro Inoculum broth for the inoculum. After 18 to 24 hours incubation at 37°C, the cultures were washed twice with physiological saline, resuspended in saline and adjusted to a turbidity reading of 70 on the Evelyn colorimeter (515 mμ filter). Four drops of this suspension were added to 20 ml of saline for the final inoculum, and one drop was used per tube. The composition of the basal medium used with *L. lactis* and *Leuc. mesenteroides* is shown in Table I. This medium, supplemented with 20 μg of riboflavin, was developed in a study of an unidentified growth stimulating factor for *L. lactis*(9). Complete growth of *L. lactis* is not reached until about 40 hours incubation if this factor is not supplied. Since no pure source of the stimulatory factor is available, a 40-hour incubation period was used in most of the *L. lactis* assays to permit the organism to synthesize the factor. The final volume was 10 ml, 5 ml of double strength medium being added to 5 ml of sample. The tubes were autoclaved for 5 minutes at 121°C, cooled, inoculated and incubated at 37°C for varying periods of time, depending on the assay. Growth was determined turbidimetrically with the Evelyn colorimeter, using the 620 mμ filter.

Results and discussion. The growth responses of *L. lactis, L. casei* and *Leuc. mesenteroides* to riboflavin and L-lyxoflavin are shown in Table II. While L-lyxoflavin promotes the growth of *L. lactis,* the rate of growth is somewhat slower than that with riboflavin. The flattened growth curve at 22 hours was obtained because, in this medium, the stimulatory factor must be synthesized by the organism. There was slight inhibition with 5 to 100 μg per tube of L-lyxoflavin at 22 hours, but no inhibition was evident at 40 hours with these doses. When the incubation period was continued to 3 or 6 days, the activity of L-lyxoflavin approached that of riboflavin. In a series of eight assays with *L. lactis,* L-lyxoflavin had an average activity per μg equivalent to 0.09 μg of riboflavin in a 20- to 24-hour assay; 0.16 μg at 40 to 44 hours and 0.43 μg in 6 days.

While *L. casei* gave a typical dosage response curve to riboflavin after 20 hours of incubation, no growth was obtained with *L-lyxoflavin* even after 72 hours' incubation.

TABLE III. Inhibition of Growth of *Lactobacillus casei, Leuconostoc mesenteroides* and *Lactobacillus lactis* by L-Lyxoflavin in Presence of Riboflavin, after 40 Hr Incubation.

Riboflavin added, μg/10 ml	L-lyxoflavin added, μg/10 ml	*Lactobacillus casei* Snell's medium GR*	II†	*Lactobacillus casei* Synthetic medium GR*	II†	*Leuconostoc mesenteroides* Synthetic medium GR*	II†	*Lactobacillus lactis* Synthetic medium GR*	II†
0	0	98		86		44		93	
0	.05			80		95		93	
0	50	97		75		69		41	
.05	0	82		80		41		67	
.1	0	72		58		43		48	
.5	0	64		52		40		42	
1	0	64		55		47		41	
.05	.05					86	.37	57	
.05	.1	76	60			92		53	
.05	.5	93				92		40	
.05	50	97				96		39	0
.1	.1	65		55		77	.33	43	
.1	1	63		52		84		39	
.1	5	78	50	55		85		38	
.1	10	93		58		85		39	
.1	50	97		92	440	93		40	0
.5	.5	61		50		39		39	
.5	1	55		50		42		40	
.5	10	57		50		48		40	
.5	50	92	50	57	>100	65	150	39	0
1	1	67		53		48		41	
1	5	60		52		50		41	
1	10	57		50		50		41	
1	50	75	50	48	0	47	0	41	0

* GR—A galvanometer reading of 100 represents no growth.

† II—Inhibition index is equal to the ratio of concentration of inhibitor to concentration of the metabolite, at which half maximum inhibition of growth of the organism occurs. The inhibition index was determined on curves drawn from the data shown in the table.

Riboflavin was not required by *Leuc. mesenteroides* at 22 hours, but at this time it had a slight stimulatory effect at low levels. L-lyxoflavin was very active in inhibiting the growth of *Leuc. mesenteroides* at this early hour, over a range of from 0.05 to 50 μg per tube. However, at 40 hours, riboflavin was still only slightly stimulatory while *L. lyxoflavin* at the higher levels produced a response which must be interpreted as growth stimulation in the light of inhibition analyses which follow.

Inhibition analyses were made with all 3 organisms and results are shown in Table III. In confirmation of Snell's report(7), *L. casei* was competitively inhibited by L-lyxoflavin when Snell's assay medium for riboflavin was used. and slight stimulation occurred at the lower levels of lyxoflavin in the presence of suboptimum riboflavin. However, when the same tests were done with *L. casei,* using the synthetic medium described in Table I, the inhibition with L-lyxoflavin was evident only when low levels of riboflavin were in the medium. When high levels of riboflavin were used, high levels of L-lyxoflavin stimulated the growth of *L. casei.* More marked growth stimulation occurred with 50 to 100 μg per tube of L-lyxoflavin in the synthetic medium containing 1 μg per tube of riboflavin when the *L. casei* inoculum was washed and diluted by the method used for *L. lactis* (data not shown). The response with a washed dilute culture suggests that adaptation occurred or that some component in the synthetic medium, in addition to riboflavin, might be required for L-lyxoflavin utilization. Since the treatment of the components of Snell's medium would tend to destroy or remove some of the vitamins present in the synthetic medium, it is possible that one of these vitamins is involved.

With *Leuc. mesenteroides,* inhibition was competitive only at the lowest levels of L-

lyxoflavin used. At high levels of L-lyxoflavin the inhibition disappeared. There was no evidence of competitive inhibition with *L. lactis*.

Among other pentose isoalloxazines or probable isoalloxazine precursors examined for growth stimulation or inhibition for *L. lactis* and *L. casei*, D-lyxoflavin, D-araboflavin(10), galactoflavin and α-ribazole did not promote growth of either organism, while 5,6-dimethylbenzimidazole was inactive for *L. lactis* (not tested with *L. casei*). Only riboflavin phosphate(11) and isoriboflavin(12) had activity, having 0.82 and 0.038 μg of riboflavin activity per μg; respectively, for *L. casei*, and 0.66 and 0.002 μg activity for *L. lactis*. The former compound was not corrected for phosphate content. All of the above compounds were tested for inhibition, using 1 μg of riboflavin in the medium per tube, in Snell's medium with *L. casei*, and in the synthetic medium with *L. lactis*. No inhibition was observed with any of the compounds, nor was there any stimulation over that due to the added riboflavin.

A microbiological assay for the presence of L-lyxoflavin in natural materials does not appear at present feasible when using either growth stimulation or competitive inhibition as a criterion. Because all of the pentose isoalloxazine compounds migrated at the same rate in several solvent systems, paper chromatographic separation of L-lyxoflavin from the other pentose isoalloxazines has not been accomplished, although this would have been of great value in a microbiological assay. The method of synthesis of L-lyxoflavin makes it highly improbable that it is contaminated with riboflavin. Therefore the growth of *L. lactis* with L-lyxoflavin must be due to a direct response to this compound. Furthermore, in other tests, it has been observed that the total riboflavin content of an *L. lactis* culture was not increased when it was grown in the presence of lyxoflavin. The fact that *L. casei* responds to L-lyxoflavin, when riboflavin is present and the medium is adequate in other nutrients, lends support to the idea that L-lyxoflavin may function in a manner different from riboflavin. The growth response in animals, which has also been observed in chicks in this laboratory,§ may be due to the utilization of L-lyxoflavin directly by the animal body or by micro-organisms. Furthermore, any inhibitory effect of L-lyxoflavin on bacteria might possibly be reversed by the presence of adequate riboflavin and other nutrients in an enriched environment such as would be found in the intestinal tract.

Summary. L-lyxoflavin had 16% of the activity of riboflavin in promoting growth of *Lactobacillus lactis* in a 40-hour assay. The activity increased to 43% after 6 days of incubation. Growth with L-lyxoflavin occurred in the presence or absence of riboflavin and there was no evidence of competitive inhibition. L-lyxoflavin inhibited riboflavin competitively in the growth of *Lactobacillus casei*, in Snell's riboflavin assay medium, with an inhibition index of 50, but the inhibition became stimulation in a synthetic medium when optimum amounts of riboflavin were present. D-lyxoflavin, D-araboflavin, galactoflavin, and α-ribazole were inactive in promoting growth of either *L. lactis* or *L. casei* while 5,6-dimethylbenzimidazole was inactive for *L. lactis*. Riboflavin phosphate and isoriboflavin had riboflavin activity for both organisms.

§ Menge, H. and Combs, G. F., unpublished data.

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