NUTRITION OF THE HETEROFERMENTATIVE LACTOBACILLI THAT CAUSE GREENING OF CURED MEAT PRODUCTS[1]

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Cured meat products are subject to a type of bacterial spoilage in which the cured meat pigment is oxidized to a greenish or faded color. The microorganisms responsible for this effect appear to be a fairly restricted group of catalase-negative, salt-tolerant microorganisms that are capable of growing at low temperatures, and are able to oxidize certain substrates in the meat product with the accumulation of hydrogen peroxide. The most common organism isolated in this laboratory that is associated with this type of spoilage has been a heterofermentative *Lactobacillus* described by Niven, Castellani, and Allanson (1949). On the basis of detailed physiological and serological studies, this organism appeared to be a hitherto undescribed species. The cultures were reported to grow rather poorly in a wide variety of laboratory media.

In view of the economic importance of this organism and its apparently unusual nutritive requirements, an investigation of its nutrition has been undertaken.

CULTURES

The cultures used in this study were isolated from naturally occurring outbreaks of surface discoloration of a variety of cured meat products, and also cases of green cores in sausages (Niven and Evans, 1950). The physiological and serological characteristics of these organisms were consistent with those of the heterofermentative lactobacilli described by Niven, Castellani, and Allanson (1949), with the exception that the majority of them failed to ferment or to produce a polysaccharide from sucrose.

METHODS

Some preliminary experiments were conducted in order to devise a laboratory medium that would support rapid and vigorous growth of these organisms. The medium derived from these studies (APT medium) contains per liter: 10 g tryptone (Difco), 5 g yeast extract (Difco), 5 g NaCl, 5 g K_2HPO_4, 5 g sodium citrate ($2Na_3C_6H_5O_7 \cdot 11H_2O$), 10 g glucose, 1 g "tween 80" (sorbitan monooleate), 0.8 g $MgSO_4 \cdot 7H_2O$, 0.14 g $MnCl_2 \cdot 4H_2O$, and 0.04 g $FeSO_4 \cdot 7H_2O$. In contrast to the laboratory media employed in previous studies the greening lactobacilli grow promptly in this medium with the production of a very heavy uniform turbidity within 24 hours after inoculation. The addition of 1.5 per cent agar results in a

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satisfactory plating medium for the routine examination of discolored cured meat products.

All glassware used in this study was cleaned in dichromate cleaning solution and repeatedly rinsed with deionized water. Tests were conducted in 18.0 mm calibrated pyrex tubes, and growth as measured by optical density was determined with a Coleman model 11 spectrophotometer at 660 mμ.

In some experiments the inoculum was washed with sterile distilled water, diluted 1:100 in distilled water, and one drop added to each tube. In other experiments the test media were inoculated directly from a 24-hour APT broth culture using a nichrome wire needle, followed by a serial transfer in the test media after good growth was achieved. Essentially the same results were obtained with either technique.

TABLE 1

*The effect of manganese and citrate upon the growth of a typical greening Lactobacillus** (Culture S40A; 48 hr at 30 C; optical density × 100)

MG OF Mn^{++} PER 100 ML OF MEDIUM†	G OF SODIUM CITRATE‡ PER 100 ML OF MEDIUM				
	0	0.1	0.5	1.0	2.0
0	24	40	69	37	0
0.1	74	107	116	113	0
1.0	76	113	136	128	1
4.0	79	115	136	128	122

* The basal medium contained 1.0 per cent tryptone, 0.5 per cent yeast extract, 0.5 per cent NaCl, 0.5 per cent K_2HPO_4, 2.0 per cent glucose, and 0.1 per cent "tween 80"; pH 7.0.

† $MnCl_2 \cdot 4H_2O$ was the source of Mn^{++}.

‡ $2Na_3C_6H_5O_7 \cdot 11H_2O$.

RESULTS AND DISCUSSION

It was noted that the addition of either manganese or citrate to a tryptone, yeast extract medium increased the intensity of growth, and that the addition of both substances exhibited an even greater effect. The results obtained with a typical strain are shown in table 1. The demonstration of a Mn^{++} requirement in a complex medium from which no metallic ions were removed in any manner was rather surprising. It should be noted, however, that a noticeable stimulation of growth by the addition of Mn^{++} to the same basal medium was also achieved with *Lactobacillus arabinosus*, strain 17-5, *Leuconostoc mesenteroides*, strain P-60, and *Leuconostoc citrovorum*, strain 8081. On the other hand, citrate did not stimulate the growth of these latter three organisms.

The addition of citrate tended to extend the lag phase of the greening lactobacilli, but in levels up to 0.5 per cent this substance increased the amount of growth obtained during a 48-hour incubation. The addition of Mn^{++} to the medium containing citrate tended to shorten the lag phase and also increased the total amount of growth.

MacLeod and Snell (1947, 1950) have studied in detail the Mn^{++} requirement of certain lactic acid bacteria in synthetic media. They reported that Zn^{++}

inhibited the growth of *Lactobacillus arabinosus* and *Lactobacillus pentosus*. The toxicity of Zn^{++} could be competitively reversed by Mn^{++}, Mg^{++}, Ca^{++}, or Sr^{++}, although these latter three ions could not replace the Mn^{++} required for growth.

The specificity of the Mn^{++} requirement for the greening *Lactobacillus* in a complex medium is illustrated in table 2. It can be seen that Ca^{++}, Cu^{++}, and Zn^{++} were inactive, and that Co^{++}, Fe^{++}, and Mg^{++} had some stimulatory effect at high levels. After 48 hours' incubation there was appreciable growth in the tubes containing no added ions, as indicated in table 1. The tubes containing Mg^{++}, Co^{++}, and Fe^{++} showed very little more growth after 48 hours than did the tubes without any added ions. A toxic effect was manifested by Cu^{++} concentrations of 0.04 mg per 100 ml and above, and by Ca^{++} and Zn^{++} at a concentration of 4.0 mg per 100 ml.

TABLE 2

*The effect of various mineral ions upon the growth of a typical greening Lactobacillus**

(Culture S40A; 24 hr at 30 C; optical density × 100)

MINERAL ION†	MG OF ION PER 100 ML OF MEDIUM				
	0	0.004	0.04	0.4	4.0
Mn^{++}	0	41	112	130	130
Mg^{++}	—	—	8	30	46
Co^{++}	—	—	10	14	94
Ca^{++}	—	—	5	3	4
Cu^{++}	—	—	2	2	2
Fe^{++}	—	—	0	4	76
Zn^{++}	—	—	0	0	10

* The basal medium was the same as table 1, except for the addition of 1.0 per cent sodium citrate and reduction of the glucose concentration to 1.0 per cent.

† The following ion sources were used: $MnCl_2 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $CaCl_2 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, and $ZnSO_4 \cdot 7H_2O$.

The results as presented in table 2 should not be interpreted as implying that high concentrations of Fe^{++}, $Co,^{++}$ or Mg^{++} specifically affect the growth of the greening *Lactobacillus*. It would appear more likely that the salts of these ions as used might have been contaminated with small amounts of Mn^{++}; or that in the presence of citrate and other chelating agents in the medium, high concentrations of these ions made available to the organism additional quantities of Mn^{++} already present. All of the salts used in these experiments were cp grade and no attempt was made to further purify them.

The effect of citrate and Mn^{++} upon the growth of the greening lactobacilli could also be demonstrated in other common laboratory media. For example, little growth of these organisms occurred in a glucose, veal infusion medium prepared in the laboratory unless it was supplemented with both these substances. A glucose, yeast extract, tryptone medium supplemented with 25 per cent filtered tomato juice afforded rather poor growth, but when fortified with additional Mn^{++} rapid and intense growth was achieved.

After successfully culturing the greening lactobacilli in complex laboratory media attempts were then made to culture them in a casein-hydrolysate medium. All strains tested (14 cultures) were found to be capable of growing promptly in

TABLE 3

A simplified medium for the growth of the greening lactobacilli

COMPONENT	PER LITER	COMPONENT	PER LITER
	g		*mg*
Acid-hydrolyzed casein (GBI)	5	Adenine·SO_4	10
Sodium citrate (hydrate)	5	Guanine·HCl	10
K_2HPO_4	5	Uracil	10
Glucose	10	Xanthine	10
"Tween 80" (sorbitan monooleate)	1	Thiamine·HCl	1
DL-Tryptophan	0.1	Nicotinic acid	5
L-Cystine	0.1	Riboflavin	1
Asparagine*	0.1	Pyridoxine	1
$MgSO_4 \cdot 7H_2O$	0.8	Calcium pantothenate	1
$MnCl_2 \cdot 4H_2O$	0.14		*μg*
$FeSO_4 \cdot 7H_2O$	0.04	Biotin	1
NaCl	0.04	Folic acid	10
		pH 7.0	

* Sterilized by Seitz filtration and added to the tubes after autoclaving.

TABLE 4

Growth factor requirements of two greening lactobacilli

SUBSTANCE OMITTED*	OPTICAL DENSITY × 100			
	S11A		S40A	
	24 hr	48 hr	24 hr	48 hr
None	112	88	44	86
Thiamine	0	0	0	0
Nicotinic acid	0	0	0	0
Riboflavin	0	0	0	0
Pyridoxine	110	85	9	78
Calcium pantothenate	0	0	0	0
Biotin	115	100	31	88
"Tween 80" (sorbitan monooleate)	107	100	0	92
Biotin and "tween 80"	0	0	0	0
Folic acid	65	88	6	82
Asparagine	0	0	0	0

* The complete medium is given in table 3. These data record the intensity of growth after one serial subculture in the respective media. Incubation was at 30 C.

a medium having the composition of that listed in table 3. As would be expected, Mn^{++} and citrate influenced growth in this medium similar to that noted in the complex media.

No unusual growth factor requirements could be demonstrated for these microorganisms. The results with 2 representative strains are presented in table 4. All 14 strains required thiamine, nicotinic acid, riboflavin, pantothenic acid,

and biotin. The lag phase of some strains was reduced by the addition of "tween 80," and in its presence biotin was no longer required. In the absence of asparagine a lapse of 3 to 6 days resulted before visible turbidity developed. Both folic acid and pyridoxine shortened the lag phase with some strains, but in most cases the 48-hour growth was not affected by the omission of these two vitamins. As shown to some extent in table 4, once these organisms achieved maximum growth a slow but steady decrease in turbidity was usually noted. However, in most cases this apparent lysis did not appear to seriously interfere with comparative growth estimates as determined turbidimetrically.

Although not investigated, it would be interesting to determine the reason for the apparent synergistic effects of Mn^{++} and citrate upon the growth of the greening *Lactobacillus*. It might be expected that citrate is metabolized by these microorganisms and that Mn^{++} is, in some manner, associated with its metabolism. In this connection it should be recalled that Hartman and Kalnitsky (1950) demonstrated that Mn^{++} was necessary for the oxidation of citrate by a dialyzed rabbit kidney cortex homogenate. The possibility should not be overlooked, however, that in the case of the greening lactobacilli citrate is merely solubilizing Mn^{++} in a more readily available form, or that it may be effectively binding other competing ions.

SUMMARY

The nutritive requirements of heterofermentative lactobacilli that have been isolated from discolored cured meat products have been studied. These organisms require the addition of manganese and citrate for optimum growth, even in complex laboratory media. In a casein-hydrolysate medium they also require thiamine, nicotinic acid, riboflavin, pantothenic acid, biotin, and asparagine. The lag phase may be shortened for some strains by the addition of "tween 80" (sorbitan monooleate), folic acid, and pyridoxine.

The extreme homogeneity in nutritive requirements of strains isolated from products from widely scattered parts of this country is in accord with their homogeneity in physiological and serological characteristics. They would thus appear to constitute a sharply defined species within the ill-defined group of heterofermentative lactobacilli.

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