A STUDY OF THE LACTIC ACID BACTERIA THAT CAUSE SURFACE DISCOLORATIONS OF SAUSAGES

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Sausages prepared from cured, comminuted meats (wieners, bolognas, etc.) are subject to several types of spoilage, the majority of which are manifested by a visual change in color of the product. Most, but not all, of these discoloration problems are known to be of microbial origin. One of the types of spoilage is due to bacterial contamination on the surface of the sausage after processing, which, under appropriate conditions, results in a change in color of the cured meat pigment to a dull green, and thence to a faded color. This change may occur on the uncut surface, as in the case of wieners or other small sausages, or on the sliced surface of such products as bolognas, luncheon meat loaves, and other cold cuts.

The first evidence of spoilage is the appearance of small greenish spots on the damp surfaces of the product, which tend to spread and cover the entire surface in due time if favorable conditions are present. Accompanying the surface discoloration, a slight "greasy" or slimy appearance of the product may be noted in most cases. The discoloration usually penetrates the sausage to a depth of 1 to 2 millimeters. A freshly cut surface invariably reveals an interior that is normal in appearance, odor, and flavor.

Bacteriological evidence indicates that this type of discoloration on the various types of cured meat sausages results from surface contamination after the sausages have been smoked and cooked. Very large numbers of bacteria are found on those areas showing discoloration, but very few viable bacteria may be found in samples obtained from the interior.

It has been reported that the bacteria found to be associated with this type of sausage discoloration are either heterofermentative lactobacilli or members of the genus *Leuconostoc* (Niven, Castellani, and Allanson, 1948). Further studies indicate that these microorganisms comprise rather homogeneous varieties or species within their respective genera and are distinct from any species hitherto described.

CULTURES

The cultures used in this study were obtained from natural outbreaks of surface discoloration on sausages. Scrapings from the discolored areas were plated onto suitable agar media and incubated for 3 days at 30 C. The most satisfactory plating media found were trypticase soy agar (B.B.L.) or blood agar. Representative colonies were picked into a glucose, beef infusion broth and streaked back onto cut surfaces of freshly steamed, normal-appearing wieners.

These inoculated wieners were then incubated in moist chambers at 30 C. Those cultures that discolored the steamed wieners within 24 hours were considered to be associated with the natural discoloration of the sample examined.

A large variety of sausages prepared from cured meats showing surface discoloration have been examined for the presence of "green-producing" bacteria. In every case in which the discoloration was of microbiological origin, the green-producing bacteria were present in very large numbers, usually in great excess over any other type of microorganism found. Thirty of these greening bacteria were selected at random from ten discolored samples for extended study. These samples originated from six widely separated geographical locations in the eastern half of the United States. Twenty of these 30 cultures were found to be members of the genus *Lactobacillus* (subgenus, *Betabacterium*). The remaining 10 cultures were members of the genus *Leuconostoc* (*Betacoccus*).

CHARACTERISTICS OF THE LACTOBACILLUS STRAINS

Morphologically the *Lactobacillus* cultures are short, straight rods (2 to 4 microns in length) with rounded ends, occurring either singly or in pairs. They are strongly gram-positive, as viewed in films prepared directly from the original samples as well as from pure cultures. Little granulation or marked variation in morphology was noted under any of the cultural conditions tested.

Growth on all plating media tested was rather slow. Colonies were either barely perceptible or not yet visible after 24 hours at 30 C. After 3 days' incubation on a glucose, beef infusion agar the colonies were smooth, compact, and approximately 0.5 millimeter in diameter. Growth on blood agar was usually inferior, but was more easily detected because of the green zone surrounding the individual colonies.

In glucose broth media, uniform turbidity, with some sediment, developed after 48 hours at 30 C. Growth was considered to be rather slow, with maximum turbidity usually inferior to that produced by some of the better known heterofermentative lactobacilli tested under the same conditions.

All cultures adhered to the characteristics recognized for the genus *Lactobacillus* in that they were nonmotile, nonpigmented, and catalase-negative and failed to reduce nitrates. Incubation under anaerobic conditions and under 10 per cent CO_2 appeared to improve growth to a slight degree.

Table 1 presents the physiological characteristics determined for the 20 cultures. Perhaps because of their slow rate of growth, a great deal of difficulty was encountered with the usual cultural methods for determining gas production by these microorganisms. However, dependable results were obtained with the use of Eldredge tubes. A basal medium consisting of 1 per cent (Difco) tryptone, 0.5 per cent (Difco) yeast extract, 0.2 per cent dipotassium phosphate, and 0.5 volume beef infusion was employed, in which 1 per cent glucose was added aseptically prior to inoculation. In this medium at 30 C, the cultures fermented 0.7 to 0.9 per cent glucose within 10 days. Calculated on a weight basis, all cultures produced 19 to 24 per cent carbon dioxide from the sugar fermented.

In a separate experiment three of the greening lactobacillus strains produced a lactic acid yield of only 36 to 40 per cent, based upon the glucose fermented.

Lactic acid was determined quantitatively by the method of Barker and Summerson (1941). Zinc salts of the lactic acid produced from glucose by these three strains were isolated for the purpose of determining the optical form produced. In each case the inactive form of lactic acid was produced. These determinations tend to confirm the heterofermentative nature of the lactobacillus strains. No

TABLE 1

Physiological characteristics of the sausage-greening microorganisms

	LACTOBACILLUS GROUP (20 STRAINS)	LEUCONOSTOC GROUP (10 STRAINS)
CO_2 from glucose	+	+
Polysaccharide from sucrose	14+; 6−	−
Greening on blood agar	+	+
Final pH, glucose broth	4.6–4.8	4.6–4.9
Litmus milk	No change	Sl. acid
Lactic acid from glucose	DL	D(−)
Growth:		
5 C	+	+
40 C	±	±
6.5% sodium chloride	+	+
10.0% sodium chloride	−	+
Hydrolysis:		
Sodium hippurate	−	−
Esculin	−	+
Arginine	−	−
Gelatin	−	−
Starch	−	−
Fermentation:		
Xylose	−	−
Arabinose	−	+
Mannose	+	+
Fructose	+	+
Galactose	−	+
Lactose	−	+
Maltose	+	+
Sucrose	15+; 5−	+
Trehalose	4+; 16−	+
Raffinose	−	+
Inulin	−	−
Glycerol	−	−
Mannitol	−	−
Sorbitol	−	−

attempts were made to identity other fermentation products of these microorganisms.

When streaked upon a 5 per cent sucrose gelatin agar medium, 14 of the 20 cultures produced large mucoid colonies very similar to those produced by certain *Leuconostoc* cultures. Of the 6 strains that failed to possess this characteristic, 5 were unable to ferment sucrose.

The optimum temperature for growth appeared to be approximately 30 to

32 C. All cultures grew at 37 C but none were able to grow at 45 C. The maximum temperature for growth appeared to be 40 C, as evidenced by the fact that the cultures grew poorly, or not at all, at this temperature. The lower temperature limit for growth was below 5 C. All cultures developed visible turbidity in broth culture at 3.5 C within 1 week.

The greening lactobacilli were relatively tolerant to salt. All strains grew in a glucose medium containing 6.5 per cent sodium chloride. Growth occurred in the presence of 8.0 per cent sodium chloride, but was markedly reduced. Higher concentrations of salt completely inhibited growth.

The fermentation pattern of these cultures was rather striking. Only glucose, fructose, mannose, and maltose were fermented by all of the strains. The majority fermented sucrose, whereas only a few fermented trehalose. None of the cultures were able to ferment the pentoses, galactose, lactose, or any of the higher alcohols tested.

CHARACTERISTICS OF THE LEUCONOSTOC STRAINS

The individual cells of the ten *Leuconostoc* cultures were either spherical (approximately 1 micron in diameter) or ovoid in shape, occurring in short to moderately long chains when viewed from broth cultures. Some chains contained as many as 20 cells. In films prepared directly from discolored sausage samples, these microorganisms appear in much shorter chain lengths. The majority may be present as individual cells or in pairs; consequently, the presence of large numbers of *Leuconostoc* microorganisms on the original product may be easily overlooked. All cultures were strongly gram-positive.

Growth on glucose agar or blood agar plating media was much more rapid for these than for the *Lactobacillus* cultures. Within 2 days at 30 C the individual colonies were smooth, compact, and approximately 1 millimeter in diameter. Blood agar colonies were surrounded by strong green zones.

Within 24 hours at 30 C, glucose broth cultures showed moderately heavy turbidity. After 48 hours the cells tended to settle out. All cultures were nonmotile, nonpigmented, and catalase-negative and failed to reduce nitrates.

The physiological characteristics of these cultures are also presented in table 1. With the same techniques described for the *Lactobacillus* cultures, the 10 *Leuconostoc* strains were found to produce 15 to 24 per cent carbon dioxide from the sugar fermented. Lactic acid yields from glucose fermentation by two of the strains were approximately 25 and 35 per cent. The lactic acid produced by these two strains was *levo*-rotatory, which is in line with the better known species within this genus.

Somewhat striking was the fact that none of the 10 greening *Leuconostoc* cultures produced mucoid colonies on sucrose agar. Repeated attempts to demonstrate this phenomenon under different conditions failed. No polysaccharide synthesis from sucrose occurred under anaerobic conditions, or in sucrose gelatin stab cultures as described by Hucker and Pederson (1930); yet, all cultures fermented sucrose.

The greening *Leuconostoc* cultures showed practically the same temperature

limits for growth as the *Lactobacillus* cultures described. They grew slowly at 3.5 C; poor or no growth occurred at 40 C. In comparison with the lactobacilli, the *Leuconostoc* cultures were tolerant to high salt concentrations. All strains grew in the presence of 10 per cent sodium chloride; some grew moderately well in concentrations as high as 12 per cent.

All of the ten strains fermented arabinose, the hexose sugars, the disaccharides, and raffinose. Xylose and the higher alcohols were not fermented.

SEROLOGICAL STUDIES

One of the *Lactobacillus* strains was inoculated into 100 ml of beef infusion broth containing 0.1 per cent glucose and incubated for approximately 30 hours at 30 C. The cells were sedimented, resuspended in 5 ml saline, and heat-killed at 56 C for 1 hour. The volume was then adjusted to 25 ml with saline. One ml of this prepared antigen was inoculated intravenously into rabbits daily for 5 days each week. Ten days after the fifth series of injections the rabbits were bled and the serum was collected in the usual manner.

Extracts of 18 of the greening lactobacilli were prepared using minor modifications of the original method of Lancefield (1933). Of the 18 extracts tested, all gave a strong precipitin test with the serum, thus demonstrating the serological homogeneity of the cultures. No cross reactions were obtained with any other cultures tested. A serum prepared by use of a second greening *Lactobacillus* culture as an antigen gave identical results.

A serum was also prepared from one of the *Leuconostoc* cultures. Of the 10 *Leuconostoc* extracts tested, five gave strong precipitin tests equal in intensity to that of the homologous strain. The remaining five extracts also gave positive precipitin reactions with the serum, though much weaker than that obtained with the homologous strain. Serum obtained by an additional four series of injections failed to produce any increase in intensity of reaction with these five extracts.

Reciprocal precipitin tests between the *Lactobacillus* and *Leuconostoc* groups failed to reveal any cross reactions.

DISCUSSION

The number of cultures representing the two groups of microorganisms described is admittedly small. However, considering the widely separated geographical sources from which the cultures were obtained, it would seem more than coincidental that they comprise such remarkably homogeneous groups within their respective genera. The majority of the cultures included in this study were isolated within a period of approximately nine months. However, the chances of their having originated from a common source would be exceedingly small.

Taxonomically, the lactobacilli would fit into the heterofermentative group characterized by their low temperature limits of growth. They are perhaps more closely related to *Lactobacillus brevis* than any other described species. However, and this is substantiated by a comparative study of several heterofermentative lactobacilli received from Dr Carl S. Pederson, these cultures are easily dis-

tinguished from any of the known species. Of the few tested, none of the known heterofermentative *Lactobacillus* strains were capable of discoloring sausages in pure culture.

Of particular interest was the ability of the sucrose-fermenting *Lactobacillus* strains to produce mucoid colonies on sucrose agar. Only one sucrose-fermenting strain failed to synthesize a polysaccharide from this disaccharide. This strain fermented sucrose slowly. In a study of the group as a whole, however, the percentage of the cultures that form mucoid colonies might be considered too low to be of great taxonomic value.

The synthesis of a polysaccharide from sucrose by lactobacilli has not been recognized to any great extent, although it may be a more common occurrence than is suspected. Dr. Pederson has called the authors' attention to the description of *Bacterium vermiforme* Ward, described in *Bergey's Manual of Determinative Bacteriology* (1948). This organism, later called *Betabacterium vermiforme* Mayer, appears to have been a slime-forming *Lactobacillus* that was isolated from the ginger beer plant fermentation. More recently, Kobayasha (1944) has described a microorganism, *Lactobacillus musicus*, isolated from tobacco leaves. This microorganism is reported to ferment sucrose into a viscous substance. The relationship of the sausage-greening lactobacilli to these slime-producing bacteria is unknown.

As mentioned previously, the greening lactobacilli grow rather slowly and have a comparatively high limiting pH in glucose culture media. On the other hand, these microorganisms appear to grow rapidly when inoculated onto sausages. This apparent discrepancy might be due to the existence of a nutritional deficiency in the artificial media used. Many different types of media and supplements have been tested in attempts to enhance the growth of these bacteria. These attempts have failed to divulge any significant information concerning the nutrition of these microorganisms. Furthermore, preliminary trials indicate that representative cultures of this group are capable of growing in a medium composed of chemically defined constituents. Therefore, it is unlikely that the cultures require an unrecognized growth factor. At the moment, no explanation can be offered for these anomalous observations.

The *Leuconostoc* cultures are taxonomically peculiar in that they ferment sucrose and yet do not form mucoid colonies on agar media containing this substrate. They also appear to be much more tolerant to sodium chloride than the described species of this genus. With the exception of the above-mentioned characters, these microorganisms might be considered to be related to *Leuconostoc mesenteroides*.

The serological studies might indicate the existence of a serological group, as well as type, relationship among the *Leuconostoc* cultures similar to that among the hemolytic and some other streptococci. However, the evidence presented is too meager to warrant serious consideration of this possibility. Also, conforming to the existing terminology for the streptococci, the 20 greening lactobacilli would appear to be members of a single type, rather than of a serological group.

The question arises as to the mechanism by which the sausages are discolored when these bacteria are allowed to grow extensively on the surface. The cured meat pigments (nitric-oxide myohemoglobin and nitric-oxide hemochromogen) can be easily oxidized, with accompanying change in color (Urbain and Jensen, 1940). Evidence has appeared (Jensen, 1944, 1945; Jensen and Urbain, 1936) which indicates that certain types of discoloration of sausages result from the microbial production of peroxides that react chemically with the cured meat pigment. Experiments of a preliminary nature indicate that the bacteria described here also discolor the sausages as the result of hydrogen peroxide production. Being members of the lactic acid group of bacteria, they are devoid of catalase. The catalase originally present in the sausage meats is inactivated by the curing ingredients and the smoking and cooking temperatures. Therefore, when these bacteria are the predominant microorganisms present on the sausage, little or no catalase may be present.

Under the conditions tested, cell suspensions of one of the greening lactobacilli were able to oxidize unknown substances in beef infusion with the production of hydrogen peroxide in concentrations up to 0.02 per cent. Experiments were conducted in which different concentrations of hydrogen peroxide were applied to freshly cut surfaces of sausages. When a liberal application of 0.03 per cent hydrogen peroxide was made, discoloration occurred after several hours similar to that produced microbiologically. Higher concentrations resulted in prompt and complete fading.

Few attempts were made to determine the substance, or substances, in beef infusion that were oxidized by the test culture. However, incubation with glucose resulted in the accumulation of low concentrations of hydrogen peroxide. In contrast to the "minute" and certain viridans streptococci that produce large quantities of hydrogen peroxide from butyrate (Niven, Evans, and White, 1945), the test *Lactobacillus* culture failed to oxidize this substrate.

If sausages are inoculated with the greening cultures and incubated anaerobically, no discoloration occurs, although abundant growth takes place. However, upon removal from the desiccator, the sausages discolor within a few hours.

Indirect evidence that the discoloration occurs as the result of hydrogen peroxide production was gained by the observation of a ham slice that had developed several green spots on the cut surface after having been incubated at room temperature at high humidity. Films prepared from the discolored areas showed the presence of large numbers of lactobacilli in apparently pure culture. Films prepared from the normally colored areas showed the presence of large numbers of micrococci, with only an occasional gram-positive rod. When droplets of 3 per cent hydrogen peroxide were applied, the discolored areas appeared to be devoid of catalase, whereas the normally colored areas gave a strong catalase test. The catalase was undoubtedly of microbial origin.

Catalase appears to protect sausages from discoloration due to microbial growth. In one experiment, freshly cut surfaces of steamed wieners were coated with a catalase concentrate of high activity. The surfaces were inoculated with a greening *Lactobacillus* and incubated for 24 hours. No discoloration took place

although extensive growth of the inoculum occurred. Inoculated control samples discolored within 16 hours. Higher dilutions of catalase failed to protect the sausage color since the enzyme appeared to be inactivated at a rapid rate.

It is not implied that the two groups of microorganisms described in this article are the only ones that are capable of causing surface discoloration of sausages. It would seem that any salt-tolerant, catalase-negative microorganism that is capable of growing at low temperatures, and that oxidizes certain substrates with the accumulation of hydrogen peroxide, might be found associated with natural outbreaks of this type of spoilage. Indeed, among the cultures tested one unidentified homofermentative *Lactobacillus* isolated from raw sausage mix was found which discolored sausages in pure culture. From other samples of raw sausage mix, four micrococcus strains were isolated which discolored sausages. The cells of these cultures existed in the form of tetrads. Fitting into the general pattern of the other sausage-greening microorganisms, three of these strains appeared to be catalase-negative under the conditions tested; the fourth strain exhibited extremely weak catalase activity.

More recently, six *Lactobacillus* strains that would discolor sausages in pure culture have been isolated from three samples of boiled ham showing greenish discoloration on the sliced surface. They appeared to be closely related to the greening lactobacilli described, but differed in that they hydrolyzed esculin and arginine and failed to ferment fructose. Of all the substances tested, only glucose, mannose, maltose, and trehalose were fermented by these strains. Unfortunately, five of the six strains were lost before any serological studies were begun. The sixth, however, failed to give a positive precipitin test with the type-specific *Lactobacillus* serum. It therefore appears that these strains represent a second variety of sausage-greening lactobacilli.

All the sausage-greening microorganisms also produced a strong greening reaction on blood agar. This characteristic is not necessarily indicative of their ability to discolor sausages, however. Other than the microorganisms already described, no other "viridans" type of lactic acid bacteria have been found which would promptly discolor sausages, even though they would grow extensively on this product.

The sausage spoilage described in this article may reach "epidemic" proportions and result in serious economic loss if proper precautions are not taken. For control purposes it would be interesting to learn the original habitat of the causative microorganisms. Thus far no light has been thrown on this question.

Although these microorganisms appear to be hitherto undescribed varieties, or species, within their respective genera, the authors hesitate to suggest any new names until our knowledge concerning the classification of the entire group of heterofermentative lactic acid bacteria is less confused.

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SUMMARY

A detailed study has been conducted on 30 cultures isolated from outbreaks of greenish discolorations on the surface of sausages. Twenty of the cultures comprised a homogeneous and distinct group of heterofermentative lactobacilli that belonged to one serological type.

The remaining 10 cultures were members of the genus *Leuconostoc*, but these cultures failed to produce mucoid colonies on sucrose agar, although this sugar was fermented.

All sausage-greening cultures were salt-tolerant and capable of growing at low temperatures. Evidence is presented which indicates that the sausage discoloration results from the production of hydrogen peroxide by the microorganisms.

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