LOW TEMPERATURE LACTIC ACID BACTERIA FOR MEAT FERMENTATION

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INTRODUCTION

The volume of fermented meat products (dry and semidry sausage) processed in the U. S. under federal inspection increased 23% over the 3 year period between 1975 and 1978. Lactic acid cultures are used by a continuously increasing number of manufacturers both in the U.S. and in Europe.

A controlled fermentation of meats, using lactic acid bacteria, has many advantages over either "natural fermentation" or "back slopping". Among others it assures tang, reduces processing time, increases yield, improves work schedules, reduces losses and controls the growth of undesirable microorganisms.

Phenolic type antioxidants, often in combinations of 2 or more, are used in meat products to protect the fat from oxidation and rancidity (USDA, 1976). The phenolic nature of the antioxidants suggest that they might also act as antimicrobial agents.

Indeed, phenolic type antioxidants alone (Robach and Pierson, 1979; Turcotte et Saheb, 1978; Robach et al., 1977; Shih and Harris, 1977; Lin et al, 1977; Chang and Branen, 1975; and Ward and Ward 1967) or in combination with either NaCl (Stern et al, 1979) or fatty acids (Saheb et al., 1978) were shown to inhibit the growth of some gram negative and gram positive bacteria. There is no information on the effect of phenolic type antioxidants on the fermentative activity of lactic acid bacteria.

In recent years coagulase positive staphylococci have attracted considerable attention with regard to the safety of fermented meats. These organisms have the ability to grow better than other pathogens under what would usually be considered adverse conditions such as those found in fermenting sausage. Coagulase positive staphylococci were involved in 33 cases of food poisoning associated with fermented meats (Genoa sausage was involved in 25 cases) between 1968 and 1977 (Bryan, 1980). Levels ranging from 2.3×10^2 to 1.7×10^4 staphylococci/g were reported in sausage mixes before fermentation (Pullen and Genigeorgis, 1977). Pork cheeks contaminated by nasal sinuses may be carrying unusually high numbers of staphylococci. The growth of Staphylococcus aureus and enterotoxin(s) production are significantly reduced at temperatures of 25°C and below, and the enterotoxin(s) production is totally inhibited at 10°C (Vandenbosch et al., 1973; Scheusner and Harmon 1971). A meat fermentation at temperatures between 27°C and 10°C is a common practice in many European countries.

The development of a relatively rapid lactic acid culture for meats fermented at temperatures of 27°C and below will reduce the energy consumption as required for some commercially available cultures (Everson et al., 1970; Rothchild and Olsen, 1971) and will further control the growth of undesirable microorganisms.

The purpose of this work was to examine the fermentative activity of a new lactic acid culture, LACTACEL® 75, (Pediococcus pentosaceus) in different meat systems, and to study the effect of NaCl and phenolic type antioxidants on this culture. In addition, the inhibitory effect of LACTACEL 75 on Staphylococcus aureus was tested in different meat systems.

MATERIALS AND METHODS

Meat - Genoa and Pepperoni were all pork (Boston Butt) products (20-22% fat). Summer sausage was prepared from 60% beef (chuck) and 40% pork (Boston Butt). Both the beef and pork were frozen (-31°C) until utilized.

Sausage formulae - The different types of sausage were prepared using the ingredients presented in Table 1.

Table 1. SAUSAGE INGREDIENTS

Ingredient	Sausage		
	Genoa	Pepperoni	Summer Sausage
	g/Kg Meat		
NaCl	28.0 - 33.0	33.0	30.0
Dextrose	5.0 - 7.0	5.0 - 7.0	10.0
$NaNO_2$	0.156	0.156	0.156
Genoa spice mix[a]	3.8	-	-
Pepperoni spice mix[a]	-	13.7	-
Summer sausage spice mix	-	-	6.8
LACTACEL® 75[b]	6.2	6.2	6.2

[a]Contains 0.003% Butylated Hydroxyanisol (BHA) and 0.003% Butylated Hydroxytoluene (BHT) and citric acid as an antioxidant system (USDA, 1976).

[b]Diluted (5.4×10^{-2}) with tap water. The same inoculation rate was used with either LACTACEL or LACTACEL MC.

Prepration of sausage - The meat was chopped in a Hobart Silent Cutter (Model 8418D) to the desired size. Cure, spices and culture were added in this order. Genoa, Pepperoni and Summer sausage were stuffed into 76, 40 and 53mm fibrous casings respectively. Links of 30-45 cm were formed.

Fermentation - The sausage products were fermented in a Controlled Environmental Chamber (model 412461, Hot Pack Corp., Philadelphia, PA) at different temperatures. In all instances, the relative humidity (RH) was kept at 80%.

pH Measurements - 30g of sausage were blended with 90 ml distilled water for 30-60 seconds. The pH of the slurry was determined using an Orion Research Digital pH meter.

Assay for the inhibitory effect of phenolic type antioxidants and NaCl on acid production by LACTACEL 75 - APT broth (Difco) was used (11.6g/L) as the assay medium. LACTACEL 75 was added to provide 3.4×10^8 cells/ml.

Phenolic type antioxidants are hydrophobic and have a slight solubility in water. BHT, for example has a solubility in water, of less than 10^{-5} Molar. Therefore, ethanolic stock solutions (10 mg/ml) of each BHA, BHT and

TBHQ were prepared, and added to the assay medium to provide the final concentration of 0.003% (30µg/ml). Due to the formation of slight percipitations, the effective amount in solution may be less. Citric acid stock solution (10 mg/ml) was prepared with 0.01N HCl. The effect of NaCl was tested at the following concentrations: 3.0, 3.3, 3.6 and 3.9% (W/V).

The assay was conducted at either 37°C (2 h) or 27°C (4 h). The pH of the medium was measured at the beginning and at the end of the testing period. The pH measurements were used to calculate the inhibition of acid production according to the following formula: $\% \text{ Inhibition} = 100 - \left[\frac{\Delta pHi}{\Delta pHc} \times 100\right]$

Where ΔpHi and ΔpHc are the differences between the initial and final pH values of the assay medium containing a particular inhibitor and the control respectively.

Staphylococcus aureus inoculated sausage - S. aureus strain 265-1 (enterotoxin A producer) was transferred daily for 3 successive days in Difco Brain Heart Infusion broth (35°C, 18-20 h). An aliquot of the third transfer was used to provide approximately 10,000 viable cells/g sausage mix.

Sampling of S. aureus inoculated sausage - 10g samples were taken only from the outer surface (0.5-1.0 cm depth) of the sausage where the most significant growth and enterotoxin production by S. aureus, take place (Barber and Deibel, 1972; Lee et al., 1977).

Enumeration of S. aureus - The sausage sample (10g) was blended for 2 min with 0.1% (w/v) Difco Peptone water. Further decimal dilutions were made with the same diluent. Aliquots of 0.1 ml of the appropriate dilutions were surface spread onto prepoured, dried surface Baird-Parker (BP) Agar plates in duplicates. The BP Agar plates were incubated at 35°C for 48 h. Plates containing 20-200 colonies were selected and only typical S. aureus colonies (Black surrounded by an opaque zone with an outer clear zone) were counted.

RESULTS

Genoa sausage - LACTACEL® 75 reduced the fermentation time (to pH 5.0) of this product (as compared to LACTACEL) from 48 to 20 h at 27°C (Table 2). Higher temperatures decreased the fermentation time(to pH 5.0), for example, only 12 h were required at 35°C. From the economical (energy saving) and safety point of views, temperatures of 21°C and below are favorable. Although it took anywhere between 37 and 71 h to bring the pH down to 5.3 or 41 to 80 h to pH 5.0 (depending on the temperature and salt concentration), it was not necessary to have a special source of energy to maintain these temperatures. The growth of pathogenic organisms, such as S. aureus, was completely, controlled (see S. aureus section). Furthermore these low temperatures are used in many European countries and are favorable for the development of the "typical" flavor of dry sausage.

Removal of the antioxidant system (BHA, BHT and citric acid) reduced the fermentation time (with 3.3% NaCl) by 1-4 h to pH 5.3 or 2-4h to pH 5.0 (depending on the temperature used). Within the temperature range of 21° to 29°C, reduction of the concentration of NaCl from 3.3 to 3.0%, and from 3.0 to 2.8% in the absence of the antioxidant system decreased the fermentation time to either pH 5.3 or 5.0 by 2 and 1 h respectively. As can be seen, (Table 2) at 24°C the addition of the antioxidant system to Genoa containing 3.3% NaCl increased the fermentation time (to pH 5.0) by 4 h as compared to increasing the concentration of NaCl in the absence of the antioxidant system from 3.0 to 3.3% (24 to 28 h vs. 22 to 24 h respectively).

The hydrophobic nature of a phenolic type antioxidant molecule promotes strong interactions with the hydrocarbon zones of cell membrane structures and causes perturbing effects on membrane associated functions such as a decrease in permeability to essential metabolites (Snipes et al., 1975; Singer and Wan, 1977). One such perturbing effect may be on the phosphoenol pyruvate (PEP): Glucose phosphotransferase system (PTS). Any degree of inactiviation of this system will hinder that transport of glucose and as a result, the fermentative activity of the lactic acid culture will be slower, increasing the fermentation time to the desired pH.

Pepperoni - The fermentation times required to reach pH 5.0 of this product were examined within the temperature range of 27° to 35°C (Table 2). The fermentation time decreased with increasing the fermentation temperature (18 h at 27°C vs. 11 h at 35°C).

Summer sausage - This product is usually fermented at a temperature range of between 32°-43°C to obtain a rapid fermentation when prepared with cultures such as LACTACEL® (Pediococcus acidilactici previously P. cerevisiae) or LACTACEL MC (P. acidilactici and Lactobacillus plantarum). LACTACEL 75 (P. pentosaceus) fermented this meat system at a faster rate than either LACTACEL or LACTACEL MC (Table 3). LACTACEL 75 at 27°C makes it possible to attain pH 5.0 only after 15 h of fermentation which is 33 and 8 h faster than LACTACEL and LACTACEL MC respectively. A short fermentation period is essential to reduce energy consumption and to shorten the growth period of non desirable organisms such as S. aureus. Fermentation temperatures below 24°C where staphylococcal enterotoxin formation is diminished (Vandenbosch et al., 1973 Scheusner and Harmon 1971) is made practical by the use of LACTACEL 75. A temperature range of 24° to 16°C reflects Summer and Winter "ambient" room temperatures. The use of LACTACEL 75 makes it possible to ferment Summer sausage without a special energy source to maintain a fixed temperature. The end result will be saving of energy, and control of S. aureus growth and enterotoxin production. The fermentation period varies according to the "ambient" temperature (31 h at 21°C vs. 75 h at 16°C).

Inhibitory effects of phenolic type antioxidants and NaCl - The most potent inhibitor of acid production, by LACTACEL 75 in APT broth, was TBHQ followed in decreasing order of potency by BHT and BHA (Table 4). Similar results were observed with LACTACEL (P. acidilactici): TBHQ inhibited 97% of the acid production while BHT only 50.3% and BHA 0.0%. This sequence of potency may be ascribed to the different lipid solubility of the various phenolic type antioxidants tested. Paquot and Cuvier (1979) found TBHQ superior to either BHA or BHT as an antioxidant in both meat fat and vegetable oils. It appears that the sensitivity to BHA varies with the bacterial species.

The combined inhibitory effects of BHA, BHT and citric acid (the antioxidant system) did not reveal synergism between BHA and BHT, but had an additive nature.

In Table 2 an increase in fermentation time (to pH 5.0) of 9.1 - 16.7% was observed with the addition of the antioxidant system. These values are much smaller than the inhibition observed in APT broth. It is conceivable that phenolic type antioxidants added to a meat system will dissolve at different rates in the fat of the system and in the bacterial cell lipids. The rate of solubility in each of these different lipids (fat) will determine the degree of potency of each antioxidant. In meat, the inhibitory effect of the phenolic type antioxidants on LACTACEL

75 was smaller than that in APT broth, suggesting that a portion of these antioxidants accumulated in the fat of the meat system leaving a smaller portion to affect the lactic acid culture. Another explanation is that the meat proteins exert a strong buffering action neutralizing, to a certain degree, the hydrogen ions formed by the lactic acid bacteria thus preventing synergism between the hydrogen ions and the phenolic type antioxidants (Stern et al., 1975). These results are in accordance with those of Robach et al., (1977) who found, for example, that 400 ppm BHA were required to inhibit the growth of Vibrio parahaemolyticus in a crab meat homogenate as compared to only 50 ppm in Trypticase Soy broth.

The inhibitory effect of NaCl on acid production by LACTACEL® 75 was concentration dependent. An increase of 45.6% in the inhibitory effect of NaCl was observed when the concentration was increased from 3.0 to 3.9%. The addition of the antioxidant system to 3.3% NaCl increased the inhibitory effect on LACTACEL 75 from 44.6 to 83.0% (Table 4), a value smaller than the sum of the effects of these chemicals suggesting that the effect in concert was not additive. No attempt was made to distinguish between the net effect of either the phenolic type antioxidants or sodium chloride on acid production by LACTACEL 75.

It has been shown that hydrogen ions are synergistic to the adverse effect of BHA (Stern et al., 1979). It may be assumed that if these tests were carried out in a buffered assay medium the results coult have been different.

Growth of S. aureus in dry sausage - Genoa sausage: LACTACEL 75 controlled the growth of S. aureus in the outer surface (0.5-1.0 cm depth) of Genoa sausage with the temperature range of 21° to 35°C (Table 5). At 21°C LACTACEL 75 was slightly bacteriocidic decreasing (41.7%) the population of S. aureus. In general, the population of the pathogen in association with LACTACEL 75 was 1000 fold lower than the minimal level ($1.4x10^7$ cells/g) for enterotoxin(s) production (Barber and Deibel, 1972). The generation time of S. aureus inoculated into Genoa sausage in the absence of LACTACEL 75 generally decreased with the increase of the fermentation temperature (15.5 h at 21°C vs. 2.1 h at 35°C). S. aureus in association with LACTACEL 75, had generation times 1.8 to 15.3 times longer (depending on the temperature) than those of the pathogen growing alone, and was able to increase in population by only 0.08 to 0.24 log. These results indicate the adverse effect that LACTACEL 75 had on the growth of S. aureus. This pathogen is salt tolerant (up to 20% NaCl), a facultative anaerobe, grows at an A_w as low as 0.83 and produces enterotoxin(s) at temperatures between 10° and 46°C (Tatini, 1973). S. aureus is not totally inhibited by NaCl, $NaNO_2$, the initial pH and A_w of the sausage mix. Therefore, it appears that the formation of enterotoxin(s) will be during the early stages of fermentation as the hydrogen ions, A_w and the lactic acid bacteria present in the finished product have an adverse effect on S. aureus growth and formation of enterotoxin(s).

It is not surprising that enterotoxin A was detected in surface samples of Genoa sausage made without added lactic acid bacteria and inoculated with 10^5 S. aureus cells/g (Lee et al., 1977). Under the same conditions, enterotoxin B was not detected. Niskanen and Nurmi (1976) reported that starter cultures prevented the production of enterotoxin A in dry sausage. Thus, a short fermentation which will rapidly turn the sausage internal environmental conditions unfavorable for S. aureus growth will also prevent the pathogen from attaining levels associated with enterotoxin(s) production.

Pepperoni: This product was prepared with 0.56% (W/V) dextrose to limit acid production and to prevent the pH from falling below 5.0. As can be seen, (Table 6) LACTACEL 75 controlled the growth of S. aureus during the 10 h fermentation period required to attain pH 5.0 The Pepperoni sticks were left for an additional 8 h at 35°C to simulate the come up time during the heat treatment for trichinae destruction. During these 8 h in the absence of LACTACEL 75, S. aureus population increased by almost 100 fold (1.93 log) and attained $1.7x10^7$ cells/g (the minimal level associated with enterotoxin(s) formation) in the surface of sausage. During the same time, S. aureus in association with LACTACEL 75 attained only $1.5x10^5$ cells/g which was 100 fold lower than the minimal level for enterotoxin(s) formation. Tatini et. (1976) showed that the population of S. aureus inoculated in meat (10^4 cells/g) being made into Pepperoni (without added lactic acid bacteria) increased by 2 log.

In Pepperoni (as in Genoa sausage) the generation times of S. aureus in association with LACTACEL® 75 were about 3 times longer than those of S. aureus alone, (Table 6) a clear indication of the antagonistic action of LACTACEL 75 against S. aureus.

In summary, LACTACEL 75 had a faster fermentative activity in different meat systems as compared to some commercial lactic acid cultures. The rapid fermentative activity of LACTACEL 75 enables the manufacturer to speed up production, saves energy and last, but not least, to control effectively the growth and enterotoxin(s) production by S. aureus.

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Table 2. Fermentation times[a] of different dry sausage.

Temperature (°C)	Genoa Sausage: With antioxidants	Genoa Sausage: W/O antioxidants		Genoa Sausage: W/O antioxidants	Pepperoni: With antioxidants
	NaCl concentration (%)				
	3.3	3.3	3.0	2.8	3.3
	Hour				
16	N.E.	N.E.	80 (71)[b]	71(65)	N.E.
18	N.E.	N.E.	53 (47)	50(42)	N.E.
21	48(45)	44(40)	42 (38)	41(37)	N.E.
24	28(25)	24(21)	22 (19)	21(18)	N.E.
27	20(18)	18(16)	16 (14)	15(13)	18(15)
29	18(15)	16(14)	14 (12)	13(11)	15(13)
32	14(12)	N.E.	N.E.	N.E.	13(11)
35	12(10)	N.E.	N.E.	N.E.	11(9)

[a]To attain pH 5.0
[b]( )= Time to pH 5.3
N.E. = Not examined.

Table 3. Fermentation times[a] of Summer Sausage.

Temperature (°C)	Culture: LACTACEL 75	LACTACEL	LACTACEL MC
	Hour		
16	75	N.E.	N.E.
18	46	N.E.	N.E.
21	31	N.E.	N.E.
24	17	N.E.	N.E.
27	15	48	23
29	N.E.	N.E.	N.E.
32	N.E.	17	20
35	11.5	N.E.	N.E.
38	N.E.	10	14
40.5	N.E.	N.E.	N.E.
43	N.E.	9	10

[a]To attain pH 5.0
N.E. = Not Examined.

Table 4. Inhibitory effects of phenolic type antioxidants and NaCl on acid production by LACTACEL® 75.

Material/Combination	Incubation temperature: 27°C	37°C
	Inhibition (%)[b]	
BHA[a]	14.0	10.7
BHT[a]	53.6	57.6
Citric acid	N.E.	2.7
BHA[a], BHT[a] & Citric acid	N.E.	65.3
TBHQ[a]	98.0	N.E.
NaCl (%)		
3.0	N.E.	36.2
3.3	N.E.	44.6
3.6	N.E.	49.5
3.9	N.E.	52.7
BHA[a], BHT[a], Citric acid & 3.3% NaCl	N.E.	83.0

[a]Added each at a concentration of 0.003% (W/V).
[b]% Inhibition = $100-\left[(\Delta pHi \div \Delta pHc)\ 100\right]$. See materials and methods.

Table 5. Effect of LACTACEL® 75 on growth of S. aureus in Genoa sausage fermented at different temperatures.

Fermentation Conditions	S. aureus count/g: Initial	Final	Generation Time (h)	Count Increase (log)[b]
21°C, 48 h				
S. aureus alone	1.0×10^4 (5.95)[a]	8.7×10^4 (5.60)	15.5	0.94
S. aureus & LACTACEL 75	1.2×10^4 (5.96)	7.0×10^3 (4.70)	N.A.[c]	N.A.
24°C, 25 h				
S. aureus alone	9.6×10^3 (5.92)	2.5×10^4 (5.67)	18.2	0.42
S. aureus & LACTACEL 75	7.6×10^3 (5.94)	9.2×10^3 (5.00)	91.3	0.08
27°C, 18 h				
S. aureus alone	1.1×10^4 (5.91)	2.9×10^4 (5.72)	13.0	0.42
S. aureus & LACTACEL 75	1.1×10^4 (5.89)	1.9×10^4 (5.00)	23.0	0.24
32°C, 14 h				
S. aureus alone	1.4×10^4 (5.99)	3.0×10^5 (5.65)	3.2	1.33
S. aureus & LACTACEL 75	1.1×10^4 (5.98)	1.4×10^4 (5.03)	40.5	0.10
35°C, 11 h				
S. aureus alone	1.0×10^4 (5.93)	3.6×10^5 (5.60)	2.1	1.6
S. aureus & LACTACEL 75	7.4×10^3 (5.90)	9.4×10^3 (4.92)	32.1	0.10

( )[a] = pH value.
[b]Count increase = Log (Final count ÷ Initial count).
[c]Not Applicable.

Table 6. Effect of LACTACEL 75 on growth of S. aureus in Pepperoni fermented at 35°C.

Treatment	Fermentation Period (h): 0	10	18	Generation time (h): 0-10	10-18	Count increase[a] (log): 0-10	10-18
	Count/g						
S. aureus alone	1.1×10^4 (5.95)[b]	2.0×10^5 (5.60)	1.7×10^7 (5.62)	1.6	1.3	1.26	1.93
S. aureus & LACTACEL 75	1.0×10^4 (6.01)	4.0×10^4 (5.00)	1.5×10^5 (5.00)	5.0	4.2	0.60	0.57

[a]Count increase = Log (Final count ÷ Initial count).
( )[b] = pH value.