IDENTIFICATION OF SUBSTANCES IN MILK CULTURES OF *PSEUDOMONAS FLUORESCENS* WHICH STIMULATE LACTIC STARTER CULTURES

by

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INTRODUCTION

Economic loss resulting from slow acid development by lactic starter cultures during the manufacture of cheese and cultured dairy products is a major problem in the dairy industry. Even under normal conditions the time required for acid production necessitates the use of prolonged procedures which contribute to manufacturing costs. Possible advances in acceleration and mechanization of cheese making are limited by the time factor involved for the lactic starter cultures to produce acid. For this reason there is a keen and growing interest in factors affecting lactic starter culture activity and in finding means of stimulating acid development.

Milk has long been considered an adequate nutritional medium for lactic starter cultures. In cases where slow acid production is experienced, it is usually attributed to other than nutritional defiencies of the milk. Sanitizers, bacteriophage, antibiotics and naturally occurring inhibitors are the agents usually suspected. However, in recent years it has been shown (5, 6, 27) that other characteristics of milk are often responsible for variation in lactic starter culture activity.

It has been observed that proteolytic organisms growing in milk stimulated acid production by lactic starter cultures (15, 21, 34). It was speculated by these workers that protein degradation products were involved and that the lactic starter cultures utilized these fractions more readily than the native proteins.

The influence of such a symbiotic relationship on acid development was clearly demonstrated in a recent occurrence of defective market milk at the Kansas State University Creamery. Rapid souring and coagulation of the milk resulted from the associative growth of a proteolytic organism and a slow acid producing streptococcus. Neither type alone, when grown in milk, produced the defect. Preliminary tests showed that the proteolytic culture, when inoculated into milk with lactic starter cultures, stimulated acid production and coagulation. Subsequent study showed that cell free filtrates of milk cultures of the proteolytic organism were also stimulatory. Further studies of the organism showed it to resemble Pseudomonas fluorescens as described in Bergey's Manual of Determinative Bacteriology (11).

The research reported in this thesis was undertaken to determine the agent or agents in milk cultures of the Ps. fluorescens strain responsible for the stimulation of lactic starter cultures. Such information is needed from the standpoint of fundamental knowledge of lactic starter culture nutrition. Furthermore, it is believed that this knowledge will contribute to a practical method of decreasing the incidence of slow starters in dairy manufacturing and also in accelerating normal manufacturing procedures. Such developments would be of marked economic importance to the dairy industry.

LITERATURE REVIEW

The difficulty of obtaining uniform activity from lactic starter cultures is well established. One of the major sources of variable starter activity has been the milk used. In the following study the need for eliminating the effect of varying milk supply was felt to be of major importance.

Various attempts at utilizing the milk from a single herd, or even a single cow have failed to produce uniform results (8). The problem of using pooled samples of milk from a large number of farmers is beset by the difficulty of regulating these additional producers. Horrall et al. (26) found that by using reconstitued skim milk powder, the problem of variable milk supply could be lessened. However, it was necessary to precheck each new batch of powder to insure the absence of inhibitory substances. The phenomenon observed recently by Wright and Tramer (47), that rising fat globules swept the lactic culture organisms from the milk and concentrated them in the cream phase, thereby causing lower acidities is not a problem when skim milk us used.

Effect of Heating Milk on Culture Response

The effect of heat treatment on milk to be used as a culture medium has been studied extensively by Foster (16). He believed that the improved rate of acid development by lactic cultures in highly heated milk was due to the release of readily available nitrogenous compounds caused by the heating. The negative results

obtained in highly heated milk, using Streptococcus liquefaciens, were explained on the basis of its strong proteolytic ability. Babel (7) found that cultures differed in response to various heat treatments of milk, but that heating at 15 lb. steam pressure for 15 min. was most favorable for acid production by the cultures studied. Green and Jezeski (18, 19, 20) showed that skim milk powder which had undergone high heat treatment supported growth better than those of low heat treatment, as long as the heat treatment was not excessive. They also showed that milk passed through cycles of stimulation and inhibition as it was heated under various time and temperature combinations. Various reasons given for the observed stimulation were lowering of Eh, destruction of heat labile inhibitors, partial denaturation and hydrolysis of the proteins, and reduction of toxic sulfides. Inhibition was due to an increased concentration of sulfides from the denatured proteins.

The effect of heat and homogenization on the nitrogenous compounds of milk have been investigated by Shahani and Sommer (36). Their results showed that pasteurizing at 143° F. for 30 min. had no appreciable effect on the nitrogen distribution of milk, but nine percent of the albumin and five percent of the globulin fraction were coagulated by this treatment. Pasteurization at 155° F. for 30 min. and then homogenization at 2,000 lb. pressure decreased the globulin nitrogen and increased the alpha amino and nonprotein nitrogen fractions.

Influence of Nitrogenous Compounds on Lactic Starter Culture Development

The bulk of research dealing with the nitrogenous requirements of lactic starter cultures has been carried out with synthetic media. Because of this, it is then difficult to correlate such work with growth requirements of lactic organisms in a complex medium like milk. Even when using a synthetic medium, the nitrogen requirements for these organisms have not been conclusively shown. This is quite understandable when one realizes the variation between strains of lactic starter organisms. When hydrolyzed casein was replaced as the nitrogen source in a synthetic medium, Niven (33) found that 14 amino acids were required by Streptococcus lactis if prompt growth was to occur. Anderson and Elliker (3), using synthetic media, showed that the requirements for a completely synthetic medium varied greatly for 35 strains of S. lactis and Streptococcus cremoris tested. They also observed that peptides or peptide-like substances played an important role in the nutrition of the lactic acid bacteria as evidenced by the effect of liver fraction L, a source of peptides, on growth.

Working with an extremely sensitive variety of lactic streptococcus, Hunter (27) was able to note changes in its activity with what appeared to be seasonal changes in the milk. During a period of drought, the addition of tryptone would return the culture to its normal activity suggesting a deficiency of some nitrogenous compound(s) during that period. Investigating the

utilization of nitrogenous compounds by lactic cultures in a basal milk serum, Walker (45) found that various lactic cultures gave an increase in growth on the addition of isoelectric casein, lactalbumin and proteose-peptone to the milk serum medium. Non-protein nitrogen fractions generally were not utilized. Pasteurization and autoclaving of the basal serum and protein fractions improved the growth of the lactic organisms.

Protein Hydrolysis by Lactic Starter Cultures

Slow acid production by lactic starter cultures in milk may be attributed to a deficiency in ability to break down the available nitrogen sources (2, 4, 24, 38). From the literature that is available on the stimulatory effect of various added nitrogenous compounds, this would seem the case. There is considerable evidence available that some lactic organisms do have sufficient proteolytic ability to supply their nutritional needs and these are capable of rapid coagulation of milk. Using cultures of S. lactis, Anderegg and Hammer (2) showed a definite increase of soluble nitrogen in milk after growth of many of the cultures tested. The addition of peptone retarded protein decomposition rather than accelerated it. They noted that the more proteolytic cultures coagulated milk faster than the slower non-proteolytic cultures. Harriman and Hammer (24) also showed that cultures of S. lactis which were proteolytic, coagulated milk more rapidly than those cultures which were non-proteolytic.

Using microbiological assay methods, Morgan and Nelson (32) noted increases in free amino acids after milk samples were incubated with cultures of S. lactis. They felt that slow lactic cultures of limited proteolytic ability may derive their amino acid requirements during early growth from the proteose-peptone fraction. Van Der Zant and Nelson (44) studied the increase of soluble nitrogen, tyrosine and tryptophane in milk cultures of S. lactis. They found a rapid increase during the first 24 hr., then a gradual increase up to 90 hr. when the pH was maintained at 6.0 - 7.5. Studying the changes in soluble nitrogen of pure and mixed cultures of S. lactis and certain coliform organisms, Collins and Nelson (14) found the greatest increase in soluble nitrogen during the early growth period of S. lactis in milk. A decrease in soluble nitrogen was noted during the period of maximum growth of Escherichia coli and Aerobacter aerogenes. Results during the first seven days seemed to indicate that in mixed cultures the coliform organisms utilized the soluble nitrogen products formed by S. lactis during their early stages of growth.

Van Der Zant and Nelson (41, 42, 43) studied the endocellular peptidase and protease action of S. lactis. They found that whole casein stimulated proteolytic activity with an optimum activity at pH 7. The peptidases were not affected by the presence of whole protein (casein) and had an optimum activity at pH values ranging from 7 - 8.5.

Effects of the Addition of Nitrogenous Compounds to Milk on Starter Culture Activity

The addition of nitrogenous compounds to media used for bacterial propagation took on even greater significance when the early work of Woolley (46) and Sprince and Woolley (40) showed the nature, occurrence and importance of peptides as growth factors for certain organisms. Since that time there has been considerable work done with the addition of peptide sources to various media. Anderson and Elliker (4) noted that the stimulatory factors were greater in peptonized milk, trypsinized milk and liver fraction L than in acid hydrolyzed casein. The stimulation that was obtained from hydrolyzed casein was attributed to the free amino acids only. A high percentage of these digested products were toxic to the organism studied, if added in excess. Slow acid producing strains of lactic organisms were observed to respond better to stimulation than faster cultures. Once a strain was slow, continued transfer with stimulatory substances failed to maintain activity, when the stimulatory material was removed. Kizer et al. (29) found that the stimulation obtained from trypsinized lactalbumin was due to the impurities accompanying the enzyme and not to the lactalbumin portion.

Sandine et al. (35), while working with pancreas tissue extract, found two stimulants for Lactobacillus casei and S. lactis which were active in the presence of strepogenin. Subsequent hydrolysis of one of the stimulants showed it to contain 10 or 11 amino acids. Garvie and Mabbitt (17), using amino acids,

peptones, various enzymes and hydrolyzates, observed increased acid production by starter cultures when these substances were added to milk. The rate and amount of acid produced by lactic cultures could be increased by the addition of yeast extract as reported by Braz and Allan (10). Kennedy and Speck (28) showed that the addition of one percent corn steep liquor to a milk medium resulted in the stimulation of various lactic cultures. Using fresh whole milks, Anderson et al. (5) showed that in most cases, as the amount of peptides present in the milk increased, acid production also increased, and that the addition of 0.1 to 1 percent trypsin hydrolyzed skim milk to Jersey and Holstein milk caused a marked increase in the initial growth rate of the cultures tested. Speck and Williamson (39) observed that the addition of stimulatory peptides to milk precluded the necessity of cultures to hydrolyze milk proteins before initiating growth.

It was suggested by Krehl and Fruton (30) that particular combinations of amino acids in peptides could be metabolized without prior enzymatic hydrolysis, and that the structure of a peptide is what affects its growth promoting properties. Hansen (23) observed that cellular extracts of S. cremoris and S. lactis stimulated the development of L. casei and Betacoccus cremoris. He noted that the activity of the extracts varied considerably and that their nitrogen content was only an approximate index of the potency of the extracts.

Presence of Amino Acids and Peptides in Milk

The presence of free amino acids and peptides in milk would enable lactic cultures to initiate prompt growth without first needing to hydrolyze the protein present. Block (9) investigated the protein-free portion of milk. He found at least eight free amino acids as well as several peptides. His study included fresh milk, dry skim milk, evaporated milk and cheddar cheese. Hetzl (25), studying the free amino acids in fresh skim milk, found considerable amounts of the following: leucine/isoleucine, methionine/valine, threonine, proline, alanine, serine, glycine, glutamic acid, aspartic acid, histidine, arginine, lysine and cystine. He also noted that there was a decrease in the amount of amino acids in the milk after sterilization as compared to a slight decrease between raw and the same milk pasteurized.

Growth of Lactic Starter Cultures in Conjunction with Proteolytic Organisms

The presence of proteolytic organisms and their effect on the growth of lactic cultures, when grown together, is not well understood. Marshall (31) in 1920, found a definite relationship between the numbers of Bacillus subtilis present in milk and the observed stimulatory effect on Streptococcus lacticus. No explanation was given for this observation. Allen (1), from observations on the ripening of cheese, attributes fast lactic culture development in cheese made from "dirty" milk to the presence of a greater number of proteolytic bacteria than is in "clean" milk

cheese. Rice (34) inoculated B. subtilis cultures concurrently with lactic cultures into milk and observed stimulated acid production. He attributed this stimulation to the release of a more readily available nitrogen source for the lactic organisms than is normally present in milk. Cox and Whitehead (15), using four pure strains of Bacterium coli, B. subtilis, Bacterium faecalis alkaligenes and a staphylococcus, obtained greater acid production from S. lactis under varying conditions, if grown in association with these organisms. Hall (21) found that the addition of B. coli cultures to various strains of lactic cultures increased acid production and Seitz filtered extracts of the B. coli culture were also stimulatory. If the extract was added to milk prior to the lactic culture, the longer the pre-incubation period before starter addition, the greater the stimulatory effect. Hall, therefore, suggested that this stimulation was enzymatic in nature.

Commercial Application of Lactic Culture Stimulation

The feasibility of stimulating lactic cultures in a commercial manufacturing process would seem justifiable if the material caused no defect in the finished product, was economical, added in some way to the overall value of the product, and resulted in a saving in time and labor. Speck and Ledford (37) reported their findings on the commercial possibility of starter culture stimulants using a pancreas extract as the stimulating agent. They observed some technical difficulties, but there was a saving

in manufacturing time. On the use of starter culture stimulants, Hammer and Babel (22) state:

> The addition of materials to milk to increase culture activity appears to have little practical value. The cost of the suggested growth stimulants prohibit their use in large quantities of milk.

Previous Work at this Station with Filtrates of *Pseudomonas fluorescens* Cultures

Data obtained prior to the study reported herein showed the following (12):

1. cell free filtrates of milk cultures of *Ps. fluorescens* stimulated acid production by all lactic starter cultures tested,

2. maximum stimulation was obtained from filtrates of milk cultures of *Ps. fluorescens* that had been incubated for ten days at 30° C.,

3. one percent added filtrate gave optimum stimulation for most cultures studied,

4. the stimulatory nature of the filtrate was partially destroyed by sterilization or heating for five min. in a boiling water bath, and

5. addition of the filtrate to milk, with incubation prior to adding the lactic starter culture, did not increase the stimulatory effect.

EXPERIMENTAL PROCEDURE

It was thought at the start of this investigation that numerous components of the Ps. fluorescens milk culture could be responsible for the observed stimulation of the lactic starter cultures. Preliminary observations and a review of literature indicated that nitrogenous compounds in the culture would be at least partially responsible for the stimulation of the lactic starter cultures. With the assumption that nitrogenous compounds were involved in the stimulation, work was first initiated in the separation and isolation of the nitrogenous fractions present in the Ps. fluorescens culture. The various fractions obtained were then assayed quantitatively for their stimulatory ability by adding them to lactic starter cultures and measuring acid development.

Bacteriological Methods

Media Used in Studies. Reconstituted skim milk was used as the substrate for the growth of the Ps. fluorescens culture and as the medium in the stimulation investigations. The skim milk powder used was from a single batch that was pretested to insure the absence of inhibitory substances. The milk was reconstituted to nine percent solids with distilled water and dispensed into 250 ml. Erlenmeyer flasks, or six-oz. screw top bottles in 100 ml. quantities. For studies involving the growth of the Ps. fluorescens culture in a medium, which was free of any

organic nitrogen sources, the following medium was prepared in 100 ml. quantities: 0.5 percent sodium acetate, 0.1 percent potassium phosphate (secondary), 0.1 percent magnesium sulfate, 0.1 percent ammonium sulfate, plus enough distilled water to bring to volume. After preparation, all media were sterilized at 15 lb. steam pressure for 20 min.

Preparation of Ps. fluorescens Culture. The stock culture of Ps. fluorescens was propagated in litmus milk at 21° C. with weekly transfer. When milk cultures of Ps. fluorescens were required in stimulation studies, transfers from the stock culture were inoculated into 100 ml. quantities of milk contained in Erlenmeyer flasks and incubated for ten days at 30° C. When a filtrate of the Ps. fluorescens organism from the synthetic medium of known composition was required, two transfers in synthetic medium on alternate days were made prior to the final inoculation into the medium used for the study. The flask was then incubated for ten days at 30° C.

In order to obtain cell free filtrates of the Ps. fluorescens culture, sterile Seitz filters with sterilizing filter sheets were used. Depending upon the conditions of the culture, from three to ten ml. of filtrate could be obtained at one time. The sterile filtrate was collected in small vials and stored at -5° C. until used.

A simple method that could be used for checking filtrates and various culture fractions for proteolytic ability was desired.

It was found that milk-agar plates were very satisfactory for this purpose. The milk-agar plates were prepared by mixing equal five ml. quantities of skim milk and melted four percent agar, then poured into petri dishes. Proteolysis could be observed as cleared zones around the samples placed on the milk agar.

Lactic Starter Cultures. The four lactic starter cultures used in this study to test the stimulatory effect of the Ps. fluorescens culture fractions were mixed-strain commercial cultures. They were transferred weekly in litmus milk and incubated at 21° C. until coagulation of the milk occurred (usually 16 to 18 hr.), then stored in the refrigerator. Sixteen hours prior to an experiment, a fresh transfer was made into milk that was to be used to inoculate samples. The fresh transfer was incubated for 12 hr. at 21° C., then refrigerated until used. It was observed that by using lactic starter cultures that had not coagulated, more uniform dispensing and dispersing of the starter culture throughout the milk samples was possible, and much closer agreement between duplicate samples was obtained.

Preparation of Stimulatory Factors

In preliminary investigations it was found that, in most cases, the addition of one percent filtrate of the Ps. fluorescens culture to the lactic starter cultures gave optimum stimulation. So that comparisons of the stimulatory ability of the various fractions obtained could be equally evaluated, the volumes of the

fractionated material tested were all adjusted to the same initial volume as the original culture material used for the fractionation. By this constant volume method, the addition of one ml. of any of the fractions prepared contained the same quantity of that fraction as was present in one ml. of the original culture. The procedures used in preparing the fractions are shown diagrammatically on page 18.

Fractionation of Milk Cultures of Ps. fluorescens. Before preparing the component fractions, a portion of the culture to be fractionated was removed, Seitz filtered and stored at -5° C. until used. This filtrate was used to determine the overall response of the starter culture to the addition of the stimulatory agents and to check the amount of stimulation that was recovered from the various fractions. In order to precipitate nonhydrolyzed milk proteins, enzymes and cells from the Ps. fluorescens culture, 40 ml. of the culture were mixed with 120 ml. of acetone and 40 ml. of ethanol and allowed to stand in a freezer for four hours. The culture-solvent mixture was then centrifuged for 15 min. at 2,000 r.p.m. The supernatant was decanted off and the precipitate was dried in the centrifuge bottle under vacuum in a 40° C. water bath. This method of precipitation was found to give a clear supernatant that would not form a precipitate of proteinaceous material when applied to the ion exchange column, as was observed when other methods of precipitating the culture were used. The dried precipitate was then resuspended in sterile distilled water to a volume of 40 ml. By using a resus-

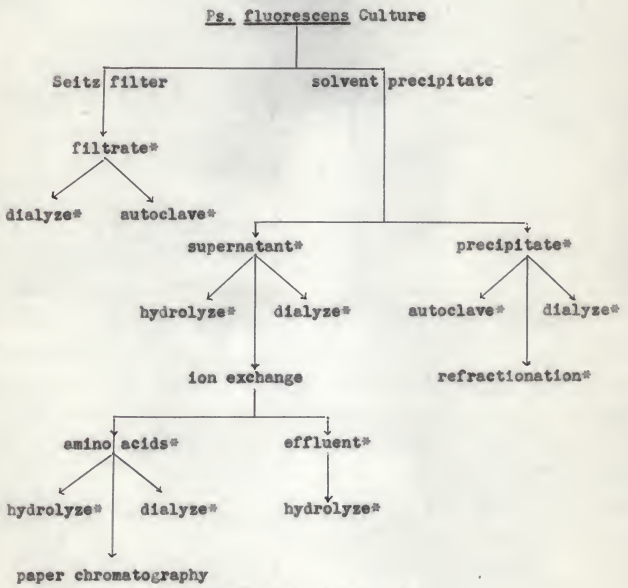
pended precipitate, the problems of quantitative recovery, time for redissolving and dispensing this material into the milk were lessened. This resuspended precipitate, when tested on milk-agar plates, showed proteolytic activity after storage at -5° C. for as long as six months. The precipitate was in most cases sterile after this treatment and was stored at -5° C. until used.

The supernatant portion of the milk culture which contained the soluble fractions, such as amino acids, peptides and carbohydrates, was reduced under vacuum to less than original volume. It was then brought back with sterile, distilled water to 40 ml. The supernatant was in most cases sterile after this treatment and was stored at -5° C. until used.

Fractionation of the Supernatant. To remove the free amino acids from the supernatant, an ion exchange column was used. The column (2 x 30 cm.) was prepared using "Amberlite" IR 120 (40 - 60 mesh) in the hydrogen cycle. The calculated quantity of ion exchange resin based on the manufacturer's specifications, plus a 15 percent excess, was weighed out and added to 4 N. hydrochloric acid and heated on a steam bath for two hours. The resin was then washed with distilled water by decantation five times and placed in the column. The resin in the column was further washed with distilled water until the effluent was pH 6.8.

Twenty ml. of supernatant was diluted with four volumes of ethanol and applied to the top of the ion exchange column. The

Plan of culture fractionation



* Tested for stimulatory ability.

rate of flow of the supernatant was adjusted through the column so that all amino acids were removed. Complete removal of the amino acids from the effluent was checked by the ninhydrin test. The supernatant was followed through the column with enough distilled water to remove the major volume of the supernatant that remained. The effluent was reduced in volume under vacuum, then adjusted to 20 ml. with sterile distilled water. The effluent was in most cases sterile and stored at -5° C. The column was further washed with distilled water to remove any remaining lactose until a negative Molisch test was obtained. The washings were discarded.

In order to elute the amino acids from the column, a two percent ammonia solution was used. The ammoniacal amino acid solution obtained from the column was reduced under vacuum to 20 ml. and stored at -5° C. The amino acid solution was in most cases sterile after this treatment.

Preparation of Hydrolyzed Samples. Portions of the supernatant, effluent and amino acid fractions were hydrolyzed to destroy any complex nitrogenous sources that might have been present. A known volume of a sample to be hydrolyzed was added to three volumes of 5 N. hydrochloric acid, then autoclaved for seven hours under 15 lb. steam pressure. After hydrolysis, the samples were repeatedly dried and wetted on a steam bath four times to remove the excess hydrochloric acid. They were then brought back to half their desired volume with sterile, distilled

water, adjusted to pH 6.5 with 0.1 N. sodium hydroxide, then brought to their original volume and stored at -5° C.

Preparation of Dialyzed Samples. When the removal of amino acids and other possible low molecular weight nutrients from the various fractions was desired, dialysis was used. Samples were dialyzed at 5° C. for three days with six changes of distilled water. Dialyzer tubing 4465-A2 from Arthur H. Thomas Company was used.

Known Amino Acid Samples. For use in stimulation studies and as chromatographic standards, solutions containing single amino acids and a composite sample were prepared using the following 20 amino acids:

glycine	DL-glutamic acid
DL-leucine	DL-tryptophane
DL-methionine	DL-isoleucine
DL-alanine	DL-valine
DL-tyrosine	DL-aspartic acid
DL-phenylalanine	L-proline
DL-histidine	L-hydroxyproline
DL-serine	L-cysteine
DL-threonine	L-cystine
DL-lysine	L-arginine

The concentration of the single amino acids was 10 mg. per ml. and the composite was prepared so that one ml. contained one mg. each of the 20 amino acids. The solutions were stored at -5° C. until used.

Assay Methods

To measure acid development of the lactic cultures, a series

of screw top bottles containing 100 ml. of sterile skim milk were prepared. Just prior to the addition of the fractions to be tested for stimulatory ability, one percent lactic starter culture was added. The materials to be tested were then added and a volume adjustment was made on the control and other samples with sterile, distilled water if needed. The added materials were then dispersed throughout the milk by inverting the bottles several times.

From each bottle, six 9 ml. samples were then pipetted into sterile, screw top tubes and the tubes were incubated at 21° C. Acid development was measured by titrating the samples directly in the tubes with 0.1 N. sodium hydroxide. Duplicate samples were titrated at 10, 12 and 14 hr. and the results reported as the average of the two titrations.

Determination of Free Amino Acids in the Ps. fluorescens Culture. Two dimensional descending paper chromatography was used to determine the amino acids present in the Ps. fluorescens culture. Whatman No. 4 chromatographic paper, 18¼ x 22¼ was used. The amino acid sample obtained from the ion exchange column was spotted 2.5" from one corner of the paper. The solvent system used for the first dimension was butanol-acetic acid-water (5-1-4 v/v). The second dimension solvent was phenol saturated with water in an atmosphere of ammonia. An aqueous solution of sodium cyanide was placed in the phenol cabinet to limit decomposition of the phenol during the separation.

Four chromatograph papers were placed in a chromatograph cabinet at one time. Three papers contained the unknown amino acid sample and one paper contained the known amino acid composite sample. The sample sizes of the unknowns were 50, 75 and 100 lambda[1] and the known composite sample size was 50 lambda. The papers were air dried between dimensions and after the second dimension until the solvent system could not be detected. The dried papers were dipped in a 0.15 percent solution of ninhydrin in acetone and allowed to develop at room temperature. Amino acids were identified by comparing the chromatograms of the unknown with that of the known standard composite sample. Tryptophane was identified by one dimensional descending paper chromatography using butanol-acetic acid-water (5-1-4 v/v) as the solvent. Samples of the unknown amino acids, known composite and standard tryptophane sample were run in parallel. The dried papers were sprayed with Erlich's reagent and the Rf value of the indole containing compounds were compared.

Investigation of the Precipitate Fraction. Attempts were made to isolate the stimulatory substance(s) from the dialyzed precipitate fraction by gradient addition of acetone or ammonium sulfate. The fractions thus obtained from the precipitate were dialyzed against distilled water for three days to remove the bulk of the precipitating agent. The samples were then tested for proteolysis, and stimulatory ability using a lactic starter culture.

1 lambda = 0.001 ml.

RESULTS AND DISCUSSION

Eight series of fractionations on milk cultures of the Ps. fluorescens organism were carried out. Each of the eight series represents one trial using a lactic starter culture as the test organism for the identification of the stimulatory fractions. Two trials with each of four lactic starter cultures were conducted over a period of four months. The data from the eight trials have been assembled to show the effect of each fraction on acid development by the lactic starter cultures.

Effect of Various Fractions on Activity of Lactic Starter Cultures

Filtrate. The cell free filtrates from the milk cultures of the Ps. fluorescens organism stimulated all lactic starter cultures tested (Table 1). The samples containing the filtrate had an average titratable acidity of 20, 30 and 36 percent greater than the control samples at 10, 12 and 14 hr., respectively. It was observed that the slowest acid producing cultures showed the greatest response to the addition of filtrate. The samples containing filtrate also showed a definite trend to earlier coagulation of the milk, but their coagulation could not be attributed to the increased acid production alone. When tested on milk agar plates, the filtrates were proteolytic. The proteolytic enzymes present in the filtrate may have accounted for the earlier coagulation that occurred in these samples.

Table 1. Effect of filtrate on acid development by lactic starter cultures

Culture	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
	Titratable acid in percent[a]					
A	0.32	0.38[b]	0.39	0.52	0.46	0.63[b]
A	0.46	0.52[b]	0.54	0.63	0.68[b]	0.83
B	0.41	0.56[b]	0.56	0.72	0.70	0.85
B	0.36	0.43[b]	0.49	0.60[b]	0.61	0.76
C	0.30	0.35	0.36	0.47[b]	0.45	0.65
C	0.37	0.39	0.43	0.60[b]	0.60	0.77
D	0.28	0.34	0.33	0.43[b]	0.37	0.61
D	0.30	0.36	0.34	0.50[b]	0.39	0.62
Av.	0.35	0.42	0.43	0.56	0.53	0.72

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Autoclaved Filtrate. As a means of testing whether enzymes were involved in the observed stimulation, the filtrates were autoclaved before addition to the milk substrate. Autoclaving the filtrates for 15 min. at 15 lb. steam pressure caused a sharp drop in their ability to stimulate acid production by the lactic starter cultures (Table 2). The only titrations that showed definite differences in acid production were those at 14 hr. where the average titratable acid of the samples containing autoclaved filtrate was 11 percent greater than the control. Coagulation of the milk occurred within the range of that normally expected when only acid coagulation is responsible. The increase in acid production due to the addition of the autoclaved filtrates might be attributed to various milk protein degradation

products produced by the Ps. fluorescens culture. The decreased stimulatory nature of the filtrates upon autoclaving as compared to the untreated filtrates would indicate that either an enzyme(s) or nutrient material(s) was modified by this treatment.

Table 2. Effect of autoclaved filtrate on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Autoclaved filtrate	Control	Autoclaved filtrate	Control	Autoclaved filtrate
	Titratable acid in percent[a]					
A	0.46	0.45	0.54	0.58	0.68[b]	0.76[b]
B	0.41	0.45	0.56	0.56	0.70	0.67
B	0.36	0.37	0.49	0.46	0.61	0.67[b]
C	0.30	0.32	0.36	0.39	0.45	0.51
C	0.37	0.34	0.43	0.45	0.60	0.66[b]
D	0.28	0.32	0.33	0.36	0.37	0.46
D	0.30	0.31	0.34	0.38	0.39	0.46
Av.	0.35	0.37	0.44	0.45	0.54	0.60

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Dialyzed Filtrate. The results on acid production of dialyzing the filtrate are shown in Table 3. Dialysis only partially removed the stimulatory effect of the filtrate, as there was an average increase of 20 percent titratable acid over the control samples at 14 hr. The dialyzed filtrate was still proteolytic when tested on milk agar plates and coagulation of the milk was noted to occur at abnormally low acidities and incubation times. The dialysis tubing used in this work had an average pore diameter

of 48 Angstrom units. Pores of this size would not only allow the passage of lactose, and free amino acids, but also large peptides. However, the removal of the low molecular weight compounds and the subsequent decrease in stimulation as compared to the nondialyzed filtrate (Table 1) would indicate that they contributed to the stimulatory ability of the filtrate. The large molecular weight compounds and enzymes that remained after dialysis, therefore, were responsible for the increased acid production from the dialyzed filtrate.

Table 3. Effect of dialyzed filtrate on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Dialyzed filtrate	Control	Dialyzed filtrate	Control	Dialyzed filtrate
	Titratable acid in percent[a]					
A	0.46	0.47[b]	0.54	0.58	0.68[b]	0.80
B	0.41	0.47	0.56	0.61[b]	0.70	0.74
B	0.36	0.35	0.49	0.47[b]	0.61	0.67
C	0.30	0.33	0.36	0.42[b]	0.45	0.58
C	0.37	0.37	0.43	0.54[b]	0.60	0.72
D	0.28	0.31	0.33	0.37	0.37	0.52[b]
D	0.30	0.32	0.34	0.42[b]	0.39	0.55
Av.	0.35	0.37	0.44	0.49	0.54	0.65

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Synthetic Medium Filtrate. Synthetic medium filtrates were not stimulatory as seen from Table 4. The filtrates, when tested on milk agar plates were not proteolytic, nor did they contribute

to early coagulation of the milk, indicating the absence of certain adaptive enzymes. It would appear then that the stimulatory nature of the Ps. fluorescens organism was dependent upon its growth in a more complex medium and not just upon its growth per se.

Table 4. Effect of a synthetic medium filtrate on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Synthetic filtrate	Control	Synthetic filtrate	Control	Synthetic filtrate
	Titratable acid in percent[a]					
A	0.40	0.40	0.49	0.50	0.62	0.62
B	0.45	0.46	0.65	0.64	0.73	0.73
C	0.36	0.36	0.44	0.44	0.58	0.57
D	0.26	0.26	0.31	0.31	0.40	0.40
Av.	0.37	0.37	0.47	0.47	0.58	0.58

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Precipitate Fraction. When an amount of the precipitate obtained from the Ps. fluorescens culture, comparable to that present in one ml. of the original culture was added to milk, stimulation was quite pronounced (Table 5). There was an average increase of 28 percent titratable acid over the control samples at 14 hr. The precipitate was proteolytic on milk agar plates and coagulation of the milk occurred at abnormally low acidities in the samples with the added precipitate. The precipitate fraction contained dead cells and enzymes as well as high molecular

weight protein compounds. It is doubtful if milk proteins enter into the stimulation since they already are present in the milk substrate. The enzymes present in the precipitate would be exoenzymes such as the proteolytic one(s) and the endoenzymes from the lysis of the cells. Preliminary work had shown that cells centrifuged from the Ps. fluorescens culture and sterilized by addition of the alcohol-acetone mixture were not stimulatory. It would therefore appear that the majority of the stimulation obtained from the Ps. fluorescens culture arose from either enzymes or complex growth products produced by the cells.

Table 5. Effect of precipitate fraction on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Precipitate	Control	Precipitate	Control	Precipitate
	Titratable acid in percent[a]					
A	0.32	0.39	0.39	0.49	0.46	0.60[b]
A	0.46	0.53[b]	0.54	0.61	0.68[b]	0.80
B	0.41	0.48	0.56	0.66[b]	0.70	0.81
B	0.36	0.41[b]	0.49	0.51	0.61	0.73
C	0.30	0.32	0.36	0.45[b]	0.45	0.57
C	0.37	0.43[b]	0.43	0.50	0.60	0.75
D	0.28	0.31	0.33	0.40	0.37	0.55[b]
D	0.30	0.36	0.34	0.42[b]	0.39	0.60
Av.	0.35	0.40	0.43	0.50	0.53	0.68

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Autoclaved Precipitate. In an attempt to determine what amount of the stimulation obtained from the precipitate fraction

was enzymatic in nature, the precipitate was autoclaved for 15 min. at 15 lb. steam pressure (Table 6).

Table 6. Effect of autoclaved precipitate fraction on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Autoclaved precipitate	Control	Autoclaved precipitate	Control	Autoclaved precipitate
	Titratable acid in percent[a]					
A	0.32	0.34	0.39	0.40	0.46	0.48
A	0.46	0.46	0.54	0.54	0.68[b]	0.69[b]
B	0.41	0.43	0.56	0.59	0.70	0.69
B	0.36	0.38	0.49	0.50	0.61	0.63
C	0.30	0.31	0.36	0.38	0.45	0.47
C	0.37	0.37	0.43	0.43	0.60	0.61
D	0.28	0.28	0.33	0.33	0.37	0.38
D	0.30	0.32	0.34	0.34	0.39	0.40
Av.	0.35	0.36	0.43	0.44	0.53	0.54

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Autoclaving the precipitate destroyed its stimulatory ability as well as the tendency for early coagulation of the milk. This would indicate that the stimulation obtained from the precipitate fraction was either enzymatic in nature or else due to a heat labile, complex compound(s) produced by the Ps. fluorescens organism. The synthetic medium filtrates, not being stimulatory, would eliminate synthesized growth products normally produced by the Ps. fluorescens organism as being the causative agents. It appeared, therefore, that the stimulation obtained from the precipitate fraction was enzymatic in nature. Presumably, the

enzyme(s) are adaptive in nature and only produced by the Ps. fluorescens culture on a more complex medium such as the milk substrate.

Dialyzed Precipitate Fraction. Table 7 shows the effect of dialyzing the precipitate on acid development.

Table 7. Effect of dialyzed precipitate fraction on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Dialyzed precipitate	Control	Dialyzed precipitate	Control	Dialyzed precipitate
	Titratable acid in percent[a]					
A	0.32	0.36	0.39	0.47	0.46	0.57[b]
A	0.46	0.50[b]	0.54	0.58	0.68[b]	0.78
B	0.41	0.44	0.56	0.61[b]	0.70	0.75
B	0.36	0.38	0.49	0.50[b]	0.61	0.67
C	0.30	0.32	0.36	0.43[b]	0.45	0.56
C	0.37	0.41[b]	0.43	0.47	0.60	0.71[b]
D	0.28	0.31	0.33	0.38	0.37	0.53[b]
D	0.30	0.34	0.34	0.38	0.39	0.53[b]
Av.	0.35	0.38	0.43	0.48	0.53	0.64

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Although still quite stimulatory, there was a decrease in acid production as compared to the nondialyzed precipitate. This reduction in stimulation after dialysis might be attributed to denaturation of enzyme(s) during dialysis as well as to removal of low molecular weight compounds that were occluded in the precipitate during its formation. There appeared to be a shift

toward a longer time required for the enzymatic coagulation of the milk with the dialyzed precipitate indicating that denaturation of the enzyme(s) did occur.

Supernatant Fraction. The results from the addition of the supernatant fraction on acid development are included in Table 8.

Table 8. Effect of supernatant fraction on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Supernatant	Control	Supernatant	Control	Supernatant
	Titratable acid in percent[a]					
A	0.32	0.37	0.39	0.48	0.46	0.58
A	0.46	0.47	0.54	0.58	0.68[b]	0.77[b]
B	0.41	0.44	0.56	0.56	0.70	0.64
B	0.36	0.36	0.49	0.41	0.61	0.66[b]
C	0.30	0.31	0.36	0.39	0.45	0.52
C	0.37	0.33	0.43	0.42	0.60	0.64[b]
D	0.28	0.30	0.33	0.35	0.37	0.43
D	0.30	0.30	0.34	0.37	0.39	0.47
Av.	0.35	0.36	0.43	0.44	0.53	0.59

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

There was an 11 percent average increase in titratable acid over the control sample at 14 hr. Early coagulation of the milk was not apparent, nor was this fraction proteolytic when tested on milk agar plates. The supernatant gave a strong positive test with ninhydrin. As can be seen from the results in Table 8, this fraction contributed a small, but noticeable, portion of the stimulation obtained from the Ps. fluorescens culture. The

increased acid production in samples containing the supernatant might be attributed to the protein degradation products which it contained.

Dialyzed Supernatant Fraction. Dialyzing the supernatant removed the stimulatory nature of this fraction. These results are summarized in Table 14. The dialyzed supernatant did not give a positive reaction with ninhydrin. This would lend support to the supposition that the stimulants contained in the supernatant were of relatively low molecular weight.

Hydrolyzed Supernatant. As summarized in Table 14, the average acidities produced by the lactic starter cultures were almost identical when hydrolyzed and nonhydrolyzed supernatants were added to the milk. This would indicate the stimulatory material in this fraction was not altered by hydrolysis and that it was most probably composed of amino acids.

Amino Acid Fraction. The amino acid fractions obtained from the ion exchange column, when added to milk, were stimulatory (Table 9). At 14 hr. there was an average increase of 13 percent titratable acid over the control. Therefore it can be assumed that the free amino acids present in the filtrates and other fractions did stimulate the lactic starter cultures. A readily available organic nitrogen source such as free amino acids would be expected to supplement those amino acids already present in the milk. This addition of amino acids would allow for more rapid synthesis of cellular material and increased metabolic

rate, with a subsequent increase in acid production.

Table 9. Effect of amino acid fraction on acid development by lactic starter cultures

Culture	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
	Control	Amino acids	Control	Amino acids	Control	Amino acids
	Titratable acid in percent[a]					
A	0.32	0.37	0.39	0.48	0.46	0.59
A	0.46	0.51	0.54	0.60	0.68[b]	0.75[b]
B	0.41	0.42	0.56	0.59	0.70	0.72
B	0.36	0.39	0.49	0.53	0.61	0.65[b]
C	0.30	0.31	0.36	0.40	0.45	0.51
C	0.37	0.39	0.43	0.46	0.60	0.64[b]
D	0.28	0.31	0.33	0.38	0.37	0.47
D	0.30	0.33	0.34	0.40	0.39	0.46
Av.	0.35	0.38	0.43	0.48	0.53	0.60

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Hydrolyzed Amino Acid Fraction. Table 14 shows that hydrolyzing the amino acid fractions had little effect on their stimulatory ability. This treatment was to insure that any larger protein fragments, that might have been held in the columns and eluted with the amino acids, did not contribute to the stimulation obtained from these fractions.

Dialyzed Amino Acid Fraction. Dialyzing the amino acid fractions completely removed their stimulatory ability (Table 14), indicating that all stimulatory substances in these fractions were of low molecular weight.

Composite Amino Acid Sample. The addition of a mixture of 20 synthetic amino acids to milk increased the acid production by 12 percent over that of the control at 14 hr. (Table 10.).

Table 10. Effect of composite amino acids on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Composite amino acids	Control	Composite amino acids	Control	Composite amino acids
	Titratable acid in percent[a]					
A	0.33	0.37	0.38	0.46	0.47	0.60
B	0.36	0.38	0.49	0.45	0.61	0.65[b]
C	0.37	0.36	0.43	0.42	0.60	0.62
D	0.30	0.32	0.34	0.35	0.39	0.44
Av.	0.32	0.36	0.41	0.42	0.52	0.58

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

The final concentration of each of the 20 amino acids added to the milk were 0.01 mg. per ml. Although the composite amino acid sample and those amino acids obtained from the Ps. fluorescens culture caused a similar response from the lactic starter cultures (and is further confirmation that the amino acids in the culture did contribute to the stimulation) the concentration and configuration of the various individual amino acids present in the two samples probably differed. These results also indicate that although large quantities of amino acids in the form of proteins are present in milk, they are not readily available to the lactic starter cultures.

Effluent Fraction. The samples containing the added effluent from the ion exchange columns and the control were identical in average percent acid at all incubation times (Table 11).

Table 11. Effect of effluent fraction on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Effluent	Control	Effluent	Control	Effluent
	Titratable acid in percent[a]					
A	0.32	0.34	0.39	0.39	0.46	0.46
A	0.46	0.44	0.54	0.53	0.68[b]	0.68[b]
B	0.41	0.41	0.56	0.56	0.70	0.67
B	0.36	0.35	0.49	0.50	0.61	0.61
C	0.30	0.31	0.36	0.37	0.45	0.46
C	0.37	0.35	0.43	0.41	0.60	0.57
D	0.28	0.28	0.33	0.33	0.37	0.37
D	0.30	0.30	0.34	0.35	0.39	0.39
Av.	0.35	0.35	0.43	0.43	0.53	0.53

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

This indicated that the stimulation obtained from the Ps. fluorescens culture was limited to the precipitate and amino acid fractions.

Hydrolyzed Effluent Fraction. The effluents were hydrolyzed to check the possibility that nutrients might be present and yet unavailable to the starter cultures. The samples containing the hydrolyzed effluents tended to develop slightly less acid than the control samples (Table 12).

Table 12. Effect of hydrolyzed effluent fraction on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Hydrolyzed effluent	Control	Hydrolyzed effluent	Control	Hydrolyzed effluent
	Titratable acid in percent[a]					
A	0.32	0.32	0.39	0.36	0.46	0.45
A	0.46	0.43	0.54	0.51	0.68[b]	0.68[b]
B	0.41	0.41	0.56	0.55	0.70	0.68
B	0.36	0.35	0.49	0.50	0.61	0.61
C	0.30	0.30	0.36	0.35	0.45	0.44
C	0.37	0.33	0.43	0.42	0.60	0.59
D	0.28	0.29	0.33	0.33	0.37	0.37
D	0.30	0.30	0.34	0.34	0.39	0.37
Av.	0.35	0.34	0.43	0.42	0.53	0.52

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

This would indicate that the effluents contained no stimulatory material made available by hydrolysis.

Recombined Precipitate, Amino Acids and Effluent Fractions. Table 13 shows the effect when the three main fractions were recombined in the amounts originally present in one ml. of the milk cultures of the Ps. fluorescens organism and tested for stimulation. There was an 84 percent average recovery of stimulation at 14 hr. as compared to the filtrate samples (Table 1). The difference in the average acidities produced by the recombined materials and by the untreated filtrates amounted to 0.03 percentage points in acid at 14 hr. and may have been due to experimental techniques.

Table 13. Effect of precipitate, amino acid and effluent fractions on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Precipitate amino acid effluent	Control	Precipitate amino acid effluent	Control	Precipitate amino acid effluent
	Titratable acid in percent[a]					
A	0.32	0.39	0.39	0.52	0.46	0.65[b]
A	0.46	0.54[b]	0.51	0.64	0.68[b]	0.81
B	0.41	0.47	0.56	0.61[b]	0.70	0.75
B	0.36	0.43[b]	0.49	0.55	0.61	0.75
C	0.30	0.34	0.36	0.47[b]	0.45	0.59
C	0.37	0.45[b]	0.43	0.52	0.60	0.77
D	0.28	0.32	0.33	0.41	0.37	0.58[b]
D	0.30	0.37	0.34	0.43[b]	0.39	0.63
Av.	0.35	0.41	0.43	0.52	0.53	0.69

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

Summarized Effects of Various Fractions. Table 14 was prepared to show the relationship between the fractions and treated fractions of the Ps. fluorescens culture on acid development by the lactic starter cultures. It can be seen that only those samples that contained free amino acids and/or the active enzyme portion were stimulatory. When the average stimulation derived from the amino acid and precipitate fractions was used in calculating recovery at 14 hr., the stimulation using these two fractions in the calculation yields an average recovery of 116 percent of that obtained from the filtrates alone. No adequate explanation for this apparent increase in acid production, when the two stimulatory fractions were added to milk separately, suggests itself.

Table 14. Effect of various fractions of the Ps. fluorescens culture on acid development by lactic starter cultures

Fraction[a]	Incubation at 21° C.		
	10 hr.	12 hr.	14 hr.
	Average titratable acid in percent[b]		
Control	0.35	0.43	0.53
Filtrate	0.42	0.56	0.72
Autoclaved filtrate	0.37	0.45	0.60
Dialyzed filtrate	0.37	0.49	0.65
Precipitate fraction	0.40	0.50	0.68
Autoclaved precipitate	0.36	0.44	0.54
Dialyzed precipitate	0.38	0.48	0.64
Supernatant fraction	0.36	0.44	0.59
Hydrolyzed supernatant	0.37	0.44	0.59
Dialyzed supernatant	0.34	0.40	0.51
Amino acid fraction	0.38	0.48	0.60
Hydrolyzed amino acids	0.38	0.47	0.58
Dialyzed amino acids	0.35	0.43	0.52
Effluent fraction	0.35	0.43	0.53
Hydrolyzed effluent	0.34	0.42	0.52
Precipitate, amino acids, effluent	0.41	0.52	0.69

a The samples contained the equivalent amount of material present in one ml. of the Ps. fluorescens milk culture.

b Averages of the results of the eight trials.

Addition of Hydrolyzed Wheat Bran. As a result of observations made on the effect of various stimulatory agents, the

possibility of utilizing a cheap, commonly available , natural product as a stimulatory agent was investigated. Hydrolyzed wheat bran was tested for its stimulatory ability (Table 15).

Table 15. Effect of hydrolyzed wheat bran on acid development by lactic starter cultures

	Incubation at 21° C.					
	10 hr.		12 hr.		14 hr.	
Culture	Control	Hydrolyzed wheat bran	Control	Hydrolyzed wheat bran	Control	Hydrolyzed wheat bran
	Titratable acid in percent[a]					
A	0.46	0.54	0.54	0.63	0.68[b]	0.83[b]
B	0.36	0.46	0.49	0.53	0.61	0.77[b]
C	0.37	0.46	0.43	0.52	0.60	0.76[b]
D	0.30	0.38	0.34	0.43	0.39	0.59
Av.	0.37	0.46	0.45	0.53	0.57	0.74

a Results are the average of duplicate samples.
b Indicates when coagulation was first observed.

There was a 30 percent average increase in titratable acid over the control sample at 14 hr. It is possible that products other than free amino acids could be responsible for the observed stimulation but amino acids, undoubtedly, were involved as the amount of hydrolyzed bran added resulted in an increase of 0.07 mg. of nitrogen per ml. of the milk. This increase was at least partially due to alpha amino nitrogen.

Analysis for Amino Acids in the *Pseudomonas fluorescens* Culture

By the paper chromatographic procedure previously described, the following 17 amino acids were identified as being present in

the amino acid fraction obtained from the Ps. fluorescens culture: aspartic, glutamic, serine, glycine, threonine, alanine, tyrosine, valine, phenylalanine, leucine/isoleucine, proline, histidine, lysine, arginine, methionine and tryptophane. An unknown substance was present on the developed chromatograms which could not be positively identified. It had an Rf. value similar to that reported in the literature for cysteine but it failed to give a positive nitroprusside test. The known samples of cystine and cysteine used as comparisons in chromatographic studies both produced a similar spot as well as various others when tested under the same condition.

Investigation of Precipitate Fraction

Various attempts at isolating the stimulatory substance(s) from the precipitate fraction were not successful. Gradient additions of both ammonium sulfate or acetone, precipitated fractions which were all proteolytic on milk agar and were stimulatory for lactic starter cultures when tested in milk. Since separation of the stimulatory substance(s) was not possible under the condition used, no conclusions could be drawn to its exact nature.

The results of this study indicate that the stimulation obtained from the Ps. fluorescens culture arose from the free amino acids and the precipitate fractions.

The observation concerning the effect of amino acids agrees with the results of numerous other workers (13, 17) who observed that the addition of a mixture of amino acids, whether synthetic or obtained from natural sources, to media of various complexities resulted in accelerated growth of certain microorganisms. No single amino acid seemed to be responsible for this phenomenon (4, 13, 17). A complex mixture of amino acids is required before measurable differences are apparent. It is also quite obvious that the ability to stimulate microorganisms is not a particular property of one substance alone but is inherent to many, if tested under the proper conditions.

The nature of the stimulation obtained from the precipitate portion of the Ps. fluorescens culture is still obscure. The stimulatory factor does not appear to act by liberating from the substrate specific substances required for growth as do many of the other reported cases of enzymatic stimulation (1, 17, 21, 34). This would be indicated by the failure of prior additions of the filtrate to the milk substrate to show a greater response over those samples that had filtrate and culture added concurrently. That is assuming that the stimulation obtained from the precipitate fraction is enzymatic in nature. The fact that autoclaving the precipitate so completely destroys its stimulatory nature would indicate that an enzyme(s) is involved. Peptides, vitamins, nucleic acids and other growth stimulants would not have their properties so drastically changed that they would become completely inactive upon autoclaving. Any assumption regarding the mode of

action between enzyme and lactic culture would be speculative at this time.

The early coagulation of the milk, when the precipitate portion or filtrate is present in milk, is undoubtedly due to a proteolytic enzyme(s) which is present. An interesting observation was made during the course of this study pertaining to coagulation of milk samples with added filtrate. When the cell free filtrates were added to uninoculated, autoclaved milk (to observe coagulation), the samples were slowly proteolyzed without coagulation. On the other hand, in pasteurized milk, with an added preservative, the samples generally coagulated. This observation might indicate a need for re-evaluation of the present methods used for determining those organisms that are able to coagulate milk.

The possible commercial utilization of starter culture stimulants appears to warrant further investigation. For as in the case of the Ps. fluorescens culture, its use would offer the added advantage of it's rennen-like action in the cheese making process. The physical and chemical changes occurring during cheese making, utilizing such enzymatic stimulants, would need to be investigated further before their true merit could be evaluated.

SUMMARY AND CONCLUSIONS

Investigations were conducted to identify the agent(s) present in milk cultures of Ps. fluorescens that stimulated acid production by lactic starter cultures.

Sterile, skim milk was inoculated with a variety of Ps. fluorescens and incubated for 10 days at 30° C. The proteolyzed milk culture was fractionated by first precipitating the protein-enzyme material with an alcohol-acetone mixture. Further separation of a stimulatory fraction was obtained from the supernatant by use of ion exchange. Sterilization, dialysis, and hydrolysis of the fractions was also employed to aid in the identification of the stimulatory substances present in the fractions. Two dimensional paper chromatography was used for the identification of the free amino acids present in the proteolyzed culture. Eight series of fractionations on milk cultures of the Ps. fluorescens organism were carried out. The fractions obtained were added to lactic starter cultures in a milk substrate. The starter cultures with the added fractions were incubated for 10, 12 and 14 hr. at 21° C. and the increase in titratable acidity over the control samples was measured.

The results of this study indicated that the free amino acids present in the Ps. fluorescens culture were partially responsible for the stimulation of the lactic starter cultures in a milk substrate. The addition of the amino acids recovered from the Ps. fluorescens culture to the milk substrate elicited

a 13 percent increase in titratable acidity over that of the control after 14 hr. when incubated at 21° C. This effect was supported by the fact that a similar response using a mixture of 20 synthetic amino acids was obtained. The identification of 17 amino acids employing two dimensional paper chromatography confirmed their presence in the Ps. fluorescens culture.

The solvent precipitated fraction was found to be responsible for the major portion of the stimulation obtained from the Ps. fluorescens culture. The addition of the precipitate fraction to milk resulted in an increase in titratable acidity 28 percent greater than that of the control sample at 14 hr. incubation. The precipitate fraction exhibited strong enzymatic characteristics. The active agent(s) in the precipitate was nondialyzable and autoclaving destroyed its ability to stimulate lactic starter cultures.

A proteolytic enzyme(s) is believed responsible for the early coagulation of the milk substrate when either a cell free filtrate or the precipitate fraction from the Ps. fluorescens culture was used as the stimulatory factor. The proteolytic enzyme(s), although involved in coagulation, did not appear to be involved in stimulation of the lactic starter cultures. The characterization of the enzyme(s) involved in the stimulation obtained from the precipitate fraction might aid in understanding many of the symbiotic relationships existing between lactic starter cultures and other organisms.

It would appear from the results of this work that stimulation of lactic starter cultures by the addition of bacterial growth products in commercial cheese production has merit. The many products which are capable of stimulating lactic starter cultures offer a wide area for further research to determine those most suitable for this purpose.

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IDENTIFICATION OF SUBSTANCES IN MILK CULTURES OF *PSEUDOMONAS FLUORESCENS* WHICH STIMULATE LACTIC STARTER CULTURES

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The purpose of this investigation was to identify the agent(s) in skim milk cultures of a variety of Pseudomonas fluorescens which stimulated acid production in lactic starter cultures.

Previous work had established that a symbiotic relationship existed between the Ps. fluorescens organism and the lactic cultures. It was also observed that cell-free filtrates from 10 day milk cultures of the Ps. fluorescens organism stimulated the lactic starter cultures to greater acid production.

To isolate the stimulatory factor(s), eight series of fractionations were made on cultures of the Ps. fluorescens organism. Solvent precipitation and ion exchange, as well as autoclaving, dialysis, hydrolysis, and paper chromatography were employed to separate and characterize the various stimulatory fractions. The fractions obtained were tested for their stimulatory effect on commercial lactic starter cultures in a milk substrate. The milk cultures of the lactic organisms with the added fractions were incubated for 10, 12 and 14 hr. at 21° C. and the increases in titratable acidity over the control samples were measured. Of the various fractions obtained and tested, only two were found to be stimulatory; the solvent precipitated fraction and the amino acid fraction.

The addition of the precipitate fraction to the lactic starter cultures resulted in a 28 percent average increase in titratable acid over that of the control samples at 14 hr. incubation. Attempts to further isolate and concentrate the active

factor(s) involved were unsuccessful. The exact mode of action involved in the stimulation obtained from the precipitate fraction is not known. The active portion was nondialyzable and was destroyed by autoclaving. It appeared to have enzymatic characteristics.

The amino acid fraction elicited a 13 percent average increase in titratable acidity over that of the control samples at 14 hr. incubation. A similar response was obtained employing a mixture of twenty synthetic amino acids. Using two dimensional paper chromatography, seventeen free amino acids were found to be present in the amino acid fraction obtained from the Ps. fluorescens culture.

In all cases where the precipitate fraction or cell-free filtrate from the Ps. fluorescens cultures were added to lactic starter cultures, coagulation occurred at low acidities and short incubation times. This phenomenon might be attributed to the presence of a proteolytic enzyme(s), since the precipitate fraction and cell-free filtrate both exhibited a strong proteolytic action when tested on milk agar plates.

The results of this investigation indicate that further study into the possible commercial utilization of starter culture stimulants of microbiol origin is warranted.