PRODUCTION AND PRESERVATION OF CONCENTRATES OF *LACTOBACILLUS ACIDOPHILUS* FOR THERAPEUTIC USE

by

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PRODUCTION AND PRESERVATION OF CONCENTRATES OF *LACTOBACILLUS ACIDOPHILUS* FOR THERAPEUTIC USE

INTRODUCTION

The use of acidophilus milk to aid in the restoration of a normal balance of intestinal flora has been practiced for many years. It has been found to be helpful in treating many cases of constipation, gastritis, diarrhea and other gastro-intestinal complaints. Its use by pediatricians is common, as is its use in Geriatrics. The most recent need for acidophilus milk is the result of prolonged antibiotic tyerapy, which has been known to cause an intestinal flora imbalance, by at least partial destruction of the normal flora. Antibiotic resistant forms of micrococci and Candida, which are gas forming and/or proteolytic, seem to become predominant. *Lactobacillus acidophilus* has been successfully implanted in the intestine, and presumably by producing an acid environment, inhibits the tryptic and other undesirable actions of the resistant forms. Normal flora can then be reintroduced along with commonly ingested materials to restore the normal flora balance. Acidophilus milk has been used successfully to relieve anorectal complications and severe proctitis or colitis which have resulted from antibiotic therapy.

Lactobacillus acidophilus has been administered in the form of fermented milk, usually a pint to a quart per day. However, many persons find the acid flavour unpalatable, especially in this quantity. In addition, production and maintenance of the product entails more time and effort than is desirable. Therefore it has been suggested that high concentrates of Lactobacillus acidophilus equivalent to the daily dose be made, and stored in a highly viable frozen state until needed. This concentrate could then be taken alone, or preferably, added to a quantity of fresh whole milk, yielding a palatable product.

This problem involved a study of nutrition of strains of the organism, development of a suitable growth medium and studies of optimum freezing preservation conditions.

HISTORICAL

Basis for Acidophilus Therapy. Intestinal flora and acidophilus therapy are reviewed by Rettger and Cheplin (48, 135p.), Kopeloff (28, 211p.), Rettger et al. (49, 203p.) and Frost et al. (12, pp.862-866).

The antecedants of acidophilus therapy go back to earliest times when man used the soured milk of many animals. Perhaps the best known preparation for intestinal treatment was the yoghurt of the Balkan Peninsula. Eli Metchnikoff (28, p.1) believed that harmful bacteria and their toxins produced in the large intestine, accounted for many human ills. He concluded that a favorable intestinal flora was necessary for health. The longevity of the Balkan peasants was believed to be the result of the widespread use of their soured milk product, which may have influenced the intestinal flora. The organism Metchnikoff considered important in this product was Lactobacillus bulgaricus. Subsequently bulgaricus therapy received considerable study, but the results obtained were largely contradictory. Kopeloff (28, p.10) terms the conclusions drawn from these studies as belonging "more to the category of speculation than to that of scientific verity". Many of the claims at establishing L. bulgaricus in the intestine may have been due to difficulty in distinguishing L. bulgaricus from

L. acidophilus. The latter organism could have been stimulated by the increased lactose intake in bulgaricus milk, as will be seen. L. bulgaricus cannot be established in the intestine (49, p.5).

It has been shown that diet can affect the nature of intestinal flora (48, pp.65-110). High protein diets result in a predominately proteolytic flora, while high carbohydrate diets stimulate an aciduric flora to predominate. Lactose and dextrin have been shown to favor the establishment of Lactobacillus acidophilus, possibly because they are more slowly absorbed and hence persist for a longer time, thereby being carried further down in the intestine (49, p.4).

It has been shown by sound experimentation (28, p.181) that Lactobacillus acidophilus can be easily established in, and recovered from, the intestinal tract. Ingestion of a large number of viable Lactobacillus acidophilus supplemented by suitable carbohydrates has been most successful in inducing a transformation of the intestinal flora from a proteolytic to an aciduric type. A pint to a quart of acidophilus milk containing at least 10 to 20 x 10^7 viable organisms per ml. has been recommended as essential. Direct clinical observations have shown the practical importance of L. acidophilus and its therapeutic possibilities in relieving cases of

constipation, diarrhea and other intestinal disorders. Further, it has been shown that the beneficial effects of acidophilus milk therapy is a bacteriological one, rather than chemical, since only the use of the organism plus suitable carbohydrate will produce the desired results (28, p.116).

Nutrition of Lactobacillus acidophilus. Lactobacilli include some of the most fastidious bacteria known. It is obvious from Snell's review of the nutrition of lactic acid bacteria (57, pp.235-241) that all of the water soluble vitamins are required by one or more of the lactobacilli. Amino acid, purine and pyrimidine requirements are also complex. Peptides, oleic acid or other unsaturated fatty acids or sterols are required by, or stimulate, certain lactobacilli. Ions required in large amounts include potassium, manganese, phosphate and often magnesium. Lactobacillus acidophilus has been reported to require pantotheine or pantothine though growth may occur in the presence of large amounts of pantothenic acid. Pyridoxamine phosphate may be required but may be replaced by large amounts of pyridoxal or pyridoxamine. These latter may not be required in media containing D-alanine. Though certain lactobacilli requirements for oleic or other unsaturated fatty acids may be replaced

by biotin, Lactobacillus acidophilus has been reported to require the fatty acid not replaceable by biotin.

Kitay, McNutt and Snell (26, pp.727-738) reported the effect of desoxyribosides and vitamin B_{12} as growth factors for lactic acid bacteria including ten strains of Lactobacillus acidophilus. Nine of the ten strains grew only when thymidine was added to the basal medium. Some of the "thymidine requiring" strains could use other desoxyribosides equally well. Other strains showed a distinct preference for one or another of the desoxyribosides. Vitamin B_{12} replaced desoxyribosides for some of these strains. One strain could not use vitamin B_{12} in place of desoxyribosides. Reducing agents replaced thymidine, or other desoxyribosides, or B_{12} for some strains--some in the presence of casein digest, some in its absence. For one strain, ascorbic acid permitted more rapid and heavier growth than that induced by thymidine. Some strains required more vitamin B_{12} than others, though the level of desoxyriboside requirements are similar.

Kitay and Snell (27, pp.49-56) reported additional nutritional requirements of ten strains of Lactobacillus acidophilus. All of the strains required oleic acid and this requirement was not replaceable by an excess of biotin. Nine of the ten strains tested required

thymidine, other desoxyriboside or vitamin B_{12}. One strain, of nine tested, required pyridoxamine phosphate. Under appropriate conditions (not described), the addition of D-alanine eliminated the requirement for pyridoxamine phosphate. Six of nine strains tested required the Lactobacillus bulgaricus factor (LBF). (pantothine). Very high levels of calcium pantothenate (30-50 micrograms per ml.) could replace the LBF requirement for many organisms. An enzymatic casein digest was not required by any of the ten strains but all were stimulated by its addition to the complete medium. Yeast extract, liver extract (reticulogen) and L-glutamine all stimulated growth in a basal medium using Henderson and Snell amino acid mixture and the known growth requirements. Lactose accelerated the growth of several cultures though its effect on Lactobacillus acidophilus strains was not mentioned.

Rogosa et al. (50, p.689) in studies of Lactobacillus acidophilus of oral origin reported that most strains require niacin, pantothenate, riboflavin, folic acid and vitamin B_{12}. The latter is replaceable by desoxyribosides. Vitamin B_6 was reported as being stimulatory.

Skeggs et al. (51, p.734) using ATCC 4963 (originally called Lactobacillus bifidus, now labelled as Lactobacillus acidophilus) studied differences in

nutritional requirements in the presence of either vitamin B_{12} or desoxyribonucleic acid (DNA). Para-aminobenzoic acid pyridoxal or pyridoxine, biotin (in the presence of oleic acid), riboflavin and thiamin were not required in either medium under the conditions used. This strain required arginine, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophan tyrosine and valine in both media. Aspartic acid and DL-alanine were stimulatory in both media. Glycine stimulated in the presence of vitamin B_{12} but was not required in the DNA medium. Threonine stimulated in the DNA containing medium but was not required in the vitamin B_{12} medium. DL-norleucine was reported as not being required.

Magnesium, potassium and phosphate were shown to be required while manganese was not required and may have even been slightly toxic. Iron salts in the ferric or ferrous state were required in the B_{12} medium but were only stimulatory in the DNA containing medium. The DNA may have contained traces of iron.

In the presence of DNA, the organism did not require purine bases. If thymidine was substituted for DNA, Lactobacillus bifidus was unable to utilize xanthine, which supported growth in the presence of vitamin B_{12}.

This strain could use glucose, galactose, levulose,

lactose and maltose, but not arabinose, xylose, sucrose, melezitose, inulin, salicin or inositol. A fatty acid requirement could not be replaced by biotin. Acetate stimulated growth of this organism.

Koser and Thomas (29, pp.287-298) determined the amino acid requirements of four oral strains of Lactobacillus acidophilus and found it to be the most exacting of oral lactobacilli studied, requiring or being stimulated by, nearly all the 18 amino acids. Though they did not obtain maximum growth in their completely synthetic medium, they determined that arginine, cystine, glutamic acid, histidine, leucine, lysine, phenylalanine, proline, tryptophan, tyrosine, and valine were essential for any growth at all. Alanine and isoleucine were stimulatory. Methionine and serine were required by some and not by others. Glycine stimulated two strains and had no effect on the other two.

Lactobacillus acidophilus (Moro) carbohydrate fermentation is described in Bergey (5, p.352) as follows: all strains ferment glucose, fructose, galactose, mannose, maltose, lactose, and sucrose; raffinose and trehalose are fermented by some cultures, while some have a slight action on dextrin, Xylose, arabinose, rhamnose, glycerol, mannitol, sorbitol, dulcitol, inositol are not fermented.

Rogosa et al. (50, p.686) describes L. acidophilus

as using fructose, maltose, sucrose, salicin, trehalose, and cellobiose. Melibiose and raffinose are fermented slowly or not at all. Inulin is fermented by about 1/2 of the strains.

Skeggs et al. (51, p.736) reported that L. acidophilus ATCC 4963 utilizes glucose, galactose, fructose, lactose, and maltose, but not sucrose nor inulin.

Wheater (63, pp.123-132) characterized Lactobacillus acidophilus, L. bulgaricus and intermediate strains by physiological tests, including carbohydrate fermentations. Lactobacillus acidophilus (29 stains) fermented glucose, galactose, lactose, fructose, mannose, amygdalin, cellobiose, salicin, and sucrose. Most strains fermented dextrin, maltose, trehalose, melibiose, and raffinose, but did not utilize glycogen. Adonitol, aesculin, arabinose, dulcitol, erythritol, glycerol, inositol, inulin, mannitol, melezitose, rhamnose, sorbitol, sorbose, starch or xylose were not fermented. Intermediate strains did not ferment trehalose, amygdalin, cellobiose, salicin, sucrose, melibiose, or raffinose.

Storage Preservation. Storage viability experiments on L. acidophilus in acidophilus milk are reviewed by Rettger et al. (49, pp.26-30). Initial acidity and storage temperature were suggested as the factors affecting viability. Most workers agreed that 16°C. was the

best storage temperature for the commercial product, though acidophilus milk of not more than 0.65% titratable acidity could be stored at 5°C. without serious loss of viability. Myers (43, pp.867-872) stored resuspended L. acidophilus cells in sterile sweet milk at 2° to 5°C. He found a "very low death rate".

Lactobacilli have been preserved by freezing or freeze drying and as compressed tablets kept under refrigeration. Harrison et al. (18, p.20) reported the survival of bacteria at various temperatures and as cultures in broth, packed cells, or as concentrated cell suspensions. Some bacteria (including Lactobacillus fermenti) survived better at temperatures below zero, while others were sensitive to freezing temperatures. Some survived better as packed cells, and for others (including Lactobacillus bifidus) storage in broth was better. Lactobacillus acidophilus survived well when frozen in broth at -22°C. (35, pp.244-249), while there was practically no death at all from freeze-thawing at -78°C. (dry ice) (16). Acidophilus milk has been frozen as a flavored sherbet at -17° to -25°C. for 7 days with "adequate survival" (46, pp.413-419).

Briggs, et al. (7, pp.503-507) preserved Lactobacilli by freeze-drying. Viable counts were used to study factors influencing survival rate, the best results

being obtained in horse serum with 8% added glucose, as the menstruum. The majority of survival rates were over 50% immediately after drying, and over 25% after 6 months storage. Some of the dried cultures were tested again after storage period of about 2 years and showed "satisfactory" survivals.

McLaughlin and Brewer (40, p.30) briefly mentioned a technique for compressing lactobacilli into tablets and storing under refrigeration (5°C.) "without appreciable loss in viability". However, many workers have shown that tablets are unsatisfactory for therapeutic uses (46, p.413).

Frozen storage of lactobacilli in a suitable menstruum would appear to be the simplest and most effective method of preservation.

Hampil (14, pp.192-196), Weiser and Osterud (62, pp. 415-418), Luyet and Gehenio (32, pp.19-27) reviewed the literature on death of bacteria at low temperatures. The research related to the mechanism of injury on freezing is of the most value here.

Effects of Freezing and of Storage Temperatures. Prudden (47, pp.341-350) was the first to distinguish between "immediate" death and "storage" death occurring in frozen bacterial suspensions.

Smith and Swingle (53, pp.481-483) observed that the mortality at freezing temperatures from near 0°C. to -195°C. was essentially the same and that the critical point at which immediate destruction took place was somewhere near 0°C. Hilliard and Davis (22, pp.423-431) stated that there is no critical temperature below freezing where the germicidal effect is greatly accelerated. Haines (13, pp.451-463) found that freezing temperatures of -5°, -20° and -70°C. produced essentially the same mortality in a suspension of Pseudomonas aeruginosa whereas prolonged storage at various temperatures from -1° to -20°C. resulted in the greatest death at the higher temperatures, -2°C. being the most lethal. Native proteins of Pseudomonas aeruginosa were found to be denatured most rapidly at -2°C.

Turner (58, pp.61-78), using protozoa and viruses, showed storage survival with no loss of titre for 6 months at -78°C. and only slight alteration after one year. However, at -10° and -20°C. no recovery was found after 2 months. Turner and Brayton (59, pp.639-650) using a spirochete observed no storage death at -78°C. after 6 months.

Weiser and Osterud (62, p.423) presented evidence that mortality due to immediate death by freezing is marked, but does not vary with the intensity of the

freezing temperature. It was established that rate of storage death at the higher freezing temperatures is very rapid under the conditions used, and is much greater at temperatures above -30°C. than at temperatures of -30°C. and below. They fluctuated repeatedly, suspensions of Escherichia coli in phosphate buffer between -1.5° and -15°, -15° and -30°, -30° and -78°, -78° and -195°C. and between -2° and -195°C. These repeated fluctuations did not exert any lethal action additional to that of storage. Storage death was detected at -30°, -45°, -60°, -78°, but not at -195°C.

Vass (60, pp.1043-1047) found better survival at -190°C. than at -15°C. Lactobacillus bifidus survived better at dry ice temperature (-50° to -70°C.) than at higher temperatures (45). Lactobacillus acidophilus and L. fermenti survived equally well at -22°C. (35, p.248), but there was practically no death at all at -78°C. from freeze-thawing (16).

Ulrich and Halvorson (6, p.197) found Lactobacillus casei and Escherichia coli survived better at -21°C. than at -2°C., in milk. Tanner and Wallace (56, pp.32-34) found that Escherichia coli survived better at -70°C. than at -40° or -6°C. McFarlane (38, pp.481-492) found E. coli to survive better at -20° than at -10°C.

Luyet and Gehenio (32, p.253) call the range 0° to -40°C. the dangerous zone for frozen living cells and refers to -40° to -273°C. (with no lower limit) as the safe zone. Lovelock (30, pp.414-426) labelled -3° to -40°C. as the critical region in which damage occurs rapidly and found decreased mortality at lower temperatures.

Effects of Solids in the Freezing Menstruum on Survival--Protective Effects. Keith (25, pp.877-879) found that greater death of bacteria occurred in water suspensions than to cells suspended in solutions such as sugar, glycerin, milk or peptone. Hilliard and Davis (22, pp.427-431) observed protection being offered by colloidal matter (milk and cream) and by glucose. Squires and Hartsell (54, pp.40-45) obtained results indicating that the interference of ice formation, by either a material which formed a true solution with alteration of colligative properties or by a substance which had no effect on colligative properties, afforded a protective action against freezing and storage death.

Vass (60, pp.1043-1047) froze Bacillus radicola at -15° and -190°C. in the presence of increasing levels of dextrose. He stated that at the temperature above the eutectic point of the sugar, the sugar exhibited a marked

protective action, raising the survival from 2% to 98% in 0.01% to 15% glucose. He also increased the concentration of glycerin in broth from 0.01% to 15% and survival rose from 4%, to 100% in 1% glycerin, when frozen at -15°C. No growth occurred in the medium containing 10% glycerin. The concentration of sugar had no effect on survival when the temperature of freezing was -190°C., which is below the eutectic of the sugar solution, Vass stated.

McFarlane (36, pp.43-57) demonstrated redistribution of soluble solids and microbial forms in a freezing water solution, into areas of concentration. He showed that microbial cells retained their viability for longer periods of time in those sections of a frozen mass which possessed a high concentration of a favorable substance such as sucrose. He reported (38, p.491) that concentrations of 30% to 50% sucrose greatly delayed freezing destruction of Escherichia coli and that distilled water offered less protection than any sucrose concentration. Tanner and Wallace (56, pp.32-34) reported that suspensions of cells frozen in distilled water with various sugar concentrations showed a very slow decrease in viable count.

Harrison (17, p.714) and Harrison and Cerroni (19, p.579), contrary to the findings of other workers in freezing studies, report best survival of E. coli in

distilled water.

Solute Toxicity Effects. Some solutes can be toxic to bacteria when concentrated during freezing. Haines (13, pp.451-463) found that native proteins from Pseudomonas aeruginosa were flocculated at high freezing temperatures. He believed that this flocculation of the cellular proteins was due to the high salt concentration and change in pH obtained in freezing. McFarlane (36, p.56) showed that HCl was concentrated with the other non-aqueous materials, and that areas of higher hydrogen ion concentration had increased bacterial mortality. Tanner and Wallace (56, pp.32-34) reported that high concentrations of NaCl seemed to cause a more rapid decrease in surviving cells, especially in the higher concentrations used. Squires and Hartsell (54, pp.40-45) believed that uneven distribution of solutes in the intercrystallic film was involved in the freezing kill. Weiser and Hargiss (61, p.78) theorized that concentration of solutes as well as mechanical crushing can cause death on freezing. Lovelock (30, pp.414-426) showed that the critical region, -3° to -40°C. where damage occurs rapidly, coincides with that region in which the cell is exposed to concentrated salt solution. In NaCl solutions between 0.8M and 2.0M, at room temperature the cells were

extremely sensitive to temperature change, mechanical shock and change in osmotic pressure. In stronger concentrations the structural integrity of the cell was destroyed. He concluded (31, pp.28-36) that damage on freezing resulted largely from the concentration of electrolytes within the cells as the cells were dehydrated by the sudden extracellular solute concentration. He found that glycerol acts as a "salt buffer" preventing excessive intercellular increase in salt concentration on freezing, since glycerol was not effective unless it permeated into the cell.

Effects of pH of the Menstruum on Survival of Frozen Bacterial Suspensions. Although the effects of concention of solutes has been reviewed and the concentrations of hydrogen ions mentioned at various times, the effects of pH on frozen cells might also be considered as a special case.

McFarlane (36, pp.43-57) showed that on freezing a solution containing HCl, the hydrogen ions were progressively concentrated with the unfrozen solution as the water froze out as pure ice crystals. When there were microorganisms in the suspension as it was frozen, greater mortality occurred in the zones of higher concentration of hydrogen ion. Tanner and Wallace (56, pp.32-

34) found a greater mortality in frozen menstrua of low pH. Reactions on the alkaline side of neutrality were not as harmful as those on the acid side. Haines (13, pp.451-463) theorized that it was the effects of change in pH as well as a high salt concentration obtained on freezing that caused flocculation of cellular proteins of Pseudomonas aeruginosa. McFarlane (37, pp.59-68) observed that when approximately neutral sucrose suspensions of cells were frozen, the cells tended to survive in the greatest numbers in those areas of the ice mass which were highest in sucrose (due to concentration by freezing water) but that this was not true in a frozen acidified medium in which the areas of high hydrogen ion concentration coincided with areas of high sugar concentration. Using buffered sucrose suspensions of pH 3.7, 5 and 6.5, Escherichia coli survived better in the more neutral suspensions, with pH 3.7 media being the most destructive (38, pp.481-492).

Appleman and Huhn (3, p.35) found total acidity and pH of the freezing menstruum to be factors affecting the survival of frozen organisms. C. K. Johns (24, p.32) reported that some frozen mixed-strain lactic starters retained a higher degree of activity when neutralized than when unneutralized, although some cultures were not greatly affected by neutralization. Prauty and Bendixen

(46, pp.413-419) froze acidophilus milk as a flavored sherbet and observed poorer survival in those products with added flavoring acids.

It is possible that the effects of concentrated solutes and pH are interrelated in their effect on cells in the frozen state. Winslow and Falk (66, p.242) have shown the relationship of NaCl and pH on Escherichia coli suspensions at room temperature. The toxicity of 0.725M NaCl appeared to be greatest below pH 6.0 and above pH 7.0.

Beard and Cleary (4, pp.141-144) found an appreciable enhancement of the bactericidal effect of the hydrogen ion by low temperatures.

Miscellaneous Factors. These might include the effect of the previous history of the culture. Harrison and Cerroni (19, p.578) and Harrison (17, p.714) have reported that Escherichia coli which had been grown in an aerated medium exhibited much more resistance to freezing than this organism grown in non-aerated broth.

Theories on the Mechanism of Protection and on Freezing Damage Based on Observed Environmental Effects. Haines (13, pp.451-463) summarized the theories of freezing destruction and protection that had been advanced up until 1937:

1. The capillary theory held that very small volumes of water freeze well below 0°C. This theory suggests that cells surviving exposure to very low temperatures have not in fact been frozen.

2. The "chemical" or dessication theory states that water may be removed from the cell, leading to concentration of solutes in the cell, and change in pH and concentration of one or more of the native proteins of the cell.

3. The mechanical destruction theory. Cells are said to be disintegrated by mechanical compression either on freezing or thawing or the surface of the cell is said to be destroyed.

Haines states that it is not easy to devise critical experiments to decide between these alternatives in bacteria. He showed that different organisms varied in their susceptibility to freezing and thawing. Spores in general were little affected by the freezing-thawing process.

Luyet and Gehenio (32, p.250) state that the capillary theory is not experimentally provable. However, they quote evidence (pp.162-164) showing that all the water in colloidal suspensions does not freeze. A temperature of -28°C. was necessary before water stopped freezing in a lampblack suspension. In egg white, cessation of freezing took place at -31°C. As the

temperature of a gelatin gel approached -20°C., unfrozen water approached a minimum of 34.8% water. It was found that 34.5% water gels did not freeze at any temperature, even in liquid air.

Before the work of Weiser and Osterud (62, pp.413-439) there were only four observations contributing to the theory of the mechanism of injury and death on freezing:

1. Freezing and thawing, as such, exert an immediate and marked lethal action.

2. Storage death occurs at higher freezing temperatures.

3. Colloids and many other non-aqueous materials serve to protect against freezing injury.

4. Repeated freezing is more destructive than a single freezing or prolonged storage at freezing temperatures.

Keith (25, pp.877-879) first observed and reported the phenomena of "immediate" and "storage" death. Wieser and Osterud (62, pp.423-424) reestablished that there occurs a rapidly acting or "immediate" death caused by freezing-thawing as such, and a "storage" death which is a direct function of time and temperature. That the freezing process as such, and not merely the effect of cold is lethal was evidenced by the much greater mortality

in frozen suspensions than in suspensions supercooled to even lower temperatures (Hilliard and Davis, 22, pp.423-431; Weiser and Osterud, 62, p.431).

The existence of ice crystal formation within frozen bacterial cells has not been seriously considered in freezing studies for some time, but Weiser and Osterud (62, pp.423-430) obtained evidence that intracellular crystals do not exert any detectable effects on frozen cells. This was shown by repeated fluctuations within every temperature range below freezing, without the enhanced mortality that would be expected if cells were fluctuated through a temperature range where internal ice crystals were formed and reformed.

Repeated freezing and thawing has been reported by several authors as being more lethal than single freezing (Prudden, 47, pp.341-350; Smith and Swingle, 53, pp.481-483; Sedgwick and Winslow, 61, p.416; Hilliard and Davis, 22, pp.423-431). Harrison (17, pp.711-715) reported that the lethal effect of the second freezing is greater than that of the first freezing or subsequent freezings. Weiser and Osterud (61, p.424) showed that repeated freezing is more lethal than a single freezing or storage in the frozen state for a similar length of time.

Weiser and Osterud (62, p.435) obtained evidence that immediate death occurs at a brief stage in the

freezing process during which extracellular ice formation is being completed. From this evidence they deduced that the mechanical action of extracellular ice was the principal cause of death of their bacteria from freezing, since the intercrystallic films presumably become so limited as no longer to accommodate the cells. This marked immediate death is apparently not due to the indirect effects of extracellular ice, such as the concentration of solutes, since if this were true the maximum rate reached at the completion of ice formation should continue at a constant rate. Contrary to this, the death was observed to diminish upon the completion of ice formation. They added that the protective action of colloids and sugar supports the concept of destructive mechanical action of extracellular ice, since the resulting intercrystallic films of frozen solutions or suspensions are sufficiently thicker to accommodate the cells.

Smith and Swingle (53, pp.481-483) observed that the critical point at which immediate destruction took place was somewhere near 0°C. and that the mortality from near 0°C. to -195°C. was essentially the same.

Keith (25, pp.877-879), Hilliard and Davis (22, pp. 423-431) also held the opinion that crushing action by ice crystals is the major factor responsible for death of bacteria by freezing, based on observed protective

action by colloids, and other compounds, and on the enhanced mortality of frozen over supercooled suspensions.

Weiser and Hargiss (61, pp.71-79) found vitromelting (conversion of vitreous or non-crystalline "glassy" ice, directly to liquid by very rapid passage through the crystalline temperature range) to be more lethal than the crystallization treatment. The devitrification treatment (slow warming of vitreous ice through the crystalline state) was more lethal than either vitromelting or crystallization. They theorized that since ice crystals do not form in bacterial cells on freezing to low temperatures, the only alternative state of water at temperatures when water cannot exist as a liquid, is in the vitreous state. It seemed likely to them that the explanation for the greater lethality of vitromelting over crystallization is that the intracellular physical state effected by vitromelting is more injurious than that effected by the crystallization treatment. They theorized that in the case of the crystallization treatment it is probable that the partial dehydration attending extracellular crystallization renders cells resistant to adverse effects accompanying further reduction in temperatures, particularly to intracellular vitrification.

Weiser and Hargiss (61, p.78) suggested a sequence of events, based on their experimental evidence, on the

freezing of a bacterial suspension: As crystallization begins and continues, concentration of solutes and material in the intercrystallic film occurs, bringing about a partial dehydration of cells. Due to their small size, the loss of water from bacterial cells probably can take place with such great speed, that no matter how rapidly the temperature is lowered, the resulting dehydration is sufficient to increase the resistance of the cell to the adverse effects attending further cooling. Death of a number of cells takes place during crystallization as the result of the mechanical action of ice crystals and the concentration of solutes in the intercrystallic film. Vitrification of the intracellular water occurs when the temperature reaches the vitrification point (about -110° C.), but does not produce injury because the cells have become partially dehydrated during the earlier crystallization. In vitromelting, appreciable amounts of extracellular ice do not form in the early stages of cooling and therefore dehydration does not take place. The devitrification treatment presumably includes both injury of vitrification on cooling and the injury of crystallization on warming, which may account for the high mortality seen after this treatment.

The workers mentioned above all theorized a mechanical crushing of cells. However, Haines (13, pp.451-463)

could find no evidence of cytological changes of cells following freezing.

Lovelock (31, pp.28-36) concluded that the main protective action of glycerol was not against mechanical injury, since he found less injury at lower temperatures where there would be more water frozen as ice crystals. He also found greater damage to frozen cells when glycerol was prevented from entering the cells. Since the extracellular glycerol concentration was about the same whether glycerol entered the cell or not, then the extracellular glycerol was not protecting the cell (i.e., against mechanical crushing). Meryman (41, p.519) states that most sugars and glycols are efficient binders of water. One mole of glycerol will prevent about 8 moles of water from freezing. The addition of a non-toxic binder that can pass through cell membranes to a biological solution, reduces the water available for crystallization and hence limits the dehydration that causes concentration of toxic salts in the cell.

Harrison and Cerroni (19, pp.577-579) claim evidence to refute the theory of crushing death in frozen bacterial suspensions. There appeared to be no correlation between the physical strength of cells in the Mickle Tissue Disintegrator and their susceptibility to the lethal effects of freezing. Therefore it seemed unlikely to

them that the lethal factor of freeze-thawing is mechanical. Harrison (17, p.715) and Major *et al.* (35, p.248) noted that *Escherichia coli* survived much better in pure water than in solutions of higher concentration. This evidence, they claimed, is contrary to the concept that non-aqueous materials protect cells by providing an intercrystallic film where ice crystals do not crush cells.

Squires and Hartsell (54, pp.40-45) believed that freezing kill is not entirely the result of mechanical destruction by ice crystal formation as such, but probably is due to an uneven distribution of solutes contained in the intercrystallic film. Freezing kill proceeds until the colloids of the bacterial cell come to equilibrium with the solutes with which it is confined. The effect of adding materials to suspending fluid is to alter the manner in which ice crystallization takes place. Additives permit a more rapid equalization of solute concentration to take place as ice formation proceeds. Results indicated that the interference of ice formation by either a material which formed a true solution with alteration of colligative properties, or by a substance which had no effect on colligative properties, afforded a protective action against freezing and storage death. If the concentration of solutes surrounding the cell during

ice formation is higher than the colloids within the bacterial cell, dehydration takes place. The overall effect would be the reduction in turgor pressure and under such conditions only limited mechanical pressure would be exerted on the cell by extracellular ice. Conversely, if the colloids within the bacterial cell were in higher concentration than the surrounding solutes, moisture could be drawn into the cell which would then increase its turgor pressure. Death from freezing could result either by puncture of the swollen cell by the ice crystals in which the bacterial cell would be confined or perhaps from freezing of the protoplasm which became dilute as moisture was drawn into the cell.

Molisch (32, pp.144-145) exposed yeast suspensions to -9°C. and observed shrinkage of cells (by 10% of their original volume).

Lovelock (31, pp.28-36) showed that glycerol functions in protecting cells on freezing by preventing the excessive increase in salt concentration which otherwise occurs on freezing. This protective action was exerted in full only when glycerol was present both within and without the cell. He showed that the destructive action of freezing could be attributed to the concentration of the electrolytes of the cell and of its suspending medium. The critical region of temperature in which

damage occurs rapidly, -3° to -40°C. was shown to coincide with that region in which the cell was exposed to a toxic concentration of salt solution which occurs as ice freezes. The temperature at which the mole fraction of NaCl is 0.014 (0.8M in water) was always the same as the temperature where damage first occurs. When the initial concentration of added glycerol exceeds 25% by weight, this critical NaCl concentration is not reached in the temperature range 0° to -40°C. In the presence of glycerol the concentration of NaCl continues to increase below -35°C. so the rapid decrease in the destructive effect of freezing at temperatures below -35°C. indicates that the lower limits of the critical range of temperatures is determined by some other factor than the external NaCl concentration. It was shown that damage occurs at a steadily decreasing rate as the temperature falls, becoming very low at temperatures below -35°C. The fact that glycerol must be present within the cell to protect, suggests that the internal concentration of some salt (i.e., KCl) is at least as important as the external NaCl concentration in causing damage. Unlike NaCl, KCl becomes less soluble as the temperature falls and at temperatures below -35°C., is insufficiently soluble to maintain the critically damaging concentration.

Smith, Polge and Smiles (53, pp.481-483), studying living cells microscopically during freezing and thawing, noted the mode of action of glycerol in protecting cells during freezing. They noted that in the presence of glycerol, the processes of crystallization and dissolution are modified. None of the cells were frozen into the ice.

Harrison and Cerroni (19, p.579) theorized that cells differing in resistance to freezing probably differed in permeability and/or concentration of intracellular metabolites, rather than physical strength. Chemical or physico-chemical phenomena were suggested to be involved in the cause of death.

Turner and Brayton (59, pp.639-650) suggested that there are two injurious factors. One acts rapidly and is associated with freezing and thawing, the other is a storage injury acting at higher freezing temperatures, but not at -78°C., and is caused by changes detrimental to cell metabolism, or to proteolytic or other enzymes.

Dubos (9, pp.101-112) proposed that autolytic enzymes released from freezing damaged Pneumococcus cells attack the substrate of cell architecture.

Luyet and Gehenio (32, p.180) state that many authors have observed that selective permeability of the cell membrane is damaged. Foulkes (11, p.505) used

repeated freezing and thawing to alter the permeability of yeast to oxidizable substrates.

Haines (13, pp.451-463) believed that either of two factors are responsible for death on freezing. One factor is unknown but apparently not mechanical and the other, some change leading to flocculation of the cellular proteins, or else there is one process leading to coagulation, but with a time lag so that coagulation is not found on immediate freezing and thawing. He believed that this flocculation of the cellular proteins is due to the high salt concentration and to the change in pH that results when a solution freezes. Squires and Hartsell (54, pp.40-45) also believed protein denaturation to be the cause of death on frozen storage. Hilliard and Davis (22, pp.423-431) found native cellular proteins of Pseudomonas aeruginosa to be denatured on freezing. Higher freezing temperatures (-2°C.) caused the most rapid denaturation.

Borgstrom (6, p.167), in a review, states that the reason why temperatures higher than -10°C. are more lethal to microorganisms is most likely connected with the processes of protein denaturation being far less disastrous at lower temperatures. In freezing mammalian and fish muscles, workers drew attention to the rapid denaturation of the protein which occurs at temperatures

just below the freezing point of the muscle, and in the region of -2° to 4°C. They attributed the rapid death of microorganisms in this temperature range to the fact that, within 8 days at -2°C. half of the coagulable protein of the organisms was precipitated. At -20°C. no such change took place. Borgstrom states that death of frozen organisms may be ascribed to the denaturation of the protein and subsequent flocculation of the cellular proteins. This concept refuted the idea that death is due to the mechanical action of ice crystals, he felt.

Nord (44, p.55) reported disaggregation of protein particles by lowering the temperature. It may be enlightening to consider the effects of concentrated hydrogen ions and cations on disaggregated protein particles at low temperatures.

Keith explains storage death (25, pp.877-879) as being the result of starvation or destructive metabolism, but that freezing preservation diminishes the destructive metabolism more at lower than at higher freezing temperatures. He felt that freezing preservation diminishes the rate of destructive metabolism. Hilliard and Davis (22, pp.423-431) theorized that low temperatures interfere with some metabolic activities which result in an exhaustion of stored energy or starvation.

Many theories have been advanced on the mechanism of freezing damage, based on comparatively few facts. It is only possible to deduct from these data, that many complex factors are involved in freezing damage. No substantiated evidence has been given that can rule out any one factor. However, most workers do agree that lower temperatures below freezing, higher non-toxic-solids freezing menstruum, and more neutral pH reactions in the menstruum all favor better survival of frozen bacterial suspensions.

PART I. NUTRITIONAL STUDIES

The study of the nutritional requirements of Lactobacillus acidophilus was undertaken as an aid in the development of a suitable growth medium for production of cell concentrates.

Experimental Methods

Stock Cultures. Six strains of Lactobacillus acidophilus were selected from various sources for this study. These were:

ATCC* 4962

ATCC 4963

ATCC 9857

A culture from The Dairy Laboratories, 23rd and Locult Streets, Philadelphia 3, Pa. (labelled "DLF" in this study)

A culture from Farr Laboratory, P. O. Box 174, Kalamazoo, Michigan (labelled "Farr" in this study)

A culture from the Oregon State College Bacteriology Department stock cultures (labelled "OSC" in this study

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* American Type Culture Collection, 2029 M Street, N.W., Washington 6, D.C.

The organisms were grown in 10% reconstituted skim milk, made from high grade non-fat spray dried milk solids. The milk was prepared weekly and sterilized at 121°C. for 12 minutes. The cultures were transferred daily in 10 ml. of milk in aluminum capped tubes, until active enough to curdle the milk in 10 hours or less. Mother cultures were frozen at -20°C., thawed and transferred periodically. Periodic platings in Tryptone-tomato juice agar (50, p.687) showed typical fuzzy colonies.

Lactobacillus arabinosus 17-5 (ATCC 8014) and Streptococcus faecalis (ATCC 8043) were used as control organisms. They were both carried in a broth consisting of Tryptone, Yeast extract, Gelatin glucose, lactose, sucrose, NaCl, and sodium acetate (15, pp.125-126).

Basal Medium. The basal medium used consisted of the amino acid and vitamin levels used in Henderson and Snell's (21, pp.15-29) uniform medium for the determination of amino acids. The constituents common to all media were in the following composition:

Constituents	Per 100 ml. double strength medium
Glucose	2 gram
Sodium acetate	0.1 gram
Adenine sulfate	1 mgm

Constituents	Per 100 ml. double strength medium
Guanine hydrochloride	1 mgm
Uracil	1 mgm
Thiamin	100 g (micrograms)
Riboflavin	100 g
Pyridoxal	100 g
Niacin	100 g
p-aminobenzoic acid	20 g
Folic acid	1 g
Biotin	1 g
Calcium pantothenate	100 g
DL alanine	10 mgm
DL aspartic acid	100 mgm
*L glutamic acid	100 mgm
L arginine	20 mgm
L lysine	20 mgm
*L histidine	10 mgm
*L isoleucine	10 mgm
*L methionine	10 mgm
*L phenylalanine	10 mgm
*L proline	10 mgm
*L threonine	10 mgm
*L tyrosine	10 mgm

*When DL amino acids were used, twice these levels were used.

Constituents	Per 100 ml. double strength medium
*L valine	10 mgm
*L tryptophane	10 mgm
*L cystine	10 mgm
*L serine	10 mgm
Glycine	10 mgm
Glutamine	10 mgm
Asparagine	10 mgm
Salts solution A	1 ml
Salts solution B	1 ml

Preparation of basal medium stock solutions. The solutions of amino acids, vitamins and salts were prepared in convenient dilutions to facilitate rapid and accurate preparation of the basal medium.

The following mineral salts concentrations were used in the initial studies but improved levels were determin ed later:

Salts solution A	grams per 100 ml. double strength medium
K_2HPO_4	0.10
KH_2PO_4	0.10

Salts solution B	grams per 100 ml. double strength medium
$MgSO_4 \cdot 7H_2O$	0.16
$MnSO_4 \cdot 7H_2O$	0.024
$FeSO_4 \cdot 7H_2O$	0.004
NaCl	0.004

The above ingredients were dissolved and brought up to 200 ml. in distilled water and stored in the refrigerator. One ml. of each was added to 100 ml. of double strength basal medium.

All of the vitamin solutions were prepared in a concentration which contained the proper quantity per ml. for 100 ml. of the double strength basal medium. The solutions were stored in Duraglass 8 ounce screw cap bottles and refrigerated at 5°C. Riboflavin, Folic acid and the B_6 vitamins were stored in paper wrapped bottles to prevent light deterioration.

Adenine, quanine and uracil were dissolved in the least amount of hydrochloric acid that would bring them into solution and diluted to a concentration containing one mgm. per ml. All three substances could be prepared in the same solution or individually.

Thiamin, riboflavin and pyridoxin were prepared separately by dissolving 20 mgm. in 200 ml. of 0.02N Acetic acid. Calcium pantothenate and niacin were

prepared separately by dissolving 20 mgm. in 200 ml. of 50% ethanol.

P-aminobenzoic acid, biotin and folic acid were all prepared in a concentration of 20 micrograms (μgms) per ml., while biotin and Folic acid were made up to contain one microgram per ml.

The amino acids were diluted in distilled water to contain in 20 ml. the amount required for 100 ml. of the basal medium. The amino acids were divided into three groups and made up in three separate solutions for most studies, usually of 200 ml. each. Asparagine and glutamine were made up separately from the other amino acids. The amino acids were stored in the frozen state in 8 ounce Duraglass prescription bottles until needed.

Twice as much of the DL-form of amino acids were used as the L-form (except for arginine, lysine and alanine) because only the L-amino acid is utilized by the organism. Tyrosine and cystine, which are relatively insoluble in neutral aqueous solution, were dissolved in a minimum quantity of 3 Normal hydrochloric acid and brought up to the proper concentration. Aspartic acid and glutamic acid were added in a dry state to the basal medium, since levels used were too high for convenient stock solutions.

Preparation of Accessory Factor Stock Solutions.

Ascorbic acid. 600 mgm. of Ascorbic acid (Nutritional Biochemical Co., 21010 Miles Ave., Cleveland 28, Ohio) in 10 ml. of distilled water, was prepared immediately before use. One ml. of the solution (60 mgm) was added to 100 ml. double strength medium, giving the concentration used by Kitay, McNutt and Snell (26, p.732).

Tween 80. Tween 80 (Hill Top Laboratories Inc., 921 Wm.H. Rd., Cincinnati 6, Ohio) is a nontoxic polyoxyethylene derivative of sorbitan monooleate. A 10% solution was prepared by dissolving 10 ml. in 90 ml. of hot water. The solution was stored in the refrigerator. One ml. was added to 100 ml. of double strength medium.

Thymidine. Thymidine (Nutritional Biochemical Co.) was made by dissolving 100 mgm. in 200 ml. of distilled water. It was stored in the frozen state until needed. To 100 ml. of double strength basal medium, 2 ml. of stock solution were added, yielding a final concentration of 50 μgms per 10 ml. as used by Kitay, McNutt and Snell (26, p.727).

Vitamin B_{12}. Fifteen microgram ampules of vitamin B_{12} (Nutritional Biochemical Co.) were diluted to 100 millimicrograms (Mμgm) per ml. as a stock solution. This

was stored in the frozen state until used. Four ml. of the stock was added to 100 ml. of double strength basal medium yeilding a final concentration of 20 mμgms per 10 ml. as used by Kitay, McNutt and Snell (26, p.735).

Pantotheine (Lactobacillus bulgaricus Factor or LBF). Pantotheine (Krichell Laboratory, 1735 S.E. Powell Blvd., Portland 2, Oregon) which is extremely deliquesant was stored at -25°C. Before making stock solutions, about 100 mgm. of the material in the form of 23,650 LBF units per mgm., was dried overnight in a vacuum desicator over P_2O_5, and then weighed. A stock solution containing 100 LBF units per ml. was made and stored in the frozen state. One LBF unit is defined as that quantity of pantotheine which will support maximum growth of the Gere A strain of Lactobacillus bulgaricus in 10 ml. of synthetic growth medium (27, p.52). The stock was dispensed at the rate of 2 ml. per 100 ml. of double strength basal medium, giving a final level of 10 LBF units per 10 ml.

Inoculum Medium. The inoculum medium used by Kitay, McNutt and Snell (26, p.727) was used in the first studies. It contained all the constituents of Henderson and Snell's (21, pp.15-29) complete medium, plus Tomato Juice serum. Later, when a medium was developed that

would support growth with no added non-synthetic materials, it was used as the inoculum growth medium.

Ten ml. of the inoculum medium was dispensed into 20 x 125 mm. screw cap tubes. These were autoclaved at 121°C. for 8 minutes, cooled in a water bath, then rotated to resuspend the slight sediment that had formed. If the sediment was shaken while the medium was still hot, a clear medium was not obtainable.

Inoculum. The tubes were inoculated with one loopful from a 24 hour old milk culture, shaken to disperse the cells and incubated at 37°C. for 24-30 hours. By this time a fairly heavy turbidity had resulted in all the tubes. They were centrifuged and washed twice with sterile M/15 phosphate buffer, pH 6.0, using a Sorvall Superspeed Angle head centrifuge. The cells were resuspended in 2 to 3 times the original volume, with the phosphate buffer, in the screw cap tubes and stored at 5°C. until used. They maintained sufficient viability for inoculation for at least two weeks.

The nutritional study media were inoculated with one drop of the inoculum and incubated at 37°C. for 72 hours. After this time they were frozen at -20°C. to preserve them until just before titration.

Dispensing the Medium. The large number of factors being studied for six strains and two control organisms, necessitated the use of an accurate and rapid method of making the determinations. The micro method for the determination of amino acids developed by Henderson et al. (20, pp.31-38) and used by Anderson and Elliker (2, pp.161-167) for lactic streptococci nutritional studies, was found to be satisfactory.

Two ml. of the test medium was dispensed into triplicate 125 x 13 mm. culture tubes (previously washed in Trisodium phosphate, rinsed in dilute acetic acid, and then distilled water). The entire test tube rack was covered with a stainless steel lid. The medium was sterilized by bringing the autoclaved up to a temperature of 250°F. for two minutes, then cooling rapidly. This short heat treatment was used by Anderson and Elliker (2, pp.161-167) and was used to minimize the effect of high temperatures on the thermolabile constituents in the medium, and to prevent as much as possible the formation of various unknown organic complexes. No difficulties due to contamination were encountered using this method. The medium was immediately cooled and inoculated with one drop of the inoculum.

Growth response after the test cultures had incubated for 72 hours at 37°C. was measured by the titratable acid

developed, using the Cannon automatic titrator equipped with a quinhydrone electrode, or a Beckman Model N-2 pH meter with a Probe Electrode assembly. The apparatus was equipped so that titrations could be carried out directly in the growth tubes using 0.05N NaOH to titrate to an endpoint of pH 8.3. The results expressed in counter numbers (which were directly proportional to the acidity produced) were used.

Results and Discussion

Henderson and Snell (21, pp.31-38) synthetic medium with amino acids, vitamins, purines and pyrimidines, glucose, acetate and oleic acid does not support the growth of any of the six strains of L. acidophilus studied here. When some of the accessory factors described by Kitay and Snell (27, pp.49-54), including ascorbic acid, vitamin B_{12}, thymidine, and pantothein (LBF) were added, however, adequate growth became possible. Attempts were made to improve this growth medium before nutritional requirements were determined.

Influence of the Length of Heat Treatment. It has been reported (26, p.731) that long autoclaving would improve a medium to support growth of fastidious strains of L. acidophilus. To determine the effect of this

treatment, growth promotion in the medium was compared after 2 minutes versus 20 minutes autoclaving at 121°C.

It appears from Table 1 that longer heat treatment results in lower total growth in the medium for all strains as measured by acid production, when compared to the results after shorter heat treatment. In addition, the two minute period of sterilization is to be preferred since it is less likely to cause the synthesis of unknown growth factors or the thermal destruction of added factors (1, p.60). No trouble with unsterile media was encountered with this short period of sterilization.

Comparison of Different Basal Media. The medium used above still supported only poorly, the growth of Lactobacillus acidophilus. In an effort to improve the medium for growth, the different salts levels suggested by Kitay, McNutt and Snell (26, p.728) were compared. The growth supported by Henderson and Snell's (21, pp.31-38) amino acid medium was also compared to a medium utilizing Casamino acids as the Nitrogen source. The salts levels used by Kitay, McNutt and Snell may be summarized in the following table:

	Per 100 ml. Double Strength Medium		
	Medium 1	Medium 2	Medium 3
Sodium Acetate	2.0 gms.	2.0 gms.	0.2 gms.
Sodium Citrate	2.0 "	---	2.0 "
KH_2PO_4	0.6 "	0.10 "	0.6 "
K_2HPO_4	0.6 "	0.10 "	0.6 "
$MgSO_4 \cdot 7H_2O$	0.56 "	0.16 "	0.56 "
$MnSO_4 \cdot 7H_2O$	84 mgm	24 mgm	84 mgm
$FeSO_4 \cdot 7H_2O$	28 mgm	4 mgm	28 mgm
NaCl	8 mgm	4 mgm	8 mgm

The Casamino acid medium may be summarized in the following table:

	per 100 ml. double strength
Casamino Acids (Difco Vitamin Free)	1 gm.
Asparagine	20 mgm.
DL tryptophane	20 mgm.
L cystine	40 mgm.
Other constituents as previously described.	

As seen in Table 2, Salts Medium 1 and 3 supported higher total growth than Medium 2. Greater total growth also resulted in the Casamino acid medium than in Henderson and Snell amino acids medium. Both media contain the same essential amino acids, therefore it was thought that some other factor was present in the Casamino acids to stimulate growth. Since Casamino acids is an hydrolysate of casein, a calcium containing complex, and because

other lactobacilli have been reported to be stimulated by calcium (10, pp.322-325), the effect of added calcium was determined as shown in Table 3. It can be seen that the addition of calcium to the Henderson and Snell amino acid medium increases the total acid production to the level of acid produced in the Casamino acid medium. A comparison of the growth in the various basal salts media of Kitay, McNutt and Snell (26, p.728), with and without added calcium in the synthetic amino acid medium is shown in Table 4.

It is evident in all these experiments that calcium stimulates growth of all strains, in all of the media tested, and that the total acid production of all strains are more uniform in the presence of calcium. It is not known at the present time whether the effect of the added calcium was a reversal of the toxicity of other ions such as Zn^{++}, as described by MacLoed and Snell (33, pp.783-792), or if the calcium had some other effect. However, MacLoed and Snell (p.785) found that less Zn^{++} was required to inhibit growth of Lactobacillus arabinosus in an amino acid medium than in a casein hydrolysate medium. Calcium, though not required, could counteract the inhibition in the presence of Mn^{++}, which was required. Eades and Womack (10, pp.322-325) found calcium to stimulate L. casei in the presence of adequate Mn^{++}, in a

completely synthetic medium. Stimulation was not observed in a medium using casein hydrolysates as amino acid source, probably because this source was high in calcium. Slow growth in completely synthetic media may have been due to small amounts of calcium in pure analytical grade mineral reagents or from glassware.

Salts Medium 1 was chosen as most suitable for growth of these strains of Lactobacillus acidophilus, in completely synthetic medium, when supplemented with calcium.

Cation Requirements in the Adopted Medium. The effect of omission of single cations from the medium used is shown in Table 5. When Mg^{++} ion is omitted, no growth of L. acidophilus occurs. This may have been due to the chelation of essential ions by the high citrate level as described by MacLoed and Snell (34, p.352), rather than to an absolute requirement for Mg^{++} ion. The high level of Mg^{++} ion used in this medium normally balances the chelation by the high citrate concentration.

The omission of Mn^{++} ion resulted in a slight decrease in total growth of all strains of L. acidophilus used. The omission of the Fe^{++} ion caused a greater loss of total growth, while the omission of NaCl resulted in a slightly greater total acid production than in its presence. Calcium omission, as found previously, results in

a decrease in total acid production.

Using ATCC 4963, Skeggs et al. (51, p.735) also found that Mg^{++} ion was required. However, Mn^{++} ion was not required and may have been slightly toxic. Ferric or ferrous salts were required in vitamin B_{12} containing medium, but were only stimulatory in desoxyribonucleic acid (DNA) containing medium. The DNA may have contained trace Fe^{++} ion contamination.

Effect of Omission of Vitamins. In the absence of riboflavin, pyridoxal, niacin, and both pantothenic acid and pantotheine (LBF) together, only minimal growth resulted as seen in Table 6. The omission of thiamin, biotin, folic acid, p-aminobenzoic acid (PABA) or the last two together did not greatly reduce growth under the conditions used. The organisms could use either pantothenic acid or LBF, but greater total acid production occurred in the presence of pantothenic acid. This may have been due to the very low level of Pantotheine used, as suggested by Kitay and Snell (27, p.52). However, this level (10 LBF units per 10 ml.) was described as being ten times the level required for maximum growth of L. bulgaricus Ga.

It seemed necessary to establish the lack of LBF requirement for these strains since several investigators

have reported the requirement for this organism (57, p. 237). Therefore the exhaustive transfer method of Kitay and Snell (27, p.50) was used. All strains of the organism were grown in 10 ml. of complete medium containing LBF for 2 days, then one drop of the growth was transferred aseptically into each of two media, one lacking LBF. If growth occurred in the LBF deficient medium, this growth was used as inoculum for a repeat of the same procedure. The exhaustive transfer was repeated three times. It became evident that none of the six strains of Lactobacillus acidophilus studied required the LBF factor under the conditions used, since growth occurred in all tubes lacking LBF even after three transfers.

Since biotin was not demonstrable as being required, by an omission of the vitamin alone, an attempt was made to establish the relationship between biotin, oleic acid, and acetate. In this experiment a lower level of acetate was used than described for Salts Medium 1, since the omission of a great quantity of acetate would cause a great decrease in buffering capacity. This would result in a very great difference between the total acid produced in acetate containing media and one with no acetate. The difference would not be so great if only a small amount of acetate were used in the control medium. In Table 7 it can be seen that the omission of acetate in

the presence of biotin and oleic acid results in reduced total acid production as expected, but acetate does not seem to be required under the conditions used.

When oleic acid is omitted, even in the presence of biotin and acetate, only minimal growth occurs. It seems, therefore, that oleic acid is required and the requirement is not completely replaceable by biotin and/or acetate.

In a biotin deficient medium, decreased total acid production was observed, but an absolute requirement was not demonstrated under the conditions used. Perhaps exhaustive transfer would show that such a requirement is present, but it seems possible that in the presence of aspartic acid, acetate and oleic acid such a requirement would not be demonstable (65, pp.619-630; 23, pp.229-230).

When acetate and oleic acid are omitted together, minimal acid production results. Omission of acetate and biotin together seem to result in decreased acid production equivalent to that caused by the omission of either one singly for 5 strains. The OSC strain, which exhibits only minimal growth, requires either acetate or biotin in the presence of oleate.

When oleic acid and biotin are omitted together, no growth occurs. Even less acid is produced than in oleic acid deficient medium, indicating that biotin may play

some role in fatty acid metabolism in some of these strains, since biotin supports very slight acid production in the absence of oleic acid.

Kitay and Snell (27, p.50) also found the ten strains of L. acidophilus they studied to require oleic acid in the presence of excess biotin. Snell (57, p.240) reports that L. acidophilus requires biotin and oleic acid. Rogosa et al. (50, p.689) found L. acidophilus to require biotin in the presence of oleic acid. Skeggs et al. (51, p.735) however, found ATCC 4963 did not require biotin in the presence of oleic acid (Tween 80).

Requirement for Vitamin B_{12} or Thymidine. As shown in Table 8 all strains require either vitamin B_{12} or thymidine in the presence of ascorbic acid, but the OSC strain does not show the same degree of requirement, since only decreased acid production is seen when growth in deficient media is compared to that in complete medium. Kitay, McNutt and Snell (26, p.731) showed that several strains of L. acidophilus can use either vitamin B_{12} or thymidine. Rogosa et al. (50, p.689) and Skeggs et al. (51, p.735) report that L. acidophilus requirement for vitamin B_{12} is replaceable by desoxyribonucleic acids or desoxyribonucleosides.

Amino Acid Requirements. In Table 9 it may be seen that all six strains demonstrate requirements for histidine, isoleucine, leucine, methionine, phenylalanine, threonine, valine, tryptophane, alanine arginine, lysine, tyrosine, cystine, aspartic acid and glutamic acid, when these amino acids are omitted one at a time. When proline or glycine was omitted singly, no decrease in acid production resulted. Asparagine and glutamine do not seem to be required in the presence of aspartic and glutamic acids. Serine was required by one strain (OSC) and omission of serine resulted in decreased acid production by the other strains.

Koser and Thomas (29, pp.287-298) and Skeggs et al. (51, p.734) studied amino acid requirements for strains of L. acidophilus, though neither group were able to obtain maximum growth of this organism in the complete media that they used. Koser and Thomas' findings are in accordance with those described above, with the following exceptions: Proline was required, isoleucine was stimulatory, methionine and serine were required by some and not by other strains, and glycine stimulated some strains but not others. Skeggs et al. (51, p.734) found ATCC 4963 to require the same amino acids as those found to be required in this study with certain exceptions. Serine was required, while aspartic acid and DL-alanine

were stimulatory. Threonine stimulated in desoxyribonucleic acid containing media, but was not required in media containing vitamin B_{12}.

Purine and Pyrimidine Requirements. In the absence of adenine (Table 10) less than maximum acid was produced with five strains while the sixth strain (ATCC 4962) showed a definite requirement for the factor. Guanine was not required by any of the strains when omitted singly. Uracil was required by five strains, while ATCC 9857 only produced less acid in its absence.

No studies on extensive purine and pyrimidine requirements for L. acidophilus have been published other than Skeggs et al. (51, p.736) who found that ATCC 4963 did not require purine bases in the presence of DNA. Xanthine would support growth in the presence of vitamin B_{12} but not in the presence of thymidine.

Carbohydrate Utilization.

Methods.

Basal medium. A sugar-free broth basal medium was used in combination with each carbohydrate. The broth consisted of the following constituents (15, pp. 125-126):

Trypticase (BBL)	2.0 %
Gelatin (Difco)	0.25%
Yeast Extract (Difco)	0.5 %
Sodium acetate	0.4 %
Sodium chloride	0.15%
Tween 80	0.05%
Brom Cresol Purple	0.04%

Procedure. A double strength broth was mixed with an equal quantity of 2% carbohydrate to make the desired strength test medium with 1% carbohydrate. Duplicate 10 ml. quantities were dispensed for each of the six strains to be tested, in aluminum capped tubes. The tubes were autoclaved 12 minutes at 121°C., inoculated with one drop of the inoculum used in the Nutrition Studies (see p.43) and incubated at 37°C. for 14 days. The tubes were observed for acid production daily and the pH of each was taken at the end of the 2 week period. No growth or acid production occurred in control tubes of the basal medium without added carbohydrate.

Results. All strains fermented glucose, fructose, galactose, mannose, trehalose, lactose, sucrose and maltose in one day to pH 4.0-4.3. No fermentation were observed from arabinose, xylose, sorbose, rhamnose, melibiose, cellobiose, raffinose, melizitose, dextrin,

inositol, dulcitol, sorbitol, mannitol, glycerol, salicin or inulin.

Discussion. These results compare favorably with Bergey's description (5, p.352) but Rogosa et al. (50, p.686) found L. acidophilus to use salicin and cellobiose. Skeggs et al. (51, p.736) found that ATCC 4963 did not ferment sucrose, which has been reported as utilized by this organism by many workers. Wheater (63, pp.123-132) found 29 strains of L. acidophilus would ferment cellobiose and salicin, which were not utilized by the six strains studied here.

Summary. In a study of the nutritional requirements of six strains of Lactobacillus acidophilus maximum growth was not obtained with media used by other workers. Efforts were made to improve the adopted medium. Long autoclaving resulted in no improvement of the growth medium for L. acidophilus. Two minutes heating time at 250°C. was more desirable for several reasons. Calcium was found to stimulate growth of all strains studied and to raise all strains to uniform levels of acid production. The possibility that calcium reverses toxicity of other ions was discussed.

Mg^{++} omission resulted in no growth in the medium used, probably because of chelation of essential

ions by the high citrate level used. When Mn^{++} ion or Fe^{++} ion were omitted, a decrease in total acid production was noted, while omission of NaCl caused a slight increase.

With the omission of either riboflavin, pyridoxal, niacin, or pantothenic acid and pantotheine together, the growth medium supported no growth of any of the strains. The organisms could use either pantothenic acid or pantotheine. Thiamin, folic acid and/or p-aminobenzoic acid were not required under the conditions used. Oleic acid requirement was not replaceable by biotin or acetate. An absolute requirement for biotin or acetate was not demonstratable for five of the strains by their omission singly or together, but one strain (Farr) requires one or the other. Omission of acetate and oleic acid together resulted in minimal growth (about 1/6 of the total acid produced in the complete medium). Omission of oleic acid and biotin together supported even less growth (about 1/10 of the total acid produced in complete medium).

All strains required vitamin B_{12} or thymidine in the presence of ascorbic acid.

Histidine, isoleucine, leucine, methionine, phenylalanine, threonine, valine, tryptophan, alanine, arginine, lysine, tyrosine, cystine, aspartic acid and glutamic

acids were required by all strains, but proline, glycine, asparagine and glutamine singly or together, were not required under the conditions used. Serine was required by one strain and was "stimulatory" for the other strains.

Adenine was "stimulatory" for all but one strain (ATCC 4962) which showed a requirement for the purine. Uracil was required by five strains and was "stimulatory" for one (ATCC 9857). Guanine was not required under the conditions used.

All strains fermented glucose, fructose, galactose, mannose, trehalose, lactose, sucrose and maltose in one day. Arabinose, xylose, sorbose, rhamnose, melibiose, cellobiose, raffinose, melizitose, dextrin, inositol, dulcitol, sorbitol, mannitol, glycerol, inulin and salicin were not fermented.

Table 1

Effect of Sterilization Time at 121°C. on Growth of L. acidophilus in the Synthetic Medium

Sterilization Time	Acid production by following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
minutes						
2	48[2]	55	36	46	34	45
20	30	44	34	30	16	23

[1] Cultures incubated 72 hours at 37°C.

[2] Average of titrator units for triplicate samples. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 2

Comparative Growth of L. acidophilus in Various Synthetic Media

Medium	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
Salts medium 1 and Casamino acids	68[2]	64	67	67	65	57
Salts medium 1 and Amino acids	34	47	48	34	59	22
Salts medium 2 and Casamino acids	65	41	42	40	44	31
Salts medium 2 and Amino acids	45	33	29	30	22	24
Salts medium 3 and Casamino acids	70	65	68	68	62	62
Salts medium 3 and Amino acids	34	47	32	31	36	25

1 Cultures incubated 48 hours at 37°C.

2 Average of titrator units for triplicate samples. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 3

Influence of Amino Acid Basal Medium on Response of L. acidophilus to Added Calcium

Amino acid basal medium	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
Casamino acids	84[2]	85	82	84	84	83
Casamino acids plus calcium	86	88	88	88	87	86
Henderson and Snell amino acids	77	65	75	77	76	76
Henderson and Snell amino acids plus calcium	83	85	87	83	83	83

1 Cultures incubated 72 hours at 37°C.

2 Average of titrator units for triplicate samples. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 4

Influence of Mineral Salt Content of Synthetic Medium on Growth of *L. acidophilus*

Mineral salts in medium	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
Salts medium 1	52[2]	58	19	62	73	53
Salts medium 1 plus calcium	78	78	79	79	80	78
Salts medium 2	29	8	10	9	21	16
Salts medium 2 plus calcium	37	27	36	36	31	34
Salts medium 3	44	67	38	53	50	44
Salts medium 3 plus calcium	71	72	61	69	68	66

1 Cultures incubated 72 hours at 37°C.

2 Average of titrator units for triplicate samples. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 5

Effect of Omission of Cations on Growth of *L. acidophilus* in a Synthetic Medium

Ions omitted	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
none	82[2]	81	84	83	82	83
Mg	3	3	3	3	4	3
Mn	76	76	76	77	75	76
Fe	47	70	57	43	63	52
NaCl	85	86	87	89	88	91
Ca	55	68	60	42	65	64

1 Cultures incubated 72 hours at 37°C.

2 Average of titrator units for triplicate samples. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 6

Effect of Omission of Vitamins on Growth of L. acidophilus in a Synthetic Medium

Vitamins omitted	Acid production by the following strains[1]: 4962	4963	9857	DLF	OSC	FARR
None	87[2]	85	86	86	77	86
Thiamin	86	85	84	89	79	84
Riboflavin	20	13	30	39	28	33
Pyridoxal	13	13	57	53	38	51
Biotin	84	84	87	87	71	82
Folic acid	81	81	85	83	74	82
P-amino benzoic acid (PABA)	82	87	84	84	72	85
Folic acid and PABA	75	84	84	81	68	84
Niacin	11	8	20	18	12	15
Pantothenic acid	57	67	76	67	50	74
Pantotheine (LBF)	83	84	85	80	70	84
Pantothenic acid and LBF	17	23	20	15	19	16

1 Cultures incubated 72 hours at 37°C.

2 Average of titrator units for triplicate samples. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 7

Effect of Omitting Oleic Acid, Acetate and Biotin, Singly or in Pairs, on Growth of L. acidophilus in a Synthetic Medium

Growth factor omitted	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
None	89[2]	88	85	79	94	92
Acetate	81	62	73	67	73	94
Oleic acid	26	10	33	26	11	26
Biotin	76	60	73	50	40	75
Acetate and oleic acid	20	11	23	14	13	16
Acetate and biotin	75	46	69	64	28	78
Oleic acid and biotin	12	9	7	8	8	6

[1] Cultures incubated 72 hours at 37°C.

[2] Average of titrator units for triplicate samples. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 8

Effect of Omission of Accessory Growth Factors on the Growth of *L. acidophilus* in Synthetic Medium

Factor omitted	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
None	70[2]	70	69	71	70	70
Vitamin B_{12}	69	70	70	70	70	71
Thymidine	68	69	70	70	70	71
Vitamin B_{12} and thymidine	8	11	27	4	52	12

1 Cultures incubated 72 hours at 37°C.

2 Average of titrator units for triplicate samples. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 9

Effect of Omission of Amino Acids on the Growth of *L. acidophilus* in Synthetic Medium

Amino acid omitted	Acid production by the following strains[1]: 4962	4963	9857	DLF	OSC	FARR
None	109[2]	114	111	113	110	111
Histidine	13	10	12	8	13	12
Isoleucine	7	6	9	6	4	6
Leucine	10	6	10	6	4	6
Methionine	16	15	18	15	15	17
Phenylalanine	18	10	14	10	6	14
Proline	106	101	101	102	100	102
Threonine	15	9	10	8	10	15
Valine	12	9	5	10	10	14
Tryptophane	12	10	12	46	38	8
Serine	59	55	53	55	16	59
Glycine	104	104	106	108	96	104
DL Alanine	14	10	9	9	9	13
Arginine	13	12	12	8	7	11
Lysine	11	7	11	7	8	9
Tyrosine	15	7	15	10	15	15
Cystine	10	10	10	10	9	10
Aspartic acid*	24	7	16	6	6	13
Glutamic acid*	10	4	6	3	3	5
Asparagine	108	108	105	109	110	110
Glutamine	113	110	109	101	108	114
Asparagine and glutamine	101	101	102	97	94	102

* Asparagine and glutamine were omitted when aspartic and glutamic acids were being determined.

1 Cultures incubated 72 hours at 37°C.

2 Average of titrator units for triplicate tubes. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 10

Effect of Omission of Purines and Pyrimidines on Growth of *L. acidophilus* in a Synthetic Medium

Factor omitted	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
None	79[2]	79	78	80	79	79
Adenine	13	60	58	55	43	60
Guanine	76	73	74	76	77	79
Uracil	5	4	64	6	7	15
Adenine, guanine and uracil	19	29	22	30	26	39

1 Cultures incubated 72 hours at 37°C.

2 Average of titrator units for triplicate tubes. One titrator unit represents acid production by 0.04 ml. of 0.05 Normal NaOH.

PART II. THE DEVELOPMENT OF A MEDIUM FOR LARGE SCALE PRODUCTION OF LACTOBACILLUS ACIDOPHILUS

A low cost source of nutrients in which L. acidophilus may grow to a moderate or large population is required. The medium must also allow efficient centrifugal removal of the cells.

This organism grows well in milk, attaining a population of 100 to 200 x 10^7 viable cells per ml. as determined by the use of T_{19} agar (15, pp.125-126) as a plating medium. Milk, however, by its nature does not lend itself to centrifugation after acid fermentation. The most obvious answer then, would be the use of a whey product, since it is quite clear and is low in coagulable proteins, which can interfere with centrifugational separation of bacterial cells.

Several forms of whey were tested in the preliminary studies to determine suitability for the desired growth medium. The forms of whey included fresh, condensed and powdered products. The powdered whey offered the most advantages with the least disadvantages. Of the three powdered wheys examined, one, a "deionized" spray dried product called whey-lac (Consolidated Dairy Products, 635 Elliott Ave., Seattle 99, Washington) was chosen. Acids and a small amount of calcium are removed from the product in an exchange column, the manufacturers state.

On reconstitution to 7% solids (64, p.4) it has a reaction of pH 6.8. It is usually translucent like fresh whey, and autoclaves to a clear brownish liquid with little or no precipitate. This product seemed best suited to the need.

Growth in 7% Whey-lac reached a maximum population of only 10 to 50 x 10^7 viable cells per ml. when a 1% inoculum from a milk culture was used. After one or two transfers of this culture, however, no more growth occurred in the Whey-lac medium. It was apparent that this product is deficient in certain factors for growth of Lactobacillus acidophilus.

Since the product is described as a "deionized" powder, it was thought that added inorganic ions might restore the whey to a complete growth medium. Addition of Henderson and Snell (21, pp.31-38) synthetic medium salts (phosphate, Mg^{++}, Mn^{++}, Fe^{++} and NaCl) resulted in only a slight growth stimulation in Whey-lac. These salts caused a similar stimulation of growth when added to 10% milk.

When a synthetic medium was developed that would support the growth of Lactobacillus acidophilus it was used in combination with Whey-lac to determine the additions necessary for growth in the whey product.

Experimental Methods

Stock Cultures. The six strains (see page 35) of Lactobacillus acidophilus used were transferred daily in reconstituted 10% spray dried, low heat milk powder in aluminum capped Pyrex tubes. The milk was made fresh weekly and sterilized at 121°C. for 12 minutes then stored at room temperature. Incubation was at 37°C. and the cultures set up in 10 hours or less.

Stock Solutions. Double strength (14%) reconstituted Whey-lac solution was made up in distilled water and stored in the deep-freeze until used. Stock solutions of amino acids, vitamins and other synthetic medium constituents were prepared as described in Part I, page 36. The synthetic medium used to supplement Whey-lac was prepared from the stock solutions and made to double strength at pH 7.0. It was added to double strength (14%) Whey-lac, giving a final concentration of 7% Whey-lac, with the synthetic medium constituents at the levels suggested by Henderson and Snell (21, pp.31-38).

Triplicate tubes of 2 ml. each, for each strain studied, were dispensed. Pyrex tubes (125 x 13 mm.), in aluminum racks with an aluminum cover, were used throughout. The medium was sterilized at 121°C. for 8 minutes, cooled and inoculated with one drop of the

inoculum described in Part I, page 43.

Incubation was at 37°C. for 39 hours, after which time the racks of tubes were placed in the deep-freeze. Titration for acid production was with a Canon Automatic Titrator. A pH 8.3 endpoint was determined by a Beckmann Probe Electrode Assembly and Model N pH meter, or a standardized Quinhydrone electrode system. Titration was carried out in the growth tubes and results were tabulated as titrator units, which were proportional to acid production.

Procedure. The complete synthetic medium of amino acids, vitamins, purines, pyrimidines, salts and accessory factors (pantotheine, vitamin B_{12} and thymidine) were added in double strength to double strength Whey-lac (14%) so as to give a final single strength medium when adjusted to pH 7.0 and made to volume. This medium and similar media with only the various groups of synthetic medium constituents added, were studied. The techniques and methods used paralleled those used in the Nutrition Studies. Lactobacillus arabinosus 17-5 and Streptococcus faecalis (ATCC 8043) were used as control organisms.

Results

Table 11 shows the results of the first experiment.

Increased Whey-lac solids had little effect. The maximum growth that resulted on the addition of all synthetic medium constituents was duplicated only when the amino acids supplemented the whey product. Addition of vitamins and inorganic ions did not improve the growth medium. Catalytic quantities of peptides, (0.1% NZ-amine, Seheffield Chemical Company Inc., Norwich, N.Y.), in the presence of amino acids stimulated greatly, but had little effect when added alone to Whey-lac.

The composition of whey includes only 0.6% protein (64, p.4); therefore it seemed likely that the whey medium was low in essential amino acids or nitrogen compounds. Table 12 shows that addition of 1% NZ-amine to 7% Whey-lac allowed excellent growth. NZ-amine, an enzymatic casein hydrolysate, was compared to Nutrient L-1 (Sheffield Chemical Company Inc.) an enzymatic lactalbumen hydrolysate. It appears that NZ-amine is not as stimulatory as Nutrient L-1 alone or NZ-amine in combination with Nutrient L-1.

Nutrient L-1 would be the medium of choice because of its greater stimulatory power and lower cost, as compared to NZ-amine. However, Nutrient L-1 is no longer available.

Another Lactalbumen hydrolysate called Lactalbumen peptone (Sheffield Chemical Company Inc.) is available

at about 1/3 the cost of NZ-amine. This is described as a calcium complex of enzymatically digested lactalbumen, but it is not water soluble. It makes up to a whitish opaque suspension at the concentrations desired.

The first sample tested could be filtered to clarity. This filtrate was compared to NZ-amine for growth promotion as shown in Table 13. It appeared to be slightly better in growth stimulation than NZ-amine, at the same concentration. Later samples and large scale attempts at filtration in the laboratory proved unsatisfactory, however, and heat treatment of Lactalbumen peptone in Whey-lac resulted in coagulation. It was necessary to sterilize the two constituents separately, therefore, and then mix. For these reasons this product was discarded.

Heating NZ-amine in Whey-lac caused no coagulum. Because of this and the simplicity and ease of handling in preparation of a clear solution of peptides at the desired levels, NZ-amine was chosen to supplement Whey-lac as a growth medium for L. acidophilus.

Increasing the NZ-amine level caused increased total growth as shown in Table 14. The addition of over 3% NZ-amine had little additional effect of growth stimulation. With a 3% level of NZ-amine, the growth medium was very viscous, though it did not coagulate on heating as it did when over 3% was added. However, 3% NZ-amine

appeared to be too viscous for efficient centrifugation. Therefore the level of 2% NZ-amine was chosen as most suitable for growth.

Any lowering of the Whey-lac solids level resulted in the decreases in total growth shown in Table 15. Whey-lac solids higher than 7% are undesirable since a precipitate often occurs on heat sterilizing of the medium.

The effects of two different levels of inorganic salts used in synthetic media (26, p.728) were determined. The salts concentrations are summarized:

	A_1	A_2
$K H_2 PO_4$	300 mgm	100 mgm
$K_2 H PO_4$	300 mgm	100 mgm
	B_1	B_2
$Mg SO_4 7 H_2 O$	280 mgm	80 mgm
$Mn SO_4 7 H_2 O$	42 mgm	12 mgm
$Fe SO_4 7 H_2 O$	14 mgm	2 mgm
Na Cl	4 mgm	2 mgm

From the results shown in Table 16, it seems that a high level of phosphate or high cation levels resulted in a slightly lower total growth than lower levels of phosphates or cations. When phosphates and cations were used together the Salts Medium 2 seemed slightly better

than Medium 1.

Table 17 shows the effect of omission of single cations and the effect of added calcium. Here, Mg^{++} ion and Mn^{++} ion appear to have had an inhibitory effect, since the omission of either one resulted in greater total growth. The omission of Fe^{++} ion or NaCl seemed to have little effect on most strains. The addition of calcium ions caused a great stimulation of all strains, but the growth medium coagulated on heating. It seems unnecessary or undesirable, then, to add any cations.

Plate counts in T_{19} agar (15, pp.125-126) of Lactobacillus acidophilus made when the culture had reached pH 4.3 (the limiting reaction where it was found that maximum population occurs in milk and whey) are shown in Table 18. The best medium of those tested seems to be 7% Whey-lac, 2% NZ-amine, and Salts A_2 (0.1% phosphates) when buffered with 0.5% sodium acetate. This level of acetate in 7% Whey-lac produces approximately the same buffering capacity as that of 10% reconstituted milk. When used in 7% Whey-lac and 2% NZ-amine, this level of acetate resulted in a doubling of the maximum viable population at pH 4.3. Counts on 10% milk and 10% milk with a stimulatory level (0.1%) of added peptides, were lower than those on the best whey medium.

The presence of the added phosphate (Salts A_2) also

seemed to aid in securing a clearer product on autoclave sterilization.

The final growth medium used in production of large quantities of Lactobacillus acidophilus consists of 7% Whey-lac, 2% NZ-amine, 0.1% phosphate and 0.5% sodium acetate.

Summary and Conclusions

In an effort to develop a fairly clear growth medium for the production and centrifugation of large numbers of Lactobacillus acidophilus, a powdered whey resuspended to 7% solids content, was studied. It was found to be deficient in the protein materials required for growth of L. acidophilus. An enzymatic casein hydrolysate was found best suited to supplement the whey product for growth of this organism. A concentration of 2% NZ-amine was found optimum, and any variation of the whey solids content from 7% was found to be undesirable.

The addition of cations was found to be unnecessary, and a level of 0.1% phosphate was more suitable than 0.3% in growth promotion. Phosphate also aided in producing a clear product on heat sterilization of the medium. The addition of 0.5% acetate increased the buffering capacity of the growth medium and resulted in a doubling in the maximum population.

The final growth medium used in production of large quantities of Lactobacillus acidophilus consisted of 7% Whey-lac, 2% NZ-amine, 0.1% Phosphate and 0.5% sodium acetate. The reaction was set at pH 7. A maximum population as high as 220 x 10^7 cells per ml. was obtained.

Table 11

Influence of Groups of Synthetic Medium Growth Factors on Growth of L. acidophilus in reconstituted Whey-lac

7% Whey-lac plus:	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
No addition	8[2]	9	7	10	9	8
1% Whey-lac	9	13	10	8	12	9
Complete synthetic medium	90	85	89	91	78	93
Amino acids and mineral salts	90	91	91	94	80	85
Vitamins and mineral salts	4	9	7	6	7	6
Mineral salts	4	10	8	8	8	7
Complete synthetic medium plus 0.1% NZ-amine	107	107	106	109	93	106
0.1% NZ-amine	9	15	13	12	15	12

1 Cultures incubated 39 hours at 37°C.

2 Average of titrator units for triplicate tubes. One titrator unit represents acid production neutralized by 0.04 ml. of 0.03 Normal NaOH.

Table 12

Comparison of the Effects of NZ-amine and Nutrient L-1 on the Growth of L. acidophilus in Whey-lac

7% Whey-lac and mineral salts plus:	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
No additives	19[2]	20	28	15	19	17
1% NZ-amine	87	81	84	80	88	83
1% Nutrient L-1	102	96	102	82	91	94
0.5% NZ-amine and 0.5% Nutrient L-1	103	95	99	100	99	103

1 Cultures incubated 39 hours at 37°C.

2 Average of titrator units for triplicate tubes. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 normal NaOH.

Table 13

Comparison of the Effects of NZ-amine and Lactalbumen Peptone on the Growth of *L. acidophilus* in Whey-lac

7% Whey-lac and mineral salts plus:	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
1% NZ-amine	68[2]	68	64	56	63	63
1% Lactalbumen peptone	93	85	88	77	82	92

1 Culture incubated 39 hours at 37 °C.

2 Average of titrator units for triplicate tubes. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 14

Effect of Increasing NZ-amine Concentrations on the Growth of *L. acidophilus* in Whey-lac

7% Whey-lac and mineral salts plus:	Acid production by following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
1% NZ-amine	84[2]	77	74	69	74	123
2% NZ-amine	108	100	105	99	100	136
3% NZ-amine	143	132	131	130	124	154
4% NZ-amine	138	131	132	135	137	151

[1] Cultures incubated 39 hours at 37°C.

[2] Average or titrator units for triplicate tubes. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 15

Effect of Decreased Whey-lac Concentrations on Growth of L. acidophilus in the presence of 1% NZ-amine

1% NZ-amine and mineral salts plus:	Acid production by the following strains[1]:					
	4962	4963	9857	DLF	OSC	FARR
7% Whey-lac	79[2]	73	75	71	78	76
5% Whey-lac	64	59	60	56	64	57
3% Whey-lac	38	35	36	37	32	33
1% Whey-lac	17	20	20	18	20	19

[1] Cultures incubated 39 hours at 37°C.

[2] Average of titrator units for triplicate tubes. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 16

Effect of Two Different Levels of Mineral Salts on the Growth of *L. acidophilus* in a Whey-lac and NZ-amine medium

Mineral salts tested	Acid production by the following strains :					
	4962	4963	9857	DLF	OSC	FARR
Salts A_1	47[2]	53	49	45	38	41
Salts B_1	70	62	66	62	64	66
Salts A_2	77	75	81	75	80	76
Salts B_2	79	72	74	71	71	72
Salts A_1 and B_1	73	69	64	66	66	56
Salts A_2 and B_2	75	72	77	72	72	72

1 Cultures incubated 39 hours at 37°C.

2 Average of titrator units for triplicate tubes. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 17

Effect of Omission of Single Cations of Mineral Salts B_2 on the Growth of *L. acidophilus* in the Whey-lac and NZ-amine Medium

Ion omitted	Acid production by the following strains[1]: 4962	4963	9857	DLF	OSC	FARR
No omission	75[2]	65	69	62	69	67
Mg	77	78	78	74	72	72
Mn	92	87	89	80	82	84
Fe	76	72	69	66	68	73
NaCl	80	73	67	65	63	69
No omission, Ca added	104	106	103	103	98	101

1 Cultures incubated 39 hours at 37°C.

2 Average of titrator units of triplicate tubes. One titrator unit represents acid production neutralized by 0.04 ml. of 0.05 Normal NaOH.

Table 18

Maximum Growth[1] at pH 4.3, in Experimental Media as Determined by Plate Count on T_{19} Agar

Growth Medium	Time to reach pH 4.3	Count at pH 4.3
7% Whey-lac plus:	hours	no. x 10^7
1. 0.1% phosphate	13.5	57
2. 1% NZ-amine	8.5	90
3. 1% NZ-amine plus 0.1% phosphate	9.0	118
4. 2% NZ-amine plus 0.1% phosphate	9.5	117
5. 2% NZ-amine plus 0.1% phosphate plus 0.5% acetate	13.5	220
6. 10% milk	13.5	154
7. 10% milk plus 0.1% NZ-amine	13.0	162

1 Inoculated with 1% inoculum.

PART III. FREEZING PRESERVATION

Published studies on freezing preservation of bacteria seem to agree that higher solids menstrua, more neutral menstruum reactions and lower freezing temperatures tend to give higher freezing and storage survival.

Preliminary experiments agreed with the findings that higher solids and more neutral reactions offered better protection to frozen cultures. These experiments involved both cultures grown and frozen in milk of increasing solids content, and broth grown cells resuspended in milk, whey, sugar solutions or colloidal suspensions. In these preliminary experiments it became apparent that great variability due to the nature of the techniques used and to many unknown factors, would necessitate statistically sound design and analysis. Therefore, a statistically designed method was developed from which conclusions might be drawn.

Materials and Methods

The method involved four or five replicates of each experiment. Within each replicate the initial count was to be determined by drawing a number of samples related to the number of subsequent samples compared to it, according to the relationship:

$I = S\sqrt{D}$

where I = number of samples drawn for the initial count

S = number of samples drawn for each subsequent determination (i.e., duplicate samples

D = number of subsequent determinations to be compared to the initial count (i.e., determinations over 5 storage periods)

In the experiments to follow, for each experimental condition, the initial count was detemined, then duplicate samples (i.e., S = 2) were taken after storage intervals of one, two, four, six and eight weeks (so here D = 5). In some experiments, the determinations for all conditions could be compared to one averaged initial count (here, D = 5x no. of conditions) while in other experiments each condition necessitated its own initial count. Each sample was analyzed by the plate count method, with triplicate or quadruplicate counts averaged, for each determination.

Procedure

Growth Medium. The growth medium was always prepared the day it was to be inoculated. Ten liters were made by suspending 700 grams of Whey-lac powder, 200 grams of

NZ-amine, 50 grams of sodium acetate, and 50 ml. of the phosphate solution (10% KH_2PO_4, 10% K_2HPO_4, ph 7.0), in about 5 liters of lukewarm water. Stirring hastened the suspension of the powdered constituents, though they would suspend in about one-half hour on standing in the lukewarm water. The reaction of the medium was checked, and adjusted to pH 7 if it was more than 0.2 units off. The medium was then poured into a 12 litre Pyrex solution bottle and diluted to 10 litres with tap water. The bottle was plugged with a two holed rubber stopper having a "breather tube" with a cotton-filled air-sterilizing filter, and a long glass siphon tube, which extended to the bottom of the bottle. The whole top and neck were covered with Kraft paper held by rubber bands, to minimize contamination when the stopper was removed for inoculation. When all foam in the growth medium subsided, the bottle was placed in the autoclave, the rubber stopper was loosened and the steam turned on. The autoclave was run for about 10 minutes with the exhaust valve open to allow time for the bottle and media to come up to a high temperature. The exhaust valve was then closed and the medium was held at 121°C. for 45 minutes. At this time the steam was shut off, leaving the exhaust valve tightly closed, and the autoclave was allowed to come down slowly to atmospheric pressure (in about 2 hours). The autoclave

door was opened and left for about 30 minutes to allow further cooling and to safeguard against "bumping" of superheated liquid when the bottle was lifted out of the sterilizer. The bottle was then placed into a deep water bath, containing a stirring device, to cool. Cold water was added to the bath occasionally to aid the cooling. In about one hour, when the temperature of the medium was about 37°C. (as judged by the temperature of the water bath and the bottle), the bottle was inoculated with 100 ml. of a 24 hour old culture. The culture medium was incubated overnight in a stirred 37°C. water bath, until the medium reached a reaction of pH 4.3, at which time it was neutralized to pH 6.5 and then centrifuged.

Inoculum. 100 ml. quantities of the inoculum medium in 8 ounce prescription bottles were made up weekly. They were of the same composition as the growth medium, but were sterilized for 15 minutes at 121°C. They were stored in the refrigerator until needed. The first inoculum medium was originally inoculated with 1 ml. from a 24 hour old milk culture, and was incubated at 37°C. for 24 hours. The subsequent inoculum media were inoculated by a transfer from each inoculum before it was used.

Centrifugation. A Sharples Laboratory Model Supercentrifuge (steam turbine model) was used to separate the

lactobacilli from the growth medium. The centrifuge bowl, feed nozzle and a long rubber tube to fit were wrapped in Kraft paper, and sterilized before each run. After sterilizing, the bowl was cooled by leaving in the refrigerator for several hours, or over night.

The centrifuge was brought up to about 40,000 RPM with a steam pressure of 24-26 psi. When sufficient speed had been attained, the siphon was started by blowing into the breather tube. The rate of flow through the centrifuge was controlled by a screw clamp on the siphon feed hose. The correct rate of flow was judged by the clarity of the centrifuge effluent, in a 10 ml. graduate cylinder. The rate of flow was usually about 500-700 ml. per minute. It was found to be of considerable importance to maintain the reaction of the culture above pH 5.8-6.0, since below this range, proteinacious material tended to flocculate and interfere with efficient centrifugation. After 10 litres of the culture had been centrifuged it was found advisable to stop the centrifuge, remove the bowl and scrape out the accumulated cellular material into a sterile, chilled Waring Blendor cup. This allowed more efficient centrifugation of the remaining culture, since the effective centrifuging diameter of the bowl was increased when the cells were removed.

After all the culture media had been centrifuged, the bowl was again removed and the cells scraped out with a large sterile spatula, into the sterile Waring Blendor cup, and a small amount of Whey-lac was added. The Blendor was run at a very slow speed until a smooth suspension was obtained. The suspension volume was adjusted in a sterile graduate cylinder using sterile menstruum so as to give an estimated concentration of 100 x 10^9 viable cells per ml. The concentrate was then treated as described in each of the experiments to follow.

Adjustment of pH. The method used to adjust the pH involved adding saturated KOH, with stirring, to the cell concentrate in a sterile beaker until the desired pH was attained as shown by a pH meter. The electrodes had been sterilized by immersing in 95% ethanol before immersion into the concentrate. Stirring the viscous or plastic mass was with a sterile spatula or spoon.

Dispensing. The concentrate was dispensed into 20 x 125 mm. screw cap tubes with a Brewer automatic dispenser, or a large mouth 10 ml. milk dilution pipette.

Plating. Appropriate dilutions were plated and the plates were poured with T_{19} agar containing Tween 80, as described by Hannesson (15, pp.125-126). The plates were

incubated at 37°C. for two days and then counted.

Quick Freezing. It was found necessary to freeze the concentrate of lactobacilli immediately after dispensing, to prevent the great rate of acid production from changing the menstruum from the reaction desired for each experiment. The method devised to accomplish this rapid freezing involved placing the racks of tubes into a large pan of a precooled eutectic salt solution before dispensing the concentrate. For tubes to be frozen at -10°C., eutectic NaCl was cooled to -15°C. It was found that when dispensing was finished this brine bath had warmed to -10°C. This series of tubes in brine was then placed in a freezer at -10°C. for several hours. Tubes to be frozen at -20°C. were placed in a larger volume of eutectic brine precooled to -22°C., the concentrate was dispensed and the tubes and brine were placed in a freezer at -20°C. The tubes to be frozen at -60°C. were placed into a $CaCl_2$ eutectic brine precooled to -58°C. The concentrate was dispensed and then the tubes alone were placed into the -60°C. Chill-chest.

After several hours the tubes were removed from the brine baths, capped and stored in labelled wire baskets. The tubes were left in the higher temperature brines to bring the temperatures of the tubes down to the

temperature to be studied as rapidly as possible. Since the tubes to be frozen at -60°C. were quick-frozen at -55-58°C. it was felt that the tubes alone in wire baskets would reach -60°C., faster than if they were left in a rather large volume of brine.

Thawing of the Tubes. At the time the samples were to be plated, the tubes were removed from the freezer, and placed into an open wire rack. The rack was placed into an agitated 32°C. water bath, and left for exactly 4 minutes. At this time the concentrate was always just melted.

Experimental

Experiment 1. Effect of Solids in the Freezing Menstruum on Survival of L. acidophilus in Frozen Concentrates

Method. A cell concentrate was made and divided into three parts. Each was diluted one-to-one with either sterile water, 7% Whey-lac, or 14% Whey-lac. The organisms were grown in 7% Whey-lac so the dilutions described should give final solis concentrations respectively of 3.5%, 7% and 11.5% Whey-lac.

Each concentration was dispensed into tubes immersed in eutectic brine at -20°C. Four unfrozen samples were taken, plated in triplicate and the plate counts were

averaged for the initial count.

During the storage period, duplicate samples were taken from each concentration block and triplicate plate counts were averaged for the surviving numbers. These values are reported as percent survival based on the initial count.

Four replicates of this procedure were made on four different days.

Results and Discussion. The Analysis of Variance and the Table of Means are shown in Tables 19 and 20. There was no statistical evidence that the differences in menstruum solids used here caused any consistent differences in survival, under the conditions used. However, larger changes in menstruum solids content gave evidence showing that the higher proteinaceous solids in the cell concentrate have some protective effect. These results were obtained in the experiment on the effect of cell concentration and the evidence is discussed in that experiment.

Various workers in the study of freezing injury to bacteria have reported that higher non-toxic menstruum solids exert a protective effect on suspended cells (25, pp.877-879; 22, pp.423-431; 54, pp.40-45; 60, pp.1043-1047; 36, pp.43-57; 38, p.491; 56, pp.32-34). A limiting concentration beyond which the addition of higher

Table 19

Analysis of Variance Table

Effect of Solids of Suspending Medium of Frozen Concentrates of L. acidophilus. Percent Survival after Storage for 1, 2, 4, 6 and 8 Weeks

Source of Variation	D.F.	Sum of Squares	Mean Square	F
Total	59	8,622.60	------	---
Replicates	3	4,235.40	1,411.95	9.12*
Media	2	313.90	156.95	1.01
Reps x Media (error a)	6	929.30	154.88	---
Storage Interval	4	610.43	152.61	2.00
Stor. Int. x Reps. (error b)	12	913.44	76.12	---
Stor. Int. x Media	8	602.77	75.35	1.78
Stor. Int. x Reps. x Media (error c)	24	1,017.36	42.39	---

* Significant at the 5% level.

Table 20

Effects of Solids of Suspending Menstruum on Survival of Frozen Concentrates of *L. acidophilus*

Table of Means

Percent Survival After 1, 2, 4, 6 and 8 Weeks Storage

storage interval in weeks	Adjusted solids of menstruum 3.5%	7%	11.5%	storage interval means
1	96.5	90.8	108.5	98.6
2	101.8	95.0	101.5	99.4
4	95.8	98.8	97.0	97.2
6	97.2	94.8	101.5	97.8
8	88.5	92.5	90.5	90.5
MEDIA MEANS	96.0	94.4	99.8	96.7

non-toxic solids has no additional protective effect, is probably reached. It is probably in this plateau region of solids concentration that the cells of this concentrate find themselves. Therefore slight dilution or the addition of a small amount of protective solids may have no significant effect on survival.

Experiment 2. Effect of pH on the Freezing Menstruum on the Survival of L. acidophilus in Frozen Concentrates

Method. A cell concentrate was made and adjusted to a given pH. The concentrate was dispensed into tubes immersed in eutectic brine at -22°C. Four unfrozen samples were taken and plated in triplicate and the plate counts were averaged for the initial value.

The remaining concentrate was adjusted to the next pH value desired and the concentrate was treated, dispensed and sampled as above. This procedure was repeated for a third pH value. The pH levels tested were 4.5, 5.5, and 7.0.

Five replicates of this procedure were made on five different days.

Results and Discussion. The Analysis of Variance and the Table of Means are shown in Tables 21 and 22. There was slight evidence of a pH effect, but this effect

was not statistically significant. The slight evidence indicated that more neutral reactions of the freezing menstruum were more favorable for survival under the conditions used. Storage death was not detectable with the methods used and there was no evidence that pH affects "storage death".

The pH range chosen for study here includes values likely to be used in the production of a frozen concentrate. Reactions beyond the range studied may cause more drastic differences in survival, but these reactions are not likely to be encountered.

The preliminary studies on effects of pH of the freezing menstruum on survival indicated a greater survival at neutral pH values than at more acid reactions. These studies, however, were with cells centrifuged from a clear broth and resuspended in Whey-lac. Five ml. amounts in tubes were frozen by placing in a deep freeze at -22°C. This method of freezing was slower than that of immersing the tubes in eutectic brine. It may be that slow freezing accentuates the effects of low reactions in the freezing menstruum. Perhaps the combination of very rapid freezing in eutectic brine and the protective action of the heavy proteinaceous material in the resuspended concentrate modifies the damaging action of an acidic

freezing menstruum on these cells. If the organisms and menstruum were more rapidly taken through the most damaging temperature range (0° to -10°C.), then cellular protein denaturation by toxic salts and hydrogen ions would have less time in which to act and cause permanent damage (41, p.519). Devik and Ulrich (6, p.168) found E. coli and Lactobacillus casei to be more readily destroyed by slow than quick freezing. Beard and Cleary (4, pp.141-144) found enhancement of bactericidal effects of hydrogen ion at low temperatures. Winslow and Falk (65, pp. 237-244) showed that toxicity of NaCl on E. coli was greater below pH 6.0. Conceivably, cold could enhance this effect even more. Meryman states that the effects of damaging factors during freezing are biochemical effects, and as such, they are time dependent (41, p.518). Therefore if the time of exposure of the organisms to deleterious factors could be minimized, survival should be enhanced.

In this, as in all these procedures, the difficulty of obtaining a uniform cell suspension in the sometimes plastic mass contributes to the large variation in survival over the storage period.

Although the results obtained did not definitely establish the necessity of neutral menstrua, it would seem advisable to use more neutral reactions in the

Table 21

Analysis of Variance Table
Effect of pH of Menstruum on Survival of Frozen Concentrates of L. acidophilus. Percent Survival after storage for 1, 2, 4, 6 and 8 Weeks

Source of Variation	D.F.	Sum of Squares	Mean Square	F
Total	74	12,983.95	---	
Replicates	4	4,888.88	1,222.22	4.04*
pH	2	1,215.23	607.62	2.01
Low vs. High	1	722.00	722.00	2.39
pH Remainder	1	493.23	493.23	1.63
Reps. x pH (error a)	8	2,419.44	302.43	---
Storage Interval	4	622.35	155.59	1.12
Stor. Int. x Reps. (error b)	16	2,216.05	138.50	---
pH x Stor. Int.	8	355.97	44.50	1.12
pH x Reps. x Stor. Int. (error c)	32	1,266.03	39.46	---

* Significant at the 5% level.

Table 22

Effect of pH of Menstruum on Survival of Frozen Concentrates of *L. acidophilus*

Table of Means

Percent Survival After 1, 2, 4, 6 and 8 Weeks Storage

storage interval in weeks	pH 4.5	pH 5.5	pH 7.0	storage interval means
1	85.6	91.6	87.4	88.2
2	82.2	95.6	95.0	90.9
4	83.8	97.4	90.6	90.6
6	80.2	83.6	86.0	83.3
8	83.6	93.4	94.4	90.5
pH means	83.1	92.3	90.7	88.7 grand mean

LSD = 11.4%

concentrate for freezing since this would facilitate handling and dispensing. Acid reactions in the proteinaceous concentrate increase plasticity. In addition the flavor is less distasteful than the more acidic product. If sufficient samples were taken it does not seem unlikely that more neutral reactions would give evidence of favoring better survival.

Experiment 3. Effect of the Freezing and Storage Temperature on Survival of Frozen Cells

Method. A cell concentrate was made and dispensed into tubes immersed in eutectic brine or $CaCl_2$ at one of three temperatures. Eight unfrozen samples were taken, quadruplicate plates counts being made and averaged for the initial count. The tubes were frozen in eutectic brine at -10°C., and at -22°C., or in eutectic $CaCl_2$ at -58°C.

During storage, duplicate samples were taken from each temperature and quadruplicate plate counts were averaged for the surviving numbers. These values are reported as percent survival based on the initial count.

Results and Discussion. The Analysis of Variance and the Table of Means (Tables 23 and 24) indicated only slight evidence that the lower freezing temperatures

(-60°C.) yielded better survival on storage under the conditions used.

Other workers in freezing death studies have found lower freezing temperatures to be less damaging to cells (13, pp.451-453; 62, p.429; 56, pp.32-34; 32, p.253). These workers used simple systems and more ideal conditions for their studies. This study, by its nature, involved great variation in results. The variation between samples and within a sample, due to the uneven cell distribution, contribute to the difficulty. Also there seems to be considerable protection offered by the heavily proteinaceous resuspended concentrate that may modify the effects of destructive factors at higher freezing temperatures. Evidence for this effect is discussed in the experiment on the effect of cell concentration (see page 108).

Table 23

Analysis of Variance Calculations
Effect of Freezing and Storage Temperatures on Survival of Frozen Concentrates of L. acidophilus. Percent Survival after Storage for 1, 2, 4, 6 and 8 Weeks

Source of Variation	D.F.	Sum of Squares	Mean Square	F
Total	59	13,910.58	---	---
Replicates	3	8,277.38	2,759.13	9.18*
Temperature	2	774.43	387.22	1.29
Reps. x Temp. (error a)	6	1,803.57	300.60	---
Storage Interval	4	811.66	202.92	4.39*
Stor. Int. x Reps. (error b)	12	544.21	46.18	---
Temp. x Stor. Int.	8	250.24	31.28	0.52
Temp. x Stor. Int. x Reps. (error c)	24	1,439.09	59.96	---

* Significant at the 5% level.

Table 24

Effect of Freezing and Storage Temperature on Survival of Frozen Concentrates of *L. acidophilus*

Table of Means

Percent Survival After Storage for 1, 2, 4, 6 and 8 Weeks Storage

storage interval in weeks	freezing temperatures			storage interval means
	-10°C.	-22°C.	-60°C.	
1	90.8	87.5	95.2	91.2
2	90.2	86.8	97.2	91.4
4	84.2	75.2	89.5	83.0
6	90.0	82.2	87.8	86.7
8	80.0	81.8	87.8	83.2
TEMP MEANS	87.0	82.7	91.5	87.1

STORAGE INTERVAL LSD. 5% level - 6.04
1% level - 8.47

Experiment 4. Effect of Cell Concentration on Survival of L. acidophilus in Frozen Concentrates

Method. A cell concentrate was made and adjusted to a neutral reaction. This concentrate (approximately 50 x 10^9 cells/ml.) was dispensed into 10 tubes immersed in eutectic brine and frozen. Four unfrozen samples were taken and triplicate plate counts were made and averaged for the initial value. An aliquot was taken from the remaining concentrate and diluted ten times. This concentrate (approximately 50 x 10^8 cells/ml.) was dispensed into 10 more tubes, frozen and samples taken as above. Another aliquot of the concentrate was taken and diluted 100 times. This concentrate (approximately 50 x 10^7 cells /ml.) was dispensed, frozen and sampled as above.

Duplicate samples were taken during storage and triplicate plate counts were averaged for surviving numbers. These values were tabulated as per cent survival based on the initial count.

Results and Discussion. The Analysis of Variance and the Table of Means are shown in Tables 25 and 26. There was a great and real difference between the undiluted concentrate and the diluted samples. There was no appreciable difference between the ten-fold and the 100 fold dilutions. Storage death was detectable in the

diluted but not in the undiluted cell suspensions.

Although the survival varied between the various experiments described in this study, the survival was in all cases much higher than survival found in preliminary experiments or in the diluted concentrates described above. Preliminary experiments were made using cultures grown and frozen in the same menstruum, or with cells grown in a clear broth then resuspended in a freezing menstruum. These final experiments were carried out using cells centrifuged from the Whey-lac growth medium. On centrifugation, considerable proteinaceous material was spun out with the cells and resuspended with them in the freezing menstruum. In a previous experiment the effect of a three-fold fluctuation in added freezing menstruum solids (Whey-lac) had little or no effect on the rate of survival of the frozen cultures. In this experiment, although its purpose was not to demonstrate the effects of menstruum solids, it can be seen that dilution of the concentrate by ten or one hundred fold results in a markedly lower rate of survival and a definite storage death. There is only slight evidence (not statistically significant) that there is a difference in survival in the two diluted cell suspensions and the indication is that the lower, not the higher cell concentrate, survives slightly better. Major et al. (35, pp.244-249) reported

Table 25

Analysis of Variance Table
Effect of Cell Concentration on Survival of Frozen Concentrates of L. acidophilus. Percent Survival after Storage for 1, 2, 4, 6 and 8 Weeks

Source of Variation	D.F.	Sum of Squares	Mean Squares	F
Total	59	15,773.93	----	----
Replicates	3	2,143.00	714.33	2.02
Cell Concentration	2	8,093.73	4,046.86	11.47*
Reps. x Cell Conc. (error a)	6	2,116.80	352.80	----
Storage Interval	4	933.43	233.36	4.66*
Stor. Int. x Reps. (error b)	12	601.50	50.12	----
Stor. Int. x Cell Concentration	8	957.77	119.72	3.10
Stor. Int. x Cell Conc. x Reps. (error c)	24	927.70	38.65	----

* Significant at the 5% level.

Table 26

Effect of Cell Concentration on Survival of Frozen Concentrates of *L. acidophilus*

Table of Means

Percent Survival After 1, 2, 4, 6 and 8 Weeks Storage

Storage interval in weeks	Approximate cell concentration per ml. 50×10^7	50×10^8	50×10^9	Storage interval means
1	90.5	75.5	85.5	83.8
2	98.8	71.5	82.0	84.1
4	96.0	72.0	80.5	82.8
6	95.0	60.0	73.5	76.2
8	98.2	59.2	67.5	75.0
Cell concentration means	95.7	67.6	77.8	80.4

Cell concentration LSD - 5% level - 14.54
1% level - 22.02

Storage interval LSD - 5% level - 6.30
1% level - 8.83

that variations in cell concentrations of Lactobacillus acidophilus had no effect on the rate of survival of the frozen cells. Therefore, there is some indication from the results of this experiment that the heavy proteinaceous material present in the high concentrate, when centrifuged and resuspended, may have been contributing to the excellent recovery and low storage death rate. This protection may be a combination of factors including "buffering" of the immediate environment surrounding the cells, by the proteinaceous material. The "buffering" action may be against toxic ions, including the hydrogen ion, that are concentrated in the unfrozen portion of the menstruum along with the cells.

Experiment 5. Survival of Lactobacillus acidophilus in Frozen Concentrates on Six Month Storage at -20°C.

Method. A cell concentrate was made and dispensed into tubes immersed in eutectic brine at -20°C. Ten ml. of the concentrate was dispensed into each of the tubes, in this experiment. Six unfrozen samples were taken and quadruplicate plate counts were averaged for the initial count.

During storage, duplicate samples were taken and quadruplicate plate counts were averaged for the surviving numbers. These values are reported as percent survival

based on the initial count. Storage survival was determined at 1, 2, 4 and 6 months.

Results and Discussion. Storage death, as shown in Table 27, is undetectable with the methods used and under the conditions of this experiment. After the "immediate" death that occurs on freezing, the concentrate remains essentially constant in viable numbers. This was also true in the other storage experiments carried out in this study. The survival attained in these concentrates is satisfactory. If the initial cell concentration in the concentrate was 100 x 10^9 per ml., then 50 to 90 x 10^9 viable cells per ml. can reasonably be expected even after 6 months storage at -20°C. Therefore 10 ml. of this concentrate would be equivalent to one litre of commercial acidophilus milk, which usually contains 50 to 100 x 10^7 viable cells per ml. It has been recommended that a pint to a quart of acidophilus milk be taken each day, when the product is used therapeutically. It is suggested then, that the concentrate be made up in either 5 or 10 ml. quantities and added to a pint or quart of milk for consumption. In this form the "acidophilus milk" is as palatable as fresh milk, and contains sufficient viable organisms for effective therapeutic use.

Table 27

Survival of *Lactobacillus acidophilus* in Frozen Concentrates on 6 Month Storage at -20°C.

Replicate Number	Percent survival after the following storage periods				Storage interval means
	1 Month	2 Months	4 Months	6 Months	
1	69	70	72	88	77
2	95	87	(91)*	90	91
3	95	98	92	121	101.5
4	88	89	51	87	79
5	52	41	45	44	46.5
Replicate survival means	80	77	70	86	

* Dummy value

SUMMARY AND CONCLUSIONS

The influence of four factors on the survival of concentrates of Lactobacillus acidophilus during frozen storage, have been studied. A three-fold variation in added menstruum solids had no effect on survival, although evidence from another experiment showed that perhaps larger variations in solids content could affect survival.

There was slight though inconclusive evidence that neutral menstruum reactions were more favorable for survival under the conditions used. The possible protective effect of the high concentrations of proteinaceous material in the concentrate was discussed.

Ten- and one hundred-fold variations in cell concentration were studied for their influence on survival. There was a great and real difference between the survival of the highest concentration and that of the two dilutions, but no difference between the survival of the two dilutions of the concentrate. A possible explanation for the differences in survival noted was made, and again the influence of proteinaceous materials in the concentrate seemed to be implicated.

Only slight but inconclusive evidence was obtained indicating that lower temperatures (-60°C.) favored survival on frozen storage.

After six months storage at -20°C., an average of 86% survival was found with five replicates. The range of survival was from 44% to 100%. This was considered good survival and made the use of frozen preservation of acidophilus concentrates feasable for therapeutic uses.

It would seem that no great precautions need be taken to ensure good survival of frozen concentrates of Lactobacillus acidophilus prepared as described in this study. Neutralization of the concentrate may not be necessary if the tubes are "quick frozen" in a eutectic brine, but a neutral reaction is to be recommended to ensure good storage survival. Lower temperatures, (-60°C. if available) may give better survival in the long run, but temperatures between -10° and -20°C. gave good survival in this study. The menstruum solids resulting from centrifugation of the cells from the growth medium seem to offer excellent protection against freezing damage, and large variations in the solids content of the concentrate would be necessary to affect the storage survival during freezing.

Five or ten ml. quantities of the concentrate containing 50 to 100 x 10^9 viable cells per ml. should be stored, and when needed, added to a pint or a quart of fresh milk for daily consumption. It has been found that such a product has no objectionable flavor.

The entire procedure of production and preservation of this concentrate requires certain special equipment and some technological or scientific training; however, the techniques involved could easily be adapted to commercial production. Sufficient concentrate could be made and frozen in one day to last for several months, thereby eliminating the need for daily preparation of small quantities of the product.

The difficulties associated with production and consumption of acidophilus milk, then, have been overcome. That is, the elimination of unpleasant flavor and of the necessity for daily production of small volumes of the product, have been accomplished.

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