ABSTRACT

TRAHAN, CAITLIN ELIZABETH. *Lactobacillus acidophilus* NCFM Survival in Acidified Yogurts. (Under the direction of Professor T. R. Klaenhammer.)

*Lactobacillus acidophilus* NCFM is a probiotic culture widely added to dairy products and dietary supplements. The bacterium has been linked with immune modulation, cold prevention in humans, and relief of gut pain via a morphine like mechanism in animals. Industrially, the target for delivery of viable cells in a 6 oz. serving of yogurt at the end of a 52 day shelf-life is $2 \times 10^6$ CFU/g. Survival studies with an industrial yogurt formulation showed that counts of NCFM fell to less than $10^2$ CFU/g at the end of shelf-life. The objective of this study was to investigate the reasons for the dramatic loss of viability during shelf-life, and define a solution that could maintain viability throughout shelf-life. Levels of NCFM were followed in fermented yogurts at pH 4.1, 4.7 and 5.0. Results showed that with inoculation levels of $10^8$ CFU/ml, NCFM added at the outset of fermentation maintained survival in yogurts acidified at pH 4.7 and 5.0. NCFM showed poor survival in yogurts acidified to pH 4.1, exhibiting a 3 log loss after 48 days. The possible effects of cell injury in lyophilized *Lb. acidophilus* NCFM cells were also investigated. Cells subjected to a 60 minute resuscitation period in MRS media, prior to addition to acidified yogurt base at pH 4.1, showed only a ~1.5 log loss after 42 days. Survival of NCFM in yogurt was compromised at acidic conditions below pH 4.7 and a recovery period of the lyophilized cells can greatly improve survival.
Lactobacillus acidophilus NCFM Survival in Acidified Yogurts

by
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BIOGRAPHY

Caitlin E. Trahan was born on June 19, 1983, in New Bedford, Massachusetts to Deborah and Robert Trahan. She attended the University of Massachusetts at Amherst where she completed her BS in Food Science in 2006. She then began her graduate program at North Carolina State University in August of 2006 under the direction of Dr. Todd R. Klaenhammer. Caitlin also competed in collegiate cycling and was the Atlantic Collegiate Cycling Conference women’s mountain biking champion in both 2006 and 2007.
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CHAPTER I

LITERATURE REVIEW

ACID RESISTANCE IN LACTIC ACID BACTERIA
INTRODUCTION

The current classification of life begins with the division of each organism amongst three domains: Eukarya, Bacteria and Archaea. Lactic acid bacteria (LAB) contain several genera which lie in the bacterial domain. These functionally related bacteria primarily produce lactic acid and are widely used in the food industry as means of preservation, flavor and texture enhancement (37).

The term lactic acid bacterium refers to “milk-souring organism”. In 1873, J. Lister obtained the first purified “milk-souring organism” termed Bacterium lactis (likely Lactococcus lactis). This core metabolic similarity resulted in the functional classification of LAB. Orla-Jensen in the early 1900s redefined the systematics of LAB by expanded phenotypic classifications that examined morphology, mode of glucose fermentation, range of growth temperatures and spectrum of fermentable sugars. With modern technologies, conserved genetic regions and sequences (e.g. 16s rRNA) are used to more precisely classify these bacteria, phylogenetically (53).

The emergence of the LAB dates to 3 billion years ago with the first recorded use around 3000 years BC. Since their use in food production, LAB have been selected, characterized and their controlled use has shaped the definition of many food products (12). LAB are characterized as gram-positive, catalase negative, nonspore forming, fastidious and acid-tolerant. They are aerotolerant and strictly fermentative producers of lactic acid. The core genera involved in food fermentations are Lactobacillus, Leuconostoc, Pediococcus, Oenococcus and Streptococcus.
However, the general group of LAB include around 20 others with current revisions in taxonomy (53).

Certain LAB are further considered to impart probiotic properties (28). Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (22). Some benefits of probiotics include (i) improving the intestinal health; (ii) enhancing the immune system; (iii) reducing the symptoms and prevalence of lactose intolerance; and (iv) reducing the risk of certain cancers (49). The majority of probiotic organisms classified to date are LAB.

The major defining characteristic of the LAB is the production of lactic acid via either homo or heterofermentative metabolism. However, the accumulation of lactic acid can be detrimental and the LAB have developed a number of strategies to cope with high acid and low pH environments (16). Moreover, probiotic LAB ingested daily must also survive the harsh conditions of the gastrointestinal tract (GIT). This involves a rapid drop in pH (via hydrochloric acid) upon entering the stomach exposure to digestive enzymes (e.g. pepsin) and bile salts in the GIT (53). Survival in acid environments involves a variety of defenses including export of protons from the cytoplasm, alkalization of the external environment, production of stress proteins and chaperones, altered metabolism and altered cell envelope composition (9, 14, 20, 46, 66). This review will focus on the mechanisms by which LAB protect themselves against acid environments.
Factors contributing to an acidic environment

Lactic acid

Metabolism

Fermentative metabolism leads to the creation of an electrochemical proton gradient across the cytoplasmic membrane which drives essential reactions. LAB do not possess a membrane-linked electron transport chain which is the most common system for creating a proton gradient in respiring organisms. Unlike respiring organisms, ATP is generated via substrate-level phosphorylation. This efflux of protons creates the proton motive force (PMF). LAB are able to consume a variety of carbohydrates and related compounds to generate this PMF. The predominant end product of carbohydrate metabolism is lactic acid. Bacteria which produce greater than 85% lactic acid are classified as homofermenters. Those that produce ~50% lactate plus equimolar amounts of CO₂, ethanol and/or acetic acid are defined as heterofermenters (53). These two fermentation pathways to lactic acid production can vary within each species (53). Homofermentative groups use glycolysis to ferment hexose sugars while heterofermentative groups use the phosphoketolase pathway to ferment both hexose and pentose sugars (33).

Glycolysis

The Embden-Meyerhof pathway is characterized by the presence of aldolase and glyceraldehyde-3-phosphate which catalyze vital steps in the production of pyruvate. The generation of pyruvate under normal conditions is reduced to lactate. This reduction is catalyzed by a NAD⁺-dependent lactate dehydrogenase (nLDH) which in turn reoxidizes the NADH
formed in the first steps of glycolysis. Since lactic acid is virtually the only end product the metabolism is referred to homolactic fermentation (Figure 1) (53).

**Phosphoketolase pathway**

Heterofermentative LAB are able to use both hexose and pentose sugars via the phosphoketolase pathway. This pathway is characterized by the formation of 6-phosphogluconate which is decarboxylated to produce CO₂ and pentose-5-phosphate which is then split by phosphoketolase into glyceraldehyde-3-phosphate and acetyl-phosphate. Glyceraldehyde-3-phosphate then proceeds to lactic acid via the same pathway steps used in glycolysis. Acetyl-phosphate is reduced to ethanol through acetyl CoA and acetylaldehyde. Since both CO₂ and ethanol are produced in significant amounts, this form of metabolism is referred to as heterolactic fermentation (Figure 2) (53).

**Generation of lactic acid**

Lactic acid is generated by the last step in the Embden-Meyerhof pathway through the conversion of pyruvate to lactate and subsequently regenerates NAD⁺ (27). Pyruvate is converted to either L(+) or D(-) lactate by the nLDH classified as EC 1.1.1.27 or EC 1.1.1.28 respectively (27). Some species have the ability to only form L(+) lactate while others can only form D(-) lactate (27). A few species contain a racemase which produce both L(+) and D(-) lactate in a ratio that can be specific to the particular strain (27).
**Deprotonation of lactic acid**

Lowering of the intracellular pH ($\text{pH}_i$) occurs during the fermentation process as acids are produced. Export of organic acids then can lower the environmental pH ($\text{pH}_e$). When the $\text{pH}_e$ reaches the pKa of lactic acid ($\text{pKa}=3.86$ at $25^\circ\text{C}$) 50% of the acid is undissociated, uncharged and able to pass freely through the bacterial membrane (20, 36). Because the $\text{pH}_i$ is more alkaline than the $\text{pH}_e$, upon entering the cytoplasm the uncharged form of lactic acid is dissociated with the release of a proton (20). Lowering $\text{pH}_i$ and accumulation of $\text{H}^+$ and lactic acid eventually leads to loss of cell function and viability (36). The concentration of undissociated lactic acid is an important inhibitory factor on microbial growth (51). Weak organic acids such as lactic and acetic acids are far more inhibitory than hydrochloric acid because of their ability to passively diffuse into a bacterial cell and lower $\text{pH}_i$ (34).

**Sensing acidic environments**

*Two-Component regulatory systems*

A response to acid inhibition is essential for survival of LAB particularly because of their presence in fermented foods where organic acids are present and act as strong preservatives (29). Survival of LAB in foods or through the stomach depends on their ability to sense and respond to the acid environment. LAB have been shown to contain two-component regulatory systems which sense acid concentration and fluctuation of pH (2).
Two-component regulatory system construction

Two-component regulatory systems can sense changes in environmental conditions via signal transduction pathways. The system generally utilizes two characteristic primary structure motifs; a membrane-associated histidine protein kinase (HPK) to detect environmental signals and a cytoplasmic response regulator (RR) which controls expression of genes in one or more operons (Figure 3) (5, 48).

The HPKs typically consist of a signal input domain and an autokinase domain. The former is characteristically an N-terminal input domain while the latter is generally a C-terminal transmitter module which can be divided into two subdomains, a histidine phosphotransferase subdomain and an ATP-binding subdomain (48). The majority of RRs are composed of an N-terminal regulatory (receiver) domain and a C-terminal DNA-binding (output) domain (48).

The signal input domain of the HPK detects the external signal which controls its activation. This active component autophosphorylates a histidine residue through ATP hydrolysis (48). The phosphoryl is transferred to an aspartate residue in the receiver domain of the RR that activates the regulatory protein and promotes either a positive or negative transcriptional response (48). Acidic conditions can be sensed through two-component regulatory systems in the LAB and these organisms can respond with a variety of mechanisms that provide some level of acid protection (2).

Response

In *Lactococcus lactis* MG1363, collectively 90 proteins were upregulated during an acid adaptation experiment exposing cells to pH 5 in both M17 and SA media. A total of 68 different
proteins were identified by mass spectrometry. Many of the genes affected were involved with sugar and amino acid metabolism, pH homeostasis and stress adaptation (10). Upregulation in sugar metabolism may help diversify sugar sources and improve metabolic efficiency in acidic environments (10). Alterations in the transcription and translation of amino acids may be due entirely to acid stress, or may reflect a general stress response (10). In order for bacteria to survive, significant levels of mRNA expression must be maintained for translation to continue (20). In *Lactobacillus reuteri* ATCC 55730, the gene *clpL* (lr1864) was upregulated during acid exposure at pH 2.7 (66). The Clp protein family are involved in degradation of damaged proteins (23). After prolonged exposure to acid environment, and accumulation of organic acids, the cells cease growth and expend energy to counter acidification while maintaining significant levels of mRNA to continue translation. Endogenous RNA catabolism occurs when catabolic activity is abolished (20).

LAB are able to tolerate a range of internal pHs from 4.4 -8.4 (36, 57). The limit of tolerable internal pH levels depends upon the particular LAB species and the ability of internal enzymes to function at lower pH ranges (36).

**Rerouted metabolism**

Many genes are down-regulated during exposure to acid. In *Lb. reuteri* ATCC 55730 the main genes down-regulated are involved in basic cellular processes such as lr0119 (DNA polymerase III, delta subunit), lr1014 (site-specific recombinase XerD), and lr1240 (recombinational DNA repair ATPase) involved in DNA replication, recombination and repair (66). As shown in *Lc. lactis*, the bacteria respond through metabolic changes such as inhibition
of enzymatic activities, diminished catabolic flux through glycolysis, and a decreased rate of biochemical energy synthesis (20). The decrease in energy expenditure helps to slow the acidification process, but then biomass synthesis becomes limited. With inhibition of glycolytic enzyme activity, growth is eventually stopped while a catabolic flux is maintained (20). Control of the catabolic flux is mainly transferred to enzymes involved in phosphoglycerate metabolism, an intermediate in glycolysis which produces two molecules of pyruvate with concomitant production of two molecules ATP (20).

**Acid Adaptation**

Adaptation to acid stress involves multiple genes and can elevate survival from acid death by several orders of magnitude. This type of adaptation can initiate the acid tolerance response (ATR) and affect protein synthesis, as seen by comparing changes in extracted protein patterns (29). The adaptation occurs in two physiological states: (i) logarithmic growth during acid accumulation; or (ii) stationary phase as a result of continued acid accumulation, general stress responses and additional factors produced during stationary phase (64). These modifications are shown through synthesis of approximately 519 proteins expressed in control cells. A majority of these showed a reduction of synthesis, however, at least 33 showed enhanced expressions compared to the control. At least one protein enhanced in acid shock, identified as U8, is also induced during heat shock, the SOS response and the H$_2$O$_2$ response (29, 30). The ATR requires presensitization to lactic acid in order to activate the stress response and precondition the cells to withstand severe acid stress. However, stationary phase cells need no pre-activation by acid, likely because the cells were adequately acid challenged, and adapted, due
to the low pH typically involved in stationary phase cultures (29). The effect of the ATR varies in degree and between species.

**Mechanisms of acid resistance**

*Main mechanisms of acid resistance*

**ATPases**

One mechanism to maintain a tolerable pH$_i$ is through an H$^+$-ATPase. An inwardly directed gradient of protons drives the influx of a solute entering via a proton symport. An outwardly directed gradient of protons is able to drive the efflux of a solute via a proton symport creating a proton motive force. F$_1$F$_0$-ATPase is a multisubunit ATP-dependent proton pump generating a PMF using ATP produced by fermentative substrate-level phosphorylation (14). This system is found across the LAB group (41). The PMF can facilitate extrusion of protons from the cell cytoplasm which results in a drop in intracellular pH (14). In oxidative respiration, the function of the F$_1$F$_0$-ATPase is used in generation of ATP (14). In LAB which utilize substrate-level phosphorylation, the sole function of the F$_1$F$_0$-ATPase is to extrude protons for pH$_i$ homeostasis (14).

Regulation of the enzyme level of F$_1$F$_0$-ATPase is dependent on the pH$_i$ in *Enterococcus hirae* (1). Most regulation occurs largely through steps at the point where the enzyme is assembled from subunits, albeit a small amount of regulation comes at translation (1). There is an increase in the activity of the ATPase in *Enterococcus faecalis* when the pH$_e$ is below pH 8.0, causing the pH$_i$ to reach below 7.6 (39). ATPases are only involved in alkalization of cytoplasmic pH to protect from acidification and exert no activity when the pH is alkaline (38).
As the pH$_i$ returns to basal levels, the amount of ATPase decreases (40). The increase in H$^+$-ATPase activity is sufficient only to maintain a constant steady-state cytoplasmic pH. However, upon large pH fluctuations the ATPase must be complemented by other systems (61). In *Lactobacillus acidophilus* regulation was shown to be at the transcription level or through mRNA degradation (41).

The *atp* operon of *Lb. acidophilus* consists of 8 genes encoding the subunits a, b, c, α, γ, δ, ε and β of the F$_1$F$_0$-ATPase (41). The F$_0$ complex is composed of subunits a, b and c and has proton-translocation activity (14). The remaining subunits belong to the F$_1$ protein and are likely involved in the assembly or gating of the channel (14). In addition, the F$_1$ protein catalyzes the interconversion of proton translocation and ATP synthesis or hydrolysis. The F$_1$ component has ATPase activity when it is released from the membranes and is attached to the F$_0$ complex in sealed membranes (Figure 4) (14).

The F$_1$F$_0$-ATPase is pH-inducible (41). The acid susceptibility of the individual strain is dependent on the active inflow and outflow of protons through proton-translocating membrane ATPases (4). Species with a high tolerance to acid are able to slow the influx of protons in acidic environments (4). The proton permeabilities in different species are dependant on the pH optima of the ATPases (4). The association of the F$_1$ complex with the F$_0$ enhances the pH range of hydrolytic activity (60).

Exposing *Oenococcus oenos* to an environmental pH of 3.1, before a lethal challenge at pH 2.6, significantly improves survivability (19). The improvement of survival depends on the strain’s initial level of acid tolerance (19). An acid tolerant *O. oenos* mutant generated by
survival screening at pH 2.6 showed higher H⁺-ATPase activity at low pH than did the parent
(19). Part of pH homeostasis is the cation transport system, K⁺-ATPase. This exchanges the K⁺
for H⁺ and creates a transmembrane pH gradient (64). The induction of H⁺-ATPase activity is
part of the ATR.

**Arginine Deiminase (ADI)**

In addition to H⁺-ATPase, LAB also utilize pathways to alkalize the pHᵢ. One such way
is through the ADI pathway and arginine catabolism (6). The ADI pathway is widely present in
many genera including *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus,*
and *Streptococcus* (3, 18, 43, 62, 68, 69), however, it does not span all LAB (17).

The catabolism of arginine serves several purposes such as providing a source of energy
and promoting higher survival in stationary phase (17, 63, 65). The ADI catalyzes the first step
of pyrimidine biosynthesis with arginine serving as a pyrimidine precursor (47). In addition to
acidic environments, carbohydrate limitation can also trigger the expression of the ADI pathway
(11).

The uptake of arginine is due to an arginine-ornithine antiport system which does not
require metabolic energy (50). Arginine deiminase, OTCase, and carbamate kinase are the
enzymes responsible for catalysis of arginine into ornithine, ammonia, and carbon dioxide (15).
ArcA is the responsible enzyme for deimination of arginine to ammonia and citrulline. ArcB
then catalyzes the conversion of ammonia and citrulline to ornithine and carbamoyl phosphate.
ArcD, an antiporter, subsequently exports ornithine. Carbamoyl phosphate is dissociated by
ArcC in ammonia and CO₂. This generates 1 mol ATP per mol of arginine consumed (9). The ammonia generated also helps neutralize the pH𝑖 (Figure 5).

The ADI system spans a seven-gene operon where only the ArcA and ArcB proteins are transcribed from the acid stress proteome (8). The gene that transcribes arcB is amongst several other genes which encode putative enzymes involved in the arginine deiminase (ADI) pathway (9). ArcA, ArcB and ArgS, present in Lactococcus lactis, are highly induced by lowering pHc. The regulation of this pathway is thought to be indirectly influenced by many factors including the proton motive force, ATP depletion, levels of enzyme synthesis, and arginine levels (8, 17, 50). It is induced in the presence of arginine, but subject to catabolite repression (65). Proteomic analysis of the ATR showed that ArcA, ArcB and ArcS are acid-inducible proteins (8, 9, 15). Transcriptional analysis showed that at least part of the arcB gene is transcriptionally regulated and that other genes in the arc operon are induced when presented with acid. Further study showed that ArcB is involved in intrinsic acid tolerance during acid challenge (9). The degradation of arginine can occur well below the pH minimum for growth and glycolysis (44). This allows cells to be able to recover from severe acid damage which may otherwise be lethal to cells (44).

**Amino acid decarboxylation reactions**

At low pH, bacteria also produce enzymes that drive decarboxylation reactions (24). This process generates metabolic energy through decarboxylation of amino acids coupled with the antiport of the decarboxylated amino acid. The process of decarboxylation requires the consumption of a proton. The charged product is transported out while a molecule with exactly
one less proton is transported in. The primary goal of amino acid decarboxylation reactions is to maintain the basal pH through proton extrusion (46).

In this process, glutamate is an important amino acid which can be decarboxylated to help maintain pH. Glutamate decarboxylase (GAD) catalyses the decarboxylation of L-glutamate to glutamate-γ-aminobutyrate (GABA). Both gadC and gadB have been identified through nucleotide sequencing and functional analysis in Lactococcus lactis (55). GadC specifies a putative glutamate-γ-aminobutyrate antiporter while GadB is a glutamate decarboxylase (55). Upstream of the promoter is GadR which is homologous to the activator Rgg from Streptococcus gordonii, a positive regulator of glucosyltransferase (55). The acid resistance mechanism is glutamate-dependent.

Glutamate decarboxylase requires the presence of chloride. This increases the expression of gadCB when the pH of the environment is at low levels (55). GadB, the glutamate decarboxylase, converts internalized glutamate to GABA. This requires the consumption of a proton and produces one molecule of CO₂ (55, 67). GadC is involved in the antiport of glutamate and GABA. Extracellular glutamate is exchanged for GABA, which is more alkaline, raising the pH (55, 67) (Figure 6).

The regulation of the GadCB system varies among bacteria. The promoter in Lc. lactis was found to be regulated by sodium-chloride (56). Growth at a neutral pH prior to extreme acid challenge is vital for the functionality of the glutamate cycle in Escherichia coli (31). Finally, in Shigella flexneri this acid resistance mechanism is RpoS regulated and requires cells to be grown to stationary phase (67).
Citrate Transport

Citrate transport is a mechanism of acid resistance in *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* (25). It is a pathway that is also essential to creating the unique flavor of particular cheeses. CitP, which is a citrate permease P, catalyzes the uptake of citrate under acidic conditions (25). At neutral pH, citrate is not able to enter the cell. The P1 promoter is responsible for the increase in transcription of CitP at low pH (25). The pH sensitive CitP mediates transport of citrate at low pH in an exchange for intracellular lactate. Metabolism of citrate produces oxaloacetate which then consumes a proton when converted to pyruvate (26).

A pH of 4.5 in the exclusive presence of glucose results in poor growth of *Lc. lactis*. Cit+ strains at the same pH with the addition of citrate show a growth advantage (25). Replacing glucose with lactose shows the same behavior (25). The growth is not stimulated by citrate alone, therefore, cometabolism of citrate with a sugar is important (25). Metabolism of citrate alkalizes the external medium whereas metabolism of the accompanying sugar acidifies the external medium (25). The net result, however, is a slow alkalization of the external medium which confers a selective advantage to Cit+ lactococci (25). The total biomass benefits greatly in the presence of citrate in systems with uncontrolled pH. Under conditions with controlled pH, acetic acid build up negatively effects survival (54). The *als, aldB*, and *aldC* genes and the *butBA* operon from the *Pals, P3aldB, PaldC*, and *PbutAB* promoters involved with citrate metabolism and generation of aromatic compounds are induced at pH 5.5 in *Lactococcus lactis* subsp. *lactis* bv. *diacetylactis* (26). Citrate metabolism of *Leuconostoc mesenteroides* results in extra energy via a PMF mechanism where CitP functions as a electrogenic citrate/lactate
exchanger (45). The energy generated by the secondary metabolism of citrate is shown to stimulate growth (45). In addition to alkalization of the pH, metabolism of citrate can lower the expenditure of ATP used in glycolysis or proton expulsion by ATPases (54). Both growth stimulation and deacidification of the external medium can confer protection under acid stress.

Additional mechanisms of acid resistance

Cell density and biofilms

The ATR was also shown to interface with quorum sensing to initiate genetic competence. High cell densities in biofilms showed greater acid resistance compared to low cell density systems. These biofilms were also more capable of growth at a lower pH (42). It is likely that the biofilm community is producing a protein-like extracellular signal molecule to enhance induction of acid adaptation. Cells able to form biofilms generally have an advantage in acidic environments (42).

Cell wall and cell surface changes

While examining *Lb. reuteri* under the acidic conditions of the human stomach it was shown that 70 genes were differentially expressed. In response to acid shock, *Lb. reuteri* can decrease the permeability of protons by changing the composition of the cell wall or membrane (66). In *Streptococcus mutans*, increased levels of monosaturated and longer-chain fatty acids were found in the cytoplasmic membrane when the bacterium was exposed to pH 5 (52). *Lb. reuteri* induces *lr1797* which encodes phosphatidylglycerophosphatase at pH 2.7, an enzyme
involved in synthesis of phosphatidylglycerol and cardiolipin, both acidic phospholipids of bacterial membranes (66). Acidic conditions also induce gene lr1516 in Lb. reuteri, a putative esterase of the penicillin-binding proteins which are typically involved in peptidoglycan synthesis (66). St. mutans with a mutation in gene dltC is highly permeable to protons compared to the wild type. This gene is involved in production and esterification of D-alanyl-lipoteichoic acid. These esters have important roles in growth including synthesis of the teichoic wall (7).

Lb. reuteri induces also rr7 (lr1804) which produces the response regulator of a two-component signal transduction system (66). This operon is homologous to the yycFG operon in Bacillus subtilis, as well as other lactic acid species, which regulate genes involved in cell wall metabolism and membrane protein synthesis (21).

In addition to changes in cell wall composition, genes associated with cell membrane synthesis and composition are also up-regulated in response to acid. Gene lr0997 in Lb reuteri encodes a conserved membrane protein which is similar to the gdmH/epiH genes found in Staphylococcus gallinarum and Staphylococcus epidermidis (66). GdmH is likely to be involved in several microbial processes (32). Since the cell wall and cell membrane are likely the first cellular components that encounter acid, the changes in composition may be important to survival.

**Urease**

Urease, present in Streptococcus salavarius, Streptococcus thermophilus and other oral streptococci, is able to catalyze urea to CO₂ and ammonia, which raises the pHₗ (13, 64). At a pH below 4.0, St. salivarius urease is outside its activity range and free enzyme is rapidly
inactivated. Enzyme remaining within intact cells is able to raise the pH_i for at least 1 hour during lethal acid exposure (58). In addition to pH regulation, *St. salivarius* 57.1 urease is upregulated in the presence of excess carbohydrate (13).

**Conclusions**

LAB are industrially important because of their production of lactic acid in many food and industrial fermentations. These bacteria have been consumed for thousands of years and have evolved to specific nutritionally rich environments, notably food substrates and the gastrointestinal tracts of animals. The production of lactic acid creates a low pH environment injurious to bacteria, both pathogenic and beneficial.

The mechanisms involved in acid resistance have allowed LAB to compete and survive in the unique niche of high acid environments. These bacteria use two component regulatory systems to sense acidic stress and signal a response. To adapt, the bacteria change their metabolic flux to attain basal pH_i levels. The most well understood forms of maintaining the pH_i as the pH_e drops, is through H^+-ATPase, the ADI pathway, and amino acid decarboxylation reactions. Several additional mechanisms exist in particular species but do not span the entire LAB classification, for example citrate metabolism. Acid resistance mechanisms are an important research area of LAB because of their importance in industrial applications, food preservation, and pathogen inhibition.

Understanding acid resistance mechanisms has allowed an improved ability to select strains harboring enhanced resistance. Understanding the individual response systems and how they complement the whole remain an important research challenge for the future.
Figure 1. The Embden–Meyerhoff pathway used by homofermentative LAB. The dashed line indicated the NAD/NADH oxidation-reduction part of the pathway (33).
Figure 2. The phosphoketolase pathway used by heterofermentative LAB (33).
Figure 3. Two Component Regulatory Systems (59).
Figure 4. Schematic diagram of the bacterial ATP synthase. The enzyme consists of two major portions, called F₁ and F₀. The F₁ head consists of five different subunits in the ratio 3α: 3β:1δ: 1γ:1ε. The α and β subunits are organized in a circular array to form the spherical head of the particle; the γ subunit runs through the core of the ATP synthase from the tip of F₁ down to F₀ to form a central stalk; the ε subunit helps attach the γ subunit to the F₀ base. The F₀ base, which is embedded in the membrane, consists of three different subunits in the apparent ratio 1α: 2b: 12c. The c subunits are thought to form a movable ring within the membrane; the paired b subunits of the F₀ base and the δ subunit of the F₁ head form a peripheral stalk that holds the α/β subunits in a fixed position; and the a subunit contains the proton channel that allows protons to traverse the membrane. (35)
Figure 5. The arginine deiminase pathway with the arginine/ornithine antiporter (AP). M denotes the cytoplasmic membrane (53).
Figure 6. Generation of ATP by transport and proton-consuming decarboxylation. The movement of glutamate (Glu2) ions into the cell, glutamate decarboxylation, and extrusion of GABA ions (GABA), the movement of malate (Mal2) ions into the cell, malate decarboxylation, and extrusion of lactate (Lac) ions (L. lactis), and the movement of citrate (HCit2) ions into the cell, oxaloacetate decarboxylation, and extrusion of Lac ions (Lac) (L. lactis) all create an electrogeneric potential. These decarboxylation reactions and the consumption of a proton increase the alkalinity of the cytoplasm. Three cycles of decarboxylation and antiport create a PMF sufficient for the synthesis of ATP via the F1F0-ATPase. Ace, acetate; Oxace2, oxaloacetate; Pyr, pyruvate. (14)
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CHAPTER II

*Lactobacillus acidophilus* NCFM survival in acidified yogurts

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INTRODUCTION

Lactic acid bacteria (LAB) metabolize carbohydrates to produce lactic acid as the primary end product (13). These functionally related bacteria are widely used in the food industry as means of preservation, flavor and texture enhancement (16). Lactic acid acts as a strong preservative which can inhibit the growth of both pathogens and spoilage microorganisms (10). Since their domesticated use in food production, LAB have been extensively selected for important fermentation and probiotic properties (6). Recent genomic characterization of the LAB has revealed their adaptive evolution to nutritionally rich environments by genome reduction and decay, resulting in a group of fermentative bacteria that are both efficient and competitive when grown in food and dairy substrates (11).

The fermentation of dairy products by LAB produce desirable sensory characteristics including a distinct sour taste from the accumulation of lactic acid. These food products are generally acidic which improves the shelf-life by inhibiting undesirable microorganisms (14). In addition, many LAB organisms are considered to elicit probiotic properties. The definition of probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (9). Manufacturers of food and dairy products containing probiotic cultures demand robust bacteria resistant to metabolic byproducts of fermentation including the accumulation of lactic acid.

Yogurt fermentations employ starter cultures comprised of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* typically in a one-to-one, rod to coccus ratio. Additional strains may be added to improve the sensory properties or marketability of the
product (12). Cultures used in yogurt fermentations are either produced via bulk cultures or added directly to the fermentation vat via frozen or dried culture concentrates. Concentrated cultures allow a safe and efficient means to inoculate the yogurt base. Lyophilization is a method of drying cultures that reduces injury when compared to alternative drying methods such as spray drying. However, this technique can also have several deleterious effects which can reduce both culture viability and activity. Optimization of concentrated cultures is dependent on conditions used for growth, resuspension, and presensitization of the cells (4).

A significant factor in the preservation of dairy products is the level of lactic acid and pH. When the pH of the environment reaches the pKa of lactic acid at 3.86, half of the acid is dissociated and half is in the undissociated state. The uncharged, protonated acid passes freely through the cytoplasmic membrane of bacteria. Upon entering the cell, the undissociated molecule encounters a pH greater than its pKa and subsequently dissociates releasing a proton, essentially contributing to the acidification of the cytoplasm. Functionality of cell components and metabolism may be rendered inactive or ineffective at the lower pH. Tolerance to acid, and therefore viability, is dependent on the strains efficiency to consume or export the internal protons and raise the internal pH (8, 15).

Well studied mechanisms of acid resistance include ATPases, the arginine deiminase (ADI) pathway and amino acid decarboxylation reactions (3, 7, 22). ATPases can facilitate the extrusion of protons from the cell cytoplasm which results in a drop in intracellular pH (7). The ADI pathway catabolizes arginine to produce 1 mol of ATP and subsequently generates ammonia which helps to neutralize the intracellular pH (3). Amino acid decarboxylation
reactions also can generate metabolic energy via decarboxylation of amino acids and consume a proton during the process. This reaction is coupled with the antiport of a charged decarboxylated amino acid (18). Additional mechanisms do exist in LAB but do not span the entire group. LAB can also employ two-component regulatory systems to sense and respond to the environmental changes (1). Notably, Lactobacillus acidophilus NCFM encodes an ATPase (17) and a two-component regulatory system that confers acid tolerance (1) by detecting specific environmental signals via a histidine protein kinase (HPK) and a response regulator (RR), which generate an adaptive response through regulation of gene expression (20).

Lb. acidophilus is widely used in yogurt fermentations with approximately 80% of US commercially available varieties adding this species as a probiotic supplement (23). This study examined the relationship between the survival of Lb. acidophilus NCFM and the environmental conditions encountered in fermented yogurt.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are listed in Table 1. Lactobacilli were propagated in MRS broth (Difco, Detroit, MI) aerobically at 37°C without agitation. MRS was supplemented with clindamycin hydrochloride for selection of Lb. acidophilus (0.2 μg/ml, Sigma, St. Louis, MO). Streptococcus thermophilus was cultivated in M17 broth (Difco, Detroit, MI) with glucose. Agar media was prepared by adding 1.5% (w/v) agar to liquid media and overlay agar was prepared with 0.75% (w/v) agar. When appropriate, 5 μg/ml erythromycin (Fisher Scientific, Fair Lawn, NJ) was added to MRS broth.
**Yogurt preparation.** For the bulk starter, 12% NFDM (MP Biomedicals, Inc., Solon, OH) was fermented by a 2% (w/v) inoculation of a lyophilized multiple-strain starter culture (Danisco USA, Madison, WI) composed of *Streptococcus thermophilus, Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Lactococcus lactis*. The initial concentration of LAB in the milk was $10^6$ CFU/ml. The milk was fermented at 42°C until a pH of 4.55 was reached. The bulk starter was immediately placed in an ice bath and stored in the refrigerator. The bulk starter was used within 24 hours for the actual yogurt fermentation. Yogurt base ingredients (Table 2) are combined in 1% fat skim milk (NCSU Creamery, Raleigh, NC) and stirred for 20 minutes prior to a 30 minute steam treatment. The yogurt base was stirred again for 2 minutes then put in an ice bath until the temperature reached approximately 42°C. The yogurt base was inoculated at 5% (v/v) (approximately $2 \times 10^8$ CFU/ml initial levels of total cultures) with bulk starter and fermented at 42°C until pH 4.55. The final yogurt was cooled in an ice bath and stored at 4°C. Inoculation points and parameters for the probiotic culture with the yogurt starters are noted in Figure 1.

**Isolation of yogurt strains.** The yogurt mixed starter culture was streaked onto M17 agar plates with 0.125% (w/v) LiCl (Fisher Scientific, Fair Lawn, NJ) for isolation of *St. thermophilus* (24). Plates were incubated anaerobically at 45°C for 48 hrs. Colonies were picked and examined by phase-contrast microscopy for the presence of cocci in small-medium chains. *Lactobacillus delbrueckii* ssp. *bulgaricus* was isolated from the multiple strain starter culture by streaking onto
MRS agar and incubated aerobically at 45°C for 48 hrs. Colonies were picked and examined by phase-contrast microscopy for the presence of small rods.

**Commercial yogurt survey.** Commercial yogurts purchased from nationwide grocery chains were plated on MRS agar with 0.2 μg/ml clindamycin. Four isolated colonies from each sample were picked and used for PCR amplification (Expand High Fidelity PLUS PCR System, Roche, Mannheim, Germany) of the 16S rRNA gene using primers based on conserved regions. The primers used were plb16 (5’ AGAGTTTGATCCTGGCTCAG 3’) and mlb16 (5’ GGCTGCTGGCACGTAGTTAG 3’) (IDT, Coralville, IA). Amplification of DNA was performed in a Bio-Rad® MyCycler Thermal Cycler (Bio-Rad, Hercules, CA) programmed for an initial denaturation of 10 min at 94°C followed by 30 cycles of 15 s at 94°C for subsequent denaturation steps, 30 s at 58°C to anneal, 1 min at 72°C for extension and 7 min at 72°C for the final extension. Reaction products were resolved by electrophoresis in a 1.0% (w/v) agarose gel (Denville, Metuchen, NJ) and visualized by staining with ethidium bromide. Sequencing of both strands of the PCR products was performed by Davis Sequencing, Inc. (Davis, CA) using primers plb16 and mlb16. Sequences were aligned using Clone Manager Professional Suite 8 (Sci-Ed software, Cary, NC) and a sequence identity matrix was generated for the alignments. The sequences were identified using the Basic Local Alignment Search Tool (BLAST) for comparison of known genes in the data base of the National Center for Biotechnology Information (NCBI).
**Bacteriocin assays.** An overnight culture was spotted (10 μl) or streaked onto an MRS agar plate. The plate was incubated anaerobically overnight at 42°C and then overlayed with a lawn of the test indicator strain in 10 ml of semisolid agar. After anaerobic overnight incubation at 37°C zones of inhibition were noted. Possible bacteriocin activity from *Lb. bulgaricus* was assayed by spotting 10 μl of an overnight culture onto MRS agar plates and incubating anaerobically overnight at 37°C. Next, nine proteolytic enzymes (listed on Table 2) (1 mg/ml) were spotted (5 μl) onto the edge of the culture spots and allowed to dry for 30 minutes. An overnight culture of the indicator, *Lb. acidophilus* NCFM, was added (50 μl) to 10 ml of semisolid MRS agar (0.75% w/v) and then poured onto the surface of the agar plate. After a 19-24 hour incubation period at 37°C, zones of inhibition in the presence and absence of the proteases was examined.

**Hydrogen peroxide production assay.** An overnight culture of *Lb. delbrueckii* ssp. *bulgaricus* was spotted (10 μl) onto an MRS agar plate. The plate was incubated anaerobically overnight at 37°C. Next, 5 μl containing 2, 20 or 200 units/ml of catalase (Sigma, St. Louis, MO) were spotted at the edge of the overnight colonies, and allowed to dry for 30 minutes. A lawn of 100 μl of *Lb. acidophilus* NCFM in semisolid agar was poured onto the surface of the agar plate. After 19-24 hour incubation at 37°C, zones of inhibition were recorded.

**Growth and survival of *Lb. acidophilus* with catalase supplement.** Yogurt base was fermented with bulk yogurt starters plus *Lb. acidophilus* NCFM (approximately 10^8 CFU/ml
initial levels) in the presence and absence of catalase (0.1% and 0.5%). Growth of *Lb. acidophilus* NCFM was enumerated every hour during the yogurt fermentation using MRS agar with 0.2 μg/ml clindamycin. Additionally, yogurt base was fermented with bulk starters and *Lb. acidophilus* NCFM (approximately 10⁸ CFU/ml initial levels) plus 0%, 0.1%, 0.5% and 1.0% catalase and stored in the refrigerator. Survival of *Lb. acidophilus* NCFM was enumerated weekly using MRS agar with 0.2 μg/ml clindamycin.

**Effect of pH.** Lyophilized *Lb. acidophilus* NCFM was added at 10⁸ CFU/ml directly to yogurt which was previously fermented to pH 4.1, 4.7 or 5.0. Samples were immediately placed in an ice bath and then were refrigerated at 4°C. Survival of *Lb. acidophilus* NCFM was monitored weekly using MRS agar with 0.2 μg/ml clindamycin. Yogurt base was directly acidified using DL-lactic acid to pH 4.2, 4.4, 4.6, 4.8 and 5.0. Lyophilized *Lb. acidophilus* NCFM cells were added at 10⁸ CFU/ml directly to acidified yogurt base. Samples were refrigerated at 4°C. Survival of *Lb. acidophilus* NCFM was monitored weekly using MRS agar with 0.2 μg/ml clindamycin.

**Cell recovery assay.** Lyophilized *Lb. acidophilus* NCFM cells were added at 10⁸ CFU/ml to 100 ml MRS and incubated at 37°C for 15 minutes. Cells were spun down and resuspended in 100 ml of phosphate-buffered saline (PBS) (21) that was acidified to pH 3.5 with DL-lactic acid (Sigma, St. Louis, MO). The cell suspension was incubated for 1 hr at 37°C and aliquots were removed every 15 minutes, diluted in 10% MRS, and plated on MRS agar. A non-resuscitated
control sample of lyophilized \textit{Lb. acidophilus} NCFM cells was added at \(10^8\) CFU/ml directly to PBS acidified to pH 3.5 (\(\text{DL-}\)lactic acid), and sampled and assayed as above.

\textbf{Adaptation studies.} Lyophilized NCFM was added at 100 ml \(10^8\) CFU/ml to MRS adjusted to pH 5.5 and 6.5 using \(\text{DL-}\)lactic acid. Samples were incubated for 1 hr at 37°C. Cells were centrifuged and resuspended at \(10^8\) CFU/ml in unfermented yogurt base at either pH 4.1 and 4.7. The pH was adjusted using \(\text{DL-}\)lactic acid at \(10^8\) CFU/ml. Samples were refrigerated at 4°C. Survival of \textit{Lb. acidophilus} NCFM was monitored weekly using MRS agar with 0.2 \(\mu\)g/ml clindamycin.

\section*{RESULTS}

\textbf{Survival of \textit{Lactobacillus acidophilus} NCFM in yogurt.} The target level of \textit{Lb. acidophilus} NCFM in commercial yogurt is \(6 \times 10^6\) CFU/ml in a 6 oz. serving or a daily dose of \(10^9\) CFU at the end of a 52 day shelf-life (25). Industrial production of yogurt (Figure 1) using a bulk starter has two primary fermentations. The bulk starter fermentation uses a multiple starter culture to inoculate 12\% (w/v) skim milk. The milk is fermented until a final pH of 4.55. At this point, lyophilized \textit{Lb. acidophilus} NCFM cells are added at 0.26\% (w/v) to achieve an initial population of \(10^6\) CFU/ml in the fermented bulk starter. This bulk starter was then cooled, stored, and used up to 72 hours later to inoculate the yogurt fermentation.

At the outset of this study, we evaluated the population of NCFM through the course of the traditional industrial process. Initially, \(10^6\) CFU/ml \textit{Lb. acidophilus} NCFM was added to the
bulk starter at the end of fermentation. This bulk starter (containing NCFM) was added at 5% to the yogurt base yield an initial level of NCFM of $10^5$ CFU/ml in the yogurt base. After fermentation of the yogurt product to 4.55 and storage at 4°C for 7 weeks, no viable NCFM cells were recovered from the industrial product (data not shown). Following these observations, the NCFM inoculation level to the fermented bulk starter was then increased to $10^8$ CFU/ml and the bulk starter was used to inoculate the yogurt base and prepare yogurt. After 7 weeks at 4°C no viable NCFM was recovered from the yogurt (Figure 2). When *Lb. acidophilus* NCFM was added directly to the yogurt mix at the beginning of the yogurt fermentation process survival was improved by 4.5 logs (Figure 2, sample B). If *Lb. acidophilus* NCFM was prepared separately as its own bulk starter, and then combined with the traditional bulk culture for inoculation of the yogurt base, survival of NCFM in the fermented and stored yogurt improved to 4.5 logs (Figure 2, sample C). When lyophilized *Lb. acidophilus* NCFM was added directly to yogurt mix at the outset of fermentation, only a 2.8 log loss in viability occurred over the 7 week shelf-life (Figure 2, sample D). If *Lb. acidophilus* NCFM was the sole species used to ferment the yogurt base, viability improved 4.5 logs over the original industrial process (Figure 2, sample E). The point at which *Lb. acidophilus* NCFM was added to the industrial yogurt process greatly affected its viability over the course of the yogurt shelf-life. Adding lyophilized *Lb. acidophilus* NCFM cells directly to the fermented yogurt base at the outset of the fermentation, as opposed to the bulk starter, improved the viability of NCFM 5.5 logs at the end of the shelf-life.
**Sequence analysis of isolates from commercial yogurt strains.** Given the poor survivability of *Lb. acidophilus* in the base industrial process above, we surveyed six commercial yogurts for the presence of *Lb. acidophilus* by plating samples on MRS agar plus clindamycin. All the yogurts stated on the label that they contained *Lb. acidophilus*. Colonies were recovered from all yogurt samples except sample F. For those samples yielding colonies on MRS + clindamycin plates, levels were always < 10⁵ CFU/ml. Four colonies were randomly selected from plates representing 5 of the 6 yogurt samples, and colony PCR was used to amplify the 16s rRNA gene. The identities of the colonies were confirmed by DNA sequencing and the results are shown in Table 4. *Lb. acidophilus* was recovered from only 2 (samples A& B) of the 5 yogurts surveyed. The other 3 yogurts (C, D & E) contained *Lb. gasseri, Lb. johnsonii*, and *Lb. rhamnosus*, respectively. Therefore, *Lb. acidophilus* appears to survive poorly in numerous commercial yogurts, which claim its presence.

**Fermentation behavior of Lb. acidophilus NCFM in yogurt base.** Unfermented yogurt base was inoculated at 10⁷, 10⁸, and 10⁹ CFU/ml with NCFM only (no yogurt cultures) and viability was monitored over a 24 hour period (Figure 3a). Higher inoculation levels resulted in faster acidification (Figure 3b), notably even as the 10⁹ population declined over the same 5 hour period. The populations monitored over a 24 hr period all showed a terminal count of ~10⁸ CFU/ml after 24 hours, irrespective of the initial inoculum level. No significant growth occurred for NCFM cells inoculated at 10⁸ CFU/ml, even though acid accumulated at an intermediate rate. When inoculating at 10⁷ CFU/ml, cell numbers slowly increased by one log cycle and acid
development was slow, eventually reaching pH 4.0 after 24 hours. Since *Lb. acidophilus* NCFM stably survived at levels of $10^8$ CFU/ml over the fermentation period, this level was chosen as the initial target population for subsequent inoculations.

**Antimicrobial activity of yogurt strains.** The precipitous decline of the probiotic culture, NCFM, in the fermented yogurt during shelf-life could potentially result from antimicrobials produced by the yogurt starter strains. Therefore, we investigated whether or not the yogurt starter strains produce inhibitory compounds active against NCFM. The *St. thermophilus* and *Lb. bulgaricus* strains were isolated and purified from the lyophilized yogurt starter. Deferred antagonism assays revealed inhibition of NCFM by *Lb. bulgaricus*, but not by *St. thermophilus* (Figure 4). To determine if the inhibition was due to bacteriocin activity, 9 proteases were spotted directly onto the edge of the producer culture, followed by an overlay of the indicator culture. None of the proteases relieved the inhibition (data not shown). Protease activity was confirmed for each protease used in this assay (data not shown), using a control bacteriocin assay of *Lb. acidophilus* NCFM as a producer of lactacin B, which is bactericidal against *Lactobacillus delbrueckii* (2). We conclude that the zones of inhibition produced by *Lb. bulgaricus* against NCFM were not the result of bacteriocin production. Overnight spots of *Lactobacillus delbrueckii* ssp. *bulgaricus* were then spotted adjacentally with 2, 20 and 200 units/μl of catalase and overlaid with a cell lawn of *Lb. acidophilus* NCFM (Figure 5). The presence of catalase did not relieve the inhibition observed on the agar plates. However, we did observe that addition of catalase to the yogurt base significantly improved NCFM growth, and pH development during
fermentation. It is likely that all the yogurt starters were stimulated by catalase to accelerate both growth and acid development. Growth of *Lb. acidophilus* NCFM added at the beginning of the yogurt fermentation improved 0.2 and 0.5 logs with the addition of both 0.1% and 0.5% catalase, figures 5 and 6 respectively. However, unexpectedly, survival of NCFM in yogurt with 0.1%, 0.5% and 1.0% catalase, decreased compared to the no catalase control at the end of a 4 week yogurt shelf-life (Figure 7). The reason for this behavior is unknown. Because catalase promotes growth of the starter cultures and NCFM, the accumulation of lactic acid is likely greater in the samples containing catalase. Therefore, we speculate that the pH would be lower and the storage stability of NCFM would be compromised. pH values were not taken for these samples.

**Effect of yogurt pH on survival of *Lb. acidophilus* NCFM.** Since lactic acid is a known inhibitor and preservative, the potential effects of lactate against *Lb. acidophilus* NCFM were further investigated. To assess the effect of lactic acid and pH on *Lb. acidophilus* NCFM, lyophilized NCFM cells were inoculated into an unfermented yogurt base acidified directly with DL-lactic acid to pH 5.0, 4.8, 4.6, 4.4 and 4.2 (Figure 8). In a separate experiment, lyophilized NCFM cells were added to finished yogurts that had already been fermented to pHs of 5.1, 4.7 and 4.1 and halted at those pH values (Figure 9). NCFM survival in the directly acidified yogurt was monitored over a 5 week shelf-life and showed that population levels decreased proportionately as the pH was reduced. The NCFM population was relatively stable at pHs 5.0 and 4.8. In this condition, pH levels remained stable at the initial values over the shelf-life
period. At pHs 4.6, 4.4 and 4.2, the NCFM population dropped 1.3, 1.7 and 2.6 logs, respectively. In traditionally fermented yogurts, NCFM losses over shelf-life were most dramatic at pH 4.1, with a 2 log decline (Figure 9). Interestingly, this was 1 log greater survival than was observed in the directly acidified yogurt at pH 4.1 (Figure 8). Similarly, the pH 4.7 sample in the fermented yogurt was also better, and showed only minor losses in viability over the 5 week shelf life. Therefore, NCFM was relatively stable at pH values higher than pH 4.7. Notably, NCFM was more stable in fermented yogurt over directly acidified, and unfermented, yogurt base.

**Injury of lyophilized *Lb. acidophilus* NCFM cells.** Given that NCFM stability in yogurt was compromised by initial pH values less than 4.7, we first investigated the level of acid injury in lyophilized NCFM cells. Attempts were made to resuscitate lyophilized NCFM cells prior to addition to fermented yogurt or directly acidified yogurt base. Azcarate-Peril et al., 2004 showed previously that acid adaptation at pH 5.5 for 1 hr prior to acid exposure results in higher acid tolerance compared to cells directly added to an acidic environment (1). In this study, cells were given a 15 minute recovery period from the lyophilized state prior to an acid challenge at pH 3.5 (acidified with DL-lactic acid). Cells which underwent the recovery period showed nearly 2 log higher survival at the end of the 2 hr exposure (Figure 10).

**Resuscitation of *Lb. acidophilus* NCFM prior to yogurt addition** Resuscitating the lyophilized cells prior to an acid challenge favorably affected their stability in an acid environment. As
noted earlier, Azcarate-Peril et al., 2004 showed that acid adaptation at pH 5.5 prior to acid exposure resulted in higher acid tolerance (1). Therefore, an acid adaptation during resuscitation of lyophilized NCFM cells was investigated. Lyophilized NCFM cells were resuscitated in MRS for 1 hr at pH 6.5, or in MRS acidified to pH 5.5, using DL-lactic acid. Cells were then added to unfermented acidified yogurt base at pH 4.7 and 4.1 (Figure 11). NCFM in yogurt at pH 4.7 maintained near complete viability during the 6 week shelf-life. Samples given a resuscitation period prior to addition to yogurt at pH 4.1 were also able to maintain higher numbers over 6 weeks. The pH of the resuscitation media did not influence survival. However, there was a marked difference in the survival of NCFM in yogurt base acidified to pH 4.1 which underwent resuscitation compared to previous experiments. Figure 11 shows an approximate 1 log loss of NCFM in yogurt acidified to pH 4.1 compared to a 3 log loss during similar exposure of NCFM which did not undergo a resuscitation period.

**DISCUSSION**

The delivery of viable probiotic cultures at target levels throughout shelf-life in various dairy products, notably yogurt, is an important objective for current food manufacturers. Intake of *Lactobacillus acidophilus* NCFM in conjunction with *Bifidobacterium lactis* Bi-07 at $10^{10}$ CFU/day provides a greatly reduced occurrence and duration of respiratory tract infection symptoms in 3-5 year old children (19). Approximately 80% of the United States yogurt products are stated to contain *Lb. acidophilus* (13).
This study examined a current method of incorporating *Lb. acidophilus* NCFM into a commercial yogurt. This bulk starter was fermented to pH 4.55 and then inoculated with lyophilized cells of *Lb. acidophilus*. It was then stored for up to 72 hours at 4°C and used to inoculate yogurt base in fermentation tanks. An inoculation of $10^6$ CFU/ml *Lb. acidophilus* into the bulk starter ($10^5$ CFU/ml initial levels in the yogurt) resulted in no NCFM cells recovered from the finished yogurt at pH 4.55 at the end of a 52 day shelf-life. According to a survey of commercial yogurts that listed the presence of *Lb. acidophilus* on the label, this probiotic species was only recovered from 2 of 6 products. While 5 of the 6 products contained reported probiotic bacteria, *Lb. acidophilus* was only recovered from 2 products. This finding highlights either the misidentification of cultures believed to be “acidophilus”, the poor survivability of *Lb. acidophilus* strains in commercial yogurts, or potentially low population levels that were below the limits of the detection method used in this study. With the apparent absence of *Lb. acidophilus* in 66% of selected yogurts, the label claims are clearly compromised.

Inhibition of the probiotic culture by the individual starter culture strains, *St. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* was investigated. Overlay assays showed that *Lb. bulgaricus* could contribute to inhibition of *Lb. acidophilus* NCFM. Neither bacteriocin nor hydrogen peroxide production were responsible for this inhibition. We concluded that acid production by *Lb. bulgaricus* is largely responsible for the on plate inhibition of *Lb. acidophilus*. The effect of the final pH of yogurt showed that samples below pH 4.7 drastically reduced the viability of NCFM over the shelf-life of the product.
Lactic acid can be inhibitory because of its weak organic acid properties. The pKa of lactic acid is 3.86 at which point half of the acid is in the undissociated form. As the closer the pH of the yogurt moves toward the pKa, the more acid is in an uncharged state and able to pass freely through the membrane. Once inside, the acid undergoes a rapid disassociation because the intracellular pH (pHi) is higher than the pKa. This results in the release of protons and acidification of the internal pHi. If the cell is unable to dispel the protons efficiently, the cell will no longer be able to perform vital functions eventually leading to mortality (8, 15).

The pH of fermented yogurt undergoes a phenomenon called “over-acidification” where the pH continues to drop over the shelf-life, as was observed in this study (Figure 8). This is likely due to slow but continuous acidification by the yogurt starter, and notably *Lb. bulgaricus*. This phenomenon should be considered when selecting yogurt starter culture strains to be used in conjunction with probiotic cultures (5). In addition, results of this study suggest that control of the terminal pH can dramatically improve this probiotic cultures’ survival over the expected shelf-life.

Hydrogen peroxide proved to be a factor in growth of *Lb. acidophilus* NCFM in the yogurt mix. Addition of both 0.1% and 0.5% catalase to fermenting yogurt slightly improved the growth of *Lb. acidophilus* NCFM when added at the beginning of yogurt fermentation. Curiously however, the addition of catalase to the fermentation ultimately reduced the viability of *Lb. acidophilus* NCFM over the shelf-life. Yogurt samples with no added catalase showed the highest survival. Hydrogen peroxide is produced through multiple mechanisms and can lead to lethal oxidative products; however, if catalase is present hydrogen peroxide is broken down to
water and carbon dioxide. Growth stimulation in the presence of catalase could result in more acid production and lower final yogurt pH which would be detrimental to NCFM survival. However, the fermentation was stopped at pH 4.55. Therefore, the reason for NCFM instability in the catalase treated fermentation is unknown.

The most significant cause of inhibition of *Lb. acidophilus* NCFM appeared due to low pH and lactic acid. This inhibition was manifested in the original industrial process by adding lyophilized cells directly to the already fermented and acidified bulk starter. Because of the freezing and drying process, lyophilization can lead to significant cell death and injury (4). This study clearly showed that lyophilized cells added directly to a low pH lactic acid suspension were susceptible to significant levels of cell death. Because *Lb. acidophilus* NCFM cells are in a suspended and potentially injured state, we investigated whether or not a resuscitation period of 15 minutes before exposure to acid conditions improved viability. The results showed that when lyophilized cells are allowed a brief recovery period they better survive acid challenge. In an additional study, a resuscitation period for lyophilized cells of 1 hr in MRS was performed prior to addition to acidified yogurt (pH 4.7 and 4.1). Under these conditions the viability of *Lb. acidophilus* NCFM in yogurt acidified to pH 4.1 was markedly improved over the shelf-life of the product. Inoculation of $2.6 \times 10^8$ of lyophilized *Lb. acidophilus* NCFM into MRS for 1hr prior to addition to yogurt saw only a 1.5 log loss after 6 weeks at 4°C. The adaptation period was performed in acidified MRS (pH 5.5) and unadjusted MRS (pH 6.5). The pH of the resuscitation media made no significant difference towards the prolonged viability, however, the
recovery period of either 15 minutes or 1 hour made a clear difference compared to previous levels of survival of *Lb. acidophilus* NCFM in yogurt.

These data suggest that a recovery period promotes maximum viability of lyophilized *Lb. acidophilus* NCFM when added to acidified yogurts. An industrially feasible method to improve survival would be to resuscitate NCFM cells in a milk or yogurt base prior to addition to the yogurt fermentation tank. Therefore, it is suggested that resuscitated *Lb. acidophilus* cells be added to the pH-neutral yogurt base at the beginning of the yogurt fermentation and that the terminal pH be halted at 4.5-4.6.

ACKNOWLEDGEMENTS

This investigation was funded by the NC Dairy Foundation and the Southeast Dairy Foods Research Center.
### Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus acidophilus NCFM</td>
<td>Commercially available probiotic strain</td>
<td>Danisco USA</td>
</tr>
<tr>
<td>Mixed starter cultures</td>
<td>Lactobacillus delbrueckii ssp. bulgaricus, Streptococcus thermophilus, Lactococcus lactis</td>
<td>Danisco USA</td>
</tr>
<tr>
<td>Lb. delbrueckii ssp. bulgaricus</td>
<td>Isolated from yogurt starter culture</td>
<td>This study</td>
</tr>
<tr>
<td>Streptococcus thermophilus</td>
<td>Isolated from yogurt starter culture</td>
<td>This study</td>
</tr>
</tbody>
</table>

### Table 2. Yogurt base formulation

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Skim Milk</td>
<td>86.88%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.17%</td>
</tr>
<tr>
<td>Modified Starch</td>
<td>2.50%</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.20%</td>
</tr>
<tr>
<td>High Fructose Corn Syrup</td>
<td>2.25%</td>
</tr>
</tbody>
</table>

### Table 3. Proteolytic enzymes used in bacteriocin assay

<table>
<thead>
<tr>
<th>Protease</th>
<th>Source</th>
<th>Supply</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsin</td>
<td>Bovine pancreas</td>
<td>Boehringer (Ingelheim, Germany)</td>
</tr>
<tr>
<td>Papain</td>
<td>Papaya latex</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
<tr>
<td>Pepsin</td>
<td>Hog stomach mucosa</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
<tr>
<td>Protease</td>
<td>Aspergillus saitoi</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
<tr>
<td>Protease</td>
<td>Bacillus licheniformis</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
<tr>
<td>Protease</td>
<td>Streptomyces griseus</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
<tr>
<td>Protease</td>
<td>Bovine pancreas</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Tritrachium album</td>
<td>Fisher (Fair Lawn, NJ)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Bovine pancreas</td>
<td>Sigma (St. Louis, MO)</td>
</tr>
</tbody>
</table>
Figure 1. Industrial yogurt process flow chart. Bulk starter was prepared by addition of mixed starter cultures to 12% milk (w/v) and fermentation was allowed to occur until pH 4.55. Yogurt base was mixed, homogenized, and pasteurized (HTST) and then bulk starters were added to start the yogurt fermentation. The yogurt base was fermented to a final pH of 4.55.

A1) Industrial method: Lyophilized *Lb. acidophilus* NCFM culture were added at 0.26% (w/v) to achieve an initial inoculum of 10^6 CFU/ml in the bulk starter at the end of fermentation. Bulk starter was added at 5% (w/w) to the yogurt base and fermented to pH 4.55.

A2) Increasing inoculation: Lyophilized NCFM culture were added at 1.2% (w/v) to achieve an initial inoculum of 10^9 CFU/ml in the bulk starter at the end of bulk starter fermentation.

B) NCFM was prepared as a separate bulk starter by fermenting NCFM in 12% NFDM overnight, and combined with traditional bulk culture to ferment the yogurt. Each were added to the yogurt base at 2.5% and fermented to a final pH of 4.55.

C) Lyophilized NCFM culture was added at 0.06% w/v (approximately 6 x 10^7 CFU/ml initial levels) directly to yogurt base as the sole fermentation species and allowed to ferment to pH 4.55.

D) Lyophilized NCFM culture was added at 0.06% w/v (approximately 6 x 10^7 CFU/ml initial levels) directly to yogurt base with the traditional bulk starter and fermented to pH 4.55.

E) Lyophilized NCFM culture was added at 0.06% w/v (approximately 6 x 10^7 CFU/ml initial levels) at the end of the yogurt fermentation at pH 4.55.
Figure 2. Survival of *Lb. acidophilus* NCFM in fermented yogurt acidified to pH 4.55. Methods include NCFM addition to bulk starter at the end of fermentation (sample A♦), addition directly to yogurt base fermentation at the outset (sample B ■), *Lb. acidophilus* NCFM as separate bulk starter and combined with the traditional fermented bulk starter (sample C ▲), addition to fermented yogurt (sample D ×), and *Lb. acidophilus* NCFM as sole fermentation species used to ferment the yogurt base (sample E ○).
Table 4. Strains isolated from commercial yogurts

Identification based on 16SrRNA sequencing from a single colony isolated on MRS with 0.2 mg/L clindamycin

<table>
<thead>
<tr>
<th>Commercial brand</th>
<th>Final pH @ end of shelf-life</th>
<th>Colony selected</th>
<th>Colony Isolated a</th>
<th>Species listed on package</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>4.1</td>
<td>1</td>
<td><em>Lb. acidophilus</em></td>
<td>Contains live and active cultures including <em>L. acidophilus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td><em>Lb. acidophilus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td><em>Lb. acidophilus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td><em>Lb. acidophilus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td><em>Lb. acidophilus</em></td>
<td></td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>4.1</td>
<td>2</td>
<td><em>Lb. acidophilus</em></td>
<td><em>L. acidophilus</em> and <em>L. bulgaricus, Bifidobacterium</em> sp. and <em>S. thermophilus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td><em>Lb. acidophilus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td><em>Lb. acidophilus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td><em>Lb. gasseri</em></td>
<td></td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>3.7</td>
<td>2</td>
<td><em>Lb. gasseri</em></td>
<td><em>S. thermophilus, L. bulgaricus, L. acidophilus, Bifidobacterium, L. casei</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td><em>Lb. gasseri</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td><em>Lb. gasseri</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td><em>Lb. johnsonii</em></td>
<td></td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>3.9</td>
<td>2</td>
<td>Uncultured bacterium</td>
<td><em>L. acidophilus, L. bulgaricus, S. thermophilus, bifidus.</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Uncultured bacterium</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>Uncultured bacterium</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td><em>Lb. rhamnosus</em></td>
<td>Contains six live active cultures including <em>L. acidophilus, bifidus, L. casei</em> and <em>L. rhamnosus</em></td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>4.0</td>
<td>2</td>
<td><em>Lb. rhamnosus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td><em>Lb. rhamnosus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td><em>Lb. rhamnosus</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>No growth on plates</td>
<td></td>
</tr>
<tr>
<td><strong>F</strong></td>
<td>N/A</td>
<td>2</td>
<td>No growth on plates</td>
<td>Active yogurt cultures including <em>L. acidophilus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>No growth on plates</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>No growth on plates</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3a. *Lb. acidophilus* viability in yogurt base at 37°C for 24 hours (no other cultures present).

Figure 3b. pH development by *Lb. acidophilus* grown in yogurt base at 37°C for 24 hours (no other cultures present).
Figure 4. Yogurt starter culture isolates plated with a *Lb. acidophilus* NCFM overlay. Clear zones represent areas where *Lb. acidophilus* was inhibited.
Figure 5. *Lb. bulgaricus* and *Lb. acidophilus* were spotted on MRS agar and incubated overnight and then overlaid with a lawn of NCFM cells. Catalase was spotted adjacent to the colony spot prior to *Lb. acidophilus* overlay. The concentration of catalase and location of spots (5 μl) are shown on figure. Clear zones represent inhibition of *Lb. acidophilus* NCFM growth.
Figure 6a. Growth and acid development of *Lb. acidophilus* NCFM during a yogurt fermentation supplemented with 0.1% catalase.

Figure 6b. Growth and acid development of *Lb. acidophilus* NCFM during a yogurt fermentation in the presence of 0.5% catalase.
Figure 7. Survival of *Lb. acidophilus* NCFM in fermented yogurt, supplemented with catalase.
Figure 8. Survival of *Lb. acidophilus* NCFM in directly acidified yogurt base stopped at pH 5.0 (♦), 4.8 (■), 4.6 (▲), 4.4 (×) and 4.2 (○), using DL-lactic acid. pH levels were maintained through experiment (data not shown).

Figure 9. Survival of *Lb. acidophilus* NCFM in fermented yogurt stopped at pH 5.1, 4.7 and 4.1. After 7 weeks storage at 4°C the final pH levels of the fermented samples went from pH 5.1 → 4.3; pH 4.7 → 4.1; and pH 4.1 → 3.8.
Figure 10. Survival of *Lb. acidophilus* NCFM added to PBS buffer at pH 3.5 (acidified with DL-lactic acid). Control sample is lyophilized NCFM added directly to buffer. Resuscitated sample is lyophilized NCFM resuscitated in MRS for 15 min prior to resuspension in the buffer.
Figure 11. Survival of resuscitated *Lb. acidophilus* NCFM in MRS and acidified MRS (pH 5.5) prior to addition to DL-lactic acid acidified yogurt (pH 4.7 and 4.1). Control sample was held in MRS for 1 hr prior to addition to either yogurt acidified to pH 4.7 (♦) or pH 4.1 (▲). Adapted sample was held in MRS acidified to pH 5.5 for 1 hr prior to addition to yogurt acidified to pH 4.7 (■) or pH 4.1 (×).
REFERENCES CITED


APPENDICES
APPENDIX I

Production of yogurt agarose plates

Caitlin E. Trahan, Dr. Todd R. Klaenhammer
**Introduction:**

The ability to analyze global gene expression in bacteria via microarrays requires the ability to harvest cells and extract mRNA immediately from the environment of interest. The concept of yogurt agar allows exposure of *Lb. acidophilus* cells to a yogurt environment (pH, nutrients, cultures) for various times and easy removal of those cells from the surface for RNA isolation and microarray analysis. The following is a protocol and formula for constructing yogurt agarose plates which could be used for future exposure of probiotic and starter bacteria to a dairy matrix, for subsequent analysis of global gene expression.

**Materials and Methods:**

**Yogurt preparation.** *Yogurt bulk starters.* Milk was fermented by the inoculation of a multiple starter culture composed of *Streptococcus thermophilus* (NCK 2028), *Lactobacillus delbrueckii* ssp. *bulgaricus* (NCK 2029), and *Lactobacillus lactis* (2% - approximately 10⁶ CFU/ml initial population) (Danisco USA, Madison, WI) into 12% (w/v) skim milk (MP Biomedicals, Inc., Solon, OH). The milk was fermented at 42°C until it reached a final pH of 4.55. The bulk starter culture was then placed in an ice bath to cool, and then stored in the refrigerator. The starter was used with in 24 hours.

*Yogurt base.* Ingredients were combined and stirred for 20 minutes prior to a 30 minute steam treatment (Table 2). Ingredients were stirred again for 2 minutes followed by immersion into an ice bath until a temperature of 42°C was reached. The industrial yogurt base was inoculated at 5% (approximately 2 x 10⁸ CFU/ml initial population) with yogurt bulk starter culture and
fermented at 42°C in a water bath until pH 4.55 (~ 3.5 hrs). This final yogurt was cooled in an ice bath and stored in a refrigerated at 4°C.

**Yogurt agarose.** A 10 ml agarose solution (5% w/v, Denville Scientific, Inc., Metuchen, NJ) was added to a 30 ml test tube and then autoclaved at 121°C, and tempered to 55°C in a water bath. The yogurt was tempered to 42°C in a 500 ml beaker and the 10 ml agarose solution was added to 40 ml of yogurt. The final agarose concentration was 1.0%. Vortex or mix immediately and pour to a 100 x 15 mm plate. The 50 ml yogurt agarose solution makes one plate. Larger volumes are possible; however, the agarose solution sets rapidly and can result in uneven textures throughout the plate. The process must be done in a timely fashion. Allow plates to cool and store at 4°C.

**Conclusion:**

The agarose solution sets rapidly upon reaching cooling. To date, the largest volume used to successfully prepare one plate was 250 ml. It was necessary to have 200 ml yogurt tempered in a 500 ml bottle, as well as a 50 ml agarose solution in a 100 ml bottle which could readily be poured into the tempered yogurt. These plates were poured while on a shaker moving at 150 RPM. To preserve the viability of the yogurt starter cultures and avoid heat damage, the yogurt is tempered to a maximum of 42°C. The agarose solution is tempered to at least 55°C to prevent rapid solidification upon mixing.
Table 1. Yogurt base composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Skim Milk</td>
<td>86.88%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.17%</td>
</tr>
<tr>
<td>Modified Starch</td>
<td>2.50%</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.20%</td>
</tr>
<tr>
<td>High Fructose Corn Syrup</td>
<td>2.25%</td>
</tr>
</tbody>
</table>

Figure 1. Yogurt agarose (250ml) in Genetix Vented QTray.
APPENDIX II

Investigation of a $msmE$ gene mutant in *Lactobacillus acidophilus* NCFM

Caitlin E. Trahan, Dr. Todd R. Klaenhammer
In an experiment involving the stability of *Lb. acidophilus* NCFM in yogurt, a selective agent allowing direct enumeration of *Lb. acidophilus* NCFM against the yogurt starter strains was necessary. Prior to using clindamycin as a selective agent, we chose to screen previously generated mutants that harbored antibiotic resistance markers. The strain selected should have no difference in acidification profiles in milk or yogurt base compared to the parent. The acidification rate of a *Lb. acidophilus* NCFM strain with a mutation in the *msmE* gene (NCK 1626) (1) was compared to the parent in 12% NFDM and showed a significantly different acidification profile below pH 5.5 (Figure 1). The *msmE* gene is an ABC-transporter induced in the presence of fructooligosaccharides (FOS). Glucose represses expression of this operon which suggests a regulation mechanism based on preferred carbohydrate utilization.

Since lactose is the major carbohydrate in milk with only trace amounts of glucose, fructose, glucosamine, galactosamine, neuraminic acid and neutral and acidic oligosaccharides, it was unlikely that this mutation should have an effect on the growth in milk (2). With these unexpected results, we speculated that this ABC-transporter may also have some affinity for additional substrates in milk, such as oligopeptides. To test this, both samples were supplemented with 1% casitone and acidification profiles were followed for 24 hours. The results showed that acidification profile in the mutant matched that of the parent when casitone was added to the NCK 1626 culture (Figure 2).

ABC-transporters have very specific affinity for substrates. In order for an ABC-transporter to transport additional compounds they must first be chemically modified to mimic the substrate specific to that transporter (4). A second method for multisubstrate affinity is the
‘flippase’ model associated with multidrug resistance (3). In order to test whether or not the FOS ABC-transporter has some affinity for oligopeptides, it would be necessary to label the individual constituents of casitone and to distinguish uptake through this specific ABC-transporter versus the other 2 oligopeptide specific transporters in NCFM.
Figure 1. Acidification profile of *Lb. acidophilus* NCFM vs. the *msmE* mutant (NCK 1626).

Figure 2. Acidification profile of *Lactobacillus acidophilus* NCFM vs. the *msmE* mutant (NCK 1626) in milk with a 1% casitone supplement.
REFERENCES CITED


