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In vitro modeling studies of *Lactobacillus acidophilus*: Antimicrobial activity and growth

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IN VITRO MODELING STUDIES OF LACTOBACILLUS ACIDOPHILUS: ANTIMICROBIAL ACTIVITY AND GROWTH

by

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TITLE

IN VITRO MODELING STUDIES OF Lactobacillus acidophilus:

ANTIMICROBIAL ACTIVITY AND GROWTH

BY

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IN VITRO MODELING STUDIES OF LACTOBACILLUS ACIDOPHILUS: ANTIMICROBIAL ACTIVITY AND GROWTH

Custodio F. Fernandes, Ph. D.
University of Nebraska, 1987

Advisor: Prof. Khem M. Shahani

Consumption of cultured dairy products results in an increase in the Lactobacillus count and a decrease in coliform count. Hence, model experiments were designed to study the antimicrobial effect of L. acidophilus and the resultant changes in fecal flora. Modeling of in vivo growth and antimicrobial activity of L. acidophilus in vitro requires its growth in the presence physiological concentrations of bile salts. L. acidophilus LA 15 antimicrobial activity was reduced from 20 mm in control to 14.1 mm (12mM of NaTCA or NaGCA) in vitro in the presence of bile salts (0-12 mM) when grown using the conventional technique. The reduction in antimicrobial activity was due to the bacteriocidal effect of accumulating deconjugated bile salts that strongly inhibit the growth of L. acidophilus LA 15 from 8.6 log # cfu/ml (control) to 4.4 log # cfu/ml in the presence of 3 mM NaTCA and 9 mM NaGCA. An "Add-back" in vitro technique has been developed, with the assumption that by periodically centrifuging out the

conjugated and deconjugated bile salts, it may be possible to simulate the physiological conditions involving diffussion of conjugated and deconjugated bile salts. With the "Add-back" technique, L. acidophilus LA 15 growth was not inhibited in the presence of 3 mM NaTCA and 9 mM NaGCA concentration, the log # cfu/ml after 24 h were 8.1 and 6.3 in the "Add-back" and conventional techniques, respectively.

In the "Add-back" technique the conjugated and deconjugated bile salts are removed periodically enabling the bacteria to grow. The individual and associative growth of E. coli and L. acidophilus was studied by activating the lactoperoxidase (LP) system in vitro. In individual grown cultures, the LP system at 30:30 ppm of hydrogen peroxide:SCN-, was more inhibitory to growth of E. coli (5.5 log # cfu/ml) than L. acidophilus (7.1 log # cfu/ml), as observed in cell counts at the end of 8 h. In associative growth studies at 30:30 ppm of hydrogen peroxide:SCN-, it was observed that the number of viable E. coli (6.0 log # cfu/ml) decreased whereas the number of L. acidophilus (7.0 log # cfu/ml) increased. This suggests that the LP system if activated in vivo may be responsible in part for the alteration in fecal flora.

THIS DISSERATION

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DEDICATED

TO

MY PARENTS

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PREFACE

The dissertation form represents a change from the conventional style and deserves special mention. The dissertation has been divided into four sections. Each section is written as a manuscript for a scientific journal, and each is complete in itself. Section one relates to the review of research work on therapeutic benefits of lactic acid bacteria. Sections two, three and four are based on experimental work and are complete with Introduction, Material and Methods, Results, Discussion and References. Section five is a summary and conclusion from sections two, three and four.

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GLOSSARY

AA:	Antimicrobial activity
CA:	Cholic acid
CBS:	Conjugated bile salts
DCBS:	Deconjugated bile salts
HDL:	High density lipoproteins
LBS:	*Lactobacillus* selection
LDL:	Low density lipoproteins
LP:	Lactoperoxidase system
MRS:	deMan, Rogosa and Sharpe
NaGCA:	Sodium glycocholate
NaTCA:	Sodium taurocholate
SCN-:	Thiocyanate
VRBA:	Violet red bile agar

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INTRODUCTION

The consumption of cultured dairy products results in the alteration of gut microbiota as a result of which there is an increase in fecal lactobacilli and a concommitant decrease in coliforms. The favorable alteration of the gut microbiota as observed in fecal counts has been attributed to the biosynthesis of broad spectrum of antimicrobial agents. These antimicrobial agents inhibit the growth of undesired coliforms and pathogens at low pH as observed in *in vitro* experiments.

In order to explain the therapeutic aspects that may be attributed to dietary lactobacilli, it is necessary either to demonstrate the benefit *in vivo* or, design *in vitro* studies that may be conducted in an environment that resembles the *in vivo* situation. Hence, during the course of this dissertation, a study of the antimicrobial activity and growth of *Lactobacillus acidophilus* in the presence of bile salts was carried out.

When bile salts, sodium taurocholate (0-12 mM) and Sodium glycocholate (0-12 mM) were added to MRS broth and *L. acidophilus* strains were inoculated there was a decrease in antimicrobial activity. The decrease in antimicrobial activity was because the bacterial growth was strongly inhibited in the presence of physiological concentrations of

bile salts. However, this is in sharp contradiction to the observed phenomenon *in vivo*, where the bacteria survive and grow at physiological concentrations of bile salts. The *L. acidophilus* fail to grow *in vitro* at physiological concentrations of bile salts due to the bacteriocidal effect of deconjugated bile salts. The bacteriocidal effect of deconjugated bile salts is eliminated *in vivo* as the conjugated and deconjugated bile salts are recovered through the enterohepatic cycle. Hence, it was necesary to develop a method that permits the growth modeling of *L. acidophilus* in the presence of physiological concentration of bile salts.

A novel "ADD-BACK" technique has been developed and is proposed to study the growth of *L. acidophilus* in the presence of bile salts at physiological concentrations of bile salts. It is assumed that by periodic centrifugation of the spent broth containing bile salts, the *in vivo* process of absorption of deconjugated and conjugated bile salts is simulated. In this technique growth of *L. acidophilus* is simulated through removal of deconjugated bile salts and spent broth, and readdition of fresh media containing conjugated bile salts. The sequential steps that are involved in the "ADD-BACK" technique simulate the *in vivo* growth. The growth modeling of *L. acidophilus* is made possible in the "ADD-BACK" technique because bile salt tolerant lactobacilli are selected and allowed to grow.

This may resemble the *in vivo* process in the small intestine, in which bile salts are removed through absorption or excretion and added back by intermittent release from the gall bladder. Hence, the "ADD-BACK" technique models the continued growth of *L. acidophilus* in the presence of bile salts. This is the first experiment that models *L. acidophilus* growth in the presence of physiological concentrations of bile salts.

Following the *in vitro* studies of growth of *L. acidophilus* in the presence of bile salts, it was essential to understand the basis of alteration in gut microbiota in a physiological environment. In *in vitro* experiments it has been demonstrated that coliforms and food borne pathogens fail to grow in association with lactic acid bacteria. The effect has been attributed to antimicrobial substances and lactic acid. However, in the intestinal tract, the digesta is highly buffered and hence the pH may not drop below pH 5.0, where the growth of *Escherchia coli* is affected. Hence, to explain the modification of gut microbiota, the individual and associative growth of *L. acidophilus* and *E. coli* was studied, following the *in vitro* activation of lactoperoxidase (LP) system. The LP system has been shown to be activated *in vivo* following feeding of the enzyme lactoperoxidase to dairy calves. Hence, it was of interest to see if this system when activated *in vitro* could be used to explain the modification of gut microbiota. In

individual growth studies it was observed that the LP system was more bacteriocidal to the growth of E. coli than L. acidophilus. In associative growth studies it was observed that the LP system decreases the E. coli count and the L. acidophilus was always present in higher numbers. This effect was observed at pH values that do not affect the growth of E. coli.

Thus in this study it was shown for the first time that L. acidophilus can grow in the presence of physiological concentrations of bile salts. Additionally, based on earlier studies of feeding lactoperoxidase enzyme to calves, where the hydrogen peroxide producing lactobacilli activate the LP system in vivo, it is suggested that the LP system may be responsible in part for alteration of gut microbiota.

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SECTION I

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THERAPEUTIC ROLE OF DIETARY LACTOBACILLI AND LACTOBACILLIC FERMENTED DAIRY PRODUCTS

A B S T R A C T

Lactic acid bacteria present in the human and animal gut are introduced through fermented milk products, food and feed supplements containing viable bacteria. Lactobacillus species, such as L. acidophilus, L. bulgaricus, L. lactis along with B. bifidum and Streptococcus faecium constitute an integral part of the healthy gastro-intestinal microecology and are involved in the host metabolism. They impart therapeutic benefits to the consumer. The antimicrobial substances produced by these bacteria control the proliferation of undesired pathogens. Their anticholesterimic properties assist in lowering serum cholesterol. It has been suggested that the tumor suppression trait of these microbes reduce the incidences of colon cancer.

I N T R O D U C T I O N

At birth the intestinal tract of infants is devoid of microbes. It gets progressively inhabited with ubiquitous microbes through food. The major bacterial genera that inhabit and proliferate in the human intestinal tract are Bacteroides, Bifidobacterium, Clostriduim, Escherichia, Lactobacillus and Streptococcus. In healthy adults there is a definite balance among these microbes. However, in the disease state the bacterial populations are altered unfavorable, resulting in overgrowth of undesirable or pathogenic bacteria (1).

The role of lactic acid bacteria in health and disease state has been documented in the literature (1-5). Metchnikoff (4) was perhaps the first to suggest that microorganisms were responsible for the beneficial effects associated with lactobacilli fermented foods. He attributed the longevity of the Bulgarians to consumption of milk products fermented with Lactobacillus bulgaricus. He postulated that the intestinal flora produces toxins that are detrimental to the host's health. The harmful effects of the undesired bacteria can be overcome by establishing a balance between intestinal flora, through ingestion of lactic organisms present in cultured dairy products.

In order that an individual may derive continued nutritional and therapeutic benefits, it is essential that the lactic acid bacteria survive the physiological environment of the gut. It is broadly accepted that Lactobacillus acidophilus will establish itself in the gastro-intestinal tract (6, 7). Sherman and Savage (8), observed in mice that lactobacilli colonize the gastric epithelial surface and adhere to the surface through the lipoteichoic acids. L. bulgaricus will establish colonies which are attached to the gut mucosal epithelial cells in germ-free mice (9). The lipoteichoic acid of Bifidobacterium bifidum subsp pennsylvanium adhere to human colonic epithelial cells (10). Lin and Savage (11) suggested that host specific cryptic plasmids may be