ANTIOXIDATIVE AND REDUCING ACTIVITIES OF SPECIES OF LACTOBACILLI AND STREPTOCOCCI

By

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CHAPTER I

INTRODUCTION

Interest in the role of lactic acid bacteria in promoting human health goes back at least as far as 1908 when Metchnikoff suggested that the consumption of milk fermented with lactobacilli would prolong life (Metchnikoff, 1908). Since then, various species of lactic acid bacteria have been reported to possess many attributes that can improve health in humans when consumed as food adjuncts. These benefits include improved digestibility, improved nutritional value, improved lactose utilization, antagonistic action towards enteric pathogens, anticarcinogenic effect, hypocholesterolemic effect, and immune modulation (Bolognani *et al*, 1997; Lin and Yen, 1999b; Hitchins and McDonough, 1989; Fernandes and Shahani, 1990; Gilliland, 1990; Sanders, 1993; Orrhage *et al*, 2000; and Gomes and Malcata, 1999). One of the most recent claimed attributes is their ability to act as reactive oxygen species scavengers. Among the bacterial species involved are lactobacilli, streptococci and bifidobacteria (Lin and Yen, 1999c; Lin and Chang, 2000; Bing *et al*, 1998; and Kinouchi *et al*, 1998).

Although oxygen is an essential component of living organisms the generation of reactive oxygen intermediates is inevitable in aerobically metabolizing cells. These reactive oxygen intermediates are relatively instable due to the existence of one or more unpaired electrons in their structure. These molecules are known as free radicals. As a

result the organism needs defense systems to cope with such potentially damaging compounds (Machlin and Bendich, 1987). Many enzymes such as superoxide dismutases, peroxidases, and catalases, as well as other components such as glutathione, vitamins and micronutrients are quite useful in disarming these free-radical species (Winston *et al*, 1998; and Cao and Prior, 1998). However, the action of these substances frequently is not enough to terminate the effect of all free radicals. The organism's antioxidant status and its role against the development of certain pathological conditions known to be associated with oxidative stress has gained potential preventive and therapeutic significance in view of the beneficial effects of free-radical scavenging drugs or antioxidants (Halliwell, 1994). Various synthetic and natural antioxidants have been reported. Nevertheless, there are doubts about the safety and long-term effects on health of synthetic antioxidants (Lin and Yen, 1999b). Antioxidants from natural sources are likely to be more desirable. Few studies have been published reporting the ability of some lactic acid or similar bacteria to produce or act as antioxidants. Among these, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii ssp. bulgaricus*, *Streptococcus salivarius ssp. thermophilus* and *Bifidobacterium longum* are the most studied concerning their ability to produce or act as antioxidants (Lin and Yen, 1999a, 1999b). Furthermore, although some differences in results have been reported in these studies, it seems that all three species perform well depending on the method used.

CHAPTER II

LITERATURE REVIEW

Characteristics of cultures included in the study

The genus *Lactobacillus* comprises 56 recognized species (Kandler, 1984). Lactobacilli can be found in the various ecological niches (plants, gastrointestinal and genital tracts) and constitute an important part of the indigenous microflora of man and animals (Hitchins and McDonough, 1989). Their distribution is affected by factors like pH, type and level of specific substrates, oxygen availability, presence of secretions and bacterial interactions (Gomes and Malcata, 1999). The genus is generically characterized as Gram-positive nonsporeforming rods. They are either aerotolerant or anaerobic, strictly fermentative and catalase negative. Glucose is fermented predominantly to lactate in the case of homofermentation and to mixtures of lactate, CO_2, and ethanol (and/or acetic acid) in the case of heterofermentation. They have complex nutrient requirements and can grow in a wide range of temperature. However, their optimum growth temperature is between 30 and 40°C (Weiss *et al*, 1983).

Lactobacillus acidophilus is the most commonly used species for use as a probiotic in dairy products. It is a Gram-positive rod with round ends that occur as single cells, as well as in pairs or in short chains. The dimensions vary from 0.6-0.9 μm in width to 1.5-6.0 μm in length. It is nonmotile, nonsporeforming, and salt intolerant. Additionally, it is microaerophilic. Most strains can ferment amygdalin, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose, trehalose and esculine. Substrates such as arabinose, gluconate, mannitol, melezitose, rhamnose, ribose, sorbitol, xylose and sometimes melebiose, raffinose and trehalose are not metabolized by this species. Although lactose is virtually the only sugar present in milk, it has been reported that *L. acidophilus* can utilize sucrose more effectively. This can eventually be explained by differences in β-galactosidase and β-fructofuranosidase activities. The latter is a constitutive enzyme in *L. acidophilus* while β-galactosidase is inducible (Gomes and Malcata, 1999). *Lactobacillus acidophilus* may grow at 45°C, but optimum growth occurs within 30-40°C. Its acid tolerance varies with an optimum between pH 5.5 and 6.2. When grown in laboratory conditions it has complex growth requirements like low oxygen tension, fermentable carbohydrates, protein hydrolysates, and minerals.

Lactobacillus delbrueckii ssp. lactis exists as rods with rounded ends, with the size varying between 0.5μm to 0.8μm width and 2μm to 9μm length. Occurrence as single or short chains is the most common. They grow well at 45°C but can sustain slightly higher temperatures (48-52°C). Optimal growth is observed between 40-44°C and they do not grow under 15°C. Their fermentation pattern is characterized by positive

reactions with amygdalin, esculin, fructose, glucose, lactose, maltose, mannose, salicin, sucrose, trehalose and most of the times also positive with cellobiose and galactose. They do not ferment arabinose, gluconate, mannitol, melezitose, rafinose, rhamnose, ribose, sorbitol or xylose. This is characteristically a homofermentative species with D-lactate as the sole end product (Weiss *et al,* 1983).

Lactobacillus delbrueckii ssp. bulgaricus is phenotypically and genotypically very similar to *L. delbrueckii ssp. lactis*. However, the fermentation pattern is quite different. *L. delbrueckii ssp. bulgaricus* ferments very few carbohydrates such as fructose, glucose and lactose. It does not ferment amygdalin, arabinose, cellobiose, galactose, gluconate, maltose, mannose, mannitol, melibiose, rafinose, rhamnose, ribose, sorbitol, sucrose or xylose (Weiss *et al*, 1983).

The genus *Streptococcus* comprises 27 species characterized by spherical or ovoid cells, with a diameter less than 2μm. They form pairs or chains in liquid media. They are Gram-positive, non-sporeforming, catalase negative, strictly or facultative anaerobes and ferment carbohydrates with no gas production. They are fastidious, mainly homofermentative producing primarily lactic acid. Their growth temperature varies with an optimum at 37°C. The majority of the species in the genus are not used in the food fermentation industry as starters or adjuncts except *Streptococcus salivarius ssp. thermophilus* and *Streptococcus lactis* (Weiss *et al*, 1983).

Streptococcus salivarius ssp. thermophilus are oval shaped cells, with a diameter varying from 0.7μm to 0.9μm, forming pairs or long chains. One of the most important characteristics of this species is the relatively high optimum temperature for growth (40-45°C). It ferments preferably sucrose and lactose but also ferments fructose, glucose, lactose and mannose. Arabinose, glycerol, mannitol, inulin, dextrin, salicin, sorbitol, starch and xylose are not fermented. It is also differentiated by its rapid growth in litmus milk incubated at 45°C. This is a homofermentative organism and is used commonly as a starter culture mainly in the milk products industry.

Free radicals

The role of free radicals in the etiology and pathogenesis of many degenerative diseases is currently gaining a great interest (Garsetti *et al*, 2000). The formation of free radicals is a normal consequence of a variety of essential biochemical processes occurring in aerobic living systems (Lin and Yen, 1999b; and Halliwell and Chirico, 1993). The range of antioxidant defenses in the living organism should be adequate to protect it from oxidative damage. However, the balance can be lost due to overproduction of free radicals by the action of sources that overwhelm the antioxidative defenses. It is believed that the potential antioxidant components of diet can counter the deleterious effect of the free radicals present in the organism either produced in the organism or obtained from the environment.

By definition, free radicals are chemical species that contain an odd number or unpaired electrons. They may be positively charged, negatively charged, or neutral

(Halliwell and Gutteridge, 1984; and Machlin and Bendich, 1987). This broad definition includes the hydrogen atom (one unpaired electron) and most of the transition metals among others.

The source of the free radical can be endogenous or exogenous. Endogenous free radicals are those which are generated and act intracellularly as well as those formed within the cell and excreted into the surrounding environment. They are products mainly of autoxidation and oxidases. Electron transport systems are a major continuous source of intracellular free radicals. Exogenous free radicals are those obtained from environment pollutants such as tobacco smoke, and some aerosols (Winston *et al*, 1998). A number of these substances including some medicines can be metabolized into active intermediates or even to form free radicals. The most common free radicals found in living organisms are hydroxyl, peroxyl, hypochlorite, superoxide and alkoxy radicals (Machlin and Bendich, 1987). Radicals vary in reactivity, and similarly to other chemical species, their reactivity is dependent on temperature and concentration of the constituents of the medium (Glazer, 1990). Free radicals can damage several crucial biological components, and prime targets include membrane lipids (unsaturated bonds) with the consequent loss of membrane fluidity and receptor alignment, proteins hampering their functionality, carbohydrates altering any of the cellular receptor functions, and DNA causing mutations (Garsetti *et al,* 2000; and Halliwell and Aruoma, 1991). Additionally, hydroxyl radicals are particularly dangerous due to their ability to participate in aromatic hydroxylations, a phenomenon potentially harmful when occurring in the colon where the presence of various hazardous chemicals (consumed with foods or drugs) can trigger their conversion through this mechanism into carcinogens.

Antioxidative action of lactobacilli and streptococci

There is not much data published about the antioxidative ability of lactic acid bacteria. However, in studies published mainly by Lin's group (Lin and Yen, 1999a; Lin and Yen, 1999b; Lin and Yen, 1999c) strains of *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus, Streptococcus salivarius ssp. thermophilus* and *Bifidobacterium longum* grown in MRS broth have shown antioxidant activity towards ascorbic acid autoxidation and hydroxyl radical scavenging both as viable cells and as cell-free extracts. The tested species demonstrated an antioxidative activity with an inhibition rate in the range of 7.2-12.3%. Furthermore, they also reported that these species inhibited linoleic acid peroxidation as well as scavenged hydroxyl radicals, hydrogen peroxide and *t*-butylhydroperoxide. All these substances are described to be active in lipid peroxidation.

Lin and Chang (2000) reported antioxidative effect of *L. acidophilus* and *B. longum* that caused 28% and 32% inhibition of linoleic acid peroxidation respectively, using intact cells. These percentages were even higher when cell-free intracellular extracts were used being 45% and 48% respectively for *L. acidophilus* and *B. longum*. This indicated that both species had a good antioxidative effect towards linoleic acid peroxidation.

Bing *et al* (1998) and Allison *et al* (1992) also reported that culture supernatants of *L. acidophilus* had a suppressing effect on ileal ulcer formation caused by nonsteroidal anti-inflammatory drugs (NSAID) in rats. This study was related to a separate study reporting that intestinal ulceration occurred in 8.4% of the NSAID users but only 0.6% in

non-users. In a similar study, Kinouchi *et al* (1998), working with rats observed that overnight cultures of *L. acidophilus* and *B. adolescentis* inhibited ileal ulcer formation by oral administration of BFMeT (5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonylphenyl) thiophene. The incidence of multiple ileal ulcers was more than 85% in non-treated animals and was only 0 and 14% in those treated respectively with water suspension of cells of *L. acidophilus* and *B. adolescentis.* Osawa *et al* (1987) showed that supernatant from broth cultures of *L. acidophilus* inhibited *in vitro* lipid peroxidation of erythrocyte membrane ghosts induced by *t*-butyl hydroperoxide (BHP). Kudoh and Matsuda (2000) reported that species of *Lactobacillus* increased the antioxidative activity of sweet potato yogurt when used has probiotics.

Hirayama and Rafter (1999), although suggesting that there might be several mechanisms to explain the anticarcinogenic activity of some lactic acid bacteria including *L. acidophilus*, reported that the antiproliferative effect shown by the cultures was not necessarily related to their physical presence. It was suggested that some extra-cellular substances produced by them were responsible for the effect (Ahotupa *et al*, 1996). According to Sanders *et al* (1995) most lactic acid bacteria have the ability to disarm oxygen radical by either using a superoxide dismutase (SOD) or a high internal Mn^{2+} concentration. Furthermore, it was also observed that although both systems are common in lactic acid bacteria, they only contain one of the systems at a time, either SOD or high levels of Mn^{2+} (Zitzelberger *et al*, 1984)

Reducing ability of starter cultures

The notion of biological activity as a continuous sequence of oxidation-reduction reactions in living cells has permitted us to comprehend many fundamental problems concerning bacterial growth and metabolism by measuring the reducing potential developed in bacterial cultures and cell suspensions. For carrying out these measurements the use of redox dyes such as methylene blue and resazurin have been of incommensurable help. However, the reversibility of the final products and sensitivity of the process to atmospheric oxygen creates some limitations on their use (Laxminarayana and Iya, 1953).

Tetrazolium chloride (2,3,5-triphenyltetrazolium chloride) has been reported to be useful in a variety of studies such as testing of seed viability and root vitality (Clemensson-Lindell, 1994; Porter *et al*, 1994; and Upadhyaya and Caldwell, 1993), studies on dehydrogenase systems in plants as well as in animal tissues (Friedel *et al*, 1994; Urban and Jarstrand, 1979; Trevors, 1984; Casida Jr., 1977; and Steponkus, 1971), bacterial viability (Liska *et al*, 1958; Eidus *et al*, 1959; Roslev and King, 1993; and Bhupathiraju *et al*, 1999) and reductase test in milk (Mustakallio *et al*, 1955), antibiotic substances in milk and bacterial taxonomy (Gunz, 1949; and Turner *et al*, 1963). The use of tetrazolium redox dyes for the direct visual determination of bacterial metabolic activity has gained increased recognition in recent years (Rodriguez *et al*, 1992; Roslev and King, 1993; Beloti *et al*, 1999; and Thom *et al*, 1993). Tetrazolium salts (redox dyes) scavenge electrons from microbial oxidation-reduction reactions. They enter the microbial cell and are reduced, transforming from a colorless water-soluble solution into

a dark red water insoluble pigment. This reduction is performed by the electron transport system (ETS) components or hydrogenases in metabolically active cells or bacteria (Roberts, 1951).

Tetrazolium salts are a large family of heterocyclic organic compounds, which were first prepared in 1894. They are derived from tetrazoles, which exist in two isomeric forms. One of the most significant characteristics is the unusual property of forming highly colored insoluble substances known as formazans when reduced. The utility of these compounds as indicators of biological reduction was not appreciated at the time of discovery. Since then, many tetrazolium salts have been discovered and later their usefulness uncovered.

According to Seidler (1991), in 1894 Pechmann and Runge first synthesized 2,3,5-triphenyl tetrazolium chloride (TTC). This is a five-membered unsaturated ring containing two double-bonds, one carbon and four nitrogen atoms. When reduced (formazan) it has an absorption maximum around 485nm (acetone solution).
Later, it was demonstrated that bacteria, yeasts and plants are capable of reducing this dye. One of the first applications of TTC was to verify the germination capacity of seeds and viability of plant cells (Lindell, 1994; and Upadhyaya and Caldwell, 1993). Since then, many other uses have been detailed and included in the field of microbiology. As an indicator of microbial redox activity, TTC was first used in 1951 by Schönberg in the indirect determination of bacterial content in milk (Mustakallio *et al*, 1955).

The reduction of tetrazolium salts by anaerobic bacteria in low oxidation-reduction potential environments was initially thought doubtful because of the potential needed to reduce them (Rodriguez *et al*, 1992). However, in a study reported by

Laxminarayana and Iya (1953) it was shown that streptococci (*S. salivarius ssp. thermophilus*) and lactobacilli (*L. bulgaricus* and *L. lactis* among others) were capable of reducing TTC when cultured in broth or milk. They also reported that the reducing activity although considerable, varied as function of the growth medium. *Streptococcus salivarius ssp. thermophilus* exhibited better reducing ability in milk than in broth. The reduction was more rapid and more intense than in broth, showing the first color changes after about 2-3 hours from the beginning of the reaction and reaching the maximum in 6 hours, by which time the milk was completely coagulated and no further changes in color were observed. According to the same study, *L. acidophilus, L. lactis* and *L. bulgaricus*, exhibited lower performance. However, the trend was similar in both media. An interesting observation was the relation of color formation and pH. Cultures which reduced TTC poorly generally showed more acid production than did those that exhibited stronger reducing activity (Atkinson, 1950).

Recent studies also demonstrated that TTC could be used in assessment of the reducing activity of anaerobic bacteria. Bhupathiraju *et al* (1999) and Thom *et al* (1993) used TTC to assess viability and activity of anaerobic bacteria. They also reported that reduction of the dye was observed in all growth stages and it was proportional to the cell counts. They hypothesized that dehydrogenase systems in the cell or ETS (electron transport system) components might be involved in the process although direct evidence was scarce.

Objectives of the study

The objectives of this study were to assess the reducing activity using the TTC method and determine if there was an apparent relationship of this activity with the antioxidative activities of *Lactobacillus acidophilus, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus delbrueckii ssp. lactis, Lactobacillus casei* and *Streptococcus salivarius ssp. thermophilus* as measured through protection of β-phycoerythrin from attack by free radicals generated by APPH (2,2'-azobis (2-amidinopropane) hydrochloride.

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CHAPTER III

ANTIOXIDATIVE AND REDUCING ACTIVITIES OF SPECIES OF LACTOBACILLI AND STREPTOCOCCI

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ABSTRACT

The reducing ability and antioxidative activity of some species of *Lactobacillus* and *Streptococcus* were compared under *in vitro* conditions.

Strains of *Lactobacillus delbrueckii ssp. lactis, Lactobacillus delbrueckii ssp. bulgaricus, Lactobacillus acidophilus, Lactobacillus casei* and *Streptococcus salivarius ssp. thermophilus* were grown at 37°C in MRS broth (Difco) or nonfat milk (10% NFDM) supplemented with 0.5% 2,3,5 triphenyl tetrazolium chloride (TTC) to evaluate reducing activity. Reduced TTC was extracted from the cultures with acetone and the intensity of the red color measured with a colorimeter at 485nm as an indication of reducing activity. Antioxidative activity was evaluated by the ability of the whole cells or the cell-free extracts from cultures to protect a protein from being attacked by free radicals. Analyses were performed using the Oxygen Radical Absorbance Capacity method (ORAC).

The lactobacilli varied significantly in relative ability to reduce TTC when grown in MRS broth for 15 hours. None of the streptococci tested reduced TTC when gown in MRS broth in the same period. When grown in 10% milk, cultures exhibited lower reducing activity than in MRS broth. However, the streptococci did reduce TTC in the milk cultures.

When cultures were analyzed for antioxidative capacity, all of them exhibited some degree of antioxidive activity. Among the treatments, the cell-free extracts from cells grown in MRS broth exhibited significantly higher values than did whole cells. Similar results were obtained from the whey with cultures grown in nonfat milk although

the antioxidative activities of the whey from sonicated milk cultures were not statistically different. There was no apparent relationship between the reducing and antioxidative activities of the cultures evaluated.

The results from this study show that these cultures can provide a source of dietary antioxidants. Furthermore, selection of cultures that produce antioxidants as starters could provide yet another health or nutritional benefit from cultured or culture containing dairy products.

INTRODUCTION

The interest of Reactive Oxygen Species (ROS) in Biology and Medicine is evident due to their strong relation with phenomenon like aging and disease processes (Cao *et al*, 1995). The concept of ROS comprises not only oxygen centered radicals such as $O_2^{\cdot-}$ and $OH^{\cdot}$ but also non-radical derivatives of oxygen such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). It is well known that free radicals and other reactive oxygen species are continuously being produced in living organisms. As a consequence, defense mechanisms have also evolved to deactivate these free radicals and/or repair the damage caused by their reactivity (Halliwell and Chirico, 1993). However these systems are not always sufficiently active to disarm the totality of metabolically produced and/or exogenous free radicals.

Most lactic acid bacteria have systems to cope with oxygen radicals. According to Stecchini *et al* (2000) the most common systems are superoxide dismutase (SOD) and high internal concentrations of Mn^{2+}. Knauf *et al* (1992) also reported that some species of lactobacilli produced a heme-dependent catalase which can degrade hydrogen peroxide at a very high rate blocking the formation of peroxyl radicals. The ability of lactic acid bacteria to create low oxidation-reduction potential needed for their optimum growth must be related to some of these systems. Reducing activity can be measured by the ability of the organisms to reduce 2,3,5-triphenyltetrazolium chloride (Laxminarayana and Iya, 1954).

Free radical scavenger properties of starter cultures would be useful in the food manufacturing industry. They could beneficially affect the consumer by providing another dietary source of antioxidants (Ouwehand and Salminen, 1998) or by providing

probiotic bacteria having potential of producing antioxidants during growth in the intestinal tract.

There are many methods to assess free radical scavenging ability, such as Trolox-Equivalent Antioxidant Capacity (Randox-TEAC) and Ferric Reducing Ability (FRAP) (Cao and Prior, 1998). Most of these methods assess one of the two components of the antioxidative process, measuring time to reach a fixed degree of inhibition or the extent of inhibition at a fixed time (Cao *et al*, 1995).

The Oxygen Radical Absorbance Capacity (ORAC) assay is, to date, the only method combining both variables (Cao *et al*, 1998; Wang *et al*, 1996; and Cao and Prior, 1998). This is basically a process where by the reaction between a reactive oxygen species, such as hydroxyl or peroxyl radicals, and a target molecule, such as low-density lipoprotein or β-phycoerythrin, can be monitored (Handelman *et al*, 1999). In the case of β-phycoerythrin, the structural change is reflected in the loss or decrease of fluorescence.

The objective of this study was to compare the antioxidative activity of various species of lactobacilli and streptococci used as yogurt starter cultures or as probiotic bacteria. A second objective was to determine if reducing activity, based on the reduction of 2,3,5-triphenyltetrazolium chloride could be used to predict relative antioxidative activity of these organisms.

MATERIAL AND METHODS

Source and maintenance of cultures

The cultures used in this study (*Lactobacillus acidophilus, Lactobacillus delbruekii ssp. lactis, Lactobacillus delbruekii ssp. bulgaricus, Lactobacillus casei* and *Streptococcus salivarius ssp. thermophilus*) were obtained from the stock culture collection of the Food Microbiology Laboratory in the Food and Agricultural Products Research and Technology Center at Oklahoma State University. Cultures were maintained by subculture in MRS broth (Difco Laboratories, Detroit, MI) using 1% innocula and incubation for 18 hours at 37°C. Between uses, cultures were stored in a refrigerator at 5°C. To make freshly prepared cultures for experiments, immediately prior to use, they were subcultured three times on successive days in MRS broth.

Plate counts

The total numbers of lactobacilli and streptococci in the cultures were determined using the pour plate method (with overlay) on MRS agar. The samples were diluted in 0.1% peptone (Sigma Chemicals Co, St. Louis, MO) dilution blanks (99ml) containing 0.01% silicone antifoamer (Sigma Chemicals Co, St. Louis, MO). Duplicate plates of the appropriate dilution were prepared and incubated at 37°C for 48 hours. The colonies were counted with the aid of a Quebec colony counter (American Optical Co., Buffalo, NY) and the colony forming units per milliliter were determined.

Screening cultures for reducing activity

A stock solution of 2,3,5-triphenyl tetrazolium chloride (TTC; Sigma Chemicals Co, St. Louis, MO) was prepared by dissolving 50mg in 10ml of distilled water and passing it through a sterile membrane filter (0.45μm pore diameter; Gelman Laboratory, Ann Arbor, MI) into a sterile tube. The solution was prepared fresh daily and kept in the dark by wrapping the tube with aluminum foil.

For the screening of reducing activity, tubes containing 9ml of sterile modified (without beef extract) MRS broth supplemented with 1ml of 0.5% (w/v) TTC (added just prior to use) were inoculated with 0.1ml of freshly prepared cultures and incubated 18 hours at 37°C. After the incubation, 0.8ml aliquots were removed from each tube and dispensed into 1.8ml micro-centrifuge tubes along with an equal volume of acetone (Pharmaco, Brookfield, CT) and shaken vigorously. The micro-centrifuge tubes were placed in an ice-water bath for 3 hours to allow maximum extraction of the formazan. Then, the tubes were centrifuged at 12,000 x g for 10 minutes. One ml of the supernatant of each tube was collected with a 1ml pipette and dispensed in a 1ml cuvette. Then, the absorbance at 485nm was read using a spectrophotometer (Spectronic 21D, Milton Roy Co., Rochester, NY) against a blank of deionized water. The remaining portions of the initial 9ml of cultures were checked for pH.

The screening process, using the same procedure was employed using sterile 10%NFDM as a growth medium. Additionally, pH of the incubated milk was measured.

Antioxidative activity of broth cultures

Freshly prepared cultures were used to inoculate (1%) 50ml volumes of sterile MRS broth and incubated for 18 hours at 37°C. After the incubation period the content of each bottle was transferred into a centrifuge bottle and centrifuged at 12, 000 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet washed by resuspending it in 50ml of deionized water and centrifuging again at the same conditions. This wash procedure was repeated three times. Finally, each washed pellet was resuspended in 50ml of 0.2M potassium phosphate buffer (pH 7.0) and aseptically dispensed (in 25ml aliquots) into two sterile vials to prepare the samples corresponding to whole cells and cell-free extracts.

The whole cells were incubated at 37°C for 30 minutes. The cell-free extract was obtained by sonicating cells suspended in buffer at setting 4 (Sonic Dismembranator, Heat Systems Ultrasonics, New York, NY) for 5 minutes. To avoid temperature damage during sonication, tubes were maintained in an ice-water mixture.

At the end of the incubation period, the whole cells and the sonicated cells were centrifuged as above for removal of cells and/or cell debris and the supernatants collected for evaluation of antioxidative activity. The buffer (0.2M phosphate buffer pH 7.0) solution without cells was used as control.

Antioxidative activity of milk cultures

Milk cultures were processed basically in a similar manner as the broth cultures. Bottles containing 50ml of sterile 10% nonfat milk were inoculated (1%) with the desired cultures (which had been subcultured three times in nonfat milk prior to the experiment) and incubated at 37°C for 18 hours. After incubation, the contents of the bottles were dispensed (in 25ml aliquots) into two separate vials. The contents of one vial were centrifuged 10min. at 12,000 x g and 4°C. The whey was collected and analyzed for antioxidative activity. The second vial was sonicated at setting 4 (Sonic Dismembranator, Heat Systems Ultrasonics, New York, NY) for 5min. and centrifuged 10min. at 12,000 x g and 4°C. The whey fraction was collected and analyzed for the antioxidative activity. As control sterile 10% NFDM (from the same batch used to prepare the cultures) was acidified (with lactic acid) to pH 4.0 and centrifuged as were the cultured samples. The whey fraction was analyzed for antioxidative activity.

Measuring the antioxidative activity

Assay for antioxidative activity

The antioxidative activity of the samples from the cultures was measured by the method described by Cao *et al* (1995). For convenience the protocol is described below.

The stock solution of β-phycoerythrin (sigma Chemicals Co. St. Louis, MI) was prepared by dissolving 1mg into 5.6ml of phosphate buffer (0.2M pH 7.0). This stock solution was kept under refrigeration. The working solution was made by mixing 300μl of the stock solution with 13.4ml of phosphate buffer in a flask just prior to use.

The 2,2'-Azobis (2-amidinopropane)dihydrochloride (AAPH; Waco Chemical USA Richmond, VA) solution was prepared fresh immediately before running the experiment. Sixty milligrams of AAPH were weighed and dissolved in 5ml of phosphate buffer.

The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Aldrich Chem, Inc. Milwalkee, WI) solution was prepared by dissolving 5mg of the substance in 200ml of 0.2M phosphate buffer as a stock solution (100μM). To obtain a working solution 1ml of the stock solution was mixed with 9ml of phosphate buffer. The stock solution was stored at 2°C.

The phosphate buffer was initially prepared by producing 0.75M solutions of K_2HPO_4 and NaH_2PO_4 mixed at 61.6: 38.9 v/v ratio. Then, the mixture was diluted 1:9 with distilled water and the pH adjusted to pH 7. This was the working solution (0.2M). Both the initial phosphate solution and the buffer were stored at 2°C.

In this assay the substrate (β-phycoerythrin) was subjected to oxidative attack from the radical generator (AAPH). To assess the antioxidative capacity of the studied cultures, diluted (100x) aliquots of the samples were added to the reaction mixture and the protection exercised by each sample against the oxidation of β-phycoerythrin was quantified by measuring the Relative Fluorescence (RF) emitted at 595nm after excitation of the protein at 535nm (Cao *et al*, 1995 and Glazer, 1990).

In this assay total ORAC (Trolox equivalents) of a sample was directly proportional to the area under the kinetic curve of the plotted Relative Fluorescence values against time. To correct any deviation due to instrument drifting, reagents or any other assay conditions, the value of the analyzed samples was expressed with reference to

known amounts/concentrations of Trolox and presented as Trolox equivalents (Cao *et al*, 1995).

All reactions were carried out in a Falcon® 48-well plate (Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ). To each well of the plate was first dispensed 20µl of the respective sample to be analyzed followed by 160µl of the working solution of β-phycoerythrin. Immediately before initiating the measurements 20µl of AAPH were added to each well to initiate the reaction. The plate was covered and placed into the analyzer.

In the assay the ability of compounds to protect β-Phycoerythrin from oxidation was monitored by its decay curve. The Relative Fluorescence of β-phycoerythrin had to decrease to 5-10% of its initial value after 70 minutes. The quantification was achieved by determining the net protection area under the quenching curve of β-Phycoerythrin in the presence of AAPH. (Appendix C; Diagram 1).

Since there was a correlation between the ORAC values and the Trolox equivalents the following equation was used in the calculation (Cao *et al*, 1998):

$$\text{ORAC value} = XK\,(S_{sample} - S_{blank})/(S_{trolox} - S_{blank})$$

where X is the sample volume in microliters,

K is the dilution coefficient, and

S is the area under the curve of the corresponding subscript.

Statistical analysis

For statistical analyses the General Linear Model (GLM) procedures from SAS® (SAS® Institute Inc., 1985) were used to determine if there was any significant interaction between strains and treatments and if any significant differences occurred among strains and treatments. LSMeans were used to separate the means.

RESULTS

Confirmation of Identity of the Cultures

The identification characteristics of the fifteen cultures used in this study are listed in Tables A.1-A.5 of Appendix A. All cultures were Gram-positive and catalase negative rods except for *Streptococcus salivarius ssp. thermophilus* which were cocci. All grew at 45°C but not at 15°C. The carbohydrate fermentation patterns of the cultures matched the characteristics of the indicated species published in the *Bergey's Manual of Systematic Bacteriology vol. 2* (Kandler and Weiss, 1986)

Reducing activities

Reducing activity of cultures grown in modified MRS

The reducing activities exhibited by the studied cultures when grown in modified (without beef extract) MRS broth supplemented with TTC are shown in Table 1. Based on results from preliminary experiments (data not shown) the cultures reached their maximum activity at 12 to 15 hours of incubation. The statistical analysis of the results, found no interaction for reducing activity between the strains and time for any species but *Lactobacilus delbrueckii ssp. bulgaricus*. *Lactobacillus delbrueckii ssp. lactis* (RM2-5 and RM5-4), *Lactobacillus acidophilus* (O16 and L-1), and *Lactobacillus casei* (E10) exhibited the highest reducing activity in the respective species and among all analyzed cultures. These cultures showed also the highest production of acid as indicated by the pH reaching values well below pH 4.0. *Streptococcus salivarius ssp. thermophilus* (1, 2 and VI) did not reduce any TTC during the 15 hours period of study.

TABLE 1

Reducing activity of selected cultures of lactobacilli and streptococci grown in MRS broth measured using the TTC method

Species	Strain[1]	Incubation time[2]			
		12 hours		15 hours	
		pH	A_{485nm}	pH	A_{485nm}
Lactobacillus delbrueckii ssp. lactis	**RM2-5**	3.7	1.193^{aA}	3.7	1.036^{aA}
	RM6-5	3.7	0.932^{aB}	3.6	0.819^{aB}
	RM5-4	3.8	1.144^{aA}	3.7	0.912^{bAB}
Lactobacillus acidophilus	**O16**	3.7	1.042^{aA}	3.6	1.098^{aA}
	NCFM	4.4	0.748^{aC}	4.0	0.802^{aB}
	L-1	3.7	0.895^{bB}	3.6	1.000^{aA}
Lactobacillus delbrueckii ssp. bulgaricus	**18**	6.3	0.268^{bA}	6.3	0.338^{aA}
	10442	6.4	0.037^{aB}	6.4	0.045^{aB}
	Y-23	6.5	0.017^{aB}	6.4	0.019^{aB}
Lactobacillus casei	**9018**	4.4	0.594^{aB}	4.3	0.688^{aB}
	E5	6.4	0.017^{aC}	6.1	0.030^{aC}
	E10	3.7	1.070^{aA}	3.7	1.104^{aA}
Streptococcus salivarius ssp. thermophilus	**1**	6.5	0.016^{bA}	6.5	0.028^{aA}
	2	6.3	0.022^{aA}	6.2	0.020^{aB}
	VI	6.5	0.017^{aA}	6.4	0.019^{aB}

[1] Subcultured three times in MRS broth prior to experiment.
[2] Each value is an average of three replications.
[a,b] Numbers with the same lower case superscript in each row are not statistically different ($P>0.05$)
[A,B,C] Numbers with the same upper case superscripts in each column within the same species are not significantly different ($P>0.05$)

Reducing activity of cultures grown in 10%NFDM

The reducing activities of cultures grown in 10% nonfat milk are given in Table 2. Their activities were assessed in the same manner as described for MRS broth cultures. These results showed some interaction between strains and time within the same species specifically for *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus salivarius ssp. thermophilus*. The remaining species did not exhibit such interactions.
In this medium, most cultures did not exhibit as high reducing ability as in the broth. Although *S. salivarius ssp. thermophilus* did not reduce tetrazolium chloride when grown in MRS broth, all three strains did reduce it when grown in 10% nonfat milk. The acid production based on pH was also lower when compared to the acid production when grown in MRS broth. However, *L. delbrueckii ssp. lactis* (RM5-4) and *L. delbrueckii ssp. bulgaricus* (10442) showed much higher activities than did the other cultures. *Lactobacillus delbrueckii ssp. lactis* RM5-4, *L. acidophilus* NCFM, *L. delbrueckii ssp. bulgaricus* 10442, *L. casei* E10, and *S. salivarius ssp. thermophilus* 2, exhibited significantly higher ($P<0.05$) reducing activity than did the other strains in each of the respective species. Culture RM5-4 began showing visual changes in color within the first 3 hours of incubation while the remaining cultures showed changes after the 6^{th} or 9^{th} hour.

TABLE 2

Reducing activity of selected cultures of lactobacilli and streptococci grown in 10% milk measured using the TTC method

Species	Strain[1]	Incubation time[2]			
		12 hours		15 hours	
		pH	A_{485nm}	pH	A_{485nm}
Lactobacillus delbrueckii ssp. lactis	**RM2-5**	5.9	0.052^{aB}	5.8	0.081^{aB}
	RM6-5	6.1	0.054^{aB}	6.1	0.054^{aB}
	RM5-4	4.7	1.022^{aA}	4.1	1.053^{aA}
Lactobacillus acidophilus	**O16**	6.0	0.090^{aA}	6.0	0.091^{aB}
	NCFM	5.9	0.100^{aA}	5.8	0.104^{aA}
	L-1	6.1	0.097^{aA}	6.1	0.093^{aB}
Lactobacillus delbrueckii ssp. bulgaricus	**18**	5.2	0.193^{bC}	4.7	0.532^{aB}
	10442	5.1	1.035^{bA}	4.5	1.255^{aA}
	Y-23	5.1	0.321^{bB}	5.1	0.542^{aB}
Lactobacillus casei	**9018**	6.1	0.101^{aA}	6.1	0.104^{aB}
	E5	6.1	0.101^{aA}	6.1	0.104^{aB}
	E10	6.0	0.095^{bA}	6.0	0.776^{aA}
Streptococcus salivarius ssp. thermophilus	**1**	5.4	0.311^{aAB}	5.2	0.521^{aB}
	2	5.6	0.222^{bB}	5.2	0.839^{aA}
	VI	5.2	0.525^{aA}	5.2	0.575^{aB}

[1] Subcultured three times in 10% nonfat milk for 18 hrs. at 37°C prior to experiment.
[2] Each value is an average of three replications.
[a,b] Numbers with the same lower case superscript in the same row are not statistically different ($P>0.05$)
[A,B] Numbers with the same upper case superscripts in the same column within the same species are not significantly different ($P>0.05$)

Antioxidative capacity/activity

The same cultures used in the previous section (reducing activity) were also studied for antioxidative activity. Their activities were assessed after growth in MRS broth (Difco) and 10%NFDM for 18 hours at 37°C using the Oxygen Radical Absorbance Capacity (ORAC) method (Cao *et al*, 1995).

Antioxidative activity after growth in MRS broth

Washed cells of each culture were resuspended in potassium phosphate buffer, incubated at 37°C for 30 minutes, and the supernatant analyzed for antioxidative activity. Washed cells resuspended in buffer also were sonicated and the cell-free extracts tested. The results of the antioxidative activity of both the whole cells and the cell-free extracts are presented in Table 3. No significant interaction ($P>0.05$) was detected among the strains and the treatments.

Both whole cells and cell-free extracts exhibited antioxidative capacity. However, in the majority of the studied cultures cell-free extracts exhibited significantly higher ($P<0.05$) total antioxidative capacity than did the intact cells. In the remaining cases, although the total antioxidant capacity of the cell-free extracts was numerically higher, they were not statistically different ($P>0.05$). The analysis of Trolox equivalents per 10^9 cells/ml produced a similar pattern. The values of most cell-free extracts were significantly higher than the respective values of the intact cells ($P<0.05$). *Lactobacillus delbrueckii ssp. lactis* RM5-4, *L. acidophilus* L-1, *L. delbrueckii ssp. bulgaricus* Y-23, *L.*

casei E5, 9018 and *S. salivarius ssp. thermophilus* VI each produced significantly higher ($P<0.05$) antioxidant activity per 10^9 cells than did other strains in the respective species. *Lactobacillius delbrueckii ssp. lactis* (Y-23) and *Streptococcus salivarius ssp. thermophilus* exhibited by far the highest activities when compared to the corrected values of the remaining cultures.

TABLE 3

Oxygen Radical Absorbance Capacity (μmol Trolox/L equivalents) of selected cultures of lactobacilli and streptococci grown in MRS broth

Species	Strain[1]	CFU/ml[1]	TROLOX/L eq.		TROLOX eq./10^9cells[4]	
			Cells[2]	Cell-free extract[3]	Cells	Cell-free extract
L. delbrueckii ssp.lactis	**RM2-5**	$6x10^8$	304^{aA}	474^{aAB}	507^{aAB}	790^{aB}
	RM6-5	$8.7x10^8$	265^{aA}	407^{aB}	304^{aB}	467^{aB}
	RM5-4	$3.7x10^8$	400^{bA}	651^{aA}	1082^{bA}	1758^{aA}
L. acidophilus	**O16**	$2.9x10^9$	511^{aA}	613^{aA}	176^{aC}	212^{aC}
	NCFM	$1.1x10^9$	451^{bA}	668^{aA}	411^{bB}	607^{aB}
	L-1	$6x10^8$	468^{aA}	562^{aA}	779^{aA}	936^{aA}
L. delbrueckii ssp. bulgaricus	**18**	$1.8x10^8$	85^{bB}	327^{aB}	474^{aB}	1817^{aB}
	10442	$6x10^8$	76^{bB}	335^{aB}	127^{aB}	559^{aB}
	Y-23	$9x10^7$	284^{bA}	546^{aA}	3152^{bA}	6070^{aA}
L. casei	**9018**	$5.7x10^8$	318^{bA}	840^{aA}	558^{bB}	1473^{aA}
	E5	$3.7x10^8$	412^{aA}	505^{aB}	1113^{bA}	1365^{aA}
	E10	$8.3x10^8$	461^{aA}	561^{aB}	556^{bB}	676^{aB}
S.salivarius ssp. thermophilus	**1**	$4.3x10^8$	233^{bAB}	584^{aA}	543^{aB}	1358^{aB}
	2	$3.2x10^8$	166^{bB}	552^{aA}	518^{bB}	1725^{aB}
	VI	$1x10^8$	379^{bA}	574^{aA}	3790^{bA}	5740^{aA}

[1] Cultures were grown in MRS; colony forming units/ml at 18 hr.

[2] Whole/intact washed cells; Each value is an average of three replications.

[3] Cell-free extract; Each value is an average of three replications.

[4] Trolox eq. / 10^9 cells = $\frac{\text{μmol Trolox/L equivalents}}{\text{CFU/ml}} \times 10^9$

[a,b] Means in the same row followed by same lower case superscript letter are not significantly different (P>0.05).

[A,B,C] Numbers followed by the same upper case superscripts in the same column within the same species are not significantly different (P>0.05)

Antioxidative activity of cultures grown in milk

Cultures also were evaluated for antioxidative activity after 18 hours growth in 10% nonfat milk at 37°C. The whey fractions from the cultured milks were assayed for activity. Wheys from sonicated cultured milks also were tested. Whey from acidified milk was tested in each case for comparison. The results are presented in Table 4. Similarly to the antioxidative capacity pattern shown by the cells grown in MRS broth, the activity of whey from sonicated cultures exhibited numerically higher antioxidative capacity than did whey from nonsonicated cultures though they are not statistically different ($P<0.05$). Exceptions were *Lactobacillus acidophilus* (NCFM and L-1), *Lactobacillus casei* (E10) and *Streptococcus salivarius ssp. thermophilus* (VI) where whole cells showed numerically higher Trolox equivalents than the cell-free extracts. The higher values of the whey from the other sonicated cultures probably results from disruption of many cells in the milk cultures. These results were difficult to interpret in part due to the antioxidative activity attributed to milk components in the whey. Comparison of corrected values (for which Trolox equivalents of the milk were subtracted from those of the cultured wheys) tend to substantiate the difficulty caused by the complexity of milk. The pH values which provide an indication of growth in the milk cultures, do not suggest that cultures with the lowest value exhibited the greatest level of antioxidative activity. For example, the pH of *L. delbrueckii ssp. lactis* RM5-4 was slightly lower than that of RM2-5 but, strain RM2-5 had significantly higher ($P<0.05$) antioxidative activity.

TABLE 4

ORAC (μmol Trolox/L equivalents) of selected cultures of lactobacilli and streptococci grown in 10% Milk

Species	Strain[1]	pH	μmol Trolox/L eq.			Corrected values[5]	
			Culture[2]	Sonicated culture[3]	Milk[4]	Culture	Sonicated culture
L. delbrueckii ssp. lactis	**RM2-5**	3.5	2388^{aA}	2458^{aAB}	1740^{aA}	648	718
	RM6-5	3.7	2737^{aA}	2777^{aA}	1740^{aA}	997	1033
	RM5-4	3.4	1683^{aB}	1767^{aB}	1740^{aA}	-57	27
L. acidophilus	**O16**	3.6	2206^{aA}	2364^{aA}	2237^{aA}	-31	127
	NCFM	3.9	2354^{aA}	2339^{aA}	2237^{aA}	117	102
	L-1	3.6	2320^{aA}	2281^{aA}	2237^{aA}	83	44
L. delbrueckii ssp. bulgaricus	**18**	4.1	1732^{aA}	2110^{aA}	1347^{aA}	385	763
	10442	3.9	1925^{aA}	2060^{aA}	1347^{aA}	578	713
	Y-23	3.9	1642^{aA}	1954^{aA}	1347^{aA}	295	607
L. casei	**9018**	3.8	1880^{aA}	2064^{aA}	1991^{aA}	-111	73
	E5	3.9	2124^{aA}	2267^{aA}	1991^{aA}	133	276
	E10	3.9	1941^{aA}	1900^{aA}	1991^{aA}	-50	-91
S.salivarius ssp. thermophilus	**1**	4.9	2147^{aA}	2152^{aA}	2219^{aA}	-72	-65
	2	4.8	2004^{aA}	2333^{aA}	2219^{aA}	-215	114
	VI	4.8	2039^{aA}	1990^{aA}	2219^{aA}	-180	-229

[1] Cultures were grown in 10%NFDM for 18 hours prior to experiment.
[2] Supernatant of cultured milk; Each value is an average of three replications.
[3] Supernatant of sonicated cultured milk; Each value is an average of three replications.
[4] Supernatant of acidified (with lactic acid) nonfat milk; Each value is an average of three replications.
[5] Corrected value = value of culture – value of milk.
[a] Means in the same row and same lower case superscript within the same species are not significantly different (P>0.05).
[A,B] Numbers with the same upper case superscripts in the column with in the same species are not significantly different (P>0.05)

DISCUSSION AND CONCLUSIONS

In this study we examined the possibility of using 2,3,5-triphenyltetrazolium chloride as an indicator of potential antioxidative capacity.

Tetrazolium chloride has been used in a number of studies to test cell viability (Liska *et al*, 1958; Eidus *et al*, 1959; and Bhupathiraju *et al*, 1999), measure dehydrogenases in animal and plant systems (Friedel *et al*, 1994) as well as in reductase testing in milk (Mustakallio, 1951). According to Seidler (1991) the water-soluble tetrazolium chloride is reduced to a water-insoluble red compound (formazan) which is trapped inside the cell.

In our study the assessment of the reducing capacity of cultures of lactic acid bacteria was made by growing the cultures in modified MRS broth and nonfat milk supplemented with 0.5% TTC. The use of a suitable concentration of TTC was essential since it can be suppressive (bacteriostatic influence) towards cell growth when in excess. The concentration used in the present study was well below the maximum (2%) used by May *et al* (1960) which caused no inhibition.

The cultures grown in MRS broth reached their maximum reducing activity between 12 and 15 hours. In most cases reducing values between the two sampling periods did not show statistical difference. *Lactobacillus delbrueckii ssp. lactis* (RM2-5, and RM5-4), *Lactobacillus acidophilus* (O16 and L-1) and *Lactobacillus casei* (E10) exhibited the best reducing activities in the studied period. Concurrently, these cultures showed the best growth in the same period (graphs A.1-A.5, appendix A) reaching their

maximum around 15th hour. It was also observed that these cultures produced more acid, dropping the pH below 4.0 (table B.1, appendix B)

None of the *Streptococcus salivarius ssp. thermophilus* cultures showed any activity although particularly culture 1 showed considerable growth by means of growth medium turbidity measured at 620nm. This is in conformity with reported data showing that *St. thermophilus* does not reduce TTC when grown in MRS broth (Laxminarayana, 1953; Laxminarayana, 1954; and Kendler and Weiss, 1986).

The trend shown by the same cultures when grown in nonfat milk was slightly different. The species, which showed considerable reducing activity in MRS broth did not perform as well in milk. *Lactobacillus delbrueckii ssp. lactis* (RM5-4) was the only culture performing relatively well in both media and also the only culture coagulating the milk during the period of study. *Lactobacillus delbrueckii ssp. bulgaricus* (10442) did not reduce TTC well in MRS broth. However, it exhibited the highest activity when grown in nonfat milk. This was accompanied by considerable production of acid. The intensity of the reducing activity was apparently related to degree of growth of the cultures in both broth and milk based on pH values. Observing the trends of the reducing activities and the acid production in both media (MRS broth and milk) it is clear that the two processes are correlated.

In this study we also assessed the antioxidative activity of lactic acid bacteria cultures grown in MRS broth and in milk.

The Oxygen Radical Absorbance Capacity (ORAC) has been found to be a relatively simple, reliable and sensitive method of quantifying the antioxidative capacity of foods and food products by protection of a protein from radical damage (Cao *et al*, 1993 and

Cao *et al*, 1995). In our study the antioxidative activity of our cultures was assessed by quantifying the protection of β-phycoerythrin from attack by AAPH in the reaction mixture. Similarly to other methods used for antioxidative assessment, the ORAC method gives the total antioxidative capacity of the analyzed sample, rather than the capacity of the individual components of the system, but it also differs in some other aspects. It is the only method including the variables inhibition time and degree of inhibition in one system. It also allows the automated analysis of enormous number of samples at the same time and in a relatively short time/period.

Various authors have reported similar findings when working with whole cells and cell-free extracts of lactic acid bacteria but using different methods. Lin and Yen (1999abc) reported that the whole cells and cell-free extracts of all their 19 studied cultures (*L. acidophilus, L. delbrueckii bulgaricus* and *S.salivarius ssp. thermophilus)* showed some degree of antioxidative activity when assessed by inhibition of ascorbate autoxidation. Lin and Chang (2000) also reported that whole cells and cell-free extracts of lactic acid bacteria (*L. acidophilus*) exhibited some antioxidative capacity when tested using the thiobarbituric acid (TBA) method. Based on the higher Trolox equivalents of the cell free extracts compared to the intact cells observed in our study, it is possible that these cultures once consumed would release antioxidants into the gut after being exposed to bile salts. In related experiments (data not shown) we attempted to determine if addition of bile salts to the cells would increase the release of antioxidants. However, the bile salts caused too much interference with the ORAC assay so that results were inconclusive.

The interference caused by some of the milk components we observed in evaluating cultured milk could be due to antioxidants in milk such as lactoferrin, urate and some vitamins like vitamin C and E (Østdal *et al*, 2000 and, Lindmark-Månson, 2000). Particular attention was drawn to sulphydryl groups by Taylor and Richardson (1980) and Tong *el at* (2000). According to these authors, sulphydryl groups are one of the main sources of antioxidative capacity in whey especially after heat treatment due to partial denaturation of the milk whey proteins. Upon measuring the values of the uninoculated milk we observed variations from one batch to another. This could have contributed to difficulty in comparing results among cultures eventhough test for each species were compared in individually prepared batches.

Comparing the values of the reducing and antioxidative activities for cultures grown in MRS broth and nonfat milk, we did not observe any apparent relationship between the two activities. Thus, the reducing activity of these cultures as measured by the TTC method would be of little or no value in predicting the intensity of the cultures' antioxidative capacity.

The results of this study show that lactic acid bacteria possess an antioxidative capacity which can be assessed quantitatively through their ability to protect β-phycoerythrin from radical oxidation. The greatest degree of antioxidant capacity was associated with the cell-free extracts of the cultures. This suggests that they may be important in delivering antioxidants to the intestines where they could be released when cells of the cultures encounter bile. Bile is known to alter the permeability of the organisms to enhance passage of substances into and/or out of the cells (Noh and Gilliland, 1993). Consumption of foods containing lactic acid bacteria may be

encouraged and may also contribute to the health effects associated with dietary antioxidants.

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APPENDIX A

Identification of Cultures

Cultures were streaked onto plates of sterile MRS agar (MRS broth plus 1.5% agar) individually which were incubated in plastic bags (flushed with CO_2 for 30sec.) for 18 hours at 37°C and checked for purity (colony uniformity). Each was Gram stained and tested for presence of catalase. Finally, for confirmation of the identity fermentation patterns were determined using API 50 CH kit reactions (BioMerieux Vitek, Inc. Hazelwood, MO). Cells were harvested from MRS broth cultures and washed with the API solution. The same solution was used to resuspend the cells and inoculate the cells of the kit containing the substrates. Then, the inoculated kits were incubated in a Gas Pak (BBL, Cockeyville, MD) anaerobic system for 48 hours at 37°C. Each bacterial fermentation pattern was compared with the identification patterns presented in 8th edition of the *Bergey's Manual of Systematic Bacteriology vol.2.*

Table A.1

API 50 CH identification pattern of *Lactobacillus delbrueckii ssp. lactis* cultures

API tests[1]	*Lactobacillus delbrueckii ssp. lactis*[2]	Strains[3]		
		RM2-5	RM5-4	RM6-5
Amygdalin	+	+	+	+
Arabinose	-	-	-	-
Cellobiose	+/-	-	+	+
Esculin	+	+	+	+
Fructose	+	+	+	+
Galactose	+/-	+	-	-
Glucose	+	+	+	+
Gluconate	-	-	-	-
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	-	-	-	-
Mannose	+	+	+	+
Melezitose	-	-	-	-
Melibiose	-	-	-	-
Raffinose	-	+	-	-
Rhamnose	-	-	-	+
Ribose	-	-	-	-
Salicin	+	-	-	+
Sorbitol	-	-	-	-
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	-	-	-	-

1 API 50 CH kit

2 Reactions as listed in the Berguey's Manual of Determinative Bacteriology

3 All cultures were Gram-positive, catalase negative rods which grew at 45°C but not at 15°C

Table A.2

API 50 CH identification pattern of *Lactobacillus delbrueckii ssp. bulgaricus* cultures

API tests[1]	*Lactobacillus delbrueckii ssp. bulgaricus*[2]	Strains[3]		
		18	10442	Y-23
Amygdalin	-	-	-	-
Arabinose	-	-	-	-
Cellobiose	+/-	+	+	-
Esculin	-	-	-	-
Fructose	+	+	+	+
Galactose	-	-	-	-
Glucose	+	+	+	+
Gluconate	-	-	-	-
Lactose	-	-	-	-
Maltose	+/-	-	+	+
Mannitol	-	-	-	-
Mannose	+	+	+	-
Melezitose	-	-	-	-
Melibiose	-	-	-	-
Raffinose	-	+	+	-
Rhamnose	-	-	-	-
Ribose	-	-	-	-
Salicin	-	-	+	-
Sorbitol	-	-	-	-
Sucrose	+	+	+	+
Trehalose	+/-	-	-	-
Xylose	-	-	-	-

1 API 50 CH kit

2 Reactions as listed in the Berguey's Manual of Determinative Bacteriology

3 All cultures were Gram-positive, catalase negative rods which grew at 45°C but not at 15°C

Table A.3

API 50 CH identification pattern of *Lactobacillus acidophilus* cultures

API tests[1]	*Lactobacillus acidophilus*[2]	Strains[3]		
		O16	NCFM	L-1
Amygdalin	+	+	+	+
Arabinose	-	-	-	-
Cellobiose	+	+	+	+
Esculin	+	+	+	-
Fructose	+	-	+	+
Galactose	+	+	+	+
Glucose	+	+	+	+
Gluconate	-	-	-	-
Lactose	+	+	+	+
Maltose	+	+	+	+
Mannitol	-	-	-	-
Mannose	+	-	+	+
Melezitose	-	-	-	-
Melibiose	+/-	+	+	-
Raffinose	+/-	+	+	+
Rhamnose	-	-	-	-
Ribose	-	-	-	-
Salicin	+	+	+	+
Sorbitol	-	-	-	-
Sucrose	+	+	+	+
Trehalose	+/-	+	+	-
Xylose	-	-	-	-

1 API 50 CH kit

2 Reactions as listed in the Berguey's Manual of Determinative Bacteriology

3 All cultures were Gram-positive, catalase negative rods which grew at 45°C but not at 15°C

Table A.4

API 50 CH identification pattern of *Lactobacillus casei* cultures

API tests[1]	*Lactobacillus casei*[2]	Strains[3]		
		9018	E5	E10
Amygdalin	+	+	+	+
Arabinose	-	+	-	-
Cellobiose	+	+	+	+
Esculin	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Glucose	+	+	+	+
Gluconate	+	+	+	+
Lactose	+/-	-	-	+
Maltose	+	+	+	+
Mannitol	+	+	+	+
Mannose	+	+	+	+
Melezitose	+	+	+	-
Melibiose	-	+	-	+
Raffinose	-	-	-	-
Rhamnose	-	+	+	-
Ribose	+	+	+	+
Salicin	+	+	+	+
Sorbitol	+	+	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Xylose	-	-	-	-

1 API 50 CH kit

2 Reactions as listed in the Berguey's Manual of Determinative Bacteriology

3 All cultures were Gram-positive, catalase negative rods which grew at 45°C but not at 15°C

Table A.5

API 50 CH identification pattern of *Streptococcus salivarius ssp. thermophilus* cultures

API tests[1]	*Streptococcus salivarius ssp. thermophilus*[2]	Strains[3]		
		1	2	IV
Arabinose	-	-	-	-
Glycerol	-	-	-	-
Inulin	-	-	-	-
Fructose	+	+	+	+
Dextrin	-	-	-	-
Glucose	+	+	+	+
Lactose	+	+	+	+
Mannitol	-	-	-	-
Mannose	+	+	+	?
Salicin	-	-	-	-
Sorbitol	-	-	-	-
Sucrose	+	+	+	+
Starch	-	-	-	-
Xylose	-	-	-	-

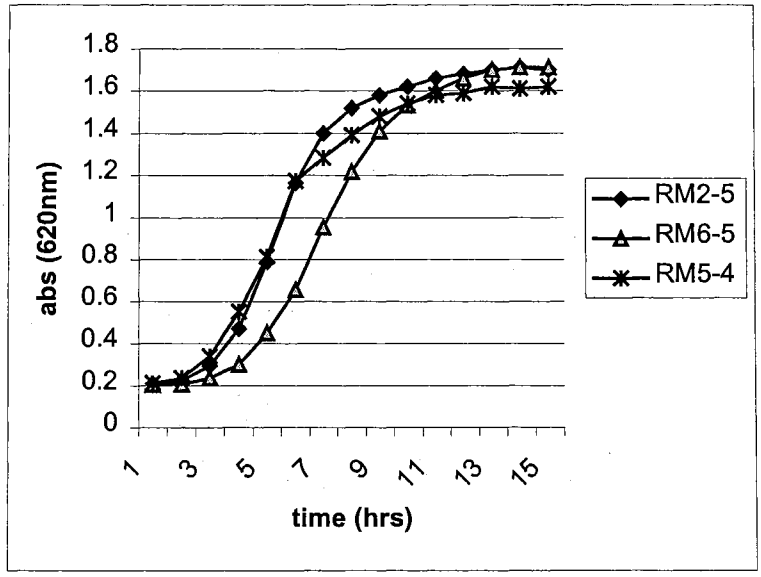
1 API 50 CH kit

2 Reactions as listed in the Berguey's Manual of Determinative Bacteriology

3 All cultures were Gram-positive, catalase negative cocci which grew at 45°C but not at 15°C

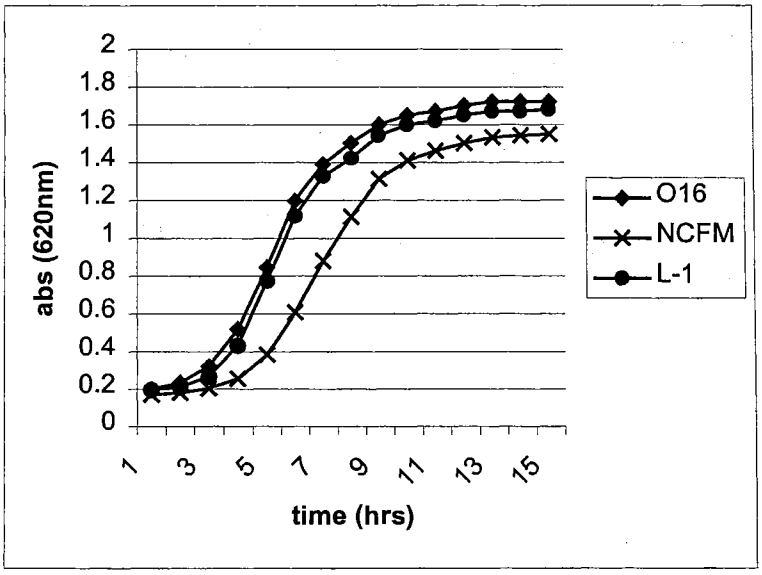
Graph A. 1

Growth curves for strains of *Lactobacillus delbrueckii ssp. lactis* grown in MRS broth at 37°C for 15 hours



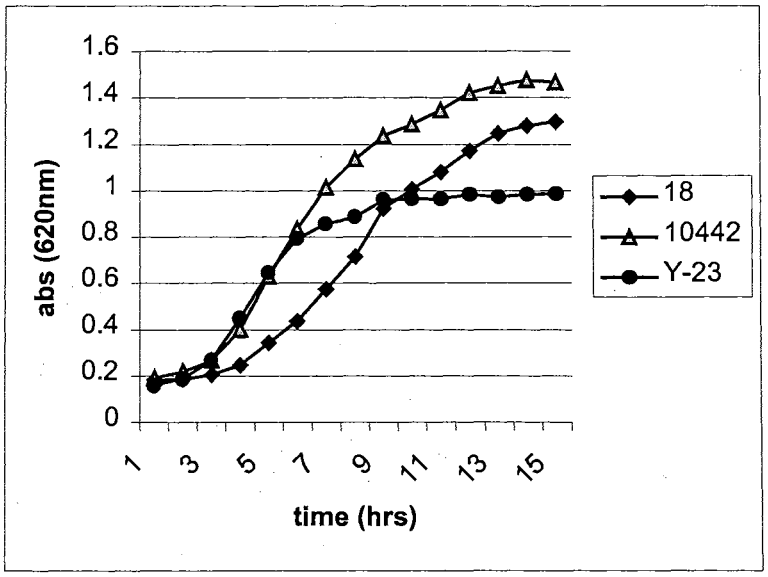
Graph A. 2

Growth curves for strains of *Lactobacillus acidophilus* grown in MRS broth at 37°C for 15 hours



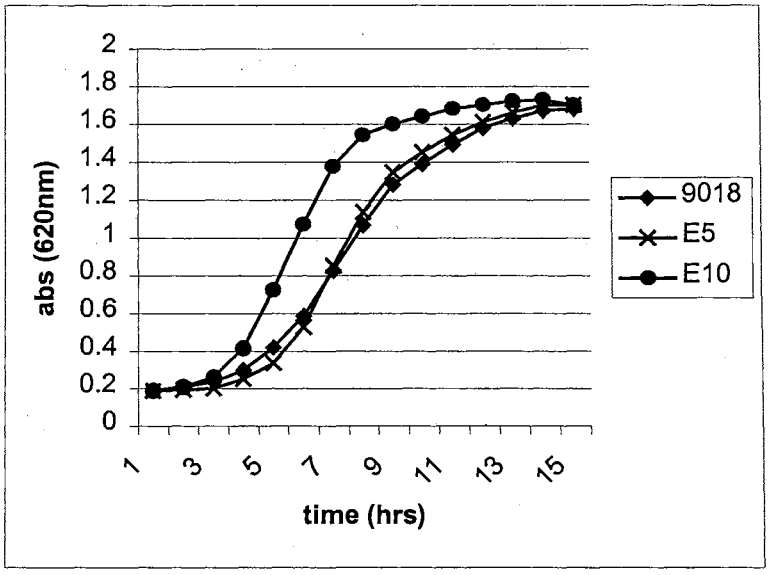
Graph A. 3

Growth curves for strains of *Lactobacillus delbrueckii ssp. bulgaricus* grown in MRS broth at 37°C for 15 hours



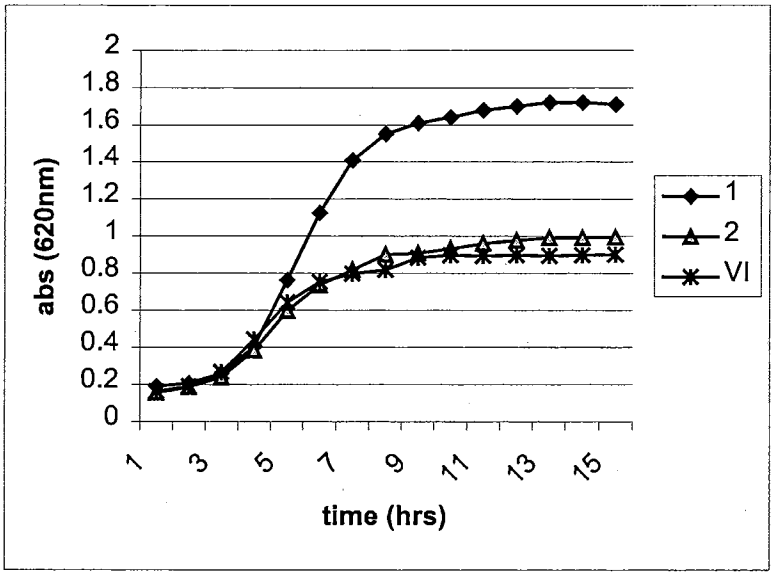
Graph A. 4

Growth curves for strains of *Lactobacillus delbrueckii casei* grown in MRS broth at 37°C for 15 hours



Graph A. 5

Growth curves for strains of S*treptococcus salivarius ssp. thermophilus* grown in MRS broth at 37°C for 15 hours



APPENDIX B

REDUCING ACTIVITY RAW DATA

Table B.1

Raw data of the pHs of the fifteen studied cultures of lactic acid bacteria grown in MRS broth at 37C for 15 hours

Culture	Strain	Time				
		3rd hour	6th hour	9th hour	12th hour	15th hour
		6.18	4.51	3.79	3.7	3.62
	O16	6.17	4.39	3.85	3.67	3.61
		6.15	4.42	3.75	3.65	3.62
		6.1666667	**4.44**	**3.7966667**	**3.6733333**	**3.6166667**
		6.44	6.28	5.61	4.4	3.96
Lactobacillus acidophilus	NCFM	6.49	6.23	5.46	4.36	3.97
		6.45	6.21	5.5	4.43	3.95
		6.46	**6.24**	**5.5233333**	**4.3966667**	**3.96**
		6.35	5.28	3.94	3.77	3.64
	L-1	6.4	5.1	4.04	3.7	3.62
		6.4	5.19	4	3.69	3.6
		6.3833333	**5.19**	**3.9933333**	**3.72**	**3.62**
		6.36	4.82	3.89	3.77	3.7
	RM2-5	6.26	4.49	3.89	3.7	3.64
		6.3	4.57	3.83	3.75	3.63
		6.3066667	**4.6266667**	**3.87**	**3.74**	**3.6566667**
		6.27	5.76	4.53	3.77	3.61
Lactobacillus delbruekii spp.	RM6-5	6.38	5.63	4.37	3.76	3.66
lactis		6.35	5.71	4.49	3.69	3.62
		6.3333333	**5.7**	**4.4633333**	**3.74**	**3.63**
		6.19	4.53	3.85	3.78	3.73
	RM5-4	5.97	4.11	3.86	3.72	3.73
		6.01	4.16	3.85	3.75	3.72
		6.0566667	**4.2666667**	**3.8533333**	**3.75**	**3.7266667**
		6.45	6.39	6.28	6.35	6.26
	18	6.5	6.37	6.29	6.28	6.32
		6.53	6.35	6.27	6.26	6.26
		6.4933333	**6.37**	**6.28**	**6.2966667**	**6.28**
		6.5	6.48	6.44	6.45	6.41
Lactobacillus delbruekii ssp.	10442	6.55	6.47	6.44	6.45	6.46
bulgaricus		6.52	6.47	6.42	6.42	6.43
		6.5233333	**6.4733333**	**6.4333333**	**6.44**	**6.4333333**
		6.53	6.49	6.42	6.46	6.44
	Y-23	6.53	6.43	6.44	6.47	6.44
		6.55	6.48	6.42	6.43	6.43
		6.5366667	**6.4666667**	**6.4266667**	**6.4533333**	**6.4366667**
		6.38	5.95	5.02	4.38	4.23
	9018	6.42	5.84	4.82	4.23	4.01
		6.36	5.7	4.87	4.44	4.52
		6.3866667	**5.83**	**4.9033333**	**4.35**	**4.2533333**

		6.46	6.48	6.37	6.35	6.11
Lactobacillus casei	E5	6.52	6.45	6.43	6.4	6.14
		6.53	6.41	6.39	6.38	6.12
		6.5033333	**6.4466667**	**6.3966667**	**6.3766667**	**6.1233333**
		6.3	5.17	3.9	3.78	3.67
	E10	6.05	5.05	3.76	3.69	3.67
		6.21	5.1	3.82	3.69	3.66
		6.1866667	**5.1066667**	**3.8266667**	**3.72**	**3.6666667**
		6.43	6.48	6.44	6.52	6.43
	1	6.54	6.46	6.47	6.49	6.54
		6.51	6.48	6.47	6.47	6.49
		6.4933333	**6.4733333**	**6.46**	**6.4933333**	**6.4866667**
		6.49	6.4	6.25	6.32	6.2
Streptococcus salivarius ssp.	2	6.49	6.33	6.29	6.25	6.28
thermophilus		6.57	6.28	6.29	6.27	6.25
		6.5166667	**6.3366667**	**6.2766667**	**6.28**	**6.2433333**
		6.37	6.48	6.43	6.49	6.42
	VI	6.56	6.48	6.45	6.44	6.47
		6.55	6.45	6.47	6.49	6.45
		6.4933333	**6.47**	**6.45**	**6.4733333**	**6.4466667**

Table B. 2

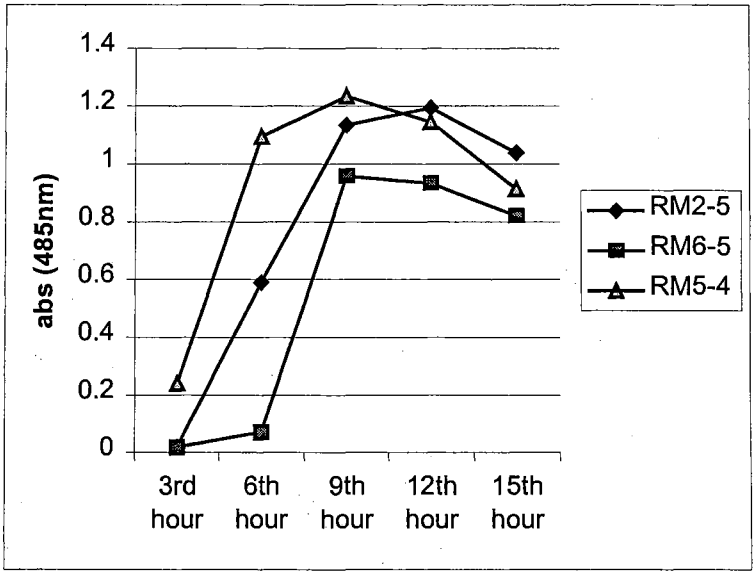
Raw data of the pHs of the fifteen studied cultures of lactic acid bacteria grown in 10% Milk at 37C for 15 hours

Culture	Strain	Time				
		3rd hour	6th hour	9th hour	12th hour	15th hour
		6.14	6.13	6.07	5.99	5.97
	O16	6.23	6.15	6.11	6	5.96
		6.19	6.14	6.08	5.99	5.97
		6.1866667	**6.14**	**6.0866667**	**5.9933333**	**5.9666667**
		6.17	6.16	6.04	5.89	5.83
Lactobacillus acidophilus	NCFM	6.16	6.16	6.05	5.87	5.84
		6.27	6.2	6.08	5.89	5.81
		6.2	**6.1733333**	**6.0566667**	**5.8833333**	**5.8266667**
		6.16	6.15	6.13	6.04	6.06
	L-1	6.29	6.25	6.18	6.1	6.08
		6.22	6.17	6.14	6.06	6.05
		6.2233333	**6.19**	**6.15**	**6.0666667**	**6.0633333**
		6.12	6.11	6.01	5.86	5.78
	RM2-5	6.14	6.12	6	5.85	5.75
		614	6.12	6.01	5.86	5.77
		208.75333	**6.1166667**	**6.0066667**	**5.8566667**	**5.7666667**
		6.17	6.16	6.14	6.09	6.08
Lactobacillus delbruekii spp.	RM6-5	6.27	6.2	6.17	6.12	6.1
lactis		6.23	6.19	6.15	6.11	6.08
		6.2233333	**6.1833333**	**6.1533333**	**6.1066667**	**6.0866667**
		6.06	5.79	5.2	4.75	4.23
	RM5-4	6.13	5.63	5.18	4.71	4.2
		6.09	5.78	5.19	4.69	4.2
		6.0933333	**5.7333333**	**5.19**	**4.7166667**	**4.21**
		6.1	6.02	5.76	5.19	4.7
	18	6.1	6.01	5.73	5.17	4.6
		6.21	6.15	5.8	5.22	4.73
		6.1366667	**6.06**	**5.7633333**	**5.1933333**	**4.6766667**
		6.15	6.15	5.85	5.15	4.48
Lactobacillus delbruekii ssp.	10442	6.19	6.16	5.87	5.16	4.46
bulgaricus		6.12	6.12	5.83	5.13	4.45
		6.1533333	**6.1433333**	**5.85**	**5.1466667**	**4.4633333**
		6.04	5.95	5.6	5.34	5.06
	Y-23	6.14	6.01	5.7	5.39	5.08
		6.2	6.08	5.79	4.44	5.12
		6.1266667	**6.0133333**	**5.6966667**	**5.0566667**	**5.0866667**
		6.19	6.18	6.15	6.07	6.08
	9018	6.21	6.19	6.19	6.18	6.19
		6.19	6.11	6.16	6.11	6.15
		6.1966667	**6.16**	**6.1666667**	**6.12**	**6.14**

		6.19	6.17	6.16	6.11	6.1
Lactobacillus casei	E5	6.18	6.18	6.1	6.16	6.17
		6.18	6.21	6.15	6.15	6.11
		6.1833333	**6.1866667**	**6.1366667**	**6.14**	**6.1266667**
		6.17	6.16	6.09	5.98	5.91
	E10	6.17	6.17	6.13	6.01	6
		6.16	6.16	6.1	6	5.98
		6.1666667	**6.1633333**	**6.1066667**	**5.9966667**	**5.9633333**
		6.05	5.87	5.75	5.41	5.22
	1	6.07	5.88	5.77	5.4	5.19
		6.08	5.89	5.77	5.41	5.19
		6.0666667	**5.88**	**5.7633333**	**5.4066667**	**5.2**
		6.11	6	5.86	5.58	5.1
Streptococcus salivarius ssp.	2	6.27	6.14	5.9	5.63	5.2
thermophilus		6.19	6.12	5.91	5.62	5.19
		6.19	**6.0866667**	**5.89**	**5.61**	**5.1633333**
		6.07	5.91	5.66	5.18	5.1
	VI	6.17	6.02	5.74	5.27	5.3
		6.15	5.99	5.71	5.26	5.21
		6.13	**5.9733333**	**5.7033333**	**5.2366667**	**5.2033333**

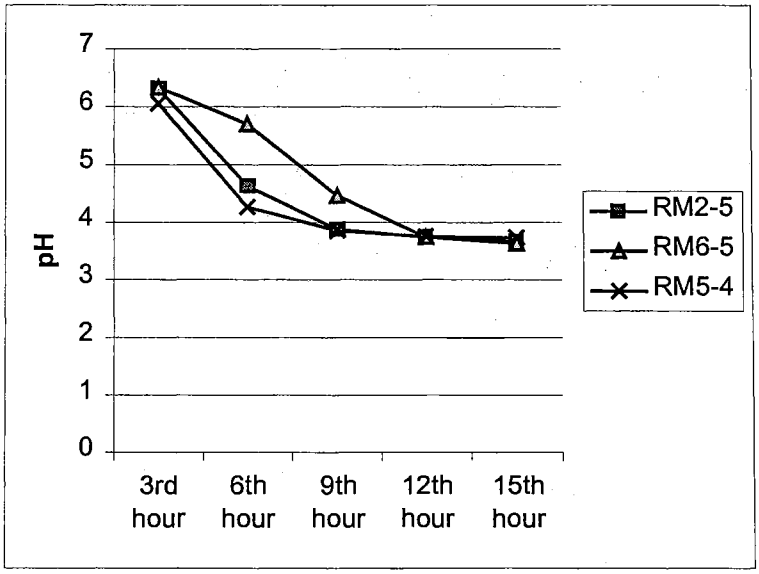
Graph B.1

Reducing activity of strains of *Lactobacillus delbrueckii ssp. lactis* grown in modified MRS broth at 37°C for 15 hours measured at 485nm



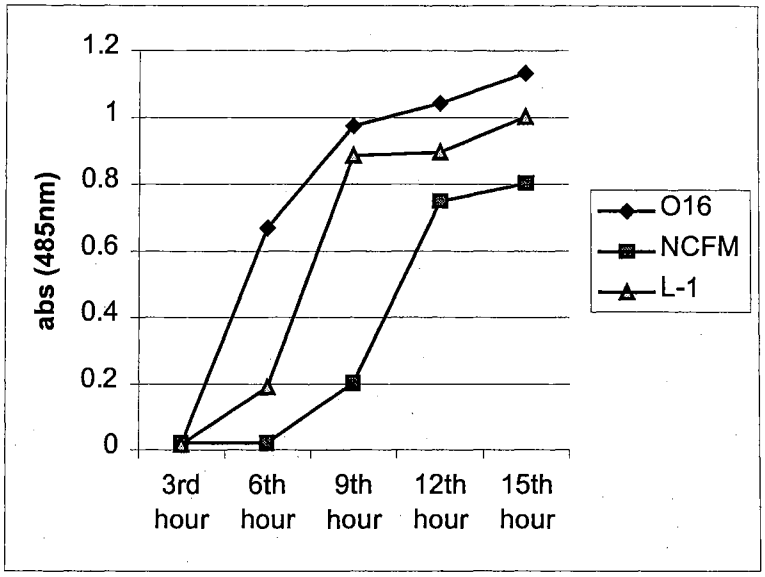
Graph B.2

pHs for strains of *Lactobacillus delbrueckii ssp. lactis* grown in modified MRS broth at 37°C for 15 hours



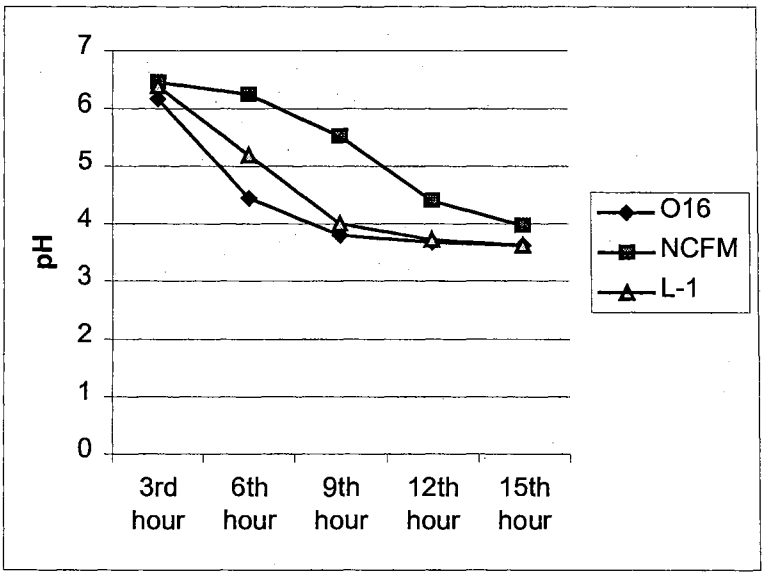
Graph B.3

Reducing activity of strains of *Lactobacillus acidophilus* grown in modified MRS broth at 37°C for 15 hours measured at 485nm



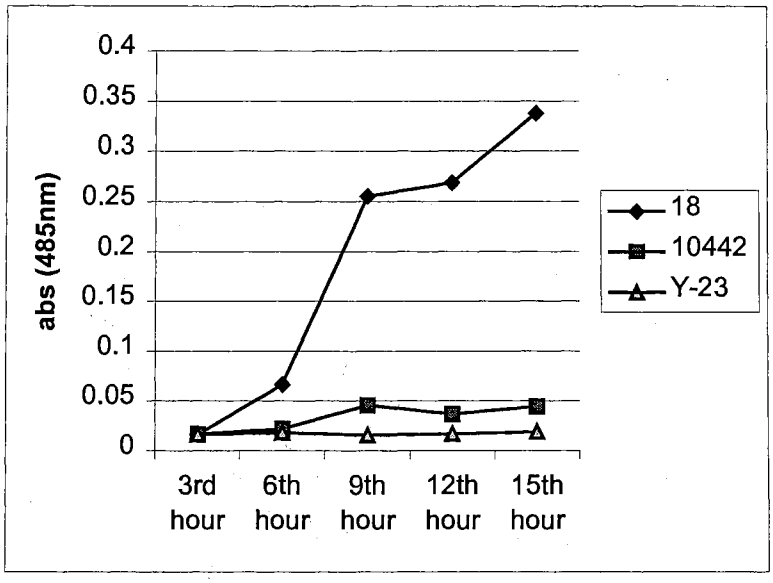
Graph B. 4

pHs for strains of *Lactobacillus acidophilus* grown in modified MRS broth at 37°C for 15 hours



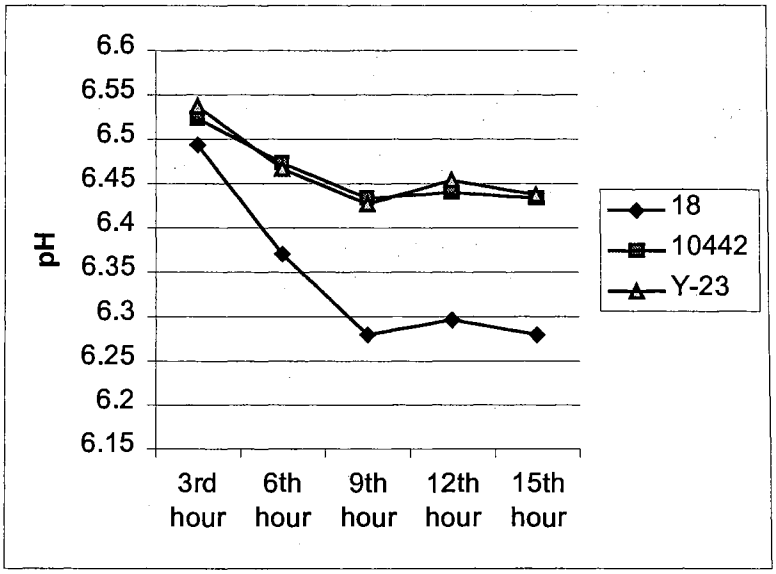
Graph B.5

Reducing activity of strains of *Lactobacillus delbrueckii ssp. bulgaricus* grown in modified MRS broth at 37°C for 15 hours measured at 485nm



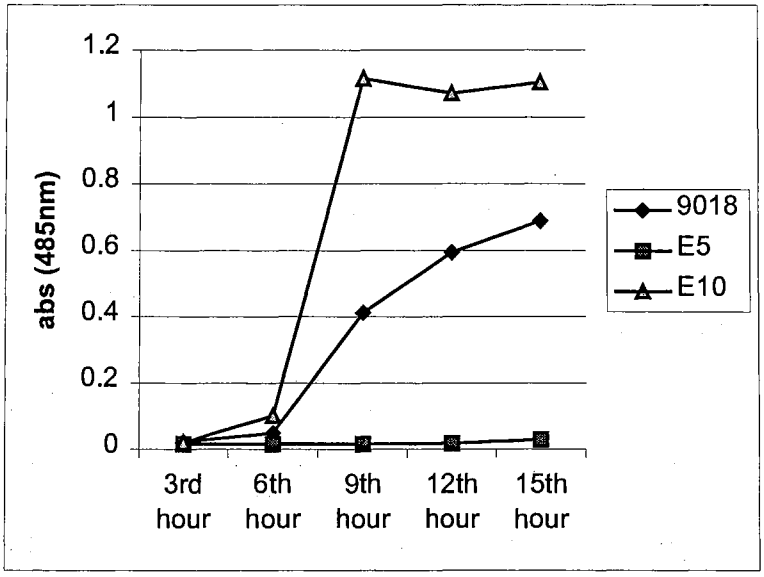
Graph B. 6

pHs for strains of *Lactobacillus delbrueckii ssp. bulgaricus* grown in modified MRS broth at 37°C for 15 hours



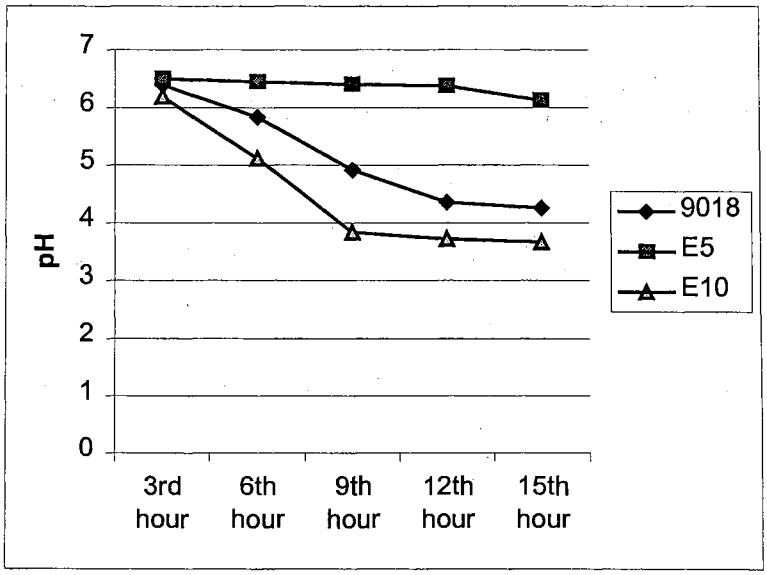
Graph B.7

Reducing activity of strains of *Lactobacillus casei* grown in modified MRS broth at 37°C for 15 hours measured at 485nm



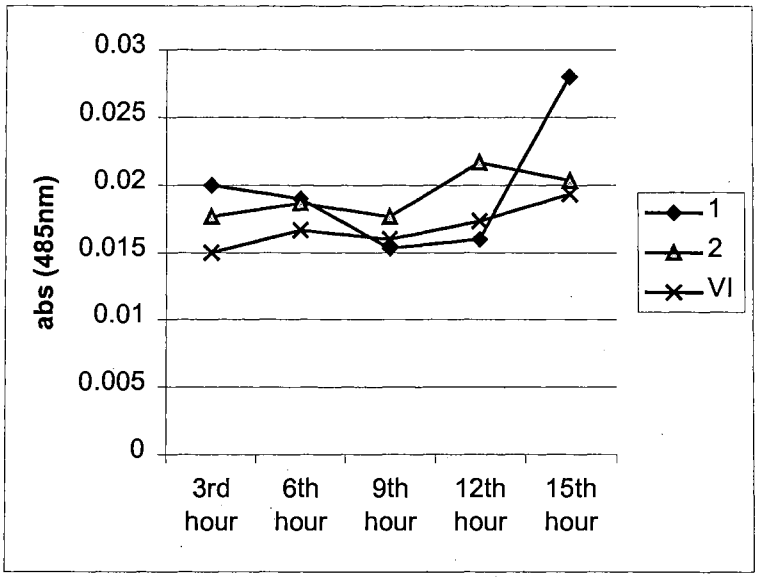
Graph B. 8

pHs for strains of *Lactobacillus casei* grown in modified MRS broth at 37°C for 15 hours



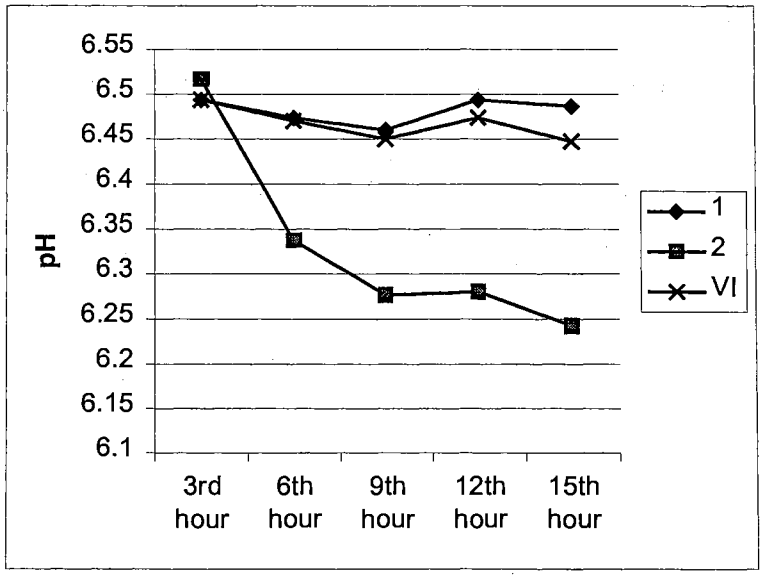
Graph B.9

Reducing activity of strains of *Streptococcus salivarius ssp. thermophilus* grown in modified MRS broth at 37°C for 15 hours measured at 485nm



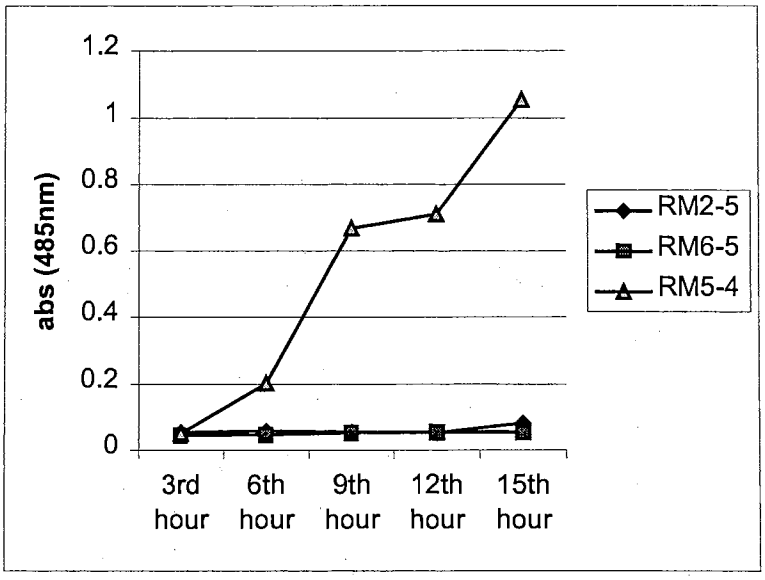
Graph B. 10

pHs for strains of *Streptococcus salivarius ssp. thermophilus* grown in modified MRS broth at 37°C for 15 hours



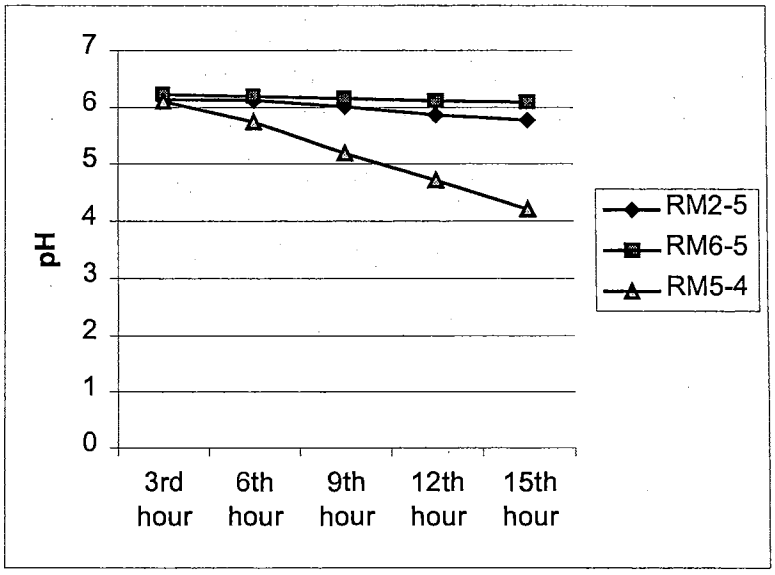
Graph B.11

Reducing activity of strains of *Lactobacillus delbrueckii ssp. lactis* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



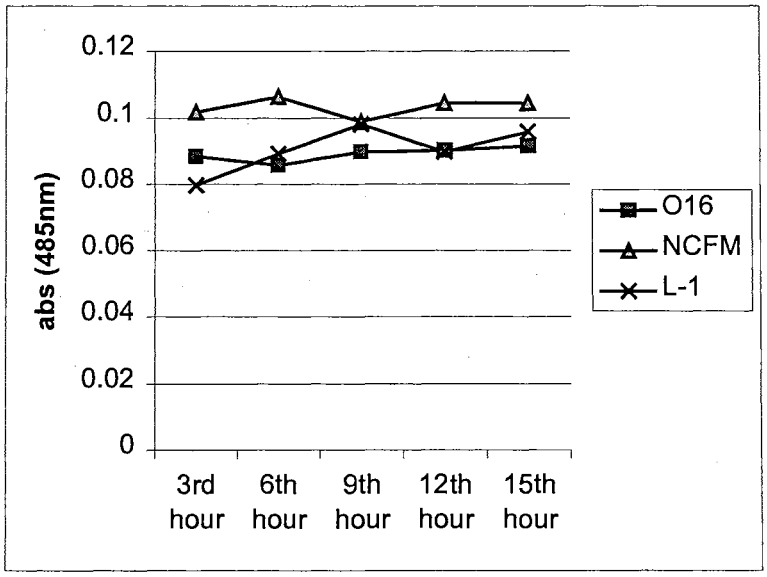
Graph B.12

pH of strains of *Lactobacillus delbrueckii ssp. lactis* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



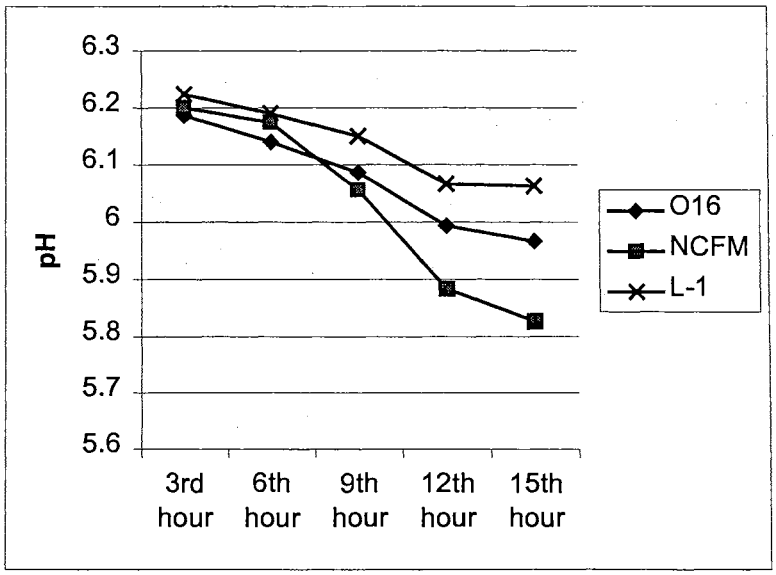
Graph B.13

Reducing activity of strains of *Lactobacillus acidophilus* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



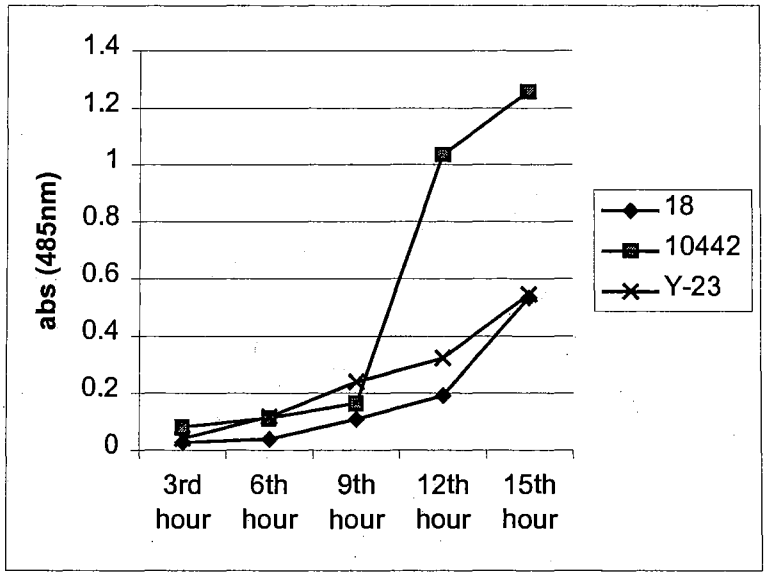
Graph B.14

pH of strains of *Lactobacillus acidophilus* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



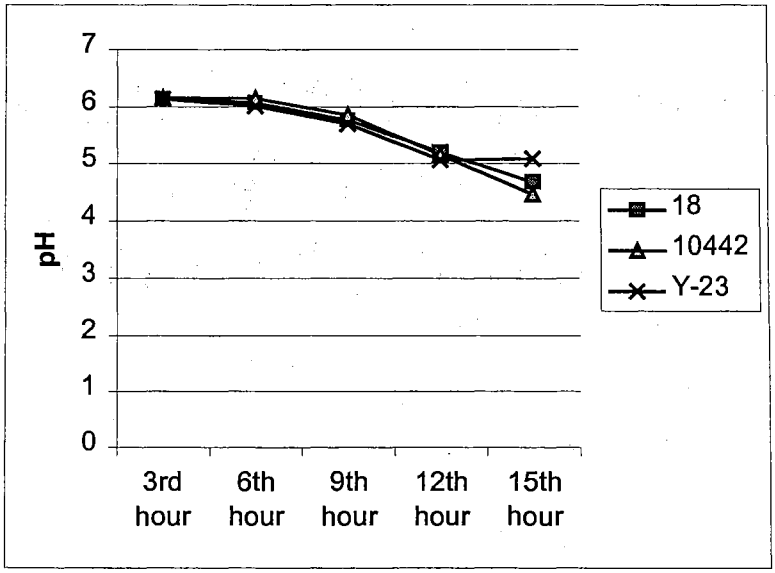
Graph B.15

Reducing activity of strains of *Lactobacillus delbrueckii ssp. bulgaricus* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



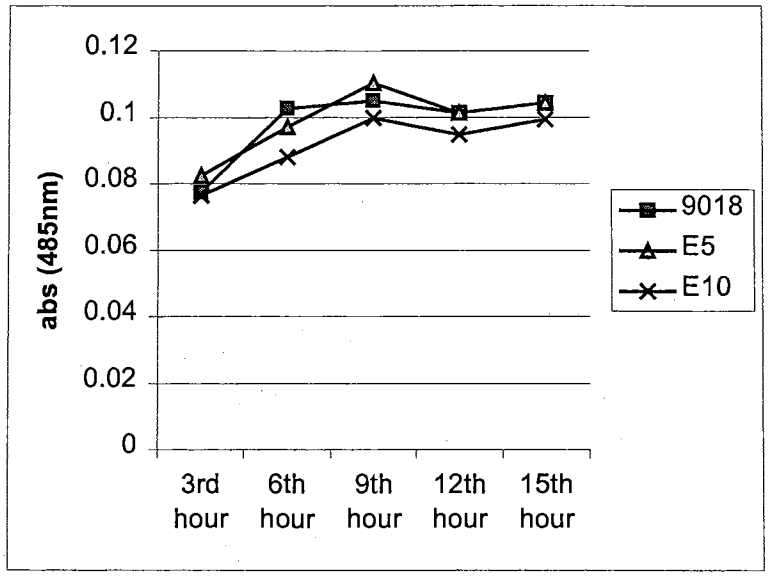
Graph B.16

pH of strains of *Lactobacillus delbrueckii ssp. bulgaricus* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



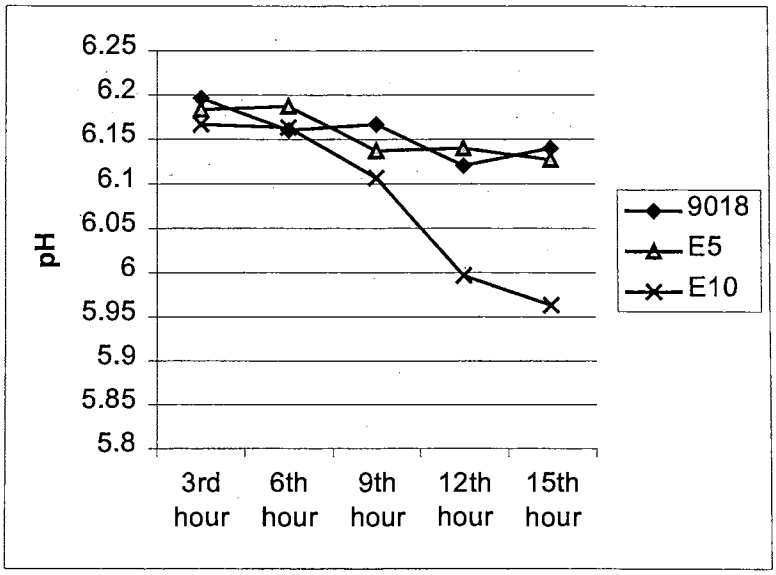
Graph B.17

Reducing activity of strains of *Lactobacillus casei* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



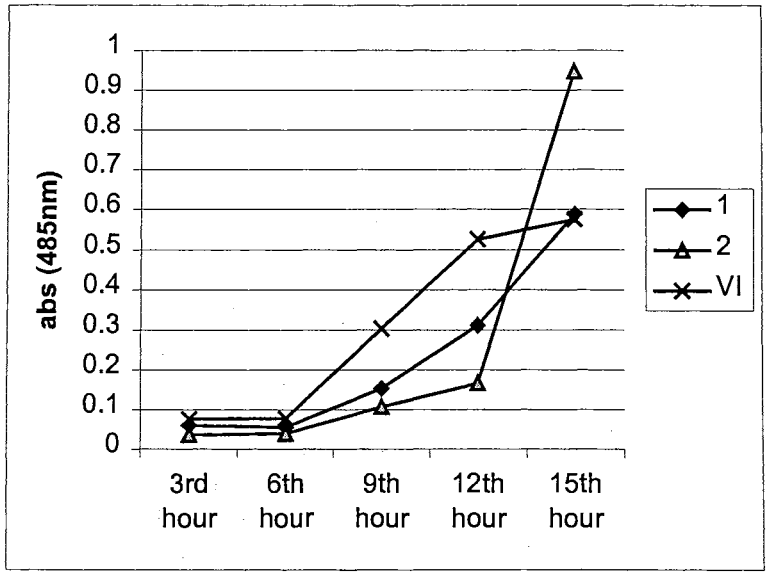
Graph B.18

pH of strains of *Lactobacillus casei* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



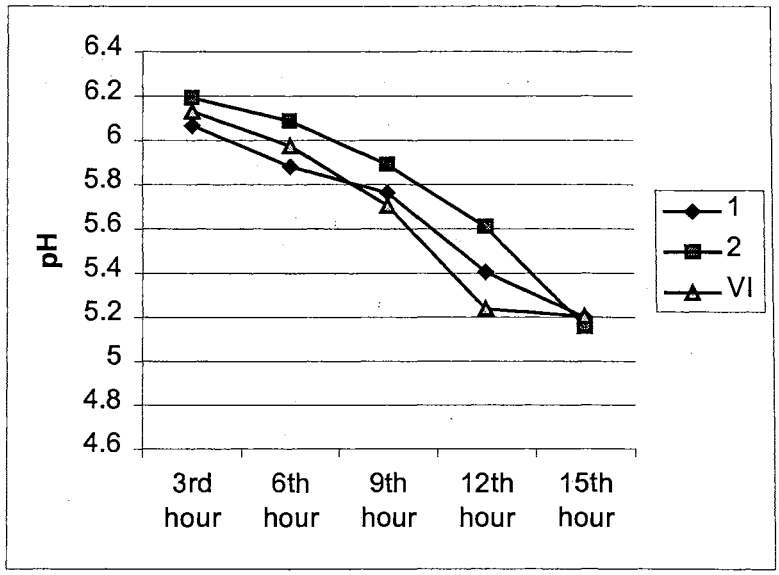
Graph B.19

Reducing activity of strains of *Streptococcus salivarius ssp. thermophilus* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



Graph B.20

pH of strains of *Streptococcus salivarius ssp. thermophilus* grown in 10% NFDM at 37°C for 15 hours measured at 485nm



APPENDIX C

ANTIOXIDATIVE ACTIVITY RAW DATA

Table C.1

Raw ORAC values for three strains of *Lactobacillus delbrueckii ssp. lactis* grown in MRS broth for 18 hours at 37C

Culture	Strain	Treatments*					
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate
		221.175	327.237	341.082	480.716	92.3015	272.853
	RM2-5	387.537	421.742	473.772	547.243	186.711	228.566
		303.962	552.07	488.365	392.863	236.072	207.359
		304.2247	**433.683**	**434.4063**	**473.6073**	**171.6948**	**236.2593**
		371.903	289.527	304.744	437.215	92.3015	272.853
Lactobacillus delbrueckii ssp. lactis	RM6-5	146.929	218.672	346.666	330.478	186.711	228.566
		275.301	311.443	354.545	452.742	236.072	207.359
		264.711	**273.214**	**335.3183**	**406.8117**	**171.6948**	**236.2593**
		378.756	361.105	419.728	465.532	92.3015	272.853
	RM5-4	419.594	443.986	297.742	977.341	186.711	228.566
		401.987	473.472	396.567	509.244	236.072	207.359
		400.1123	**426.1877**	**371.3457**	**650.7057**	**171.6948**	**236.2593**

Table C.2

Raw ORAC values for three strains of *Lactobacillus acidophilus* grown in MRS broth for 18 hours at 37C

Culture	Strain	Treatments*					
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate
		591.56	817.713	660.192	438.729	373.975	310.311
	O16	432.173	702.69	566.477	715.094	231.038	256
		509.739	689.473	557.002	686.291	198.406	205.333
		511.1573	**736.6253**	**594.557**	**613.3713**	**267.8063**	**257.2147**
		510.067	695.657	801.021	578.417	373.975	310.311
Lactobacillus acidophilus	NCFM	433.573	634.553	723.139	615.989	231.038	256
		409.933	512.745	527.687	811.053	198.406	205.333
		451.191	**614.3183**	**683.949**	**668.4863**	**267.8063**	**257.2147**
		470.61	483.061	399.17	436.323	373.975	310.311
	L-1	531.454	587.818	496.403	666.737	231.038	256
		401.348	511.388	532.978	582.022	198.406	205.333
		467.804	**527.4223**	**476.1837**	**561.694**	**267.8063**	**257.2147**

Table C.3

Raw ORAC values for three strains of *Lactobacillus delbrueckii ssp. bulgaricus* grown in MRS broth for 18 hours at 37C

Culture	Strain	Treatments*					
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate
		111.776	209.166	106.573	214.358	120.906	247.509
	18	16.617	95.471	217.908	410.098	96.3552	57.0076
		127.323	198.241	147.891	357.02	114.297	135.221
		85.23867	**167.626**	**157.4573**	**327.1587**	**110.5194**	**146.5792**
		74.511	255.06	327.731	306.497	120.906	247.509
Lactobacillus delbrueckii ssp. bulgaricus	10442	59.77	39.325	161.463	361.619	96.3552	57.0076
		93.112	187.344	199.023	333.577	114.297	135.221
		75.79767	**160.5763**	**229.4057**	**333.8977**	**110.5194**	**146.5792**
		190.964	244.482	339.524	486.415	120.906	247.509
	Y-23	232.459	227.498	277.671	387.404	96.3552	57.0076
		427.688	305.322	354.214	438.443	114.297	135.221
		283.7037	**259.1007**	**323.803**	**437.4207**	**110.5194**	**146.5792**

Table C.4

Raw ORAC values for three strains of *Lactobacillus casei* grown in MRS broth for 18 hours at 37C

Culture	Strain	Treatments*					
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate
		307.0508	401.8643	679.163	793.522	311.768	291.303
	9018	350.9668	445.5483	734.3572	909.4505	226.674	253.401
		295.8067	475.1248	413.7584	815.9114	188.415	231.693
		317.9414	**440.8458**	**609.0929**	**839.628**	**242.2857**	**258.799**
		372.313	695.633	689.075	544.696	311.768	291.303
Lactobacillus casei	E5	520.3251	381.7137	544.4969	543.8608	226.674	253.401
		343.6208	343.9752	480.4112	425.5416	188.415	231.693
		412.0863	**473.774**	**571.3277**	**504.6995**	**242.2857**	**258.799**
		330.977	612.592	573.79	656.365	311.768	291.303
	E10	618.5508	623.5318	452.4815	560.226	226.674	253.401
		433.2176	415.2989	473.1367	467.2988	188.415	231.693
		460.9151	**550.4742**	**499.8027**	**561.2966**	**242.2857**	**258.799**

Table C.5

Raw ORAC values for three strains of *Streptococcus salivarius ssp. thermophilus* grown in MRS broth for 18 hours at 37C

Culture	Strain	Treatments*					
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate
		142.486	725.893	576.531	491.304	251.372	355.898
	1	303.457	691.734	407.353	597.811	190.757	273.225
		255.071	599.118	488.346	663.021	201.011	293.537
		233.6713	**672.2483**	**490.7433**	**584.0453**	**214.38**	**307.5533**
		137.344	567.099	390.527	613.799	251.372	355.898
Streptococcus salivarius ssp. thermophilus	2	58.05	579.996	580.204	555.194	190.757	273.225
		302.077	412.333	451.249	487.053	201.011	293.537
		165.8237	**519.8093**	**473.9933**	**552.0153**	**214.38**	**307.5533**
		435.474	780.584	601.784	459.536	251.372	355.898
	VI	387.232	699.436	522.319	673.989	190.757	273.225
		315.056	707.392	573.213	588.071	201.011	293.537
		379.254	**729.1373**	**565.772**	**573.8653**	**214.38**	**307.5533**

Table C.6 Raw ORAC values for three strains of *Lactobacillus delbrueckii ssp. lactis* grown in 10% milk for 18 hours at 37C

Culture	Strain	Treatments*						
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate	Milk
		2593.37	2505.201	2431.784	2611.543	76.431	157.019	1944.58
	RM2-5	2709.195	2836.005	2862.041	3080.269	279.876	223.272	1573.303
		1863.132	1941.476	1759.754	1683.476	152.907	141.664	1702.101
		2388.5657	**2427.5607**	**2351.193**	**2458.4293**	**169.738**	**173.985**	**1739.9947**
		3092.554	2339.517	2664.17	2842.898	92.3015	272.853	1944.58
Lactobacillus delbrueckii ssp. lactis	RM6-5	3133.361	3377.925	2770.187	3444.854	186.711	228.566	1573.303
		1983.848	1788.798	1912.798	2043.735	236.072	207.359	1702.101
		2736.5877	**2502.08**	**2449.0517**	**2777.1623**	**171.69483**	**236.25933**	**1739.9947**
		2019.366	1421.105	1428.553	1690.762	92.3015	272.853	1944.58
	RM5-4	1841.746	2000.488	2188.054	2483.771	186.711	228.566	1573.303
		1188.678	986.9142	1186.036	1126.884	236.072	207.359	1702.101
		1683.2633	**1469.5024**	**1600.881**	**1767.139**	**171.69483**	**236.25933**	**1739.9947**

Table C.7 Raw ORAC values for three strains of Lactobacillus acidophilus grown in 10% milk for 18 hours at 37C

Culture	Strain	Treatments*						
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate	Milk
		2174.395	2362.908	2971.211	2889.391	115.436	479.992	2112
	O16	2507.007	2302.474	2089.889	2114.985	148.714	576.944	2306.073
		1936.679	1978.939	1967.628	2087.385	106.003	385.469	2290.587
		2206.027	**2214.7737**	**2342.9093**	**2363.9203**	**123.38433**	**480.80167**	**2236.22**
		2673.507	2534.313	2404.585	2777.366	115.436	479.992	2112
Lactobacillus acidophilus	NCFM	2373.437	2188.745	1731.747	2069.9	148.714	576.944	2306.073
		2014.26	1819.974	1967.319	2168.737	106.003	385.469	2290.587
		2353.7347	**2181.0107**	**2034.5503**	**2338.6677**	**123.38433**	**480.80167**	**2236.22**
		2373.164	1910.322	2280.078	2465.156	115.436	479.992	2112
		2115.449	1620.731	1949.564	2135.923	148.714	576.944	2306.073
	L-1	2471.944	1598.547	1703.163	2241.202	106.003	385.469	2290.587
		2320.1857	**1709.8667**	**1977.6017**	**2280.7603**	**123.38433**	**480.80167**	**2236.22**

Table C.8 Raw ORAC values for three strains of *Lactobacillus delbrueckii ssp. bulgaricus* grown in 10% milk for 18 hours at 37C

Culture	Strain	Treatments*						
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate	Milk
		1983.689	2060.697	1854.021	2061.473	109.235	217.11	1462.237
	18	2055.395	2411.908	3084.211	2996.391	139.576	916.971	1277.029
		1157.595	1215.427	1249.337	1272.204	202.448	481.657	1300.883
		1732.2263	**1896.0107**	**2062.523**	**2110.0227**	**150.41967**	**699.314**	**1346.7163**
		2015.306	1781.128	2022.561	2073.879	109.235	217.11	1462.237
Lactobacillus delbrueckii ssp. bulgaricus	10442	2794.307	2646.313	2561.585	2870.366	139.576	916.971	1277.029
		964.959	1019.392	1109.471	1110.495	202.448	481.657	1300.883
		1924.8573	**1815.611**	**1897.8723**	**2018.2467**	**150.41967**	**699.314**	**1346.7163**
		1828.086	1427.528	696.1761	1945.365	109.235	217.11	1462.237
	Y-23	2388.449	1941.981	2341.227	2455.156	139.576	916.971	1277.029
		710.1231	491.9944	679.394	1461.944	202.448	481.657	1300.883
		1642.2194	**1287.1678**	**1238.9324**	**1954.155**	**150.41967**	**699.314**	**1346.7163**

Table C.9 Raw ORAC values for three strains of *Lactobacillus casei* grown in 10% milk for 18 hours at 37C

Culture	Strain	Treatments*						
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate	Milk
		2307.818	2315.925	2153.87	2335.818	313.456	332.174	1981.303
	9018	1773.576	1915.067	1887.556	1857	316.781	287.092	2112.768
		1558.361	1876.624	1724.046	2000.423	99.0359	346.887	1702
		1879.9183	**2035.872**	**1921.824**	**2064.4137**	**243.09097**	**316.9895**	**1932.0237**
		2315.225	2451.131	2340.113	2418.955	313.456	332.174	1981.303
Lactobacillus casei	E5	1992.582	2125.352	2060.813	2031.982	316.781	287.092	2112.768
		2063.143	2170.809	1915.725	2349.578	99.0359	346.887	1702
		2123.65	**2249.0973**	**2105.5503**	**2266.8383**	**243.09097**	**316.9895**	**1932.0237**
		2268.463	2085.288	2208.231	2194.046	313.456	332.174	1981.303
	E10	2080.234	1577.142	2027	2011.901	316.781	287.092	2112.768
		1476.164	1214.546	1216.271	1291.675	99.0359	346.887	1702
		1941.6203	**1625.6587**	**1817.1673**	**1832.5407**	**243.09097**	**316.9895**	**1932.0237**

Table C.10 Raw ORAC values for three strains of *Streptococcus salivarius ssp. thermophilus* grown in 10% milk for 18 hours at 37C

Culture	Strain	Treatments*						
		Cells & buffer	Cells & cholate	Cells & glycholate	Sonicated cell	Cholate	Glyco-cholate	Milk
		2254.154	2613.788	2269.698	2396.136	208.703	186	2491.312
	1	1579.319	1620.917	2052.452	1994.374	249,622	227.455	2045.631
		2608.284	1916.187	2344.078	2066.083	129.077	296.51	2119.901
		2147.2523	**2050.2973**	**2222.076**	**2152.1977**	**83319.927**	**261.9825**	**2082.766**
		2165.087	2002.434	2229.54	2559.256	208.703	186	2491.312
Streptococcus salivarius ssp. thermophilus	2	1812.75	1856.26	1987.248	2084.333	249,622	227.455	2045.631
		2034.237	1898.567	1802.331	2355.879	129.077	296.51	2119.901
		2004.0247	**1919.087**	**2006.373**	**2333.156**	**83319.927**	**261.9825**	**2082.766**
		2605.471	2221.737	2195.667	2159.172	208.703	186	2491.312
	VI	465.6098	584.4549	932.349	764.9676	249,622	227.455	2045.631
		2045.871	1935.884	2075.918	2045.023	129.077	296.51	2119.901
		1705.6506	**1580.692**	**1734.6447**	**1656.3875**	**83319.927**	**261.9825**	**2082.766**

Table C.11. Processed ORAC values for three cultures of *Lactobacillus delbrueckii ssp. lactis* after 18 hours incubation in MRS broth at 37C exported from HTSoft into Excell

RM2-5©	RM2-5©	RM2-5©	**RM2-5©**	RM2-5(ch)	RM2-5(ch)	RM2-5(ch)
14810	14888	14526	**14741.33333**	15366	14097	13881
15105	15206	14945	**15085.33333**	15812	14748	14563
14745	15149	14597	**14830.33333**	15276	14376	14043
14128	14621	14189	**14312.66667**	14956	13847	13850
13307	13754	13696	**13585.66667**	14067	13307	13269
12293	13056	12841	**12730**	13212	12604	12504
11384	11820	11714	**11639.33333**	11989	11763	11552
9996	10581	10721	**10432.66667**	10683	10623	10528
8726	9327	9391	**9148**	9463	9509	9368
7661	8164	8158	**7994.333333**	8354	8261	8272
6385	7076	7004	**6821.666667**	6958	7061	7117
5476	6030	5944	**5816.666667**	6059	5926	5974
4725	5136	5135	**4998.666667**	5262	5060	5157
4053	4391	4385	**4276.333333**	4368	4322	4335
3511	3929	3777	**3739**	3832	3717	3779
2979	3344	3250	**3191**	3277	3162	3164
2623	2853	2818	**2764.666667**	2800	2707	2811
2232	2463	2415	**2370**	2421	2298	2273
1954	2173	2112	**2079.666667**	2056	1927	1899
1643	1844	1855	**1780.666667**	1782	1682	1682
1450	1569	1569	**1529.333333**	1552	1446	1448
1264	1345	1368	**1325.666667**	1312	1203	1234
1111	1204	1158	**1157.666667**	1144	1064	1039
943	1023	1022	**996**	981	889	861
810	906	819	**845**	844	795	754
740	794	789	**774.3333333**	738	689	664
636	680	665	**660.3333333**	619	584	557
545	598	603	**582**	565	507	514
485	531	508	**508**	509	475	444
486	481	458	**475**	452	431	388
431	461	436	**442.6666667**	421	405	392
410	427	375	**404**	397	386	341
385	411	377	**391**	347	369	343
373	391	361	**375**	350	362	347
376	380	349	**368.3333333**	344	355	335

Area	**23.73593524**
Net area	**1.103654333**
ORAC	**0.110587287**
Correected ORAC	**221.1745743**

RM2-5(ch)	RM2-5(gl)	RM2-5(gl)	RM2-5(gl)	RM2-5(gl)	RM2-5(so)	RM2-5(so)	RM2-5(so)
14448	14441	14363	14033	**14279**	14319	14500	14093
15041	14815	14839	14777	**14810.33333**	14718	14960	14829
14565	14525	14638	14388	**14517**	14505	14633	14516
14217.66667	13963	14299	13976	**14079.33333**	14002	14522	14337
13547.66667	13311	13596	13331	**13412.66667**	13250	13837	13844
12773.33333	12547	12848	12835	**12743.33333**	12613	13199	13166
11768	11527	11972	11944	**11814.33333**	11617	12320	12280
10611.33333	10290	10888	10995	**10724.33333**	10484	11351	11349
9446.666667	8951	9533	9754	**9412.666667**	9275	10111	10167
8295.666667	7733	8466	8391	**8196.666667**	8070	8969	9096
7045.333333	6546	7313	7315	**7058**	6884	7648	7878
5986.333333	5575	6138	6103	**5938.666667**	5787	6617	6555
5159.666667	4778	5145	5328	**5083.666667**	4950	5557	5657
4341.666667	4013	4417	4375	**4268.333333**	4277	4613	4846
3776	3420	3766	3783	**3656.333333**	3610	4005	4105
3201	2920	3170	3233	**3107.666667**	3115	3393	3503
2772.666667	2536	2735	2774	**2681.666667**	2710	2919	3019
2330.666667	2095	2365	2352	**2270.666667**	2269	2485	2462
1960.666667	1916	2010	1966	**1964**	1904	2079	2122
1715.333333	1563	1717	1699	**1659.666667**	1618	1733	1849
1482	1373	1482	1467	**1440.666667**	1436	1510	1575
1249.666667	1140	1284	1213	**1212.333333**	1200	1265	1307
1082.333333	979	1061	995	**1011.666667**	1004	1071	1108
910.3333333	852	883	865	**866.6666667**	870	964	976
797.6666667	720	776	739	**745**	741	775	817
697	657	675	643	**658.3333333**	639	675	706
586.6666667	582	590	537	**569.6666667**	567	572	603
528.6666667	496	542	513	**517**	523	520	548
476	449	484	456	**463**	435	450	461
423.6666667	424	442	422	**429.3333333**	385	415	433
406	378	429	398	**401.6666667**	412	395	380
374.6666667	357	404	355	**372**	374	351	385
353	384	385	356	**375**	333	332	339
353	356	383	334	**357.6666667**	342	349	349
344.6666667	354	380	355	**363**	328	322	326
24.26518088				**24.33426711**			
1.632899973				**1.7019862**			
0.163618239				**0.170540749**			
327.2364775				**341.081498**			

RM2-5(so)	RM5-4©	RM5-4©	RM5-4©	RM5-4©	RM5-4(ch)	RM5-4(ch)	RM5-4(ch)
14304	14106	14495	14380	**14327**	14577	14237	13203
14835.66667	14493	14948	14813	**14751.33333**	15145	14564	13735
14551.33333	14213	14627	14713	**14517.66667**	14621	14448	13595
14287	13824	14296	14329	**14149.66667**	14203	13934	13204
13643.66667	13220	13865	13719	**13601.33333**	13717	13684	12710
12992.66667	12375	13204	13182	**12920.33333**	12887	12910	11975
12072.33333	11440	12284	12445	**12056.33333**	11798	11979	11039
11061.33333	10383	11146	11379	**10969.33333**	10591	11049	10227
9851	9003	9861	9961	**9608.333333**	9293	9713	9113
8711.666667	7759	8727	8761	**8415.666667**	8080	8511	7893
7470	6518	7358	7600	**7158.666667**	6879	7173	6828
6319.666667	5553	6362	6378	**6097.666667**	5774	6002	5789
5388	4674	5318	5529	**5173.666667**	4926	5174	4814
4578.666667	4019	4469	4553	**4347**	4229	4430	4025
3906.666667	3383	3860	3975	**3739.333333**	3605	3776	3425
3337	2877	3285	3349	**3170.333333**	2999	3191	2998
2882.666667	2468	2754	2874	**2698.666667**	2563	2699	2511
2405.333333	2074	2343	2452	**2289.666667**	2287	2259	2163
2035	1788	1946	2067	**1933.666667**	1917	1959	1766
1733.333333	1517	1658	1777	**1650.666667**	1637	1616	1540
1507	1328	1418	1512	**1419.333333**	1434	1439	1327
1257.333333	1099	1260	1255	**1204.666667**	1140	1200	1097
1061	936	1074	1019	**1009.666667**	1025	1022	949
936.6666667	799	849	921	**856.3333333**	833	880	799
777.6666667	704	758	774	**745.3333333**	742	736	709
673.3333333	591	653	674	**639.3333333**	681	670	565
580.6666667	537	543	603	**561**	562	557	531
530.3333333	475	490	497	**487.3333333**	537	502	485
448.6666667	429	455	458	**447.3333333**	465	447	399
411	408	407	403	**406**	428	437	416
395.6666667	408	391	389	**396**	403	409	385
370	362	384	357	**367.6666667**	383	395	358
334.6666667	353	350	339	**347.3333333**	351	362	365
346.6666667	355	328	341	**341.3333333**	360	383	360
325.3333333	362	322	348	**344**	341	377	380
25.03104027				**24.51229613**			
2.398759362				**1.880015225**			
0.240358129				**0.188379438**			
480.7162578				**376.758877**			

RM5-4(ch)	Cholate	Cholate	Cholate	Cholate	Buffer	Buffer	Buffer
14005.66667	14212	13777	14327	14105.33333	13999	12908	14331
14481.33333	14544	14349	14711	14534.66667	14617	13284	14674
14221.33333	14190	14096	14540	14275.33333	14137	12962	14416
13780.33333	13750	13721	14139	13870	13818	12579	13901
13370.33333	13155	13275	13574	13334.66667	12922	11922	13290
12590.66667	12247	12437	12797	12493.66667	11870	11042	12476
11605.33333	11225	11337	11925	11495.66667	10581	10003	11580
10622.33333	9930	10348	10668	10315.33333	9342	8861	10212
9373	8636	9056	9343	9011.666667	7993	7463	8934
8161.333333	7462	7708	8158	7776	6791	6420	7656
6960	6280	6457	6953	6563.333333	5793	5446	6496
5855	5302	5357	5729	5462.666667	4825	4531	5446
4971.333333	4466	4565	4962	4664.333333	4163	3788	4571
4228	3849	3795	4062	3902	3476	3208	3850
3602	3258	3247	3542	3349	2940	2718	3325
3062.666667	2738	2777	2888	2801	2510	2362	2832
2591	2418	2361	2481	2420	2140	1962	2435
2236.333333	2042	1912	2134	2029.333333	1831	1644	2077
1880.666667	1701	1671	1748	1706.666667	1554	1416	1714
1597.666667	1509	1460	1470	1479.666667	1340	1160	1473
1400	1249	1153	1307	1236.333333	1144	1049	1286
1145.666667	1083	1007	1040	1043.333333	975	845	1091
998.6666667	956	833	911	900	848	735	928
837.3333333	770	707	757	744.6666667	741	661	772
729	681	598	650	643	620	560	665
638.6666667	579	533	547	553	551	481	578
550	513	491	525	509.6666667	470	457	522
508	453	448	458	453	444	410	457
437	417	420	426	421	420	389	429
427	408	384	396	396	372	393	386
399	386	365	352	367.6666667	390	348	366
378.6666667	360	364	348	357.3333333	343	333	359
359.3333333	357	356	352	355	362	339	364
367.6666667	347	332	336	338.3333333	329	329	334
366	323	347	334	334.6666667	327	339	333
24.4341814				23.56588524			
1.801900497				0.933604338			
0.180552263				0.093548105			
361.1045265				187.0962093			

Buffer	RM6-5©	RM6-5©	RM6-5©	RM6-5©	RM6-5(ch)	RM6-5(ch)	RM6-5(ch)
13746	14705	14460	14532	**14565.66667**	13987	14017	14469
14191.66667	15126	14899	14952	**14992.33333**	14479	14650	14798
13838.33333	14665	14543	14442	**14550**	14266	14202	14337
13432.66667	14320	14037	13936	**14097.66667**	13893	13800	13842
12711.33333	13977	13348	13034	**13453**	13376	13046	13153
11796	13026	12620	11972	**12539.33333**	12504	12415	12457
10721.33333	12507	11625	10743	**11625**	11635	11472	11351
9471.666667	11459	10621	9636	**10572**	10451	10606	10384
8130	10537	9294	8363	**9398**	9130	9253	9044
6955.666667	9264	7944	7377	**8195**	7883	7984	7869
5911.666667	8292	6744	6272	**7102.666667**	6774	6872	6750
4934	7128	5800	5180	**6036**	5653	5818	5779
4174	6218	5086	4471	**5258.333333**	4854	4974	5022
3511.333333	5276	4325	3930	**4510.333333**	4036	4217	4363
2994.333333	4703	3750	3370	**3941**	3465	3619	3692
2568	4041	3205	2964	**3403.333333**	2926	3074	3243
2179	3588	2844	2587	**3006.333333**	2531	2641	2824
1850.666667	3081	2387	2218	**2562**	2105	2201	2394
1561.333333	2758	2060	1920	**2246**	1796	1942	2088
1324.333333	2330	1817	1628	**1925**	1517	1662	1794
1159.666667	2185	1595	1438	**1739.333333**	1309	1399	1608
970.3333333	1832	1323	1272	**1475.666667**	1051	1189	1375
837	1648	1158	1084	**1296.666667**	891	1006	1215
724.6666667	1435	1013	898	**1115.333333**	785	891	1059
615	1284	877	806	**989**	673	770	904
536.6666667	1116	771	704	**863.6666667**	587	647	772
483	968	676	609	**751**	544	577	690
437	873	580	527	**660**	465	511	573
412.6666667	769	522	493	**594.6666667**	418	453	543
383.6666667	722	449	465	**545.3333333**	390	411	506
368	633	439	424	**498.6666667**	368	397	450
345	612	415	405	**477.3333333**	351	350	422
355	545	398	344	**429**	366	344	390
330.6666667	526	363	341	**410**	333	354	384
333	505	350	353	**402.6666667**	338	318	365

22.63228091

24.48806554

0

1.855784636

0.185951509

371.9030177

RM6-5(ch)	RM6-5(gl)	RM6-5(gl)	RM6-5(gl)	RM6-5(gl)	RM6-5(so)	RM6-5(so)	RM6-5(so)
14157.66667	13996	14031	14584	**14203.66667**	14329	14109	14695
14642.33333	14497	14528	14892	**14639**	14924	14613	15278
14268.33333	14218	14317	14627	**14387.33333**	14630	14246	14817
13845	13953	13823	14135	**13970.33333**	14405	13852	14283
13191.66667	13207	13309	13357	**13291**	13850	13560	13680
12458.66667	12525	12506	12576	**12535.66667**	13304	12594	12856
11486	11650	11711	11320	**11560.33333**	12316	11823	11562
10480.33333	10762	10671	10239	**10557.33333**	11484	10622	10493
9142.333333	9558	9407	9032	**9332.333333**	10234	9589	9445
7912	8278	8244	7836	**8119.333333**	8954	8319	8108
6798.666667	7066	6974	6605	**6881.666667**	7738	6962	6863
5750	5889	6021	5676	**5862**	6669	6175	5890
4950	5086	5026	4915	**5009**	5761	5134	5063
4205.333333	4256	4315	4062	**4211**	4771	4456	4376
3592	3620	3617	3574	**3603.666667**	4177	3743	3681
3081	3107	3082	3056	**3081.666667**	3545	3182	3214
2665.333333	2610	2600	2674	**2628**	3050	2791	2842
2233.333333	2255	2220	2273	**2249.333333**	2587	2478	2351
1942	1924	1926	1941	**1930.333333**	2223	2034	2089
1657.666667	1593	1652	1688	**1644.333333**	1904	1710	1778
1438.666667	1374	1385	1461	**1406.666667**	1655	1482	1540
1205	1125	1180	1254	**1186.333333**	1400	1228	1320
1037.333333	995	1022	1061	**1026**	1163	1067	1080
911.6666667	842	834	908	**861.3333333**	1000	907	941
782.3333333	714	759	769	**747.3333333**	860	796	886
668.6666667	628	655	693	**658.6666667**	745	698	749
603.6666667	553	539	578	**556.6666667**	606	597	634
516.3333333	517	481	535	**511**	570	516	571
471.3333333	415	439	489	**447.6666667**	479	455	490
435.6666667	390	402	401	**397.6666667**	428	420	433
405	380	410	395	**395**	422	393	416
374.3333333	346	364	391	**367**	401	368	387
366.6666667	328	365	357	**350**	376	349	339
357	342	351	351	**348**	349	368	359
340.3333333	345	334	350	**343**	324	319	352
24.07701363				**24.15294173**			
1.444732726				**1.520660823**			
0.144763689				**0.152371762**			
289.5273784				**304.7435235**			

RM6-5(so)	RM5-4(gl)	RM5-4(gl)	RM5-4(gl)	**RM5-4(gl)**	RM5-4(so)	RM5-4(so)	RM5-4(so)
14377.66667	14122	15316	14596	**14678**	14434	14765	16229
14938.33333	14580	15828	14981	**15129.66667**	15020	15472	16637
14564.33333	14292	15358	14758	**14802.66667**	14746	15046	16286
14180	14195	14864	14394	**14484.33333**	14590	14639	15701
13696.66667	13522	14420	13833	**13925**	13945	14104	14918
12918	13125	13373	13175	**13224.33333**	13213	13356	14121
11900.33333	12019	12306	12256	**12193.66667**	12325	12587	12816
10866.33333	11046	10956	11075	**11025.66667**	11382	11489	11669
9756	9933	9712	10024	**9889.666667**	10208	10022	10321
8460.333333	8839	8518	8736	**8697.666667**	9027	8957	9015
7187.666667	7566	7227	7470	**7421**	7737	7650	7747
6244.666667	6470	6209	6291	**6323.333333**	6636	6511	6639
5319.333333	5405	5306	5417	**5376**	5596	5629	5741
4534.333333	4560	4514	4595	**4556.333333**	4843	4770	4922
3867	3909	4085	3893	**3962.333333**	4092	4058	4315
3313.666667	3389	3343	3353	**3361.666667**	3533	3588	3705
2894.333333	2872	2920	2900	**2897.333333**	3061	2953	3220
2472	2464	2587	2496	**2515.666667**	2609	2596	2809
2115.333333	2018	2176	2078	**2090.666667**	2165	2189	2396
1797.333333	1708	1856	1790	**1784.666667**	1857	1864	2104
1559	1491	1646	1479	**1538.666667**	1593	1654	1886
1316	1286	1393	1278	**1319**	1371	1441	1608
1103.333333	1130	1192	1070	**1130.666667**	1181	1186	1354
949.3333333	934	995	988	**972.3333333**	961	1001	1197
847.3333333	809	871	779	**819.6666667**	826	886	1047
730.6666667	704	773	698	**725**	709	749	925
612.3333333	614	689	559	**620.6666667**	627	628	841
552.3333333	558	610	561	**576.3333333**	553	578	680
474.6666667	504	496	480	**493.3333333**	469	504	609
427	469	463	418	**450**	444	463	582
410.3333333	428	434	415	**425.6666667**	408	404	508
385.3333333	401	399	363	**387.6666667**	377	396	474
354.6666667	417	360	373	**383.3333333**	349	378	428
358.6666667	391	351	369	**370.3333333**	348	370	370
331.6666667	372	368	359	**366.3333333**	341	345	373
24.81397074				**24.72671118**			
2.181689836				**2.094430272**			
0.218607541				**0.209864044**			
437.2150829				**419.7280887**			

RM5-4(so)	Glycocholate	Glycocholate	Glycocholate	Glycocholat	Trolox	Trolox	Trolox
15142.66667	14825	14552	14350	**14575.66667**	13718	14066	14771
15709.66667	15360	15061	14653	**15024.66667**	14166	14526	15177
15359.33333	15021	14607	14304	**14644**	13968	14104	14742
14976.66667	14699	14260	13784	**14247.66667**	13821	14194	14584
14322.33333	14188	13767	13138	**13697.66667**	13562	13980	14384
13563.33333	13248	12956	12350	**12851.33333**	13392	13907	14247
12576	12379	12007	11207	**11864.33333**	13377	13675	14282
11513.33333	11283	10971	9802	**10685.33333**	13342	13647	14043
10183.66667	9709	9538	8759	**9335.333333**	12893	13321	13914
8999.666667	8489	8318	7511	**8106**	12731	13096	13557
7711.333333	7018	7167	6449	**6878**	12007	12512	12735
6595.333333	6047	5961	5334	**5780.666667**	10868	11550	11740
5655.333333	5148	5087	4663	**4966**	9044	9956	10106
4845	4296	4287	4056	**4213**	7531	8318	8451
4155	3615	3705	3410	**3576.666667**	6237	6972	7099
3608.666667	3050	3112	2965	**3042.333333**	5005	5931	6185
3078	2614	2703	2527	**2614.666667**	4289	4848	5190
2671.333333	2242	2274	2244	**2253.333333**	3492	4105	4432
2250	1853	1938	1837	**1876**	2986	3492	3817
1941.666667	1554	1643	1588	**1595**	2512	3026	3295
1711	1312	1425	1344	**1360.333333**	2160	2611	2821
1473.333333	1110	1205	1201	**1172**	1846	2189	2438
1240.333333	945	1012	1011	**989.3333333**	1568	1971	2169
1053	771	857	841	**823**	1363	1652	1891
919.6666667	689	772	788	**749.6666667**	1110	1413	1664
794.3333333	580	641	650	**623.6666667**	936	1220	1468
698.6666667	488	581	571	**546.6666667**	789	1079	1244
603.6666667	459	518	528	**501.6666667**	707	907	1087
527.3333333	414	422	458	**431.3333333**	615	778	951
496.3333333	401	414	452	**422.3333333**	532	707	850
440	373	403	419	**398.3333333**	489	581	725
415.6666667	332	376	372	**360**	446	542	637
385	322	358	356	**345.3333333**	390	481	577
362.6666667	335	330	346	**337**	377	434	492
353	331	353	341	**341.6666667**	365	410	456
24.95526988				**23.77627095**			
2.322988972				**1.143990048**			
0.232765859				**0.114628967**			
465.5317173				**229.2579337**			

Trolox	Time
14185	2
14623	4
14271.33333	6
14199.66667	8
13975.33333	10
13848.66667	12
13778	14
13677.33333	16
13376	18
13128	20
12418	22
11386	24
9702	26
8100	28
6769.333333	30
5707	32
4775.666667	34
4009.666667	36
3431.666667	38
2944.333333	40
2530.666667	42
2157.666667	44
1902.666667	46
1635.333333	48
1395.666667	50
1208	52
1037.333333	54
900.3333333	56
781.3333333	58
696.3333333	60
598.3333333	62
541.6666667	64
482.6666667	66
434.3333333	68
410.3333333	70

32.61221948

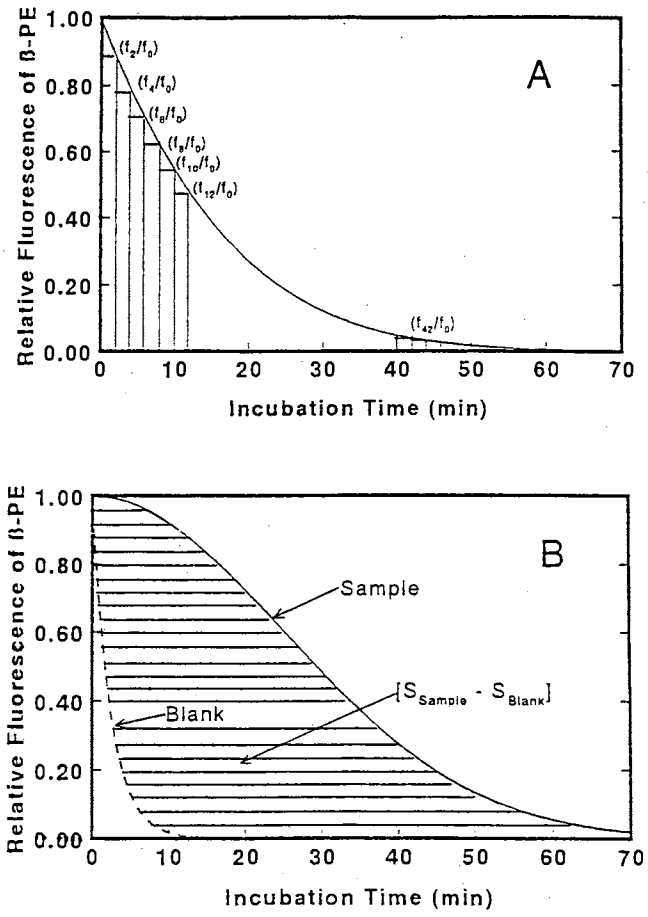
9.979938575

1

2000

Figure C.1 Example of calculation of ORAC values

A – relative fluorescence of β-phycoerythrin at different incubation time points; and
B – ORAC value calculated as $(S_{sample} - S_{blank})/(S_{trolox} - S_{blank})$



$$\text{ORAC value} = X K (S_{sample} - S_{blank})/(S_{trolox} - S_{blank})$$

where X is the sample volume in microliters,
K is the dilution coefficient, and
S is the area under the curve of the corresponding subscript and is:
$S = (0.5 + f_2/f_0 + f_4/f_0 + f_6/f_0 + \ldots + f_{60}/f_0 + f_{70}/f_0) \times 2$
where f_0 is the initial fluorescence at 0 min. and f_i is the fluorescence at time i.

VITA

Joaquim Saide

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Doctor of Philosophy

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