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Effect of Nutrient Supplements on Cucumber Fermentation by Lactic Acid Bacteria

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EFFECT OF NUTRIENT SUPPLEMENTS ON CUCUMBER FERMENTATION BY LACTIC ACID BACTERIA

EFFECT OF NUTRIENT SUPPLEMENTS ON CUCUMBER FERMENTATION BY LACTIC ACID BACTERIA

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By

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ABSTRACT

Lactic acid bacteria (LAB) are important industrial microorganisms involved in fermentation of food and beverage products. The strict fermentative growth of LAB has complex requirements of various nutrients including amino acids, vitamins and minerals. Information about the effect of these nutrients on the growth of LAB in cucumber fermentation is not readily available. It is evident, from previous research that certain nutrients like; leucine, isoleucine, tryptophan, valine, biotin, nicotinic acid, pantothenic acid, riboflavin, manganese and magnesium are beneficial for LAB growth, but are not provided in sufficient quantities by the cucumber in the brine.

The objective of this study was to determine the efficacy of (1) the above mentioned nutrients (2) the most effective concentrations of biotin, isoleucine and valine alone and (3) combination of biotin, isoleucine and valine with cucumber fermentation brine, on the production of LAB and lactic acid in brine.

The yield of LAB was determined from microscopical counts using a hemacytometer, lactic acid concentration, dry weight and the final sugar concentration in the brine was determined. The first three trials established that biotin, valine and isoleucine improved LAB growth. Efficacy of five different concentrations each of biotin, valine and isoleucine in cucumber juice was determined in trial four. Biotin and isoleucine treatments at three different concentrations, in trial four, were equally effective on LAB growth; hence the lowest concentrations of 614 nM of biotin and 0.76 mM of isoleucine were selected; whereas valine treatments showed a relative small increase in the LAB growth with increase in concentration and 1.17 mM of valine was the most effective of all the valine treatments. These optimized

concentrations of nutrients were used in trial five, in different combinations. Of these, biotin and valine treatments when used individually or in combination showed a significant increase in LAB growth and the rate of production and concentration of lactic acid in the brine. These results indicated that the addition of biotin and valine potentially increased the number of LAB. Additional research is required using whole cucumbers to develop effective treatments.

This thesis is approved for recommendation

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DEDICATION

I would like to dedicate this work to all my teachers. I am indebted to many of my teachers who inspired me throughout my academic career. My heartfelt thanks to all my teachers for instilling all the important values of life.

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INTRODUCTION

The lactic acid bacteria (LAB) present in small numbers on fresh cucumbers produce lactic acid by fermentation. The lactic acid inhibits the growth of undesirable microorganisms. Sugar is the most essential nutrient for fermentation where sugars, glucose and fructose are converted to lactic acid by LAB. Previous research on the micronutrient requirements of LAB growth has established that amino acids, vitamins and minerals increase the population of LAB and the concentration of lactic acid produced in the brine. These nutrients have to be provided to LAB by the substrate or they need to be supplemented.

Some work has been carried out on determining the nutritional requirements of LAB, predominantly *Lactobacillus plantarum* as it is the major contributor of lactic acid in cucumber fermentation (Pederson and Albury 1969; Costilow and others 1956). Previous researchers have demonstrated that *L. plantarum* depends on cucumber as a source of amino acids (Leucine, Isoleucine, Typtophan and Valine), vitamins (Biotin, Nicotinic acid, Pantothenic acid and Riboflavin) and minerals (Manganese and Magnesium) which are essential for its growth (Costilow and Fabian 1953 a, b, c; Kashket 1987; Moretrow and others 1998; Lu and others 2002; Weymarn and others 2002). de Man, Rogosa and Sharpe (MRS) basal medium is a selective media for LAB growth which contains amino acids, vitamins and minerals in required concentrations. For the LAB fermenting cucumbers the above mentioned micro nutrients are not provided in sufficient concentrations by the cucumber fruit in comparison with their concentration in MRS basal agar. Brine was supplemented with the above nutrients based on their concentration in MRS basal agar (Appendix: Table 2) as it is a selective growth media for LAB and promotes its luxuriant growth. The primary goal of this study was to find the effect of different concentrations of the nutrients supplemented on LAB growth. Increase in the LAB

numbers may increase the concentration and the rate of lactic acid production. Any of these nutrients could be a potential supplement to increase the LAB number and the lactic acid concentration in cucumber fermentation.

CHAPTER I

LITERATURE REVIEW

OVERVIEW OF CUCUMBER FERMENTATION

Fermentation of cucumbers is advantageous over processing techniques like pasteurization and refrigeration since it provides preservation and bulk storage of seasonal production (Fleming 1984). Lactic acid bacteria (LAB) metabolize sugars in cucumber fermentation and make them unavailable for spoilage/pathogenic organisms and extend cucumber shelf life.

During industrial fermentation, the harvested cucumbers are placed in large outdoor open tanks with salted brine to equilibrate to approximately 5-10% sodium chloride concentration and are allowed to ferment naturally by indigenous LAB (Breidt and others 2007). After placing the cucumbers in the brine, sugar and water soluble nutrients diffuse out of the cucumber tissue into the surrounding brine within a period of 24 hrs and salt diffuses into the cucumber tissue until equilibrium is attained (Costilow and Fabian 1953 b). The nutrients in the brine from the cucumber favor the growth of LAB which are acid and salt tolerant.

Homofermentative strains of LAB metabolize glucose in cucumber producing about 85% of the lactic acid (Kandler and Weiss 1986; Mundt 1984) and heterofermentative strains produce lactic acid, carbon dioxide, ethanol and/or acetic acid. Fructose in cucumber is oxidized by homofermentative bacteria to lactate via lactate dehydrogenase (Li 2006) and heterofermentative bacteria metabolize fructose to mannitol via mannitol dehydrogenase (Hammes and others 1991), which is converted to lactic acid, ethanol acetate and carbon dioxide (Li 2006).

The high sodium chloride concentration and low pH prevents growth of spoilage causing microorganisms. The fermentation tanks are left open to the atmosphere for the ultraviolet rays

to penetrate the brine and prevent oxidative yeast and mold growth (Fleming and others 1988). Carbon dioxide in the brine responsible for bloater formation in cucumbers is reduced by sparging with air (Fleming and others 1988).

By the end of fermentation of cucumbers, the brine pH is reduced to 3.3-3.5 with most reducing sugars converting to lactic acid, and there may be a change in the appearance of their flesh from opaque to translucent. The product is removed and salt is generally leached to a lower salt concentration of 3-5% (Fleming and others 1987), then the fermented cucumbers are packaged with acidified (vinegar) brine and flavoring.

MICROORGANISMS INVOLVED IN CUCUMBER FERMENTATION

Lactic acid bacteria are non-sporing, cocci or rods in shape and ferment sugars to produce lactic acid (Axelsson 2004). Sodium chloride concentration of the brine controls type and number of microflora including LAB, yeast, and coliform participating in fermentation (Axelsson 2004; Costilow and Fabian 1953b). The microorganisms indigenous to cucumber fermentation are *Lactobacillus plantarum, Pediococcus cerevisiae, Leuconostoc mesenteroides, Streptococcus faecalis and Lactobacillus brevis* (Maki 2004).

The micro-floral population of fresh cucumbers is dominated by gram-negative aerobic bacteria, coliforms and yeast LAB is present in insignificant numbers initially (Nout and Rombouts 1992; Font de Valdez and others 1990; Etchells and others 1975) and proliferate with rapid production of lactic acid. Acid produced acts on the spoilage and pathogenic organism's cytoplasmic membrane and interferes with the maintenance of the membrane during fermentation (Caplice and Fitzgerald 1999). The high NaCl concentrations suppress the growth of gram-negative aerobic bacteria. Coliform and yeast counts decrease within the first 5 days of

fermentation (Costilow and Fabian 1953a) and LAB being acid tolerant multiplies and outnumbers the initial microflora.

Among the LAB species growing in cucumber fermentation, *L. plantarum* is the major contributor of lactic acid and not *L. mesenteroides* or other gas producing species of *Lactobacillus* (Etchells and Jones 1946; Costilow and others 1956). *L. plantarum* also completes the final state of cucumber fermentation (Pederson and Albury 1969). The comparatively low growth-limiting internal pH of *L. plantarum* and ability to maintain a pH gradient across the cell membrane at high organic acid concentration may contribute to its acid-tolerance (McDonald and others 1990). It produces energy and lactic acid via Embden-Meyerhof pathway on metabolizing hexose; with energy produced used for cell maintenance and cell division (Lelong and others 1991; Kemp and others 1989). In general, *L. plantarum* is auxotropic for many vitamins and amino acids (Koser 1968; Ledesma and others 1977; Morishita and others 1981). Strains isolated from cucumber fermentations have also shown a positive effect on their growth rate in presence of these growth factors (Rosen and Fabian 1953; Rogosa and others 1961). Considering that *L. plantarum* is the rate determining factor in cucumber fermentation, it is important that the study on nutritional requirements of the micro-flora in cucumber fermentation should be designed based on its nutritional requirements.

FACTORS AFFECTING LACTIC ACID BACTERIA GROWTH AND METABOLISM IN CUCUMBER FERMENTATION

According to Orla-Jensen (1942) the mode of sugar fermentation (homofermentation or heterofermentation), morphology (cocci or rods) and variation in the growth temperature and the degree of sugar utilization are the basis of LAB classification. The physiology of LAB has always been of interest as these bacteria are involved in the acidification of food. But LAB can change their metabolism to suit different conditions leading to different end-products. In general, LAB growth is primarily influenced by various factors such as pH of the media, fermentation temperature, media composition (sugars and nutrients) and their mode of fermentation (Etchells and others 1975; Hofvendahl and Hahn-Hägerdal 2000; Mussatto and others 2008).

pH

LAB are active over a wide pH range (Axelsson 2004). LAB maintain an alkaline cytoplasm when compared to its medium of growth. The ability to regulate the cytoplasmic or intracellular pH is one of the most important physiological characteristics of LAB which makes them acid tolerant when compared to numerous bacterial species (Hutkins and Nannen 1993; McDonalds and others 1990). The organic acids produced by acid tolerant LAB enter the cells of acid-non-tolerant microflora consequently collapsing the energy generated by the transfer of proton and electron across an energy- transducing membrane used for chemical or mechanical work in a cell. Collapsing this energy inhibits nutrient transport in the cell hence killing the microbes (Freese and others 1973; Hunter and Segel 1973).

The optimum internal pH for *L. plantarum* growth in cucumber fermentation is 6.0-6.5 (Li 2006). It stops multiplying at an internal pH 4.6 to 4.8 irrespective of the growth media

(McDonald and others 1990) which gives it an advantage over less acid tolerant microorganisms. *L. plantarum* maintains a pH gradient despite high lactic and acetic acid concentrations; which contributes to its ability to complete cucumber fermentation (McDonald and other 1990).

The buffering capacity of the vegetables affects the extent of multiplication of the predominant culture fermenting the substrate (Li 2006). In cucumber fermentation when the brine pH (*L. plantarum* external pH) declines to around 3.3 to 3.5, LAB fermentation ceases (Passos and others 1994). Preventing the external pH of the medium (brine in this study) from falling to 3.5 and maintaining a pH above 4.0 might extend the period of fermentation and increase the number of *L. plantarum* in the brine. Several methods have been adopted including using sodium acetate and calcium acetate as pH buffers (Fleming and others 1978) to assure complete fermentation of sugars in cucumber.

Fermentation Temperature

Cucumber fermentation is carried only in the warm months of March through September in the US, and it is important that all the fermentation is carried out when the brine temperature is around 16 to 30°C (Etchells and Jones 1946). Temperature for vegetable fermentation is optimized depending on the predominant culture growing during fermentation. *L. plantarum* predominant in cucumber fermentation rapidly grows at 18°C (Raccach 1982). The optimum temperature for cucumber fermentation is 24-30°C favoring rapid lactic acid production and increased rate of LAB growth. The curing and fermentation is retarded to a large extent at 7-10ºC (Pederson and Albury 1950).

Salt Concentration

Sodium chloride in cucumber fermentation is responsible for three important purposes; it acts as a preservative, enhances the flavor of the product and also maintains the firm texture of the fruit (Fleming and others 1987).

To prevent non-lactic fermentations and inhibit the growth of coliform bacteria (Etchells and others 1975) it is important to maintain a high concentration of sodium chloride in the brine, but high concentrations of salt (12-16% after equilibration) in the brine retards the production of lactic acid by suppressing LAB growth (Etchells and Jones 1946) and favors the growth of halophilic microorganisms similar to species in the genus Aerobacter (Etchells and others 1975). Therefore, Etchells and Hontz (1972) have suggested that lactic acid concentration should be at least 0.6% before increasing the brine strength for storage stability. Thus, concentrations of 5-10% after equilibration of salt in the brine are maintained commercially, which is favorable for LAB growth and inhibits *Enterobacter* (Etchells and others 1975).

Salt also suppresses the growth of microorganisms producing enzymes which act on the pectic substances and soften the pickles. To ensure desirable level of acid production, the salt strength at the early part of the fermentation should be controlled. Once fermentation is completed, salt concentration may be increased to 10-12% after equilibration for higher osmotic pressure to ensure product stability by preventing the growth of yeast and *Enterobacter* (Maki 2004).

Sugar

Lactic acid bacteria have complex nutritional requirements, including sugars, amino acids, peptides, fatty acids, salts, nucleic acid derivatives and vitamins (He`bert and others 2004). Glucose and fructose are the major sugars in the cucumber fruit; raffinose and stachyose, are the transport sugars present in small amounts (Hendrix 1982; McCombs and others 1976). Glucose and fructose are metabolized by LAB through the glycolytic cycle where one molecule of glucose or fructose liberates 2 molecules of lactic acid (Gunsalus and others 1955). Lu and others (2002) concluded that glucose is metabolized faster than fructose in cucumber fermentation, but glucose fermentation terminates before all of the glucose has been converted to lactic acid. However, fructose is completely fermented to lactic acid (Lu and Fleming 2001).

Cucumbers contain around 2-3% fermentable sugars (Handley and others 1983; McCombs and others 1976) and the metabolism of the glucose and fructose is regulated by different mechanisms of LAB in the transport step and the subsequent steps of glycolysis (Lu and Fleming 2001). In *L. plantarum*, glucose and fructose are transported and metabolized into the cells via the phosphoenolpyruvate (PEP)- dependent sugar phosphotransferase system (PTS) and glycolysis respectively to produce lactic acid (Thompson 1987). Homolactics metabolize glucose to pyruvate via the glycolytic pathway which is further reduced to lactic acid via lactate dehydrogenase. In contrast to homolactics, heterolactics produce lactic acid via phosphoketolase pathway (Li 2006). Carbon dioxide splits from the glucose molecule and the pentose splits into three carbon and two carbon fragments, which are reduced to lactic acid and ethanol respectively (Li 2006). Fructose is reduced to fructose-6-phosphate which is reduced to glucose-6-phosphate and then reduced to pyruvate via glycolysis. Pyruvate is reduced to lactic acid through the same pathway as followed in glucose metabolism. On the other hand, heterofermentative bacteria in

cucumber fermentation convert fructose to mannitol by mannitol dehydrogenase (Hammes and others 1991) which is then converted to lactate, ethanol, acetic acid and carbon dioxide (Li 2006).

Costilow and Fabian (1953a) indicated that supplementing the brine with sugar increased the number of LAB without any significant increase in lactic acid. Presence of cations and anions in the fermentation media affects the extent of sugar utilization by LAB. Also addition of organic acids increases fructose utilization and has no effect on the glucose conversion to acids. Similarly Mn^{2+} significantly increased both glucose and fructose utilization; however nitrates inhibited the growth of starter culture (Lu and others 2002).

Lactic acid and residual sugar after fermentation can serve as substrates for yeast growth resulting in carbon dioxide production, which leads to bloater formation, resulting in texture defects and economic losses to the manufacturer (Li 2006; Fleming and others 1995). Growth of spoilage micro-organisms is another problem faced by pickle producers due to incomplete conversion of fermentable sugars to lactic acid (Daeschel and others 1988).

Influence of Nutrients on Lactic acid Bacteria

Lactic acid bacteria require multiple micronutrients including amino acids (Costilow and Fabian 1953a,b and d; Morishita and others 1981), minerals (Weymarn and others in 2002) and vitamins (Costilow and others 1953a,b). Cucumber fruit contains certain vitamins and minerals essential for LAB growth in required concentrations. The nutritional value of cucumbers based on USDA (2010) reports is given in Appendix: Table 1. Micronutrients in which cucumber is deficient could be supplemented in the brine to improve LAB activity.

Amino Acids

LAB can metabolize all amino acids, but the ability to synthesize protein greatly varies among different LAB species (Williams and others 2001; Liu and others 2003). Leucine (Leu), isoleucine (Ile), valine (Val), glutamic acid (Glu), cystine (Cys), and tryptophan (Trp) are the essential amino acids for *L. plantarum* growth (Costilow and Fabian 1953b, c; Saguir and Nadra 2007; Meretro and others 1998). These essential nutrients are not present in cucumber juice in sufficient concentrations and need to be supplemented (Appendix: Table1). According to a study conducted by Saguir and Nadra (2007), no growth of *L. plantarum* was observed in the absence of Ile, Val and lowering the concentration of Leu decreased its growth by 85%. Trp is also among the essential amino acids for *L. plantarum* growth, but in the presence of high yeast activity an increase in the rate of lowering Trp concentration from the brine was observed (Costilow and Fabian 1953c). The yeast could have been destroying or utilizing it making it unavailable for *L. plantarum* (Costilow and Fabian 1953c). Reduction in Trp concentration was critical for the growth of *L. plantarum*, further reducing the lactic acid levels in the brine (Costilow and Fabianc). Therefore as Trp is important for *L. plantarum* growth and not present in required concentration it needs to be supplemented in the brine.

Studies conducted to determine the role of threonine (Thr), Cys and Glu in *L. plantarum* growth concluded that *L. plantarum* did not require Thr (Lyman and others 1947; Stokes and Gunness 1943). However Cys was utilized by *L. plantarum*, but only within the first three days of fermentation (Costilow and Fabian 1953c, d). Similarly Glu was also required for *L. plantarum* growth in small concentrations; but its addition favored coliform growth (Ragheb and Fabian 1956), which is undesirable in cucumber fermentation. Hence in my research Thr, Cys and Glu were not used as nutrient supplements.

Vitamins

Peterson and Peterson (1945) reported that the major micronutrients promoting the growth of about 50 microorganisms including *L. plantarum* were biotin, nicotinic acid, pantothenic acid and riboflavin. Pantothenic acid (Hills 1943), nicotinic acid (Dorfman and others 1939) and riboflavin (Klein and Kamin 1942; Snell and Strong 1939) increased respiration of microbial cells by increasing metabolism of substrates like glucose, lactate and pyruvate.

Costilow and Fabian (1953a, d) have shown that *L. plantarum* required biotin, niacin, and pantothenic acid for enhanced acid production during cucumber fermentation. Nicotinic acid and pantothenic acid were indispensible for the growth of all species of LAB (Kashket, 1987). Rogosa and others (1961) studied the nutritional requirements of oral isolates of LAB and recognized two types of nutritional requirements; one required nicotinic acid and pantothenic acid and the second type required nicotinic acid and riboflavin, although the bacterial growth was subdued in the absence of pantothenic acid. The second type of strains was further divided based on their fermentation reactions and the strains requiring nicotinic acid and pantothenic acid had a standardized fermentation reaction. It was determined that the second type was identical to *L. plantarum* in their fermentation reaction (Rogosa and others 1961).

Pantothenic acid is present in sufficient amounts in cucumber for LAB growth (Rosen and Fabian 1953) and *L. plantarum* significantly decreased the concentration of pantothenic acid synthetically added to the brine (Costilow and Fabian 1953c, d). Even when small concentrations of pantothenic acid were added to the cucumber brine, LAB growth increased qualitatively and quantitatively (Snell 1946). Pantothenic acid played a major role as growth enhancer in rumen strains of LAB where growth was limited without added vitamin B (Ford and others 1958).

Studies conducted earlier conveyed that nicotinic acid (niacin) greatly stimulated the growth of acid producing species of LAB and was essential for their growth (Snell and others 1939; Snell and Wright LD 1941; Snell 1946). Nicotinic acid was labeled essential for twenty-two organisms and stimulated the growth of four others (Russell and others 1954). Kligler and others (1943 a,b) reported that in order for LAB to metabolize glucose it was essential to have nicotinic acid in the medium.

Biotin exists in two forms whereby β-biotin is twice as active as α-biotin (Peterson and Peterson 1945, Rosen and Fabian 1953). Biotin is another essential vitamin for LAB growth; it is present in abundance in fermentation brine within 12hrs of brining along with nicotinic acid (Rosen and Fabian 1953). In a study conducted by Rosen and Fabian (1953) when *L. plantarum* was inoculated in a synthetic media supplemented with biotin, the biotin content in the media was drastically reduced and a significant increase in the growth rate was observed in comparison with the control, which indicated that biotin was essential for of *L. plantarum* growth. But when a similar study was conducted using cucumber juice supplemented with biotin as the media for *L. plantarum,* its growth rate in cucumber juice was similar to that in a synthetic media supplemented with biotin. However, there was very small change in the biotin concentration of the cucumber juice, indicating that certain components in cucumber juice have similar biological activity as biotin, which prevented biotin from being depleted (Costilow and Fabian 1953c, d).

Snell and Strong (1939) reported that several species of LAB require riboflavin for growth. Riboflavin is an essential component of cell membrane metabolism as it is the precursor of the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), it carries hydrogen in biological redox reactions (Capozzi and others 2011). Riboflavin when added as a growth supplement stimulated LAB growth and improved the rate of acid production (Snell 1946).

All the strains of *L. casei* require folic acid and pyridoxal for their growth whereas *L. plantarum* grows well in their absence (Rogosa and others 1961; Costilow and others 1956). Shankman and others (1947) reported that folic acid was not essential for twenty-three species of LAB. Hence, folic acid and pyridoxal will not be included in this study.

Minerals

Minerals needed for LAB growth have been studied using a mixture containing ions of potassium, sodium, magnesium, manganese, iron, chlorine and phosphate. Of these magnesium and manganese stimulated LAB growth (MacLeod and Snell 1947). Manganese and magnesium ions function as essential co-factors for enzymes which aid primary sugar metabolism of LAB, more importantly for the transport and reduction of fructose (Weymarn and others 2002).

Magnesium is required LAB and it is essential for the growth of *L. casei, L. lactis, L. delbrueckii, L. helveticus* and *L. acidophilus* (Macleod and Snell 1947, Boyaval 1989; Weymarn and others 2002; Lu and others 2002). It acts as the cofactor in many enzyme involving transport and metabolic processes such as fructokinase, phosphoketolase and acetate kinase (Raccach 1985; Weymarn and others 2002). According to Weymarn and others (2002) sulfates from the salt $MgSO_4.7H_2O$ aid catabolism and act as essential co-factors for enzymes in the primary sugar metabolism. Activation of LAB strains which require Mg^{2+} can also be accomplished by Mn^{2+} (Nilsson and others 1942), but some enzyme reactions associated with growth and metabolism cannot take place when Mg^{2+} is substituted by Mn^{2+} (Boyaval 1989).

In a study conducted by Woolley (1941) using several metallic salts, it was determined that Mn^{2+} was the most effective cation in stimulating the growth of LAB. Mn^{2+} can act as a cofactor for several important functions including sugar transport and metabolic processes; it aids

enzymes in the pathway from glyceraldehydes-3-P to pyruvate and lactate dehydrogenase (Raccach 1985; Weymarn 2002), it acts as a detoxifying agent and stabilizer for sub-cellular entities. It specifically displays defense mechanism against oxygen toxicity for *L. plantarum* which are deficient of the enzyme superoxide dismutase; and provides protection from OH^- damage and H_2O_2 toxicity (Archibald and Fridovich 1981a, b; Archibald 1986). LAB can accumulate large concentrations of intracellular Mg^{2+} and Mn^{2+} cations (Lu and others 2002). Mn^{2+} cation significantly increased the utilization of sugar by LAB which increased the rate on fermentation (Lu and others 2001); however high Mn^{2+} concentrations of 120 mM or higher displayed an inhibitory effect on the utilization of the sugars, fructose and glucose by LAB (Lu and others 2002).

OBJECTIVES

Many researchers have demonstrated the growth of *L. plantarum* in the presence of amino acids, vitamins and minerals as nutrient supplements. However very little is known regarding supplementing the cucumber juice fermentation brine with micronutrients and their effect on LAB growth. Basis for the selection of the nutrients to be tested for their effect on LAB growth were:

a. Studies conducted in the past to determine the effect on LAB growth and percentage acid produced by supplementing certain nutrients in different growth substrates.
b. Concentration of certain nutrients in cucumber juice.
c. Providing nutrients in required concentrations based on the composition of MRS basal agar which is a selective media for LAB growth.

Specific objectives of the study were:

1. Identification of certain amino acids, vitamins and minerals that enhance lactic acid bacteria fermentation of cucumber juice: Determine the effectiveness of Leu, Ile, Trp, Val, biotin, nicotinic acid, pantothenic acid, riboflavin, manganese and magnesium in enhancing lactic acid fermentation of cucumber juice.
    - Determine the nutrient supplements which effectively increase the rate of lactic acid bacterial fermentation in cucumber juice.
    - Evaluate the yield of lactic acid in the brine during cucumber juice fermentation.
    - Screen the nutrients which effectively enhance the yield of LAB growing in cucumber juice fermentation.

Based on the results from objective 1; nutrients which proved to be effective in increasing the number of LAB taking part in the fermentation and the concentration of lactic acid were selected and used in objective 2.

2. Further examination of concentrations of biotin, Ile and Val that enhance lactic acid bacteria fermentation of cucumber juice.
    - Determine the lowest and the most favorable concentration of screened nutrient supplements which effectively increase lactic acid and LAB yield in cucumber juice fermentation.
    - Evaluate the lowest concentration that effectively increases the rate of fermentation.

Based on the results from objective 2 the most effective concentrations of biotin, Ile and Val were used in objective 3

3. Determine the effect of biotin, Ile and valine alone and in combinations on lactic acid bacteria fermentation of cucumber juice.
    - Evaluate the effect of adding two or more nutrients to the brine in their most effective concentration in enhancing the rate of fermentation and the yield of lactic acid and LAB.

CHAPTER II

METHODS AND MATERIALS

PREPARATION OF FERMENTATION STOCK SOLUTION

Sterilized Cucumber Juice

Cucumbers were obtained from local pickle industries; they were sorted for absence of decay and mechanical damage and homogenized using a blender (Waring commercial blender, Kent city, MI). The homogenate was strained through Miracloth (EMD Biosciences, Inc. La Jolla, CA) and sterilized at 121^{o}C for 15 minutes in an autoclave (Amsco 8816A, Erie, PA). The sterilized cucumber juice was stored at 7^{o}C until used for fermentation. Immediately before using the juice, residues which separated out from the juice after sterilizing were discarded and the clear juice was mixed with stock fermentation brine in equal portions for the treatments.

Stock Fermentation Brine

Composition of stock fermentation brine was 12% (±0.5%) sodium chloride, 0.1% potassium sorbate and 0.4% acetic acid in water. Potassium sorbate was added to prevent surface yeast and mold growth (Gates and Costilow 1981), similarly acetic acid was added to retard the growth of coliform bacteria (Etchells and others 1964). The 1:1 mixture of the stock brine and cucumber juice established the fermentation brine for testing the effects of selected nutrients.

TREATMENTS

Five fermentation trials were conducted as shown in table 2.1. In each trial, cucumber juice and nutrient (amino acids, vitamins and minerals) supplemented brine formulation in glass jars were randomly assigned to each treatment group. Each treatment was in replications of five. MRS basal agar is a selective medium which supports the good growth of *Lactobacilli* (de Man and others 1960). Concentrations of selected nutrients used in the brines for the first three trials were decided based on the nutrient concentrations in de Man, Rogosa and Sharpe (MRS) basal agar (Appendix: Table 1). First concentration for each nutrient treatment in trial I, II and III were the same concentration as in the basal agar and second and third concentrations were half and double the first concentration respectively. Trials I, II and III were conducted to determine the efficacy of the nutrients in objective 1 in increasing the yield of lactic acid and the number of LAB growing during fermentation. Treatments in the following trials included:

Trial-I: A control and Leu (Alfa Aesar, Meysham, Lancs), Ile (Alfa Aesar, Meysham, Lancs), Trp (Fisher Scientific Company, Fair Lawn, NJ) or Val (Calbiochem, San Diego, CA) added to the brine in three concentrations (Table 2.1).

Trial-II: A control and biotin (Calbiochem, San Diego, CA), pantothenic acid (ICN Pharmaceuticals, Inc. Cleveland, OH), riboflavin (Eastman Kodak Company, Rochester, NY) or nicotinic acid (ICN Pharmaceuticals, Inc. Cleveland, OH) added to the brine in three concentrations (Table 2.1).

Trial-III: A control and magnesium ($MgSO_4$, Fisher Scientific Company, Fair Lawn, NJ) or manganese ($MnSO_4.H_2O$, Fisher Scientific Company, Fair Lawn, NJ) added to the brine in three concentrations (Table 2.1).

TRIAL IV and V: Trial IV was conducted to accomplish objective 2. In trial IV nutrient treatments; biotin, Ile and Val which significantly increased the lactic acid yield and LAB count during fermentation in objective 1 (trial I, II and III) were used in five different concentrations. All three biotin and Ile treatments were significantly different from the control but comparable to each other hence two more concentrations; one above the highest and one below the lowest of the concentrations from objective 1 were added to the treatments in trial IV. Whereas Val treatments followed a different trend; increase in the concentration of Val in the treatments increased the number of LAB. Therefore two higher concentrations were added to the existing concentrations in trial IV to determine the lowest but the most effective concentration of the nutrients. The most effective nutrient concentrations of biotin, Ile and Val from objective 2 (trial IV) were used in combinations in trial V which was conducted to determine the results of objective 3.

Stock brine treatments were with nutrients as shown in Table 2.1.1. Val, Ile, biotin, pantothenic acid, nicotinic acid, riboflavin and salts of manganese and magnesium were dissolved in the stock fermentation brine. Leu and Trp had poor water solubility; therefore, stock fermentation was made separately for these treatments. Leu and Trp were dissolved in 100 ml of hot water at 70ºC; this solution was made up to 1000 ml with water. Nutrient dissolved water was used in place of normal tap water while making the stock fermentation brine for these nutrients with 12% sodium chloride, 0.1% potassium and 0.4% acetic acid. Sterilized cucumber juice (100 ml) and treatment fermentation brines (100 ml) were mixed together in 200 ml glass jars for each treatment sample. A separate set of treatments with 100 ml were also placed for fermentation which prepared for determining LAB yield based on dry weight.

Table 2.1: Trials consisting of treatments of fermentation brine with different concentrations of selected amino acids, vitamins, minerals and nutrients for influencing the growth of lactic acid bacteria

	Nutrients	Units	Concentrations
Trial-I	**Amino acid screening**		
	Leu	mM	0, 0.38, 0.76, 1.14
	Ile	mM	0, 0.38, 0.76, 1.14
	Trp	mM	0, 0.25, 0.49, 0.75
	Val	mM	0, 0.43, 0.85, 1.28
Trial-II	**Vitamin screening**		
	Biotin	nM	0, 205, 409, 614
	Nicotinic acid	µM	0, 3, 5, 8
	Pantothenic acid	µM	0, 4, 8, 12
	Riboflavin	µM	0, 2, 4, 6
Trial-III	**Mineral screening**		
	Magnesium	µM	0, 127, 253, 380
	Manganese	µM	0, 415, 830, 1245
Trial-IV	**Effective nutrients from Objective 1, 2, 3**		
	Bio	nM	0, 103, 205, 409, 614, 819
	Ile	mM	0, 0.19, 0.38, 0.76, 1.14, 1.52
	Val	mM	0, 0.43, 0.85, 1.28, 1.71, 2.56
Trial-V	**Effective nutrients in combination**		
	Bio	nM	614
	Ile	mM	0.76
	Val	mM	1.71
	Bio + Val	mM	614 + 1.71
	Bio + Ile	mM	614 + 0.76
	Val + Ile	mM	1.71 + 0.76
	Bio + Val + Ile	nM + mM + mM	614 + 1.71 + 0.76

CULTURE INOCULATION AND FERMENTATION

Inoculum for fermentation trials were developed from active fermentation by LAB associated with cucumber. Whole cucumbers were placed in equal volume of fermentation brine containing 12% sodium chloride, 0.7% calcium chloride, 0.1% potassium sorbate, 0.2% acetic acid and 0.02% $CaNa_2EDTA$, for fermentation at 25±0.5°C. Live LAB count in the whole cucumber fermentation was monitored using microscopic counting and confirmed using MRS agar plate counts during the fermentation. When the live LAB count was around 10^8 cells/ml the fermentation brine was used as an inoculum for the treatment brines. The active fermentation brine (0.1 ml) was diluted to 100 ml with deionized water. 200 ml of the treatment samples were inoculated with 1 ml of the diluted active fermentation brine and then placed at 25-27^{o}C for fermentation. The cell count was diluted to around 10^5 cells/200 ml of fermentation brine.

CHEMICAL ASSAYS

pH Assay

According to the research conducted on the brine conditions unfavorable for LAB growth in vegetable fermentation, when the brine pH (LAB external pH) falls to 3.3 to 3.5, the cucumber fermentation is completely terminated (Passos and others 1994; Etchells and others 1975; McDonald and others 1990). Therefore, it was important to maintain the external pH at 4.00 to prevent the ceasing of *L. plantarum* growth. pH was measured every other day using Barnant-30 digital pH meter with a VWR Symphony gel filled, combination pH electrode (Symphony SP7OP). When the sample pH was below pH 4.0±0.2 it was adjusted using 1 N NaOH. Sometimes for small (around pH 0.1), pH adjustment was made with 0.1 N NaOH. The amount

of NaOH added to the samples was recorded and taken into account when calculating the percent lactic acid in the samples.

Lactic Acid Assay

Alkaline Titration

Lactic acid was assayed using titration method and verified using HPLC. In the titration method 10 ml of the treatment samples was brought to 50 ml using the deionized water. The sample was then titrated with 0.1 N NaOH using phenolphthalein as indicator, to first appearance of pink color (Lees 1971). Concentration of lactic acid was calculated as below:

$$\% \text{ Lactic acid} = \frac{(\text{ml NaOH}) \times (\text{Normality NaOH}) \times (\text{Molecular wt. Lactic acid}) \times (100)}{\text{ml of sample} \times 1000}$$

$$\% \text{ Lactic acid} = \frac{(\text{ml NaOH}) \times (0.1) \times (90.2\text{g / mole}) \times (100)}{10 \times 1000}$$

HPLC

Lactic acid concentration was verified weekly using the Waters Liquid Chromatography (LC) Module I (Millipore Corporation, Waters Chromatography division). The LC system consisted of a Waters 600E pump, an autoinjector Waters 715 with 10 µl sample loop injection valve, and C18 'Econosphere' column (300 x 3.9 mm I.D., 10 µm) from Alltech (Deerfield, IL). The column contained 80A pore. Waters 486 UV absorbance detector was operated at 224 nm. A steady-state mobile phase was used which comprised of acetonitrile-water (70:30). The mobile phase was filtered through a 0.45 µM syringe filter (Alltech Associates, Inc. Deerfield, IL). The column flow rate was maintained at 20.0 ml / min. The response was recorded in SRI model 202 (four channel serial port) chromatography data system.

Preparation of Standard Curve

Lactic acid concentrations of 2, 4, 8 16 and 32 mM were established in the fermentation stock brine for standard curves using DL-lactic acid 99% purity (Sigma Chemical Co, St. Louis, MO). Concentrations of lactic acid in millimoles were plotted versus peak areas of DL-lactic acid. The standard curve with correlation coefficient around $R^2 = 0.998$ was established. The equation of this line was used for the quantification of unknown samples.

Sodium Chloride Assay

Sodium chloride was assayed by modified titration method of Sigma Technical Bulletin No.830, in which 1ml of brine sample was mixed with 10 ml of deionized water, 4 drops of 66% sulphuric acid and two drops of diphenylcarbazone and then titrated with 0.141N $Hg(NO_3)_2$ to first appearance of a slight, but permanent violet color. The concentration of NaCl was calculated as follows:

$$\% \text{ NaCl} = \text{ml } Hg(NO_3)_2 \times \frac{\text{Normality } Hg(NO_3)_2 \times 58.44}{1000} \times 100$$

Reducing Sugar Assay

Reducing sugars in the fermentation brines were determined by the Nelson-Somogyi method (Nelson 1944; Somogyi 1952) and expressed as µmoles glucose per ml reaction mixture. For every fermentation trial, a standard curve was developed using fermentation stock brine with 12% NaCl spiked with concentrations of 27.75 mM, 13.88 mM, 6.94 mM, 3.47 mM, 1.74 mM and 0.87 mM of D-(+)-glucose (Sigma Chemical Co. St. Louis, MO).

A 0.4 ml sample was added with equal volume of Nelson's alkaline copper reagent in a glass tube (12.5 ml Nelson's reagent A: 12.5 g anhydrous $NaCO_3$, 12.5 g potassium tartrate, 10 g

$NaHCO_3$ and 100g Na_2SO_4 made up to 500 ml with H_2O; mixed with 0.5 ml Nelson's reagent B: 7.5g $CuSO_4.5H_2O$ dissolved in 50 ml H_2O with 1 drop of H_2SO_4 added). The contents in the glass tubes were mixed well and placed in boiling water bath for 20 min. After boiling the tubes were removed from the water bath and cooled. 0.4 ml of Arsenomolybdate reagent (Arsenomolybdate reagent: 25g $(NH_4)_6Mo_7O_{24}.4H_2O$ dissolved in 450 ml H_2O and added with 21 ml conc H_2SO_4 producing acid molybdate which mixed with 3 g of $Na_2HAsO.7H_2O$ dissolved in 25 ml H_2O. It was stored at 37º C for 24 hrs before use) was added and mixed for five minutes to dissolve Cu_2O and to reduce the arsenomolybdate. The volume was brought to 10 ml with distilled water and absorbancy was read at 520 nm using Varian CARY 50 Bio UV-Visible spectrophotometer.

MICROBIOLOGICAL ASSAYS

Quantifying Live And Dead Lactic Acid Bacteria

Microbial numbers were determined every second day throughout the fermentation period and until the live LAB declined to 10^3 cells/ml less. LAB were enumerated by microscopic and plating techniques. Each treatment was assayed in triplicate by each method of analysis.

Sample Preparation

The dead LAB which had settled at the bottom and the walls of the container were introduced back into the brine for even distribution of the live and dead LAB. The distribution was carried on by vortexing the glass jars they were fermented. One ml of brine samples was diluted with 9 ml of PBS (phosphate buffered saline: 8 g NaCl, 0.2 g KCl, 0.24 g KH_2PO_4 and

1.44 g Na_2HPO_4 in 1 L of deionized water). Samples were diluted with 10X serial dilutions of PBS until the LAB count was within 25 to 200 in number.

Microscopic Technique

One ml of the sample diluted with 10X serial dilution of PBS was mixed with 100 µl of 10% Tween 20 and vortexed for 3 hrs to separate clusters of the bacteria. The vortexed sample was stained with one drop of Trypan Blue (Sigma Chemicals Co, St. Louis, MO), a vital stain which selectively colors dead tissues and cells blue. After staining, the dead bacteria were completely blue in color and the live bacteria had a blue outline with transparent white cell body. Samples were placed onto a Spencer Bright-Line hemacytometer (American Optical Corporation, Buffalo, NY) and viewed under a compound microscope (Model 06.16.62, 115 volts, 60 cycles, 15 watts, American Optical Co, Buffalo, NY) with 45X magnification. The hemacytometer consisted of nine 1 mm squares divided into smaller squares. Cells were counted in four corner squares and the count was averaged. Each 1 mm square represented a volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1cm^3 is equivalent to 1 ml, the number of cells per ml was determined using the formula:

Cells / ml = (The average count per square) x (The dilution factor) x 10^4

Plating Technique

To verify reliability of the microscopic technique, weekly plate counts of LAB were determined. MRS agar plates (Difco Lactobacilli MRS agar, Detroit, MI) were inoculated by spread plate method; every plate was divided into two parts and two 0.1 ml of PBS dilutions of every brine sample were spread onto MRS plates in triplicate and incubated in incubator (Fisher

Scientific Isotemp 655D, Phoenix, AZ) at 35 to 37° C for 48 hrs under aerobic conditions followed by colony counting in CFU / ml.

LAB Dry Weight Assay

After fermentation the samples especially prepared for measuring LAB dry weight yield were centrifuged at 10,000 rpm for 20 mins in pre-weighted tubes (Beckman Coulter) and the residue was washed with deionized water and re-centrifuged three times. Residues were dried at 50^{o} C for 12 hrs and weighed to determine the weight of the residual LAB. The difference in the weight of the residue from the fermented treatments and unfermented samples determined the net dry mass of LAB produced in the fermentation.

STATISTICAL ANALYSIS

All treatments were performed with five replications. Lactic acid concentrations were modeled as a quadratic function of time allowing the regression coefficients to differ by nutrient type and concentration. Lactic acid concentrations and viable LAB counts on several days throughout the experiment and reducing sugar and total bacterial weight on the final day of the experiment were analyzed as a two factor factorial (nutrient type x concentration) analysis of variance. Significant differences among the means were determined using a protected least significant difference (LSD).

The logarithms of the viable LAB were modeled as a function of time allowing the regression coefficient to depend on nutrient types and concentration. The function consisted of two segments, the first of which was a quadratic function of time and the second was a quadratic or a horizontal line, depending on the nutrient and concentration. The estimated maximum log units were determined from the fitted model.

All statistical analyses were carried out using SAS®, version 9.2. A significance level of 5% was used in all analysis.

CHAPTER III

RESULTS

IDENTIFICATION OF CERTAIN AMINO ACIDS, VITAMINS AND MINERALS THAT ENHANCE LACTIC ACID BACTERIA FERMENTATION OF CUCUMBER JUICE:

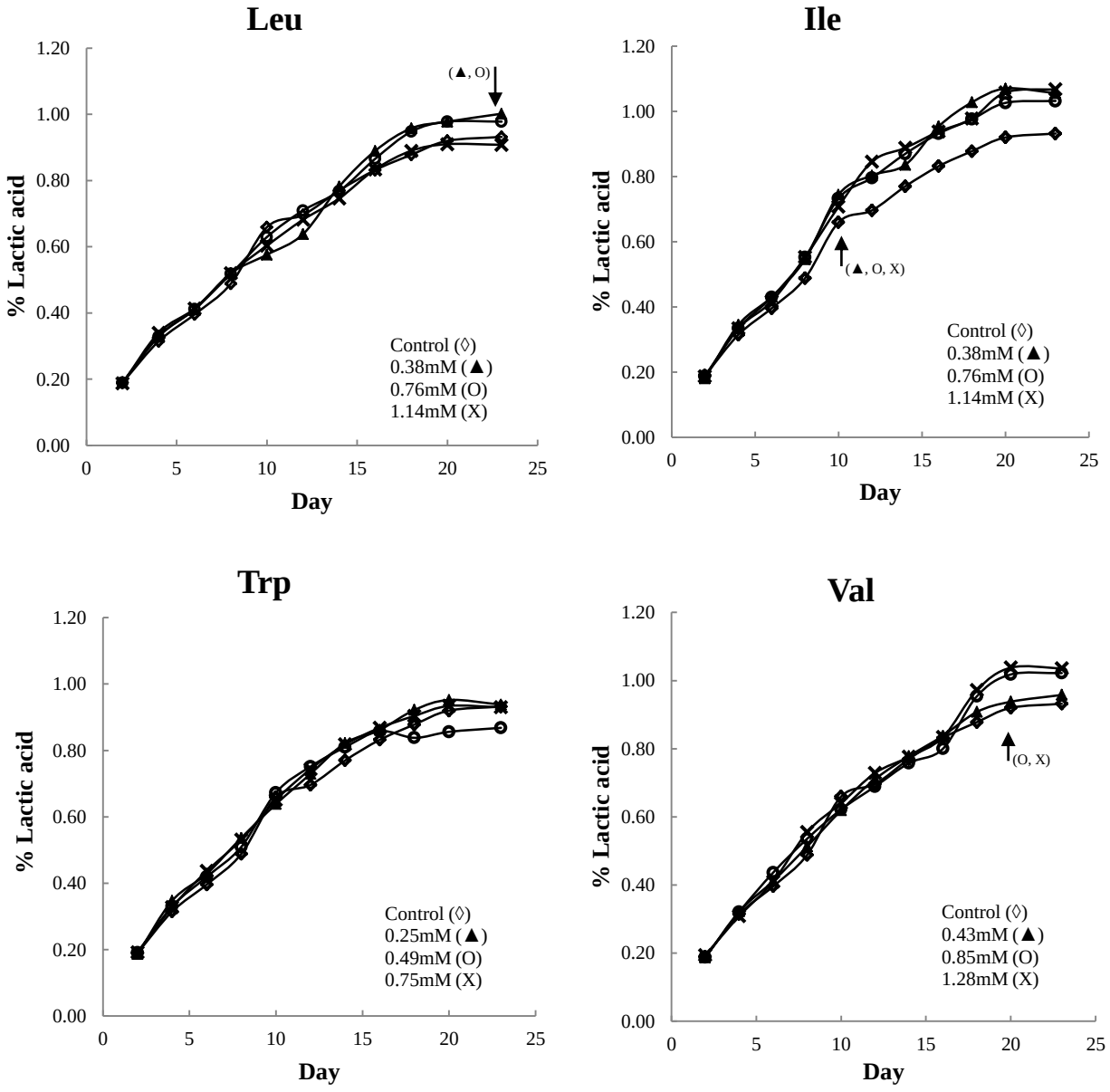
AMINO ACIDS

PRODUCTION OF LACTIC ACID

Enhanced production of lactic acid as influenced by supplementation of cucumber juice with Leu, Ile, Trp and Val was tested. Lactic acid concentrations of treatment brines during fermentation were plotted and the results are illustrated in Figure 3.1. The results revealed that except for Trp treatments all other amino acid treatments were effective in increasing the lactic acid concentration in the brines. Among the effective treatments, addition of 0.38 mM and 0.76 mM Leu in the treatment brines effectively raised the final lactic acid concentration by 7.3% (Figure 3.1 Leu). Lactic acid concentrations of the brines containing 0.38 mM 0.76 mM and 1.14 mM of Ile were higher than the control from day 11 onwards and it continued to rise until fermentation was complete (Figure 3.1 Ile). Final lactic acid concentration of Ile treatment brines was 13.2% higher than the control which was significantly different ($P<0.05$). Lactic acid levels in the Val treatments brine were similar to the control until day 18. From day 20 onwards acid levels in treatments of 0.85 mM and 1.28 mM Val were higher compared to the control and a 10% higher concentration of lactic acid was observed on day 23 (final day of fermentation) (Figure 3.1 Val).

Lactic acid concentration in the treatments during fermentation was also measured using HPLC on day 2, 8, 16 and 23. These concentrations were compared with the ones determined using titration method to confirm the data collected using titration method. Lactic acid levels reported from the titrations on day 2 and 23 were 3.1% lower than the concentrations determined using HPLC. The difference in the reported lactic acid levels determined using the two methods was not significant ($P>0.05$) (SEM: ± 0.05).

Figure 3.1: Effect of leucine, isoleucine, tryptophan and valine on lactic acid concentration determined throughout cucumber juice fermentation



Note:

- Leu- leucine, Ile- isoleucine, Trp-tryptophan and Val- valine
- Arrow indicates the day when the lactic acid concentration in the treatment brines was significantly different from the control as estimated using LSD ($P<0.05$).
- Symbols of the treatment concentrations which significantly differ from the control are indicated next to the arrow.

DEPLETION OF REDUCING SUGARS

Amount of sugars utilized in the amino acids supplemented treatments was determined by comparing the initial and final sugar concentration in the fermentation brine (Table 3.1). Treatments of 0.38 mM Ile and 0.25 mM and 0.75 mM Trp had significantly lower ($P<0.05$) concentrations of residual sugars in comparison to the control and other treatments.

Table 3.1: Concentration of reducing sugar after complete fermentation in the treatments supplemented with leucine, isoleucine, tryptophan and valine

Leu (mM)	RG* (mM)	Ile (mM)	RG* (mM)	Trp (mM)	RG* (mM)	Val (mM)	RG* (mM)
0	2.15 ^{a}	0	2.15^{a}	0	2.15^{a}	0	2.15^{a}
0.38	1.98^{ab}	0.38	1.54^{c}	0.25	1.57^{c}	0.43	2.13^{a}
0.76	1.77^{bc}	0.76	1.73^{b}	0.49	1.89^{b}	0.85	2.03^{a}
1.14	2.04^{a}	1.14	1.81^{b}	0.75	1.85^{bc}	1.28	1.83^{b}

Note:

- Leu- leucine, Ile- isoleucine, Trp-tryptophan and Val- valine
- RG* - Residual glucose
- Means with no common letters differ significantly from control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data is ±0.06.
- The concentration of reducing sugars in unfermented cucumber juice and brine mixture before inoculation was 26.72 mM.

LACTIC ACID BACTERIA PRODUCTION

NUMBER OF LIVE, DEAD AND TOTAL CELLS

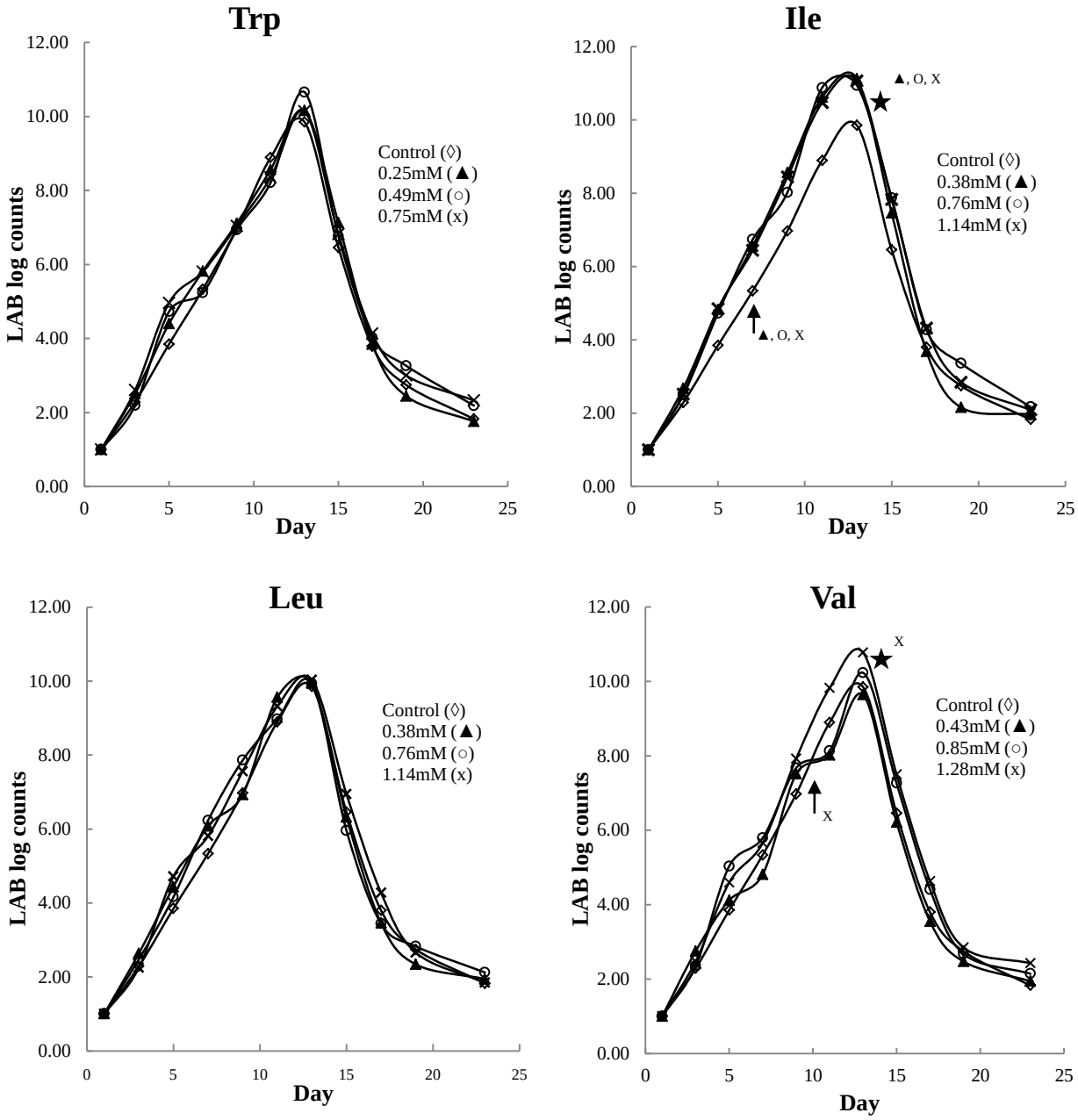
The effect of adding Leu, Ile, Trp and Val individually to the brine on the viable (live) cell count of LAB during cucumber juice fermentation was estimated using a microscope and is shown in Figure 3.2. All the treatments of Ile and a few Val treatments were effective in increasing the number of LAB. Ile treatments promoted growth of the fermenting organisms; this

action became evident considerably before maximum population of LAB was achieved. A minimum of two log units' difference in the viable LAB count was observed between the Ile treatments and the control all through the log and stationary phase of LAB growth cycle (Figure 3.2 Ile). Ile treatments showed highest log cfu/ml of LAB. A rise of 9.6% in the maximum viable LAB cell count in the Val treatment of 1.28 mM was observed, which was significantly higher ($P<0.05$) than the maximum viable cell count in the control (Figure 3.2 Val). In the death phase viable LAB units in Val treatments and the control were comparable. Trp and Leu were less active in increasing the number of viable LAB cells in the fermentation brine. Live LAB count in these treatments was not significantly different ($P>0.05$) from the control.

Along with the viable cell count of LAB, non-viable (dead) cells were also counted using the microscopic technique. The viable and non-viable cell counts together were considered as the total number of cells at different stages of fermentation. Total number of cells which were part of the cucumber juice fermentation is illustrated in Figure 3.3. Considering that Ile was effective in increasing the viable LAB cell count it is evident that the total LAB count was also significantly higher ($P<0.05$) than the control. Total LAB count in these treatments was around 14.20 log units/ml which is 9.3% higher than the control. Val treatment at 1.28 mM also promoted LAB growth; the total number of LAB cells in this treatment at the end of fermentation were 13.88 log units/ml. Trp in the fermentation brine was slightly effective in promoting LAB growth which increased the viable cell count in the treatments, but the rise was not significant. However the small increase in the number of viable cells at every stage of fermentation summed significantly increased ($P<0.05$) the total number of cells at the end of fermentation in these treatments. Unlike in Trp treatments; no change in the total LAB counts of the Leu treatments was observed in comparison to the control.

Although the viable LAB cells were counted using microscopic technique, the confirmation of the results from this technique was done using plating technique. Samples were plated on day 3, 9, 17 and 23 and the counts were compared with the viable LAB counts from the microscopic technique. The difference in the LAB count from the two techniques was around 3.1% which was significantly low ($P<0.05$), confirming the result from microscopic technique with standard error of the mean (± 0.08).

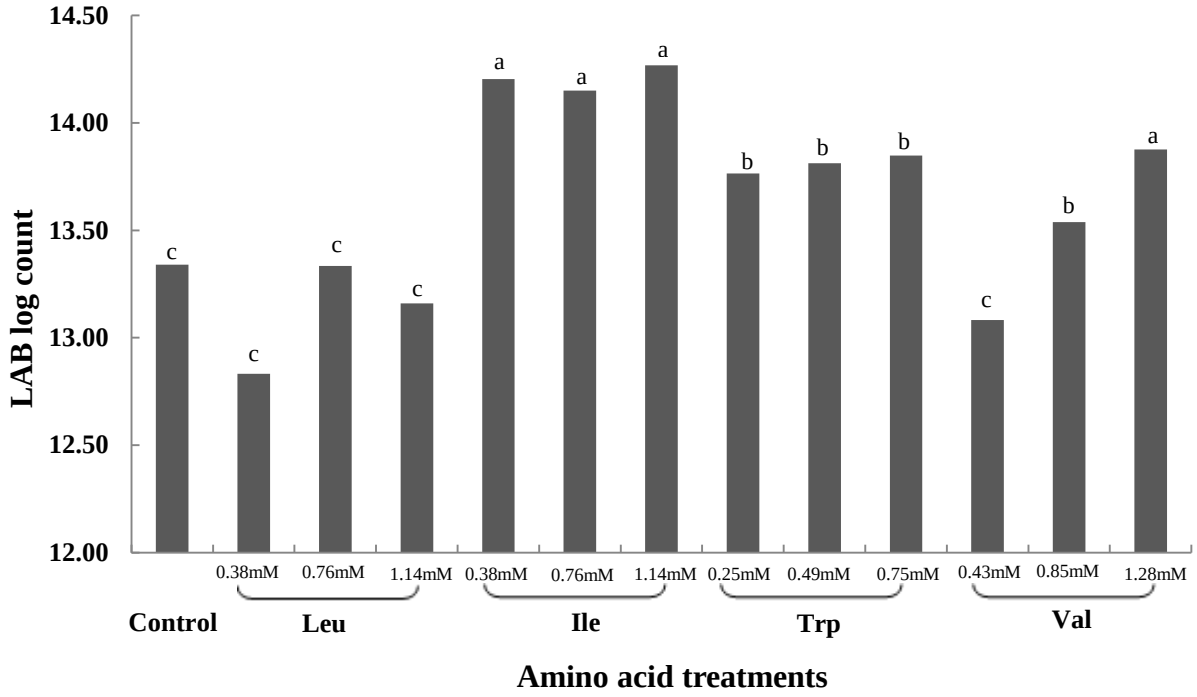
Figure 3.2: Effect of leucine, isoleucine, tryptophan and valine on the live lactic acid bacteria microscopic count determined throughout cucumber juice fermentation



Note:

- Leu- leucine, Ile- isoleucine, Trp-tryptophan and Val- valine
- Arrow indicates the day when the live bacterial count in the treatment brines was significantly different from the control as estimated using LSD ($P<0.05$).
- Star indicates the day when the live bacterial count among the treatments was not significantly different as estimated using LSD ($P>0.05$).
- Symbols of the treatment concentrations which significantly differ from the control are indicated next to the arrow and the star.

Figure 3.3: Effect of leucine, isoleucine, tryptophan and valine on the total number of lactic acid bacteria in fermented cucumber juice on the last day of fermentation



Note:

- Leu- leucine, Ile- isoleucine, Trp-tryptophan and Val- valine
- The standard error of the means for this data was ± 0.01.
- Bars with no common letter differ significantly from the control as estimated using LSD ($P<0.05$)

LAB DRY WEIGHT

Dry weight of the total LAB taking part in the fermentation was also measured to confirm the effect of the selected amino acids on the yield of these microorganisms. The results from this assay are reported in Table 3.2. As expected Ile treatments of 0.38 mM and 0.76 mM stood out from the rest of the treatments with the highest dry weight of LAB which were significantly ($P>0.05$) higher than the control. However same was not the case with the Val treatment of 1.28 mM which promoted LAB number; but the value was comparable to the control. Another observation was the significantly low ($P<0.05$) dry weights of LAB in Trp and Leu treatments in comparison to the control.

Table 3.2: Dry weight (DW) of bacteria (live and dead together) measured after complete fermentation in the treatment samples supplemented with leucine, isoleucine, tryptophan and valine

Leu (mM)	DW (mg/ml)	Ile (mM)	DW (mg/ml)	Trp (mM)	DW (mg/ml)	Val (mM)	DW (mg/ml)
0	4.46^{c}	0	4.46^{c}	0	4.46^{c}	0	4.46^{c}
0.38	4.39^{dc}	0.38	4.84^{a}	0.25	4.35^{d}	0.43	4.25^{e}
0.76	3.90^{g}	0.76	4.89^{a}	0.49	4.65^{bc}	0.85	4.14^{f}
1.14	4.25^{e}	1.14	4.73^{b}	0.75	3.99^{g}	1.28	4.55^{c}

Note:

- Leu- leucine, Ile- isoleucine, Trp-tryptophan and Val- valine
- Means with no common letters differ significantly from the control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data was ± 0.02.

VITAMINS

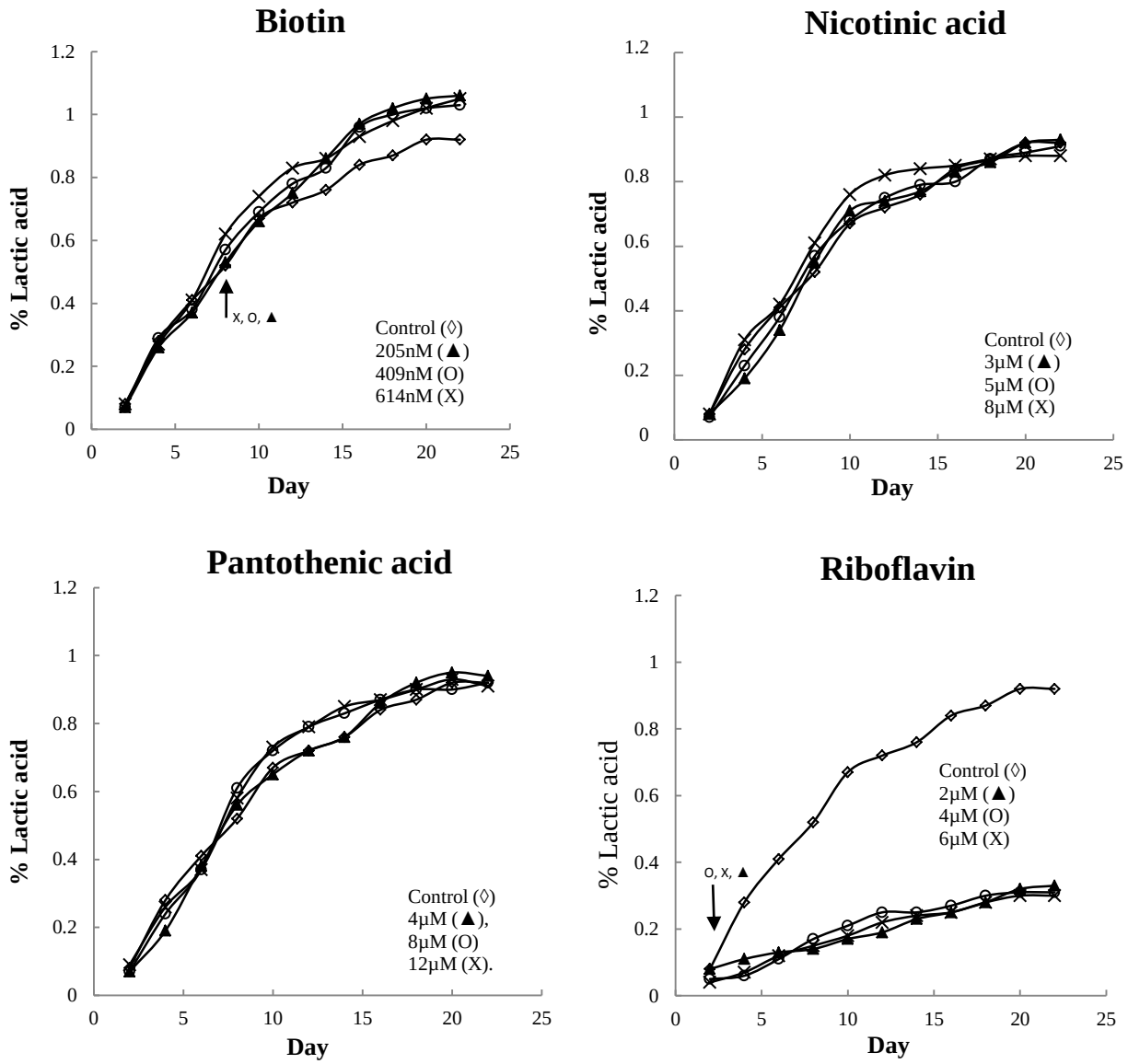
PRODUCTION OF LACTIC ACID

Efficacy of the supplemented vitamins biotin, nicotinic acid, pantothenic acid and riboflavin on the concentration of lactic acid produced in the brine was tested. Lactic acid production by LAB was measured by titration method as illustrated in Figure 3.4. Biotin supplemented brines were the only treatments which effectively increased the lactic acid concentration throughout the observation period (24 days). This activity was evident first after 8 days of incubation and continued to develop rapidly until the fermentation was completed. The final lactic acid concentration in the biotin supplemented treatment brines was 13.8% higher than the control which was significant ($P<0.05$) (Figure 3.4 Biotin). No significant difference ($P>0.05$) was observed on the lactic acid levels in pantothenic acid and nicotinic acid treated brine in comparison with the control (Figure 3.4). Addition of riboflavin to the brine adversely affected lactic acid production. The lactic acid levels in riboflavin treated brines were around 0.34 % by the end of fermentation which was three times lower than the lactic acid concentration

in the control which was significant ($P<0.05$). Although fermentation was completed in other vitamin treatments and the control on day 24, conversion of sugars to lactic acid continued in riboflavin treatments and fermentation did not cease in these treatments.

Lactic acid concentration in the treatments during fermentation was also measured using HPLC on day 2, 8, 16 and 24 to confirm the lactic acid values determined using titration. Around 2.6 % higher lactic acid concentration values was observed from alkaline titrations which will not be taken into consideration as the difference was not significant ($P>0.05$) (SEM: ±0.02).

Figure 3.4: Effect of biotin, nicotinic acid, pantothenic acid and riboflavin on lactic acid concentration determined throughout cucumber juice fermentation



Note:

- Arrow indicates the day when the live bacterial count in the treatment brines was significantly different from the control as estimated using LSD ($P<0.05$).
- Symbols of the treatment concentrations which significantly differ from the control are indicated next to the arrow.

DEPLETION OF REDUCING SUGARS

The residual sugars in the vitamin supplemented brines are reported in Table 3.3. Biotin and nicotinic acid promoted effective depletion of sugars. The final sugar concentration after complete fermentation was the lowest in treatments of 205 nM and 5 µM biotin and nicotinic acid respectively. Considerable utilization of sugars also took place in other biotin and nicotinic acid treatments which had significantly lower ($P<0.05$) sugar content compared to the control. From Table 3.3 it is evident that pantothenic acid treatments did not enhance sugar depletion and contained the same amount of sugar at the end of fermentation as the control. The extent of conversion of sugars to lactic acid was significantly lower ($P<0.05$) than the control in riboflavin treatments (Table 3.3) which also reflected in the low lactic acid concentrations in riboflavin treatment brines (Figure 3.4).

Table 3.3: Concentration of reducing sugar after complete fermentation in the treatments supplemented with biotin, nicotinic acid, pantothenic acid and riboflavin

Bio (nM)	RG* (mM)	Nic (µM)	RG* (mM)	Pan (µM)	RG* (mM)	Rib (µM)	RG* (mM)
0	2.45^{d}	0	2.45^{d}	0	2.45^{d}	0	2.45^{d}
205	1.34^{f}	3	1.74^{e}	4	2.21^{d}	2	24.42^{a}
409	1.53^{e}	5	1.42^{ef}	8	2.53^{d}	4	18.69^{c}
614	1.58^{e}	8	1.68^{e}	12	2.45^{d}	6	21.82^{b}

Note:

- RG* - Residual glucose
- Bio- biotin, Nic- nicotinic acid, Pan- pantothenic acid and Rib- riboflavin
- Means with no common letters differ significantly from the control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data was ± 0.04.
- The concentration of reducing sugars in unfermented sterilized cucumber juice and brine mixture before inoculation was 28.19 mM.

PRODUCTION OF LACTIC ACID BACTERIA

NUMBER OF LIVE, DEAD AND TOTAL CELLS

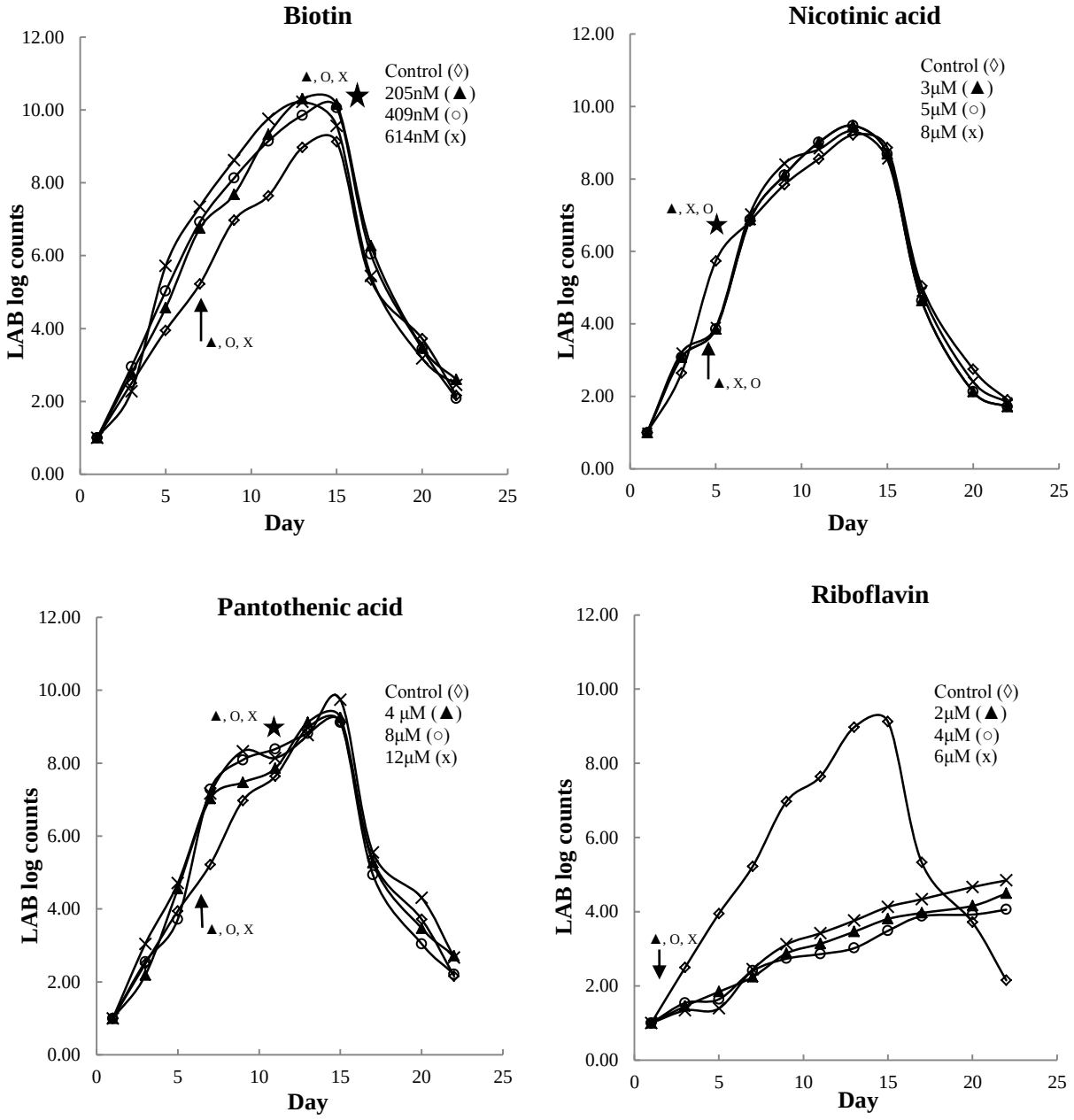
The viable LAB numbers in treatment brines with biotin, nicotinic acid, pantothenic acid and riboflavin are illustrated in Figure 3.5. Biotin increased the number of LAB in all three concentrations tested. This activity was evident immediately after initial lag phase (6 days after incubation) and continued to develop rapidly throughout the observation period (24 days). Biotin treatments reached their maximum population within 13 days along with the control (Appendix: Figure 4). The counts of viable LAB in the three biotin treatments of 205 nM, 409 nM and 614 nM when the maximum population was achieved in these treatments were 10.32, 10.06 and 10.22 log units/ml, respectively (Figure 3.5 Biotin). These counts were around 12% higher than the maximum population count in the control, but during the death phase from day 16 until the end of fermentation the number of LAB growing in all the treatments including the control were comparable. The LAB count in nicotinic acid treatments was similar to the control. Nicotinic acid treatments on day 5 had fewer LAB than the control; but from day 9 onwards all the treatments had comparable LAB counts (Figure 3.5 Nicotinic acid). Similarly pantothenic acid treatments of 8 µM and 12 µM promoted rapid increase in the number of viable LAB from day 5 through day 12, but from day 13 onwards a steady increase the LAB count similar to the control was noticed. The reason behind the sudden boost in the LAB count for a short period in these treatments was unknown. The number of viable LAB in nicotinic acid treatments of 8 µM and 12 µM when LAB reached its maximum population were 9.25 and 9.74 log units/ml, respectively (Figure 3.5 Pantothenic acid). Maximum population in these treatments was comparable to the control. There was a significant ($P<0.05$) inhibitory effect of riboflavin on LAB growth, which was evident from day 2 and continued throughout the observation period (24 days). LAB growth

in the riboflavin treatments slowly increased even after fermentation was completed in the control. The LAB count in the riboflavin treatments when the fermentation was completed in the control (day 24) was around 4.48 logs units/ml (Figure 3.5 Riboflavin).

The total number of LAB cells (live and dead) in biotin, nicotinic acid, pantothenic acid and riboflavin treated brines were also counted on the last day of observation (day 24) and the counts are illustrated in Figure 3.6. Although the biotin treatments promoted growth of LAB all through the log and stationary phase, the count of total LAB on the last day of observation was not significantly different ($P>0.05$) from the control. Pantothenic acid and nicotinic acid treatments were also comparable to the control in their total LAB count on day 24 of fermentation. Such a trend was expected as the viable cell counts in these treatments were also close to the viable cell counts of the control throughout the fermentation. The number of viable cells in riboflavin treatments were significantly higher ($P<0.05$) than the control on day 24 but the total number of bacterial cells were significantly lower ($P<0.05$).

To confirm the microscopic technique, samples were plated on day 3, 9, 17 and 24 of fermentation. Viable LAB counts from the plating technique were 4.1% higher than the counts from the microscopic technique with a standard error of (±0.032). The difference in the results from the two techniques was not significant ($P>0.05$) confirming the results from the microscopic technique.

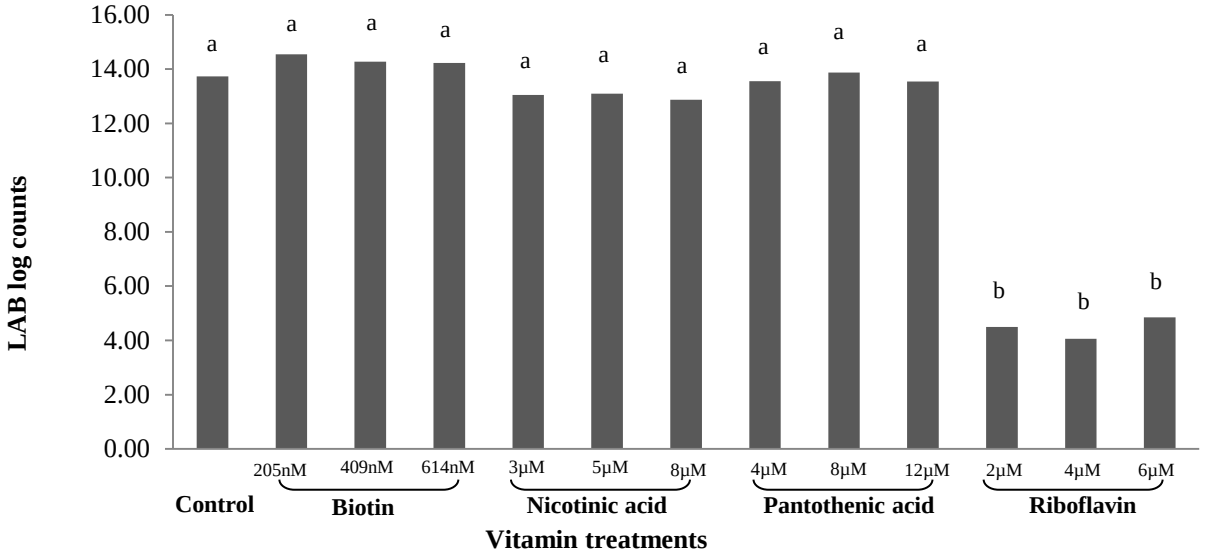
Figure 3.5: Effect of biotin, nicotinic acid, pantothenic acid and riboflavin on the live lactic acid bacteria microscopic count determined throughout cucumber juice fermentation



Note:

- Arrow indicates the day when the live bacterial count in the treatment brines was significantly different from the control as estimated using LSD ($P<0.05$).
- Star indicates the day when the live bacterial count among the treatments was not significantly different as estimated using LSD ($P>0.05$).
- Symbols of the treatment concentrations which significantly differ from the control are indicated next to the arrow and the star.

Figure 3.6: Effect of biotin, nicotinic acid, pantothenic acid and riboflavin on the total number of lactic acid bacteria in fermented cucumber juice on the last day of fermentation



Note:

- The standard error of the means for this data was ±0.01.
- Bars with no common letter differ significantly from the control as estimated using LSD ($P<0.05$)

LAB DRY WEIGHT

Dry weight of the total number of LAB taking part in the fermentation was measured and the results from this assay are reported in Table 3.4. Although the total LAB count in the biotin treatments was slightly but not statistically higher than the control, the dry weight of the microbes in the treatment brine at the end of fermentation was significantly higher ($P<0.05$) (Table 3.4). A significant increase in the total LAB number in treatment of 205 nM biotin was observed which explains the higher LAB dry weight in this treatment in comparison to the other treatments. Dry weight of LAB in pantothenic acid and nicotinic acid treatments was comparable to the LAB dry weight in the control. Significantly lower ($P<0.05$) LAB dry weight in riboflavin treatments was expected as riboflavin inhibited LAB growth.

Table 3.4: Dry weight (DW) of bacteria (live and dead together) measured after complete fermentation in the treatment samples supplemented with biotin, nicotinic acid, pantothenic acid, and riboflavin

Bio (nM)	DW (mg/ml)	Nic (µM)	DW (mg/ml)	Pan (µM)	DW (mg/ml)	Rib (µM)	DW (mg/ml)
0	1.21^{c}	0	1.21^{c}	0	1.21^{c}	0	1.21^{c}
205	1.68^{a}	3	1.27^{c}	4	1.10^{dc}	2	0.05^{e}
409	1.39^{b}	5	1.02^{d}	8	1.08^{d}	4	0.03^{e}
614	1.42^{b}	8	1.09^{d}	12	1.09^{d}	6	0.03^{e}

Note:

- Bio- biotin, Nic- nicotinic acid, Pan- pantothenic acid and Rib- riboflavin
- Means with no common letters differ significantly from the control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data was ±0.01.

MINERAL

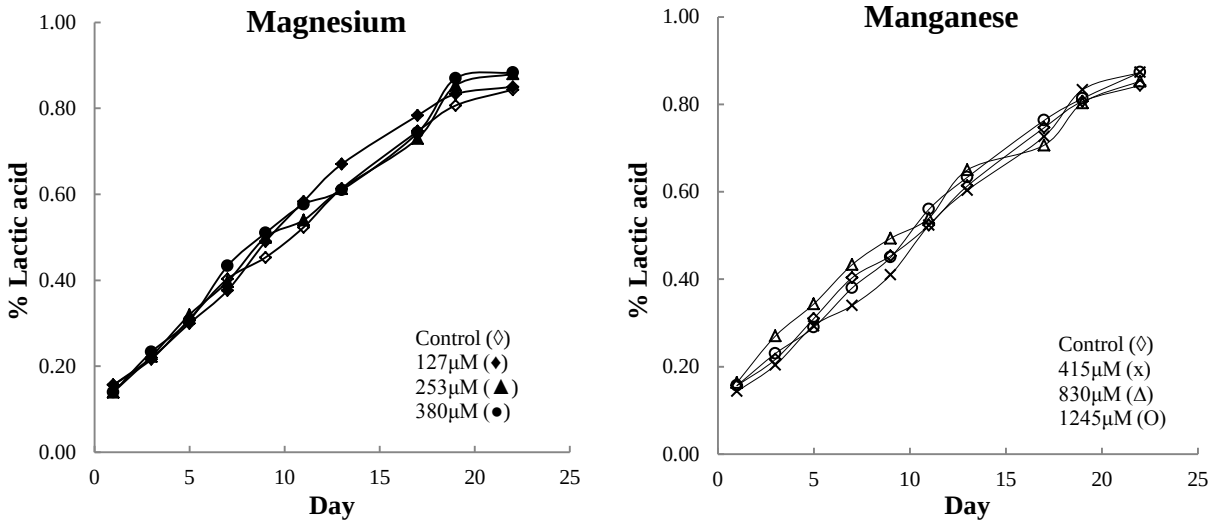
PRODUCTION OF LACTIC ACID

Lactic acid levels during fermentation in the treatment brines are plotted for each treatment and the results are illustrated in Figure 3.7. From the graph it is clear that manganese and magnesium cucumber juice treatments followed the same trend in lactic acid production as the control during fermentation. A slight decrease in the lactic acid concentration of magnesium treated brines was observed from day 13 of incubation and continued throughout the observation period (23 days). However the change was not of any statistical importance. Fermentation in the treatments were not completed within 23 days for fermentation but the number of LAB had declined to 10^3 cells/ml therefore lactic acid assay had been stopped at that point.

Lactic acid concentration in the treatments during fermentation was also measured using HPLC on day 2, 9, 16 and 23 and these concentrations were compared with the ones determined using titration method to confirm the data collected using titration method. Lactic acid levels

reported from the titrations were 1.4% lower than the concentrations determined using HPLC. The difference in the reported lactic acid levels determined using the two methods was not significant (P>0.05) (SEM: ±0.02).

Figure 3.7: Effect of manganese and magnesium on lactic acid concentration determined throughout cucumber juice fermentation



DEPLETION OF REDUCING SUGARS

Amount of residual sugars in the mineral supplemented brines at the end of fermentation are reported in Table 3.5. Although there was no significant difference in the lactic acid concentrations among the mineral treatments and the control, the amount of residual sugars was statistically different for some treatments. Treatments of 415 mM and 1245 mM of manganese and 380 mM magnesium caused lowering of residual sugar concentration to a higher extent, although all treatments had low levels.

Table 3.5: **Concentration of reducing sugar after complete fermentation in the treatments supplemented with manganese and magnesium**

Mn (µM)	RG* (mM)	Mg (µM)	RG* (mM)
0	1.74^{ab}	0	1.74^{ab}
415	1.17^{c}	125	1.54^{b}
830	1.90^{a}	253	1.57^{b}
1245	1.03^{c}	380	1.24^{c}

Note:

- Mn- manganese, Mg- magnesium
- RG* - Residual glucose
- Means with no common letters differ significantly from the control using LSD ($P<0.05$).
- The standard error of the means for this data was ±0.04
- The concentration of reducing sugars in unfermented sterilized cucumber juice and brine mixture before inoculation was 31.41mM.

PRODUCTION OF LACTIC ACID BACTERIA

NUMBER OF LIVE, DEAD AND TOTAL CELLS

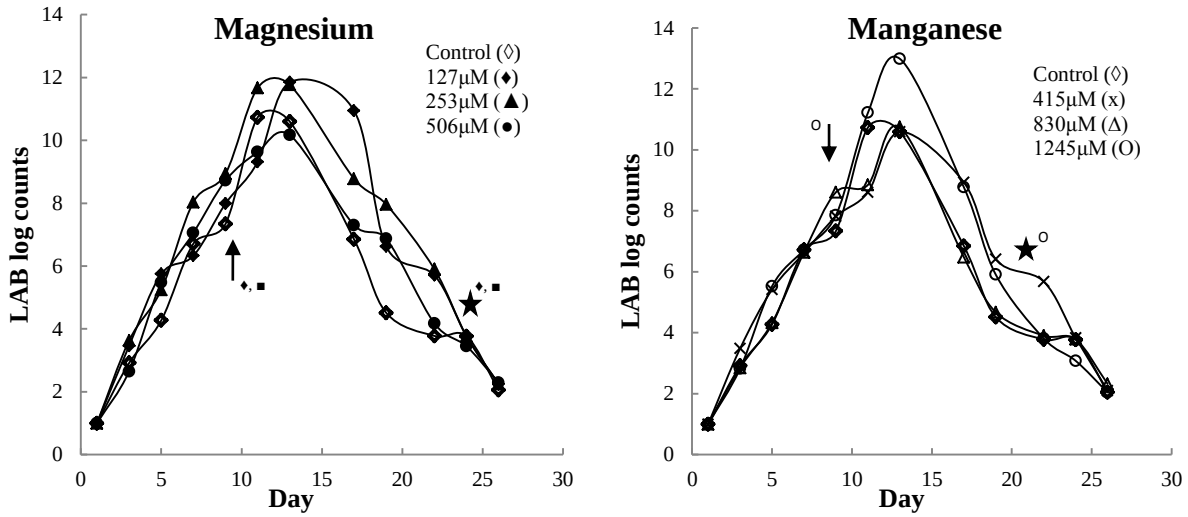
The number of viable LAB cells in fermentation brines supplemented with magnesium and manganese individually are illustrated in Figure 3.8. Minerals added to the brine were effective in increasing the number of viable LAB starting from the stationary phase through the death phase which was different from the trend followed by the vitamin and amino acid treatments where viable LAB cell count was higher than the log and stationary phase. Manganese at a concentration of 1245µM increased the number of LAB from day 12 and continued throughout the fermentation period until fermentation was completed on day 23 (Appendix: Figure 5) (Figure 3.8). Treatment brine with 1245µM of manganese had 12.98 log units of LAB in its stationary phase when the viable LAB count in the control treatments was only 9.72 log units. Treatments brines with 127µM and 253µM of magnesium increased LAB

starting from day 13 (stationary phase) and counts where consistently higher than the control until day 23.

The total number of cells (live and dead together) in the mineral supplemented brines on the final day of fermentation are illustrated in the Figure 3.9. As the viable LAB counts during fermentation were higher than the control in 127μM and 253 μM manganese and 1245 μM magnesium supplemented brines, the total LAB counts were also significantly higher in these treatments. Total LAB counts in these treatments were around 9.3%, 8.3% (127μM, 253 μM manganese) and 14.2% (1245 μM magnesium) greater than the control (Figure 3.9). Brine supplemented with 380 μM of manganese was lower than the control in its total number of LAB and all other manganese and magnesium treatments were comparable to the control.

Viable LAB cell counts from the microscopic technique were confirmed by comparing the counts with the plate counts of the same samples on day 2, 8, 16 and 22. The difference in the LAB count from the two techniques was around 2.6% which was not significantly different ($P>0.05$), confirming the result from microscopic technique with standard error of the difference (±0.03).

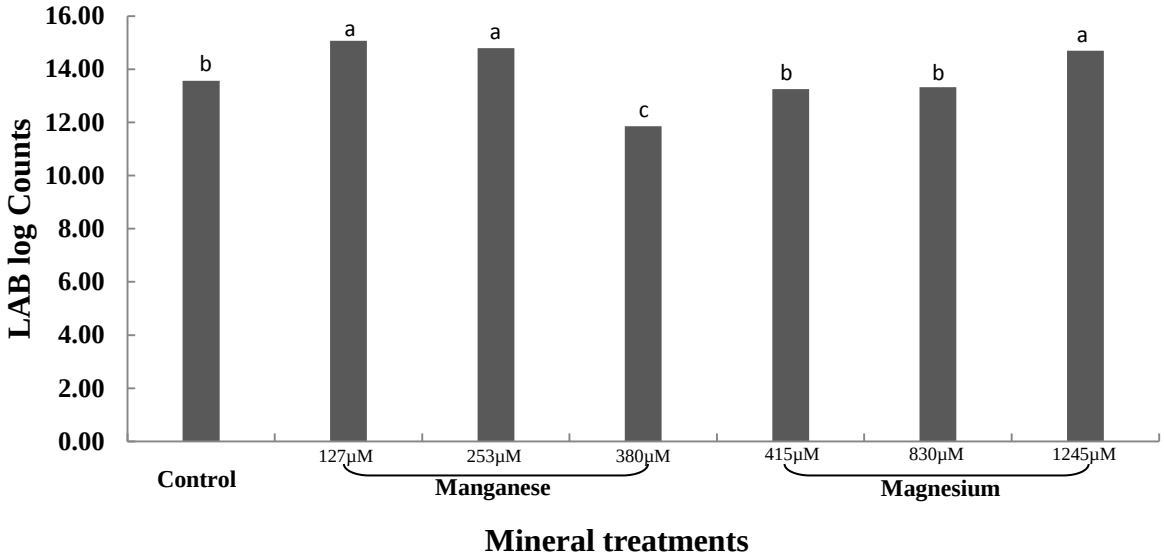
Figure 3.8: Effect of manganese and magnesium on the live lactic acid bacteria microscopic count determined throughout cucumber juice fermentation



Note:

- Arrow indicates the day when the live bacterial count in the treatment brines was significantly different from the control as estimated using LSD ($P<0.05$).
- Star indicates the day when the live bacterial count among the treatments was not significantly different as estimated using LSD ($P>0.05$).
- Symbols of the treatment concentrations which significantly differ from the control are indicated next to the arrow and the star.

Figure 3.9: Effect of manganese and magnesium on the total number of lactic acid bacteria in fermented cucumber juice on the last day of fermentation



Note:

- The standard error of the means for this data was ±0.07.
- Bars with no common letter differ significantly from the control as estimated using LSD ($P<0.05$)

LAB DRY WEIGHT

Dry weight of the total (live and dead) LAB in the treatment brines at the end of fermentation was determined to confirm the effect of the selected minerals on the yield of LAB in cucumber juice fermentation. The results from this assay are reported in Table 3.6. Only manganese treatments of 415µM and 1245µM were effective in increasing the dry weight of LAB at the end of fermentation these treatments were significantly higher than the control ($P<0.05$). All other treatments were comparable to the control.

Table 3.6: Dry weight (DW) of bacteria (live and dead together) measured after complete fermentation in the treatment samples supplemented with manganese and magnesium

Mn (µM)	DW (mg/ml)	Mg (µM)	DW (mg/ml)
0	0.28^{c}	0	0.28^{c}
415	0.67^{b}	127	0.34^{c}
830	0.21^{c}	253	0.35^{c}
1245	1.35^{a}	380	0.24^{c}

Note:

- Mn- manganese, Mg- magnesium
- Means with no common letters differ significantly from the control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data is ±0.01.

CHAPTER IV

RESULTS

FURTHER EXAMINATION OF CONCENTRATIONS OF BIOTIN, ILE AND VAL THAT ENHANCE LACTIC ACID BACTERIA FERMENTATION OF CUCUMBER JUICE

Three nutrient supplements Bio (biotin), Ile and Val were selected for further studies based on their favorable response in objective 1 of promoting lactic acid production and the number of LAB taking part in cucumber juice fermentation. It was important to find the lowest yet, the most effective concentration of these nutrients that would enhance acid production and LAB growth to a greater extent in LAB fermentation of cucumber juice. The concentrations tested in this fermentation trial were decided based on the observation from objective 1. The assays were conducted similar to the ones in objective 1. Each nutrient was used in five concentrations and each concentration was considered a treatment, the concentration of the nutrients in each treatment is defined in table 2.1. As all the concentrations in Bio and Ile treatments were significantly different from the control, but not significantly different from each other in their activity to enhance cucumber juice fermentation, two new concentrations were added to the previous concentrations. Concentrations lower and higher than tested in previous trials were added. Val treatments displayed an increasing trend in their activity, where their intensity to enhance LAB fermentation increased with increasing concentration. Hence two new higher concentrations were added as treatments.

RESULTS

PRODUCTION OF LACTIC ACID

Lactic acid concentration during fermentation of the treatment brines are plotted for each treatment and the results are illustrated in Figure 4.1. Four concentrations of the Bio treatments except 103 nM of Bio supported production of lactic acid in higher levels compared to the control. This activity was evident from day 8 and continued through the last day of fermentation (day 23). A rise on 7.8% was observed in the final lactic acid concentration of the Bio treatments 205 nM, 409 nM, 614 nM and 819 nM. These treatments reached their maximum lactic acid concentration within 20 days of fermentation (Figure 4.1 Bio). Bio treatment 205nM supported slight growth, but was not as effective as the treatments 409 nM, 614 nM and 819 nM in increasing the lactic acid level of the brine. Conversion rate of sugar to lactic acid in Bio treatment 103 nM was lower than rest of the Bio treatments. The response obtained in this treatment was not different from the control.

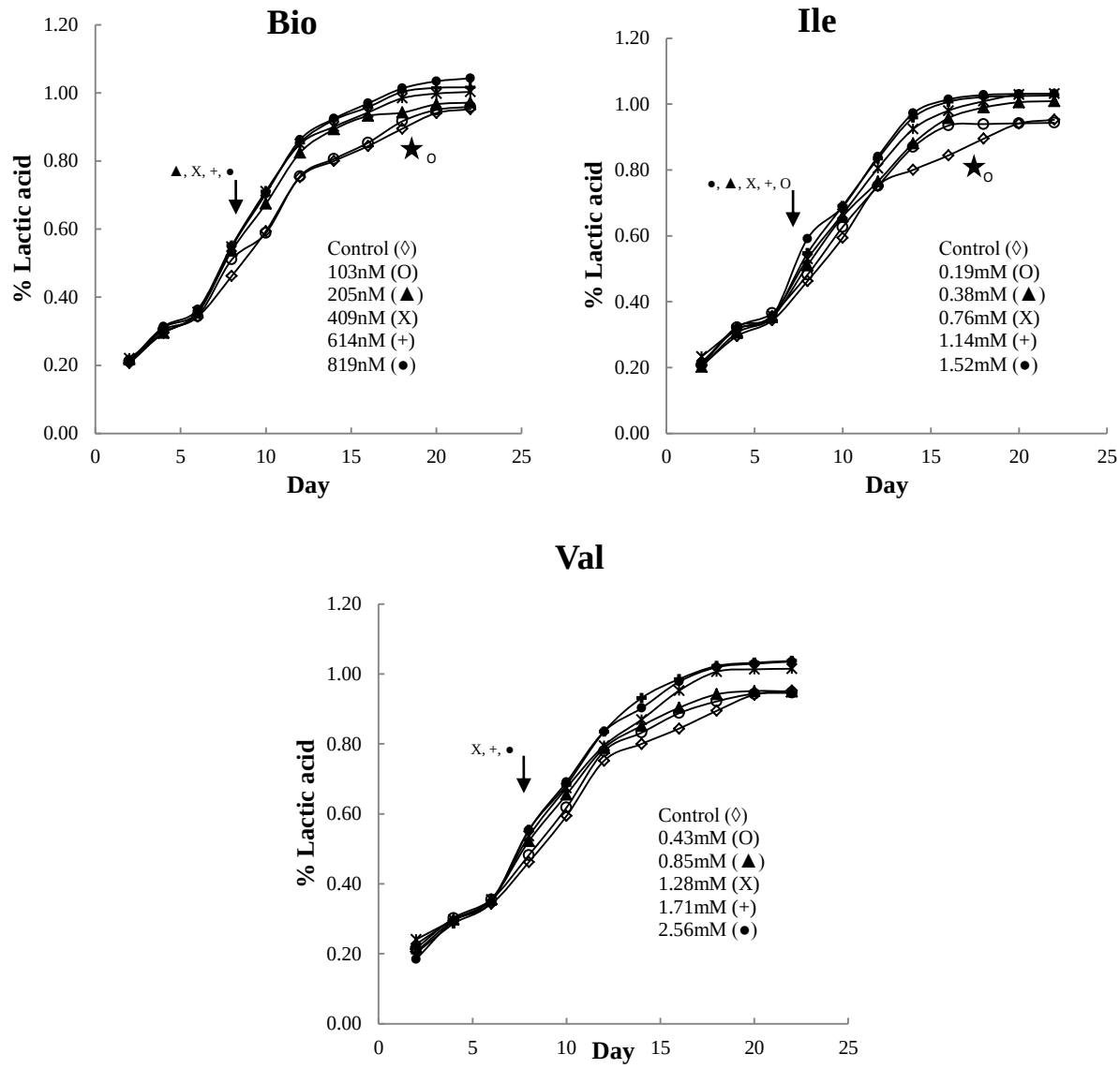
Ile treatments were not effective in increasing lactic acid concentration until day 13 and were close to the control in their lactic acid levels. Rapid production of lactic acid was observed from day 13. Among all the Ile treatments the final lactic acid concentration of 0.76 mM, 1.14 mM and 1.52 mM Ile was higher than the control and the rest of the Ile treatments (Figure 4.1 Ile). The final lactic acid levels were 8.2% higher in these treatments in comparison with the control, but they were not significantly ($P>0.05$) different from each other.

Val treatments followed the same trend as the Bio treatments. They promoted rapid production of lactic acid from day 8 onwards (Figure 4.1 Val). As observed in objective 1 the treatments 0.43 mM and 0.85 mM of Val had the same lactic acid levels as the control. But with the increase in the Val concentration in the treatments the intensity to enhance acid levels also

increased in treatments 1.28 mM, 1.71 mM and 2.56 mM of Val. 8.4% increase in the final lactic acid concentration was observed in these treatments as compared to the control.

HPLC was used on day 2, 8, 16 and 22 to determine concentration of lactic acid in the brine on those days and the data was compared with the lactic acid concentrations from alkaline titrations. The concentrations from the HPLC method were significantly higher ($P<0.05$) than the data collected using titrations by 3.1% with a standard deviation of the means (±0.06).

Figure 4.1: Effect of biotin, isoleucine and valine on lactic acid concentration determined throughout cucumber juice fermentation



Note:

- Bio- biotin, Ile- isoleucine and Val- valine
- Arrow indicates the day when the live bacterial count in the treatment brines was significantly different from the control as estimated using LSD ($P<0.05$).
- Star indicates the day when the live bacterial count among the significantly different from the control as estimated using LSD ($P>0.05$).
- Symbols of the treatment concentrations which significantly differ from the control are indicated next to the arrow and the star.

DEPLETION OF REDUCING SUGARS

Treatments that increased lactic acid production (Figure 4.1) were also effective in depleting sugar in cucumber juice. Also treatments 0.19 mM and 0.38 mM of Ile were effective in depleting sugar which was not evident from the lactic acid concentration in these brines (Table 4.1).

Table 4.1: Concentration of reducing sugar after complete fermentation in the treatments supplemented with biotin, isoleucine and valine

Bio (nM)	RG* (mM)	**Ile (mM)**	RG* (mM)	**Val (mM)**	RG* (mM)
0	1.56^{a}	0	1.56^{a}	0	1.56^{a}
103	1.54^{a}	0.19	1.11^{b}	0.43	1.43^{a}
205	1.40^{a}	0.38	0.99^{c}	0.85	1.31^{a}
409	1.23^{b}	0.76	1.01^{bc}	1.28	1.17^{b}
614	1.11^{b}	1.14	0.96^{c}	1.71	1.02^{bc}
819	1.11^{b}	1.52	0.96^{c}	2.56	0.98^{c}

Note:

- Bio- biotin, Ile- isoleucine and Val- valine
- RG*- residual glucose
- Means with no common letters differ significantly from the control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data is ±0.03.
- The concentration of reducing sugars in unfermented sterilized cucumber juice and brine mixture before inoculation was 33.76 mM.

PRODUCTION OF LACTIC ACID BACTERIA

NUMBER OF LIVE, DEAD AND TOTAL CELLS

The three nutrients had proved to increase LAB number in objective 1. They were added in five different concentrations in objective 2 to find out the most effective concentration that could increase the number of LAB in cucumber fermentation. Once again from this study it was confirmed that Bio, Ile and Val supported increase in LAB number. Treatments of 409 nM, 614

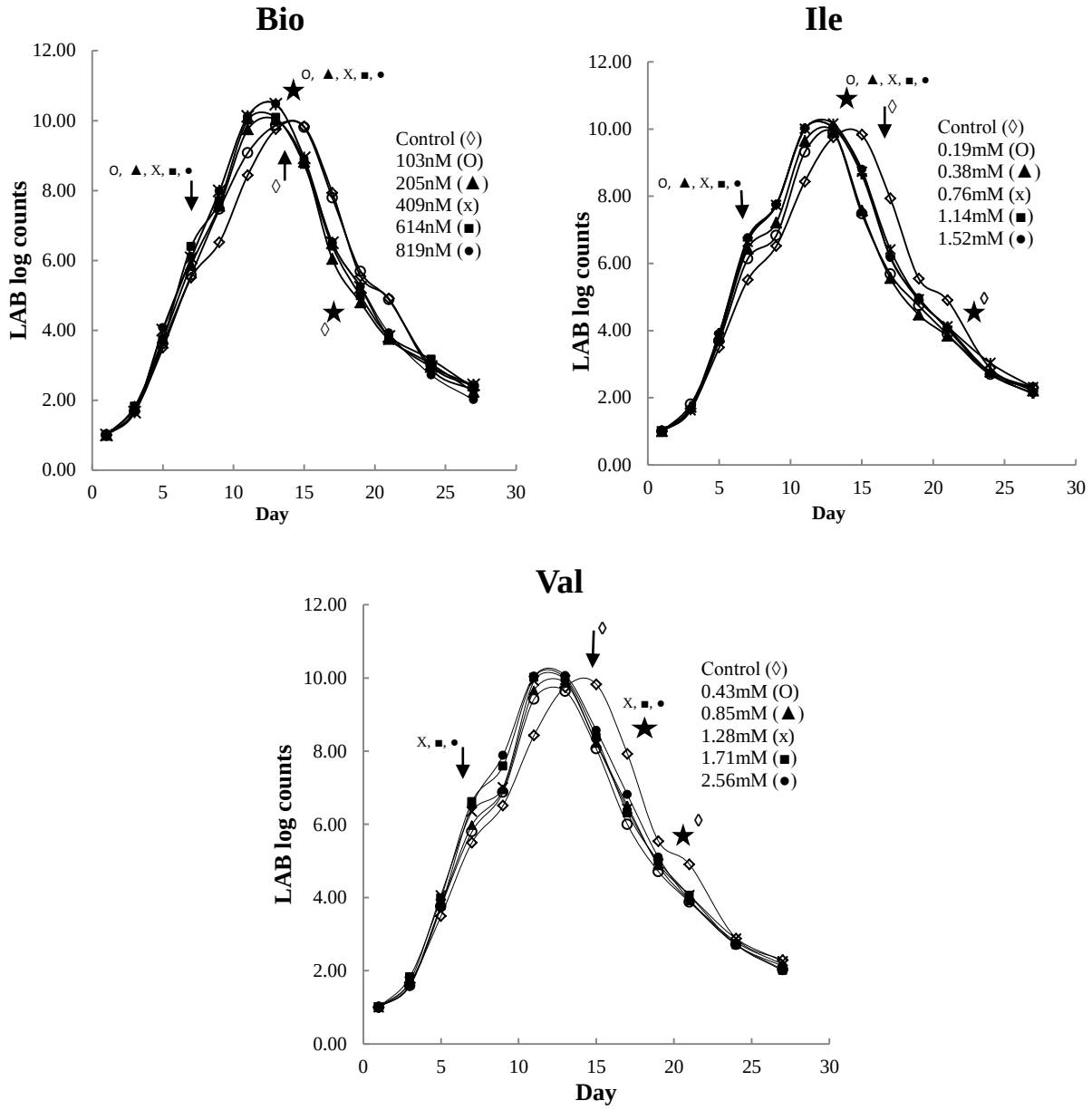
nM and 819 nM Bio were equally effective and proved to be active in increasing the viable cell number from day 8 onwards. These treatments had significantly larger number of LAB growing at any point of time in comparison with other Bio treatments and the control (Figure 4.2 Bio). Treatments of 0.19 mM and 0.38 mM Ile reached their maximum population of LAB within 12 days of fermentation as compared to 15 days in the control (Appendix: Figure 6) (Figure 4.2 Bio). However the number of bacteria growing in these treatments throughout the fermentation was similar to the control (Figure 4.3). Treatments of 0.76 mM, 1.14 mM and 2.56 mM Ile also reached their maximum population of LAB within 10 days of fermentation and had significantly higher ($P<0.05$) number of live bacteria from day 10 to day 16 (end of stationary phase) (Figure 4.2 Ile). In the death phase from day 16 onwards the viable LAB count in the control was significantly higher ($P<0.05$) than the Ile treatments (Figure 4.2 Ile). Treatments of 1.71 mM and 2.56 mM Val supported rapid multiplication of LAB until day 16 (Figure 4.2 Val). The number of viable LAB till day 16 were significantly higher ($P<0.05$) than the control in these Val treatments. From day 18 onwards the number of viable LAB in the control was higher in comparison with the Val treatments.

The total (live and dead) number of LAB in treatments of 614 nM and 819 nM Bio were around 13.64 and 13.61 log units/ml respectively, which were higher than any other Bio treatments (Figure 4.3 Bio). These Bio treatments were considered the most effective of all the Bio treatments in increasing the total number of LAB. The total number of LAB in treatments of 0.76 mM, 1.14 mM and 2.56 mM Ile at the end of the fermentation were around 13.68, 13.93 and 13.95 log units/ml. On the final day of fermentation the total LAB in the control brine was around 12.43 log units/ml (Figure 4.3), which was significantly lower ($P<0.05$) than in treatments of 0.76 mM, 1.14 mM and 2.56 mM Ile. The total number of LAB in treatments of

1.28, 1.71mM and 2.56 mM Val were around 13.74, 13.86 and 13.94 log units/ml respectively (Figure 4.2).

Viable LAB cells were quantified using plating technique at regular intervals to confirm the viable cell results from the microscopic technique. Samples were plated on day 2, 9, 15 and 21 and compared to the observations on the viable cell count using microscopic technique on the same days. Results for the two techniques were close, but a difference of 2.3% was observed. The difference in the two techniques had a standard deviation of (± 0.27)

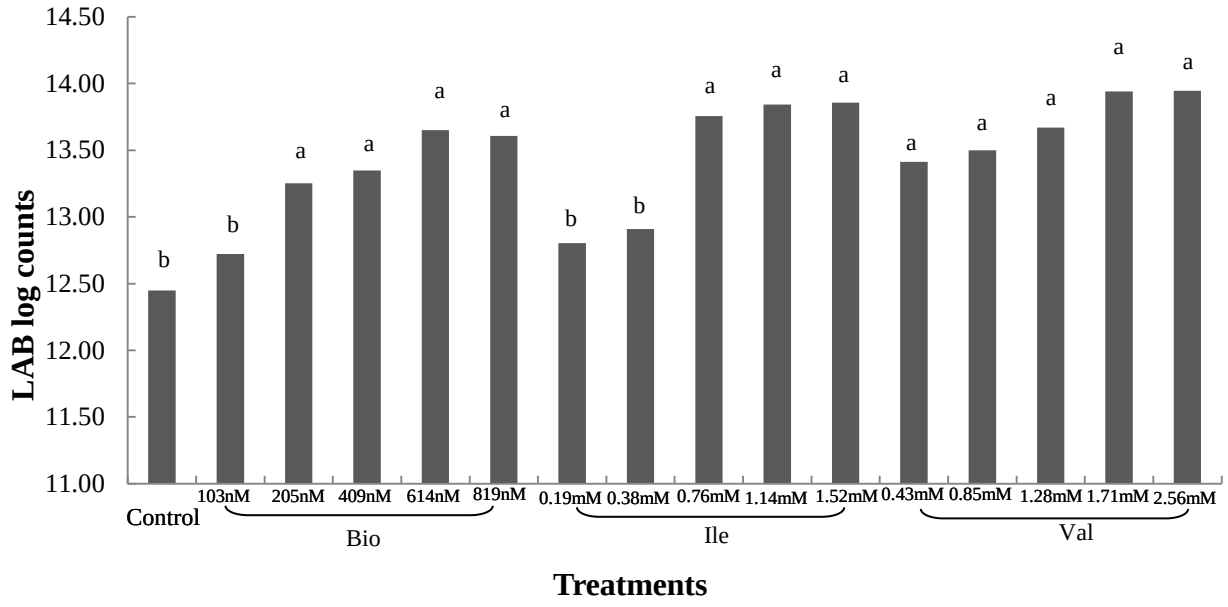
Figure 4.2: Effect of biotin, isoleucine and valine on the live lactic acid bacteria microscopic count determined throughout cucumber juice fermentation



Note:

- Bio- biotin, Ile- isoleucine and Val- valine
- Arrow indicates the day when the live bacterial count in the treatment brines was significantly different from the control as estimated using LSD ($P<0.05$).
- Star indicates the day when the live bacterial count among the treatments was not significantly different as estimated using LSD ($P>0.05$).
- Symbols of the treatment concentrations which significantly differ from the control are indicated next to the arrow and the star.

Figure 4.3: Effect of biotin, isoleucine and valine on the total number of lactic acid bacteria in fermented cucumber juice on the last day of fermentation



Note:

- Bio- biotin, Ile- isoleucine, Val- valine
- The standard error of the means for this data is ±0.02.
- Bars with no common letter differ significantly from the control as estimated using LSD ($P<0.05$)

LAB DRY WEIGHT

LAB dry weight was measured on the final day of fermentation and reported in Table 4.2. Treatments 103 nM of Bio, 1.14 mM and 2.56 mM of Val were estimated to be the most effective among all the treatments in increasing LAB dry weight. Treatments 614 nM and 819 nM of Bio, 1.28 mM and 0.85 mM of Val closely followed. Some treatments which increased the number of LAB as discussed earlier did not show a significant increase in the LAB dry weight as compared to the control.

Table 4.2: Dry weight (DW) of bacteria (live and dead together) measured after complete fermentation in the treatment samples supplemented with biotin, isoleucine and valine

Bio (nM)	DW (mg/ml)	Ile (mM)	DW (mg/ml)	Val (mM)	DW (mg/ml)
0	1.18 ^{d}	0	1.18^{d}	0	1.18^{d}
103	2.87^{a}	0.19	1.48^{c}	0.43	1.52^{c}
205	1.53^{c}	0.38	1.65^{bc}	0.85	1.63^{bc}
409	1.54^{c}	0.76	1.09^{d}	1.28	1.80^{b}
614	1.89^{b}	1.14	1.37^{c}	1.71	2.62^{a}
819	1.77^{b}	1.52	1.07^{d}	2.56	2.12^{a}

Note:

- Bio- biotin, Ile- isoleucine and Val- valine
- Means with no common letter differ significantly from the control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data is ±0.01.

CHAPTER V

RESULTS

EFFECT OF BIO, ILE AND VAL ALONE AND IN COMBINATIONS ON LACTIC ACID BACTERIA FERMENTATION OF CUCUMBER JUICE

TREATMENTS

Lactic acid bacteria activity enhancing micronutrients were tested and selected for their effect on cucumber juice fermentation in trial I through IV. Improved acid fermentation was observed in Bio, Ile and Val treatments in their respective concentrations of 614 nM, 0.76 mM and 1.71 mM. These treatments were successful in increasing the number of LAB taking part in the fermentation. They also supported increased lactic acid levels in fermentation brine. Trial V was conducted to determine the effect of these selected nutrients supplemented in combinations in brine (Table 2.1) on the number of viable LAB cells and lactic acid production in cucumber juice fermentation.

RESULTS

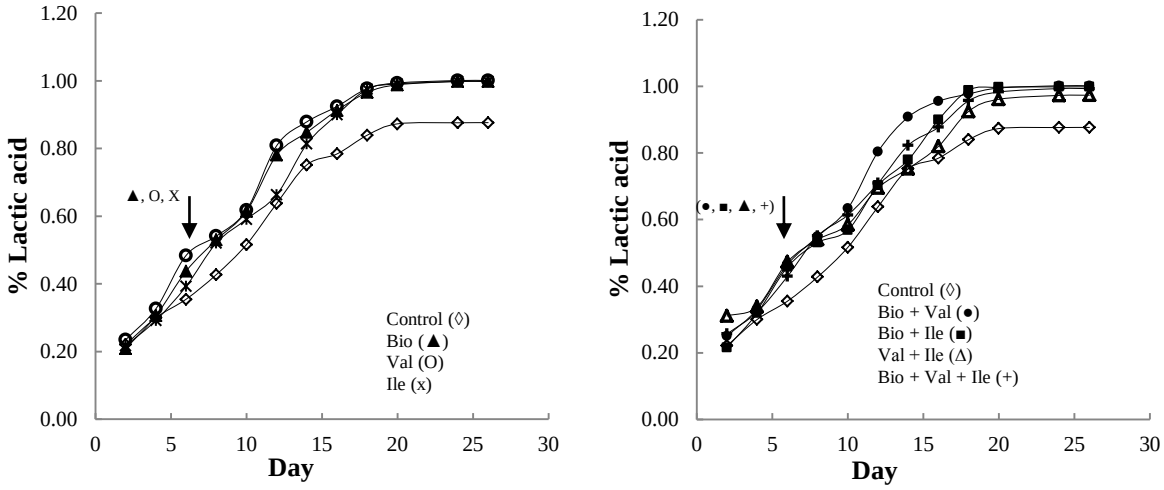
PRODUCTION OF LACTIC ACID

The effect of Bio, Ile and Val when used in combinations on lactic acid production by LAB are illustrated in Figure 5.1. Treatments with Bio and Val in combination (Bio + Val) and only Val increased lactic acid production in the brine which helped these brines reach their maximum concentrations (1.02% and 1.00% respectively) faster than other treatments. These treatments reached their maximum lactic acid concentration on day 18. Other nutrient supplemented treatment also promoted higher lactic acid levels and their final lactic acid

concentration was around 0.97% on day 21 which was also significantly higher than the control at 88% on day 23.

No notable difference was observed in the acidity values reported using the two acidity determining methods. A rise of around 1.3% was observed in the values reported using titration method which was not significant (P>0.05). Therefore the titration method was proved to be efficient in this study with a standard error of (±.0.02).

Figure 5.1: Lactic acid concentration in cucumber juice treated with biotin, isoleucine and valine in different combinations determined throughout fermentation



Note:

- Bio- biotin, Ile- isoleucine and Val- valine
- Arrow indicates the day when the live bacterial count in the treatment brines was significantly different from the control as estimated using LSD (P<0.05).
- Symbols of the treatment concentrations which significantly differ by LSD (P<0.05) from the control are indicated next to the arrow.

DEPLETION OF REDUCING SUGARS

The amount of residual sugars in the treatment brines is reported in Table 5.1. All treatments proved to be effective in depleting higher concentrations of sugar in comparison to the control. But the residual sugar concentration in treatment brines (Bio, Bio + Val, Val + Ile and Bio + Ile + Val) was lower than the rest. Greater ($P<0.05$) utilization of sugar was observed in these treatments in comparison to other treatments.

Table 5.1: **Concentration of reducing sugar after complete fermentation in the treatments supplemented with biotin, isoleucine and valine**

Treatments	Concentration	RG* (mM)
Control	0	1.68^{a}
Bio (nM)	614	1.06^{c}
Val (mM)	0.76	1.19^{b}
Ile (mM)	1.71	1.39^{b}
Bio (nM) + Val (mM)	614 + 1.71	0.99^{c}
Bio (nM) + Ile (mM)	614 + 0.76	1.24^{b}
Val (mM) + Ile (mM)	1.71 + 0.76	1.16^{c}
Bio (nM) + Val (mM) + Ile (mM)	614 + 1.71 + 0.76	1.10^{c}

Note:

- Bio- biotin, Ile- isoleucine and Val- valine
- RG* - Residual glucose
- Means with no common letters differ significantly from the control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data is ±0.01.
- The concentration of reducing sugars in unfermented sterilized cucumber juice and brine mixture before inoculation was 27.76 mM.

LACTIC ACID BACTERIA PRODUCTION

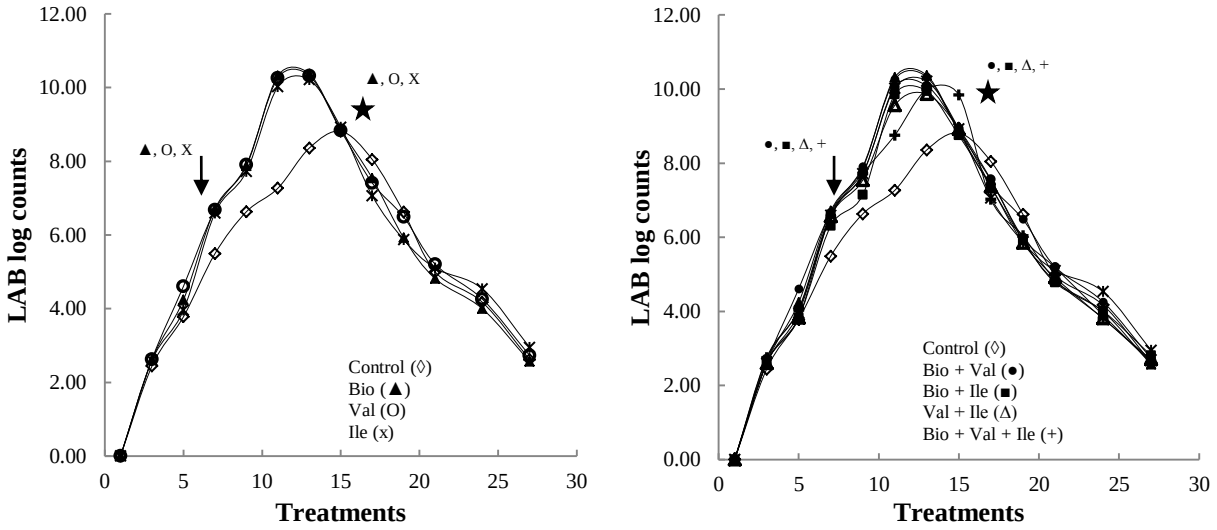
LAB in cucumber juice fermentation rapidly multiplied in the presence of Bio, Val and Ile and the results are illustrated in Figure 5.2. All treatment combinations boosted LAB numbers but some were highly active compared to the others. Treatments (Bio + Val), (Bio), (Ile) and (Val) increased LAB numbers. These treatments reached their maximum population within 12 days of inoculation (Appendix: Figure 6) with viable LAB numbers of 10.86 log units/ml, 10.37 log units/ml, 10.32 log units/ml and 10.21 log units/ml respectively. Control reached its maximum LAB population on day 15 with viable LAB count of 8.81 log units/ml (Appendix: Figure 6).

As the number of viable cells in the nutrient supplemented treatments was significantly higher ($P<0.05$) than the control, the total (live and dead) number of LAB taking part in the fermentation process in these treatments was also significantly higher. The results from the total number of LAB on the final day of fermentation are illustrated in Figure 5.3. Although all the treatments promoted LAB growth the total count on the final day of fermentation was the highest in treatments (Val) and (Bio + Val) with maximum population of 13.43 log units/ml and 13.40 log units/ml respectively (Figure 5.3). Rest of the treatments were comparable to each other with an average of 13.00 log units/ml, which was also significantly higher than the control with 10.88 log units/ml of total LAB on the final day of fermentation.

Viable LAB were quantified using two techniques (microscopic and plating) on day 2, 7, 14, 21. The results from the two techniques were compared to confirm the efficiency of the microscopic technique. Viable number of LAB determined using the microscopic technique were significantly lower ($P<0.05$) than those determined using plating technique by 7.5% on day 14

Appendix: Figure 8). The reason behind such this difference could be that the samples were stained for more than 5 seconds before viewing them under the microscope.

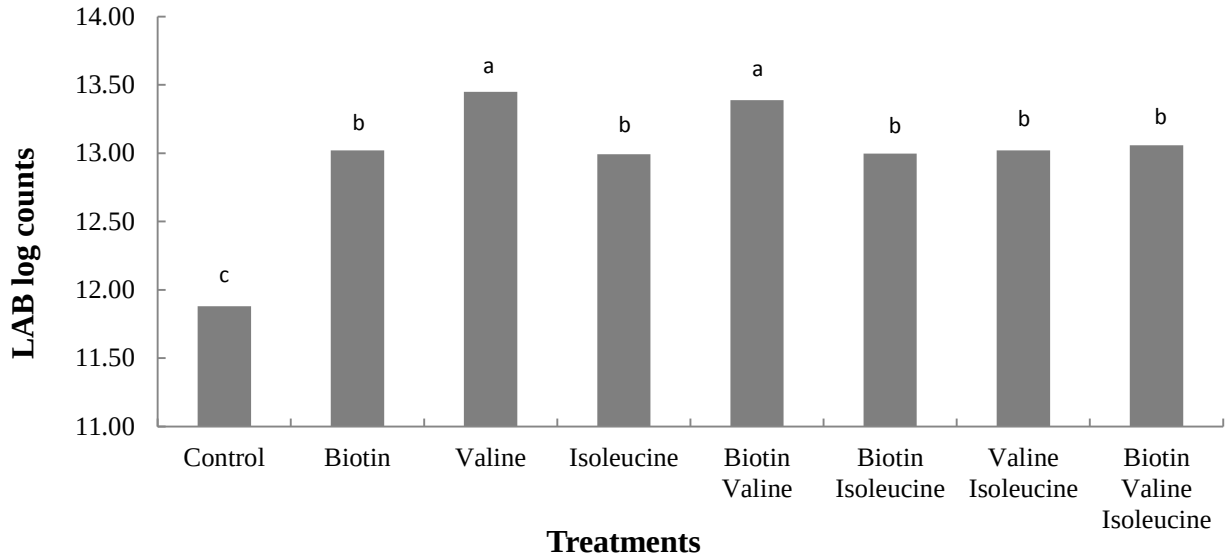
Figure 5.2: Effect on the live lactic acid bacteria microscopic count in cucumber juice treated with biotin, isoleucine and valine in different combinations determined throughout fermentation



Note:

- Bio- biotin, Ile- isoleucine and Val- valine
- Arrow indicates the day when the live bacterial count in the treatment brines was significantly different from the control as estimated using LSD ($P<0.05$).
- Star indicates the day when the live bacterial count among the treatments was not significantly different as estimated using LSD ($P>0.05$).
- Symbols of the treatment concentrations which significantly differ from the control are indicated next to the arrow and the star.

Figure 5.3: Effect on the total number of lactic acid bacteria in cucumber juice treated with biotin, isoleucine and valine in different combinations determined on the last day of fermentation



Note:

- The standard error of the means for this data is ±0.02
- Bars with no common letters differ significantly from the control as estimated using LSD ($P<0.05$)

LAB DRY WEIGHT

Dry weights of the total number of LAB taking part in the fermentation present in the treatments on the last day of fermentation are reported in Table 5.2. Bio and Val treatments when added alone or in combination (Bio, Val, Bio + Val, Bio + Ile) weighed significantly ($P<0.05$) more than the control and the Ile treatments (Table 5.2).

Table 5.2: Dry weight (DW) of bacteria (live and dead together) measured after complete fermentation in the treatment samples supplemented with **biotin, isoleucine and valine in different combinations**

Treatments	Concentration	Units	DW (mg/ml)
Control	0	0	1.55^{b}
Bio	614	nM	1.96^{a}
Val	0.76	mM	1.85^{a}
Ile	1.71	mM	1.69^{b}
Bio + Val	614 + 1.71	nM + mM	1.91^{a}
Bio + Ile	614 + 0.76	nM + mM	1.82^{a}
Val + Ile	1.71 + 0.76	mM + mM	1.69^{b}
Bio + Val + Ile	614 + 1.71 + 0.76	nM + mM + mM	1.66^{b}

Note:

- Bio- biotin, Ile- isoleucine and Val- valine
- Means with no common letters differ significantly from the control as estimated using LSD ($P<0.05$).
- The standard error of the means for this data is ±0.07.

CHAPTER VI

DISCUSSION AND SUMMARY

DISCUSSION

In this investigation, the effect of adding fermentation brine with optimized concentration of amino acids, vitamins and minerals during cucumber juice fermentation was analyzed. The basis for use of selected concentrations was drawn from the literature on substrate consumption by lactic acid bacteria during fermentation (Archibald 1986; Costilow and Fabian 1953b, c, d; Fabiana and Nadra 2007; Ruiz-Barba and Jimenez-Diaz 1994; Ragheb and Fabian 1956).

Of the four amino acids Leu, Ile, Trp and Val considered to be essential for *L. plantarum* growth (Costilow and Fabian 1953b, c; Saguir and Nadra 2007; Meretro and others 1998), only Ile and Val were effective in increasing the number of LAB taking part in the fermentation and the concentration of lactic acid in the brine. Addition of Leu and Trp showed no effect on the titratable acidity and microbiological activity of cucumber juice fermentation. These results were consistent with the findings of Costilow and Fabian (1953c), and the reason for such an observation could be that the levels of native Leu and Trp from cucumber in the brine were sufficient for LAB growth and added Trp may not be stimulating the conversion of sugar to lactic acid (Ragheb and Fabian 1956). Therefore, providing them with higher levels of Leu and Trp did not improve their growth.

L. plantarum used mainly the essential amino acid Ile and Val for their better incorporation in cell material (Saguir and Nadra 2007, Costilow and Fabian 1953d). During the preliminary screening all the three concentrations of Ile improved cucumber juice fermentation. However, in case of Val, only the treatment with highest Val concentration (1.28 mM) had

higher LAB count than the control. Although the LAB count at 0.85 mM Val was higher than the LAB count at 0.43 mM Val, these treatments increased the number of LAB growing but did not differ significantly from the control. These results indicated that LAB growth improved with increase in Val concentration, this trend was in agreement with earlier study (Moretro and others 1998). The initial amino acids levels in the brine during commercial cucumber fermentation may be sufficient for initiation of lactic acid fermentation, but concentrations decline from the first week of fermentation between 41 and 68% at the end of growth (Saguir and Nadra 2007). Therefore these amino acids were tested to determine their smallest, yet the most effective concentration for the increase in acid levels and LAB numbers. Treatment concentrations 0.76 mM Ile and 1.71 mM Val were selected to be used in combination in objective 3. Val is essential for *L. plantarum* growth (Fabiana and Nadra 2007) and the carbon skeleton of the amino acids Val and Ile can be converted to lactate or lipids (Kask and others 1999). This could be the reason that only the final lactic acid concentration of Val treatments was higher than the control as it promoted *L. plantarum* growth.

Previous investigations determined that biotin levels in the brine after one day of fermentation were higher than those required by *L. plantarum* (Costilow and Fabian 1953b,c). Biotin is required by LAB for the synthesis of aspartic acid and oleic acid, but when these nutrients are present in required quantities then biotin is required in very small concentrations (Broquist and Snell 1951). But in this study, fermentation brine showed considerable increase in the number of LAB and lactic acid concentration when the brine was added with biotin. Biotin enhanced LAB numbers, was effective in depleting sugars and yielded more lactic acid among all the vitamin treatments. These results were slightly different from the results reported by Rosen and Fabian (1953) who concluded that cucumber juice contained substances that could

substitute for biotin requirement in *L. plantarum* resulting in less biotin depletion. Studies conducted by Ruiz-Barba and Jimenez-Diaz (1995) using green olives as fermentation substrate reported that biotin, nicotinic acid and pantothenic acid were not considered growth limiting factors of *L. plantarum*. Of all the concentrations tested, biotin treatment at 614 nM proved to be most effective and was used in combination with Ile and Val in objective 3.

Though Scientists earlier (Snell and others 1939; Snell 1946) reported that supplementing fermentation brine with nicotinic acid and pantothenic acid stimulated LAB growth, similar effect was not observed in this study. However, the sugar content in the brines supplemented with nicotinic acid was significantly lower than the control; leading to a conclusion that nicotinic acid could aid the depletion of sugars to other fermentation products rather than lactic acid. Insignificant differences in the number of LAB and lactic acid concentration in pantothenic acid and nicotinic acid treatment brines could be because the cucumber juice immediately provided LAB with 3 to 17% of nicotinic acid and pantothenic acid respectively (Costilow and Fabian 1953b; Rosen and Fabian 1953) which could be sufficient for their growth.

Riboflavin was in marked contrast to the behavior of the other vitamins examined; it can be synthesized by certain species of LAB (Snell 1946) and was also toxic to the microorganisms growing in the brine when supplemented synthetically. These findings were in accordance with my results where LAB growth was inhibited and acid production was also suppressed.

Although the initial pool of nutrients should be provided to the microorganisms either from the cucumber or supplemented in the brine, several authors reported an increase in the vitamin content of vegetable and dairy products that had undergone LAB fermentation (Salih and Dilleau 1990; McFeeters 1988). In most cases, the increase could be attributed to the activity of

LAB and different microorganisms which grow during fermentation (Costilow and Fabian 1953b; Rosen and Fabian 1953).

The fermentation brine supplemented with magnesium and manganese resulted in no significant change in LAB numbers, or the conversion of reducing sugars to lactic acid. Although magnesium showed minor increase in the acid levels during fermentation, the final concentration of the acid was the same as in the control. A similar study conducted using cucumber juice reported that addition of magnesium in higher concentrations than used in this study decreased sugar utilization as it inhibited the enzymes involved in sugar transport and metabolism (Lu and others 2002). Generally, cucumbers contain only 4% manganese of what is present in the MRS basal for cultivation of LAB (USDA 2010). In studies conducted earlier (McDonalds and other 1990) addition of manganese in the cucumber media during fermentation greatly increased the sugar utilization and lactic acid production. These results were not in accordance with the results obtained in my study, which did not find any increase in lactic acid production. High concentration of manganese has an inhibitory effect on the starter culture (Lu and others 2002).

Effective concentrations of biotin, Ile and Val were added in different combinations to the fermentation brine to determine their effect on LAB numbers, dry LAB weight, lactic acid concentrations and the final concentration of reducing sugar. Treatments with Bio and Val as stand-alone or in combination (Bio, Val, Bio + Val) were the most effective. The results from all the trials have indicated that Bio stimulated LAB growth and is a potential cucumber fermentation enhancer (Costilow and Fabian 1953b, d; Rosen and Fabian 1953). Val and Ile are closely related aliphatic amino acids but on comparing their activity (Kask and others 1999), Val as a supplement promoted higher LAB numbers. This increase in the number of LAB increased the concentration of sugar converted to lactic acid and hence increased the concentration of lactic

acid in cucumber juice fermentation (Costilow and Fabian 1953c). Although Ile in trial I and IV proved to be effective in enhancing LAB fermentation in cucumber juice wasn't equally effective in combination with Bio and/or Val, for unknown reasons.

SUMMARY

- The effects of supplementing cucumber juice fermentation brine with amino acids (Leu, Ile, Trp and Val), vitamins (biotin, nicotinic acid, pantothenic acid and riboflavin) and minerals (manganese and magnesium) on LAB growth and lactic acid production were investigated.
- Bio, Ile and Val were determined to be the most effective nutrients among the nutrients tested. The most effective concentration among the five concentrations tested for each nutrient was determined.
- Of all the nutrients tested in different concentrations, 614 nM Bio, 0.76 mM Ile and 1.71 mM Val were the most effective in increasing the lactic acid levels and the number of LAB cells in cucumber juice fermentation.
- Riboflavin was the only nutrient, in this study, which inhibited the growth of LAB and resulted in low lactic acid production.
- When selected concentrations of Bio, Ile and Val were used in various combinations; it was found that Bio and Val treatments, either separate or together, could be used as potential supplements to enhance the LAB fermentation in cucumber juice. These treatments increased the number of LAB, LAB dry weight and the lactic acid concentration in the brine.

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APPENDIX

Table 1: Nutrient content of cucumber (raw and peeled, raw and unpeeled)

USDA National Nutrient Database for Standard References, Release 23 (2010)

Nutrient	Units	Value per 100g of edible portion	
Proximates		**Cucumber with peel, raw**	**Cucumber peeled, raw**
Water	g	95.23	96.73
Energy	kcal	15.00	12.00
Energy	kj	65.00	52.00
Protein	g	0.65	0.59
Total lipid (fat)	g	0.11	0.16
Ash	g	0.38	0.36
Carbohydrate, by difference	g	3.63	2.16
Fiber, total dietary	g	0.50	0.70
Sugars, total	g	1.67	1.38
Sucrose	g	0.03	0.00
Glucose (dextrose)	g	0.76	0.63
Fructose	g	0.87	0.75
Lactose	g	0.00	0.00
Maltose	g	0.01	0.00
Galactose	g	0.00	0.00
Starch	g	0.83	0.08
Minerals			
Calcium, Ca	mg	16.000	14.000
Iron, Fe	mg	0.280	0.220
Magnesium, Mg	mg	13.000	12.000
Phosphorus, P	mg	24.000	21.000
Potassium, K	mg	147.000	136.000
Sodium, Na	mg	2.000	2.000
Zinc, Zn	mg	0.200	0.170
Copper, Cu	mg	0.041	0.071
Manganese Mn	mg	0.079	0.073
Fluoride, F	mcg	1.300	1.300
Slenium, Se	mcg	0.300	0.100

Vitamins			
Vitamin C, total ascorbic acid	mg	2.800	3.200
Thiamine	mg	0.027	0.031
Riboflavin	mg	0.033	0.025
Niacin (Nicotinic acid)	mg	0.980	0.037
Pantothenic acid	mg	0.259	0.240
Vitamin B-6	mg	0.040	0.051
Folate, total	mcg	7.000	14.000
Folic acid	mcg	0.000	0.000
Folate, food	mcg	7.000	14.000
Folate, DFE	mcg-DFE	7.000	14.000
Choline, total	mg	6.000	5.700
Betaine	mg	0.100	0.100
Vitamin B-12	mcg	0.000	0.000
Vitamin B-12, added	mcg	0.000	0.000
Vitamin A, RAE	mcg-RAE	5.000	4.000
Retinol	mcg	0.000	0.000
Carotene, beta	mcg	45.000	31.000
Carotene, alpha	mcg	11.000	8.000
Cryptoxanthine, beta	mcg	26.000	18.000
Vitamin A, IU	IU	105.000	72.000
Lycopene	mcg	0.000	0.000
Lutein + zaexanthin	mcg	23.000	16.000
Vitamin E (alpha - tocopherol)	mg	0.030	0.030
Vitamin E, added	mg	0.000	0.000
Tocopherol, beta	mg	0.010	0.000
Tocopherol, gamma	mg	0.030	0.020
Tocopherol, delta	mg	0.000	0
Vitamin D (D_2 + D_3)	mg	0.000	0
Vitamin D	IU	0.000	0.000
Vitamin K (phylloquinone)	mcg	16.400	7.200
Dihydrophylloquinone	mcg	0.000	0.000

Lipids			
Fatty acids, total saturated	g	0.037	0.013
4 : 0	g	0.000	0.000
6 : 0	g	0.000	0.000
8 : 0	g	0.000	0.000
10 : 0	g	0.000	0.000
12 : 0	g	0.000	0.000
14 : 0	g	0.005	0.002
15 : 0	g	0.000	0.000
16 : 0	g	0.028	0.010
17 : 0	g	0.000	0.000
18 : 0	g	0.005	0.002
20 : 0	g	0.000	0.000
22 : 0	g	0.000	0.000
24 : 0	g	0.000	0.000
Fatty acids, total monounsaturated	g	0.005	0.002
14 : 1	g	0.000	0.000
15 : 1	g	0.000	0.000
16 : 1 Undifferentiated	g	0.000	0.000
17 : 1	g	0.000	0.000
18 : 1 Undifferentiated	g	0.005	0.002
20 : 1	g	0.000	0.000
22 : 1 Undifferentiated	g	0.000	0.000
20 : 2 n-6 c, c	g	0.000	0.000
Fatty acids, total polyunsaturated	g	0.032	0.003
18 : 2 Undifferentiated	g	0.028	0.002
18 : 3 Undifferentiated	g	0.005	0.002
18 : 4	g	0.000	0.000
20 : 3 Undifferentiated	g	0.000	0.000
20 : 5 n-3 (EPA)	g	0.000	0.000
22 : 5 n-3 (DPA)	g	0.000	0.000
22 : 6 n-3 (DHA)	g	0.000	0.000
Cholesterol	mg	0.000	0.000
Amino acids			
Tryptophan	g	0.005	0.007
Threonine	g	0.019	0.012
Isoleucine	g	0.021	0.012
Leucine	g	0.029	0.025

Lysine	g	0.029	0.025
Methionine	g	0.006	0.012
Cystine	g	0.004	0.007
Phenylalanine	g	0.019	0.031
Tyrosine	g	0.011	0.002
Valine	g	0.022	0.012
Arginine	g	0.044	0.031
Histidine	g	0.010	0.002
Alanine	g	0.024	0.031
Aspartic acid	g	0.041	0.037
Glutamic acid	g	0.196	0.204
Glycine	g	0.024	0.025
Proline	g	0.015	0.012
Serine	g	0.020	0.025
Others			
Alcohol, ethyl	g	0	0
Caffeine	mg	0	0
Theobromine	mg	0	0

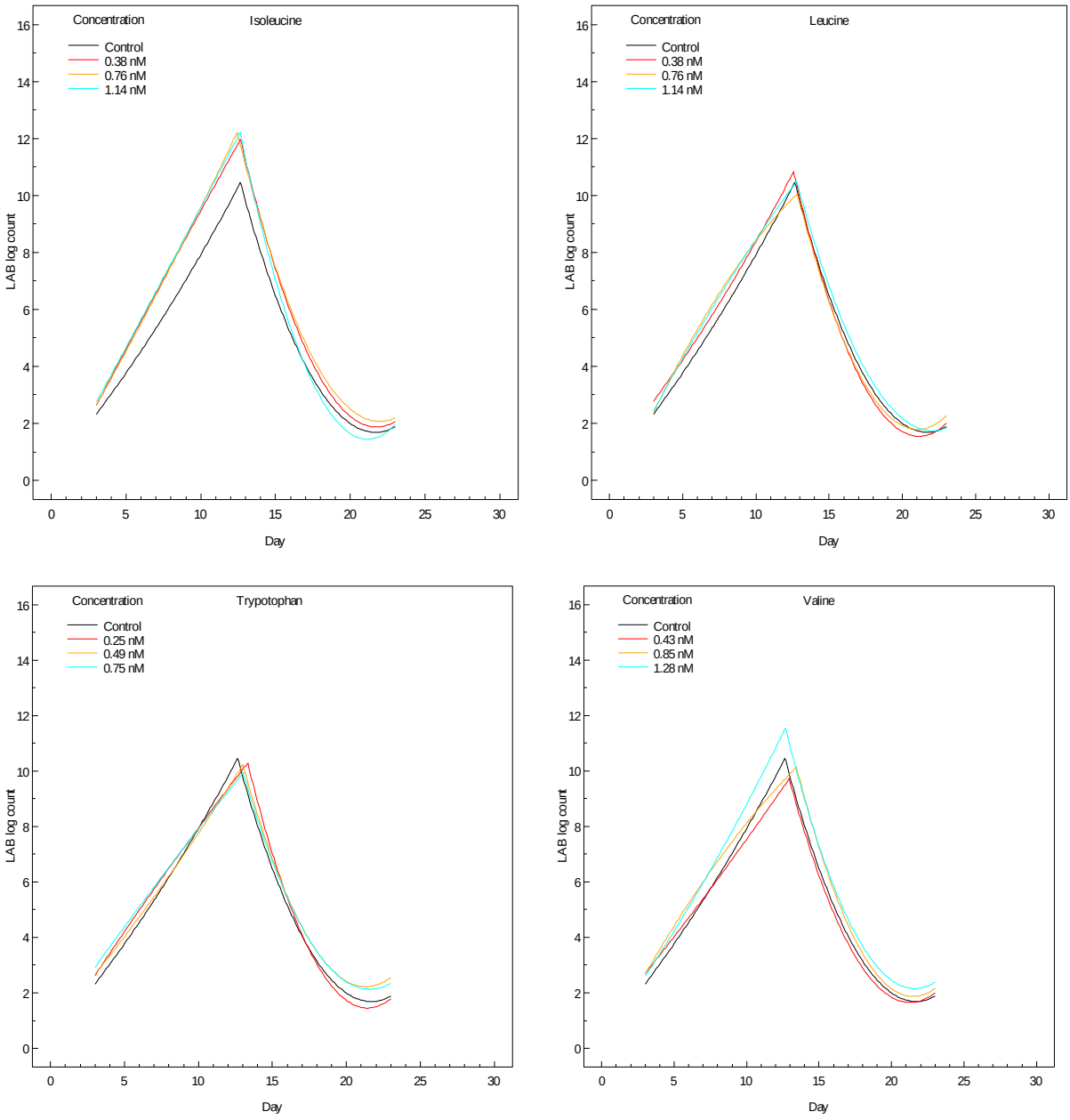
Table 2: Nutrient composition of MRS basal medium

(Fabiana and Nadra 2007)

Constituent	Concentration (g/L)
Glucose	10
Potassium acetate	10
KH2PO4	2
Sodium thioglycollate	0.5
MgSO4.7H2O	0.15
MnSO4.4H2O	0.02
FeSO4.7H2O	0.01
Tween80	0.001
Adenine	0.05
Cytidylic acid	0.05
Deoxyguanosine	0.05
Guanine HCl	0.05
p-Aminobenzoic acid	0.00001
Vitamin B12	0.000001
Calcium pantothenate	0.001
D-Biotin	0.00001
Folic acid	0.0001
Nicotinic acid	0.001
Piridoxal ethyl acetal HCL	0.0005
Riboflavin	0.0005
Thiamine HCl	0.001
DL-Alanine	0.2
L-Arginine	0.005
L-Asparagine	0.2
L-Aspartic acid	0
L-Cysteine-HCl	0.2
L-Glutamic acid	0.15
L-Glycine	0
L-Histidine-HCl	0.05
L-Isoleucine	0.05
L-Leucine	0.06
L-Lysine-HCl	0.05
L-Methionine	0.05
L-Phenylalanine	0.04

L-Proline	0.04
L-Serine	0.1
L-Threonine	0.004
L-Tryptophan	0.05
L-Tyrosine	0.05
L-Valine	0.03

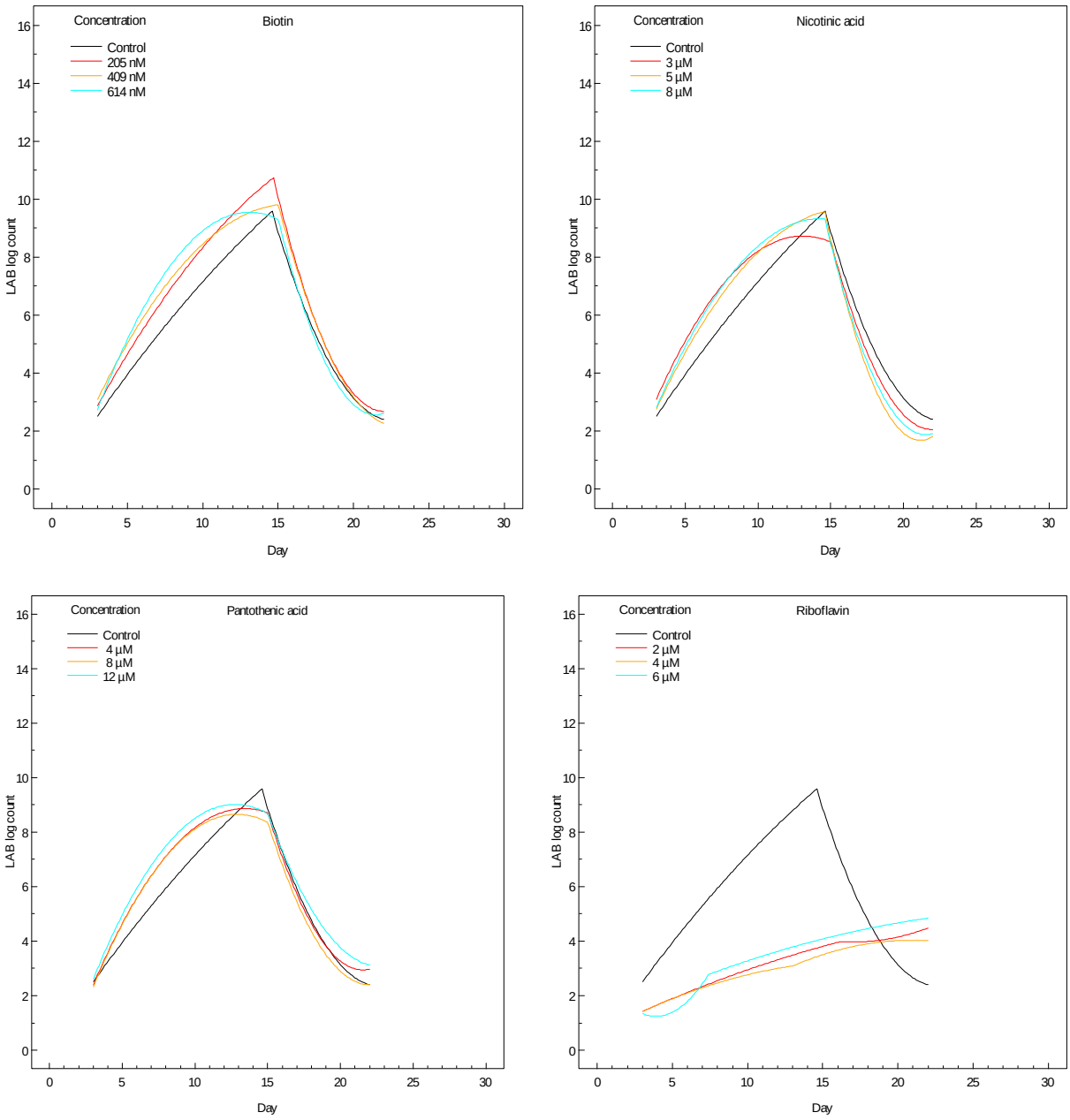
Figure 3: Effect of Leu, Ile, Trp and Val on LAB maximum population



Note:

The logarithms of the viable LAB units were modeled as a function of time allowing the regression coefficient to depend on nutrient types and concentration. The function consisted of two segments, the first of which was a quadratic function of time and the second was a quadratic or a horizontal line, depending on the nutrient and concentration. The estimated maximum log units were determined from the fitted model.

Figure 4: Effect of Biotin, Nicotinic acid, Pantothenic acid and riboflavin on LAB maximum population



Note:

The logarithms of the viable LAB units were modeled as a function of time allowing the regression coefficient to depend on nutrient types and concentration. The function consisted of two segments, the first of which was a quadratic function of time and the second was a quadratic or a horizontal line, depending on the nutrient and concentration. The estimated maximum log units were determined from the fitted model.

Figure 5: Effect of magnesium and manganese on LAB maximum population

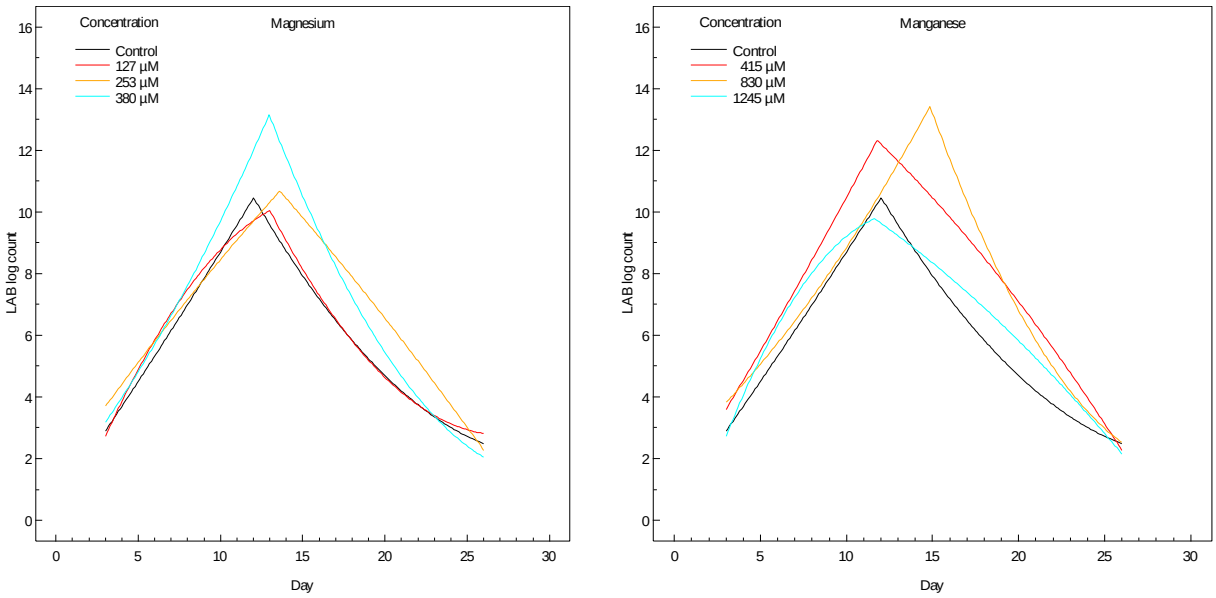
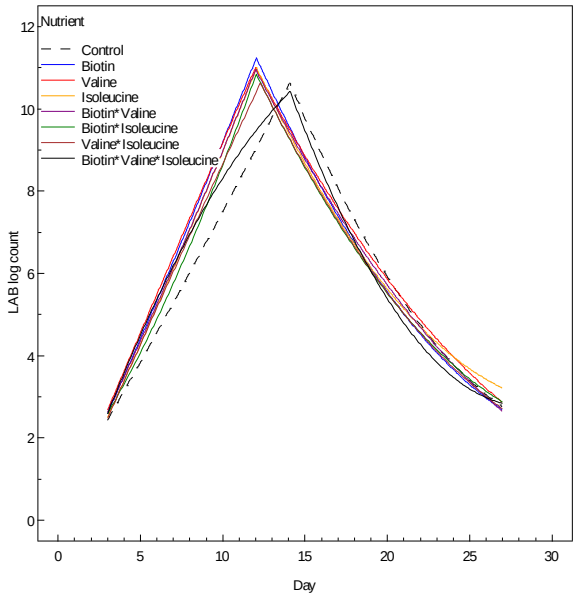


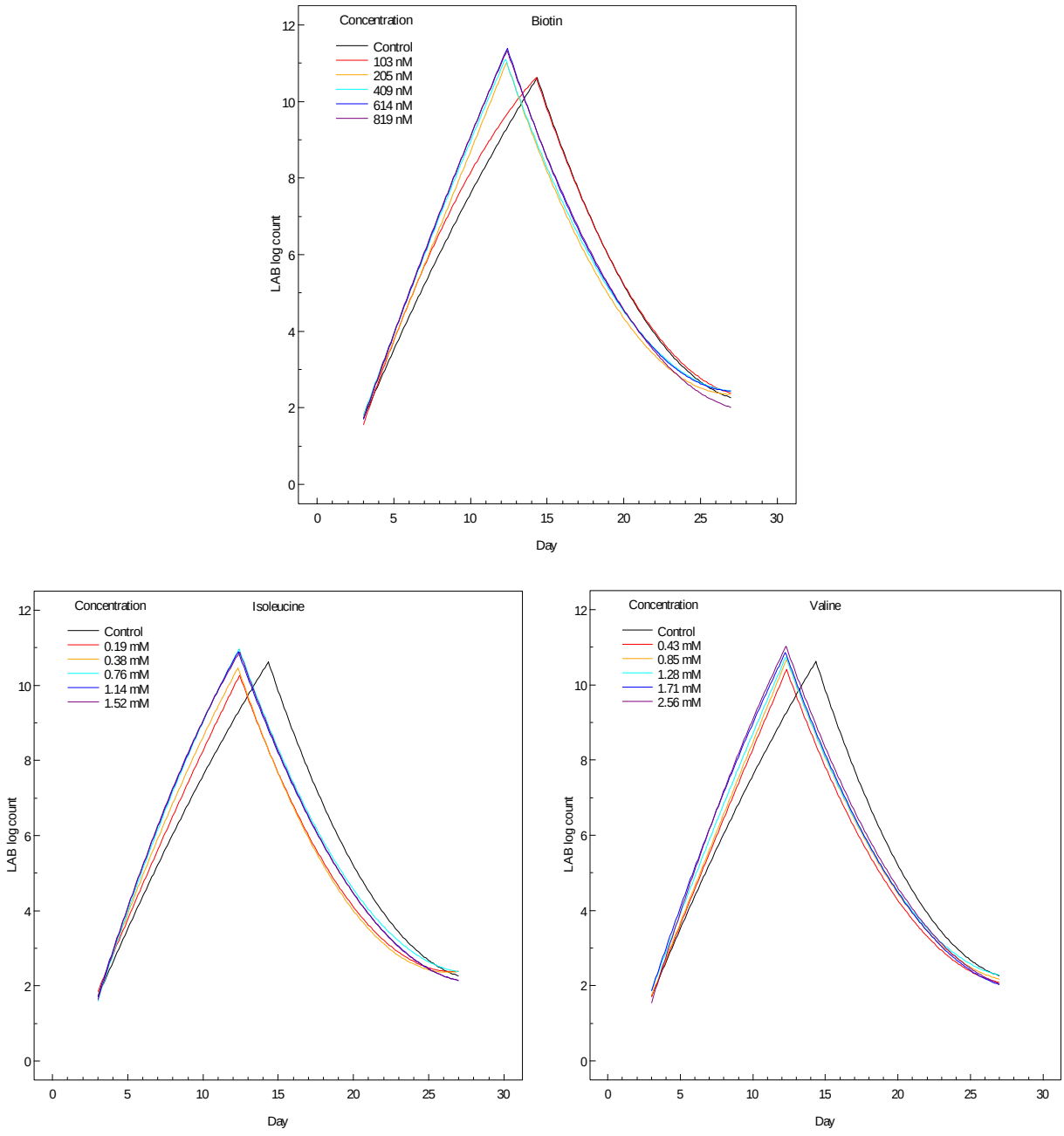
Figure 6: Effect of Bio, Ile and Val used in combination on LAB maximum population



Note:

The logarithms of the viable LAB units were modeled as a function of time allowing the regression coefficient to depend on nutrient types and concentration. The function consisted of two segments, the first of which was a quadratic function of time and the second was a quadratic or a horizontal line, depending on the nutrient and concentration. The estimated maximum log units were determined from the fitted model.

Figure 7: Effect of Bio, Ile and Val on LAB maximum population



Note:

The logarithms of the viable LAB units were modeled as a function of time allowing the regression coefficient to depend on nutrient types and concentration. The function consisted of two segments, the first of which was a quadratic function of time and the second was a quadratic or a horizontal line, depending on the nutrient and concentration. The estimated maximum log units were determined from the fitted model.

Figure 8: Comparing data form microscopic count technique (MC) and plate count technique (PC), the effect of Bio, Ile and Val on the total lactic acid bacteria count on complete fermentation of cucumber juice.