THE INCLUSION AND SURVIVAL RATE OF A BIFIDOBACTERIAL STRAIN ADDED TO A FERMENTED MILK -WHEY BEVERAGE

By

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ABSTRACT

A fermented milk-whey beverage was developed using previously standardized formulations, namely 100% milk and 0% whey and 75%-25% milk-whey respectively. Titratable acidity (TA) and pH of the beverages was measured to asses the fermentation process and the quality of the beverage. The fermentation process was stopped once the pH reached an approximate value of 4.5. The TA was measured as percentage of lactic acid using a solution of 0.1N NaOH and final TA was comparable to previously reported values for fermented milk beverages. The evaluation of this beverage as a potential probiotic food product was assessed by incorporating probiotic bacteria from the genus *Bifidobacteirum* and monitoring it's survival during refrigerated storage of 21 days. The strain used, *Bifidobacterium animalis* (Bb12) is among the most resistant to acidity, one of the factors known to adversely affect its survival next to oxygen toxicity. No significant difference was observed in the survival of bifidobacteria between the formulations. Results obtained showed that bifidobacteria maintained cell counts above those required for the label of "probiotic" (6 Log_{10}) up until the 18 days. The mean cfu ml^{-1} was of 1.03×10^7 for the 100% formulation and 5.73×10^6 for the 75-25% formulation on day 18. It can be concluded that there was no significant difference between treatments throughout the storage period.

The bifidogenic effect of the recognized prebiotic inulin was assessed by the supplementation with 1% of this oligosaccharide in fresh milk, UHT milk and MRS lactobacilli broth. The results obtained showed that even at this low supplementation level a bifidogenic effect was still achieved. Bacterial cell counts for the UHT with prebiotic and MRS with prebiotic evidenced a significant increase in log counts for day 3. For UHT an increase to 8.43 Log_{10} was achieved, which was significantly higher than MRS with no prebiotic for the same day which was 2.08 Log_{10}. Thus the supplementation of UHT milk with 1% inulin represents a viable growth media for bifidobacteria.

The identification to genus and species level of several presumptive bifidobacterial isolates was attempted. Using a genus specific primer set (Bifid-F and Bifid-R), all isolates tested were found to be from the genus *Bifidobacterium*. The ribosomal DNA of the isolates was extracted and purified and PCR amplified the digested using *EcoRI* and *DdeI*. Results obtained were comparable to the results obtained insilico. All isolates showed bands of the same length comparable to the control *Bifidobacterium animalis* therefore it can be concluded all isolates are *Bifidobacterium lactis ssp animalis* or *Bifidobacterium lactis ssp lactis*.

RESUMEN

Una bebida fermentada a base de leche y suero de leche fue elaborada usando unas formulaciones previamente estandarizadas, 100% leche y 0% suero y 75%-25% leche-suero respectivamente. La acidez titulable (AT) y el pH de las bebidas fue medida para asegurar el debido proceso de fermentación y la calidad general de la misma. El proceso de fermentación se detuvo una vez se alcanzó un pH de 4.5. La AT fue medida como porciento de ácido láctico usando una solución de 0.1N NaOH. Esta presentó un valor final comparable con valores previamente reportados para este tipo de bebidas. La evaluación de esta bebida como un alimento potencialmente probiótico fue analizada mediante la incorporación de una bacteria probiótica del género *Bifidobacterium* y el monitoreo de su sobrevivencia durante almacenamiento refrigerado (4^{o}C) durante 21 días. La cepa usada, *Bifidobacterium animalis* (Bb12) es una de las más resistentes a las condiciones de alta acidez, uno de los factores que afectan adversamente la sobrevivencia de bífido, después del oxígeno. No se observó diferencia significativa en la sobrevivencia de bífidobacteria entre las formulaciones. Los resultados obtenidos demuestran que bífidobacteria mantuvo conteos de células por encima de lo requerido para poder ser etiquetada como una bebida "probiótica" hasta el día 18. La media de ufc ml^{-1} fue de 1.03 x 10^{7} para la formulación 100% y 5.73 x 10^{6} para la formulación 75-25% en el día 18. Se puede concluir que no hubo diferencia significativa entre tratamientos para el período de almacenamiento.

El efecto bifidogénico de la inulina, un prebiótico reconocido, fue evaluado mediante la suplementación con 1% de este oligosacárido en leche fresca, leche esterilizada comercialmente "UHT" y caldo MRS. Los resultados obtenidos demuestran un efecto bífidogénico aún a este bajo porciento de suplementación. El conteo de células bacterianas para UHT con prebiótico y MRS con prebiótico demuestra un incremento a significativo para el día 3. Para UHT se observó un aumento hasta 8.43 Log_{10}, el cual fue significativamente mayor que para caldo MRS sin prebiótico para el mismo día (2.08 Log_{10}). La suplementación de leche UHT con 1% de inulina representa una opción viable como medio de cultivo para bífidobacteria.

La identificación a nivel de género y especie para varios especímenes presuntivos de bifidobacteria fue llevada a cabo. Se usó un set de "primers" específicos para género (Bifid-F

and Bifid-R). Todos los especímenes fueron positivos para el género de *Bifidobacterium*. El DNA de los especímenes fue extraído y purificado para llevar a cabo una doble digestión usando *EcoRI* and *DdeI*. Los resultados obtenidos fueron comparables a aquellos obtenidos del análisis insílico. Todos los especímenes mostraron bandas del mismo tamaño que el control positivo, *Bifidobacterium animalis* asi que se puede concluir que todos los organismos son o bien *Bifidobacterium lactis ssp animalis* or *Bifidobacterium lactis ssp lactis*.

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DEDICATION

To God

Let us hear the conclusion of the whole matter: Fear God, and keep his commandments: for this is the whole duty of man.
Ecclesiastes 12:13

To my family
Papi, Mami and Vani

In memory of my loving paternal grandmother

Luz Delia Paoli Crespo

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1 INTRODUCTION

The importance of milk and dairy products has been recognized since the early times of men, evidence of the consumption of fermented milks dates back to biblical times (Leahy et al., 2005). Bovine milk is a rich liquid nutrient secreted by the mammary gland of female mammals to feed their newborns. Generally, it contains 87.2 % of water, 4.95 % of lactose, 3.35 % of proteins (casein 2.75 % and albumin 0.60 %), 3.80 % of fat and 0.70 % of minerals (Marth and Steele, 2001). The intake of milk has always been associated with health benefits, like the provision of calcium to maintain a healthy bone structure. However in recent years the Dairy Industry is facing a hard reality, the consumption of milk around the world has decreased (Boor, 2001). An Agricultural Information Bulletin of the USDA confirms that "milk consumption is low and that soft drink consumption outstrips the former in virtually all age and gender categories, except those ages 1-8" (Frazao, 2005).

Today, milk is being replaced by soft drinks and juices that claim to provide calcium, once the traditional role of milk. In a study by Boor (2001), the author concluded that in order for the dairy industry to survive it must be able to produce fluid products that can compete with shelf-stable products available in the market. Although fluid milk consumption is lower, an increasing line of milk and dairy products have gained public interest. Yogurt and fermented milks are more shelf-stable and the variety and innovation are attractive to the consumer. New markets aim to provide milk in a way that maintains its nutritional value but is innovative in convenience and presentation. As a result, there has been an increasing effort to produce flavored milk beverages or dairy products that include probiotic bacteria (Vinderola et al., 2000).

In the last decade the scientific community and more recently the Food Industry, have acknowledged the fact that daily diet has an influence and marked effect on human health. A study performed by Cienfuegos et al. (2004) reveal that the major causes of death are related to genetic and environmental factors, of which the diet is the most relevant source. Thus in late years there has been a surge of food products that address,

not only the nutritional needs for the body's daily functions, but also provides the consumer well beyond the requirements for survival, promoting health and well being. These products are known as functional foods, and the Institute of Food Technologists described them as, "food and food components that provide a health benefit beyond basic nutrition (for the intended population)" (IFT Expert Report, 2005).

Products claiming to include beneficial bacteria, more commonly known as probiotics, are on the rise in the market and the public is beginning to be aware of the health benefits these microorganisms provide. Probiotics have been described as "a live microbial supplement that is beneficial to health" and to provide this health benefits a concentration of 10^6 cfu/g of product has been suggested (Tamine, 2002 and Shah, 2000). These bacteria are widely accepted and generally regarded as safe (GRAS) (IFT Expert Report, 2005). Traditional routes for administering probiotics are through fermented dairy products (Tamine, 2002). In the past these were often used therapeutically, before the existence and benefits of microorganisms were recognized (Leahy et al., 2005). More recently they are being provided in capsule form, in combination with prebiotics, and as live active cultures in many commercial food products. Prebiotics are defined as non digestible food ingredients that may beneficially affect the host by selectively stimulating the growth of specific bacteria in the colon (Leahy et al., 2005).

With an increasing number of health and nutrition conscious consumers, the Food Industry is being driven to provide products that will not only target the consumers organoleptic (taste and attractive presentation) expectations, but provides a healthy eating choice as well. In meeting these demands, universities and research institutions play a major role in making new discoveries and properly addressing the needs of a changing society. Recently there has been an increased interest in the development and commercialization of fermented milk beverages, due mostly to their association with health benefits (FAO/WHO, 2001). There has also been an increase in the use of hard chesses as a venue for probiotic organisms, but the production of chesses faces the problem of wastewater and whey effluent disposals.

In Puerto Rico white cheese is prepared by precipitating the casein with acetic acid and as a result large amounts of acid whey are produced. One of the reasons for developing a fermented whey-milk beverage is to make use of as much of this whey effluent as possible. In Puerto Rico the Dairy Industry had a total of 69, 928 million quarts of milk surplus for the fiscal year 2005-2006 (Informe Annual ORIL, 2005-2006). In the year 2005, 6 million quarts of surplus milk were used for the production of artisanal white cheese. As a result of this process 4 million quarts of acid whey were produced. Whey effluents contain 5% of lactose, 0.8% proteins, minerals and vitamins which make it a rich nutritional source. In Puerto Rico only a small quantity of these effluents are employed for animal feedstock, the remaining is an environmental contaminant that must be properly disposed of by the farmer. The high costs involved in disposing these wastes, and the strict environmental regulations of government agencies, make it necessary to find alternate methods to deal with this waste material.

In attempts to provide an alternative solution to the problem of whey disposal, common in cheese elaboration plants, an innovative use for whey permeates is needed. The elaboration of a fermented milk beverage that partially utilizes whey as one of its major components was proposed. The main purpose of this thesis was the inclusion of Bifidobacterium species, to act as probiotic, in a fermented acid whey-milk beverage and the determination of its survival rate throughout the shelf life of the product. The addition of a probiotic organism can make this beverage attractive to consumers looking for healthy alternatives to regular milk or soft drinks. The secondary aim of this project was to evaluate prebiotic supplemented milk as a possible growth media for bifidobacteria. Lastly, to properly identify previously isolated presumptive bifidobacteria isolates to the genus and species level by traditional and molecular methods.

2. LITERATURE REVIEW

2.1 Milk

Milk is the secretion of the mammary gland of female mammals and its main purpose is to provide nutrition and immunological protection for the newborn of the species (Robinson, 2002). The major components in milk are water, lactose (carbohydrate), proteins (casein and whey proteins) and fat, but some of these can vary depending on feed composition and breed of the animal (Marth and Steele, 2001). Other minor components present in milk are vitamins, minerals or salts and enzymes (**Appendix 1**). Due to the high nutritious components available in milk and its high water activity (Aw) it is a very perishable food product. Therefore several treatments are applied in order to minimize spoilage and lengthen its shelf life. Among these, heat treatments like pasteurization or sterilization by ultra high temperature (UHT) are often employed. According to the International Dairy Federation pasteurization of milk involves "minimizing health hazards from pathogenic microorganisms" while sterilization also destroys spores (Britz and Robinson, 2008). The disadvantage of heat treatments, especially sterilization, is the loss of some of its nutritional value and the perceptible organoleptic changes (cooked flavor).

The microbiology of raw milk can vary in numbers and type of microorganisms present, depending on production and storage conditions. Initial micro flora found in milk range from psychrotrophs, mesophilic to thermoduric which survive pasteurization. In pasteurized milk psychrotrophic microorganisms, often the result of post contamination, are able to survive and reproduce under refrigerated conditions and are responsible for its spoilage (Robinson, 2002).

2.2 Whey

Whey is the separated watery portion of milk obtained by acid precipitation aided by heat or milk coagulation with rennet (Corre et al., 1992). The most commonly used in the United States (US) for cheese manufacture is the rennet, and the result of this is the production of sweet whey. In contrast the use of any acid (acetic or citric) results in acid whey. It contains all the soluble components of milk; minerals, vitamins, some nitrogen compounds and lactose. Whey concentrate has long been used as feed for bovines and small ruminants, but some of the whey generated industrially becomes a waste effluent. The Clean Water Act (CWA) from 1972 gave the Environmental Protection Agency (EPA) the task of developing effluent limitation guidelines that provided a minimum technology based threshold for improvement in effluent quality (EPA, 2000 and Federal Register 2008). Under the CWA, EPA has revised the National Pollutant Discharge Elimination System (NPDES) and set Effluent Limitations Guidelines (ELGs) for concentrated animal feeding operations (CAFOs) (40 CFR Parts 9, 122 and 412, 2008). CAFOs are categorized by size depending on the amount of animals in a given agricultural plant (large CAFO 700-1000 cows). In a personal communication with a Puerto Rican cheese industry representative, it was confirmed that an approximately $140USD is spent about 3 times weekly for waste disposal in this small scale (350 litters per lot) cheese producing enterprise (Rosa Avalo, Quesos Vaca Negra™). If production in this industry were to expand cost for waste disposal would also rise significantly.

Whey has lately become a focal point of interest for many scientists in the last decade. Recent publications have shown that whey protein concentrates (WPC) and bioactive peptides might have beneficial activities in human health (Etzel, 2004 and Krissansen, 2007). Thus it has become an attractive option for inclusion in products claimed as nutraceuticals and as a prebiotic adjunct to act in synergy with probiotics. Akalin et al. (2007) reported increased viable counts of yogurt bacteria and probiotic *Bifidobacterium animalis* in reduced fat yogurt during storage with the addition of WPC. Results obtained by Gomes et al. (1998), suggest that the poor growth of bifidobacteria in milk is due in part by the lack of small peptides and free amino acids. Although milk

contains all of the essential nutrients for growth, these are not always present in optimal amounts or in a form that can be readily used by the organism. Several of the amino acids known to be essential for the growth of bifidobacteria and other lactobacilli are present in insufficient amounts (e.g. arginine, leucine, isoleucine, valine, glutamic acid, tryptophan, tyrosine, and cysteine) therefore available nitrogen becomes a limiting factor (Shah, 2002 and Gomes 1998.).

The soluble proteins of the whey fraction are albumin (α-lactoalbumin, β-lactoglobulin and serum-albumin), immunoglobulins, lactoferrin, lactoperoxidase, proteose peptone, glycomacropeptide (by chymosin action on casein) and minor proteins (Krissansen, 2007). A nitrogen source in the form of peptides and amino acids from whey concentrate and acid casein hydrolisate has been shown to be responsible for improvement in the viability of bifidobacteria (Shah, 2002.)

2.3 Fermented Milk

Fermented milks have been manufactured for long generations, believed to have originated on what is today known as Russia and Turkey. They have become increasingly popular in the western world (Tamine, 2002). The advantages of fermented milks include the extension of its shelf-life, the enhancement of its digestibility and flavor improvement (Tamine, 2006). The fermentation processes in the past were the result of environmental factors and different mixtures of lactic and non lactic acid bacteria (LAB). As a result the first fermented milks varied in flavor from the ones made today. In recent years due to better scientific knowledge, fermentations are conducted in controlled environments and with the addition of specific known starter cultures.

Fermented Milks are produced by three different types of fermentation, namely lactic fermentation, mold-lactic and yeast-lactic fermentation. Products such as yogurt and Skyr are produced via lactic fermentation, while Villi is produced by mold-lactic fermentation. Kefir and Koumiss are produced by yeast-lactic fermentation and involve mesophilic and thermophilic LAB and yeasts. This type of fermented milk is mildly

alcoholic and has an effervescent characteristic as a result of CO_2 production (Robinson, 2002).

Kefir originated in the Caucasus region where it has been produced by a traditional method (Tamine, 2006) that involves the addition of kefir grains, a complex microbiological flora containing a blend of lactic acid bacteria, yeasts, acetic acid bacteria and possibly a mold. Their microflora is very stable and they retain activity for years if preserved in appropriate culture and physiological conditions. The exact microflora of the grains is not well known and depends on variables such as origin of starter culture, growth conditions and type of milk used (Tamine, 2006). Nevertheless some microorganisms have been associated and identified including, *Saccharomyces spp*., *Mycotorula spp*. *Lactococcus spp*., *Streptococcus thermophilus*, *Geotrichum spp*. and *Acetobacter acetirasens*. There are also commercially available kefir starter cultures which differ from the original in form and composition. These ensure a more controlled and less difficult elaborating process of kefir (Tamine, 2006).

2.4 Bifidobacteria

2.4.1 Brief History

Bifidobacteria were first discovered in the 1900's by Henry Tissier who isolated the bacteria from infant feces and later suggested their use to aid in the restoration of healthy gut flora (Balongue et al., 1993 and Tissier et al., 1906). He described it as gram-positive, non gas-producing, non-motile anaerobic rod shaped bacteria with bifid morphology (Y and V shapes) and named it *Bacillus bifidus* (Balongue et al., 1993 and Tissier et al., 1900 and Leahy et al., 2005). Following Tissier's discovery, there was much controversy in the scientific community regarding the proper naming and classification of this new found bacterium. In 1924 Orla-Jensen, a Danish microbiologist recognized *Bifidobacterium* as a separate taxon (Mitsuoka et al., 1977). Bifidobacteria are prokaryotes, with a high guanine + cytosine (G+C) content (55-67%) that naturally colonize the gastrointestinal tract (GIT) of humans, other warm blooded animals and honey bees (Balongue et al., 1993 and Biavati *et al*, 2006).

The pathway of hexose fermentation in bifidobacteria is the fructose-6-phosphate shunt which results on the production of lactic acid and acetic acid in the theoretical ratio 1.0:1.5 respectively (Biavati et al., 2006).They are catalase negative, obligate anaerobes whose optimum growth temperature is between 37^{o}C and 41^{o}C (Biavati et al., 2006). Bifidobacterial human strains grow at an optimum temperature of 36-38^{o}C, while animal strains have a higher optimum growth temperature of 41-43^{o}C (Biavati et al., 2006). Bifidobacteria are acid-tolerant microbes and their optimum growth occurs at pH of 6.5-7.0 (Mahalakshmi et al., 2000 and Shah, 2000). Currently an approximate 34 species of Bifidobacteria are recognized (Bottacini et al., 2010) and they are mostly isolated from the gastrointestinal tract of a variety of animals, some of these species are host specific (Leahy et al., 2005). Of these, thirteen are isolated from humans, fourteen are from animal source, two are isolated from sewage waters and one is found in fermented milk (Leahy et al., 2005, Kaufmann et al., 1997).

Bifidobacterium spp. are very fastidious microorganisms, they have been reported to grow poorly on milk (Abu-Taraboush et al., 1998 and Gomes et al., 1998) and are sensitive to acidic conditions (Matsumoto et al., 2004). Therefore they present a problem when used as probiotic agents. In an attempt to improve their growth conditions, some researchers have occasionally supplemented milk with fructooligosaccharides (FOS) and other nutrients that have been proved to be bifidogenic factors, like lactulose and meat extract (Hopkins et al., 1998; Nebra and Blanch, 1999 and Shah, 2000). The presence of oxygen and the redox potential have been shown to affect the growth and viability of bifidobacteria (Jayamanne and Adams, 2009). The addition of ascorbic acid can serve as an oxygen scavenger and the addition of L-cysteine has been proved to decrease redox potential (Shah, 2000). Others have reported an improvement in the viability of some bifidobacteria species by incorporating 0.05% of L-cysteine to the medium (Biavati et al., 2000 and Shah, 2000). The following are the more common intrinsic parameters which make difficult the growth and use the genus Bifidobacteria as probiotic:

2.4.1 a pH Sensitivity

Optimun pH for sustained growth is between the ranges of 6.5 and 7.0 and no growth is reported at pH < 4.0 and pH >8.5 (Biavati et al., 2000). Acid tolerance has been found to vary by strain and developers of probiotic products will have to take into account not only the acidic conditions of the product but post-acidification of fermented dairy products during storage (Shah, 2002).

2.4.1 b Oxygen Toxicity

Oxygen toxicity results from the effects of activated oxygen compounds including superoxide, hydrogen peroxide and hydroxyl radicals. Superoxide dismutase and catalase (key enzymes that scavenge oxygen metabolites) are absent in bifidobacteria (Jayamanne and Adams, 2009). It has been reported that bifidobacteria posses the enzyme NADH-peroxidase, which converts H_2O_2 into H_2O but its activity varies among the particular strains (Shimamura et al., 1992). Jayamanne and Adams (2009) concluded that the higher survival of *B. animalis ssp. lactis* could be due to higher activity of the NADH-peroxidase.

2.4.2. Use as Probiotics

Although many challenges are faced in the development of probiotic products their use has steadily increased in recent years (Sanders and Marco, 2010). Bifidobacteria constitute about 95% of the total gut bacteria in healthy breast fed infants, but their number decreases with age (Desjardins et al., 1989 and Satokari et al., 2003). Their presence in the human gastrointestinal tract has been associated with a number of health benefits. They have been known to exert biological activities related to host health (Biavati et al., 2000 and Leahy et al., 2005). One major recognized contribution of these friendly bacteria is their antagonistic effect on other microorganisms in the gut, including potential pathogenic bacteria. Their mechanism of inhibition is thought to be related to the production of acetic and lactic acids and to the excretion of bacteriocins by some species (Biavati et al., 2000). In an *in vitro* study performed by Gibson and Wang (1994) they described the antibacterial activity of a *Bifidobacterium* strain against *Salmonella*, *Listeria*, *Campylobacter*, *Shigella* and *Vibrio cholerae* highlighting the importance that

inhibitory mechanisms have in preventing gastroenteritis. The use of *B. bifidum* to treat infant diarrhea induced by infection with rotavirus was reported to reduce the incidence of infection and a *B. lactis* strain has been used to treat acute diarrhea in healthy children (Leahy et al., 2005 and Saavedra et al., 1994). Bifidobacteria have also been used to treat people suffering from lactose intolerance and is believed to alleviate constipation (Leahy et al., 2005).

Therefore the concept of probiotics is by no means new, the use of live microorganisms in products like kefir and koumiss originated centuries ago in Turkey, Russia and Asia (Tamine, 2002, Leahy et al., 2005). These products were often used as therapeutics before any knowledge of beneficial microorganisms was scientifically recognized. The term probiotic meaning "pro life" is derived from the Greek and was introduced in 1965, but it was not until 1989 that it became popularized (Leahy et al., 2005). Today the Food Organization of the United Nations (FAO) and the World Health Organization (WHO) describes probiotic as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO., 2001). In order for probiotics to perform their health related claims, they must be present throughout the shelf-life of the product and must be viable at all times (Velázquez and Feirtag, 1997). It has been suggested that the concentration probiotic bacteria necessary in order to provide health benefits be equal to or over 10^6cfu/g (Shah, 2000).

2.4.3. Identification

Even when it is believed that bifidobacteria exerted a beneficial role in the host, the means by which it does so is still largely unknown (Cronin et al., 2011). Prior to the age of molecular biology, the surest way to assign a bacterial strain to the genus *Bifidobacterium* is by the use of the Fructose-6-Phosphoketolase Test as described by Scardovi and Trovatelli (1969). The fructose-6-phosphoketolase is a key enzyme unique to bifidobacteria species; this enzyme splits the hexose phosphate to erythrose-4-phosphate and acetyl phosphate. Through the successive action of transaldolase and transketolase, the tetrose and hexose phosphates form pentose phosphates. These phosphates then via the 2-3 cleavage give rise to lactic and acetic acid in the theoretical ratios of 1.0:1.5 (Balongue et al., 1993 and Biavati et al., 2006). The use of

morphological characteristics, phenotypic traits and end product formation, such a carbohydrate fermentation patters is time consuming and not discriminatory enough (Matsuki et al., 2003).

More recently new and improved methods have been developed to aid in the fast identification of the genus, thus making it possible to accurately discriminate between species at the genetic or molecular level. Matsuki et al. (2003) developed genus and species-specific primers based on the 16SrRNA sequences for the identification of bifidobacteria. The purpose is to be able to use tools such as PCR to quickly identify bifidobacteria from other organisms in a complex environment and also to be able to distinguish one strain from another. This is important since many of the probiotic traits associated with bifidobacteria are believed to be strain dependant (Cronin et al., 2011).

Other molecular methods have used hybridization probes such as lm3 (Kaufmann et al., 1997) oligonucleotid probes (Ben Amor et al., 2007), housekeeping genes such as atpD and groEL (Ventura et al., 2007). The identification of putative orthologs (Cronin et al., 2011) has led to the construction of a Bifidobacteria supertree that is in agreement with the 16SrRNA phylogenetic tree (Bottacini et al., 2010 and Ventura et al., 2007). Kaufmann et al. (1997) established a method for identification from food by using a polymerase chain reaction (PCR) assay by breaking the cells with proteinase K. By the PCR method all bifidobacterial species lead to a distinct band of 1.35kb. Recently, Briczinski and Roberts (2006) improved a Pulsed-Field Gel Electrophoresis (PFGE) method in order to analyze bifidobacteria within 24 hours. PFGE is a highly discriminatory molecular typing method that compares fragment patterns of restriction-digested chromosomal DNA.

There is also an ongoing effort to sequence the genome of various species and strains of bifidobacteria, since this will provide an insight into the molecular foundations of the human-bacterial symbiosis and will provide further knowledge regarding the interaction of these microbes in the host's GIT (Leahy et al., 2005). In 2004 two genomes of *B. longum* were completed and made publicly available. To date eleven bifidobacterial strains have been sequenced and some genomes are partially available (Cronin et al.,

2011). Several others are partially completed such as *B*. *breve* (Leahy et al., 2005) and others available at GenBank. Since different species of bifidobacteria are associated with different health benefits, the analysis and elucidation of each species and strains distinguishing features will impart important data regarding their probiotic nature.

2.5 Prebiotics

Prebiotics are defined as non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve health (Rossi et al., 2005). These complex non digestible food ingredients are derived from various sources (onions, asparagus and leeks or commercially synthesized) and offer the target organism an advantage against others who can not utilize this rich source. Most recognized prebiotics are carbohydrates. Those who have consistent evidence for prebiotic effects are nondigestible oligosaccharides (NDOs) like inulin and lactulose (Crittenden and Playne, 2009). Bifidobacteria are able to utilize a wide range of carbohydrate sources, including mono-, oligo- and polysaccharides as energy sources (Vernazza et al., 2006).

Supplementation with prebiotics is a strategic way to manipulate intestinal microbiota and has several advantages over the use of probiotics alone, such as stable shelf life and physicochemical properties useful to the food industry. A more popular way to provide prebiotics is in combination with probiotics in a synergistic form as with New Chapter's Probiotic Immunity® capsules.

3. MATERIALS AND METHODS

3.1 Acquisition Bacterial strain.

Bifidobacteria isolates were obtained from a commercial brand of probiotic capsules (Kyo-Dophilus 9®) sold in health stores claiming to contain the following, *Bifidobacterium longun* strain B., *B. longum* strain M., *B. bifidum*, *B. infantis*, *B. breve* and *B. lactis*. The capsules were reconstituted in 0.1% peptone water and grown on MRS broth supplemented with L-cysteine. Serial dilutions were made and plated unto MRS agar plates and individual colonies were picked. The capsules were found to be contaminated by un-identified Gram + cocci strains and were thus discarded from future use. Those colonies that were presumptive bifidobacteria were frozen in cryogenic vial (-80°C) for future confirmation awaiting a second F6PPK assay. The reference strain used for this study was *Bifidobacterium animalis subsp. animalis* marketed as Bb-12 (Bb-12®, Chr. Hansen, Hønsholm, Denmark), kindly donated by Kraft Foods North America, Tarrytown, NY.

3.2 Growth Media and Culture Conditions

Commercial lyophilized *Bifidobacteium animalis susp. animalis* (Bb-12®, Chr. Hansen, Hønsholm, Denmark) was reconstituted in 0.1% peptone water then grown on de Man Rogosa Sharpe (MRS) Lactobacilli Broth (Difco, BD Diagnostics, USA) + L-cysteine and incubated in an anaerobic jar with GasPak Plus gas generating system (BBL, Becton Dickinson& Co.) at 37^{o}C for 48h. Bb12 was continuously subcultured every 72 hours in MRS to maintain viability until used. A modified MRS medium was prepared containing; lithium chloride and propionic acid as a selective agent, lactulose (a bifidogenic factor, promotes Bifidobacterium growth) and vitamin B (riboflavin) (Shah, 2000). The pH of the medium was adjusted to 6.0 with sterile NaOH 2.5N. A 500mls overnight grown Bb12 fresh stock culture was inoculated on 4L of the previously autoclaved modified medium and incubated anaerobically, via the continuous flow of Nitrogen (N^2), at 37˚C on a Bioreactor (BIOFLO 3000, New Brunswick Scientific, Edison, N.J., U.S.A.) for a period of approximately 48h. Cells were then harvested in polypropylene Nalgene bottles and centrifuged at 15,000rpm or 18,347rcf (Hirayama

Centrifuge) x 25min at 4°C. The pellets were then washed with the sterile peptone water (pH 7.0) and immediately added to the fermented beverages.

3.3 Growth Curve

A growth curve was performed in order to establish the specific generation time of Bb12. An overnight stock culture grown on MRS + L-cysteine incubated anaerobically at 37^{o}C was used to obtain the inoculum. Bifidobacteria was inoculated in sterile media described previously and incubated in anaerobic glass bottles where oxygen had been displaced by nitrogen gas injection. Samples were taken out using a sterile syringe to avoid introducing oxygen to the medium. Optical density (OD) measures (in duplicate) were taken at intervals of 30 minutes and read at 600nm.

3.4 Prebiotic Supplementation Assay

A prebiotic solution was prepared from Orafti HP (Beneo, Orafti Chile, S.A.) a high yield inulin extracted from the artichoke plant. It is composed of inulin (>99.5%) and glucose, fructose and sucrose (<0.5%) with a degree of polymerization (DP) >5 (>99%). The solution was prepared by slowly adding the fine powder to sterilized distilled water and warmed until it was completely dissolved. The solution was then filter sterilized (0.22μm) and stored at room temperature for immediate use. An overnight stock culture of Bb-12 was grown on MRS lactobacilli broth supplemented with 0.05% of L-cysteine and incubated at 37^{o}C in an anaerobic bottle. A volume of 1mL of this bacterial stock solution was used to inoculate each 100mL growth media to be analyzed, namely fresh milk (F), UHT milk (U) and MRS (M). Each treatment bottle contained 10mL of prebiotic (Orafti HP) solution (10%), 1mL of cysteine-sulfide solution (2.5%) and 88mL of the growth medium to be analyzed and a control with no prebiotic solution for each of the treatments. All bottles were incubated at 37^{o}C, aliquots of 1ml were taken at 0h, 24h and 48h of incubation. Serial dilutions in peptone water were performed to obtain plate dilutions of 10^{-2}-10^{-3} (0h), 10^{-4}-10^{-6} (24h) and 10^{-4}-10^{-7}(48h). Samples were plated in MRS/X-gal plates and incubated in anaerobic jars at 37^{o}C for 48h and when opened left for 1h in contact with oxygen prior to counting blue colonies. (Figure in **Appendix 5**).

3.5 Identification of Presumptive Isolates

3.5.1 Traditional Methods

Phenotypical Identification

Isolates were characterized based on colony morphology, cell morphology and Gram staining reaction. Presumptive bifidobacteria colonies were chosen and further analyzed by the Fructose-6-phosphate phosphoketolase assay (Biavati et al, 2006 and Velázquez and Feirtag, 1997). Bb-12 was a pure culture obtained in lyophilized form from a commercial source and used as a positive control.

F6PPK Assay

Presumptive bifidobacterial colonies were analyzed by the Fructose-6-Phosphoketolase Test as described by Scardovi and Trovatelli (1969) with a modification by Velázquez and Feirtag (1997). Briefly, samples were grown in a 10% inoculum in MRS for 48h at 37˚C, under anaerobic conditions, using the Gas Pack Plus System® (BBL-71040 Anaerobic System Envelope with Palladium Catalyst H_2 + CO_2; Becton Dickinson, Cockeysville, MD). Cells were harvested by centrifuging at approximately 8,000rpm for 10 minutes. Pellets were washed once with 1.5ml of 0.05M (500mg/L) KH_2PO_4 (Butterfield's Phosphate Buffer) buffer (ph 6.5). Supernatant was removed and the pellet was resuspended in 1.0ml of the same buffer. Permeabilization was achieved by adding 0.25ml of 5% Triton X-100 with 0.6% (6mg/ml) NaF and 1% (10mg/ml) Na-iodoacetate. Then 0.25ml of iodoacetate-NaF and 0.25ml (70%) of fructose-6-phosphate was added. A control with cells but no fructose-6-phosphate was prepeared. Samples were incubated at 37˚C for 30 minutes to allow the enzymatic reaction to occur. The reaction was stopped by adding 1.5ml (13.9g/100ml) of hydroxylamine-HCl. After 10 minutes, 1 ml of 15% trichloroacetic acid and 4M HCl was added, followed by 1ml (5% in 0.1M HCl) of ferric chloride hexahydrate ($FeCL3 \cdot 6\ H_2O$). A change of color from yellow to a purple-reddish brown denoted a positive reaction for the *Bifidobacterium* genus.

3.5.2 Molecular Methods

Genus Identification

The following table (**Table 1**) shows the genus specific primer set used in this investigation and their target sequence. The primers were those used by Matsuki et al. (2002). The primers were obtained from IDT Integrated DNA Technologies (Iowa, U.S.).

Table 1. Genus specific Primer Set

Primer Set	Sequence[a]	Target Site[a]	Product Size (bp)[b]
Bif-F	5' CTCCTGGAAACGGGTGG 3'	153-169	549 to 563
Bif-R	3' CATCTATAGCCCTTCTTGTGG 5'	699-720	

[a] Matsuki et. al (2002) and [b] Matsuki et al. (2003).

Species-Specific Identification by PCR and Restriction Enzyme Method

Several enzymes were used to digest the PCR product in order to generate a pattern of bands that would make it possible to differentiate between species. The following enzymes were used in the following combinations; *EcoRI-DdeI*. All enzymes used were obtained from New England Bio Labs (NEB). PCR products were subjected to double digestion in a thermocycler at 37^{o}C for 90 minutes. PCR products were subjected to electrophoresis in 2% agarose gels (IBI Ultra Sieve), stained with ethidium bromide, visualized under UV light and photographed with Versa Doc Imaging System (BioRad) Quantity One (. A 100bp DNA ladder (NEB) was employed as a molecular mass marker.

DNA Extraction Protocol

Individual 1.5mL micro centrifuge tubes were filled with approximately 1mL of an overnight culture grown on MRS + L-cysteine broth. Then contents were centrifuged at 13,000rpm for 5 minutes. After removal of the supernatant a volume of 500µL of cetyl trimethylammonium bromide (CTAB) buffer was added to each tube. The contents were mixed thoroughly in a vortex machine. The 1.5mL tubes were then heated at 80^{o}C for 5 minutes then frozen in -80^{o}C for an additional 5 minutes, this process was repeated 3

consecutive times. Afterwards the tubes content were mixed by vortex and a sterile pipette was used to help break the bacterial cell wall by mixing the content several times. The contents were then centrifuged at 13,000rpm for 10 minutes and the supernatant was transferred to a new 1.5mL tube to which 500µL of chloroform was added. The contents were vigorously mixed in the vortex and centrifuged at 13,000rpm for 15 minutes. The supernatant was then transferred again to a new sterile 1.5mL tube to which a volume of cold isopropanol was added and the contents were gently mixed by inversion. The tubes were then stored at -80^{o}C for 30 minutes after which it the contents were centrifuged and the supernatant was discarded. The pellet was washed with 100µL of 70% cold ethanol and centrifuged at 13,000rpm for 30 seconds. The supernatant was discarded and the pellet was left to dry and finally it was re-suspended in 50µL of Tris-EDTA (TE) buffer and stored at -20^{o}C.

PCR Conditions and Analysis of PCR products

The PCR conditions employed are those described by Venema and Mathuis (2003) with some minor modifications, briefly; a reaction mixture of (50µL) contained 1µL (25pmol) of each primer, 4 µL (0.2mM of each deoxyribonucleotide triphosphate) of dNTP's, 10µL of PCR buffer, 5 µL of $MgCl_2$, 2.5U of GoTaq DNA polymerase (Promega®) and 1µL of isolated bacterial DNA. DNA fragments were amplified by initial denaturation at 94^{o}C for 5 minutes, followed by 5 cycles consisting of denaturation at 94^{o}C for 60s, annealing at 55^{o}C for 30s, extension at 72^{o}C for 60s, followed by 35 cycles of denaturation at 91^{o}C for 30s, annealing at 55^{o}C for 30s, extension at 72^{o}C for 60s and 7 min final extension at 72^{o}C. Products were stored at -20^{o}C until needed.

Aliquots of 5µL of the PCR product were subjected to electrophoresis in 1.5% agarose gels (IBI) in Tris-acetate-EDTA (TAE) buffer at 100 volts for 1 hour. Gels were stained with ethidium bromide and watched under visualized under UV light. A 100bp DNA ladder (NEB) was employed as a molecular mass marker.

3.6 Fermented Acid Whey-Milk Elaboration

3.6.1 Standardization of Milk

Milk was obtained from the Experimental Station of the College of Agricultural Sciences of the University of Puerto Rico, Mayaguez Campus located in Lajas, PR. The milk was pasteurized using the low temperature, long time (LTLT) method, which is at a temperature of 63°C for a period of 30 minutes (upon reaching temperature) in a stove water bath. After pasteurization milk was skimmed using a cream separator (Milk Tech, Inc.). Milk was skimmed in order to standardize its fat content to a 3.1 - 3.2%. The percentage of fat was determined with the Mojonier method approved by the Association of Official Analytical Chemists (AOAC 989.05) and mass balance was used to determine the correct amount of fat to incorporate in the formulations. The milk, cream and a portion of the skim milk were homogenized separately using a micro homogenizer (Microfluidics™ HC-5000, Newton, Ma.) at ~63ºC and ~2,300 psi to reduce of their fat globules and ensure proper mixture of their fat content (Tamine, 2006). After the proper mixture and homogenization of the constituents their fat content was verified by performing a final Mojonier test.

3.6.2 Acid Whey Elaboration

Artisanal cheese was produced by heating the milk to a temperature of 85°C and adding approximately 20mls of acetic acid (8%) (in portions of 5mls) and stirring to precipitate the casein out of solution. The resulting acid whey was collected, filtered and homogenized (Microfluidics™ HC-5000, Newton, Ma.), then stored under sanitary conditions at a temperature of 4°C to 5°C until used.

3.6.3 Neutralization of Acid Whey Prior to Fermentation

In order for the lactic bacteria to ferment the beverage, the acid whey must be neutralized to the same pH of the milk to be used in the preparation of the beverage. The pH of fresh milk is approximately 6.6. Therefore, 0.5ml to 1ml of a solution of 6N sodium hydroxide

(NaOH) was used to neutralize the acid whey (Accumet Basic, AB15 pH meter, Fisher Scientific).

3.6.4 Beverage Formulations

3.6.4.1 Inclusion of Acid whey to Milk

The following formulations were prepared, 100%milk/0%whey (100%) and 75% milk/25% whey (75-25%). For each treatment the fermented milk-whey beverage was distributed in 500mls Erlenmeyer flasks with a proper cap. These formulations were found to be the most suitable for the development of fermented milk in a previous and related experiment (Itara-Rodríguez, 2007).

3.6.4.2 Crude Fat

Fat content in the fermented beverages was determined using the Mojonnier method (AOAC, 989.05). For the Mojonnier method, the flasks were weighed on an analytical balance (Accu-124, Fisher Scientific) and loaded with 10g of the fermented beverages. Then 1.5ml of ammonium hydroxide (NH_4OH) was added in order to neutralize any acid and casein present in the product. Three drops of phenolphthalein indicator (0.5%wt/vol) were added to observe the interface between the inorganic (water) phases and organic (fat) during the extraction. An addition of 10ml of ethyl alcohol (99 % of purity), 25ml of diethyl ether (HPLC grade) and 25ml of petroleum ether (99% of purity) induced the separation of the fat from the solution. Between every addition of reagents, flasks were agitated vigorously for approximately 1 min. After the addition of all reagents, the flasks were left to rest for 30 minutes. After that time, the organic phase was extracted and put in clean and previously weighed glass plates. This extraction process was done twice in order to remove all the available fat. The glass plates were placed in a previously heated oven (100°C) to evaporate the solvent and afterwards let to dry for 30 minutes. Plates were then taken out of the oven and put in a desiccator to cool down for 15 minutes. Plates were weighed and the percent of crude fat was determined by mass difference.

3.6.4.3 Kefir Culture Addition

The beverage was prepared using a commercial Kefir starter culture (Body Ecology, Bogart, G. A., U.S.A.) following the instructions provided by the manufacturer. The formulations were incubated (Fisher Scientific Isotemp Incubator) at 25°C in the dark, until a final pH of 4.5 to 5.0 was achieved. Afterwards, the fermentation was stopped by storing the beverage at a temperature of 4ºC to 5ºC in a commercial refrigerator (GE™, USA). The level of inclusion for the formulations was of 2.0%, the beverages were stirred to ensure proper distribution of the kefir inoculum.

The bifidobacteria strain did not participate in the fermentation process of the acid whey beverage as it interferes with other lactic acid bacteria. Also bifidobacteria are heterofermentatives and their metabolism of lactose results in the production of lactic and acetic acids, the latter has a harsh flavor undesirable in milk products (Tamine, 2002). Their production of bacteriocins, antibacterial substances also known as "colicin-like" inhibitory substances, has also been known to interfere with some starter cultures (Tamine, 2002). Therefore Bb 12 was added after the fermentation process of the milk-whey beverage was concluded. Vinderola et al. (2000) reported growth inhibition of the starter cultures by the presence of the probiotic bacteria during the fermentation process. A medium consisting of x-α-Gal, previously described by Chevalier et al. (1991), was used to monitor the viability of bifidobacteria during the storage time of the acid-whey milk beverage.

3.6.4.4 Titratable Acidity and pH

To evaluate the fermentation process pH and titratable acidity were measured (AOAC 947.05). The pH was measured (Accumet Basic, AB 15 pH Meter, Fisher Scientific) at the initial stage (0h) of the fermentation and throughout, until a pH of approximately 4.5 to 5.0 was achieved, then the fermentation was stopped. Titratable acidity was measured as a percentage of lactic acid, using a solution of 0.1N NaOH and phenolphthalein as an indicator.

3.7 Survivability Experiment Protocol

3.7.1 Addition of the probiotic strain and Sampling

The bifidobacteria (Bb-12) was suspended in a volume of sterile peptone water and added to the fermented milk-whey beverage, such that, the populations reach the required level (10^6 CFU/ml) of inclusion. The fermented beverage had the following proportions; 100% milk and 75-25% milk-whey. The fermentation process was stopped once a final pH of approximately 4.5 was reached (final pH of 4.46 and 4.48 respectively) after an approximate 24 hours of fermentation. The probiotic populations were monitored for survival and viability at the time of inoculation (baseline level) and through the product's storage time (21 days). Samples were taken out from individual sealed vials, containing 30mls of the fermented beverage, of the corresponding formulation every 3 days. A total of 8 samples were drawn out for each treatment on days, 0, 3, 6, 9, 12, 15, 18 and 21st. Samples were diluted in 9mls of peptone water to obtain dilutions of 10^{-1} to 10^{-6} and triplicate plates of MRS and x-α-Gal with dilutions 10^{-2} to 10^{-7}. Plates were incubated anaerobically at approximately 37°C and then exposed to oxygen until blue colonies developed (Chevalier et al., 1991).

3.7.2 MRS and x- α -Gal Medium

Enumeration of blue colonies, positive for bifidobacteria, was done on MRS medium supplemented with 0.05% L-cysteine and with the addition of the x-α-Gal component, to create a differential medium. This medium facilitated the recognition of bifidobacterial strains, since the x- α-Gal component allows bifidobacteria colonies to develop a blue color (Chevalier et al., 1991). X-α-Gal is a chromogenic substrate which can be used to demonstrate α-galactosidase activity. MRS agar with the addition of X- α -Gal was prepared following the manufacturers indications for the product. Briefly, MRS medium was prepared and autoclaved prior to the addition of x-α-Gal. The medium was then cooled to a temperature of 55°C before adding 1ml of x-α-Gal stock solution (20mg/ml in dimethyl formamide or DMF) per 1 liter of medium. The medium was aseptically poured in petri plates and left to harden at room temperature. Cells were

plated and incubated in anaerobic jars at 37°C, for 48 hours and then exposed to oxygen until blue colonies were formed.

3.8 Statistical Analysis

All the data obtained from the experimental phase of the project was analyzed using a statistical analysis system (SAS) version 9.5 (2005). Anova and Tukey test were performed for the prebiotic assay data and the results are shown in Appendix 11.

4. RESULTS AND DISCUSSION

4.1 Bb12 Growth Curve

The probiotic pure culture of Bb-12 showed a gradual increase in population from the outset, reaching its stationary phase around 10 to 12 hours of incubation. The generation time (g) was calculated to be around 1 hour (60 minutes), which corresponds well to what is found for this specific organism elsewhere in the literature (Martinez-Villaluenga and Gomez, 2006). The rapid growth of this species and the addition of L-cysteine as redox reducer are well documented in the literature (Tamine, 2002).

Abu-Taraboush et al. (1997) reported the appearance of two log phases for several strains of bifidobacteria grown in whole camel milk, where in *B. angulatum* 27535 the two log phases occurred after 6.5 h post inoculation (1998).

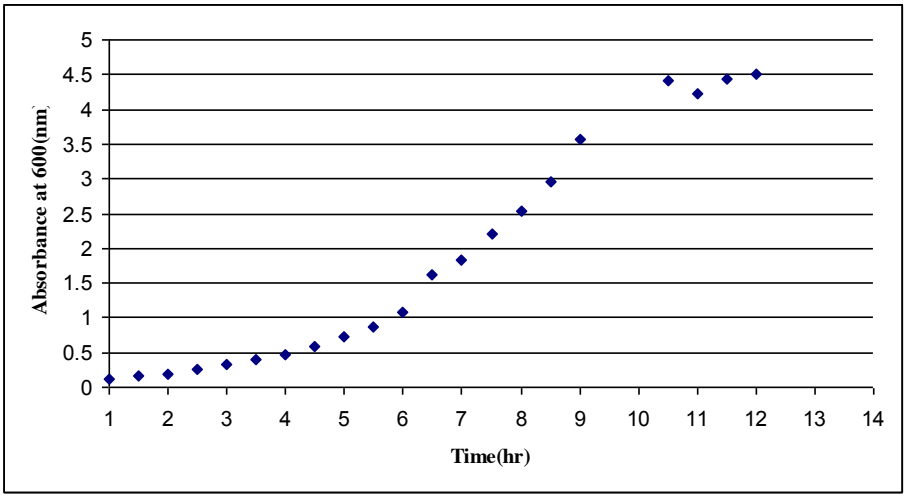

Figure 1. Growth Curve for *B. animalis* in MRS + L-cysteine absorbance at 600nm

Bifidobacterium animalis (Bb-12) exhibits a rapid growth under favorable conditions and by 18 hours it has completed its growth curve.

4.2 Prebiotic Supplemented Growth Assay

A Tukey Statistical ANOVA (confidence interval set at $\alpha = 0.05$) was performed on the acquired data and is presented on **Table 2**. No significant differences in growth were observed between fresh milk with or without prebiotic (pF and bF respectively), UHT with or without prebiotic (pU and bU respectively) and for MRS with or without prebiotic (pM and bM respectively) for day one (0h) and for pF and bF on day two (24h). There was significant difference in cell counts between the above mentioned and pU, bU (for day three or 48h), pF, pM and bM treatments for day two (24h) but not a significant difference between these treatments for the same day. There was no significant difference for pF, bF and bM for day three but there was a significant difference for these treatments with treatment pM on day three, with pM having a higher count. There was no significant difference on day three for any of the treatments except for bU which had lower cell count.

Table 2. Prebiotic Supplement Growth Assay ANOVA

Treatments	Day	Repetition
b_M[a]	1	2
b_F [a]	1	3
p_F [a]	1	2
p_U [a]	1	3
p_M	1	3
b_U [a]	1	3
b_F [a]	2	1
b_U [a]	2	3
p_U [ab]	2	3
p_F [ab]	2	3
b_U [ab]	3	3
p_M [ab]	2	3
b_M [ab]	2	3
b_F [abc]	3	3
p_F [abc]	3	3
B_M [abc]	3	2
p_M [bc]	3	3
p_U [c]	3	3

In the table below (**Table 3**) means for each treatment are in 6 log cfu/ml and in the **Table 2** treatments with a significant difference are denoted by a different letter. There were no significant differences for any of the treatments in any of the days, except for treatment of UHT milk and MRS medium with the inclusion of the prebiotic on day 3.

These two treatments showed higher ($p<0.05$) bacterial cell counts as evidenced in Table 2. UHT milk with prebiotic had an apparent ($p>0.05$) higher cell count than MRS with prebiotic, 843 Log_6 CFU/ml to 779 Log_6 CFU/ml respectively.

Table 3. Bifidobacterial Cell Counts for Prebiotic Assay

Day	Treatment *					
	Fb	Fp	Ub	Up	Mb	Mp
1	0.02	0.07	0.15	0.09	0.01	0.12
2	1.02	12.00	2.48	9.63	63.90	32.50
3	124.00	162.00	228.00	843.00	208.00	779.00
average	41.60	58.00	8.49	284.24	90.64	270.54

*Mean averages for each treatment. Bacterial cell counts expressed in Log_6 cfu/ml.

Results observed for growth of Bb12 on Inulin prebiotic supplemented media were consistent with what others (De Souza Oliveira et al., 2011 an Lopez-Molina et al., 2005) have found as to the bifidogenic factor of inulin. But results are in contrast to those found by Rossi et al. (2005) which stated that "most bifidobacteria in fecal cultures containing inulin as the carbon source were not able to grow when the hydrolytic activity of other intestinal bacteria were excluded". The authors stated that at least 8 of the bifidobacterial strains used were able to grow on inulin at some level, but that only *B. thermophilum* preferred it as carbon source. Results obtained in this study were consistent with those found by De Souza Oliveira et al. (2011) as a 1% inulin supplementation was enough to act as a bifidoenic factor in a medium not suitable for the cultivation of bifidobacteria as previously discussed. Therefore it can be concluded that UHT milk supplemented with 1% inulin serves as a perfectly acceptable growth media comparable to available synthetic media (MRS).

As shown in **Figure 2** there was a marked difference between supplemented UHT (p_U) milk versus the control synthetic media (unsupplemented MRS or b_M) for day 3. In p_U a growth form 9.13×10^4 cfu/ml (day 1) to 8.43×10^8 cfu/ml (day 3) versus 6.20×10^3 cfu/ml (day 1) to 2.08×10^8 (day 3), showing a clear bifidogenic factor in an otherwise less suitable growth media (UHT) versus the preferred MRS lactobacilli broth.

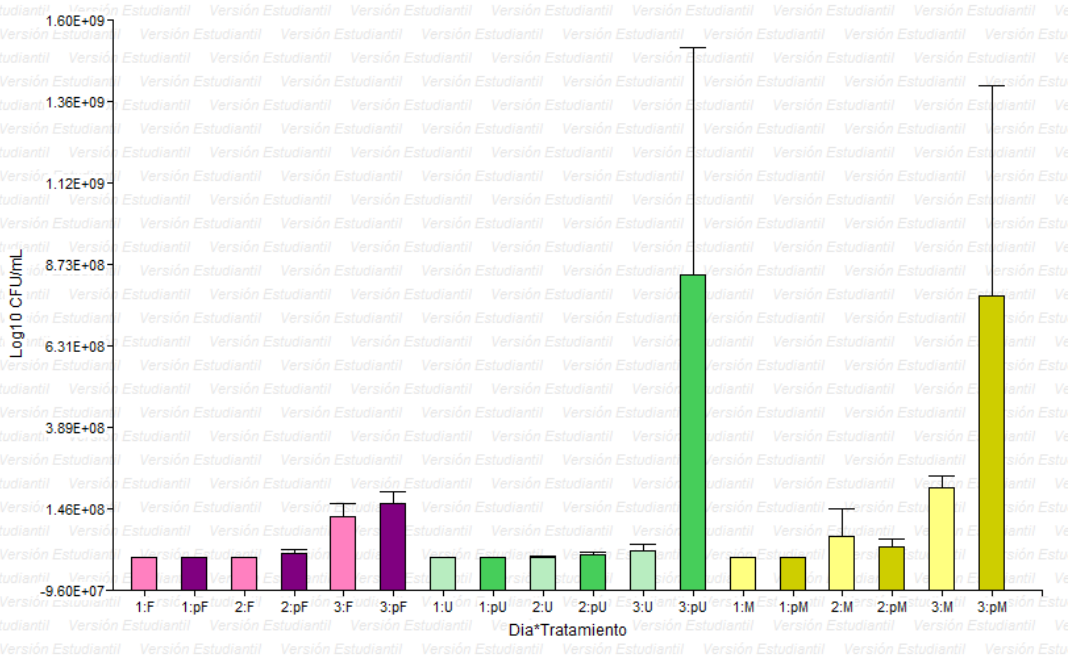

Figure 2. Bifidobacterial Growth on Prebiotic (p) Supplemented Media – Fresh Milk (F), UHT (U) and MRS (M). Where b stands for no prebiotic supplementation.

De Souza Oliveira et al. (2011) however found that *B*. *lactis sp*. *Animalis* (Bl) in both pure culture and binary culture on inulin supplemented fermented skim milk showed a marked increased in growth. They reported Bl increased from 7.23 to 7.25 LogCFU/mL to 8.02 to 8.03 Log cfu/mL ($p < 0.05$) in pure culture. Differences observed could be due to the level of supplementation as Rossi et al. (2005) utilized 10g litter^{-1} (1%) and De Souza Oliveira (2011) utilized a proportion of 4g/ 100mL of media (4%). The percentage of inclusion used in the present research was of 1% and bifidogenic activity was still perceived. Rossi et al., (2005) grew the bacteria in semi synthetic medium, while De Souza et al (2011) used reconstituted skim milk. In the present study pasteurized fresh whole milk, commercial sterile milk and synthetic media (MRS) were used.

4.3 Presumptive Isolate Identification

Several bifidobacterial presumptives isolated from a probiotic capsule and infant feces were evaluated using a genus specific primer (Matsuki et al., 2003). All 9 isolates and the control (Bb12) showed a distinctive band pattern at approximately 560bp product size (**Figure 3**). It can be concluded that all nine isolates are of the genus *Bifidobacterium*.

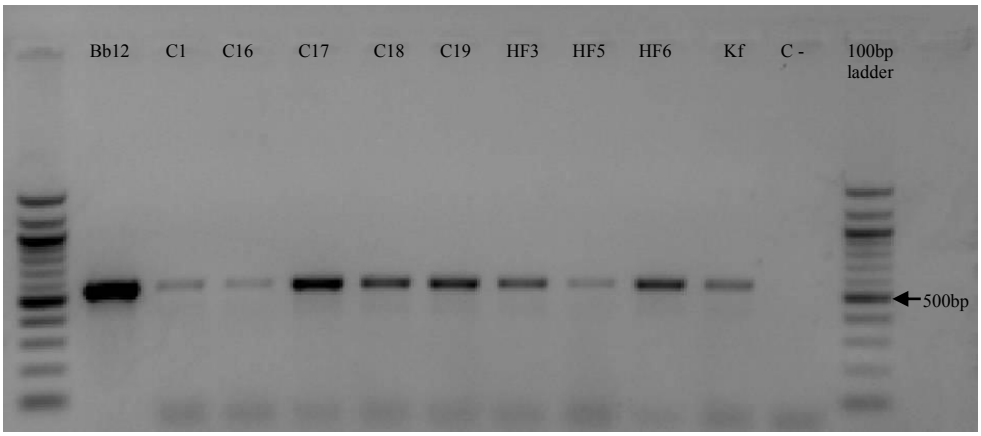

Figure 3. Genus Level PCR of Isolates – distinctive band observed at approximately 560bp

The pattern of bands obtained can be seen in **Figure 4** below. The double digestion was performed in silico using the molecular tool NEB Cutter available at the NEB website. Using *B. animalis* (Bb12) as a positive control, a double digestion was performed using *EcoRI* and *DdeI*. Results obtained with the molecular tool online (insilico) showed expected bands of 147bp and 346bp length for *DdeI* and *EcoRi-DdeI* respectively. In the figure below it can be observed that bands close to 147bp are observed for all isolates. Also in some isolates a band between 340 to 400bp can also be observed. The first row is occupied by Bb12 and is the organism used as a positive control. It can be concluded that all isolates are positive for *Bifidobacterium lactis ssp animalis* or *Bifidobacterium lactis ssp lactis*.

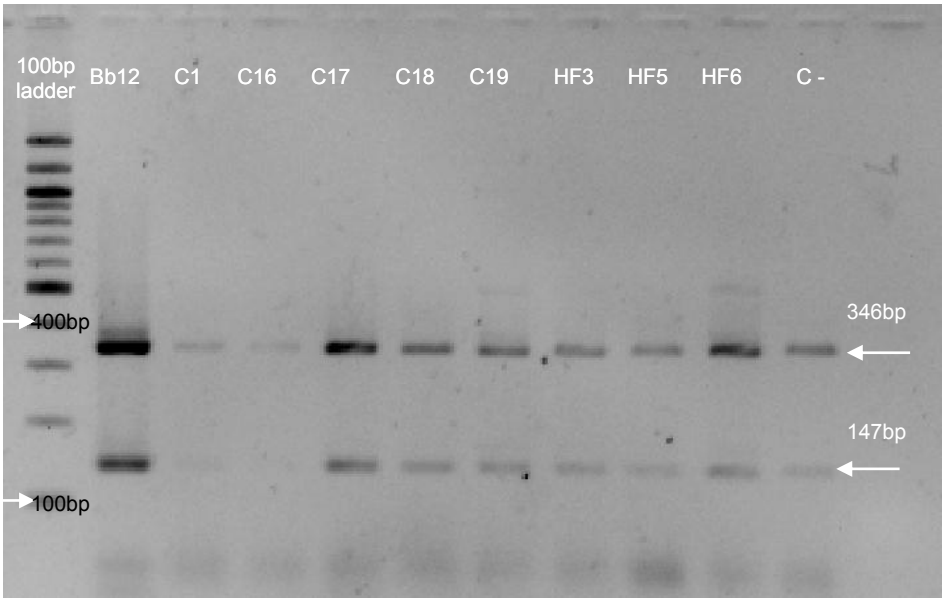

Figure 4. Double Digestion 2% Ultra Sieve Agarose Gel – Using *EcoRI-DdeI* Enzymes (NEB)

Venema and Mathuis (2003) used a similar approach to identify isolates to genus and species level, some differences were the primers used for genus identification (Bif164 and Bif662) and various other restriction enzymes. Similar to this experiment results obtained insilico were used to distinguish between species. In this experiment a set of different enzymes were used in double digestions and therefore a different band pattern was expected.

4.4 Fermented Beverage Elaboration

The pH of the two beverage formulas was measured at the beginning and at the end of the fermentation process. The results presented in **Table 4**, are a means of three experimental measures with their corresponding standard deviation. Significant difference ($p<0.05$) was found between the initial pH of the two formulations and their final pH and proves the fermentation process was carried out as expected. There were no significant differences ($p>0.05$) between the two formulations for initial or final pH. Both formulations were monitored periodically (every 8 h) and stopped once they had reached a pH of 4.4±2, which was around 25 h.

Table 4. pH of the Fermented Beverages

Beverage Formulation	Initial pH	Final pH
100%	6.40 ± 0.100^{a}	4.45 ± 0.010^{c}
75-25%	6.39 ± 0.010^{a}	4.50 ± 0.100^{c}

Values in the same column with the same upper script do not differ in significance ($p>0.05$). Values are given as means with their standard deviation. Tukey's test $\alpha = 0.05$

Titratable acidity (TA) is expressed in terms of lactic acid and in fresh milk it is a measure of its quality. Fresh milk has a titratable acidity of 0.14%-0.16% (Walstra *et al*, 2006). The TA for the formulations was measured at the beginning and at the end of the fermentation process. It can be observed, in **Table 5** that for both formulations the initial TA is within the range attributed to fresh milk and thus was of good quality. The TA measure also gives an indirect understanding of the fermentative activity of the kefir starter culture as the lactic acid bacteria present in the culture convert lactose into lactic acid (Jay and Golden, 2005).

Table 5. Titratable Acidity of the Fermented Beverages

Beverage Formulation	Initial % TA	Final % TA
100%	0.134 ± 0.006^{a}	0.589 ± 0.024^{b}
75-25%	0.144 ± 0.085^{a}	0.518 ± 0.022^{b}

Values in the same column with the same upper script do not differ in significance ($p>0.05$). Values are given as means with their standard deviation. Tukey's test at $\alpha = 0.05$

According to Tamine (2006) a commercial kefir's TA should be no less than 0.6% but for both formulations final TA was below this percentage. The 100% formulation was close to this range and it is unknown what may have caused both formulations to have a lower TA than expected. Siberio-Perez (2009) reported TA's of 0.8% and 0.6% for both 100% (control) and 75-25% respectively, but also reported lower pH for the formulations (pH 4.4 for both).

4.5 Survival Rate of Bb 12

Fermented dairy products are the preferred vehicle for the inclusion of probiotic cultures (Shah, 2002). Nonetheless these fermented dairy (yogurt, milk beverages and cheese) products are oftentimes inhospitable environments for the growth and survival of many probiotc bacteria and can in turn affect their probiotic functionality (Sanders and

Marco, 2010). These are especially detrimental to the genus Bifidobacteria due to their intolerance of high acidity and the presence of dissolved O_2 in the product (Jayamanne and Adams, 2009).

In **Figure 5** it can be observed that general Log_{10} cfu ml^{-1} did not significantly decreased from day to day, bacterial cell counts remain in the 6 Log_{10} to 7 Log_{10} cfu /ml throughout the last days of the experiment, except for a marked decrease in day 21. It can be observed that the 100% formulation maintained an apparent higher cell count than the 75-25% formulation. Data for day 9 was not available but it can be theorized that bacterial cell count continued to increase as observed in day 12. The higher cell counts obtained for day 0 can be due to various reasons such as; investigator error by not mixing the bottle contents enough prior sample selection or due to the fact that cells had been recently added therefore the acid conditions had not yet had a detrimental effect on the population. In day 2 and 3 however bacterial cell counts decreased and this can be as a result of acid and temperature shock and it is apparent that bifidobacteria developed some resistance to this harsh environment after day 3.

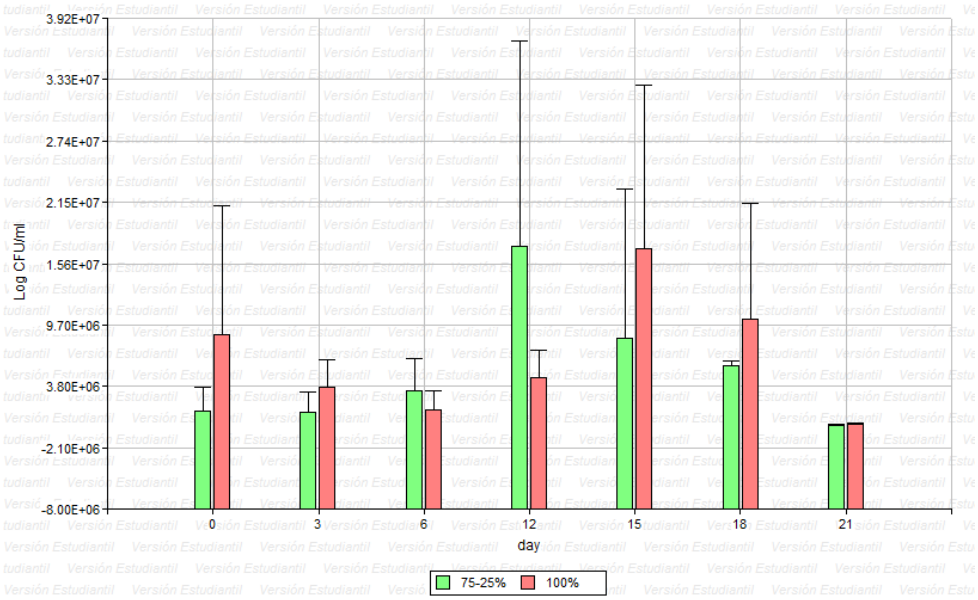

Figure 5. Population of Bb-12 in the fermented milk-whey beverage (cfu/ml) during the 21 days of refrigerated (4^0C) storage.

It has been widely reported in the literature that the genus *Bifidobacterium* does not survive well at pH lower than 5.0 and that no growth occurs below pH 4.5 (Biavati,

2000). Moreover it has been proved that survival in strong acidic conditions is largely strain dependant, among which the best survivor is *Bifidobacterium lactis* Bb 12 (Vernazza et al., 2006). Bb 12 strain has been shown to have a higher tolerance for strong acidic conditions when compared to other strains such as *Bifidobacterium longum* and *Bifidobacterium adolescentis* (Jayamanne and Adams, 2009 and Matsumoto et al., 2004). It has been reported that the activity of H^+-ATPase (an enzyme responsible for pH homoestasis) is high in Bb12 (Matsumoto et al., 2004), thus it offers an increased resistance to lower pH levels making it ideal for fermented products such as yogurt and kefir. The pH of the fermented beverages (pH 4.5) is above that reported by Jayamanne and Adams (2009), where at pH 4.25 this strain was capable of surviving in refrigerated storage for 2.1 weeks in Bio-yogurt. Vernazza et al., (2006), concluded that a pH 2 was lethal for all bifidobacterial strains studied, except for Bb 12, where survival rate was similar in all the pH conditions tested. It can be concluded that a low pH 4.5 can perhaps reduce the population of Bb 12 but it is not lethal.

There were significant differences ($p<0.05$) among days 0 and 21, between days 3 and 15 and between day 15 and day 21 for the 100% formulation. Differences among day 0 and 21 were the expected outcome, as cell counts decreased due to the inhospitable environment of the fermented beverage, and the probable decrease in the pH of the beverage as the microorganism produced lactic and acetic acid as well as its metabolic waste.There were no significant differences ($p >0.05$) in bacterial cell counts between days 0 and 21 for the 75-25% fermented milk-whey beverage. There were no significant differences for this treatment among any of the monitored days. There is however an apparent decrease in cfu ml^{-1} for both the 100% and 75-25% as evidenced in **Table 6.**

Table 6 . Enumeration of Bb-12 in the fermented milk-whey beverage.

Formulation	Mean	Mean	α = 0.05
	100% Log* CFU/ml	**75-25%** Log* CFU/ml	
Day			
0	8.73	1.37	
3	3.77	1.27	
6	1.53	3.39	
12	4.67	**17.2**	
15	**17.0**	**8.46**	
18	**10.3**	5.73	
21	0.14	0.006	
P-value	0.0054	0.2966	

* Log cfu expressed as 6Log cfu/ml.

However throughout the 21 days of storage both formulations maintained high enough cell counts (6 Log_{10}) up until the 18 d. The mean cfu ml^{-1} was of 1.03 x 10^7 for the 100% formulation and 5.73 x 10^6 for the 75-25% formulation on day 18. The 100% formulation showed apparent higher cfu ml^{-1} for the 21d storage period, except for day 12, where the 100% formulation had a mean cfu ml^{-1} of 4.67 x 10^6 whereas the 75-25% had a mean cfu ml^{-1} of 1.72 x 10^7. It can be concluded that there was no significant difference between treatments throughout the storage period. Survival rates for bifidobacteria were higher than expected; this could be partially explained by the specificity of this strain to survive in acidic conditions as marketed by the producing company in their cited literature (Study summaries BB-12®, 2011). Additionally O_2 content (not measured) in the food matrix could have been lower than anticipated due to the presence of oxygen consuming starter cultures, such as *Streptococcus thermophilus* (de Vrese and Schrezenmeir, 2008).

4.6 Factors Affecting Viability

When it comes to delivering the probiotic live and in sufficient amounts to sustain these claims, there are as one author put it, "technical hurdles" to be overcome (Ross et. al, 2005). Investigators have claimed a variety of factors that can affect the viability of the probiotic organism such as, cold storage, interactions between species present, final acidity, availability of nutrients, dissolved oxygen and oxygen permeation (Sanders and Marco, 2010, Shah, N. P., 2002). In the following **Figure 6**, there is an excellent example

of the hurdles involved in the development of a probiotic food product, some of which were confronted in this research.

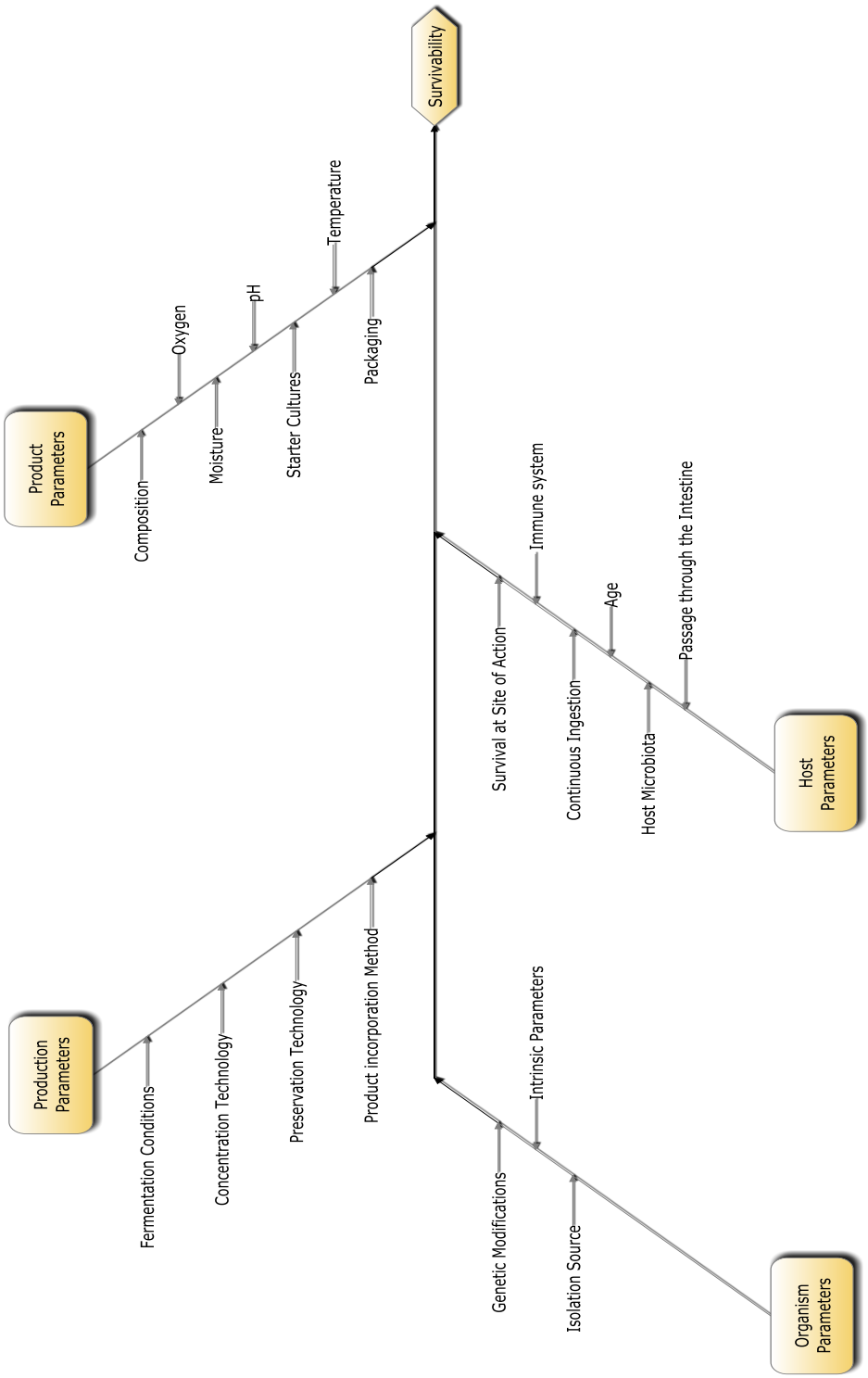

Figure 6. Potential Factors Affecting Probiotic Survivability and Functionality (modified from Sanders and Marco, 2010) inside the host and prior, during and after food production.

Fermented dairy products have been traditionally used as carriers of probiotic microorganisms. Recently a varied range of food and beverages are becoming popular options as carriers of probiotic cultures (such as salad dressing, bread and butters). Products like yogurt and fermented milks are still the most popular food carriers, where the probiotics used are mainly from the *Lactobacillus* and *Bifidobacterium* genera (Sanders and Marco, 2010 and Tamine, 2002). The fermented milk prepared for this study was made according to the instructions provided by the manufacturer (BodyEcology®) and those found in the literature (Tamine, 2006). For the 75-25% formulation the method used was that of Itara-Rodriguez (2007).

The delivery vehicle used is likely to influence probiotic functionality and the main objective should be that cells retain this throughout the shelf life of the product (Sanders and Marco, 2010). In an *in vivo* study by Pochart et al. (1992) concluded that *Bifidobacterium sp*. survived transit through the GIT of healthy adults when ingested in fermented milk. Taking into account that the results of this study show that by day 18 the bifidobacteria strain was still viable in needed amounts, the suitability of the formulations can be ascertained. Some milk and/or whey components may account for the sustained viability of the probiotic microorganism during storage.

As it has been previously discussed, bovine milk is a rather poor growth medium for Bifidobactera as it lacks the necessary amount of vital growth nutrients. But there have been recent studies regarding the beneficial effects of dairy proteins and bioactive peptides both in human health and as prebiotics. In a study by Etzel (2004) the properties of whey protein isolate (WPI) where studied under various pH conditions. Results showed that at pH 4.6 WPI were heavily precipitated (insoluble sediments), but that by the addition of a food-grade lauryl sulfate the problem was avoided. Another solution to their problem was the use of whey fractionated protein glycomacropeptide instead of WPI, and this protein had been shown to increase growth of bifidobacteria. Whey protein concentrate (WPC) contains 34-88% protein, which is considerably higher than the amount found in whey (Sodini et al., 2005 and Etzel, 2004).

A short refrigerated survivability study of six days duration was made in order to view the behavior of the organism at storage temperatures. Though the ecology of the organism requires an optimal temperature of 37°C in order to grow, no clear reduction of viable cells was perceived. The organism maintain a favorable amount of 7 Log_{10} cfu/ml with minimal variations in cell counts during the period (data not shown), which showed a sustained survivability between day 0 and day 6. But it must be observed this short trial was carried out in a synthetic medium which offers plenty of nutrients in contrast to milk or fermented milk which have less than the needed amount of nutrients for the bacteria to grow.

In a study by Jayamanne and Adams (2009) it was observed that there was no significant difference for survival of *B. animalis ssp. Lactis* for two of the 3 temperature parameters (4, 8 and 12°C), where they concluded the optimum survival temperature to be a range of temperatures from 4 to 8°C. Motyl and Libudzisz (2000) also reported similar finds and concluded that all their tested bifidocteria strains where characterized by a high viability in fermented milk during refrigerated storage. Others have reported similar results (Abu-Taraboush, 1997). Storage temperature throughout the study was constant at 4°C±2 and is the expected storage temperature of market and consumer's refrigeration appliances. As can be seen in **Figure 5** cell counts remained mostly constant throughout the 21 d of refrigerated storage but as evidenced in **Table 6** colony counts decreased significantly after 15 d for the 100% formulation and there was an apparent decrease in colony counts after 18 d for the 75-25% formulation. Therefore it can be concluded that Bb 12 was not adversely affected under the refrigerated temperature (4-5°C) for this study.

5 CONCLUSIONS

The growth of *Bifidobacterium animalis* on prebiotic supplemented growth media namely; fresh milk (F), commercially sterilized milk (UHT) and a synthetic media (MRS) was evaluated. Results obtained showed that a supplementation with 1% of the prebiotic inulin was enough to exert a bifidogenic effect on the growth of bifidobacteria. Results obtained for the supplemented UHT milk (pU) were comparable with growth on supplemented and non supplemented MRS media on day 3. A possible factor contributing to the better growth observed in UHT and MRS media may be due to the lower levels of dissolved oxygen expected, since this media is sterilized and therefore oxygen evaporation may have occurred. There were no significant differences observed in any of the other treatments for days 1, 2 and 3. The use of prebiotic supplemented UHT is recommended as a suitable growth media for bifidobacterium when elaborating food products containing bifidobacteria, such as a fermented milk beverage. The advantage of using UHT milk over synthetic media is the ability to incorporate it to the food product without having to wash and harvest the bacterial cells.

The identification of several bifidobacteria presumptive isolates was performed using a traditional and a molecular tool namely, the F6PPK assay and the use of genus specific primers as previously described. Results for the F6PPK for all of the isolates (with a C denomination) were negative except C18. The assay could not be repeated and performed on the last isolates (HF3, HF5, HF6 and Kf) due to unavailable materials. Using *B. animalis* (Bb12) as a positive control, a double digestion was performed using *EcoRI* and *DdeI*. The results obtained insilico showed expected bands of 147bp and 346bp length for *DdeI* and *EcoRi-DdeI* respectively. Expected bands close to 147bp could be observed for all isolates. In some isolates a band between 340 to 400bp could also be observed. It appeared all of the isolates were positive for *Bifidobacterium animalis*.

The survival of a probiotic strain of bifidobacteria (Bb12) on a fermented milk-whey beverage (100% and 75-25%) was analyzed at the end of 21 days refrigerated storage. The objectives were first to determine if the strain could survive in both formulations at the required levels (10^6) in order for the product to be labeled as a

probiotic dairy beverage. Secondly to analyze if there was any significant difference on the strain's survivability due to the inclusion of whey (75-25%) when compared to the control (100%). No significant difference in log cfu was observed between the 100% and 75-25% formulations. Cell counts remained fairly constant in both formulations during the experiment, but significant differences were observed for day 15, 18 and 21 of the 100% formulation. Despite the fact that a low pH is considered detrimental to the survival of bifidobacteria, cell counts remained in sufficient amounts (except for day 21) in order for both formulations to be considered a "probiotic beverage". This study shows that the addition of whey in the 75-25% (milk-whey) formula did not detrimentally affect the survival of bifidobacteria and is comparable with the 100% milk (control) formula.

One of the parameters that could have adversely affected the survival of Bb 12, was oxygen toxicity as it has been previously stated bifidobacteria are strict anaerobes, with some species being able to tolerate minimal amounts of O_2. But as observed in the presented data, even with the presence of oxygen in the food matrix bifidobacteria survival was not detrimentally affected. The crystal bottles containing the fermented beverage had a headspace where the oxygen was present and throughout the beverage the oxygen permeability might have been high. No attempt was made to maintain the beverages under anaerobic conditions as this would detract from real life expectations for the product in terms of storage and use in the consumer's households.

The results obtained show that the bifidobacteria strain Bb12 survived in sufficient amounts (10^6cfu/ml) in both fermented beverage formulations until day 18. The fermented beverage could be potentially marketed as a probiotic beverage. But further efforts should be made to grow this strain in bovine milk prior to inclusion on the formulations, since synthetic media is expensive and cell recuperation methods could adversely affect bifidobacterial survival.

Recommendations for future investigations could be the addition of other bifidobacterial species or lactic acid bacteria in conjunction with Bb12 and observe the effects on survivability. Evaluate the addition of prebiotic for use as stabilizers to the finished milk whey fermented beverage and its effects on the survaivability of bífidobacteria.

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APENDIX

Appendix 1. Milk Major Components

Milk Components	Approximate %
Water	87
Fat	3.5
Triglycerides	98

Diglycerides	0.3
Phospholipids	0.8
Cholesterol	0.3
Proteins	3.5
Caseins	80
Whey	19.3
α-Lactalbumin	3.7
α-Lactoglobulin	9.8
BSA	1.2
Lactose	4.8
Salts	0.8

Appendix 2. List of current Bifidobacterial Species

Current Recognized Bifidobacterium Species	
B. angulatum	*B. longum*
B. adolescentis	*B. magnum*
B. asteroides	*B. minimum*
B. animalis	*B. mongoliense*
B. breve	*B. dentium*
B. bifidum	*B. pseudocatenulatum*
B. bombi	*B. pseudolongum*
B. boum	*B. psychraerophilum*
B. cuniculi	*B. pullorum*
B. catenulatum	*B. ruminantium*
B. choerinum	*B. saeculare*
B. coagulans	*B. scardovii*
B. coryneforme	*B. simiae*
B. crudilactis	*B. subtile*
B. gallicum	*B. thermacidophilum*
B. gallinarum	*B. thermophilum*
B. indicum	*B. tsurumiense*
B. kashiwanohense	

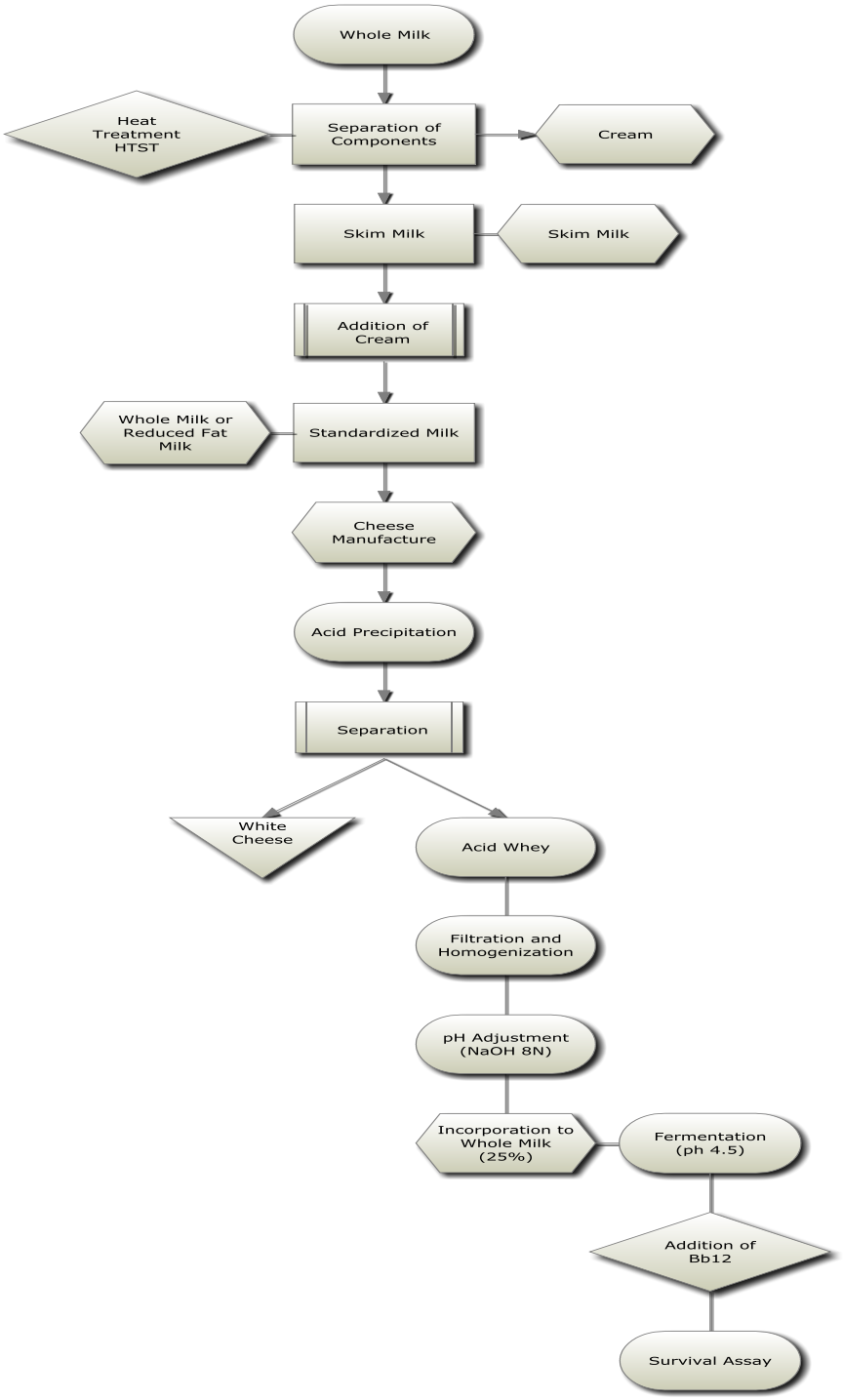
[1]Leahy et al., [2] http://www.ncbi.nlm.nih.gov/Taxonomy

Appendix 3. Presumptive Bifidobacterial Isolates

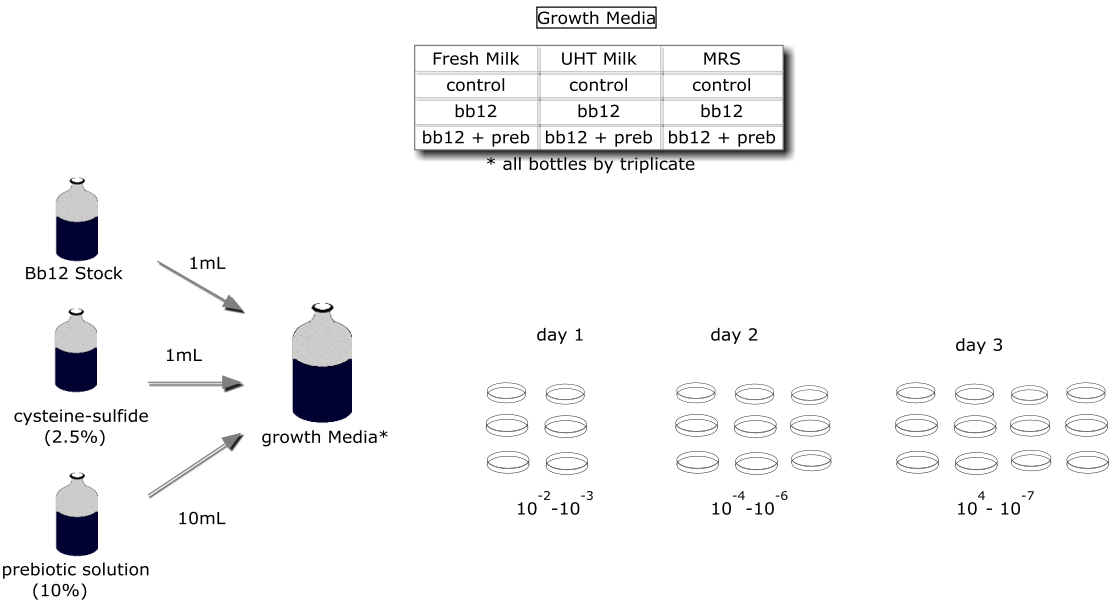
Isolate	Colony Description	Gram Stain	Cell Morphology	F6PPK
C1	Circular, white, lustrous, smooth and flat.	+	Bacilli, singles and chains and bifid morphology	-
C3	Irregular/Circular, white, flat and opaque.	+	Short bacilli, single, pairs and chain arrangement.	-

C4	Circular, lustrous, white, flat and somewhat concave in the middle.	+	“	-
C7	Irregular/Circular, lustrous white and reaised.	+	Bacilli, club shaped, single rods and bifid morphology	-
C16	Circular, white, opaque and flat.	+	Short and long bacilli in chain arrangement	-
C17	Circular (big), white, lustrous and raised	+	Short rod shaped bacilli, single, bundle and Y shape	-
C18	Circular, white, lustrous and raised.	+	short bacilli, single V and Y shape	+
C19	Very small bacilli, single, pairs, chains with V shape	+		-
Hf		+		n/a
Kf		+		n/a

Appendix 4. Fermented Milk Elaboration Flowchart



Appendix 5. Prebiotic Supplemented Growth Assay Flowchart



*all plates where done by triplicate, cultivated in MRS/x-gal agar and incubated anaerobically at 37°C in an anarobic jar

Appendix 6. Bioreactor with Sterile Supplemented MRS

Apendix 7. Bioreactor Control Panel

Apendix 8. Bioreactor after 48h

Apendix 9. Fat Content Analysis

Apendix 10. EPA Guidelines For Waste Waters in Dairy Plants

Effluent Characteristic[a]	Effluent limitations	
	Maximum for any 1 day	Average of daily values for 30 consecutive days shall not exceed—
	Metric units (kilograms per 1,000 kg of BOD 5 input)	
BOD 5	0.976	0.488
TSS	1.462	0.731
pH	-1	-1
	English units (pounds per 100 lb of BOD 5 input)	
BOD 5	0.098	0.049
TSS	0.146	0.073
pH[b]	-1	-1

[a] For plants processing 100,000 lb/day or less of milk equivalent (less than 10,390 lb/day of BOD 5 input).
[b] 1Within the range 6.0 to 9.0.

Appendix 11. Bb12 Fermented Beverage Survivability ANOVA

Medias generales: pH y Acidez

Substrato=10 ╤ Leche (paso a)

Variable	N	Mean	Std Dev	Minimum	Maximum
ph	3	6.5100000	0.0100000	6.5000000	6.5200000
acidez	3	0.1273333	0.0085049	0.1190000	0.1360000

Substrato=11 ╤ Leche antes de fermentar (paso b)

Variable	N	Mean	Std Dev	Minimum	Maximum
ph	3	6.4000000	0.1000000	6.3000000	6.5000000
acidez	3	0.1343333	0.0066583	0.1270000	0.1400000

substrato=12 ╤ Leche después de fermentar (paso c)

Variable	N	Mean	Std Dev	Minimum	Maximum
ph	3	4.4500000	0.0100000	4.4400000	4.4600000
acidez	3	0.5896667	0.0248261	0.5610000	0.6040000

Substrato=20 ╤ Suero (paso a)

Variable	N	Mean	Std Dev	Minimum	Maximum
ph	3	5.1800000	0.0100000	5.1700000	5.1900000
acidez	3	0.2320000	0.0127671	0.2210000	0.2460000

Substrato=21 ╦ 75/25 antes de fermentar (paso b)

Variable	N	Mean	Std Dev	Minimum	Maximum
ph	3	6.3900000	0.0100000	6.3800000	6.4000000
acidez	3	0.1440000	0.0850000	0.0590000	0.2290000

Substrato=22 ╦ 75/25 Después de fermentar (paso c)

Variable	N	Mean	Std Dev	Minimum	Maximum
ph	3	4.5000000	0.1000000	4.4000000	4.6000000
acidez	3	0.5183333	0.0226789	0.5010000	0.5440000

ANOVA ╦pH y Acidez ╦ DCA ╦ Tratamientos = Substrato

The GLM Procedure

Class Level Information

Class	Levels	Values
substrato	6	10 11 12 20 21 22

Number of Observations Read	18
Number of Observations Used	18

Dependent Variable: ph

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	14.38885000	2.87777000	846.40	<.0001
Error	12	0.04080000	0.00340000		
Corrected Total	17	14.42965000			

R-Square	Coeff Var	Root MSE	ph Mean
0.997172	1.046536	0.058310	5.571667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Substrato	5	14.38885000	2.87777000	846.40	<.0001

Dependent Variable: acidez

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.65191028	0.13038206	90.59	<.0001
Error	12	0.01727067	0.00143922		
Corrected Total	17	0.66918094			

R-Square	Coeff Var	Root MSE	acidez Mean
0.974191	13.03929	0.037937	0.290944

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Substrato	5	0.65191028	0.13038206	90.59	<.0001

Tukey's Studentized Range (HSD) Test for ph

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.0034
Critical Value of Studentized Range	4.75015
Minimum Significant Difference	0.1599

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	substrato
A	6.51000	3	10
A			
A	6.40000	3	11
A			
A	6.39000	3	21
B	5.18000	3	20
C	4.50000	3	22
C			
C	4.45000	3	12

Tukey's Studentized Range (HSD) Test for acidez

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.001439
Critical Value of Studentized Range	4.75015
Minimum Significant Difference	0.104

Means with the same letter are not significantly different.

Tukey Grouping		Mean	N	substrato
	A	0.58967	3	12
	A			
	A	0.51833	3	22
	B	0.23200	3	20
	B			
C	B	0.14400	3	21
C	B			
C	B	0.13433	3	11
C				
C		0.12733	3	10

ANOVA: CFUs (Log); Trat = dias de fermentación

Class Level Information

Class	Levels	Values
dia	8	0 3 6 9 12 15 18 21

Data for Analysis of LCFUS1

Number of Observations Read	24
Number of Observations Used	19

Data for Analysis of LCFUS2

Number of Observations Read	24
Number of Observations Used	15

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Dependent Variable: LCFUS1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	9.15701796	1.52616966	5.65	0.0054
Error	12	3.24228426	0.27019035		
Corrected Total	18	12.39930222			

R-Square	Coeff Var	Root MSE	LCFUS1 Mean
0.738511	8.202281	0.519798	6.337242

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dia	6	9.15701796	1.52616966	5.65	0.0054

Tukey's Studentized Range (HSD) Test for LCFUS1

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.27019
Critical Value of Studentized Range	4.94954

Comparisons significant at the 0.05 level are indicated by ***.

dia Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
15 - 0	0.4628	-1.3565	2.2820	
15 - 18	0.5682	-1.0925	2.2289	
15 - 3	0.9469	-0.7138	2.6076	
15 - 12	1.1004	-0.5603	2.7611	
15 - 6	1.4292	-0.2315	3.0899	
15 - 21	2.3534	0.6927	4.0141	***
0 - 15	-0.4628	-2.2820	1.3565	
0 - 18	0.1055	-1.5553	1.7662	
0 - 3	0.4842	-1.1765	2.1449	
0 - 12	0.6377	-1.0230	2.2984	
0 - 6	0.9665	-0.6942	2.6272	
0 - 21	1.8906	0.2299	3.5513	***
18 - 15	-0.5682	-2.2289	1.0925	
18 - 0	-0.1055	-1.7662	1.5553	
18 - 3	0.3787	-1.1067	1.8641	
18 - 12	0.5322	-0.9532	2.0176	
18 - 6	0.8610	-0.6244	2.3464	
18 - 21	1.7852	0.2998	3.2706	***
3 - 15	-0.9469	-2.6076	0.7138	

3 - 0	-0.4842	-2.1449	1.1765	
3 - 18	-0.3787	-1.8641	1.1067	
3 - 12	0.1535	-1.3319	1.6389	
3 - 6	0.4823	-1.0031	1.9677	
3 - 21	1.4065	-0.0789	2.8919	
12 - 15	-1.1004	-2.7611	0.5603	
12 - 0	-0.6377	-2.2984	1.0230	
12 - 18	-0.5322	-2.0176	0.9532	
12 - 3	-0.1535	-1.6389	1.3319	
12 - 6	0.3288	-1.1566	1.8142	
12 - 21	1.2530	-0.2324	2.7383	
6 - 15	-1.4292	-3.0899	0.2315	
6 - 0	-0.9665	-2.6272	0.6942	
6 - 18	-0.8610	-2.3464	0.6244	
6 - 3	-0.4823	-1.9677	1.0031	
6 - 12	-0.3288	-1.8142	1.1566	
6 - 21	0.9242	-0.5612	2.4095	
21 - 15	-2.3534	-4.0141	-0.6927	***
21 - 0	-1.8906	-3.5513	-0.2299	***
21 - 18	-1.7852	-3.2706	-0.2998	***
21 - 3	-1.4065	-2.8919	0.0789	
21 - 12	-1.2530	-2.7383	0.2324	
21 - 6	-0.9242	-2.4095	0.5612	

Dependent Variable: LCFUS2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	2.83758777	0.47293130	1.48	0.2966
Error	8	2.55845944	0.31980743		
Corrected Total	14	5.39604721			

R-Square	Coeff Var	Root MSE	LCFUS2 Mean
0.525864	8.768181	0.565515	6.449630

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dia	6	2.83758777	0.47293130	1.48	0.2966

Tukey's Studentized Range (HSD) Test for LCFUS2

NOTE: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.319807
Critical Value of Studentized Range	5.39912

Comparisons significant at the 0.05 level are indicated by ***.

dia Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
12 - 18	0.2711	-1.4917	2.0339
12 - 0	0.4156	-2.0774	2.9086
12 - 6	0.6485	-1.1143	2.4114
12 - 3	1.0038	-0.9671	2.9747
12 - 21	1.0310	-0.9399	3.0019
12 - 15	1.4373	-1.0557	3.9303
18 - 12	-0.2711	-2.0339	1.4917
18 - 0	0.1445	-2.3485	2.6375
18 - 6	0.3774	-1.3854	2.1402
18 - 3	0.7327	-1.2382	2.7036
18 - 21	0.7599	-1.2110	2.7308
18 - 15	1.1662	-1.3268	3.6592
0 - 12	-0.4156	-2.9086	2.0774
0 - 18	-0.1445	-2.6375	2.3485
0 - 6	0.2329	-2.2601	2.7259
0 - 3	0.5882	-2.0560	3.2324
0 - 21	0.6154	-2.0288	3.2596
0 - 15	1.0217	-2.0316	4.0750
6 - 12	-0.6485	-2.4114	1.1143

6	- 18	-0.3774	-2.1402	1.3854
6	- 0	-0.2329	-2.7259	2.2601
6	- 3	0.3552	-1.6156	2.3261
6	- 21	0.3825	-1.5884	2.3534
6	- 15	0.7888	-1.7042	3.2818
3	- 12	-1.0038	-2.9747	0.9671
3	- 18	-0.7327	-2.7036	1.2382
3	- 0	-0.5882	-3.2324	2.0560
3	- 6	-0.3552	-2.3261	1.6156
3	- 21	0.0272	-2.1318	2.1862
3	- 15	0.4335	-2.2107	3.0778
21	- 12	-1.0310	-3.0019	0.9399
21	- 18	-0.7599	-2.7308	1.2110
21	- 0	-0.6154	-3.2596	2.0288
21	- 6	-0.3825	-2.3534	1.5884
21	- 3	-0.0272	-2.1862	2.1318
21	- 15	0.4063	-2.2379	3.0505
15	- 12	-1.4373	-3.9303	1.0557
15	- 18	-1.1662	-3.6592	1.3268
15	- 0	-1.0217	-4.0750	2.0316
15	- 6	-0.7888	-3.2818	1.7042
15	- 3	-0.4335	-3.0778	2.2107
15	- 21	-0.4063	-3.0505	2.2379

Regresiones: CFUs (Log) = variable dependiente; Dias = variable independiente; Efecto lineal (dia) y efectos cuadráticos (dia2)

The GLM Procedure

Data for Analysis of LCFUS1

Number of Observations Read	24
Number of Observations Used	19

Data for Analysis of LCFUS2

Number of Observations Read	24
Number of Observations Used	15

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Dependent Variable: LCFUS1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.93453319	0.93453319	1.39	0.2553
Error	17	11.46476903	0.67439818		
Corrected Total	18	12.39930222			

R-Square	Coeff Var	Root MSE	LCFUS1 Mean
0.075370	12.95859	0.821217	6.337242

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dia	1	0.93453319	0.93453319	1.39	0.2553

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	6.676662424	0.34443023	19.38	<.0001
dia	-0.030709452	0.02608753	-1.18	0.2553

Dependent Variable: LCFUS2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00455515	0.00455515	0.01	0.9181
Error	13	5.39149206	0.41473016		
Corrected Total	14	5.39604721			

R-Square	Coeff Var	Root MSE	LCFUS2 Mean
0.000844	9.984999	0.643995	6.449630

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dia	1	0.00455515	0.00455515	0.01	0.9181

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	6.420287594	0.32562993	19.72	<.0001
dia	0.002573852	0.02455924	0.10	0.9181

Data for Analysis of LCFUS1

Number of Observations Read	24
Number of Observations Used	19

Data for Analysis of LCFUS2

Number of Observations Read	24
Number of Observations Used	15

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

Dependent Variable: LCFUS1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1.79538087	0.89769043	1.35	0.2861
Error	16	10.60392135	0.66274508		
Corrected Total	18	12.39930222			

R-Square	Coeff Var	Root MSE	LCFUS1 Mean
0.144797	12.84615	0.814092	6.337242

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dia	1	0.43965729	0.43965729	0.66	0.4273
dia2	1	0.86084767	0.86084767	1.30	0.2712

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	6.331375992	0.45647442	13.87	<.0001
dia	0.086081809	0.10568844	0.81	0.4273
dia2	-0.005424433	0.00475953	-1.14	0.2712

Dependent Variable: LCFUS2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.46872442	0.23436221	0.57	0.5797
Error	12	4.92732278	0.41061023		
Corrected Total	14	5.39604721			

R-Square	Coeff Var	Root MSE	LCFUS2 Mean
0.086864	9.935280	0.640789	6.449630

Source	DF	Type III SS	Mean Square	F Value	Pr > F
dia	1	0.45924602	0.45924602	1.12	0.3111
dia2	1	0.46416927	0.46416927	1.13	0.3086

Appendix 12. Prebiotic Supplemented Growth ANOVA

Análisis de la varianza

Variable	N	R^2	R^2 Aj	CV
Conteo	49	0.66	0.47	175.23

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	3.30001095431958E18	17	194118291430563000.00	3.52	0.0012
Tratamiento	616037239014789000.00	5	123207447802958000.00	2.23	0.0760
Dia	1.27253518083037E18	2	636267590415183000.00	11.52	0.0002
Tratamiento*Dia	1.24262201762333E18	10	124262201762333000.00	2.25	0.0410
Error	1.71153062822712E18	31	55210665426681300.00		
Total	5.0115415825467E18	48			

Test:Tukey Alfa=0.05 DMS=776113267.13174

Error: 55210665426681280.0000 gl: 31

Tratamiento	Dia	Medias	n	E.E.			
b_M	1	6200.00	2	166148526.06	A		
b_F	1	22900.00	3	135659703.46	A		
p_F	1	75000.00	2	166148526.06	A		
p_U	1	91333.33	3	135659703.46	A		
p_M	1	115000.00	3	135659703.46	A		
b_U	1	155000.00	3	135659703.46	A		
b_F	2	1020000.00	1	234969498.93	A		
b_U	2	2476666.67	3	135659703.46	A		
p_U	2	9633333.33	3	135659703.46	A	B	
p_F	2	12033333.33	3	135659703.46	A	B	
b_U	3	22843333.33	3	135659703.46	A	B	
p_M	2	32466666.67	3	135659703.46	A	B	
b_M	2	63900000.00	3	135659703.46	A	B	
b_F	3	123666666.67	3	135659703.46	A	B	
	C						
p_F	3	162000000.00	3	135659703.46	A	B	
	C						
b_M	3	207500000.00	2	166148526.06	A	B	
	C						
p_M	3	778666666.67	3	135659703.46		B	
	C						
p_U	3	843333333.33	3	135659703.46			
	C						

Medias con una letra común no son significativamente diferentes(p<= 0.05)

Análisis de la varianza

Variable	N	R^2	R^2 Aj	CV
Conteo	49	0.66	0.47	175.23

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	3.30001095431958E18	17	194118291430563000.00	3.52	0.0012
Tratamiento	616037239014789000.00	5	123207447802958000.00	2.23	0.0760
Dia	1.27253518083037E18	2	636267590415183000.00	11.52	0.0002
Tratamiento*Dia	1.24262201762333E18	10	124262201762333000.00	2.25	0.0410
Error	1.71153062822712E18	31	55210665426681300.00		
Total	5.0115415825467E18	48			

Test:LSD Fisher Alfa=0.05 DMS=237894918.30692

Error: 55210665426681280.0000 gl: 31

Tratamiento	Medias	n	E.E.			
b_U	8491666.67	9	78323166.31	A		
b_F	41569855.56	7	101114772.91	A	B	
p_F	58036111.11	8	84598688.59	A	B	C
b_M	90468733.33	7	90439802.31	A	B	C
p_M	270416111.11	9	78323166.31		B	C
p_U	284352666.67	9	78323166.31			C

Medias con una letra común no son significativamente diferentes(p<= 0.05)

Test:LSD Fisher Alfa=0.05 DMS=167727709.77007

Error: 55210665426681280.0000 gl: 31

Dia	Medias	n	E.E.		
1	77572.22	16	59820306.38	A	
2	20255000.00	16	63950597.50	A	
3	356335000.00	17	57644289.59		B

Medias con una letra común no son significativamente diferentes(p<= 0.05)

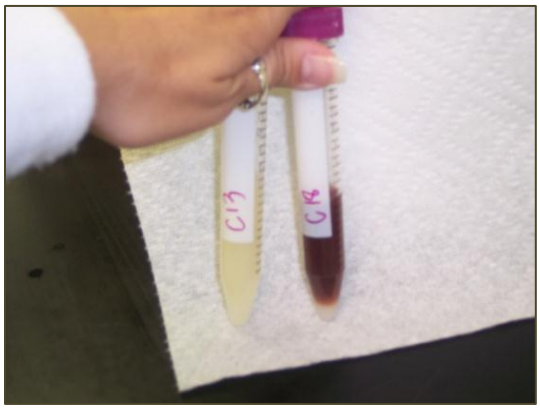
Test:LSD Fisher Alfa=0.05 DMS=417272704.96589

Error: 55210665426681280.0000 gl: 31

Tratamiento	Dia	Medias	n	E.E.		
b_M	1	6200.00	2	166148526.06	A	
b_F	1	22900.00	3	135659703.46	A	
p_F	1	75000.00	2	166148526.06	A	
p_U	1	91333.33	3	135659703.46	A	
p_M	1	115000.00	3	135659703.46	A	
b_U	1	155000.00	3	135659703.46	A	
b_F	2	1020000.00	1	234969498.93	A	
b_U	2	2476666.67	3	135659703.46	A	
p_U	2	9633333.33	3	135659703.46	A	
p_F	2	12033333.33	3	135659703.46	A	
b_U	3	22843333.33	3	135659703.46	A	
p_M	2	32466666.67	3	135659703.46	A	
b_M	2	63900000.00	3	135659703.46	A	
b_F	3	123666666.67	3	135659703.46	A	
p_F	3	162000000.00	3	135659703.46	A	
b_M	3	207500000.00	2	166148526.06	A	
p_M	3	778666666.67	3	135659703.46		B
p_U	3	843333333.33	3	135659703.46		B

Medias con una letra común no son significativamente diferentes(p<= 0.05)

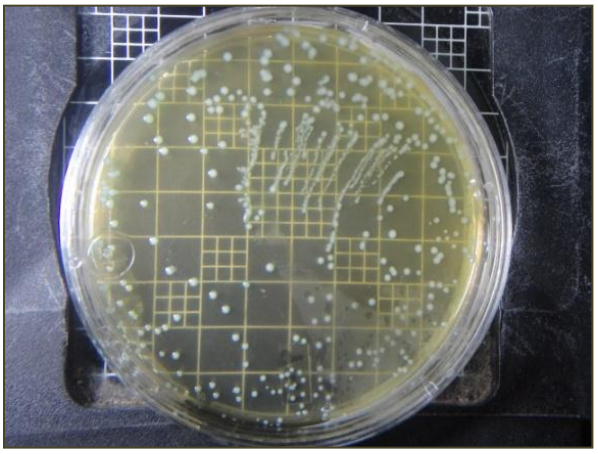
Appendix 13. F6PPK Test for C18 Isolate



Appendix 14. Anaerobic Jar System

Appendix 15. Isolates Grown on Anaerobic Tubes

Appendix 16. Photograph of Blue Colonies



Appendix 17. Prebiotic Assay on Anaerobic Bottles

Appendix 18. Photograph of Bb12 with Contrast Microscope (Emphasis on the "Y" bifid morphology)