CPQ Nutrition (2018) 1:6
Research Article

CIENT PERIODIQUE

Null Evidence of Ammonium Consumption by *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* BI-07, Commercial Probiotic Bacteria, in a Fermentative System

Solís Pacheco, J. R., Pulido Mateos, E. C., Rodríguez Arreola, A., González Reynoso, O., Balcázar López, E., González Quezada, E., Córdova López, J. & Aguilar Uscanga, B. R.*

University Center of Exact Sciences and Engineering, University of Guadalajara, Department of Pharmacobiology, Industrial Microbiology Laboratory, México

***Correspondence to:** Dr. Aguilar Uscanga, B. R., University Center of Exact Sciences and Engineering, University of Guadalajara, Department of Pharmacobiology, Industrial Microbiology Laboratory, México.

Copyright

© 2018 Dr. Aguilar Uscanga, B. R., *et al.* This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received: 06 November 2018

Published: 06 December 2018

Keywords: *Probiotics; Prebiotics; Ammonium Sulfate; Hepatica Encephalopathy*

Abstract

Ammonia is a toxic metabolite capable of causing hepatic encephalopathy in people with liver problems. This is a syndrome characterized by a series of clinical manifestations derived from attention deficit as well as cognitive deterioration, joined with the risk of fulminant hepatitis or liver cirrhosis caused by a significant reduction of hepatic parenchyma. The applications of probiotics and prebiotic in functional food is increasingly important in the prevention of diseases as the hepatic encephalopathy. Nevertheless, there is a little or null evidence of study about ammonium consumption by probiotic bacteria, in a fermentative system. Therefore, the aim of this work is to analyze the capacity of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* Bi-07 (commercial strains), for consuming ammonium in different culture media and at different concentrations of ammonium sulfate (7 and 20g/L), using two carbon sources (glucose and fructan) for producing biomass. The results show that the bacteria are able to grow in different conditions and culture media. However, not all of the ammonium sulfate was consumed by bacteria.

This research allows verifying the null capacity of these probiotic bacteria for ammonium consumption, but not to fructan consumption as source of carbon, so we consider that this *in vitro* study is important, considering that, the hypotheses established by other authors show a beneficial effect to people with liver encephalopathy problems.

Introduction

The beneficial effects of probiotic bacteria have been well-established in the treatment of gastrointestinal diseases and also have the potential to reduce the risk of developing inflammatory bowel diseases, they activate of the immune system, prevention of cancer cell growth, maintenance of mucosal integrity and promote of an antagonistic environment against pathogens [1-3]. The anticancer activity through induction of apoptosis of cancer cells seems to be promising approach for use of some probiotic strains as a support therapy or this disease prevention. Nevertheless, *in vivo* studies are necessary to ascertain if results obtained experiments from *in vitro* can be translated to the clinical practice [4].

New evidence suggest that probiotics affect beneficially the intestinal microbiota influencing in gut permeability, systemic inflammation levels preventing the production and/or uptake of lipopolysaccharides in the gut [5], and therefore reducing levels of low-grade inflammation, and host metabolism, thereby contributing to obesity [6] and fatty liver disease [7-9]. The probiotics they act by modulating visceral and hepatic fatty deposition via the gut-liver axis. Consequently, they may be proposed as add-on non-alcoholic fatty liver disease (NAFLD), treatment complementary to standard dietary and behavior strategies [10,11]. Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome and the leading cause of chronic liver disease in pediatric and adult individuals living in industrialized countries [10,12,13].

Modulation of gut microbiota by probiotics is supported by a number of studies conducted with nonalcoholic fatty liver disease animal models and in several pilot pediatric and adult human studies. Globally, this approach appears to be a promising add-on therapeutic tool to be used in the context of a tailored multi-target therapy especially in cases where standard dietary and lifestyle changes have failed. The hepatic fat metabolism also seems to be influenced by the presence of commensal bacteria, and potentially by probiotics [11]. Although the mechanisms by which probiotic might act on the liver are still unclear. However, this could be of great importance in the future because the infiltration of liver fat and hepatitis may be more prevalent as a result of high fat consumption, and may cause liver failure complicating hepatic encephalopathy [7].

Xu *et al* (2012) [14] comparing two probiotics (*Lactobacillus acidophilus* and *Bifidobacterium longum*) in rats with HFD induced NAFLD, *Bifidobacterium longum* was superior in attenuating liver fat accumulation. The lack of changes in intestinal permeability in treated mice was attributed to the effect of peptidoglycan-polysaccharide polymers rather than to endotoxin-induced stimulation of TNF-α release. This concept is supported by a human study in which levels of antibodies to peptidoglycan polysaccharide polymers significantly decreased after administration of *Lactobacillus* GG in pediatric NAFLD [15]. In the other hand, a treatment with *Bifidobacterium longum* plus the prebiotic (FOS) induced a significant improvement in serum inflammatory, metabolic, and liver enzyme parameters. End-of-study repeat liver biopsies showed improved fibrosis scores in 70% of patients and a decrease in the NASH activity index [16].

Probiotics, like other bacteria, have a set of molecules known as molecular patterns that interact with specific recognition receptors, present on the surface or membrane of the organelle of epithelial and dendritic cells. This interaction is responsible not only for some of the beneficial effects attributed to probiotics, but also for the normal interaction of the gut microbiota with the human host [17]. To be considered a probiotic bacterium it must have a high capacity of adhesion to the intestinal epithelium, produce antimicrobial substances such as bacteriocins and short-chain fatty acids, inhibiting the growth of potential pathogens [16].

Hepatic encephalopathy (HE) is a common neuropsychiatric manifestation in liver diseases. A dysfunctional liver is unable to purge ammonia from the bloodstream, which builds up to toxic levels in the central nervous system [18]. Certain studies to show the effectiveness of consumption of probiotics in improving HE, have used probiotic bacteria such as *Lactobacillus acidophilus, Enterococcus lactis, Lactobacillus paracasei subspecies paracasei, Lactobacillus plantarum, Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bi-07 [19-21]. However, knowledge about the regulation of nitrogen metabolism in lactic acid bacteria is limited. Bajaj *et al*., (2008) [21] analyzed twenty-five patients and 17 yogurt marks. The patients demonstrated a significant improvement in the number of connection test-A (NCT-A), block design test (BDT), and digit symbol test (DST) compared to baseline/no Rx group. No adverse effects or change in covariates was observed. This trial demonstrated a significant rate of HE reversal and excellent adherence in cirrhotic after probiotic yogurt supplementation with potential for long-term adherence.

Lactobacillus rhamnosus HN001 is a well-characterized probiotic strain, identified by Prasad *et al*. (1998) [22], in the New Zealand Dairy Research Institute, and was selected in studies in animals and humans for their ability to survive at low pH and relatively high concentrations of bile, actually is marketed by the company DANISCO [23]. In order to evaluate the safety of *Lactobacillus rhamnosus* HN001 several authors have worked supplying different doses of these bacteria to test mice. Shu *et al*. (1999) [24], provided *Lactobacillus rhamnosus* HN001 ($5x10^7$, $5x10^9$ or $5x10^{10}$ CFU/mouse/day) to mice for 7 days. There were not abnormal clinical signs or differences in food intake, water or weight gain compared with a controlled group (no probiotic supplement). *L. rhamnosus* HN001 was not detected in the spleen of animals.

Good *et al*., (2014) [25], they showed that oral administration of live or UV-inactivated *Lactobacillus rhamnosus* HN001 attenuates Necrotizing enterocolitis (NEC) severity in newborn mice and premature piglets, as manifest by reduced histology score, attenuation of mucosal cytokine response, and improved gross morphology. Strikingly, DNA of *Lactobacillus rhamnosus* HN001 reduced the extent of proinflammatory signaling in cultured enterocytes and in samples of resected human ileum ex vivo, suggesting the therapeutic potential of this probiotic in clinical NEC. Taken together, these findings illustrate that *Lactobacillus rhamnosus* HN001 is an effective probiotic for NEC via activation of the innate immune receptor TLR9 and that *Lactobacillus rhamnosus* DNA is sufficient for its protective effects, potentially reducing concerns regarding the infectious risk of this novel therapeutic approach.

Loguercio *et al*. (1987) [19] conducted a pilot study in patients with hepatitis of different causes (HCV, alcoholism, and NASH), which received a probiotic mix (Lactobacillus acidophilus, *L. bifidus, L. rhamnosus, L. plantarum, L. salivarius, L. bulgaricus, L. lactis, L. casei, L. breve*, more fructooligosaccharides and vitamins).

Interestingly, the authors reported that the strongest effect of probiotics was seen in patients with alcoholic liver cirrhosis, where all parameters of liver function improved, as happened with TNF-α and malondialdehyde. From these studies in humans, it appears that the microbiota is an important cofactor in the etiology of chronic liver diseases, and that probiotics might have a therapeutic role.

On the other hand, another group of probiotic bacteria are *Bifidobacteria* which are natural inhabitants of the human gastrointestinal tract and from different animals, which are present in varying amounts throughout life, appearing a few days after birth [26]. The beneficial intestinal flora, represented mainly by the genera *Lactobacillus* and *Bifidobacterium*, contributes significantly to the health status of the host, by its functions: (i) metabolic, intervening in the assimilation of nutrients from the diet; (ii) protective, contributing to the barrier effect and to the displacement of pathogenic microorganisms, and (iii) trophic, intervening in the modulation of the immune system and in cell development and proliferation [5,27].

Bifidobacterium lactis is of human origin, were discovered in the faeces of breast-fed infants, causing special interest to scientists as these bacteria are typically the most abundant species in the intestine of breastfed infants and regarded as a primary reason for the infants' greater resistance to disease. These bacteria were originally described by Meile *et al*., in 1997 [28] and was later re-classified as *B. animalis* subsp. *lactis* [29]. *Bifidobacterium lactis* Bi-07 is marketed by the company DANISCO and was deposited at the "American Type Culture Collection" as SD5220. *Bifidobacterium lactis* Bi-07 has been properly classified as *Bifidobacterium lactis*, using modern genotypic methods, including the sequencing of the rRNA 16S gene [30]. Today *bifidobacteria* are broadly recognized for their key role in the human intestinal microbiota throughout life. A high proportion of *bifidobacteria* in the intestinal tract is considered beneficial to health.

Some species as *Bifidobacterium longum*, *B. bifidum* are capable to use as only nitrogen source, ammonium salts, and when grown in the absence of a source of organic nitrogen, secrete large amounts of amino acids such as threonine, alanine, valine and aspartic acid, resulting in inhibition of the synthesis of harmful products including ammonia [31].

Deguchi *et al*. (1993) [32] selected two *B. bifidum* strains that prefer to use ammonia than any other organic nitrogen compounds by measuring incorporation of the stable isotope ammonia by the cells. They estimated that fermentable carbohydrates might enlarge the caecum and enhance a blood flow in the aorta around the caecum, resulting in a large flux of urea to the caecum and higher absorption of ammonia into the portal vein. This increased level of ammonia was reduced by oral administration of *B. bifidum* YIT4069, suggesting that *B. bifidum* YIT4069 must assimilate ammonia in the caecum. The oligofructose has a stimulating effect on the growth of *bifidobacteria*, which are metabolized by bacteria as they are not degraded by human digestive enzymes [33].

Due to the ability of probiotics to consume ammonium, the effect of the concentration of carbon and nitrogen respect to survival of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* BI07, was studied. These bacteria were grown in synthetic media (YPD and YPF), supplementing with ammonium sulfate, in order to observe the kinetic behavior and consumption of ammonium of these bacteria.

Materials and Methods

Bacterial Strains

Lactobacillus rhamnosus HN001 and *Bifidobacterium lactis* Bi-07 were isolated from DuPont™ Danisco® commercial probiotic cultures. For long-term maintenance these bacterial strains were stored lyophilized in a -80°C freezer.

Culture Media

The MRS broth (BD Difco, USA) was rehydrated according instructions of manufacturer. YPD broth media contained 2g of bovine casein peptone (BD Bioxon, USA), 5g of yeast extract (BD Bioxon, USA), 1g of dipotassium phosphate (JT Baker, USA), 20g of dextrose (BD Difco, USA) and 7 or 20g of ammonium sulfate (Baker Analyzed, USA) per liter of culture media. YPF broth media contained 2g of bovine casein peptone (BD Bioxon, USA), 5g yeast extract (BD Bioxon, USA), 1g of dipotassium phosphate (JT Baker, USA), 20g of fructans from *Agave tequilana* Weber (Fructoreal, MEXICO) and 7 or 20g of ammonium sulfate (Baker Analyzed, USA). YPD and YPF culture media were prepared by suspending all the ingredients in 1L of distilled water and boiling until the ingredients were completely dissolved. The pH of the mixture was adjusted with NaOH 1N and HCl 1N to 7 ± 0.1. The mixtures were autoclaved at 121°C for 15 min and cooled to 37°C prior to use.

Propagation of Strains and Inoculum Preparation

To carry out the fermentation kinetics, the lyophilized strains were reactivated in 50mL of sterile MRS broth. The flasks were incubated at 37°C, without agitation for 32h. Subsequently the fermented media were transferred to sterile 50mL Falcon tubes and centrifuged at 3500rpm for 5min. The supernatants were discarded and the recovered biomass was added to an Erlenmeyer flask containing 200mL of each tested medium. Thereafter, to obtain exponential-phase cells, the flasks were incubated at 37°C, without agitation for 15h.

Fermentation Kinetics of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* Bi-07 in MRS Culture Medium

Fermentation kinetics of *L. rhamnosus* HN001 and *B. lactis* Bi-07 in MRS broth were performed in duplicate in Erlenmeyer flasks containing 500mL of culture medium. Flasks were inoculated with the recovered biomass from the inoculum, adjusting to an optical density (OD) of approximately 0.3. Fermentations were performed at initial pH of 7 at 37°C and without agitation. During exponential growth phase, a sample of 10mL was taken out every two hours to measure cell growth (biomass), substrate utilization (dextrose, agave fructans, ammonium and protein) and metabolites production (organic acids).

Fermentation Kinetics of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* Bi-07 in YPD and YPF Synthetic Media with Ammonium Sulfate

Fermentation kinetics of *L. rhamnosus* HN001 and *B. lactis* Bi-07 were performed in duplicate in Erlenmeyer flasks containing 500mL of YPD broth (2g/L of bovine casein peptone, 5g/L yeast extract, 1g/L of

dipotassium phosphate and 20g/L of dextrose) and for YPF medium (2g/L of bovine casein peptone, 5g/L yeast extract, 1g/L of dipotassium phosphate and 20g/L of fructans from *Agave tequilana* Weber) additional 7 or 20g/L of ammonium sulfate respectively. Flasks were inoculated with the recovered biomass from inoculum, adjusting to OD approximately 0.3. Fermentations were performed at initial pH of 7 at 37°C without agitation. During exponential phase, a sample of 10mL was taken out every two hours to measure cell growth (biomass), substrate utilization (dextrose, total protein and ammonium) and metabolites production (organic acids).

Biomass Determination

Biomass was determined by a calibration curve relating optical density to cell density (evaluated as dry weight) for each bacteria and each tested medium. Optical density was measured at 600nm using a Jenway 6305 spectrophotometer. 1ml culture sample was taken out from the exponential phase and then diluted to OD lower than first one. Dry weight of bacterial cells was obtained by centrifuging at 3500rpm for 5min, 1mL culture sample placed in Eppendorf tubes previously dried to a constant weight. After removing the supernatant, the tubes were located in a hermetically sealed oven at 70°C for 24h and subsequently put in a desiccator containing dried silica gel for 15min. The tubes were weighed in analytical balance (AE ADAM NBL 84e), the dry weight was recorded and the dry biomass concentration was calculated using the following formula:

$$\text{Biomass } (gL^{-1}) = (PTB - PTV)/\text{sample volume in L}$$

Where:
PTB is the tube weight with dry biomass
PTV is the weight of the empty tube

Analysis of Substrate Consumption and Metabolite Production by Chromatography (HPLC)

A Varian ProStar HPLC with a Refractive Index Detector (VARIAN model 356-LC) was used for measuring substrate consumption and metabolite production, including glucose, fructose, ammonium sulfate, butyric acid, propionic acid, lactic acid, acetic acid and ethanol. A Varian MetaCarb H Plus column of 300mm × 7.8mm was used for analytical separation. The adopted chromatographic conditions were: mobile phase of 0.01N sulfuric acid solution (prepared with HPLC grade water), flow rate of 0.6mL/min, temperature of 54°C and an injection volume of 20µL. A calibration curve was performed using a standard solution containing 2g/L glucose (Difco, USA), 2g/L of fructose (Sigma, USA), 2g/L of ammonium sulfate (Baker Analyzed, USA), 8g/L of lactic acid (Sigma, USA), 1g/L of acetic acid (Reasol, USA), 1g/L of propionic acid (Sigma, USA), 1g/L of butyric acid (Aldrich, USA) and 1g/L of ethanol. The following concentrations of sulfate, glucose and fructose were prepared and used for the calibration curve: 4, 8, 10, 12, 16 and 20g/L. For lactic acid, the concentrations were the following: 0.2, 0.4, 0.5, 0.6, 0.8, 1g/L. Finally, for acetic acid, propionic acid, butyric acid and ethanol, the concentrations were the following: 0.2, 0.4, 0.5, 0.6, 0.8 and 1g/L.

Determination of Ammonium Sulfate

The ammonium concentration was determined according to the method described by Weatherburn (1967) [34]. For the analysis, two reagents were prepared; reagent "A" was prepared dissolving 5g of phenol and 25mg of sodium nitroprusside (SIGMA, USA) in distilled water at 500mL volume was reached. Reagent "B" was prepared dissolving 4.2mL of sodium hypochlorite (Golden Bell, USA) and 2.5g of sodium hydroxide (JT BAKER, USA) in distilled water at 500mL. After preparing the reagents, each 20μL sample was treated with 5mL of reagent "A" and 5mL of reagent "B" and subsequently incubated for 30 min at 37°C. The optical density was measured at 625nm using a Jenway 6305 spectrophotometer. A calibration curve was performed for each assay using a standard solution (1.8g/L) of ammonium sulfate (Baker Analyzed, USA) for preparing the following concentrations: 0.1, 0.3, 0.5, 0.7, 1.2 and 1.8g/L.

Determination of Protein

The consumption of protein was determined according to the Lowry *et al.* method (1951) [35]. For the analysis, each 100μL sample was treated with 1mL of Lowry reagent and subsequently incubated for 30minutes at room temperature. After incubation, 100μL of Folin reagent (diluted 1:1 with distilled water) was added to the tubes containing the samples which were vortexed and incubated for 30minutes at room temperature. The optical density was measured at 595nm using a Jenway 6305 spectrophotometer. A calibration curve was performed for each assay using a standard solution (1g/L) of bovine serum albumin (Equitech-Bio-Inc, USA) for preparing the following concentrations: 0.1, 0.2, 0.3, 0.5, 0.75 and 1g/L.

Experimental Design

A 2^2 full factorial experimental design with two replications was used to study the influence of *Agave Tequilana fructans* and ammonium concentration (in YPF and YPD culture media) on kinetic parameters of two probiotic bacteria. The response variables were the following: produced biomass, maximum growth rate, substrate consumption rate and yield. The controlled factors were the following: type of carbon source (glucose or agave fructans) and ammonium sulfate concentration (7 and 20g/L). Statistical analysis of data was performed through analysis of variance (ANOVA) using Statgraphics Centurion XV Software. Results are expressed as means ± S.D. (standard deviation), the calculations were performed using Excel 2007 (Microsoft, Redmond, WA, USA) program.

Results and Discussion

Fermentation Kinetics of *L. rhamnosus* HN001 and B. Lactis Bi-07 Cultured in MRS Broth

Fermentations in MRS broth were carried out in order to meet the growth kinetic parameters of the studied bacteria under no nutritional stress conditions. In this medium *L. rhamnosus* and *B. lactis* grew exponentially during 10 to 11h (Figure 1). However, *L. rhamnosus* showed a maximum growth rate (μ_{max}) of 0.32h^{-1} and a generation time of 2.2h, achieving at the end of its exponential growth phase a maximum biomass content of 2.97g/L.

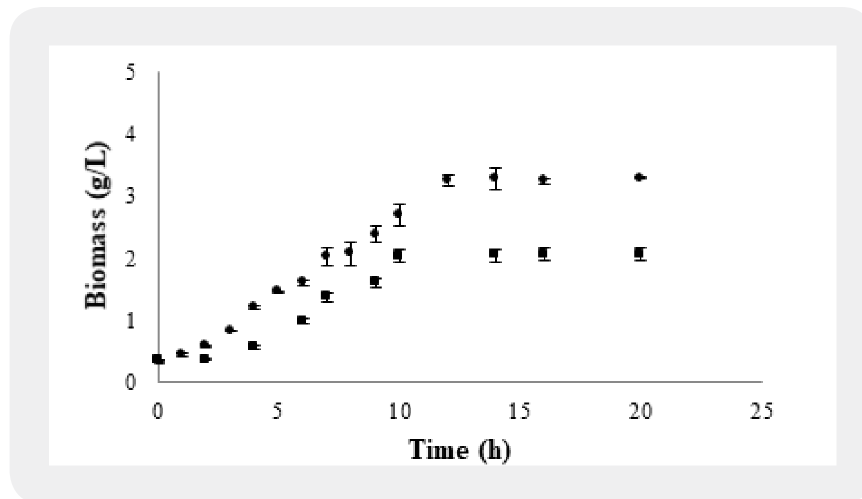

Figure 1: *Growth kinetics of L. rhamnosus HN001 (●) y B. lactis Bi-07 (■) in MRS medium. The values represent the mean and standard deviation +/- of three assays (n=3).*

According results, the amount of ammonium sulfate is capable of reducing the growth rate of *L. rhamnosus* HN001, whereas the fructan, as carbon source, is able to promote it. Regarding the increase in the growth rate due to the presence of fructan, it is noteworthy that these results are similar to those obtained by Salminen *et al*. (2006) [33] which reported that some heterofermentative *lactobacilli* exhibit a faster growth rate with fructose than with glucose. It is important to mention that in this work did not use fructose as carbon source, but a polysaccharide consisting mostly of fructose with glucose in a ratio of 8:1.

Lactobacillus then had to break fructan molecule through an enzyme β-1,2-β-fructofuranidase for getting the monosaccharides contained in the molecule. Fructose is capable to play a role both as a substrate for growth as the electron acceptor; and it is fermented by 6-PG/PK, but part of the carbohydrate are reduced to mannitol by a NAD^+ dependent mannitol dehydrogenase enzyme [36]. This allows to cells to use ATP via acetate kinase reaction. This way is less efficient that fermentation of glucose in terms of ATP formed per mole of consumed carbohydrate. However, *L. rhamnosus*, being heterofermentative *lactobacillus*, could have used glucose as an energy source and fructose as an electron acceptor, thus obtaining a higher growth rate, similar to reported by Gobbetti *et al*. (1995) [37].

Whereas in the same culture conditions, *B. lactis* was able to grow at a μmax of $0.26h^{-1}$, showing a generation time (GT) of 2.6h and achieving at the end of its exponential growth phase a maximum biomass content of 2.14g/L. The growth of both bacteria was directly correlated to their substrate utilization, obtaining a substrate consumption rate (qs) of $2.7g/g^*h^{-1}$ by *L. rhamnosus* and $2.6g/g^*h^{-1}$ by *B. lactis*; indicating that these bacteria consumed approximately 3g of glucose per h to support their rapid growth. The yield of conversion of glucose into biomass was $0.12g/g^{-1}$ by *L. rhamnosus* and $0.10\ g/g^{-1}$ by *B. lactis*. Moreover, glucose was almost completely exhausted after both microorganisms reached their stationary phase. On the other hand, proteins were not completely utilized during 24h of fermentations (Table 1), observing 66% of its consumption by *B. lactis* and 41% by *L. rhamnosus*.

Table 1: *Biomass production, glucose and protein consumption of bacteria cultured in MRS medium.*

Time (h)	Biomass production (g/L)		Glucose consumption (g/L)		Protein consumption (g/L)	
	B. lactis Bi-07	*L. rhamnosus* HN001	*B. lactis* Bi-07	*L. rhamnosus* HN001	*B. lactis* Bi-07	*L. rhamnosus* HN001
0	0.36±0.00	0.33±0.01	19.0±0.38	21.8±0.47	27.2±0.18	21.1±0.14
24	1.99±0.17	2.63±0.14	0.3±0.03	0.47±0.09	9.4±0.16	12.5±0.01

* The values in the table represent the mean and standard deviation +/- of three assays (n=3).

Fermentation Kinetics of *L. rhamnosus* HN001 Cultured in YPD and YPF Media Supplemented with Ammonium Sulfate (AS)

The results of the fermentation kinetics of *L. rhamnosus* in YPF and YPD media supplemented with 7 and 20g/L of AS are shown in Figure 2. In these media, *L. rhamnosus* tended to grow exponentially for about 5 to 6.5h. Furthermore, its μ_{max} was greatly influenced by the AS concentration (P>0.0000), observing faster growth when it was cultured in the media with the lowest AS content (Table 1). The type of carbon source also influenced the rate in which this microorganism grew (P>0.0000), noting in the 45hour fermentation that AF had a positive effect on it (Table 2 and 3). Additionally, a statistical interaction was found between both factors (P=0.0445), where L. rhamnosus had greater growth rates when it had in its media 7g/L of AS and AF as carbon source (μ_{max} 0.21h^{-1}). The GT was also affected by these factors, observing lower GTs (3.23 and 3.98 versus 6.22 and 8.96h) in the fermentations performed with the lowest AS supplementation (7g/L).

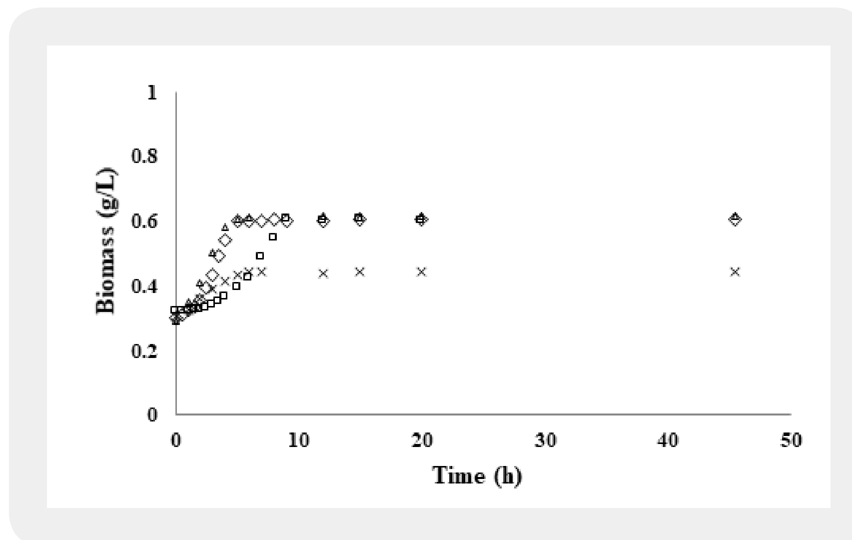

Figure 2: *Growth kinetics of L. rhamnosus HN001 in YPF media at 10g/L (◊), 20g/L (□) and YPD media at 10g/L (Δ), 20g/L (x). The values represent the mean and standard deviation +/- of three assays (n=3).*

Table 2: *Ammonium sulfate, glucose and protein consumption of bacteria cultured in YPD medium.*

Time (h)	Ammonium sulfate consumption (g/L)		Glucose consumption (g/L)		Protein consumption (g/L)	
	B. lactis Bi-07	*L.rhamnosus* HN001	*B. lactis* Bi-07	*L.rhamnosus* HN001	*B. lactis* Bi-07	*L.rhamnosus* HN001
0	8.65±0.12	8.63±0.24	19.7±0.22	18.3±0.07	7.54±0.15	8.84±0.08
45	8.52±0.11	8.68±0.02	15.3±0.13	14.7±0.13	4.36±0.11	7.40±0.39

* The values in the table represent the mean and standard deviation +/- of three assays (n=3).

Table 3: *Ammonium sulfate, glucose and protein consumption of bacteria's cultured in YPF medium.*

Time (h)	Ammonium sulfate consumption		Frutan consumption (g/L)		Protein consumption (g/L)	
	B. lactis Bi-07	*L. rhamnosus* HN001	*B. lactis* Bi-07	*L. rhamnosus* HN001	*B. lactis* Bi-07	*L. rhamnosus* HN001
0	21.3±0.72	21.7±0.16	21.6±0.31	21.7±0.13	9.83±0.05	9.19±0.06
45	20.7±0.27	21.4±0.52	18.8±0.13	17.5±0.06	8.51±0.32	8.21±0.10

* The values in the table represent the mean and standard deviation +/- of three assays (n=3).

In these fermentations carbon sources were not completely utilized, observing a residual glucose content approximately 79% in the fermentations performed in YPD medium, and approximately 82% of residual fructan content in the ones performed in YPF (Table 3). The AS content significantly affected the rate in which *L. rhamnosus* consumed its carbon source, observing higher q_s (2.29 to 2.83 versus 1.52 to 1.93g/g*h^{-1}) with the lowest AS supplementation (7g/L) (P=0.0019). The yield of conversion of glucose into biomass by *L. rhamnosus* in these media ranged from 0.04 to 0.08g/g^{-1}, showing relatively low yield compared to results obtained with MRS medium. Interestingly, in these media *L. rhamnosus* showed a preference for utilizing protein over ammonium as nitrogen source, observing 9 to 15% of protein consumption, and nearly 0% of ammonium utilization.

It is also important to mention that in the kinetics of *L. rhamnosus* HN001 on YPF medium with 7 and 20g/L of ammonium sulfate, a similar amount of biomass was obtained. However, in the case of the kinetic with 20g/L of ammonium sulfate, an increased generation time was observed because the biomass production increased at lower speed. In MRS medium, MRS *L. rhamnosus* HN001 showed a higher growth rate compared with YPD and YPF medium. This because of the lack of nutrients in the culture media that in a way limited the growth of these bacteria.

No one of the experimental kinetic studies performed with *L. rhamnosus* HN001 was observed that bacteria consumed ammonium sulfate; probably due to that bacteria metabolism is inhibited by the organic acids produced, and probably some other metabolites. It is known that glutamine synthetase (glnA) is the main enzyme responsible for the assimilation of ammonium ions in *L. rhamnosus*, and this enzyme is regulated by the transcription factors GlnR and TnrA.

In response to high concentrations of available nitrogen source in the medium, the transcriptional factor GlnR downregulates the synthesis of glutamine synthetase; also, transcription of the glnA gene is higher when the cells are grown with low nitrogen source [38]. In our case it is probably that during fermentation with *L. rhamnosus* HN001 has caused an inhibition of the activity of glutamine synthetase by the action of the transcription factor GlnR, in response to high concentration of ammonium sulfate in the culture medium provided.

Throughout these fermentations, *L. rhamnosus* caused a rapid drop of the media pH due to big amount of lactic acid that it is able to produce; observing at the end of exponential growth phase pH= 4.46 $\pm$ 0.14. The primary metabolite produced was lactic acid, which was synthetized during both, exponential and stationary growth phases, observing significantly more production when the culture media had glucose as carbon source ($P>0.0000$) (7.77 and 8.08g/L versus 2.58 and 2.73g/L). Likewise, the concentration of AS in the culture media affected this bacteria's ability to produce acetic acid ($P>0.0000$), showing that only produced it when it was cultivated in the media with the lowest AS content (7g/L). Acetic acid was exclusively produced in the stationary growth phase, reaching a maximum of 0.13g/L at the end of the fermentation performed in YPD medium supplemented with 7g/L of AS. Furthermore, Solga (2003) [18] proposed a mechanism which is possible to promote the excretion of ammonia in patients with encephalopathy, which have high concentrations of this toxic metabolite in their system; this mechanism is based on the promotion of the ionization of the ammonium molecule, result that is obtained by acidifying the pH of the intestinal lumen. In this respect if *L. rhamnosus* HN001 consume glucose as carbon source, is capable of synthesizing a high amount of lactic acid, both in low and high concentrations of ammonia, indicating an increasing in ammonium ionization to ammonia in the intestine.

Instead, it can be said that if *L. rhamnosus* HN001 consumed fructan as a carbon source, has a lower capacity for synthesis of lactic acid in comparison to glucose consumption; suggesting that *L. rhamnosus* HN001 can have greater beneficial effect in terms of small intestine rather than colon, mainly in the duodenum where the lactobacillus may dispose of the glucose. However, it is important to emphasize that in all tested media *L. rhamnosus* HN001 demonstrated survivability, growth and synthesis of organic acids, despite the high concentrations of ammonium in the medium.

Fermentation Kinetics of *B. Lactis* Bi-07 Cultured in YPD and YPF Media Supplemented with Ammonium Sulfate (AS)

The results of the fermentation kinetics of *B. lactis* Bi-07 in YPD and YPF media supplemented with 7 and 20g/L of AS are shown in Figure 3. In YPD medium *B. lactis* tended to grow exponentially during 14 to 19h showing a μ_{max} of 0.08 h^{-1}; while in YPF media this bacteria presented a shorter exponential growth phase (~ 8h) and a μmax ranging between 0.11 and 0.14h^{-1}. The type of carbon source had a great impact on *B. lactis* growth, noting a faster growth when this bacteria had AF in its culture media ($P=0.0001$). In addition, a statistical interaction was found between factors, the ammonium content and the type of carbon source ($P=0.0047$), observing that AS supplementation played an important role promoting this *bifidobacteria* growth when it had fructan as carbon source (fermentation carried out for 45hours). The GT was also influenced by these factors observing a lower GT in the fermentation performed in YPF medium with 20g/L of AS (4.99h^{-1}) (Table 2 and 3).

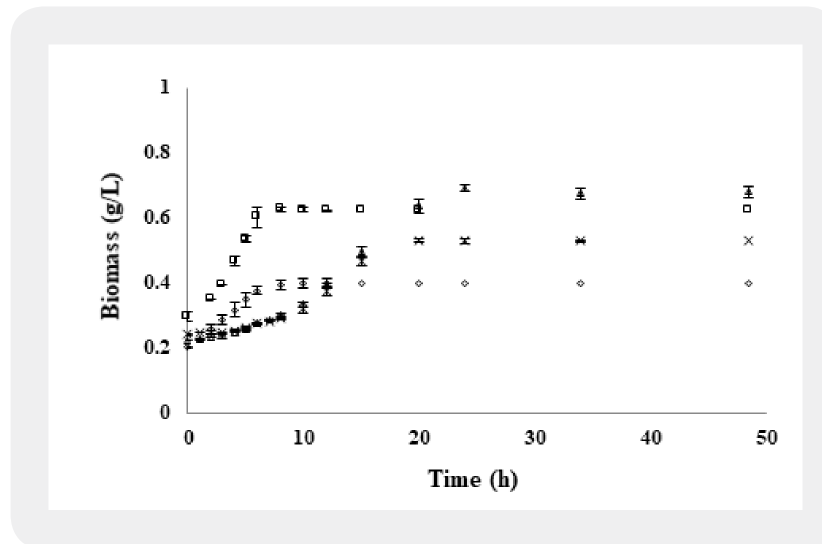

Figure 3. *Growth kinetics of B. lactis Bi-07 in YPF media at 7g/L (◊), 20g/L (□) and YPD media at 7g/L (Δ), 20g/L (x). The values represent the mean and standard deviation +/- of three assays (n=3).*

During these fermentations *B. lactis* was not able to use completely its carbon source, consuming only 22 to 25% of glucose in the fermentations performed in YPD medium and 14 to 20% of fructans in the ones performed in YPF (Table 2 and 3). In addition, *B. lactis* presented a q_s of 1g/g* h^{-1} and a yield of 0.06 to 0.09g/g^{-1} when it was cultivated in YPD medium, as well as a q_s of 2g/g* h^{-1} and a yield of 0.05 to 0.08g/g^{-1} when it was cultivated in YPF. On the other hand, protein consumption was directly correlated with biomass production, observing approximately 40% of its utilization in the fermentations with greater biomass production. It was not observed ammonium consumption throughout these fermentations.

In these media, the primary metabolites produced by *B. lactis* were lactic and acetic acids, which were generated in both, exponential and stationary growth phases. Production of lactic acid was greatly influenced by both factors, the AS content (P>0.0000) and the type of carbon source (P>0.0000); observing greater production when media had more ammonium (1.8 versus 2.3g/L, and 3.1 versus 7.1g/L) and fructans as carbon source (1.8 versus 3.1g/L, and 2.3 versus 7.1g/L). Interestingly, a statistical interaction was found between both factors (P>0.0000) noticing that the best conditions for lactic acid production were 20g/L of AS supplementation plus AF, achieving maximum of 7.1g/L of this acid after 72h of fermentation. Furthermore, acetic acid production by *B. lactis* was also influenced by the type of carbon source (P>0.0000), observing a greater production when this bacteria had glucose in its media (1.1 versus 0.3g/L). Surprisingly it was not detected acetic acid production in fermentations performed in YPF media supplemented with 20g/L of AS.

Regarding the growth of B. lactis Bi-07 in MRS media YPD and YPF, the variable that most influenced biomass production was the carbon source. It is well known the bifidogenic effect exerted by inulin-type fructans in the intestine [39]; however, it has recently been proposed that degradation of the different size fractions of these fructans is through a joint work between different species of Bacteroides genus and various species of *Bifidobacterium* genus, which exhibit diverse degradative capacity for these molecules [11,39], so these results would not be applicable "*in vivo*".

It was observed that the bacteria decrease growth when fermentation was carried out in the YPD medium at an ammonium sulfate concentration of 20g/L, since in this medium there was a maximum biomass, while in YPD medium supplemented with 7g/L of ammonium sulfate produced less amount of biomass. When the growth of *B. lactis* in YPF medium is compared at both ammonium sulfate concentrations, is observed that the ammonium sulfate production influences biomass production. Moreover, we found that maximum growth was obtained with YPD medium supplemented with 7g/L of ammonium sulfate, using glucose as a carbon source. In contrast, the culture medium where less amount of biomass produced was in the YPF supplemented with 7g/L ammonium sulfate. Surprisingly the same amount of biomass was obtained in the media with 20g/L of ammonium sulfate, with fructans or glucose as a carbon source. It is also important to mention that bacteria growth in MRS medium was greater than in YPD and YPF media; this behavior is due in part to the nutritional content of MRS medium described by Man *et al*. (1960) [40], which contains glucose, peptone, yeast extract, meat extract, magnesium, manganese, acetate, citrate and polysorbate 80, which facilitate the growth of *bifidobacteria*.

In this study is possible to consider that in the experimental media YPD and YPF did not obtain similar amount of biomass, due that these media contained less amount of nutrients, in addition, the organic acids produced by microorganisms may have inhibited their growth. In this respect, Desjardins *et al*. (1990) [41], reported that in fermentations performed with distinct species of *Bifidobacterium* with pH controlled, *bifidobacteria* stopped growth inclusive without having consumed completely its carbon source (up to 50% residual). However, in our case, the pH was a limiting, since the required pH for optimum growth of this bacterium oscillates between 6.5-7; and no exists growth to pH value of lesser of 4.0 [42]. Another important issue is that our culture media exclusively contained yeast extract and peptone that is not sufficient stimulus for growing in this strain. Similarly, attending to results found in our experiments, it is possible that concentration of ammonium sulfate present in all experimental culture medium influenced biomass production by the *bifidobacteria*.

Furthermore, the enzyme β-fructafuranosidasa in *B. lactis* necessary for the hydrolysis of fructan has an optimum pH to 6 [43] and it has been found in other species this enzyme does not act to pH lower than 4.5 [44]. So it is possible to assume that when medium has low pH, the carbon requirements (glucose and fructose) in *bifidobacteria* were not maintained free in medium, limiting their growth.

On the other hand, just as *L. rhamnosus* HN001, there was not consumption of ammonium sulfate in any of the fermentations with *B. lactis* Bi-07, so that, the enzyme responsible for the assimilation of ammonium, the glutamine synthetase and glutamate dehydrogenase, may have stronger affinity for ammonia than for the ammonium, as in other species of *Bifidobacterium* is reported activity of these enzymes in the presence of ammonia or hydroxylamine [43]. In the experiments a preference of proteins over the ammonium sulfate in medium was observed in *bifidobacteria*, assuming that under these conditions, it is difficult to consume ammonium sulfate in order to satisfy their needs for nitrogen. In this regard, it is important to mention that experimental mediums used in this work (YPD and YPF) about 99% of ammonium was in ionized form (NH_4^+), our results apply primarily to this source of nitrogen in ionized state (NH_4^+) and not for ammonia (NH_3).

Deguchi and co-workers (1985) [44] reported the complete clearance of ammonia in a biological system by the addition of a fermentable substrate and strain of *B. bifidum*, specifically chosen for its high capacity to incorporate ammonia into their cells. However, these *In vivo* results obtained, are due to the decrease in pH caused by the release of organic acids produced by the probiotic, promoting ionization of ammonia to ammonium, which is much easier to be excreted *In vivo*.

Accordingly, it is possible that *B. lactis* Bi-07 produces more amount of lactic acid when it has fructan as a carbon source and high concentration of ammonium sulfate, thus, it is believed that it might be ideal to promote ionization of ammonia at colon level, main habitat of *bifidobacteria* [18]. Also, in this studied showed that ammonium sulfate is capable of promoting the growth of *bifidobacteria* when it consumes fructan as carbon source. About it, we do not know if this is due to change in the metabolism of the microorganism or the incorporation of small amounts of ammonium on *bifidobacteria* cells. Regarding this result is important to mention the scope of our analysis, since both the chromatographic as colorimetric method, important standard deviations were found; so that a lower consumption of these may not have been detected, due to the sensitivity of the methods [45-48].

Conclusions

This work provides guidelines to analyze the capacity of probiotic bacteria to consume different carbon sources and ammonium. The *In vitro* study of fermentations certainly has its limitations regarding extrapolation *In vivo*, and never takes into account the complexity of the intestinal microbiota. Despite these limitations, the studies serve as an important reference for furthering research about bacterial metabolism in people with hepatic encephalopathy problems. The type of carbon source had a great impact on *Bifidobacterium lactis* Bi-07 growth, observing a faster increase when these bacteria consume fructan from agave in the culture media. From the obtained results, we recommend to consume food with *Bifidobacterium lactis* Bi-07 and include in their formulation fructan of agave. The ammonia in the presence of fructan, as carbon source, promotes of growth *B. lactis* Bi-07, producing more amount of lactic acid, which helps to further acidification of intestinal pH, which *In vivo* would mean increased excretion of ammonia (toxic) in the ammonium form. Although are evidence the beneficial effects of probiotic consumption in people with HE, there are no criteria to choose these strains. There is a great variability between strains selected for these studies, which causes a great diversity in their metabolic activity. Therefore, it is necessary to study in depth the mechanisms that may be involved in this process for further identification of probiotic strains with more specific metabolic potential of these diseases.

Acknowledgements

We thank CONACYT and PRODEP for the support provided to carry out this work.

Conflicts of Interests

There is no conflict of interest between the authors. We all agree that the results of this work be published.

Bibliography

1. Amraii, H. N., Abtahi, H., Jafari, P., Reza, M. H., Fakhroleslam, M. R. & Akbari, N. (2014). *In Vitro* Study of Potentially Probiotic lactic Acid Bacteria Strains Isolated From Traditional Dairy Products. *Jundishapur J. Microbiol., 7*(6).

2. Ouwehand, A., Forssten, S., Lehtinen, M., Galbraith, E. & Davis, E. (2013). Probiotic lactic acid bacteria vs bacilli: pros and cons. *Agro Food Ind Hi Tec., 24*, 13-18.

3. Arthure, C., Ouwehand, A. C. & Salminen, S. (2002). Probiotics: an overview of benefical effects. *Antonie Van leeuwenhoek, 82*(1-4), 279-89.

4. Daniluk, U. (2012). Probiotics, the New Approach for Cancer Prevention and/or Potentialization of Anti-Cancer Treatment? *J Clin Exp Oncol., 1*(2).

5. Guarner, F. & Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet, 361*(9356), 512-519.

6. Zhang, H., DiBaise, J. K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., Parameswaran, P., *et al*. (2009). Human gut microbiota in obesity and after gastric bypass. *Proc Natl Acad Sci U S A., 106*(7), 2365-2370.

7. Paolella, G., Mandato, C., Pierri, L., Poeta, M., Di Stas, I. M. & Vajro, P. (2014). Gut-liver axis and probiotics: Their role in non-alcoholic fatty liver disease. *World J Gastroenterol., 20*(42), 15518-15531.

8. Chassaing, B. & Gewirtz, A. T. (2014). Gut microbiota, low-grade inflammation, and metabolic syndrome. *Toxicol Pathol., 42*(1), 49-53.

9. Armougom, F., Henry, M., Vialettes, B., Raccah, D. & Raoult, D. (2009). Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. *PLoS One., 4,* 7125.

10. Miele, L., Marrone, G., Lauritano, C., Cefalo, C., Gasbarrini, A., Day, C. & Grieco, A. (2013). Gut-liver axis and microbiota in NAFLD: insight pathophysiology for novel therapeutic target. *Curr Pharm Des., 19*(29), 5314-5324.

11. Wilson, G. S., Mykkanen, H. & El Nezami, H. S. (2010). Probiotics and gut health: A special focus on liver diseases. *World Journal of Gastroenterology, 16*(4), 403-410.

12. Mouzaki, M., Comelli, E. M., Arendt, B. M., Bonengel, J., Fung, S. K., Fischer, S. E., McGilvray, I. D. & Allard, J. P. (2013). Intestinal microbiota in patients with nonalcoholic fatty liver disease. *Hepatology, 58*(1), 120-127.

13. Compare, D., Coccoli, P., Rocco, A., Nardone, O. M., De Maria, S., Cartenì, M. & Nardone, G. (2012). Gut-liver axis: the impact of gut microbiota on non alcoholic fatty liver disease. *Nutr Metab Cardiovasc Dis., 22*(6), 471-476.

14. Xu, R. Y., Wan, Y. P., Fang, Q. Y., Lu, W. & Cai, W. (2012). Supplementation with probiotics modifies gut flora and attenuates liver fat accumulation in rat nonalcoholic fatty liver disease model. *J. Clin Biochem Nutr., 50*(1), 72-77.

15. Vajro, P., Mandato, C., Licenziati, M. R., Franzese, A., Vitale, D. F., Lenta, S., *et al.* (2011). Effects of Lactobacillus rhamnosus strain GG in pediatric obesity-related liver disease. *J Pediatr Gastroenterol Nutr., 52*(6), 740-743.

16. Malaguarnera, M., Vacante, M., Antic, T., Giordano, M., Chisari, G., Acquaviva, R., *et al.* (2012). Bifidobacterium longum with fructooligosaccharides in patients with non alcoholic steatohepatitis. *Dig Dis Sci., 57*(2), 545-553.

17. Bouskra, D., Brézillon, C., Bérard, M., Werts, C., Varona, R., Boneca, I. G. & Eberl, G. (2008). Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. *Nature, 456*(7221), 507-510.

18. Solga, S.F. (2003). Probiotics can treat hepatic encephalopathy. *Medical Hypotheses, 61*(2), 307-313.

19. Loguercio, C., Del Vecchio, B. C. & Coltorti, M. (1987). Enterococcus lactic acid bacteria strain SF68 and lactulose in hepatic encephalopathy: a controlled study. *Journal of International Medical Research, 15*(6), 335-343.

20. Liu, Q., Zhong, P. D., Da, K. H., Bengmark, S., Kurtovic, J. & Riordan, S.M. (2004). Synbiotic modulation of gut flora: effect on minimal hepatic encephalopathy in patients with cirrhosis. *Hepatology, 39*(5), 1441-1449.

22. Prasad, J., Gill, H., Smart, J. B. & Gopal, P. K. (1998). Selection and characterization of lactobacillus and bifidobacterium strains for use as probiotics. *International Dairy Journal, 8*(12), 993-1002.

23. DANISCO (2008). *Bifidobacterium lactis Bi-07*. Technical Memorandum. TM 55-Ie.

24. Shu, Q., Zhou, K. S., Rutherfurd, K. J., Birtles, M. J., Prasad, J., Gopal, P. K. & Gill, H. S. (1999). Probiotic lactic acid bacteria (*Lactobacillus acidophilus* HN017, *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019) have no adverse effects on the health of mice. *International Dairy Journal, 9*(11), 831-836.

25. Good, M., Sodhi, C. P., Ozolek, J. A., Buck, R. H., Goehring, K. C., Thomas, D. L., Vikram, A., *et al.* (2014). Lactobacillus rhamnosus HN001 decreases the severity of necrotizing enterocolitis in neonatal mice and preterm piglets: evidence in mice for a role of TLR9. *Am. J. Physiol Gastrointest Liver Physiol., 306*(11), G1021-G1032.

26. Lee, Y. K. & Salminen, S. (2009). *Handbook of probiotics and prebiotics*. Second edition. New Jersey: A John Willey and Sons Publication. (pp. 101-389).

27. Collins, M. D. & Gibson, G. R. (1999). Probiotics, prebiotics and synbiotics: approaches for modulating the microbial ecology of the gut. *Am. J. Clin. Nut., 69*(5), 1052-1057.

28. Meile, L., Ludwig, W., Rueger, U., Gut, C., Kaufmann, P., Dasen, G., Wenger, S. & Teuber, M. (1997). Bifidobacterium lactis sp. nov., a moderately oxygen tolerant species isolated from fermented milk. *Syst. Appl. Microbiol., 20*(1), 57-64.

29. Masco, L., Ventura, M., Zink, R., Huys, G. & Swings, J. (2004). Polyphasic taxonomic analysis of Bifidobacterium animalis and Bifidobacterium lactis reveals relatedness at the subspecies level: reclassification of Bifidobacterium animalis subsp. animalis subsp. nov. and Bifidobacterium lactis as Bifidobacterium animalis subsp. lactis subsp. nov. *Int. J. System. Evol. Microbiol., 54*(Pt 4), 1137-1143.

30. Ventura, V. & Zink, R. (2002). Rapid identification, differentiation, and proposed new taxonomic classification of *Bifidobacterium lactis*. *Appl. Environ. Microbiol., 68*(12), 6429-6434.

31. Araya, K. T., Yaeshima, T., Ishibashi, N., Shimamura, S. & Hayasawa, H. (1995). Inhibitory effects of Bifidobacterium longum BB536 on harmful intestinal bacteria. *Bifidobacteria and Microflora, 14*(2), 59-66.

32. Deguchi, Y., Makino, K., Iwabuchi, A., Watanuki, M. & Yamashita, T. (1993). Selection of ammonia-assimilating bifidobacteria and their effect on ammonia levels in rat caecal contents and blood. *Microbial Ecology in Health and Disease, 6*(2), 85-94.

33. Salminen, S., Wright, A. & Ouwehand, A. C. (2006). *Lactic Acid Bacteria: Microbiological and Functional Aspects. 3rd edition. Lars Axelsson*. New York: Marcel Dekker, Inc. Cap., (pp. 22-383).

34. Weatherburn. (1967). Phenol-Hypochlorite Reaction for Determination of Ammonia. *Analitical Chemistry, 39*(8), 971-974.

35. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry, 193*(1), 265-75.

36. López, M. G., Mancilla, M. N. A. & Mendoza, D. G. (2003). Molecular structures of fructans from agave tequilana Weber var. azul. *Journal of Agricultural and Food Chemistry, 51*(27), 7835-7840.

37. Gobbetti, M., Corsetti, A. & Rossi, J. (1995). Maltose-fructose co-fermentation by Lactobacillus brevis subsp. lindneri CB1 fructose-negative strain. *Applied Microbiology and Biotechnology, 42*(6), 939-944.

38. Varmanen, P., Savijoki, K., Avall, S., Palva, A. & Tynkkynen, S. (2000). X-Prolyl dipeptidyl aminopeptidase gene (Pepx). Is part of the glnra operon in Lactobacillus rhamnosus. *Journal of Bacteriology, 182*(1), 146-154.

39. Van der Meulen, R., Makras, L., Verbrugghe, K., Adriany, T. & De Vuyst, L. (2006). *In Vitro* Kinetic Analysis of Oligofructose Consumption by Bacteroides and Bifidobacterium spp. Indicates Different Degradation Mechanisms. *Applied. Environm. Microbiol., 72*(2), 1006-1012.

40. Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology, 23*(1), 130-135.

41. Desjardins, M. L., Roy, D. & Toupin, C. (1990). Uncoupling of growth and acids production in Bifidobacterium ssp. *Journal of Dairy Science, 73*(6), 1478-1484.

42. Scardovi, V. & Trovatelli, L. D. (1965). The fructose-6-phosphate shunt as peculiar pattern of hexose degradation in the genus Bifidobacterium. *Annals of Microbiology, 15,* 19-29.

43. Warchol, M., Perrin, S. & Schneider, F. (2002). Characterization of a purified β-fructofuranosidase from Bifidobacterium infantis ATCC 15697. *Appied Microbiology, 35*(6), 462-467.

44. Deguchi, Y., Morishit, T. & Mutai, M. (1985). Comparative studies on synthesis of water soluble vitamins among human species of bificobacteria. *Agricultural and Biological Chemistry, 49,* 13-19.

45. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analitical Chemistry, 28*(3), 350-356.

46. Janer, C., Rohr, L. M., Peláez, C., Laloi, M., Cleusix, V., Requena, T. & Meile, L. (2004). Hydrolysis of oligofructoses by the recombinant β-fructofuranosidase from Bifidobacterium lactis. *Systematic and Applied Microbiology, 27*(3), 279-285.

47. Salminen, S., Von Wright, A., Morel, L., Marteau, P., Brassart, D., de Vos, W.M., *et al.* 1998. Demonstration of safety of probiotics: a review. *International Journal of Food Microbiology, 44*(1-2), 93-106.

48. Summer, J. B. (1921). Dinitrosalicylic acid: A reagent for the estimation of sugar in normal and diabetic urine. *The Journal of Biological Chemistry,* 47-55.