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HPLC Characterization of Lactic Acid Formation and FTIR Fingerprint of Probiotic Bacteria during Fermentation Processes

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Abstract

Lactic acid production during growth of several probiotic bacteria (*Lactobacillus plantarum*, *Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve* and mix of them) in model MRS media has been monitored using HPLC and the bacterial fingerprint was detected using FTIR. FTIR spectroscopy was able to fingerprint the bacteria identify specific markers located at 900-1300 cm^{-1} (carbohydrates and proteins, nucleic acids from DNA of bacteria), 1300-1900 cm^{-1} (C=O stretching vibrations) and large band (for water) at 3100-3700 cm^{-1}. HPLC evaluation showed that *Lactobacillus plantarum* achieved lactic acid concentration (after 78 h of fermentation) close to 6.08g L^{-1}, *Bifidobacterium infantis* 7.09 g L^{-1}, *Lactobacillus casei* 6.16 g L^{-1}, *Bifidobacterium breve* 6.17 g L^{-1}, while mix of them 7.12 g L^{-1} Pearson's analysis showed that the level of lactic acid in mix of probiotics bacteria was positively and significantly correlated, with lactic acid level of *Lactobacillus plantarum* (r^2=0.965), *Bifidobacterium infantis* (r^2=0.9846), *Lactobacillus casei* (r^2=0.9904), *Bifidobacterium breve* (r^2=0.9958), when P<0.0001.

Keywords: HPLC, FTIR, probiotics, lactic fermentation

Introduction

Lactic acid is one of the most important organic acids produced by lactic acid bacteria (LAB), discovered by Swedish scientist C.W. Scheele in 1780 from sour milk. Lactic acid exists in two optically active stereo-isomers, the L(+) and the D(-). Lactic acid has a wide range of beneficial uses in the sectors relating to food preservation, flavor enhancement etc. Since elevated levels of D(-) lactic acid is harmful to humans, L(+) lactic acid is the preferred isomer in food and pharmaceutical industries as humans have only L-lactate dehydrogenase that metabolizes L(+) lactic acid (Akerberg *et al*., 1998; Hofvendahl *et al*., 2000). Lactic acid can be manufactured either by chemical synthesis or by microbial fermentations using LAB.

Lactic acid bacteria (LAB) can be homofermentative or heterofermentative and can produce either L(+) or D(-) or racemic mixture of lactic acid. LAB have the property of producing lactic acid from carbohydrates through fermentation. Lactic acid bacteria are used in the food industry for several reasons. Their growth lowers both the carbohydrate content of the foods that they ferment, and the pH due to lactic acid production. It is this acidification process which is one of the most desirable effects of their growth. The pH may drop to as low as 4.0, low enough to inhibit the growth of most other microorganisms including the most common human pathogens, thus allowing these foods to prolong shelf life. The genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella* are the main members of the LAB (Axelsson, 2004; Davidson *et al*., 1995; Ercolini *et al*., 2001; Jay, 2000; Holzapfel *et al*., 2001; Stiles and Holzapfel, 1997).

Lactobacillus is the largest genus, facultative anaerobic, comprising around 80 recognized species (Axelsson, 2004). Lactic acid bacteria can grow at temperatures from 5 to 45°C and not surprisingly are tolerant to acidic conditions, with most strains able to grow at pH 4.4. The growth is optimum at pH 5.5-6.5 and the organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates. The genus is divided into three groups based on fermentation patterns:

Homofermentative: produce more than 85% lactic acid from glucose. They ferment 1 mol of glucose to 2 mol of lactic acid, generating a net yield of 2 mol of ATP per molecule of glucose metabolized. Lactic acid is the major product of this fermentation.

Heterofermentative: produce only 50% lactic acid. These ferment 1 mol of glucose to 1 mol of lactic acid, 1 mol of ethanol, and 1 mol of CO_2. One mole of ATP is generated per mole of glucose, resulting in less growth per mole of glucose metabolized.

"Facultatively" heterofermentative, meaning they will produce CO_2 and other by-products only under certain conditions or from specific substrates. These strains would include *Lb. plantarum*, *Lb. casei* and *Lb. curvatus.*

Bifidobacterium is a genus of gram-positive, non-motile, strictly anaerobic bacteria, inhabiting the gastrointestinal tract and vagina. Bifidobacteria are one of the major genera of bacteria that make up the gut flora, the bacteria that reside in the colon. Bifidobacteria aid in digestion, are associated with a lower incidence of allergies and also prevent some forms of tumours growth. Some bifidobacteria are used as probiotics. *Bifidobacterium infantis* is a probiotic bacterium that inhabits the intestines of both infants and adults. This type of bacteria is considered beneficial because of the acids it produces. *Bifidobacterium breve* produces beneficial lactic and acetic acids. It has been shown to repress the growth of ulcer-inducing bacteria.

Lactic acid occurs in fermented products as a result of hydrolysis, biochemical metabolism, and microbial activity. Quantitative determination of lactic acid is important in fermented products for technical, nutritional, sensorial, and microbial reasons. Thitrimetric methods, gas chromatography, colorimetric analysis and enzymatic methods are examples of techniques that are used for analyses of organic acids (Andersson and Hedlund, 1983). However, because simplicity and speed of analysis, the HPLC techniques is an attractive method, witch requires a minimum of sample preparation prior to separation and permits quantitative determination of organic acids in short time.

FTIR spectra of bacteria are specific to a given strain and show the spectral characteristics of cell components, such as fatty acids, membrane and intracellular proteins, polysaccharides, and nucleic acids.

We aimed to study lactic acid production during growth of several probiotic bacteria (*Lactobacillus plantarum*, *Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve* and mix of them*)* in basel MRS media, using HPLC and the bacterial fingerprint was detected using FTIR.

Materials and methods

Microorganism and Fermentation Condition

Freeze dried strains *Lactobacillus plantarum, Bifidocaterium infantis, Lactobacillus casei, Bifidobacterium breve* and mix of them were purchased form THTSA Science Park of the University of Gembloux, Belgium. After a preliminary inoculation (1g bacterial dry mass) in 10 ml MRS (de Man, Rogosa, Sharpe) broth (Merck, Germany) and incubation for 24h at 37°C under anaerobic conditions for *B. breve* and *B. infantis* and aerobic conditions for *L. casei* and *L. plantarum*, the bacterial suspension was sub-cultured into 90 mL sterile MRS broth. The fermentations were carried out at temperature of 37°C, an agitation speed of 200 rpm, with no aeration in a 200 mL Erlemayer flask.

HPLC Characterization

Aliquots of the fermentation liquid were taken every 2 h to determine lactate, concentration. Samples were thermal treated at 95°C for 20 minutes, and store at 18 °C. HPLC (Agilent Technologies 1200 Series) chromatograph coupled with UV-VIS detector and an HPLC column Acclaim OA 5 μm, 4 X 250 mm. The mobile phase was sodium sulfate (100mM) solution (pH 2.65 adjusted with MSA) using an isocratic elution with a flow rate of 0.6 ml/min. The detection of lactic acid was set at λ=210 nm. The calculation of lactic acid was made from the peak area registered at specific retention time for lactic acid, and considering the regression curve factor.

FTIR Spectroscopy and Measurement

The FTIR spectra using Attenuated Transmission (HATR) and an internal reflection accessory made of Composite Zinc Selenide (ZnSe) and Diamond crystals were obtained with Schimatzu IR Prestige- 21 equipment. Each spectrum was registered from 4000 to 500 cm^{-1}. The FTIR spectra were recorded for all samples. Three spectra were acquired for each variant at room temperature. Each spectrum was composed of an average of 128 separate scans. Measuring time was about 9 minutes per sample (*n=3*) and which depends on the number of scans per spectrum. Accordingly, as the average scans increases, the measuring time increases.

Statistical Analyses

Analysis of variance (ANOVA) was applied to all data for the production of lactic acid in all trials, followed by Pearson's correlation coefficient. The statistical evaluation was carried out using Graph Prism Version 4.0 (Graph Pad Software Inc., San Diego, CA, USA).

Results and discussion

HPLC Quantification

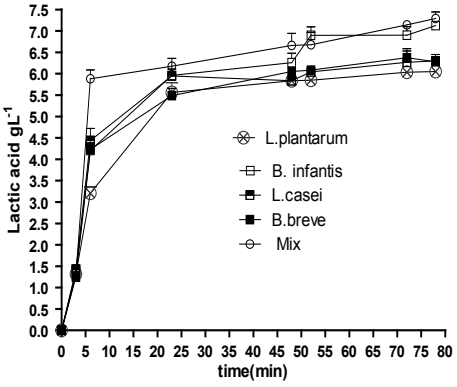

Fig. 1. Time course of lactic acid formation in fermentation processes by probiotic strains *(Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve,* and mix of them) on model MRS media. The fermentations were conducted in a 200 ml flask with 100 ml working volume at 37°C, and 200 rpm

Fig.1, showed the typical time course of lactic acid production by *Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve*, Mix of bacteria *(Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve)* on MRS model media. The cells were well adapted to MRS media and product formation began immediately after adding the inoculum. At the end of the fermentation process (after 78 hours) lactic acid production was 6.08 gL^{-1} for *Lactobacillus plantarum,* 7.09 gL^{-1} for *Bifidobacterium infantis*, 6.16 gL^{-1} for *Lactobacillus casei,* 6.17 $gL^{-1}$for *Bifidobacterium breve,* 7.12 gL^{-1} for Mix *(Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve).*

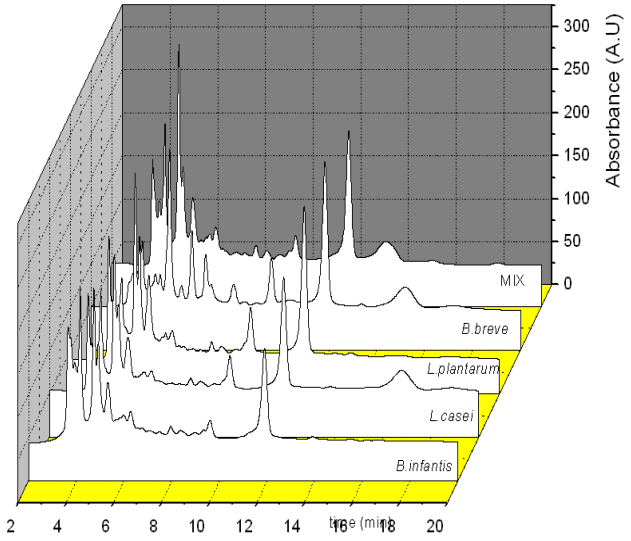

Fig. 2. HPLC Chromatograms of fermented samples by *Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve*, Mix of bacteria *(Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve)* after 78 hours of fermentation at 37°C, 200 rpm

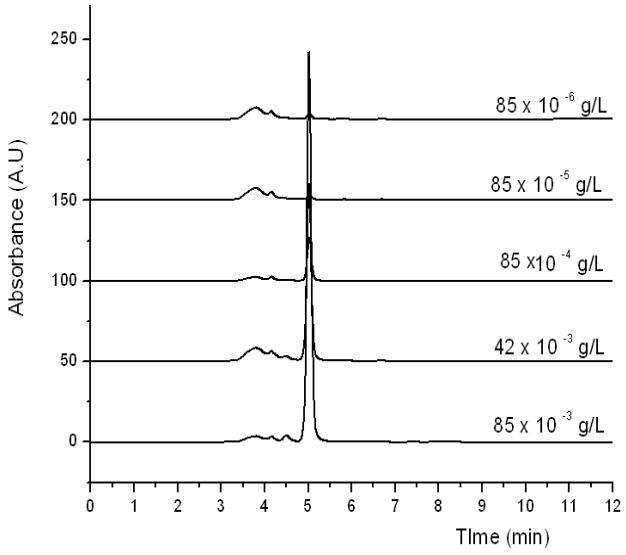

Fig. 3. Standard Chromatograms for lactic acid. (t_R=4.59 min)

The maximum lactate production was achieved after 3 hours of fermentation and before 6 hours of fermentation. *B. infantis* and Mix achieved maximal lactate concentration up to 7.1 gL^{-1} while the lowest concentration was registered by *L. casei*. The mix of bacteria has a faster growth dynamic of cells in first 6 hours while *L. plantarum* needed a longer time to adapted to media. The results correspond well to the literature for similar conditions with other lactic acid bacteria (Berry *et al*., 1999; Vodnar *et al.,* 2010).

In Fig. 2 are plotted the chromatograms of each trial, at the end of the process (after 78 hours), and show that trial with Mix *(Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve)* cumulated the maximum lactic acid formation (7.127 g L^{-1}). Retention time (t_R) for lactic acid was 4.59 min (Fig. 3).

Person's analysis showed that the level of lactic acid on MRS medium fermented by mix of bacteria was positively and significantly correlated with level achieved by *Lactobacillus plantarum* (r^2=0.965), *Bifidobacterium infantis* (r^2=0.9846), *Lactobacillus casei* (r^2=0.9904), *Bifidobacterium breve* (r^2=0.9958), when P<0.0001.

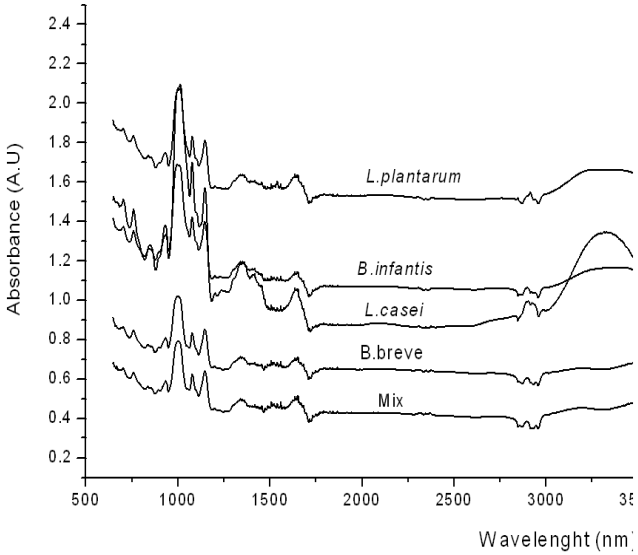

Fig. 4. Comparative FTIR fingerprints of *Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve,* Mix of bacteria (*Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve)* in water suspension

FTIR Spectroscopy

Two distinctive bands specific to probiotic bacteria are located around 3000 cm^{-1} (~ 2845 and ~ 2929 cm^{-1}) (Fig. 4). These bands are mainly due to asymmetric stretches of methyl and methylene groups respectively (Kummerle *et al*., 1998; Kansiz *et al*., 1999). These are characteristic to the bacterial cell wall fatty acids (Schmitt and Flemming,

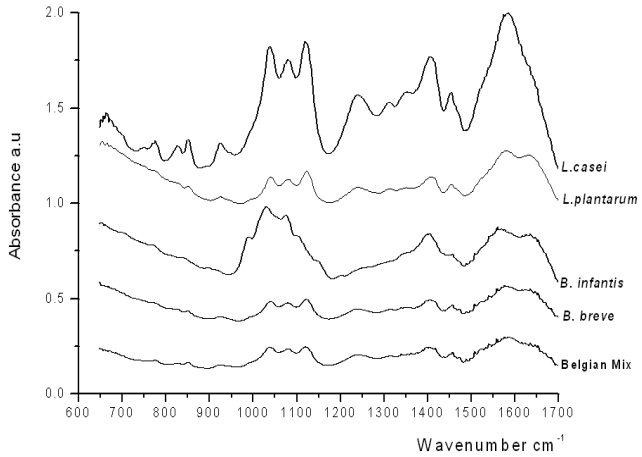

Fig. 5. FTIR Fingerprint of fermented samples (at the end of the processes-after 78h) by *Lactobacillus plantarum Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve,* Mix of bacteria *(Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve*)

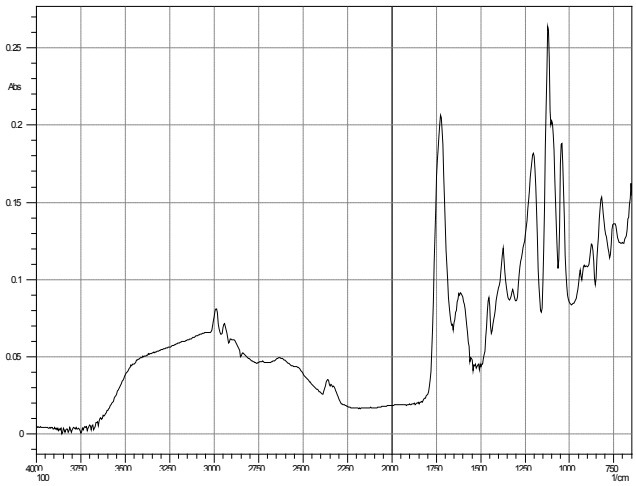

Fig. 6. FTIR fingerprint of lactic acid

1998). In addition, the absorption peak at 1730 cm^{-1} is likely due to the C=O stretching vibrations of esters functional groups primarily from lipids and fatty acids (Kansiz *et al*., 1999). The region located between 1790 and 1310 cm^{-1}, containing C=O stretching vibrations of amides linked to proteins (first amide band ~1620 cm^{-1}) (Choo-Smith *et al*., 2001), N-H deformation of amides linked to proteins (second amide at ~1530 cm^{-1}), and CH_3-, CH_2- asymmetric and symmetric deformations (bending) of proteins (~ 1430 and ~1372 cm^{-1}) (Filip and Hermann 2001). The "fingerprint region" located between 1300 - 900 cm^{-1} (Fig. 5) is characterized by vibrational features of protein, nucleic acids, cell membrane and cell wall components (Goodacre *et al*., 1996) and can be linked to single molecular bond or to a particular functional group. The specific peak for lactic acid was located at 1127 cm^{-1}(Fig. 6). The FT-IR band assignments, which specify the bacterial fingerprint region, include P=O asymmetric and symmetric stretches of the phosphodiester backbone of nucleic acids at ~1190 and ~ 1030 cm^{-1} respectively, and C-O-C stretching vibrations of polysaccharides (1200-900 cm^{-1}) associated with cell wall glycopeptides and lipopolysaccharides (Kansiz *et al*., 1999; Choo-Smith *et al*., 2001; Filip and Hermann, 2001).

Conclusions

Four probiotic bacteria (*Lactobacillus plantarum, Bifidobacterium infantis, Lactobacillus casei, Bifidobacterium breve)* and mix of them were examined for their capacity to produce lactic acid on model MRS media during fermentation process (37^0C, 200 rpm, 78 hours) using HPLC, and fingerprint of bacteria was evaluated using FTIR. HPLC results, illustrated that *Lactobacillus plantarum* achieved lactic acid concentration (after 78 h of fermentation) close to 6.08g L^{-1}, *Bifidobacterium infantis* 7.09 g L^{-1}, *Lactobacillus casei* 6.16 g L^{-1}, *Bifidobacterium breve* 6.17 g L^{-1}, while mix of them 7.12 g L^{-1} FTIR spectroscopy was able to fingerprint the bacteria identify specific markers for probiotics located at 2845 and 2929 $cm^{-1}$and one specific absorption peak at 1127 cm^{-1} for lactic acid.

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