Application of Conductimetry for Evaluation of Lactic Starter Cultures in Soymilk

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ABSTRACT: Soymilk (SM), a potential culture medium for applying conductimetric techniques to evaluate the behavior of probiotic lactobacilli and bifidobacteria was examined. Media LAPTg, LM, and SM was standardized for the growth of *Lactobacillus fermentum* and *Bifidobacterium longum*. A dilution of 1/100 for *L. fermentum* and 1/1 for *B. longum* were considered optimal to obtain a detection time (DT) between 5 and 10 min. The relationship between the initial number of cells and the DT was linear throughout the incubation period considered (40 h). The effect of temperature (30, 37, and 42 °C) on the metabolic activity of the cells was also determined after 40 h of fermentation. The higher metabolic changes were observed at 42 °C with conductance values from 600 to 800 μS and DT of 5-7 min. Results obtained were confirmed by viable counts.

Keywords: conductimetry, soymilk, lactobacilli, bifidobacteria

Introduction

MICROORGANISMS GROWN IN A SUITable medium produce charged metabolites that change the conductance and impedance of the substrate. The conductimetric technique is an interesting alternative to traditional techniques to measure microbial growth. The metabolic activity of microorganisms can be estimated in terms of detection time (DT), which is the time elapsed from inoculation to a pre-selected change in the electrical parameter. Once the DT is calibrated against the population of microorganisms determined with conventional plating methods, electrometry can be used for the prediction of microorganism populations. High number of cells give shorter DT values. Results can be obtained in 10 to 30% of the time necessary for conventional plating techniques using electrometry (Gibson 1989). For lactic acid bacteria (LAB) the direct conductimetric technique has been proposed to measure acidifying activity (Latrille and others 1992; Nieuwenhof 1984; Tsai and Luedecke 1989), estimate populations (Lanzanova and others 1993; Yoshida and others 1987), and detect bacteriophages (Carminati and Neviani 1991; Waes and Bossuyt 1984), bacteriocin activity (Giraffa and others 1990) and other inhibitory substances (Lanzanova and others 1991). In the dairy industry, the conductimetric technique has also been used successfully for assessment of performance of different starter cultures. However, little information is available on the application of this method by using soymilk as substrate.

Soymilk is a quite satisfactory growth medium for most LAB, depending on the ability of the strains to use the carbohydrates available (Angeles and Marth 1971; Garro and others 1994). In previous works growth characteristics and end-products formation by *Lactobacillus fermentum*, *L. casei*, *Streptococcus thermophilus*, and *Bifidobacterium longum* have been reported, using traditional microbiological techniques (Garro and others 1998, 1999).

The present work was carried out to evaluate if soymilk can be used as culture medium for conductimetric techniques, and to determine the behavior of *Lactobacillus* and *Bifidobacterium* strains in soymilk for their application in probiotic foodstuffs.

Materials and Methods

Microorganisms

The strains *Lactobacillus fermentum* CRL 251 and *Bifidobacterium longum* CRL 849 used in this study were obtained from the culture collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA). Both species are able to reduce α-1,6-galactosides from soy beans.

The following culture media were used:

1) Soymilk (SM). Commercial SM was used in this study which was kindly supplied by ADES, Tucumán, Argentine. This medium contains: 3% protein, 2.5% lipids, 3.5% sugars (2.8% sucrose and 0.7% stachyose), 0.5% ash, and 90.5% water (approximate composition). SM was sterilized prior to use at 115 °C for 20 min and final pH was 6.8-7.0.

2) General-Purpose Medium Plus (GPM Plus, bioMerieux, 69280 Marcy-l'Etoile, France) contained per liter: biotrypcase, 9 g; bio-soyase, 3 g; bio-gelytone, 7 g; yeast extract, 5 g; glucose, 2 g; sodium chloride, 6 g; L-arginine, 1 g; sodium pyruvate, 1 g; ammonium sulfate, 1 g; sodium hydrogen carbonate, 1 g; calcium chloride, 0.1 g; hemin, 0.005 g; vitamin K3, 0.0005 g; pH 7.8±0.3. The medium was autoclaved at 121 °C for 15 min.

3) Lactic medium (LM, bioMerieux, 69280 Marcy-l'Etoile, France) contained per liter: bacto peptone, 10 g; tryptone, 5.0 g; peptonized milk, 10.0 g; dextrose, 4.0 g; yeast extract, 7.5 g; pH 6.5.

4) LAPTg broth (Raibaud and others 1961) contained per liter:glucose, 10 g; yeast extract, 10 g; peptone, 10 g; tryptone, 10 g; Tween 80, 1 ml; pH 6.5.

LM and LAPTg were autoclaved at 121 °C for 15 min and supplemented with: 2% (w/v) sucrose, 0.0005% (w/v) hemin and 0.00005% (w/v) vitamin K (final concentration, filter-sterilized prior to addition). LM and GPM Plus (hereafter referred to as GPM only) were used as control media because of their common use in conductance measurements.

Culture conditions

Stock cultures were maintained in SM with 20% glycerol and stored at –20 °C. Working cultures were propagated by weekly transfers to LM. Active cultures were inoculated at 2% (v/v) in LM and incubated at 37 °C for 16 h.

Traditional method

Viability assays: Cell viability was determined by the plate dilution method using LAPTg agar (Raibaud and others 1961) for lactobacilli and TPY agar (Biavati and others 1992) for bifidobacteria. Serial dilutions of each sample were plated out in duplicate and incubated anaerobically at 37 °C for 48 to 72 h. Results are expressed as logarithmic colony forming units (CFU) per milliliter of culture.

The pH of the samples was measured using potentiometric methods (pH-meter Orion model 720A, Orion Research, Inc., Beverly, Mass., U.S.A.).

Conductimetric method

Changes in conductance were monitored using a Bactometer Microbial Monitoring System (BioMerieux, 69280 Marcy-l'Etoile, France) and are expressed as micro Siemens (μS). Bacterial cultures grown at 37 °C for 16 h were diluted to obtain different concentrations, and 0.1 ml of each dilution was added to 2.0 ml of each medium in the wells of sterile disposable Bactometer modules. All samples were analyzed in duplicate and incubated at 30, 37, and 42 °C for 40 h. Changes in conductance were monitored automatically and graphically represented using BPS R03-1 software. The values corresponding to uninoculated media (reagent blanks) were automatically subtracted from the microbial cultures.

Statistical analysis

All results presented in this study are the mean of 2 independent experiments with 3 replicates each. Data were analyzed with the general linear model procedures of the Statistical Analysis System (SAS Institute, Inc., Cary, N.C., U.S.A.) and $P < 0.05$ was considered significant.

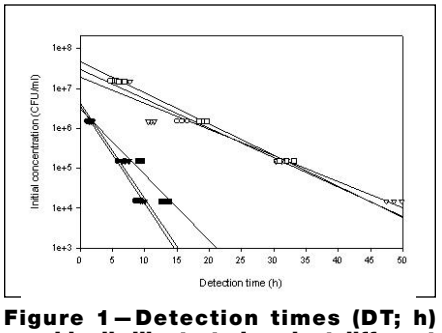

Figure 1—Detection times (DT; h) graphically illustrated against different initial concentrations of *Lactobacillus fermentum* (closed symbols) and *Bifidobacterium longum* (open symbols) grown at 37 °C in 3 culture media: LM (○), LAPTg (▽) and SM (□).

Results and Discussion

CONVENTIONAL DIRECT CONDUCTIMETRY is a quite satisfactory method for many applications but requires the use of specific culture media that enhance electrical changes resulting from microbial growth. As a consequence, soymilk (SM) was assessed as a potential culture medium for conductimetric assays.

The conductivity of a culture medium at any time is given by the concentration of each ionic species and its molar conductivity (Owens 1985). Soymilk is a very complex medium and it is difficult to assess each of the individual parameters. Therefore, comparative studies were carried out with 10% (w/v) reconstituted nonfat dry milk (NDM) (commonly used in conductrimetry), LAPTg (growth medium for LAB), GPM (culture medium recommended by Bactometer for microorganisms with high nutritional requirements) and LM (control medium for LAB recommended by Bactometer). Conductance was determined for each uninoculated medium at 30, 37, and 42 °C for 40 h. Table 1 shows the initial conductance values (μS) of the 5 uninoculated culture media tested, which remained unchanged throughout the experiment (40 h). The initial value for SM was in between the values for the 2 media recommended by bioMerieux (GPM and LM). This fact would mean that any increase in conductance due to microbial growth would be within measurable ranges of the equipment. Comparing GPM with other media assayed, it gave a much higher initial conductance which is directly related

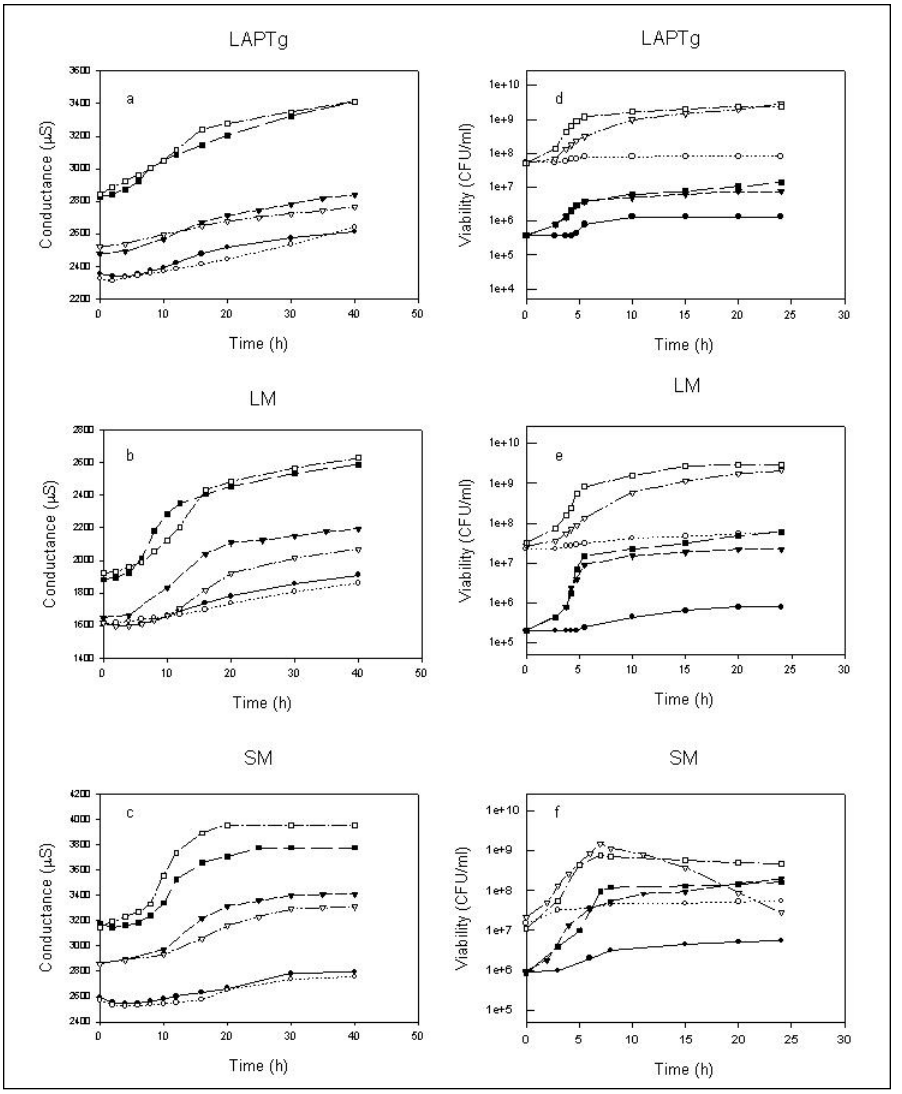

Figure 2—Growth patterns of *Lactobacillus fermentum* (closed symbols) and *Bifidobacterium longum* (open symbols) in 3 culture media: LAPTg (a, d), LM (b, e), and SM (c, f) incubated at 30 °C (○), 37 °C (▽) and 42°C (□). Changes in conductance (a, b, c) and viability (d, e, f).

Table 1—Initial conductance values (μS) for 5different culture media: soymilk (SM), reconstituted non-fat dry milk (NDM), LAPTg, lactic medium (LM) and general-purpose medium plus (GPM). Incubation temperatures were 30, 37, and 42 ºC.

Culture medium	Assay Temperature 30 °C	37 °C	42 °C
SM	2597	2957	3213
NDM	2302	2519	2763
LAPTg	2390	2579	2879
LM	1599	1695	1964
GPM	5524	5848	6214

to the number of ionic species in suspension. It was also observed that the initial conductivity of each medium is higher with increasing incubation temperature, probably due to a decrease in viscosity (Owens 1985).

SM has physico-chemical properties similar to cow milk and it is an adequate substrate for growth of LAB (Garro and others 1994). However, opacity of both media makes it difficult to use common and fast techniques to assess bacterial growth (such as turbidity measurement). In conductimetry, on the other hand, optical clarity is not a requirement, and thus opaque samples are monitored as easily as optically clear samples. Based on the results obtained SM could be used as a culture medium for conductimetry assays. Application of this technique in fermented products and probiotics would be an interesting alternative to detect metabolic changes produced by growth of microorganisms in soymilk.

Lanzanova and others (1993) reported the need to determine the initial inoculum for conductrimetric assays, as changes in conductance are related to the initial number of microorganisms. Generally, diluted cultures are used as inocula, because too high a number of microorganisms can cause very short detection times (DT) that cannot be detected by the equipment, but excessive dilutions give DT values that are too long. However, changes in conductance and DT are not only related to the number of bacteria but also to their metabolic characteristics. Similar initial inocula of different strains of lactic acid bacteria give DT values that vary from minutes to hours (Lanzanova and others 1993). Therefore it is necessary to determine the initial inoculum for each microorganism in order to obtain comparable DT values in successive conductrimetric assays.

The experiment temperature was set at 37 °C, because this is the average growth temperature for the microorganisms assayed. Five dilutions (1/1000, 1/100, 1/10, 1/1, and 10/1) of each microorganism were examined and used as initial inocula for each of the 5 culture media. Cell counts were carried out in each case. The relationship between DT and the log of the number of cells with respect to the different concentrations of a single-strain culture in the culture media assayed was linear (Figure 1). GPM is not illustrated because the strains assayed did not grow in this medium. The correlation coefficient between the 2 parameters was > 0.95 for both strains in the 3 remaining media tested. According to the results obtained DT values between 5 and 10 min would be optimum to attain a good correlation for growth of both microorganisms. A dilution of 1/100 (1.5×10^5 CFU/ml) was used for *Lactobacillus fermentum* and an undiluted concentration of 1/1 (1.5×10^7 CFU/ml) for *Bifidobacterium longum* throughout the assays. With highly concentrated inocula (more than 10/1) the time necessary for metabolite production was not detected by the device.

After determining and standardizing the initial microbial inocula metabolic changes due to growth of *L. fermentum* and *B. longum* used as starter cultures in SM and incubated at 30, 37, and 42 °C for 40 h were measured using conductrimetry. LM and LAPTg were used as control media and pH and cell viability (CFU/ml) were also measured.

The conductance values were compared with the different kinetic parameters in order to evaluate the relationship between metabolic activity and changes in conductance. Figure 2 shows growth curves obtained through conductimetric techniques (a, b, and c) and viable cell counts (d, e, and f) for both microorganisms and incubated at the different temperatures in LAPTg, LM and SM. The first 2 media were supplemented with sucrose (2% final concentration) so that the carbon source available in each medium would be comparable. Figure 2 (a, b, and c) shows real conductance values for each of the culture media.

In LAPTg (Figure 2 a) the change in conductivity for *L. fermentum* and *B. longum* was similar. At 30 and 37 °C growth was slow, while at 42 °C growth was higher for both microorganisms, showing an increase in conductance of 600 μS after 40 h of incubation with a DT less than 5 min for both strains.

In LM (Figure 2 b) low metabolic activity was observed for both microorganisms at 30 °C, whereas at 37 and 42°C activity was higher with differences between both strains. At 42 °C the lag phase for *B. longum* was longer than for *L. fermentum*, but after 16 h of incubation conductance values were similar. The maximum rate of conductance changes throghout the time (from 8 to 16 h) were 0.020 μS x h^{-1} and 0.031 μS x h^{-1} for both microorganisms, respectively. After 40 h of incubation the increase in conductivity due to microorganism growth was more than 600 μS.

In SM at 42 °C (Figure 2-c) growth of *L. fermentum* was less than that of *B. longum*. The latter 1 presented a behavior similar to that shown in LM (maximum rate of conductance changes of 0.029 μS x h^{-1}) The difference in conductance was 800 μS after 40 h with a DT of 7.2 min. At 37 °C both organisms demonstrated less metabolic activity and at 30 °C only little change in conductivity was observed in the culture medium.

The results obtained with the conductimetric method were confirmed by measuring cell viability (Figure 2 d, e, f). Results are given for 24 h of incubation because after this time no variation in the number of cells was observed. In some cases, the curves of both methods show differences, which would be due to the different measuring parameters. The conductance would reflect the change due to the formation and excretion into the medium of metabolites during microorganism growth, whereas the traditional method assesses the change owing to an increase in the number of cells. Growth of *B. longum* in SM at 42 °C, for example, diminished after 7 h measuring cell viability, while the curve for conductance was still exponential. Other cases can be observed (Figure 2 b and e) in which cell viability was constant, whereas conductance still increased (between 10 and 20 hours). These facts could be due to a decoupling of growth and acid production. Turner and Thomas (1975) reported that a low pH can cause growth inhibition of lactic acid bacteria without a reduction in the carbohy-

drate metabolism, thus leading to the formation of acids. The decrease in viability of bifidobacteria due to pH was reported previously by Garro and others (1999). These observations were confirmed by pH and acidity measurements (data not shown). The difference in pH at 37 and 42 °C after 24 h of fermentation varied from 2.0 to 2.5 units for all culture media, while at 30 °C the change was always less than 0.6 units.

Monitoring conductance as an indicator of starter activity in soymilk is advantageous over monitoring pH or titratable acidity, since conductance changes can be evaluated from the beginning of growth (that is, after 1 or 2 h of inoculation). In contrast changes in pH or titratable acidity may be minimal due to the soymilk buffering effect

Conclusions

BASED ON THE RESULTS OBTAINED, SM IS A suitable medium for conductimetry, which allows measuring of metabolic changes due to microorganism growth. The correlation between DT and the initial cell number (Figure 1) allowed us to estimate the initial number of cells in the culture in a relatively short time (3 to 10 h) compared with the plate count technique (48 to 72 h). However, the conductimetric method was difficult to use to evaluate the exact number of microorganisms in a culture. In fact, this method was more affected by the metabolic activity of strains than by the number of colony-forming units. A calibration curve allowed us to determine the initial number of microorganisms in a pure strain of known and constant composition. The stage of growth (that is, exponential or stationary phase) from which cells are taken for inoculation the culture media would influence the results obtained.

The conductance technique allowed us to study simultaneously 3 culture media at different temperatures, which is an advantage with respect to the time- and cost-consuming traditional methods.

Conductimetric analysis of the growth of LAB in soymilk was highly reproducible and can therefore be considered as a valid method (fast, sensitive, and repeatable) to assess the activity and stability of a culture and to characterize different strains with respect to their physiology and metabolism.

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