Symbiotic, a combination of short chains-carbohydrates with prebiotic properties e.g. fructooligosaccharides (FOS) and biotherapeutical microorganisms, provides positive health effects and well-being in humans and animals. However, the lack of accurate screening methods hampers the possibility to get new fructo-oligosaccharides (FOS)-fermenting yeast or bacterial strains to design potential symbiotic combinations useful for health and food industries. A valuable screening procedure to visually evaluate pure cultures of bacterial and yeast strains able to ferment FOS in liquid or solid rich media supplemented with a pH indicator is described. Using this procedure, 15 FOS-consuming strains isolated from different sources were successfully assayed using this method. This screening procedure is a useful tool in rapid large-scale detection of potential FOS fermenting-strains.

Keywords: Bromothymol blue, fructoligosaccharides, fructooligosaccharides fermenting microorganisms, prebiotics, probiotics, screening, symbiotic

Introduction

Symbiotic provides positive desirable clinical effects in humans and animals [1-3]. For this reason the selection of bacterial or yeast strains able to efficiently ferment short chains-carbohydrates with prebiotic properties e.g. fructooligosaccharides (FOS), is a subject of permanent interest for health and food industries.

However, the lack of simple and cost-effective direct screening methods hampers the rapid identification of such important microorganisms. Main targets for FOS consume in the colon are Bifidobacteria and Lactobacilli [1]. These intestinal bacteria have shown several positive effects upon human or animals well-being [4, 5]. Also some yeast strains used for medical therapy consume prebiotics [6].

According to that explained above, if certain carbohydrates, such as fructooligosaccharides, are fermented by only specific beneficial microbial strains, then a liquid or solid growth medium containing these “prebiotic” substrates as unique carbon sources could efficiently and directly select for those useful FOS-fermenters microbial strains.

However, two main drawbacks make it difficult to establish an accurate screening procedure based on FOS consumption. Firstly, FOS commercial preparations in general, may have different polymerization degrees and/or contain contaminants glucose, fructose, sucrose, or other fermentable sugars being rather difficult to establish if microbial growth in FOS-containing medium is really due to FOS metabolism.

This fact makes necessary the use of additional time-consuming confirmatory analytical techniques such as thin layer chromatography (TLC) or High Pressure Liquid Chromatography (HPLC). Secondly, the selection of FOS-fermenter strains by using continuous culture in rich media such as De Man, Rogosa and Sharpe (MRS) [7] to evaluate FOS consumption by bacteria or yeast undoubtedly could mask the final results. To overcome this problem, instead of a rich media like MRS or any similar, the use of minimal media like MRS or any similar, the use of minimal...
medium might be the best choice to evaluate FOS fermentation, but another problem arises. Contrary to yeasts, Lactobacillus and Bifidobacterium strains for example, need elevated nutritional requirements in the media, and their growth in minimal medium is almost null.

Following this line we developed a new feasible and simple pH shift-based procedure for rapid screening of pure cultures of FOS-fermenting yeast and bacterial strains growing in rich media. As the principle, FOS fermentation by bacteria and yeast provokes acidification of the culture medium (pH below 6) due to production of organic acids. By contrast, microbes unable to metabolize FOS, for example enteric bacteria like Escherichia coli or Salmonella sp., use the nitrogen-containing components in yeast extract or peptone causing alkalization (pH above 7) due to ammonia release.

The slightly pH switches from acidic to basic conditions can be detected by the addition of the innocuous pH indicator Bromthymol blue (transition interval pH 6.0-7.6; yellow-blue, Sigma Co., USA). At this stage, a simple visual inspection is enough to detect color change in liquid or solid media allowing a rapid and accurate screening and detection for those microorganisms able to metabolize FOS.

The screening of several potential FOS-consuming strains can be carried out in covered multwell plastic dishes with 12 or 24 wells (Linbro, Flow Laboratories Inc. USA or Costar, Corning Inc. USA) containing low quantities of the adequate growth medium.

**Material and methods**

**Microorganisms**

*Bifidobacterium bifidum* 15696, *Bifidobacterium dentium* 27678 were obtained from the American Type Culture Collection (ATCC) and *Escherichia coli* TOP10F. *Pichia pastoris* GS115, *Pichia pastoris* X33 were purchased from Invitrogen SA.

Other bacterial and yeast strains such as: Saccharomyces boulardii L/254/96, Saccharomyces cerevisiae L/25-7-82, Saccharomyces cerevisiae L/25-7-76, Kluyveromyces fragilis L/12-8-1 Kluyveromyces fragilis L/12-8-6, Lactobacillus acidophilus B/103-5, Lactobacillus rhamnosus B/103-1-5, Lactobacillus reuteri B/108-1, Lactobacillus fermentum B/103-11-3, Lactobacillus casei B/103-11-6, Lactobacillus paracasei B/103-11-7, Lactobacillus bulgaricus B/103-12-6, Streptococcus thermophilus B/103-12-7, used in this research were isolated from different sources and belongs to the microorganism collection of the Cuban Research Institute for Sugarcane Derivates (ICIDCA) Havana, Cuba.

**Culture media**

The commonly used minimal Yeast Nitrogen base (YNB) and rich media YP and LB (Luria bertani) for yeast and bacterial growth respectively, were prepared from Sigma Co. (USA) catalog number: M6030 and two additional FOS-consumers strains used as controls of this experiment, failed to grow in minimal media supplemented with FOS. As the principle, FOS fermentation by bacteria and yeast provokes acidification of the culture medium (pH below 6) due to production of organic acids. By contrast, microbes unable to metabolize FOS, for example enteric bacteria like *Escherichia coli* or *Salmonella sp.*, use the nitrogen-containing components in yeast extract or peptone causing alkalization (pH above 7) due to ammonia release.

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**Microbial growth under anaerobic conditions**

COY Chambers (COY Laboratory Products Inc.) were used to create and maintain anaerobic conditions as needed during growth of some of the microorganisms mentioned above in the different experiments.

**Sugars separation by High Performance Liquid Chromatography (HPLC)**

Sugar composition in the enriched FOS solution used in this research were separated in an Aminex HPX-42C column (0.78 by 30 cm; Bio-Rad Laboratories, Hercules, Calif.) and detected with a RI-410 (Waters) detector. The column temperature during the analysis was kept constant at 85 °C, and water was used as the mobile phase at a flow rate of 0.6 mL/min.

**Results and discussion**

As the first step of this research we found that unlike yeasts, some lactobacilli and bifidobacteria strains were not able to grow in minimal media supplemented with FOS. Previously, syrup composed of 4.6% nystose (GF3), 56% 1-kestose (GF2), 21% sucrose (GF), 17% glucose (G), and 1.4% fructose (F) was produced during sucrose transformation by recombinant *P. pastoris* cells entrapped in Ca-alginate beads. After sugars separation, an enriched FOS solution containing 1-kestose (96%), nystose (3%) and sucrose (1%) was obtained, as judged by HPLC analysis (Figure 1A).

To evaluate microbial FOS fermentation, the use of minimal media appears to be ideal because of the lack of carbohydrates or proteins as alternative energy sources for cell growth. On this basis, the enriched filter-sterilized FOS solution was incorporated to a final concentration of 2 and 3% into solid or liquid minimal Yeast Nitrogen Base (YNB, Invitrogen Co., USA) or minimal M9 (Sigma Co., USA) medium supplemented with 0.05% L-cysteine. Two yeasts (*S. boulardii* and *K. fragilis*) and 2 bacterial strains (*B. bifidum* and *B. dentium*) were streaked on YNB-FOS or M9-FOS plates and incubated 48 h at 30 °C or 37 °C respectively, under anaerobic conditions. The assayed yeasts were able to grow in solid or liquid YNB-FOS demonstrating that FOS was equally as good substrate as glucose (BDH), fructose (BDH) and sucrose (BDH) in supporting growth (Figure 1B). Additionally, HPLC analysis confirmed the complete depletion of the FOS fraction after microbial growth (Figure 1C). Unlike yeasts, the two *Bifidobacteria* and two additional FOS-consumers *Lactobacillus* strains used as controls of this experiment, failed to grow in solid or liquid M9-FOS (results not shown).

Lactobacilli and Bifidobacteria, organisms that are generally considered to be desirable members of the colonic microbiota, release mainly lactic and acetic acids during FOS catabolism under anaerobic conditions. These acids are generally considered to be desirable members of the colonic microbiota, release mainly lactic and acetic acids during FOS catabolism under anaerobic conditions [1] so, inactivating the culture medium to low pH values. As shown above, these bacteria are unable to grow in minimal media due to their high nutritional requirements. These characteristics prompted us to test whether the addition of a prebiotic such as lactulose would allow growth of the bacterium *Lactobacillus* and streptococcus strains for FOS utilization [8, 9].

The enteric bacterium *E. coli* and the methanotrophic yeast *P. pastoris*, which are unable to use FOS, turned the medium color to blue raising the pH values about 8 (Table 1) due to utilization of the nitrogen-containing carbon sources in yeast extract and peptone. FOS depletion was further verified by HPLC analysis demonstrating that the assay bacteria and yeasts consumed totally the GF2 (1-kestose) and GF3 (nystose) fractions (results not shown).

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The results support the idea of using prebiotics to enhance the growth of desirable bacterial strains in the gut. As shown in table 1 or figures 2A and B, the growth of 15 of the screened bacterial strains, in solid and liquid LB media, turned the medium color from initial green (pH 6.5) to yellow indicating acidification, pH below 6, due to FOS consumption (Table 1). On the other hand, when FOS was not added, the medium color turned to blue revealing alkalinization due to ammonia release from utilization of the nitrogen-containing carbon sources in yeast extract and peptone so, pH values raised up from initial 6.5 to 8 (Table 1). The same results were obtained when five yeast strains were assayed (figures 3A and B). Additionally, the use of YNB-FOS minimal medium supplemented with Bromothimol blue revealed also, by simple visual inspection, the capacity to consume these short chain carbohydrates by these yeasts (results not shown).

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It has been established that the diet, host phylogeny and gut morphology influence the microbial ecology of the gastrointestinal tract [11, 12]. The beneficial potential of lactic acid bacteria and yeast has been shown to be enhanced by the introduction of prebiotic carbohydrates mainly of fructo- and galactooligosaccharide nature [6]. In recent studies other authors concluded that prebiotics selectively enhance lactobacilli and bifidobacteria populations and reduce colonization by pathogenic bacteria [13-15]. However, there are no reports regarding to rapid and accurate qualitative detection methods to get new potential fructooligosaccharides fermenting yeast or bacterial strains based on consume of these short chain carbohydrates widely used as prebiotics. Previous studies have been aimed to apply in vitro methods for selection of suitable bacterial and yeast strains for human or animal health improvement [16, 17]. In this particular report the addition of the innocuous pH shift-based screening of FOS fermentation

**Figure 1.** FOS utilization by two yeasts currently used as probiotic. A) HPLC profile of a 96% pure FOS solution, B) culture media samples containing 2% FOS before and C) after microbial growth used in this and subsequent experiments. The remaining peak in C corresponds to GF3. Retention time for sucrose (GF): 10.37 min; 1-kestose (GF2): 9.68 min; and nystose (GF3): 9.12 min; respectively.

Table 1. pH values reached after growth in FOS-LB or YP rich media of bacterial and yeast strains screened with this method able or not to metabolize FOS as unique carbon source

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Source</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FOS(+)</td>
</tr>
<tr>
<td>Saccharomyces boulardii L/25/4/96</td>
<td>Commercial preparation</td>
<td>3.44 ± 0.02</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae L/25-7-82</td>
<td>Honey</td>
<td>3.55 ± 0.06</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae L/25-7-76</td>
<td>Honey</td>
<td>3.30 ± 0.04</td>
</tr>
<tr>
<td>Kluyveromyces fragilis L/12-8-1</td>
<td>Sugarcane industrial process</td>
<td>3.44 ± 0.06</td>
</tr>
<tr>
<td>Kluyveromyces fragilis L/12-8-6</td>
<td>Sugarcane industrial process</td>
<td>3.40 ± 0.03</td>
</tr>
<tr>
<td>Lactobacillus acidophilus B/103-5</td>
<td>yogurt</td>
<td>4.14 ± 0.06</td>
</tr>
<tr>
<td>Lactobacillus rhamnosus B/103-5</td>
<td>pigs fecal blend</td>
<td>4.45 ± 0.02</td>
</tr>
<tr>
<td>Lactobacillus reuteri B/108-1</td>
<td>human fecal blend</td>
<td>4.09 ± 0.02</td>
</tr>
<tr>
<td>Lactobacillus fermentum B/103-11-3</td>
<td>human fecal blend</td>
<td>4.01 ± 0.05</td>
</tr>
<tr>
<td>Lactobacillus casei B/103-11-6</td>
<td>human fecal blend</td>
<td>4.51 ± 0.02</td>
</tr>
<tr>
<td>Lactobacillus paracasei B/103-11-7</td>
<td>Cheese process</td>
<td>4.48 ± 0.04</td>
</tr>
<tr>
<td>Lactobacillus bulgaricus B/103-11-7</td>
<td>Cheese process</td>
<td>5.19 ± 0.01</td>
</tr>
<tr>
<td>Streptococcus thermophilus B/103-12-07</td>
<td>Cheese process</td>
<td>5.31 ± 0.03</td>
</tr>
<tr>
<td>Bifidobacterium bifidum 15696</td>
<td>ATCC</td>
<td>4.35 ± 0.04</td>
</tr>
<tr>
<td>Bifidobacterium dentium 27678</td>
<td>ATCC</td>
<td>4.32 ± 0.02</td>
</tr>
<tr>
<td>Escherichia coli TOP10F</td>
<td>Invitrogen SA</td>
<td>8.52 ± 0.02</td>
</tr>
<tr>
<td>Pichia patoris GS115</td>
<td>Invitrogen SA</td>
<td>8.44 ± 0.01</td>
</tr>
<tr>
<td>Pichia patoris X33</td>
<td>Invitrogen SA</td>
<td>8.46 ± 0.04</td>
</tr>
</tbody>
</table>

Figure 2. Growth in liquid minimal YNB medium supplemented with 2% sucrose, 2% fructose, 2% glucose, or 2% FOS. Culture growth of Saccharomyces boulardii, A) and Kluyveromyces fragilis, B) was followed by measuring dry cell weight (DCW) at different time points for 35 h.

Figure 3. Growth of six of the assayed bacteria: L. paracasei, L. rhamnosus, L. acidophilus, B. bifidum, B. dentium and E. coli under anaerobic conditions at 37 °C in a covered 12 wells plates with solid and liquid LB medium supplemented or not with 2% FOS and 0.025% (final concentration) of the pH indicator Bromothymol blue and initial pH 6.5. The FOS non-consumer enteric bacterium E. coli were used as negative control.
Figure 4. Growth of four of the assayed yeasts: *S. cerevisiae*, *S. boulardii*, *K. fragilis* and *P. pastoris* at 30 °C under anaerobic conditions in a 12-well plates with solid and liquid YP medium supplemented or not with 2% FOS and 0.025% (final concentration) of the pH indicator Bromothymol blue. Initial medium pH was 6.5. The FOS non-consumer yeast *P. pastoris* was used as negative control.

indicator like Bromothymol blue (transition interval pH 6.0-7.6: yellow-blue) to any of the used media supplemented with 2% FOS produced rapid and visible changes in the medium color depending on the bacterium or yeast ability to consume or not FOS as unique carbon source. This pH indicator has not previously been used for this purpose. On the other hand the enriched 96% FOS preparation used in this work composed mainly by GF2 (1-kestose) and GF3 (nystose) is useful as a carbon source since guarantee that microbial growth is mainly due to FOS consume as unique carbon source.

**Conclusion**

According to the results described above, the proposed screening method is a valuable tool in large-scale detection of pure cultures of potential FOS fermenters-yeast or bacterial strains for further symbiotic designs.

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