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Effect of different complex carbon sources on growth and bacteriocin synthesis of *Enterococcus faecium*

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Abstract

Different criteria are followed in order to select bacteria to be used in probiotic and symbiotic supplements. A new parameter to choose strains could be fermentation by intestinal bacteria of some complex carbohydrates because they are prebiotics and promote the development of beneficial microorganisms in the intestinal environment. An *Enterococcus faecium* strain, isolated from the crop of a free-range chicken, was assayed in order to determine the utilization of commercial sugars and/or crude carbohydrate samples from a sugar mill. The production of antimicrobial substances, under these conditions, was also considered. *Ent. faecium* CRL1385 grew well in the presence of complex carbohydrates and its ability to produce bacteriocin, active against poultry pathogens such as *Ent. hirae*, *Salmonella pullorum* and *Listeria monocytogenes*, was not significantly modified. These results are promising because the trend today is to employ eubiotic or symbiotic products and their use in the poultry industry could be a natural way to protect the flocks against potential pathogens.

Keywords: Prebiotic; Symbiotic; *Enterococcus faecium*; Poultry; Probiotic

1. Introduction

Probiotics are feed-microbial supplements, which beneficially affect the host because they improve the balance of its intestinal bacterial ecosystem (Fuller, 1989, 1995). However, sometimes these important changes are temporary and the establishment of exogenous bacteria could be limited. On the other hand, prebiotics are non-digestible ingredients of some feeds, which stimulate a selective development or antimicrobial activity of a certain number of colonic microorganisms. For this reason, their intake could significantly modulate the colonic microbiota. In general, oligosaccharides and fructooligosaccharides are prebiotics. Fructooligosaccharides (FOS) are naturally occurring carbohydrates found in garlic, banana, honey, wheat, onion, brown sugar and molasses. They are non-toxic and do not have secondary effects (Roberfroid, 1993; Gibson and

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Roberfroid, 1995). FOS act as prebiotics because they can promote the growth of beneficial intestinal microorganisms such as *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *Enterococcus faecium*. They can also limit *Escherichia coli* and *Clostridium perfringens* growth because these bacteria are unable to employ FOS as an energy source (Roberfroid, 1998; Saris et al., 1998).

The symbiotic or eubiotic concept rises from the combination of probiotics and prebiotics. This idea is used to describe some colonic feeds or supplements with important nutritive characteristics as 'functional foods' (Roberfroid, 1998; Salminen et al., 1998; Zoppi, 1998).

Since these assays were carried out in Tucumán, an Argentinean region where there are many important sugar mills, we analyzed the possibility of employing their products and residual substances as prebiotic or FOS sources. *Enterococcus faecium* CRL1385 isolated from free-range chicken is a strain with probiotic properties (Audisio et al., 1997a,b, 1999; Audisio, 1999). Therefore, the main purpose of this work was to study the growth of this microorganism and its antimicrobial substance production in a culture medium containing FOS as the main carbon source. This strain can inhibit *Salmonella pullorum*, an important pathogen for chickens (Snoeyenbos, 1991), both in vitro and in vivo (Audisio et al., 1999, 2000). In addition, in order to determine its feasibility as a symbiotic or eubiotic feed supplement for chickens, mixed cultures of *Ent. faecium* and pathogenic strains were assayed using FOS as energy source.

2. Materials and methods

2.1. Bacterial strains

Enterococcus faecium CRL1385 was isolated from the crop of an adult, free-range broiler chicken in Tucumán, Argentina. This strain in previous papers was identified as *Ent. faecium* J96 (Audisio et al., 1997a; Audisio, 1999). *Salmonella pullorum* M97 was provided by Instituto de Microbiología Dr Carlos Malbrán, Buenos Aires, Argentina; *Listeria monocytogenes* was supplied by Instituto de Microbiología Dr Luis Verna, Universidad Nacional de Tucumán, Argentina. *Enterococcus hirae* ATCC8043 came from the American Type Culture Collection. These strains were employed as indicator strains of antimicrobial substance production by *Ent. faecium* CRL1385.

2.2. Determination of fructooligosaccharide chemical composition

The different carbon sources employed in this work were two commercial sugars: brown and white sugar, and many subproducts from the sugarcane process: bagasse, mixed juice, first pressure juice and molasses. Solutions at 20% w/v of each fructooligosaccharide (FOS) were prepared with distilled water and sterilized by filtration and heat (121°C during 15 min). The chemical composition of FOS solutions, without enzymatic modifications, was analyzed by HPLC (high pressure liquid chromatography). The sample amount injected was 20 μl and the flow-rate of the mobile phase (acetonitrile/water, 80:20, v/v) was 1.0 ml min^{-1}. A refraction index detector (Shimadzu) was employed. The chromatography column (–NH_2, 5 μm particle diameter) had a diameter of 4.6 mm and a length of 250 mm. Peak profiles, integration, and quantification were obtained with a Shimadzu (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan) chromatopac integrator. Sucrose (5% w/v), fructose (3% w/v) and glucose (2.5% w/v) standards (Sigma) were employed.

2.3. Culture media and growth conditions

All bacterial strains were activated in LAPTg broth at 37°C. This culture medium was prepared according to Raibaud et al. (1961): meat peptone, 1.5 g; tryptein, 1 g; yeast extract, 1 g; glucose, 1 g; Tween 80, 0.1 ml to a final volume of 100 ml; pH 6.5. The LAPTg broth without glucose (LAPT) was employed as base medium and was supplemented with different complex carbohydrates to a final concentration of 2% w/v.

Bacterial cells from logarithmic phase cultures were kept at −20°C in skim milk (1.0% w/v) with the addition of yeast extract (0.5% w/v).

2.4. Pure and mixed cultures

Ent. faecium and *S. pullorum* pure and mixed cultures (12–14 h at 37°C) were prepared by inoculating 100 ml of fresh LAPT medium, sup-

plemented with either brown sugar or molasses, to obtain an initial number of $\sim 1 \times 10^7$ cfu ml^{-1} of each microorganism. Then, they were incubated for 6 h at 37°C. Growth of these cultures was followed by measuring absorbance at 560 nm, pH and determination of viable microorganisms. At different times, samples were saved for later analysis and quantification of bacteriocin production. *Ent. faecium* pure cultures were also studied with glucose (LAPTg), white sugar and without a carbon source (LAPT). In these cultures, viable cells of *Enterococcus* on *Streptococcus* selective agar (Merck) and of Salmonellae on MacConkey (Merck) agar, were determined. All plates were incubated at 37°C for 24 h prior to counting.

2.5. Detection of bacteriocin production

Titer of bacteriocin suspension was determined by serial two-fold dilution and it was expressed in arbitrary units by milliliter (AU ml^{-1}) (Daba et al., 1991). Antimicrobial activity of free-cell supernatants (adjusted to pH 6.00 with sterile 1 N NaOH) from different culture media was measured by well-diffusion assay (Tagg and McGiven, 1971). *Ent. hirae* and *L. monocytogenes* were used as indicator strains.

2.6. Statistical analyses

All analyses were carried out according to the Tukey test and they were considered significant at the $P < 0.05$ level. Assays were performed in triplicate.

3. Results and discussion

3.1. Growth of Ent. faecium on complex carbon sources

Glucose is considered the main carbon source by all microorganisms due to its size, rapid uptake, utilization and cellular energy conversion. However, some bacteria have a complete enzymatic machine that allows them to use complex carbohydrates; for example, *Ent. faecium* shows a variable sucrose fermentation pattern (Barnes, 1964). Brown sugar, molasses and other complex carbohydrates can be considered as prebiotics (Roberfroid, 1998).

Table 1
Chemical composition of fructooligosaccharides (FOS)[a]

FOS	Fructose	Glucose	Sucrose	Other
White sugar	1.20	1.30	38.2	ND
Brown sugar	1.01	1.16	42.0	ND
1st Pressure juice	1.23	–	7.56	ND
Bagasse	0.10	–	0.41	ND
Mixed juice	1.56	0.88	3.79	0.50
Molasses	8.00	8.10	26.0	4.10

[a] Expressed as w/v percentage. 'Other', i.e. carbohydrates, such as trisaccharides. ND, non detected.

The prebiotic molecules used in this work, showed different composition and concentrations in carbohydrates. As shown in Table 1, the main carbohydrate present in both commercial sugars was sucrose while fructose and glucose also were present in very low concentrations. Crude substances obtained from a local sugar mill had a variable concentration of carbohydrates. Molasses showed the highest sucrose concentration, but it also contained glucose, fructose and certain trisaccharides in minor amounts. The poorest sample in carbohydrate was bagasse (Table 1).

Ent. faecium grew in all culture media assayed: LAPT base medium (without sugar), LAPT supplied with brown or white sugar and LAPTg, supplemented with glucose (Fig. 1). Although absorbance at 560 nm was variable, the population of viable cells were similar in the four media studied after 6 h incubation at 37°C ($\sim$8.5–9.0 log cfu ml^{-1}). No significant differences ($P > 0.05$) in the growth rate values ($\sim \mu = 1.04$ h^{-1}) were observed; however, the highest number of viable cells was reached in presence of brown sugar (Fig. 1.b). The origin of the carbon source did not significantly affect the growth of *Ent. faecium*, for this reason the production of metabolites, mainly bacteriocin, was studied.

3.2. Bacteriocin synthesis in presence of complex carbon sources

Although *Ent. faecium* grew (Fig. 1) without carbon source (base medium LAPT), there was no bacteriocin production. This growth may be derived from the utilization of residual sugar or another nutrient, such as yeast extract or meat peptone,

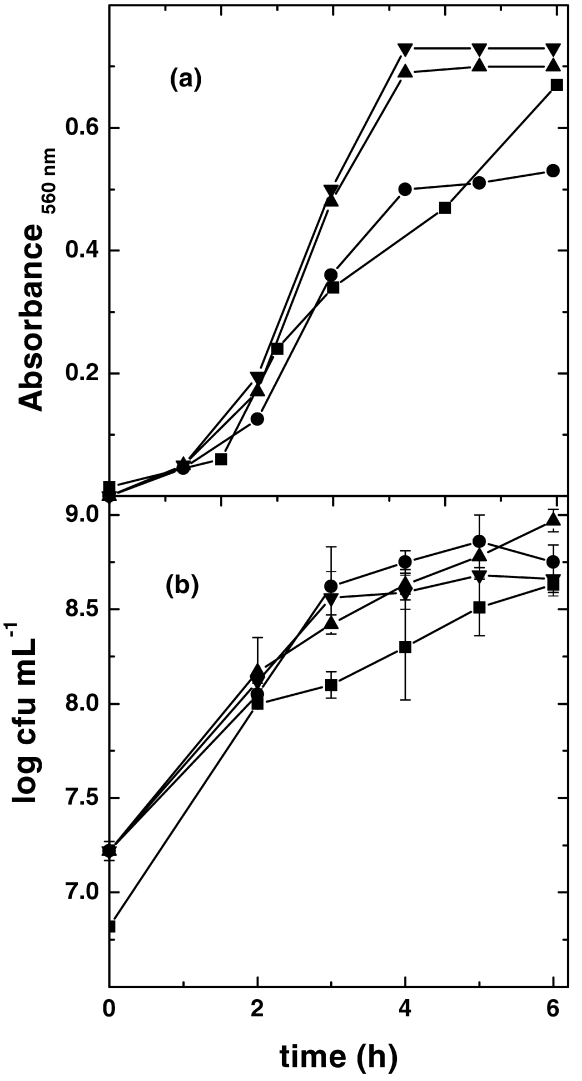

Fig. 1. (a) Measurement of changes in absorbance at 560 nm and (b) log counts in cfu ml^{-1} of *Ent. faecium* at 37°C in LAPT-base medium (▼); LAPT + brown sugar (▲); LAPT + white sugar (●); LAPT + glucose − LAPTg (■).

present in LAPT medium in sufficient quantities to allow *Ent. faecium* growth but insufficient for normal synthesis of bacteriocin. In the presence of brown and white sugar, the antimicrobial activity against *Ent. hirae*, a pathogenic strain for chicks (Kondo et al., 1997), was observed after 3 h incubation at 37°C. However, with white sugar, bacteriocin production and its titer (11 636 AU ml^{-1}) remained high even after 6 h incubation, while with brown sugar, the titer (11 636 AU ml^{-1}) showed a progressive reduction as time elapsed.

On the other hand, bacteriocin production by *Ent. faecium*, in presence of crude potential prebiotic samples (supplied by a local sugar mill), was studied from supernatants obtained after 5 h incubation. In this period, the highest synthesis of bacteriocin with activity against *Ent. hirae* (1600 AU ml^{-1}) was observed when the carbon source was glucose (Audisio et al., 1999; Audisio, 1999). The antibacterial substance production by *Ent. faecium*, in the different media studied, was variable. In presence of bagasse, no bacteriocin synthesis was proved. However, when samples of molasses, mixed juice and first pressure juice were analyzed, bacteriocin active against *Ent. hirae* and *L. monocytogenes* was detected. Bacteriocin activity was higher in presence of molasses than other complex carbon source (Table 2). These results could be related to the chemical composition of molasses that proved to have not only the highest percentage of free monosaccharides such as glucose and fructose, but also more complex sugar (Table 1).

The results obtained showed that bacteriocin was synthesized only when there was a carbon source in the medium. Bacteriocin concentration was variable, but it was highest with brown sugar. Apparently there would be a dependence between bacteriocin synthesis and presence of fructose and sucrose and concentration of the latter (Tables 1 and 2). These observations are in agreement with Pitt and Gaston (1988), who hold that the type and amount of carbon source may affect bacteriocin production.

Table 2
Bacteriocin[a] titer synthesized by *Ent. faecium* CRL1385 in presence of fructooligosaccharides (FOS)

Indicator strains	FOS[b]	Titer (AU ml^{-1})
Ent. hirae ATCC80433	Molasses	1454
	Mixed juice	727
	1st pressure juice	364
	Bagasse	–
L. monocytogenes	Molasses	1454
	Mixed juice	727
	1st pressure juice	182
	Bagasse	–

[a] Samples analyzed after 5 h incubation at 37°C.
[b] LAPT was the base medium.

3.3. Mixed cultures between Ent. faecium and S. pullorum

Ent. faecium and a mixed culture of *Ent. faecium* and *S. pullorum* M97 had similar growth rates when molasses was used as the main carbon source, while *S. pullorum* had low absorbance and pH values (Fig. 2a). When cellular viability was determined, *Ent. faecium* presented the same behavior in both pure and mixed cultures (Fig. 2b). *S. pullorum* showed a lag phase (~2 h) both in pure and mixed cultures. After this period, the pathogen strain adjusted to the medium and it continued its growth in pure culture while its viability, in mixed culture, was affected. The inhibition observed on pathogen strain can be due to a combined action of lactic acid and bacteriocin derived of *Ent. faecium* growth as was demonstrated when glucose was used as carbon source (Audisio et al., 1999).

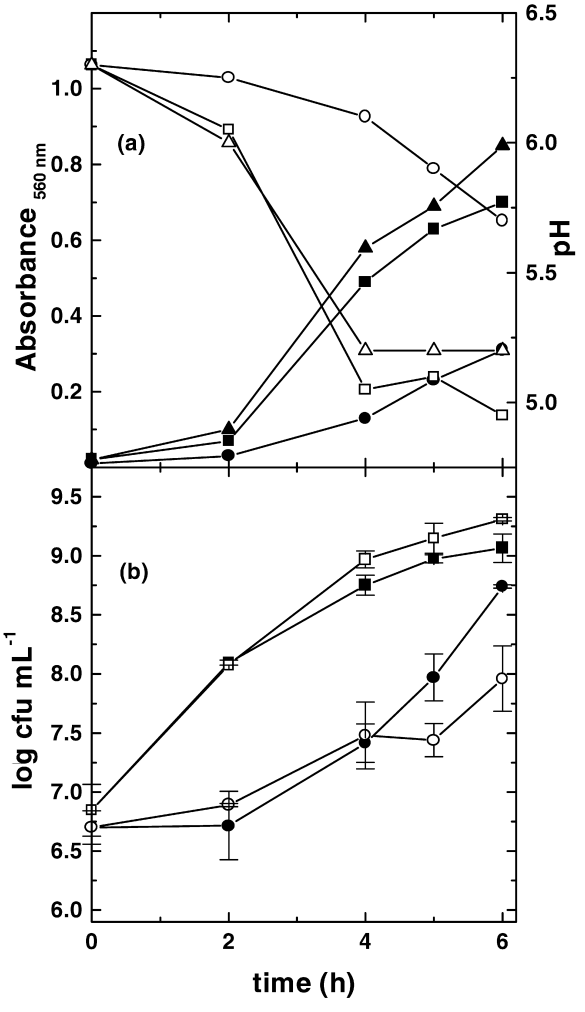

Fig. 2. (a) Measurement of changes in absorbance at 560 nm (black symbol) and pH (white symbol) (●, ○: *S. pullorum*; ■, □: *Ent. faecium*; ▲, △: mixed culture) and (b) log counts in cfu ml^{-1} in LAPT medium supplemented with 2.0% w/v molasses at 37°C of pure (black symbol) and mixed (white symbol) cultures (●, ○: *S. pullorum*; ■, □: *Ent. faecium*).

When the growth of mixed culture on brown sugar as the main carbon source was analyzed, the behavior of *Ent. faecium* was similar to the one with molasses (Figs. 2 and 3). However, the final pH values obtained from molasses were higher than those reached from brown sugar (Figs. 2a and 3a, respectively). In this case, both viability and growth rate did not show differences between pure and mixed cultures (Fig. 3b). On the other hand, *S. pullorum* cells presented a lag phase of about 2 h in the mixed culture after which the growth was inhibited (Fig. 3b). This inhibition would not be due to an adaptation to the nutrients in the growth medium because the lag phase was absent in the pathogen pure culture. The antagonistic effect could be related to the low pH value reached at this time and/or the bacteriocin synthesized by enterococcus strain.

These results indicate that *Ent. faecium* can inhibit the avian pathogen in mixed culture independently of the carbon source employed. However, the prebiotic molecule used determined variability in the antagonic activity because lactic acid and bacteriocin production were carbohydrate nature dependent.

4. Conclusions

Ent. faecium CRL1385 can grow in presence of prebiotics such as brown and white commercial sugars and molasses. However, the nature and the concentration of the carbon source were critical for bacteriocin production. It was significantly higher with brown sugar than with glucose or crude complex carbohydrate samples (11 636, 1600 and 1454 AU ml^{-1}, respectively). The enterococcus strain, in mixed culture with brown sugar or molasses, inhibited *S. pullorum*, an important avian pathogen.

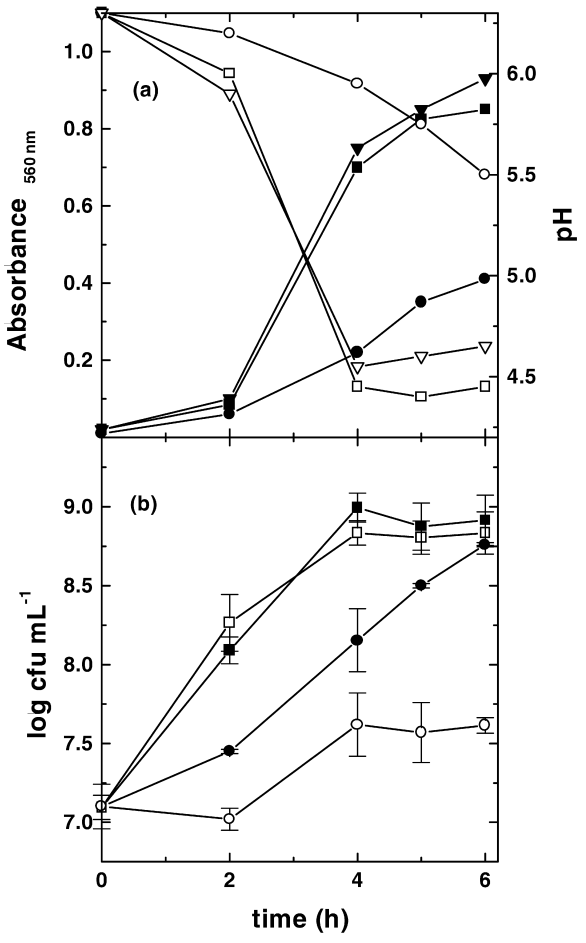

Fig. 3. (a) Measurement of changes in absorbance at 560 nm (black symbol) and pH (white symbol) (●, ○: *S. pullorum*; ■, □: *Ent. faecium*; ▼, ▽: mixed culture) and (b) log counts in cfu ml^{-1} in LAPT medium supplemented with 2.0% w/v brown sugar at 37°C of pure (black symbol) and mixed (white symbol) cultures (●, ○: *S. pullorum*; ■, □: *Ent. faecium*).

These results may open a path towards a symbiotic feed or supplement for poultry in the near future.

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