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Full Length Research Paper

Selection of local extremophile lactic acid bacteria with high capacity to degrade lactose: Potential use to reduce intolerance to lactose *in vitro*

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This study is related to the isolation and identification of strains of local thermophilic lactic acid bacteria belonging to the species, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. These bacteria can exist under extreme conditions of the digestive tract (acidity and high concentration of bile salts) and have a high capacity to degrade lactose. The aim is to produce yoguort with the bacteria that remain viable and active in the digestive tract, so as to enhance lactase activity at the intolerant lactose. The results also show considerable variations between genera, species and strains of the same species in the decomposition of lactose in pure and mixed cultures ($Sc.t_5$ and $Sc.t_3$, respectively, with 48 and 42%) ($YSLB_2$, $YSLB_4$ and $YSLB_1$, respectively, with 90.44, 87.22 and 84.28%) of initial lactose after 6 h of incubation at 37°C while keeping a level of viability higher than 10^7 cells/ ml. In the presence of pH (2.5, 4.5 and 6.5), put with or without 0.3% of bile salts, the results are also unmatched. The best cultures will be used to manufacture fermented milks (yoghurts) in order to correct intolerance with lactose in man after having to test them, in a second part, *in vivo*.

Key words: Lactic acid bacteria, extremophile, intolerance of lactose, digestive tract.

INTRODUCTION

The lactose is a diholoside present in significant quantity in the milk of mammals. In human digestive tract, it is degraded by intestinal lactase (β galactosidase) to glucose and galactose.

Approximately, 70% of the adult world population shows a lactase deficiency, which is the cause of digestive disorders when they ingest milk. The reduction of the excessive lactose rates in the intestine can decrease the risks of these disorders (distension, diarrheas, vomiting, etc) (Cerf-Bensussan, 2002).

The assumption that certain micro-organisms are able to decrease lactose in the intestine and to improve the nutritional properties- dietetic and therapeutic is being investigated by many researchers (Dilmi and Sadoun, 2002a; Bertazoni et al., 2004; Lin et al., 2005; Dilmi et al., 2007). Among the claimed effects, the modulation of the lactasic activity, due to the digestion of lactose in yoghourt is carried out by bacterial enzymes, including the β galactosidase which converts glucose to galactose during the transit in the digestive tract (Drouault et al., 2002).

In order for these micro-organisms to exert beneficial roles on human health, they must be both viable and active during the intestinal transit (Klebling et al., 2002; Corthier, 2004; Marcelli et al., 2004) by crossing the physiological barriers of the digestive tract. The fermentation itself must also be able to cross without being irreversibly damaged by the acid barrier of the stomach, or inhibited by the bile salts (Dilmi and Sadoun, 2002b; Oyetayo et al., 2003; Dilmi, 2006).

The aim of this study is to isolate lactic strains, of the two species, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* from cows' milks, and to select strains that have a higher capacity to degrade lactose in pure and mixed cultures within the extreme conditions of the digestive tract (bile salts and acidity).

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MATERIALS AND METHODS

Cows' milk

Milk samples of cows were collected from 8 different areas (altitude and microclimate). Three aseptic collections were carried out for each area and over the four seasons of the year.
The milk samples were stored in sterile bottles and at 4°C.

Dried milk

Dried milk used is of commercial nomination CELIA (France), containing 38.34 g/L lactose; this content is a useful reference for the calculation of the amount of lactose degraded by the bacteria in pure and mixed cultures.

Cultures broths

M17 broth (reference: AEB 140592) is used for screening streptococci; primarily *Streptococcus salivarius* ssp. *thermophilus* and the MRS broth (reference: AEB 140652) are used for screening thermophilous Lactobacilli mainly *Lactobacillus delbruekii* ssp. *bulgaricus*.

The strains are stored at -20°C in the presence of 3% glycerol.

Microbiological analyses

Isolation

Selective isolations of the 2 species were carried out according to the methods described by the International Federation of milk (Fil, 1996).

Identification and purification

The identification of the two species is carried out with API20A strips and with the traditional methods recognized by the committee of taxonomy. They are based on the identification of morphological (macroscopic and microscopic), physiological and biochemical traits for each species.

Gram$^+$, catalase $^-$ non-sporulating bacteria that are able to grow at 45°C were kept for further screening (Samelis et al., 1994; Joffin and Leyral, 1996). Only bacterial cultures that grow at $\geq 10^7$cells/mL were used for the degradation of lactose assay.

Dosage of lactose

Hydrolysis of lactose was measured by the method of polarimetry described by Le coq (1965) at different time points.

The concentrations of lactose in milk were determined on milk samples inoculated with pure strains (6 strains of *Streptococcus salivarius* ssp *thermophilus* and 06 strains of *Lactobacillus delbruckii* ssp. *bulgaricus*) at a rate of 3% of inoculum, and in mixed cultures (36 combinations of type $Sc.t_X + Lb.b_{y)}$.

The cultures were incubated at 37°C at different times.

Determination of milk Dornic acidity

The acidity of milk is expressed in Dornic degree (1 Dornic degree corresponds to 100 mg of lactic acid in 1 liter of milk).
Dornic acidity is calculated at t = 0 (initial acidity of milk) and at different time points, following inoculation in pure and mixed cultures.

Conditions of growths

Culture media (MRS and M17) are freshly prepared. They are divided into two fractions, one of is supplemented with 0.3% of bile salts (reference: 4054, MERCK). After adjustment of the pH by addition of pure hydrochloric acid (2.5; 4.5 and 6.5), the solutions are sterilized with 121°C for 15 min.
pH 2.5 represents the pH of the stomach fasting for several hours; pH 4.5 represents the pH of the stomach at or just after lunch; pH 6.5 is the pH in the intestines.

This concentration of bile salts is largely higher than the maximum concentration that can be secreted by bile within the duodenum (Drouault et al., 2002).

Each sample is inoculated with 3% of pure and mixed cultures, and then incubated at 37°C for 6 h, which corresponds to the length of time for the transit through the human intestinal tract.

The growth of the cultures is checked by colony counting on solid media at different times.

Statistical analysis of results

The statistical analysis of the results is carried out using the program "Anova". On one hand, we carried out an analysis of the variance, to show the significance of our results; and on the other hand, a comparison of the averages by the tests of Newman-Keuls and of the probability (P).

RESULTS AND DISCUSSION

Isolation, identification and characterization of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*

We have successfully purified 6 strains of *Streptococcus thermophilus* ($Sc.t_1$, $Sc.t_2$, $Sc.t_3$, $Sc.t_4$, $Sc.t_5$ and $Sc.t_6$) and 6 strains of *Lactobacillus bulgaricus* ($Lb.b_1$, $Lb.b_2$, $Lb.b_3$, $Lb.b_4$, $Lb.b_5$ and $Lb.b_6$).

Degradation of lactose

The results from the pure cultures indicated a great variation in the percentages of degradation of lactose; there is a considerable and a statistically significant variation ($P < 0.05$) not only between the different species, but also between the strains of the same species.

Indeed, the streptococci in pure cultures degrade large amount of lactose during the first three hours, notably $Sc.t_5$ and $Sc.t_3$ degrading 48 and 42% of initial lactose, respectively. According to Drouault et al. (2002), *Streptococcus salivarius* ssp. *thermophilus* is able to produce an active β galactosidase during the intestinal transit time.

However, the amount of lactose degraded by the lactobacilli is lower than that of streptococci, at 36 and 31% for $Lb.b_5$ and $Lb.b_2$, respectively, during the first hours; and then increases later. This is in agreement with the work of Zourari et al. (1992) which established the necessity for prior acidification of culture media for successful fermentation.

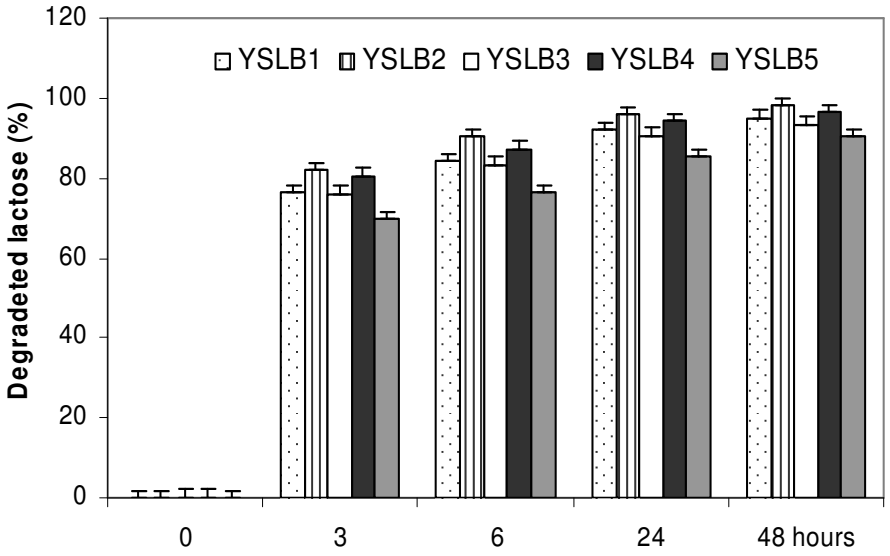

Figure 1. Evolution of the decomposition of lactose by mixed cultures YSLB1, YSLB2, YSLB3, YSLB4 and YSLB5 in the course of time.

The mixed cultures (yoghourts) which showed the best rates of lactose degradation at bacterial concentrations higher than 10^7 cells/ml are $YSLB_1$ ($Sc.t_3$ + $Lb.b_2$), $YSLB_2$ ($Sc.t_5$ + $Lb.b_5$), $YSLB_3$ ($Sc.t_4$ + $Lb.b_4$), $YSLB_4$ ($Sc.t_3$ + $Lb.b_5$) and $YSLB_5$ ($Sc.t_5$ + $Lb.b_2$) with 3.54 x 10^7, 3.46 x 10^7, 3.23 x 10^7, 2.63 x 10^7 and 2.29 x 10^7 cells/mL, respectively. Mixed cultures that have weak bacterial contents were excluded from this study. Indeed several earlier studies (Bouhnik, 2000; Corthier, 2004) confirmed that a bacterial culture can be used as probiotic only when starting at 10^7cells/mL.

The degradation of lactose by the mixed cultures is more obvious and highly significant ($P < 0.05$) than those of the pure cultures. After 3 h of incubation at 37°C, $YSLB_2$, $YSLB_4$ and $YSLB_1$ degraded 81.91, 80.33 and 76.29% of lactose, respectively; these rates increased to reach 90.44, 87.22 and 84 28%, respectively after 6 h of incubation (Figure 1).

Dornic acidity of milk

The acidifying effect and the capacity to degrade lactose are inversely proportional for all the mixed cultures; when acidity increases, the rate of lactose degradation decreases with respect to time. The same observation is made for the pure cultures, but the acidity remains lower than those of the mixed cultures; this is true for all the strains and at the same times of incubations.

The best rate of Dornic acidity, 80°D, was noted with $YSLB_2$ at the end of 3 h of incubation at 37°C, whereas it was 43°D and 37°D for $Sc.t_5$ and $Lb.b_5$, respectively, which are its constituent strains. After 24 h of incubation at 37°C, we noticed that the mixed cultures produced a high quantity of lactic acid, reaching 145°D, for the same yoghourt; and 61°D and 73°D for $Sc.t_5$ and $Lb.b_5$, respectively.

The increase in the acidity of the mixed cultures is due to the development of the growth of *S. salivarius ssp. thermophilus*, which profits from the growth factors (free amino acids and small peptides) produced by the proteolytic action of the lactobacilli (Loones,1994).

Similarly, a favorable initial ratio of *Lb. delbruckii ssp. Bulgaricus* allows the maximization of the Dornic acidity, and hence the production of acid by each strain can have a considerable influence on the shape of the curve of acidification (Beal et al., 1994).

Survival of the bacteria of yoghourt in the extreme conditions of the digestive tract

The local fermentation of yoghourt, in isolated cultures or in associations, shows a significant variation in growth in acidified synthetic media in the presence and absence of bile salts. The same result was found with the imported leavens (Dilmi Bouras, 2002).

Five mixed cultures, $YSLB_1$, $YSLB_2$, $YSLB_3$, $YSLB_4$ and $YSLB_5$ which gave the best rates of lactose degradation were exposed to the extreme conditions of the digestive tract (low pH and high concentration of bile salts).

A good growth is obtained for the five mixed cultures, in particular $YSLB_2$ and $YSLB_1$ reaching 1.65 x 10^7 and 1.41 x 10^7 cells/mL, respectively, in the absence of bile salts at pH 2.5 and after 3 h of incubation. This growth improves for all the mixed cultures and exceeds 4.57 X 10^8cells/mL for $YSLB_2$ after 6 h of incubation at 37°C (Figure 2A).

The results were almost identical when 0.3% of bile

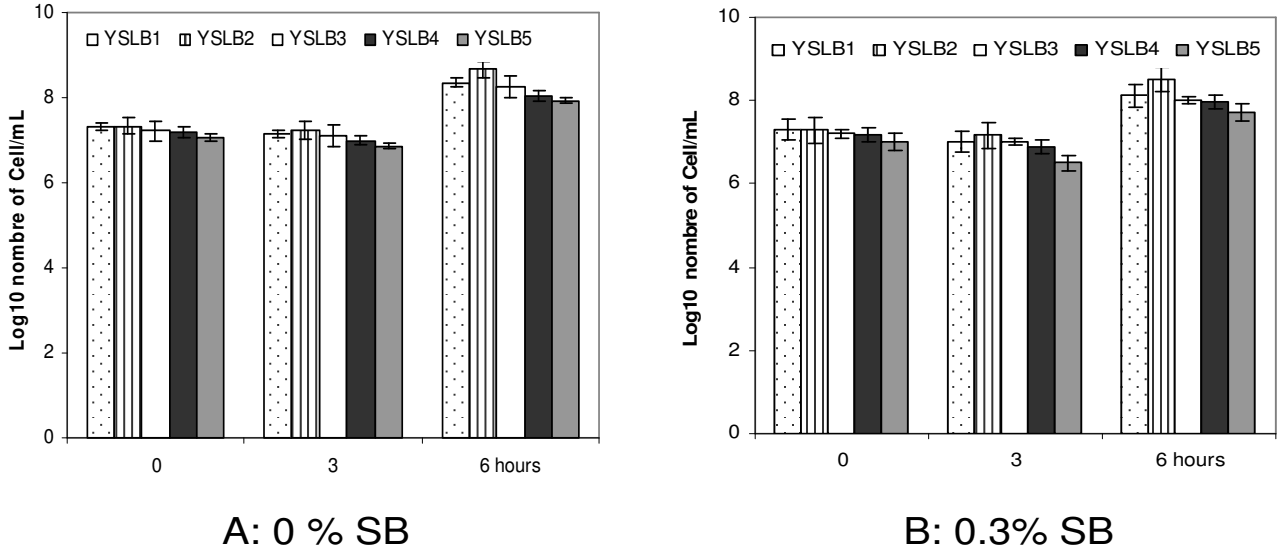

Figure 2. Evolution of the growth at pH 2.5 in absence (A) and presence (B) of bile salts.

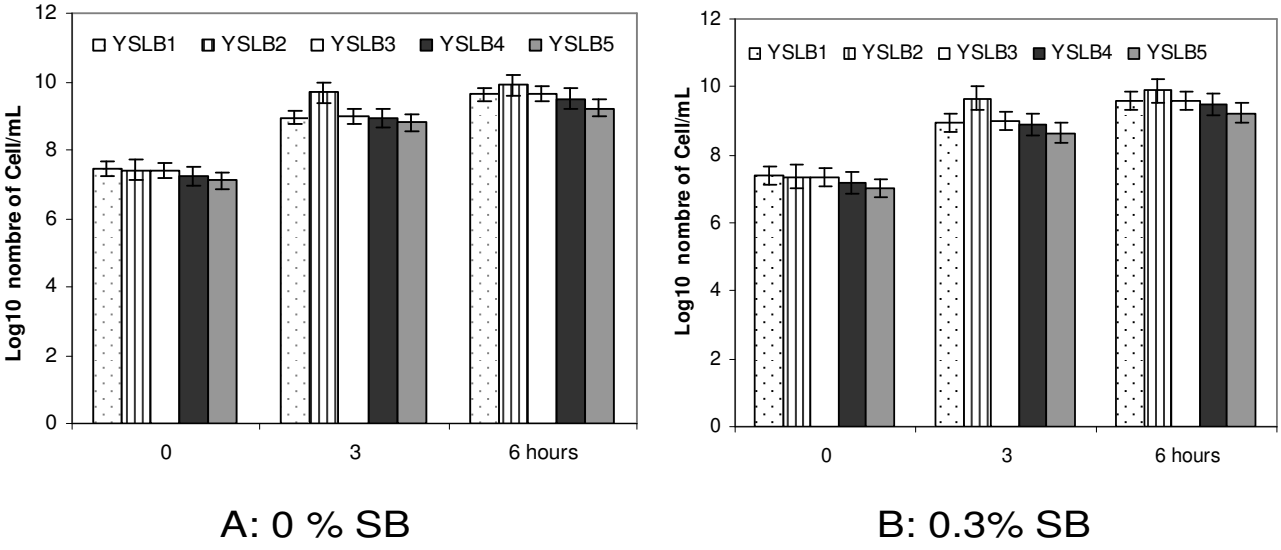

Figure 3. Evolution of the growth at pH 4.5 in absence (A) and presence (B) of bile salts.

salts was added (Figure 2B). A better growth is recorded when the pH is fairly acid (4.5) and the bacterial concentrations increase according to time; they were 8.03 x 10^9 cellules/mL for $YSLB_2$, and 4.47 x10^9 cells/mL for $YSLB_3$ after 6 h of incubation in absence of bile salts (Figure 3A).

The bacterial concentrations remained very high and almost identical when 0.3% of bile salts was added (Figure 3B).

At pH 6.5, in the absence of bile salts the ferment of the 5 yoghourts showed the best growth rates, after 6 h of incubation at 37°C (Figure 4A), considering that it is the pH optimum of the culture media of the bacteria. They were 1.23 x 10^{10}, 9.83 X 10^9 and 9.69 X 10^9 cells/mL for $YSLB_1$, $YSLB_2$ and $YSLB_3$, respectively,

When 0.3% of bile salts was added, the bacterial growth of 5 yoghourts remained significant, especially for $YSLB_1$, $YSLB_2$ and $YSLB_3$, reaching 9.81 X 10^9; 9.52 X 10^9 and 8.92 X 10^9 cells/mL after 6 h of incubation, respectively (Figure 4B).

These results are interesting and highly significant ($P< 0.05$) and thus make it possible to say that strains of *S. thermophilus* and *Lb. bulgaricus* of 5 yoghourts used, and especially $YSLB_1$, $YSLB_2$ and $YSLB_3$ (*Sc.*t_3 + *Lb.*b_2; *Sc.*t_5 + *Lb.*b_5 and *Sc.*t_4 + *Lb.*b_4), are less sensitive to very low pH (2.5); they grow better at pH of 4.5 and 6.5, and they can tolerate high concentrations of bile salts during 6 h of incubation at 37°C, which corresponds to the duration of

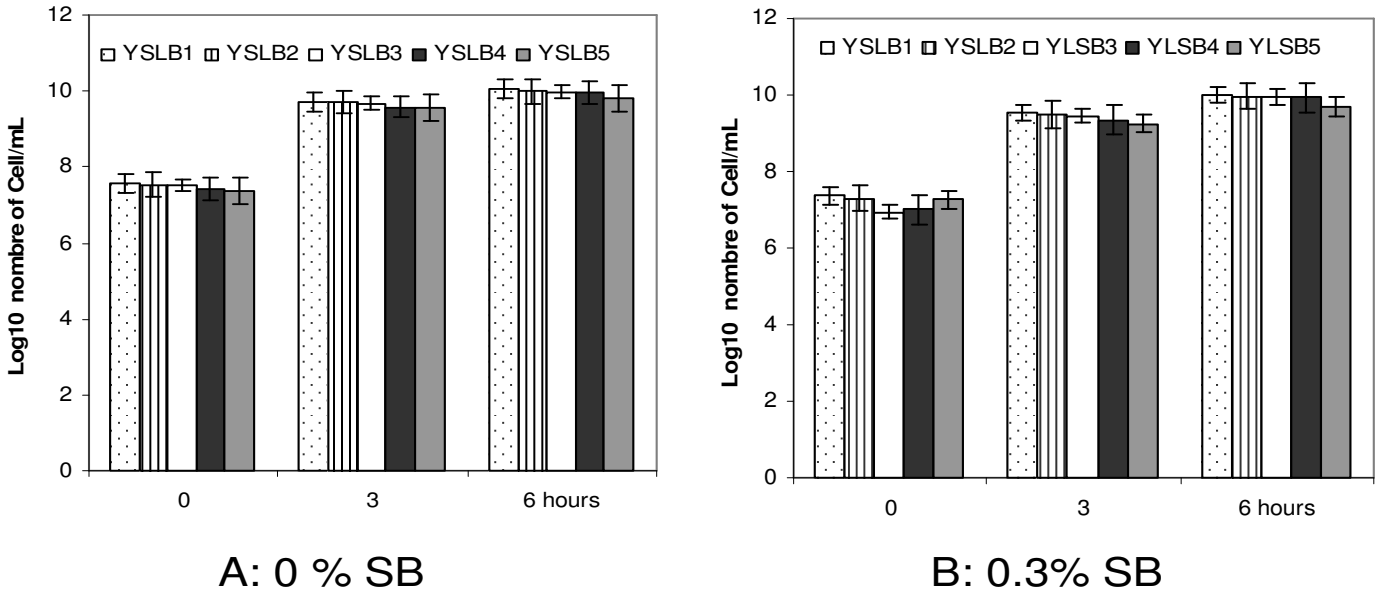

Figure 4. Evolution of the growth at pH 6.5 in absence (A) and presence (B) of bile salts.

the intestinal transit time.

These results are in agreement with those found by Dilmi (2002), which showed that the strains of *S. thermophilus* and *Lb. bulgaricus*, when in association, can survive the inhibiting action of bile salts and can resist well the gastric acidity, in particular at low pH. And according to Jin et al. (1998), that is due to the buffering effect of the yoghourt which helps the two ferment to resist at the low pH.

Conclusion

The testing of the mixed cultures of the local bacteria enabled us to select five combinations $YSLB_1$, $YSLB_2$, $YSLB_3$, $YSLB_4$ and $YSLB_5$ that have given best rates of lactose degradation with bacterial concentrations higher than 10^7 cells /mL. The best lactose degradation in mixed cultures of bacteria was obtained by $YSLB_2$ with a rate of 90.44% after 6 h of incubation at 37°C.

These results make it possible to conclude that the yoghourts-forming strains $YSLB_1$, $YSLB_2$ and $YSLB_3$ resist and grow better at relatively low pH in the presence of a high concentration of bile salts, for example they can reach 4.07 X 10^9 cells/mL for $YSLB_2$ at pH 4.5 in the presence of 0.3% of bile salts after 6 h of incubation; whereas it reaches 9.83 x 10^9 cells/mL under the same conditions with pH 6.5.

These results are very encouraging in cases where these yoghourts have bacteria that remain viable and active in the digestive tract; they can be used like alicaments for those who can not tolerate lactose, so as to enhance lactase activity, and we are planning to test these findings on animals (*in vivo*).

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