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ASSIMILATION (*IN VITRO*) OF CHOLESTEROL BY YOGURT BACTERIA

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Abstract: A considerable variation is noticed between the different species studied and even between the strains of the same species, in the assimilation of cholesterol in synthetic media, in presence of different concentrations of bile salts and under anaerobiosis conditions. The obtained results show that certain strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* resist bile salts and assimilate appreciable cholesterol quantities in their presence. The study of associations shows that only strains assimilating cholesterol in a pure state remain active when they are put in associations, but there is no additional effect. However, the symbiotic effect between *Streptococcus thermophilus* and *Lactobacillus bulgaricus* of yogurt, with regard to bile salts, is confirmed. The lactic fermenters of yogurt (Y2) reduce the levels of total cholesterol, HDL-cholesterol and LDL-cholesterol, in a well-balanced way. In all cases, the assimilated quantity of HDL-cholesterol is lower than that of LDL-cholesterol. Moreover, yogurt Y2 keeps a significant number of bacteria, superior to 10^8 cells ml^{-1}, and has a good taste 10 days after its production.

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INTRODUCTION

Cholesterol acts as a risk factor in different diseases such as cardiovascular, colon cancer and hypercholesterolemia [21, 25]. Results in recently published works indicate that the reduction of excessive levels of cholesterol in the blood decreases the risks of these diseases. In later years, a particular accent was placed on fermented products with specific microorganisms to which nutritional, dietetics and therapeutics properties were attributed [2, 10, 17, 23].

The purpose of this study is 3-fold: first, to search yogurt bacteria capable of assimilating cholesterol in specific synthetic media, in presence of cholesterol and bile salts and studying the effect of the latter on the growth and assimilation of cholesterol; second, to observe the association effect of lactic bacteria strains on the growth and assimilation of cholesterol; third, to determine which of the cholesterol fractions (HDL- and LDL-cholesterol) is preferentially assimilated by these lactic bacteria in pure strains or in associations.

MATERIALS AND METHODS

The bacteria strains used. *Streptococcus thermophilus*1 (*Sc.t$_1$*), *Lactobacillus bulgaricus*1 (*Lb.b$_1$*) are used for the making of yogurt Y1 (source: Boll-France) and *Streptococcus thermophilus*2 (*Sc.t$_2$*), *Lactobacillus bulgaricus*2 (*Lb.b$_2$*) are used for the making of yogurt Y2 (source: Laboratory of Microbiology, University HB Chlef). Different combinations have been made from these strains in the MRS broth.

Cultures broth and growth conditions. The MRS broth (reference: 0881-01, Difco) is used for the lactobacilli and the mixed cultures; whereas, the M17 broth (reference: 150-29, Diagnostica-Merck) is used for the streptococci.

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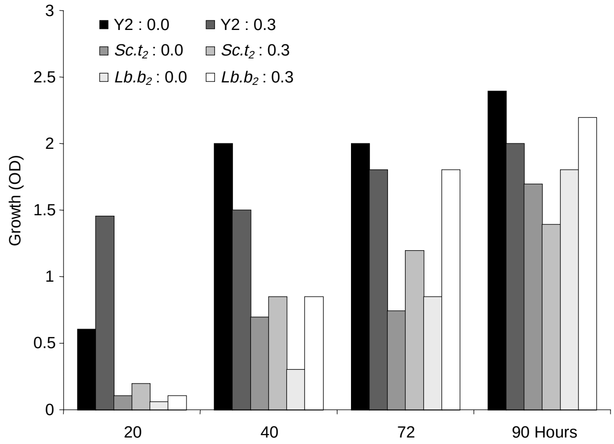

Figure 1. Growth of Y2, *Sc.t*$_2$ and *Lb.b*$_2$ (0.0 and 0.3% of bile salts).

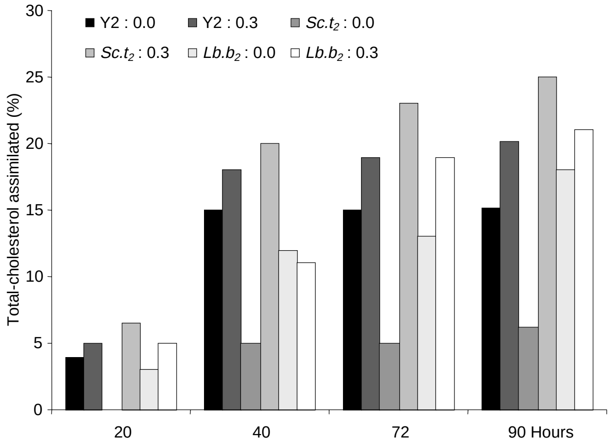

Figure 2. Total cholesterol assimilated by Y2, *Sc.t*$_2$ and *Lb.b*$_2$ (0.0 and 0.3% of bile salts).

The culture media of different strains are freshly prepared. They are divided into 2 fractions, each of which is distributed in subfractions, with 2 different concentrations (0.0 and 0.3%) in bile salts (BS) (reference: 4054, Merck) and a stationary concentration of cholesterol (Ci) of 40.0 ± 1.0 ml . l^{-1} (standard cholesterol, reference: 139050, Boehringer Mannheim GmbH). The growth of lactic bacteria is controlled by enumeration in a 10 × 90 mm plastic dishes, and by the optical density measurement using spectrophotometry at 660 nm at different time intervals (0, 20, 40, 72, 90 h), in order to follow the growth's evolution of different strains in the presence and absence of different concentrations in bile salts.

Total cholesterol analysis. The enzymatic colorimetric method of Sera-Pak test is used: US. overt 291.121 and Foteigh extensions [26].

HDL-cholesterol analysis. The analysis of this fraction is determined by the method Chod-PAP: reference 14210, Diagnostica-Merck [12].

LDL-cholesterol analysis. The method of precipitation by heparin (100,000 IU/l.) with citrate of sodium (0.064 mol.l^{-1}), as a stabilizer, is used: reference 14992, Diagnostica-Merck [28].

Life duration of yogurt Y2. After the making of yogurt Y2, we have observed the evolution of the Dornic acidity as well as the number of living cells, by the enumeration of *Sc.t*$_2$ and of *Lb.b*$_2$ during the storage at 4ºC.

The microbiologic analysis purpose, on 10 × 90 mm plastic dishes, is to determine the number of *Sc.t*$_2$ and of *Lb.b*$_2$ living in yogurt. After a microscopic exam of the dilution 1/100^e of yogurt, some successive decimal solutions are achieved. Then, 1 ml of the dilution is seeded in depth in the MRS-Agar broth for the culture of *Lb.b*$_2$ and on the surface in the M17-Agar broth for the culture of *Sc.t*$_2$. The cultures are incubated for 48 h at 37ºC. In the curves, the number values of viable organisms are given in $\log_{10}$.

The technique used to determine the Dornic acidity consists of introducing 10 ml of yogurt in a test tube with a sterile pipette. Then we add 0.1 ml (2 drops) of phenolphthalein. Titrate by using NaOH (N/9) until a pink color is obtained. The poured millilitres volume corresponding to Dornic degree (ºD). The results are expressed in Dornic degree or in gram of lactic acid per litre.

Statistical analysis of results. The statistical analysis of results is accomplished with the help of "Anova" programmes. We have performed, on the one hand, an analysis of variance in order to show the significance of our results and on the other hand, the average comparisons by Newman-Keuls' test and probability (P).

RESULTS AND DISCUSSION

Growth. The lactic bacteria in a pure culture and in association show an important variation when growing in a synthetic broth and in presence of bile salts. The isolated strains of *Sc.t*$_2$ and of *Lb.b*$_2$, initially sensitive or partially sensitive to bile salts, multiply normally when placed in association and in the presence of 0.3% of bile salts. This result shows the symbiotic effect, in relation to the bile salts, between *Sc.t*$_2$ and *Lb.b*$_2$. For the association Y2, the growth increases very significantly in the absence of the bile salts ($p<0.001$) and it is moderately significant in the presence of 0,3 % of bile salts and in relation to time ($p<0.01$) (Fig. 1). The growth of the mixed culture (Y2) is superior to that of *Sc.t*$_2$ and *Lb.b*$_2$ taken separately, both in the absence and in the presence of 0.3% of bile salts. No action against bile salts was observed, neither by *Sc.t*$_2$ nor by *Lb.b*$_2$.

These results confirm the works of Pacini *et al.* [24] and Dilmi-Bouras [9] showing that some strains of *Lb.b*$_2$ and of *Sc.t*$_2$ in association survive to the inhibitory action of some bile salts, until a concentration of 0.25% of desoxycholic acid. This concentration is normally superior to that found in the human coecum (0.05–0.20%). Similar results with different strains, were found *in vitro* [4, 5] and *in vivo* [11, 22].

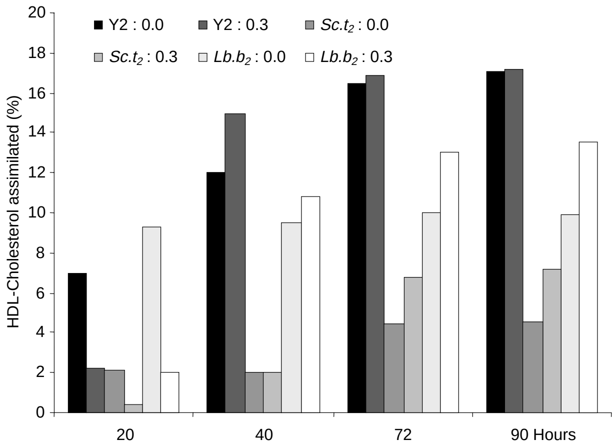

Figure 3. HDL-cholesterol assimilated by Y2, *Sc.t*$_2$ and *Lb.b*$_2$ (0.0 and 0.3% of bile salts).

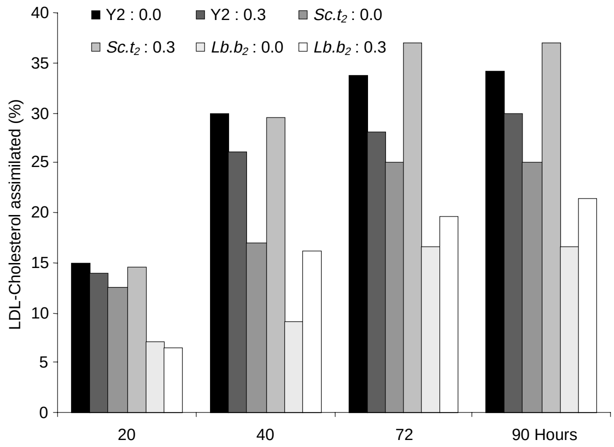

Figure 4. LDL-cholesterol assimilated by Y2, *Sc.t*$_2$ and *Lb.b*$_2$ (0.0 and 0.3% of bile salts).

Previous works [13, 8] show that certain lactic bacteria strains are very resistant, some less so, resistant and others are sensitive to acidity and bile salts; whereas others [20, 13] affirm that *Lb. acidophilus* strains can be developed only in the presence of bile salts.

This variation in the obtained results is probably due to the use of ferment strains, for these are very important variations between different species and even between strains of the same species in the resistance to acidity and bile salts.

Assimilated Cholesterol

Total cholesterol. The different strains in isolated culture and in association were placed in the presence of a known quantity of cholesterol (40.0 ± 1.0 mg/l), and after incubation in variable time, the analysis of cholesterol rest is realised by the enzymatic method.

An important variation is noticed between the different tested species and even between the strains of the same species in the assimilation of cholesterol in synthetic broth in the presence of different concentration of bile salts and under anaerobiosis. The associations (*Sc.t*$_2$ + *Lb.b*$_2$) assimilate different amounts of cholesterol in the presence of 0.3% of bile salts and after 90 h of incubation. These quantities of assimilated cholesterol are as follows: Y1: 18.0%, Y2: 21.0%. Y2 association seems to have the most important action towards cholesterol, in the presence of 0.3% of bile salts, and it has been kept as an example with these isolated strains.

Sc.t$_2$ does not assimilate cholesterol in 20 h and very little between 40–90 hours in the absence of the bile salts (Fig. 2); whereas the assimilation of cholesterol by *Sc.t*$_2$ increases significantly according to time and the concentration in bile salts ($p<0.001$), in order to attain nearly 25% in 90 h and in presence of 0.3% of bile salts. In other respects, *Lb.b*$_2$ assimilates important quantities of cholesterol which augment significantly according to time and concentration in bile salts ($p<0.001$). Y2 reacts in the same way as *Lb.b*$_2$ and assimilates more than 21% of cholesterol during 90 h and 0.3% of bile salts. Moreover, no action towards the bile salts has been noticed neither by *Sc.t*$_2$ nor by *Lb.b*$_2$.

This action towards cholesterol is confirmed even after many subculturing and two successive fermentations. We notice that during the second fermentation, and in the presence of 40.0 ± 1.0 mg/l of cholesterol, *Sc.t*$_2$, *Lb.b*$_2$ and Y2 always stay active and assimilate cholesterol quantities nearly similar to the first one in presence of bile salts. Therefore, the criterion of cholesterol assimilation by *Sc.t*$_2$, *Lb.b*$_2$ and Y2 always remains valid and cannot be lost during subculturing and successive fermentation.

These results confirm the work of Dilmi-Bouras, [9] with other strains of *Lb. bulgaricus* and of *Sc. thermophilus* (Boll-France). Similar results [10] show that *Lb. bulgaricus* and of *Sc. thermophilus* (Boll-France) diminish cholesterolemia of hypercholesterolemic rabbits. However, others [15, 16, 25] have found that yogurt ferments have no effect on the reduction of blood cholesterol level. Also, Klebling *et al.* [17] and Desreumaux [7] have not recorded any reduction of total cholesterol level in man, and De Roos and Katan [6] have concluded that hypocholesterolemic action of probiotic is not completely elucidated. This difference in results is due to experimental subjects and to different strains of ferment used.

HDL and LDL-cholesterol. This analysis was carried out to show which fraction of cholesterol is preferentially assimilated by yogurt ferments and in which proportion. The quantities of different fractions of cholesterol assimilated by the different strains and their associations are different. *Sc.t*$_2$ and *Lb.b*$_2$ in isolated cultures assimilate low quantities of HDL-cholesterol (Fig. 3) and high quantities of LDL-cholesterol (Fig. 4). This action is very important when *Sc.t*$_2$ and *Lb.b*$_2$ are in presence of 0.3% of bile salts. The association Y2 of these 2 ferments seems to give satisfactory and balanced results; it assimilates 17.20% of HDL-cholesterol and 29.70% of LDL-cholesterol in presence of 0.3% of bile salts.

This mixed culture (Y2) assimilates almost the sum of HDL-cholesterol from the 2 strains taken separately. But for LDL-cholesterol, the assimilated quantity represents

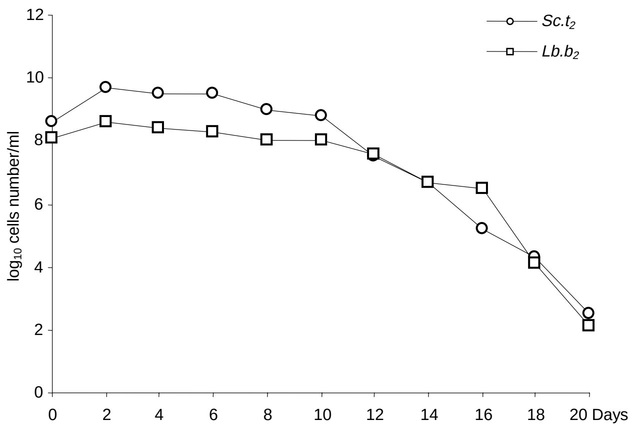

Figure 5. Growth of Y2, *Sc.t*$_2$ and *Lb.b*$_2$ in relation to time at 4ºC.

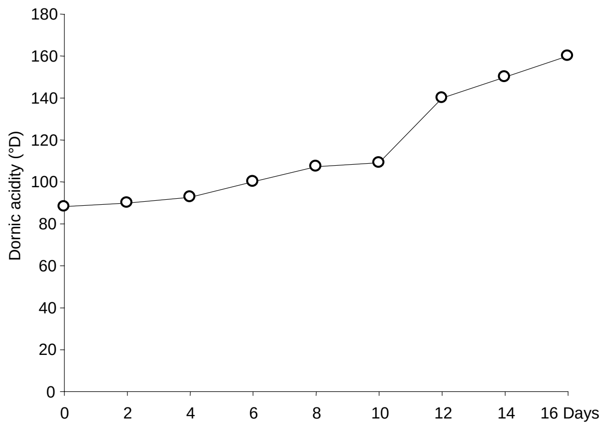

Figure 6. Dornic acidity evolution of Y2 in relation of time at 4ºC.

the average of the quantities assimilated by the 2 ferments in the isolated state. These results show clearly the moderate action of *Sc.t*$_2$, *Lb.b*$_2$ and their association (Y2) on HDL-cholesterol level, since the assimilated level of the latter remains always inferior to total cholesterol level or LDL-cholesterol. The same findings have been noted with different strains, *in vitro* [9] and *in vivo* [10]. However, Tamaï *et al.* [27] have not noticed any variation of sanguine HDL-cholesterol level of rats after the consumption of the fermented milk. In an other way, some researchers [7, 14, 17] have found that adding probiotics to the diet does not diminish LDL-cholesterol level; however, it diminishes LDL/HDL ratio. The results are different for Y1 which assimilates a low quantity of HDL-cholesterol (6.2%) and a large amount of LDL-cholesterol (28.0%). The assimilated quantities of LDL-cholesterol is very high and represents nearly 5 times the assimilated of HDL-cholesterol quantity. In this case, the equilibrium will be disrupted. According to bibliographic data, LDL-cholesterol is 3 times higher than HDL-cholesterol. For all strains and associations used, the statistic analysis shows that the assimilation of HDL-cholesterol and LDL-cholesterol in relation to time is significant until incubation time of 40 h ($p<0.01$), but beyond this period of time, the values remain nearly the same; therefore, there are no significant differences ($p>0.05$). The results will be very interesting if *Sc.t*$_2$ and *Lb.b*$_2$ are used by individuals facing problems of hypercholesterolemia.

Duration of life of yogurt Y2. During this study, we have followed the evolution (growth and Dornic acidity) of refrigerated yogurt Y2 in function of time. Figure 5 shows that the number of living bacteria increases so that to achieve 3.7×10^9 cells ml^{-1} of *Sc.t*$_2$ and 6.0×10^8 cells ml^{-1} of *Lb.b*$_2$ at the end of the second day. After the fourth day, we notice a rapid diminution of bacteria which attain less than 2.0×10^4 cells . ml^{-1} by the end of the eighteenth day. It seems to us that it is more reasonable to attribute this diminution of viability, on the one hand, to the sensitivity of *Sc.t*$_2$ to the low pH, and on the other hand, to the sensitivity of *Lb.b*$_2$ to cold weather. This result confirms other previous works, showing that the development of *Sc. thermophilus* is rapidly affected by the pH low [1, 11] and *Lb. bulgaricus* evaluates slowly at 4ºC [1]. Another writer has already shown that in yogurt fermentation the bacterium multiplication is very rapid and the number of microorganisms go beyond 10^8 . ml^{-1} [19] and after the thirtieth day at 6ºC there remain finally 10^8 cells . ml^{-1}. This variation in the found result is due to the difference of the used strains in yogurt fabrication. Generally, yogurt's life duration is nearly 3 weeks, because after this period there is yeast proliferation and mould [18].

However, Dornic acidity increases progressively with time, to attain 160ºD at the end of the sixteenth day (Fig. 6). This effect of acid production is connected to the stimulating action of *Lb.b*$_2$ on *Sc.t*$_2$.

Thus, it is important to know at what time of yogurt life we are situated, in order to evaluate and use it in a judicious way. According to the obtained results, this yogurt can be used as a "health" product, and more preferably in the 10 days following its production, mainly for 2 reasons: average Dornic acidity (90–130ºD) giving soft yogurts with a good taste and cells number of *Lb.b*$_2$ and of *Sc.t*$_2$ (more than 10^8 cells . ml^{-1}).

CONCLUSION

The isolated strains of *Sc. thermophilus* and those of *Lb. bulgaricus*, initially sensitive or little sensitive to bile salts, normally multiply when they are placed in association with and in the presence of a high bile salts concentration (0.3%). This result shows the symbiosis effect, in the presence of bile salts, between streptococcus and Lactobacillus used for the production of yogurts. The action, in the presence of 0.3% of bile salts, of *Sc.t*$_2$, of *Lb.b*$_2$ and that of Y2 on the cholesterol seems to be very interesting. These 2 strains and their association reduce cholesterol total level, of HDL cholesterol and LDL cholesterol, in a balanced way. In all cases, the assimilated quantity of HDL cholesterol is inferior to that of LDL cholesterol. However, no action has been noticed towards bile salts, neither by *Sc.t*$_2$ nor by *Lb.b*$_2$. Thus, to preserve the "health" aspect of yogurt Y2 and to profit from its lactic flora, it seems reasonable to consume the product in the 10 days that follow its production, a date from which germs' number has the tendency to regress.

Finally, these different observations need to be pursued *in vivo* studies on human model the most sensitive (the subject at risks), in order to better define the potential activity of the existing strains of lactic bacteria on the diminution of sanguine cholesterol level.

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