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Microbial interactions between *Lactobacillus Bulgaricus* and *Streptococcus Thermophilus* in milk

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- ✓ Interactions,
- ✓ *Lactobacillus Bulgaricus*,
- ✓ *Streptococcus Thermophilus*,
- ✓ co-culture,
- ✓ monoculture,
- ✓ synergy,
- ✓ proto-cooperation,
- ✓ lactic acid.

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Abstract

In this paper, we investigate the growth of *Streptococcus thermophilus* (IST) in milk in the presence (co-culture) or absence (monoculture) of *Lactobacillus bulgaricus* (ILB). The strains used in this study are isolated from a concentrate (ISTLB) cultured in a fresh milk from a freeze-dried mixture provided by the dairy: "Cooperative Laitière du Maroc Oriental (COLAIMO)". The obtained results of the acidification of fresh milk are preferable than the reconstituted milk for both IST and ILB strains. i.e., The IST strain acidified milk better than ILB strain.

A synergistic effect (proto-cooperation) is demonstrated in the two culture media. The acidity produced in the fresh milk by the mixed culture (IST + ILB) is 101° D ±3.2, while each of the two pure culture strains produced only 92° D ±2.7. In the reconstituted milk, the same results are observed but with less acidity, which are 63° D ±1.2 and 52° D ±1.9 respectively for mixed and pure culture strains. These differences are significant at 99% threshold according to the *Student* test. The filtrate of the ILB strain culture stimulates the lactic acid production of IST strain. No synergistic effect is observed on the proteolytic activity of the two strains. The IST strain seems to have an important proteolytic capacity than ILB strain.

1. Introduction

Mixed cultures of lactic acid bacteria are widely used in the dairy industry for the cheese-making, fermented milk, and yogurts [1]. Several studies about the interactions between different strains of intra- or interspecies or genera of lactic acid bacteria have been carried out. [2,3,4]. For the yogurt production, a mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* is used to obtain the acidity, viscosity, and the necessary aroma to reach a good quality of the final product [5,6,7]. Indeed, *S. thermophilus* as a lactic acid bacteria has nutritional demands [8]. The milk does not constitute an optimal growth medium for this microorganism because of its insufficient content of amino acids and peptides [9]. For this reason, it is conceivable that the addition of certain growth factors or the symbolic activity of bacteria combined with *S. thermophilus* in a mixed culture can exert a significant stimulating effect on both the development and the acidifying activity of this thermophilic streptococcus cultured in milk [10]. Moreover, the production of bacteriocins, antibiotics or other substances which modify the physicochemical conditions of the medium or also the competition against the substrate could be at the origin of the competitive interactions [11]. Thus, it is possible that a bacterial strain inhibits the growth of the other. In the dairy industry, the incubation of *S. thermophilus* and *L. bulgaricus* strains is generally carried out at a temperature between 42° C and 45° C to promote synergy between the two strains [12,13,14,1]. This combination is called protocooperation when it is beneficial to both species. The interaction between *S. thermophilus* and *L. bulgaricus* is based on the exchange of several metabolites that provide mutual growth stimulating effects [15,16]. These effects increase the rate of acidification of the milk [17,18,19,20,21,22] and the

bacterial populations at the end of growth [21,22,23]; whereas, they decrease the final pH of the product [21,23]. Yet, they stimulate the production of aromatic compounds such as acetaldehyde [24,25,26], a better production of extracellular polysaccharides (EPS) [27], and a stability of the final product [25].
Eventually, *S. thermophilus* stimulates the growth of *L. bulgaricus* by the production of pyruvic acid, formic acid, folic acid, ornithine, long-chain fatty acids, and CO_2 [28,29,30,31]. The production of formic acid depends on the strain, the medium, and the temperature [32,33]. Further, the lactic acid produced by *S. thermophilus* reduces the pH of the milk to an optimal level for *L. bulgaricus*.
L. bulgaricus exhibits high proteolytic activity enabling it to provide peptides, free amino acids, and putrescine which stimulate the growth of *S. thermophilus* [34,35,10].The aim of this work is to study the synergistic effect between *L. bulgaricus* and *S. thermophilus* strains used for industrial production at the "Cooperative Laitière du Maroc Oriental (COLAIMO)" and to determine the nature of the factors involved in this co-culture.

2. Materials and methods

2.1. *Provenance of strains*

In the present study, we used two strains of *S. thermophilus* (IST) and *L. bulgaricus* (ILB) isolated from a concentrate, cultured in fresh milk, of a mixture of *L. bulgaricus* and *S. thermophilus* (ISTLB) strains used for the direct seeding of production tanks at COLAIMO.

2.2. *Isolation and purification of strains*

A preculture in the milk of the concentrate ISTLB has been provided by COLAIMO. This concentrate is aseptically diluted in a sterile Ringer's solution from 10^{-1} to 10^{-6}. The isolation media are seeded from each of these dilutions. The IST strain of *S. thermophilus* is isolated on M17 medium [36] and The ILB strain of *L. bulagaricus* on the MRS medium [37]. Colonies with the criteria of *S. thermophilus* and *L. bulgaricus* are sub-cultured into the same media used for the isolation. At this stage, purification by streaking is performed. The purified colonies IST and ILB are sub-cultured on TPPY agar medium [38].

2.3. *Storage of the strains*

The microorganisms used in this work are stored in the refrigerator at 4° C, after culture on TPPY agar medium. Before each use, a preculture is carried out in 10 ml of reconstituted milk (10%) with three colonies and then incubated overnight at 42° C. The culture medium, a powdered milk free of antibiotic reconstituted (10%), is seeded at 1% from the preculture [39].

2.4. *Determination of Dornic acidity*

The production of lactic acid from the pure cultures of *S. thermophilus* IST, *L. bulgaricus* ILB, and a combination of IST with ILB in a reconstituted milk (autoclaved at 110 °C for 20 minutes) and in a fresh milk (heated to 80° C for 30 minutes), is followed in terms of incubation time. At each instant, an aliquot of 10 ml is removed aseptically. The growth is stopped by placing the aliquot in an ice-water bath. The total acidity developed is determined by sodium hydroxide N/9 (NaOH) in the presence of three drops of phenolphthalein. The equilibrium point is indicated by the turn of the color indicator to pink. The results are expressed in Dornic degree (°D) [40].

2.5. *Study of the filtrate cultures effect*

In order to determine the proteolytic activity of the studied strains and their effects on acidification, the following steps are carried out:

2.5.1. *Preparation of filtrate culture*

The pure culture of IST or ILB strains and IST+ILB mixture is incubated for 24 hours at 42° C in 10% reconstituted milk; then, they centrifuged at 5000 × g at 4 °C for one hour. The pH of the supernatant was previously adjusted to seven with NaOH 0.1N and its volume reduced to the initial volume of the culture using sterile distilled water. The supernatant is then sterilized by filtration using a 0.45-µm-pore-size Millipore filter [19]. In the case of non-immediate use, the supernatant is stored immediately in the freezer.

2.5.2. *Research of the filtrates effect*

In order to display the effect of filtrate culture, 1 ml of the filtrate to test of the IST or ILB strain is added to 9 ml of milk seeded with 1% of ILB or IST strain, respectively. For the control, 1 ml of lactolserum obtained by acidifying the milk at pH 4.6 using lactic acid and then adjusted to pH 7.0 that added instead of the filtrate.

2.6. *Measurement of proteolytic activity*

250 ml of the reconstituted milk is seeded at 1% from a 12-hours preculture of IST or ILB or the mixture ISTLB strains. At different incubating times (3, 6, and 24 hours), a sample is taken and immediately precipitated by trichloroacetic acid (TCA) 12% (final concentration 6%) to determine the soluble nitrogenous compounds. For the coloring method, ninhydrin is used [41]. After precipitation with TCA, filtration on WHATMAN paper No. 42 and staining with ninhydrin, the quality of the soluble nitrogenous substances is estimated by comparison with a calibration curve prepared with pure leucine.

3. Results

3.1. *Acidifying activity*

The acidifying power, in monoculture or co-culture, of isolated strains, is determined in reconstituted milk (10%) and fresh milk.

3.1.1. *In reconstituted milk*

From the obtained results, the ILB strain starts the acidification of the culture medium with one hour delay compared to IST strain. In addition, the ILB strain reaches its maximum acidification, which is 20° D ±0.6 after seven hours of incubation. The IST strain, however, reaches its maximum acidification, which is 32° D ±2.1 after eight hours of incubation.

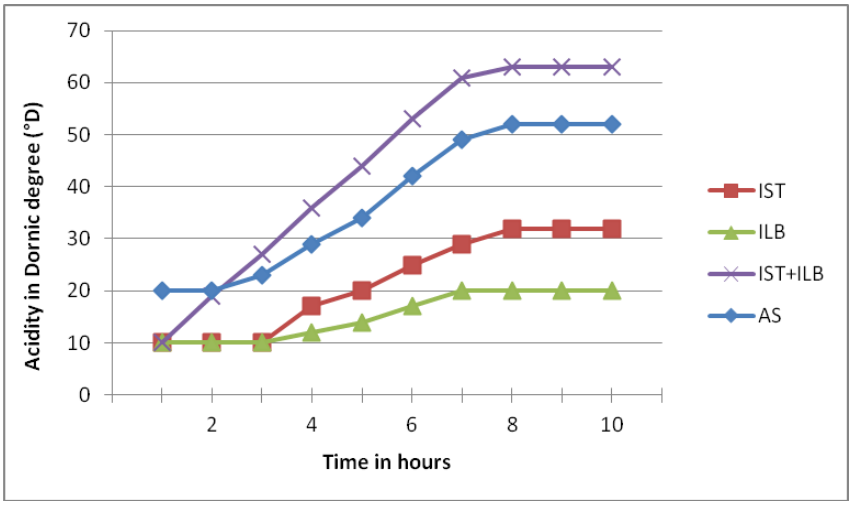

Figure 1: The evolution of the Dornic acidity during the growth of *S. thermophilus* and *L. bulgaricus* in the pure and mixed culture in the reconstituted milk.
IST: pure culture of *S. thermophilus*;
ILB: pure culture of *L. bulgaricus*;
IST+ ILB: mixture culture of 0.5% IST and 0.5% ILB of precultures;
AS: Arithmetic sum of acidities of pure cultures of IST and ILB.

A statistical study of the results for six essays according to the *Student* test (Table 1) shows that the difference in lactic acid production between the two strains becomes significant at the 99% threshold from three hours of incubation.

Furthermore, a significant synergistic effect between the two strains IST and ILB has been observed. In fact, the quantities of acid produced by the mixed culture (IST + ILB) are clearly higher than the arithmetic sum of the quantities of acid produced by each of these two strains in pure culture (Figure 1). After seven hours of incubation,

the acidity produced by each of the two strains in pure culture is only 49° D ±1.7. The results of the statistical study compiled in Table 1 show that the difference between the quantities of acid produced by the mixed culture and the sum of the quantities of acid produced by each of the two strains in pure culture is significant at the threshold of 95% until 2 hours 30 minutes of incubation. Beyond this time, the difference becomes significant at 99%. A significant difference at the threshold of 99% is observed between the acid production by the culture combining IST and ILB strains and that of ISTLB concentrate from 6 hours 30 minutes of incubation.

Table 1: Variance analysis of the lactic acid produced by IST and ILB strains, at each point of pure or mixed culture in reconstituted milk.

	IST and ILB		IST+ ILB and acidity of IST+ acidity of ILB	
Time (in h)	$Sd.t_{0,05}$	$Sd.t_{0,01}$	$Sd.t_{0,05}$	$Sd.t_{0,01}$
1	1,29	1,84		
2	0,18	0,25	1,89 *	2,69
3	0,29 *	0,41 *	1,58 *	2,25 *
4	0,68 *	0,89 *	0,57 *	0,82 *
5	1,39 *	1,99 *	0,99 *	1,42 *
6	1,14 *	1,62 *	0,79 *	1,11 *
7	1,80 *	2,56 *	0,98 *	1,39 *
8	1,93 *	2,72 *	1,63 *	2,31 *
9	2,01 *	2,85 *	1,92 *	2,73 *
10	2,01 *	2,85 *	1,44 *	2,03 *

Sd = Standard deviation at two confidence intervals (5% and 1%);
$t_{0,05}$ et $t_{0,01}$ = Limit values which have respectively 5 and 1 chance in 100 to be exceeded (They are given in the *student* table according to the number of degrees of freedom);
*Indicates that the difference is significant at the corresponding probability threshold.

3.1.2. *In fresh milk*

Figure 2 presents results of acidification in a fresh milk (heated to 80° C for 30 min), pure cultures of IST, ILB strains, and a combination of IST and ILB. Whatever the culture is, the acidification of the fresh milk is more important than the acidification of the reconstituted milk. De-facto, *S. thermophilus* (IST) starts the fresh milk acidification from one hour of incubation, whereas it does in reconstituted milk from 3 hours 30 minutes. At the end of the culture, lactic acid production reaches 62° D ±2.7 in the fresh milk (Figure 2), whereas in reconstituted milk the acid production is only 32° D ±2.1 (Figure 1) (half of that in the fresh milk). *L. bulgaricus* strain (ILB) starts the acidification slightly earlier in the fresh milk than in the reconstituted milk. At the end of the culture (i.e., after seven hours of incubation), the lactic acid production of this strain reaches 30° D ±2.2 in the fresh milk (Figure 2), whereas it is only 20° D ±0,6 in the reconstituted milk (Figure 1). This difference is significant at the 99% threshold. The combination culture of ILB and IST strains increases the lactic acid production equivalent respectively to 38 and 51° D (Figures 1 and 2) compared to the reconstituted milk.

As in the case of the reconstituted milk culture, the IST strain is more effective than the ILB strain in the fresh milk. The variability between these two strains in lactic acid production from one hour of incubation (Table 2) is significant at the 99% threshold. The IST strain starts the fresh milk acidification from one hour of incubation, while the ILB strain starts the acidification from three hours of incubation (Figure 2). In addition, the lactic acid produced by IST strain reaches 62° D ±2.7, whereas ILB strain produced only 30° D ±2.2. From another point of view, the amount of acid produced by the mixed culture of the two strains in the fresh milk (heated at 80° C for 30 minutes) is clearly greater than the sum of the amounts of acid produced by each of the two strains in pure culture (Figure 2). At the end of the culture, the acidity produced by the mixed culture is 101° D ±3.2, while the sum of acidity produced by each of the two pure culture strains is only 92° D ±2.7. This difference is significant at the 99% threshold (Table 2).

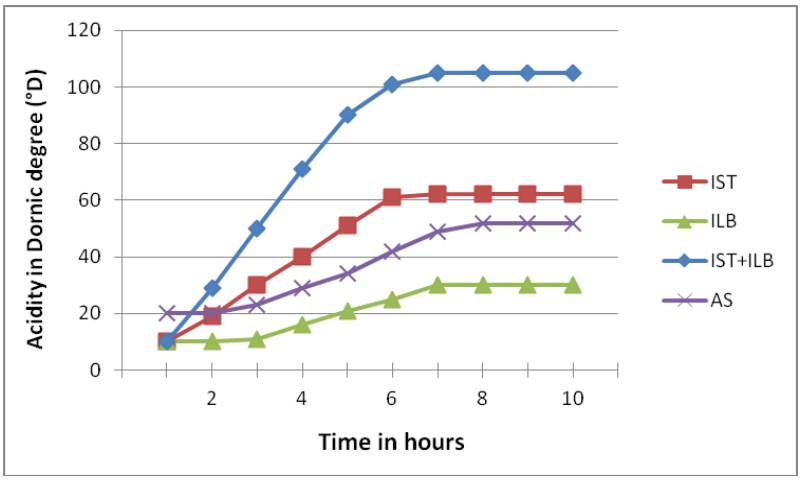

Figure 2: The evolution of Dornic acidity during the growth of *S. thermophilus* and *L. bulgaricus* in pure and mixed culture in the fresh milk.
IST: pure culture of *S. thermophilus*;
ILB: pure culture of *L. bulgaricus*;
IST+ ILB: mixture culture of 0.5% IST and 0.5% ILB of precultures;
AS: Arithmetic sum of acidities of pure cultures of IST and ILB.

Table 2: Variance analysis of lactic acid produced by IST and ILB strains, at each point of pure or mixed culture in the fresh milk.

	IST and ILB		IST+ ILB acidity of IST+ acidity of ILB	
Time(in h)	$Sd.t_{0,05}$	$Sd.t_{0,01}$	$Sd.t_{0,05}$	$Sd.t_{0,01}$
1	1,07	1,52	0,49 *	0,69 *
2	0,14 *	1,62 *	2,35 *	3,33 *
3	1,65 *	2,35 *	2,62 *	3,71 *
4	2,21 *	3,13 *	4,64 *	6,59 *
5	0,00 *	0,00 *	4,07 *	5,78 *
6	1,97 *	2,78 *	3,72 *	5,26 *
7	2,32 *	3,51 *	1,74 *	2,47 *
8	3,06 *	4,34 *	2,27 *	3,23 *
9	2,51 *	3,58 *	2,98 *	4,25 *
10	0,98 *	1,39 *	0,55 *	0,79 *

Sd = Standard deviation at two confidence intervals (5% and 1%);
$t_{0,05}$ et $t_{0,01}$ = Limit values that have respectively 5 and 1 chance in 100 to be exceeded (They are given in the student table according to the number of degrees of freedom);
*Indicates the difference is significant at the corresponding probability threshold.

3.2. *Action of filtrate culture on lactic acid production*
3.2.1. *In reconstituted milk*
Figure 3 shows the evolution of Dornic acidity according to the incubation time of *S. thermophilus* IST in the reconstituted milk, previously sterilized at 110° C for 20 minutes with the addition of 10% of filtrate from ILB strain culture. A stimulating effect of *S. thermophilus* IST acidifying activity is noted when the filtrate is added. At the end of the culture (eight hours of incubation), the acidity produced by IST strain is 69° D ±3, whereas this acidity reaches 80° D ±2 in the filtrate. This difference is significant at the 99% threshold. Besides, the filtrate of IST strain exerts no measurable stimulatory effect on the production of acid by *L. bulgaricus* ILB (Figure 4).

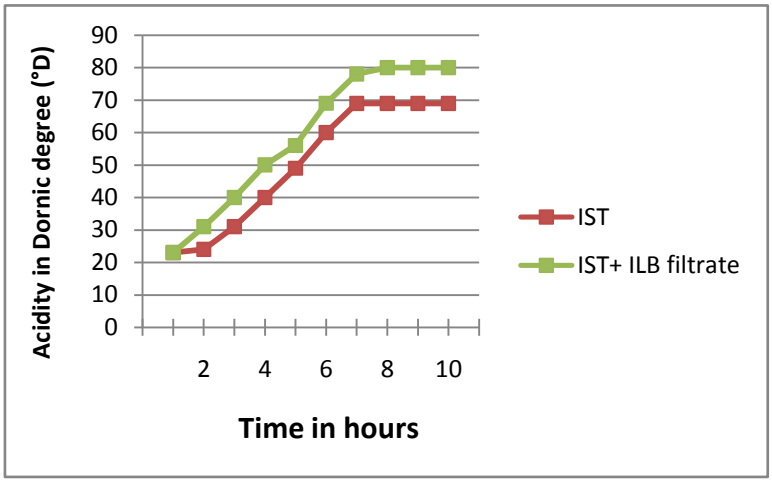

Figure 3: The action of ILB strain filtrate culture on the acid produced by *S.thermophilus* (IST) strain in the reconstituted milk.
Inoculum: 1%
Filtrate added: 10%
Incubation: 42°C.

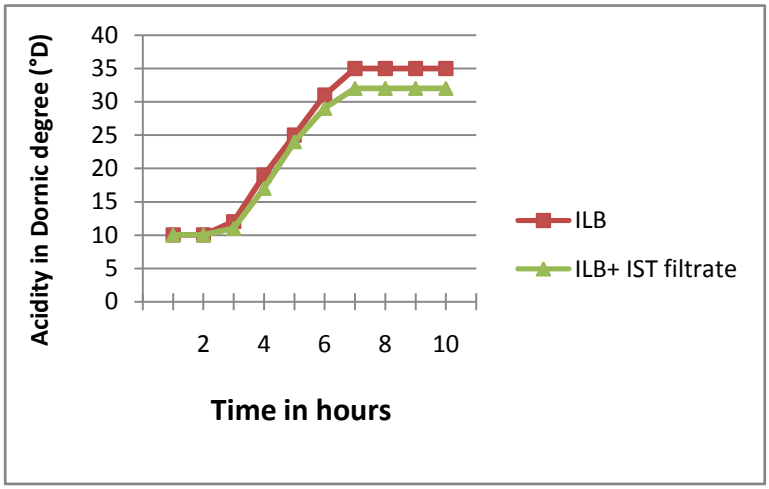

Figure 4: The action of IST strain filtrate culture on the acid produced by *L.bulgaricus* (ILB) strain in the reconstituted milk.
Inoculum: 1%
Filtrate added: 10%
Incubation: 42°C.

3.2.2. *On fresh milk*

Figure 5 shows the evolution of Dornic acidity regarding the incubation time of IST strain in the fresh milk, previously heated at 80° C for 30 minutes with the addition of 10% of filtrate from ILB strain culture. As in the reconstituted milk, a stimulating effect of the acidifying activity of *S. thermophilus* is observed in the presence of the filtrate. For the ILB strain, the filtrate of IST strain culture induces no effect on its acidification (Figure 6).
To sum up, in both cases, reconstituted and fresh milk, the addition of filtrate from the culture of the ILB strain stimulates the activity of *S. thermophilus* IST, contrary to IST strain filtrate on *L. bulgaricus*.

3.3. *Proteolytic activities of strains and strain mixture supplied to the Moroccan dairy industry*

The obtained results at different culture times are shown in Table 3. The IST strain is more proteolytic than the ILB strain. Indeed, after three hours of microbial development, the compounds quantity soluble in TCA is six times greater when the milk is inoculated with the IST strain. Unlike the acidification of milk by mixing the two strains, there is no synergy in the proteolytic activity.

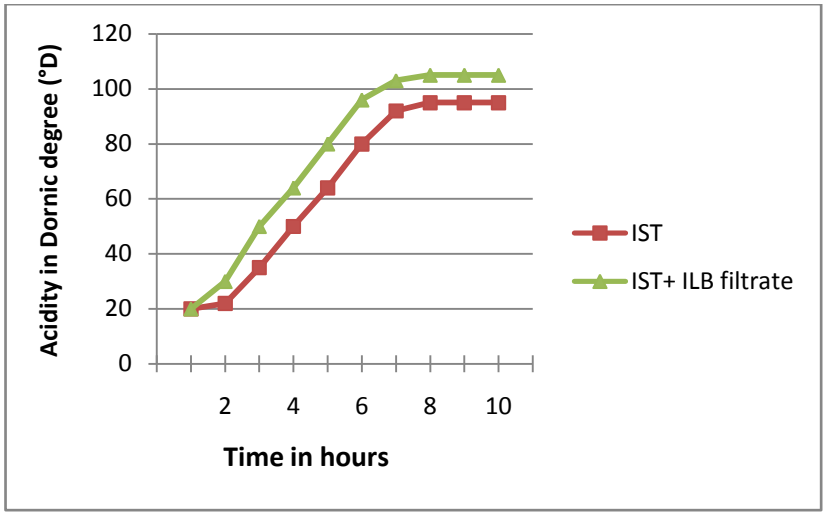

Figure 5: The action of ILB strain filtrate culture on the acid produced by *S.thermophilus* (IST) strain in the fresh milk.
Inoculum: 1%
Filtrate added: 10%
Incubation: 42°C.

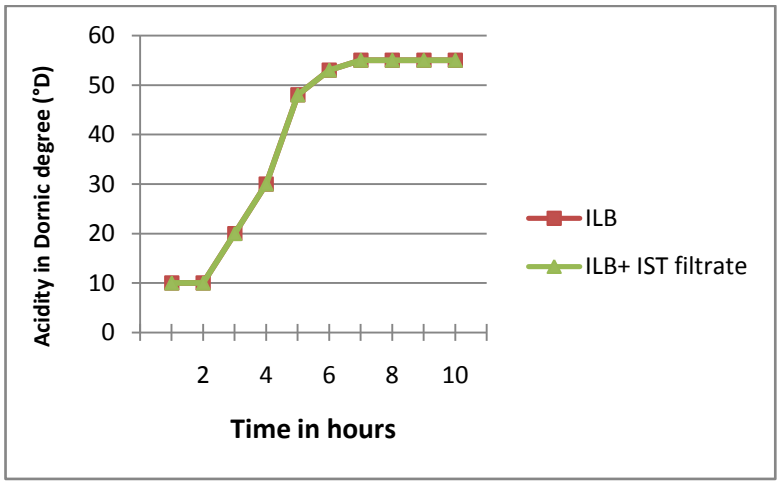

Figure 6: The action of IST strain filtrate culture on the acid produced by *L.bulgaricus* (ILB) strain in the fresh milk.
Inoculum : 1%
Filtrat ajouté : 10%
Incubation : 42°C

Table 3: The proteolytic activity in ppm equivalent of Leucine.

Time in hours	*S. thermophilus* (IST) strain		*L. bulgaricus* (ILB) strain		Mixture ISTLB	
3	635	±11	95	±5	650	±13
6	1100	±15	490	±8	1400	±18
24	1150	±19	800	±12	1750	±24

4. Discussion

Streptococcus thermophilus as a lactic acid bacteria has nutritionally demands. This microbial specie has a preference for lactose as a source of energy relative to glucose [42,43,17]. It is auxotrophic for at least six amino acids; but the amino acid and peptide concentrations are insufficient in the milk to ensure a good growth of this bacteria [44,11]. To ensure its growth, *S. thermophilus* needs an exogenous supply of peptides and amino acids [45,46,47,48,49]. The peptides are transported inside the cell and then degraded into amino acids [50,51,52]. These needs are confirmed by this study's results. Indeed, a synergistic effect (proto-cooperation) is highlighted in milk (reconstituted or fresh). The acidity produced by the mixed culture (IST + ILB) is 101° D ±3.2 in the fresh

milk whereas the sum of acidity produced by each of the two monoculture strains is only 92° D ±2.7 in the same medium. Similarly, the acidity produced by the co-culture is 63° D ±1.2 in the reconstituted milk, whereas the acidity produced in monoculture is only 52° D ±1.9. There is an increase in the acidification of the culture medium in mixed and isolated cultures. This could be due to the stimulation of the growth and/or the production of lactic acid of proteolytic activity products from one strain to another. These differences are significant at the 99% threshold according to the *Student* test. Béal et al.,[53] Observed that in a mixed culture of *S. thermophilus* strain 404 and *L. bulgaricus* strain 398 there was an increase in growth and acidification of *S. thermophilus.* Several authors have shown a stimulation of the acidification of the culture medium in a co-culture of the two strains, it results in an increase in the rate of acidification of the milk [17,19,18,20,21,22]. This can be explained by the addition of peptides and amino acids introduced into the medium by the proteolytic activity of the ILB strain. Several authors have been demonstrated proteolytic activities of lactobacilli isolated from different milks [54,55,56]. Furthermore, the filtrate action of ILB strain culture stimulates the production of lactic acid of IST strain. The filtrate culture of IST strain has no effect on the production of acid by the ILB strain. These results comply with those of Béal et al.,[53] which has noted a positive effect on the growth and acidification of the culture medium in co-culture on *S. thermophilus* and not on *L. bulgaricus.* This stimulation of *S. thermophilus* acidification either when it is co-cultured with the lactobacillus or in the presence of its filtrate may be caused due the fact that *L. bulgaricus* exhibits high proteolytic activity which enables it to provide peptides, Free amino acids, and putrescine that stimulate the growth of *S. thermophilus* [34,35,10,57]. Ultimately, no synergistic or syntrophic effect is observed on the proteolytic activity of the two strains. The IST strain seems to have a greater proteolytic capacity than ILB strain.

Conclusion

The aim of this work is to study the metabolism of S. *thermophilus.* As part of the combination of this microorganism with *L. bulgaricus* in milk, a contribution to the study of the growth stimulation phenomenon of *S. thermophilus* by the lactobacillus is effective. The obtained results confirm that *L. bulgaricus* can stimulate the growth of *S. thermophilus.* These observations confirm the stimulation of *S. Thermophilus* by the amino acids and the peptides released by the lactobacillus.

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