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LACTIC ACID BACTERIA. II : INTERACTIONS AMONG STRAINS OF *Lactobacillus* AND *Lactococcus* ISOLATED FROM RAW MILKS OF WESTERN ALGERIA

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Abstract

This work deals with the bacterial interactions among 16 members of *Lactococcus* genera and 11 of *Lactobacillus* genera isolated from raw milk of cow or she-camel from Algeria and identified thanks to biochemical and physiological tests. We looked for inter-inhibitions on 729 intra-specific, inter-specific or inter-generic couples, the co-cultures being realized in a phosphate buffered (pH=7) or non-buffered solid medium, or with catalase. 16.5% cases of inhibition, 2.5% of which are cases of self-inhibition were observed among the couples of *Lactobacillus*, and 74% cases of inhibition, 4.7% of which correspond to cases of self-inhibition among *Lactococcus* couples. Among the couples *Lactobacillus* - *Lactococcus* we noticed 2.3% cases of inhibition when *Lactobacillus* strains were used as indicator strains and 82.5% when *Lactococcus* strains were used as indicators strains. Some cases of inhibition but not all cases can be attributed to acidity or hydrogen peroxide production. No inhibition due to temperate phage was detected.

Keywords: *Lactic acid bacteria; Interaction; Lactobacillus; Lactococcus.*

Résumé

Ce travail décrit les interactions bactériennes entre 16 souches de *Lactococcus* et 11 de *Lactobacillus* récemment isolées de laits crus de vache ou de chamelle en Algérie et identifiées à l'aide de tests biochimiques et physiologiques. Nous avons recherché les inter-inhibitions chez 729 couples de souches inter- ou intra- espèce ou genre, les co-cultures étant réalisées en milieu solide en présence ou en absence de tampon phosphate (pH=7) ou de catalase. 16.5% cas d'inhibition, dont 2.5% de cas d'auto inhibition, étaient observés parmi les couples de *Lactobacillus*, et 74% cas d'inhibition, dont 4.7% de cas d'auto inhibition parmi les couples de *Lactococcus*. Parmi les couples *Lactobacillus*-*Lactococcus* 2.3% de cas d'inhibition étaient trouvés avec *Lactobacillus* comme souches indicatrices. Le pourcentage était de 82.5% avec *Lactococcus* comme souches indicatrices. Quelques cas d'inhibitions peuvent être attribués à la production d'acide ou de peroxyde d'hydrogène. Aucun cas d'inhibition par un phage tempéré n'a été détecté.

Mots clés: *Bactéries lactiques; Interaction; Lactobacillus ; Lactococcus.*

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ملخص

يقوم هذا العمل بوصف التفاعلية البكتيرية بين 16 سلالة من جنس *Lactococcus* و 11 سلالة من جنس *Lactobacillus* ، عزلت مؤخرا من الحليب الطازج لدى أبقار وناقات بالجزائر ثم صنّفت باستعمال الاختبارات البيوكيمياوية والفيزيولوجية.

تم البحث عن التفاعلية لدى 729 زوج من السلالات بين كل نوع و بين الجنسين، كما تمت عملية النمو بالنسبة لهذه السلالات على وسط صلب في حالة وجود وغياب لمنظم الـ pH الفوسفاتي (pH=7) وكذلك بالنسبة لوجود و غياب الكتلاز. قد لوحظت 16.5 % حالة تثبيط، من بينهم 2.5 % حالة تثبيط ذاتي لدى الأزواج من جنس *Lactobacillus* و 74 % حالة تثبيط، من بينهم 4.7 % حالة تثبيط ذاتي بين الأزواج من جنس *Lactococcus* .

لوحظت من بين الأزواج *Lactobacillus*- *Lactococcus*، 2.3 % حالة تثبيط مع استعمال الجنس *Lactobacillus* كسلالة مرشدة للتثبيط و 82.5 % حالة تثبيط عند استعمال الجنس *Lactococcus* كسلالة مرشدة للتثبيط.

بعض حالات التثبيط قد تكون راجعة إلى إنتاج الحموضة أو بيروكسيد الهيدروجين في الوسط مع غياب أي حالة تثبيط راجعة إلى وجود **لاقمات بكتيرية معتدلة.**

الكلمات المفتاحية: *بكتيريا الحليب، التفاعلية، Lactobacillus، Lactococcus.*

The farm-produce industry has always been using lactic starters for a wide range of manufacturing industries such as the transformation of milk, bread making, winemaking or the conservation of animal and plant products. These starters are sometimes made up of only one strain but are more often made up of two or three or more different strains of *Lactococcus*, *Lactobacillus*, *Pediococcus* or *Leuconostoc*. Precise composition of commercial starters often remains unknown. In the dairy industry, one can mainly distinguish thermophilic starters and mesophilic starters [1]. These selected starters generally contain *Lactobacillus* and *Lactococcus* strains, cultivated in association, enable to get a fermentated product which organoleptic qualities are rather well defined and reproducible.

The interactions existing between strains have an essential part in the obtaining of a good quality product. The knowledge of these interactions appears to be necessary in the making of controlled starters whose composition is controlled.

The interactions between bacterial strains may be direct or indirect [2-3] and are classified according to their profitable effects (or stimulation) or unprofitable effects (or inhibition) [3-4-5]. Several works have been devoted to the study of inhibitions of spores or bacteria such as *Clostridium*, *Leuconostoc*, *Pediococcus* [6], *Listeria monocytogenes* [7-8] or food borne bacterial pathogens [9] due to lactic acid bacteria. Inhibitive factors produced by lactic acid bacteria have been reviewed [10-11-12].

Nevertheless, there are no or very little information about inter-inhibitions between strains of lactic acid bacteria that are usually co-cultured in dairy starters cultures, *i.e.* mainly *Lactococcus* and/or *Lactobacillus* strains. This lack of information brought us to investigate inter-bacterial inhibitions among *Lactococcus* couples, *Lactobacillus* couples and *Lactobacillus*-*Lactococcus* couples.

MATERIALS AND METHODS

Organisms

The 31 strains studied were from the collection of our laboratory. They were isolated from raw milks of Algerian cows and she-camels and pre-identified thanks to biochemical and physiological tests. They are Gram-positive bacteria, non-motile, non-spore forming, without catalase activity. Citratase and arginine dihydrolase activities were established as well as homo- or hetero-fermentation, and for the *Lactococcus* strains, the growth was tested at 45°C or in a medium containing 6.5% NaCl. The identification was clarified by comparison of biochemical profiles obtained with known lactic acid bacteria thanks to API 50 CH strips (La Balme Les Grottes 38390 Montalieu Vercieu, France).

The strains are divided into B (*i.e.* bovine) or C (*i.e.* camel) as follows:

Lactococcus lactis B1, B2, B3, B4, B5 ; *Lactococcus diacetylactis* B6, B7, B8, B9, B10, B11, B12, B13, B14 ; *Lactococcus cremoris* B15 ; *Lactococcus* sp. C1, B16 ; *Lactobacillus plantarum* B17, B18, B19, B20, B21, B22, B23, C2, C3 ; *Lactobacillus casei* B24, B25, B26 . Two lysogenic strains were used as a reference for research of lysogeny : *Lactococcus lactis* B27 and *Lactobacillus plantarum* B28.

Bacterial cultures and storage

Lactococcus strains were grown in M17 medium [13] and *Lactobacillus* strains in MRS medium [14]. Co-cultures of *Lactobacillus* and *Lactococcus* were realized either in MRS or in M17. Plates were incubated at 30°C. Anaerobiosis was obtained inside jars with a BBL GasPak Anaerobic Systems (BBL Microbiology Systems, Cockeysville, Maryland 21030, USA).

Bacterial cells were grown to mid-log phase and stored at –80°C in MRS medium or M17 medium supplemented with 20% sterile glycerol or in sterile 10% skimmed milk.

Bacterial interactions

The bacterial interactions were searched for according to the methods described by [15] and by [16]. According to the first method, the bacteria were grown in liquid medium for 18 hours. These cultures were used to inoculate a solid medium. Inoculation was done with a home-made multipoint inoculator that allowed testing several dozens of strains with the same indicator strain. The spot-inoculates were let dry for 1 hour in an ambient temperature (about 24°C) and then at 30°C for 4 hours. 0.5ml of a culture of the indicator strain was added to 8ml of semi-solid medium (0.7% agar-agar) kept at 45°C and then the mixture was spread on the top of the plate-culture. After the solidification of the second layer the plates were incubated at 30°C for 24 or 48 hours.

Experiments were also conducted in solid medium supplemented with 1mg/ml catalase (Sigma), or Na/K_2 phosphate buffered at pH=7.

Negative interactions led to growth-inhibition of the indicator strain and a ring of growth-inhibition was easily detectable around the spot-inoculated strain. The intensity of the inhibition was estimated by the width (w) of the inhibition ring. It allows to classify the detected inhibitions into strong inhibitions when w>1.5mm and weak inhibitions when w<1.5mm to be in accordance with the method described by [17].

Phage detection

Pieces of the growth inhibition ring were sampled and mixed to 1ml of sterile medium. After it had settled, 0.1ml were added to 8ml of semi-solid medium and 0.5ml of an 18 hour-indicator culture and the mixture was spread on a solid medium. The plates were incubated at 30°C for 24 to 48 hours.

RESULTS AND DISCUSSION

Detection of the interactions between lactic acid bacteria

According to the method of [16] the indicator strain was inoculate in the medium and when the medium became solid, the inhibitive strains were spot-inoculated. In our hands, this technique didn't allow us to get clear results nor to quantify the intensity of the inhibitions.

According to the method of [15] the inhibitive strains were spot-inoculated on the surface of a solid medium and overlayed with semi-solid medium containing the inhibitor strain. With this technique we were able to clearly bring into fore the inhibitions between different strains (Fig.1). The same results were obtained with a good reproducibility either in MRS medium or in M17 medium for *Lactococcus* as well as for *Lactobacillus*.

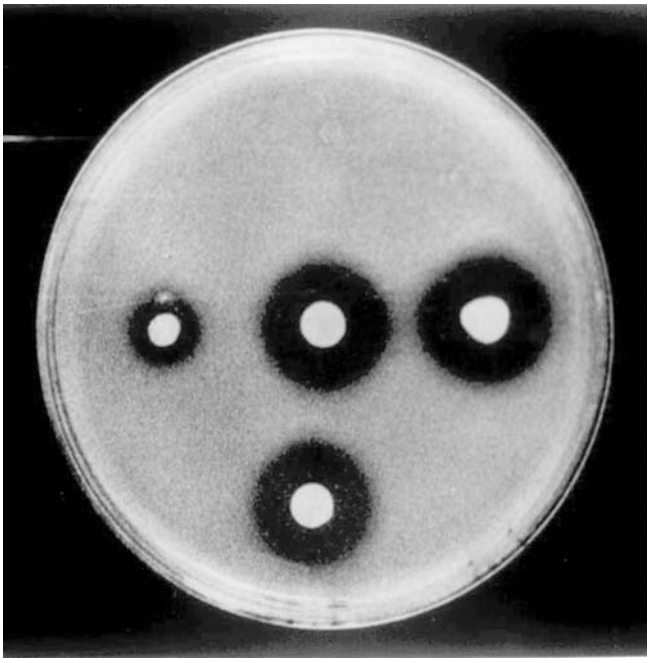

Figure 1: Interactions between lactic acid bacteria.
Bacteria were grown in liquid medium. The 18 hours-cultures were then used to spot-inoculate a plated medium according the method described by [15]. The indicator culture was added and the plates were incubated as described in Materials and Methods. The indicator strain was *Lactococcus lactis* B14 and the spot-inoculated strains were from top of the plate *Lactococcus cremoris* B15 which had no detectable inhibitive effect and clockwise from this strain, *Lactococcus lactis* B2, *Lactococcus diacetylactis* B6, *Lactococcus diacetylactis* B10 and in the middle of the plate, *Lactococcus lactis* B1.

Table 1: Interactions between *Lactococcus* strains.

Strains S1 / S2		I				II	III								IV	
		B1	**B2**	**B4**	**B5**	**B15**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**	**B14**	**C1**	**B16**
I	**B1**	1.5	1.5	0	1	0.5	2	0.5	1	0	1	0.5	2.5	2	2	0	0.5
	B2	1.5	2	1	1	0	2	0	0.5	0	1	0	2	1.5	2	0	1
	B4	0.5	0	0.5	0.5	0	1	0.5	1	0.5	0	0	1	0	0.5	0	0.5
	B5	2	2.5	0.5	1.5	0.5	2.5	0.5	0.5	0.5	1	0.5	5	3	2	0.5	1.5
II	**B15**	3	4.5	0.5	0.5	1	1	0	1	0	1	1	1	1	1.5	1.5	2
III	**B6**	1	1.5	0	1	0	1.5	0	1	1	1	0.5	2	2	1.5	0.5	0
	B7	0.5	6	0.5	0.5	0	0	0	0	0	0	0	0	0	0.5	0	0
	B8	0.5	4	1.5	3	0.5	0.5	0	1.5	1.5	1.5	2	2	2	2	1.5	3
	B9	0.5	0	0.5	2	0	0.5	0	0.5	0	0	0	1	0	0	0	0
	B10	2	3	0.5	0.5	1	1	1	0	1.5	0.5	1	1	1	0.5	1	4.5
	B11	0	0	0	0	0.5	0	0.5	1	1	0	0	0	0	0	1	2.5
	B12	1.5	1	0	1	1	2	0	1	0.5	1	0.5	2	2	1.5	1	0
	B13	2	2	0	1	1	2	1	0	1	0.5	1	2	2	2	0.5	1
	B14	3	3	0	2.5	0	2	0.5	0	0	0.5	0	4	5	2	0	0
IV	**C1**	1	1	0	1	0.5	1	1	1.5	1	0.5	1	1	1	1	0	1.5
	B16	1.5	2	1	2	0.5	1	1.5	2	1.5	2	2	1	1.5	1.5	2	0.5

The inhibitions were searched according to the method described by [15]. The width of the ring of inhibition is expressed in mm.
S1 = spot-inoculated strain **S2** = overlay-inoculated strain
I = *Lc. lactis* ssp *lactis* **II** = *Lc. lactis* ssp *cremoris*
III = *Lc. lactis* ssp *diacetylactis* **IV** = *Lc.* sp

Interactions between *Lactococcus* strains

Table 1 shows the results got with *Lactococcus*. Among 256 co-cultured couples we got 76% or so cases of inhibition and 24% lacks of interactions. We noticed 78 cases of strong inhibitions and 112 cases of weak inhibitions. The inhibitions were intra-subspecific (*Lactococcus lactis* ssp *lactis* B2 - *Lactococcus lactis* ssp *lactis* B5 for example) or inter-subspecific (*Lactococcus lactis* ssp *lactis* B2 - *Lactococcus lactis* ssp *diacetylactis* B7 for example). Three types of responses were observed:

(***i***) For some couples the spot-inoculated strain inhibited the overlay-inoculated strain: however it was not inhibitive any more in the opposite combination and it was even inhibited by that it previously inhibited. For example it is the case for the combination *Lactococcus lactis* ssp *lactis* B1 - *Lactococcus lactis* ssp *diacetylactis* B13.

(***ii***) For some other couples the spot-inoculated strain inhibited the overlay-inoculated strain but in the opposite combination the latter strain was of no effect on the first. For example it is the case for the couple *Lactococcus lactis* ssp *lactis B2* - *Lactococcus lactis* ssp *diacetylactis* B7.

(***iii***) 12 strains among the 16 strains studied in this work showed a curious phenomenon of self-inhibition. These are for example *Lactococcus lactis* ssp *lactis* B2 or B5, *Lactococcus lactis* ssp *cremoris* B15, *Lactococcus lactis* ssp *diacetylactis* B12 or B14. Similar results were recorded by [18] and by [19].

Inhibitions between *Lactobacillus* strains

The three same categories of responses like for *Lactococcus* strains were found for *Lactobacillus* strains. For example the couple *Lactobacillus casei* B24 - *Lactobacillus plantarum* B21 belongs to the first category (***i***), the couple *Lactobacillus plantarum* B17 – *Lactobacillus plantarum* B26 belongs to the second category (***ii***) and the strains *Lactobacillus plantarum* B20 or B22 and *Lactobacillus casei* B25 belongs to the third category (***iii***). 3 strains of *Lactobacillus* out of 11 were concerned by self-inhibition. These results are written down in Table 2.

Table 2: Interactions between *Lactobacillus* strains.

Strains S1 / S2		V								VI		
		B17	**B19**	**B20**	**B21**	**B22**	**B23**	**C2**	**C3**	**B24**	**B25**	**B26**
V	**B17**	0	0	0	0	0	0	0	0	0	0	0
	B19	0	0	0	0.5	0	0	0	0.5	0	0	0
	B20	0	1	2	1.5	2	1	1	0	0	0	1
	B21	0	0	0	1	0	0	0	0	0.5	0	0
	B22	0	0	0	1	0	0	0	0	0.5	0	0
	B23	0	0	0	0.5	0	0	0	0	0	0	0
	C2	0	0	0	2	0	0	0	0.5	0	0	0
	C3	0	0	0	0	0	0	0	0	0	0	0
VI	**B24**	0	0	0	0.5	0	0	0	0	0	0	0
	B25	0	0	0	0	0	0	0	0	0	0.5	0
	B26	0.5	0	0	0.5	0	0.5	0	0	0	0	0

The inhibitions were searched according to the method described by [15]. The width of the ring of inhibition is expressed in mm.
S1 = spot-inoculated strain. **S2** = overlay-inoculated strain
V = *Lb. plantarum.* **VI** = *Lb. casei*

Inter-generic inhibitions

Table 3 shows results of the 176 "spot-inoculated strains of *Lactobacillus* / overlay-inoculated strains of *Lactococcus"* combinations. 150 cases of inhibition could be noticed among which 114 show strong inhibitions.

Among the opposite combinations (*i.e*. "spot-inoculated strains of *Lactococcus* / overlay - inoculated strains of

Table 3: Interactions between Lactobacillus and Lactococcus strains.

Strains		V								VI		
S1 / S2		B17	B19	B20	B21	B22	B23	C2	C3	B24	B25	B26
I	B1	2	2	4.5	4	1.5	2	3	2	0	0	0.5
	B2	4	2	5	5	2	3	4	2.5	0	0	0.5
	B4	3.5	3.5	0.5	3.5	2	3	2	3	0	1.5	3
	B5	2	0.5	6	2	0.5	0.5	1	0.5	1	1	2
II	B15	1	0	1	3	3.5	2	0	0.5	0	0	0
III	B6	1.5	3	4	2	2.5	2.5	2	1.5	0	1	2.5
	B7	4	0	0	5	4	4	0	2	0	0	3
	B8	3.5	4	1.5	5	3	4	4.5	4	1.5	0	0
	B9	2	3	1	5	2.5	3	2.5	2.5	0	0	2
	B10	3	2.5	0.5	3	2.5	2.5	2	3	0.5	0	1.5
	B11	2	5	0	2.5	2	2.5	2	3	0	1	2
	B12	3	3.5	4	2	2.5	3.5	3	2.5	0	1	2.5
	B13	2	2	4	0	3	3	4	3	0.5	2	0
	B14	2	2	6	1.5	2	2	2	1	0	1	1
IV	C1	2	1	1	0.5	1.5	1	1	0.5	0.5	1	0.5
	B16	4	4	1	4.5	2.5	2.5	4	2.5	0.5	2.5	0.5

The inhibitions were searched according to the method described by [15]. The width of the ring of inhibition is expressed in mm.
S1 = spot inoculated strain. **S2** = overlay inoculated strain.
I = *Lc. lactis* ssp *lactis.* **II** = *Lc. lactis* ssp *cremoris.*
III = *Lc. lactis* ssp *diacetylactis.* **IV** = *Lc.* sp.
V = *Lb.plantarum.* **VI** = *Lb. casei.*

Lactobacillus") we noticed only 4 cases of inhibition among 176 combinations : *Lactococcus lactis* ssp *diacetylactis* B8 and B12 were able to inhibit *Lactobacillus plantarum* B20 while *Lactococcus lactis* ssp *lactis* B2 inhibited *Lactobacillus plantarum* B21 and *Lactococcus* sp. C1 inhibited *Lactobacillus plantarum* B22.

This study allowed us to select 15 strains with the strongest inhibitory effect as it was defined by [17] with as a criterion, the number of inhibited strains, the width of the zone of inhibition and also the rapid growth and the good viability of the strains. The selected strains were *Lactococcus lactis* ssp *lactis* B1, B2, B5, B6, B8, B14, B15, *Lactococcus lactis* ssp *diacetylactis* B12, B13 and *Lactobacillus plantarum* B17, B19, B22, B23, C2.

Influence of aerobiosis

The same inhibitions were recorded with cultures either with aerobiosis or with anaerobiosis, which shows that oxygen seemed to have no effect in the nature of the observed phenomenon.

Influence of acidity

No inhibition was abolished in a buffered medium (Tab. 4), although in some cases the intensity of inhibition was weakened. In some cases it seemed that acidity plays a part

Table 4: Interactions between some strains on buffered or non-buffered medium.

S1 / S2	B1		B2		B5		B6		B12		B13		B14		B20	
	M	BM	M	BM	M	BM	M	BM	M	BM	M	BM	M	BM	M	BM
B1	1.5	1.5	1.5	2	1	1.5	2	2.5	2.5	1.5	2	1	2	2	4	3
B2	1.5	1.5	2	1	1	1	2	1.5	2	2	1.5	2	2	2	3	4
B5	2	1	2	2	1.5	2	2.5	3	3	4	2	3	2	2	6	5
B6	1	1	1.5	1.5	0.5	1	1.5	1.5	2	1	2	2	1.5	1.5	4	4
B12	1.5	1	2	2	1	1.5	2	1.5	2	1	2	1	1.5	2	4	4
B13	2	2	2	2	1	1	2	2	2	2	2	2	2	2	4	4
B14	3	3	2.5	4	2	3	3	4	4	5	4	4	2	3	6	5
B20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5	2

The inhibitions were searched according to the method described by [**15**] but either in a phosphate buffered medium, pH=7, or in non-buffered medium.. The width of the ring of inhibition is expressed in mm.
S1 = spot inoculated strain. **S2** = overlay inoculated strain
M = non buffered medium **BM** = phosphate buffered medium

Table 5: Effect of catalase on the interactions between different strains.

S1 / S2	B1		B2		B5		B6		B12		B13		B14		B20	
	M	MC	M	MC	M	MC	M	MC	M	MC	M	MC	M	MC	M	MC
B1	1.5	1	1.5	1	1	0.5	2	1.5	2.5	2	2	1.5	2	1	4	3
B2	1.5	0	2	1	1	0	2	1	2	1	1.5	1	2	0	5	4
B5	2	2	2	1	1.5	1	2.5	1.5	3	2.5	2	2	2	2	6	5
B6	1	1	1.5	1	0.5	0	1.5	1	2	1	2	1	1.5	0	4	4
B12	1.5	1	2	1	1	0	2	1	2	1	2	1	1.5	0	4	4
B13	2	0	2	1	1	0	2	2	2	1	2	1	2	0	4	4
B14	3	2	2.5	2	2	2	3	3	4	4	4	3.5	2	2.5	6	5
B20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5	1.5

Bacterial cultures were realized according to the method of [**15**] in a solid medium containing or not containing catalase (1 mg/ml). The width of the inhibition zone is expressed in mm.
S1 = spot-inoculated strain **S2** = overlay-inoculated strain.
M = medium without catalase **MC** = medium containing catalase.

in the phenomenon. Measures of the pH in the inhibition zone and in the zone where bacteria grew did not show any important variation: pH inside the inhibition zone was 4.6 and its value outside this zone was 4.7. This result cannot be compared with the results reported by [20] but it is in line with those of [5] and [21].

Influence of hydrogen peroxide

The adding of catalase in the culture medium as described by [22] removed the inhibition in 8 cases. This type of results was also described by [5]. However in our experiments, inhibition persisted in 11 cases without any qualitative modification and in 45 cases it persisted with a slight diminution of intensity (Tab. 5).

Influence of temperate phage

None of the selected inhibitive strains seemed to be lysogenic for no phage was detected in the samples of the inhibition zones.

CONCLUSION

A lot of studies of interactions between microorganisms have been described. Most of them concern interactions between lactic acid bacteria and bacteria undesirable in fermentation industries [23] or favourable to men [24-25-26] or favourable to animals [27-28]. Relatively few works were devoted to inhibitions between lactic acid bacteria whose industrial importance can't help growing. Our study shows that this phenomenon is frequent since we found 363 cases of inhibition among 729 couples of lactic acid bacteria. The frequency of the phenomenon highly varies from members of one genera to the other one. That must be taken into account when lactic acid bacteria are to be used in the different fermentation industries.

Some inhibitions have been well defined: they have been attributed to the production of acidity [29-31] or to the production of hydrogen peroxide [18]. In our study, though we can attribute our results to these two causes in some cases, some other cases remain where the cause of inhibition is different.

Lysogeny has often been recorded [32-34] to explain why fermentation stopped, especially in dairy industry. [35-37] reported that 85% of the *Lactobacillus* strains are lysogenic whereas [38] found only 1% and [39] only 10%; those different results can be explained by the difficulty to bring into fore phages of lactic acid bacteria in a solid medium [13-40] though the spontaneous induction of those phages is observed [41-42]. In our study searching for spontaneously induced phages among 15 strains was eventually negative. Observation with an electronic microscope is often made necessary [13].

Inhibitions can also be triggered off by bacteriocins secreted in the culture medium [11-43]. We noticed that the culture medium of some inhibitory strains inhibits the growing of some other strains. We are now looking forward to clearing up the nature of this (or these) inhibitory agent(s).

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