

Asian Journal of Food and Agro-Industry

ISSN 1906-3040

Available online at www.ajofai.info

Research Article

Plasmid profile of lactic acid bacteria with antifungal properties

Naresh Kumar, Rita Narayanan*, N. Kavitha and B. Dhanalakshmi

Department of Dairy Science, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai -51, Tamil Nadu, India.

*Author to whom correspondence should be addressed, email: ritanarayanan@yahoo.com

Abstract

The present investigation was carried out to determine if metabolites of lactic acid bacteria can be used as a natural preservative in the dairy industry. Four standard lactic cultures *Lactococcus lactis* ssp *lactis* (94), *Lactococcus lactis* var. *diacetylactis* (60), *Lactobacillus acidophilus* (014) and *Lactobacillus acidophilus* (015) were found to have inhibitory activity towards one or two of the mould cultures tested. Among the lactic cultures tested, *Lactobacillus acidophilus* (015) was found to be the most promising culture. Plasmid profile analysis showed *Lactococcus lactis* ssp. *lactis* (94) and *L. lactis* var *diacetylactis* (60) to have a single plasmid of size 26.69kb. *Lactobacillus acidophilus* (014) and *Lactobacillus acidophilus* (015) showed a single plasmid of size 19.91 kb. In order to establish the involvement of a particular plasmid with a specific phenotype in the lactic cultures in vitro curing experiments were conducted by physical and chemical methods. In physical method of curing, the cultures were grown at elevated temperature (45⁰C) and it was found that there was loss of plasmid in all the cultures.

Subsequently the cell free supernatant of the cured cultures did not show inhibitory activity towards the moulds tested. In the chemical method of curing using intercalating dye - ethidium bromide at 4, 6, 8, 10 µg / ml of broth the cell free supernatant of all the cured cultures did not exhibit antimycotic activity. Intergeneric mating by conjugation was tried between *L. lactis* var. *diacteylactis* (60) also showing antibacterial activity and *L. acidophilus* (015) showing antifungal activity using filter mating technique. The results revealed non transfer of plasmid between the two strains suggesting that probably the plasmid responsible for that particular phenotype is not of conjugative nature.

Keywords: dairy, milk, fermentation, mould, lactobacillus, lactococcus, India

Introduction

Lactic acid bacteria constitute an important group of organisms which are extensively used in the preparation of fermented milk products. Although they are recognized for their health and nutritional benefits, these industrially important organisms can also be used for the preservation of dairy products. These organisms by virtue have the ability to bring about transformation of milk constituents into a variety of useful and desirable metabolites. Production of primary metabolite viz. lactic acid and the resultant decrease in pH is the main factor in the preservation of foods. In addition, some strains may contribute to food preservation by producing inhibitory substances such as antifungal substances and bacteriocin, which are of protein moiety that have inhibitory activity towards food spoilage moulds and bacteria.

Moulds found in milk and its various derivatives are of increasingly great concern to investigators involved in microbiological analysis of dairy products. They are able to grow and multiply at reduced water activity levels, reduced pH and wide range of temperatures. They not only cause off flavour and discolouration, but elaborate toxic metabolites and mycotoxins which are of great concern from public health point of view.

The possible use of lactic acid bacteria producing broad spectrum antifungal substance as a bio preservative is becoming increasingly interesting as many of them are able to inhibit the growth of food contaminating moulds and bacteria. Hence screening of lactic cultures producing antifungal substance that exhibit a broad spectrum of activity is one approach in finding functional strains of value as dairy adjuncts. The present work was researched with an attempt to study if the antifungal property of the lactic acid bacteria was on plasmids.

Materials and Methods

Samples comprised of milk, dahi, silage, fruit and yoghurt were collected aseptically from households and restaurants and brought to the laboratory in sterile containers containing ice and stored at 5°C until further use. The standard cultures of *Lactobacillus acidophilus* (014), *Lactococcus lactis* ssp. *lactis* (94), *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis* var. *diacetylactis* (60) and *Lactobacillus acidophilus* (015) were procured from the National Collection of Dairy Cultures, NDRI, Karnal, India. The mould cultures of *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillium citrinum*, *Mucor*, *Rhizopus* were obtained from Dairy Science College, Hebbal, Bangalore.

Assay of antifungal activity

The antifungal activity of the isolated and standard lactic cultures were tested against *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillium citrinum* *Mucor* and *Rhizopus* species by agar well diffusion method outlined by Batish *et al* [1].

Mould spore suspension was prepared by the method outlined by Batish *et al* [1].

The cell free supernatant was tested for antifungal activity as per the procedure outlined by Batish *et al* [1].

Qiagen plasmid mini kit was used for the plasmid DNA isolation.

In order to establish the involvement of a particular plasmid with a specific phenotype, in vitro curing experiments using physical method viz. elevated temperature and chemical method of curing using intercalating dyes such as ethidium bromide were employed.

Physical method

Actively growing (18 hours old) cultures were inoculated into Elliker's broth at the rate of 1% and incubated at elevated temperature (45⁰C).

Chemical method

Ethidium bromide was tried for curing after making a stock solution of 2 mg/ml. Aliquots of 5 ml of Elliker's broth tubes were inoculated with 18 hour old cultures at the rate of 1%. Ethidium bromide was added at the rate of 4, 6, 8 and 10 µl of broth. The tubes were incubated for 24 hours at 37⁰C.

Plasmid DNA

The cured cultures were subjected to plasmid DNA isolation as well as agar well assay method for antifungal properties and to determine if these properties are plasmid mediated.

Conjugation

Filter mating technique according to Thompson *et al* [2], was adapted for conjugation.

Results and Discussion

In the present study *Lactococcus lactis* ssp *lactis*, *Lactococcus lactis* ssp *cremoris*, *Lactococcus lactis* var. *diacetylactis*, *Lactobacillus delbrueckii* ssp *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Lactobacillus casei* were isolated from samples of dahi, milk, yoghurt, fruit and silage but they did not show any antifungal properties when tested. In case of standard cultures, all the four cultures *Lactococcus lactis* ssp *lactis* (94), *Lactococcus lactis* var. *diacetylactis* (60), *Lactobacillus acidophilus* (014) and *Lactobacillus acidophilus* (015) were found to have inhibitory activity towards one or two of the mould cultures tested.

Lactococcus lactis ssp *lactis* (94) inhibited *Aspergillus flavus* and *Penicillium citrinum* (Fig. 1). *Lactococcus lactis* var. *diacetylactis* (60) inhibited *Aspergillus fumigatus* and *A.flavus* (Table 1).

Similar results were obtained for *A. flavus* by Reddy and Ranganathan [3], who had tested with cell free supernatant of *Lactococcus lactis* var. *diacetylactis* strain (S₁- 67/C). *Lactobacillus acidophilus* (015) showed inhibition against *Aspergillus flavus* and *Penicillium citrinum* *Lactobacillus acidophilus* (014) was found to be inhibitory against *Aspergillus flavus*. These results are in close agreement with the findings of earlier work [1, 4, 5].

Fig 1
Agar well assay method-
ANTIFUNGAL ACTIVITY OF *L. LACTIS* SSP. *LACTIS* AGAINST

- ***ASPERGILLUS FLAVUS*** • ***PENICILLIUM CITRINUM***

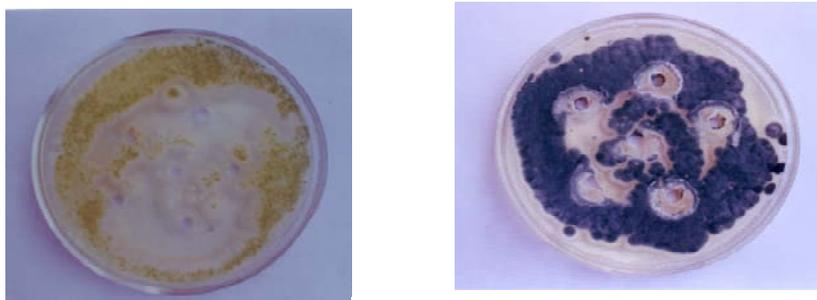


Figure 1. Antifungal activity of *L. lactis* spp. *lactis* against (A) *A. flavus* and (B) *P. citrinum* using the agar well assay method.

Table 1. Various lactic cultures showing their antifungal activity.

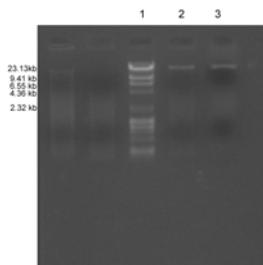
Lactic cultures	Fungal cultures					
	Diameter of zone of inhibition (mm)			AFU/ml of Cell Free Supernatant(CFS)		
	<i>A.flavus</i>	<i>A. fumigatus</i>	<i>P.citrinum</i>	<i>A. flavus</i>	<i>A.fumigatus</i>	<i>P.citrinum</i>
<i>Lactococcus lactis</i> ssp <i>lactis</i> (94)	11.80	-	10.34	9.44		8.27
<i>Lactococcus lactis</i> var. <i>diacetylactis</i> (60)	-	12.81	-		10.25	
<i>Lactobacillus acidophilus</i> (014)	10.30	-	-	8.24		
<i>Lactobacillus acidophilus</i> (015)	12.86		12.12	10.29		9.70

Plasmid analysis

The plasmid DNA profiles of the culture *Lactococcus lactis* ssp. *lactis* (94), *L. acidophilus* strains (014), (015) and *Lactococcus lactis*, var *diacetylactis* (60) demonstrated by agarose gel electrophoresis can be seen in Fig. 2.

Fig.2
Plasmid profile Analysis

Plasmid profile showed, *L.lactis* ssp.*lactis* and *L. lactis* var *diacetylactis* to have a plasmid of size 26.69kb



Agarose Gel Electro Phoresis Showing Plasmid Profile of :

- 2. *Lactococcus lactis* var *diacetylactis*
- 3. *Lactococcus lactis* ssp. *lactis*

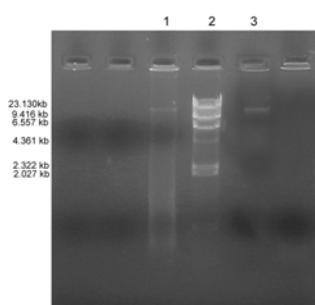
1.Lambda DNA- Hind III/φ X-Hae III

Figure 2. Plasmid profile analysis.

Electrophoresis of plasmid DNA on agarose gel showed that the cultures harboured one plasmid each. Plasmid profile analysis showed *Lactococcus lactis* ssp.*lactis* (94) and *L. lactis* var *diacetylactis* (60) to have a single plasmid of size 26.69kb. *Lactobacillus acidophilus* (014) and *Lactobacillus acidophilus* (015) showed a single plasmid of size 19.91 kb (Fig. 3). The number of plasmids and molecular sizes obtained in this study was within the range as described by Wang and Lee [6].

Fig-3

L.acidophilus (14) & (15) had a plasmid of size 19.91kb



Agarose Gel Electro Phoresis Showing Plasmid Profile of :

- 1. *Lactobacillus acidophilus* (14)
- 3. *Lactobacillus acidophilus* (15)

2.Lambda DNA- Hind III

Figure 3. Plasmid profile for *Lactobacillus*.

Curing studies

Physical method: elevated temperature

For the physical method of curing, the cultures were grown at elevated temperature (45⁰C) and it was found that there was loss of plasmid in all the cultures. The CFS of the cured lactic cultures was also tested for antifungal activity. All the cultures did not show any zone of inhibition towards the moulds tested. This is in accordance with the work of Fortina and Silva [7], who found that elevated temperature resulted in curing a 14.3 kb plasmid in *Lactobacillus helveticus* strain ILC 54.

Chemical method: ethidium bromide

It was found that the cultures behaved differently when treated with intercalating dye, ethidium bromide, at concentrations of 4, 6, 8 and 10 µg/ml dye - ethidium bromide and did not show any plasmids at 8 and 10µg/ml. Subsequently the CFS of *L. acidophilus* (014) was tested for antifungal activity. It was noticed that the CFS of the culture treated with 4, 6 µg/ml of ethidium bromide showed zone of inhibition against the respective molds. However, the 8 and 10 µg/ml treated broth samples which had lost the plasmid failed to show any zone of inhibition against moulds. Similarly, *Lactobacillus acidophilus* (015) when treated to the same concentration levels of ethidium bromide did not show plasmid. *Lactococcus lactis* ssp. *lactis* (60) showed plasmid in the presence of 4, 6 and 8µg/ml, but did not show any plasmid at 10µg/ml. The antifungal activity also was similarly correlated. *L. lactis* var *diacetylactis* (94) showed presence of plasmid in all concentrations and presence of zone of inhibition.

All the strains except *Lactococcus lactis* ssp *lactis* (94) showed loss of plasmid when cured with ethidium bromide at concentrations of 8 and 10µg/ml of broth. *Lactococcus lactis* ssp *lactis* (94) showed loss of plasmid at concentration at 10 µg/ml of broth. Cell free supernatant of all the cured cultures did not exhibit antimycotic activity. The results obtained are in agreement with those of Morelli *et al* [8], as they reported that ethidium bromide is effective in curing *Lactobacillus delbrueckii* ssp *bulgaricus* strains. Similar observations were also noted by Sharma *et al* [9].

Conjugation

Intergeneric mating by conjugation was tried between *L. lactis* var. *diacetylactis* (60) which also possessed antibacterial activity and *L. acidophilus* (015) showing antifungal activity using filter mating technique. Several trials were carried out and the resultant strain after conjugation was not showing either antibacterial or antifungal activity. The results revealed non transfer of plasmid between the two strains. Failure of the conjugation experiment between the two lactic cultures may be due to the reason that the plasmid responsible for that particular phenotype is not of conjugative nature. Non- conjugative plasmids are not characterized by the ability to promote sexual conjugation between the two bacterial cells.

Since there is every possibility for plasmid involved in the antifungal activity to be of non-conjugative type, conjugation between *L. acidophilus* (015) and *L. lactis* var *diacetylactis* (60) by filter mating technique was not effective.

Conclusion

From the foregoing discussion, it is evident that the economically important characters of dairy lactic acid bacteria, namely antifungal properties, are plasmid encoded. The use of these plasmids in genetic engineering techniques for the dairy industry will go a long way towards constructing a strain with all desirable characters. Among the lactic cultures tested *Lactobacillus acidophilus* (015) was found to have the highest potential. Hence there is no doubt that this particular strain in future will find a place in the bio preservation of dairy products.

References

1. Batish, V.K., S. Grover and R. Lal (1989). Screening lactic starter cultures for antifungal activity. **Cultured Dairy Products Journal**, 21-25.
2. Thompson, J.K., K.J. McConville, C. McReynolds and M.S. Collins (1999). Potential of conjugal transfer as a strategy for the induction of recombinant genetic material into strains of *Lactobacillus helveticus*. **Applied Environmental Microbiology**, 65: 1910-14.
3. Reddy, N.S. and B. Ranganathan (1985). Preliminary studies on antimicrobial activity of *Streptococcus lactis* subsp. *diacetylactis*. **Journal of Food Protection**, 47:222-225.
4. Karunaratne, A., E. Wezenberg and L.B. Bullerman (1990). Inhibition of mold growth and aflatoxin production by *Lactobacillus* spp. **Journal of Food Protection**, 53(3): 230-236.
5. Gourama, H. and L.B. Bullerman (1995). Inhibition of growth and aflatoxin production of *Aspergillus flavus* by *Lactobacillus* species. **Journal of Food Protection**, 58:1249-1256.
6. Wang, T.T. and B.H. Lee (1997). Plasmids in *Lactobacillus*. **Critical Reviews in Biotechnology**, 17: 227-272.
7. Fortina, M.G. and M. Silva (1996). Mapping of two plasmids from *Lactobacillus helveticus* ILC54, plasmid curing and preliminary studies on their involvement in lactose metabolism and peptidase activity. **Biotechnology Letters**, 18: 1003-06.
8. Morelli, L., M. Vescovo and V. Bottazzi (1983). Plasmids and antibiotic resistance in *Lactobacillus helveticus* and *Lactobacillus bulgaricus* isolated from natural whey culture. **Microbiologica**, 6: 145-154.
9. Sharma, V., V.K. Batish and S. Grover (1992). Evidence of plasmid linked phenotypic characters in two wild *Lactobacillus casei* strains. **Microbiologie-Ailments-Nutrition**, 10: 167-175. Cited from Medline.