SUSCEPTIBILITY OF *Enterococcus faecalis* AND *Lactobacillus casei* STRAINS ISOLATED FROM PALMITA-TYPE VENEZUELAN CHEESE TO BACTERIOPHAGES

Susceptibilidad a bacteriófagos de cepas de *Enterococcus faecalis* y *Lactobacillus casei* aisladas de queso tipo Palmita venezolano

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ABSTRACT

Ten fresh cheese whey samples from local plants were analyzed to detect bacteriophages and evaluate the phage effects on the titratable acidity production in 10% sterile skim milk of eleven *Enterococcus faecalis* and seven *Lactobacillus casei* strains, which can be used as starters for the manufacture of Palmita-type Venezuelan cheese. The results showed that four whey samples were positive for phages, with lytic activity demonstrated by the presence of plaques from 0.2 to 0.3 mm in diameter on both M17 and MRS agar plates. The fermentative activity tests showed that 91% of the cultures with *E. faecalis* strains and 57% with *L. casei* strains were resistant to the isolated phages. Variations were observed between species as well as between strains of the same species. Such variability suggests the use of strains resistant to bacteriophages in order to guarantee the cheese quality.

Key word: Bacteriophage, *Enterococcus, Lactobacillus*, milk, Palmita-type cheese.

RESUMEN

Diez muestras de suero de queso fresco provenientes de plantas queseras de la región, fueron analizadas en su contenido de bacteriófagos. Las muestras de suero a su vez, fueron utilizadas para evaluar el efecto de fagos sobre la actividad fermentativa medida en producción de acidez titulable de once cepas de *Enterococcus faecalis* y siete cepas de *Lactobacillus casei* en leche descremada estéril al 10%, aisladas del queso tipo Palmita Venezolano. Los resultados mostraron que cuatro de las diez muestras de suero resultaron positivas en bacteriófagos, con actividad lítica evidenciada por la presencia de calvas de 0,2 a 0,3 mm de diámetro, en placas con medio M17 y MRS. Como resultado de las pruebas de actividad fermentativa, se obtuvo que el 91% de los cultivos con cepas de *E. faecalis* resultaron resistentes a los fagos aislados, mientras que un 57% de resistencia se observó en los cultivos con cepas de *L. casei*. Hubo variaciones en cuanto a resistencia tanto de especies como de cepas de la misma especie. Dicha variabilidad sugiere el uso de cepas resistentes, capaces de mantener los procesos fermentativos en cultivos iniciadores, aún en presencia de agentes inhibidores, como los bacteriófagos.

Palabras clave: Bacteriofagos, *Enterococcus*, *Lactobacillus*, leche, queso tipo Palmita.

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INTRODUCTION

The formation of lactic acid by starter bacteria has an utmost importance in fermented dairy products manufacture [5, 11]. Bacteriophage attack is a factor most often encountered as responsible for starter activity failure [5, 10, 26, 30], which causes an acidity development reduction and ultimately a complete starter growth inhibition. This situation is very common in the cheese industry [41]. As a consequence, strategies such as the use of starters resistant to phage attack are now used in the industry [25], and currently, lactic acid cultures characterization and selection include bacteriophage resistance tests [12, 13, 21, 28].

Enterococci and lactobacilli play a major role in the manufacture of several dairy products, mainly cheese, in which they are responsible for the acid and flavor development [5, 6, 17, 18, 32]. The attack of these microorganisms by

bacteriophages has a great economical impact on the dairy industry [19, 21, 30, 38]. Lodics and Steenson [26], and Bhimani and Freitas [5] have pointed out the lactic acid bacteria susceptibility to phages isolated from butter, curd, cheese and cheese whey, getting low acid formation from cultures in skim milk. This information has been useful to select resistant strains as starters.

Enterococcus faecalis and *Lactobacillus casei* strains have been found as responsible for the typical mild acid flavor of the commercial Palmita-type Venezuelan cheese [6, 17] and are currently being tested as starters for the elaboration of such cheese with pasteurized milk [6, 29]. This work evaluates the resistance of the *Enterococcus faecalis* and *Lactobacillus casei* strains, isolated from Venezuelan Palmita-type cheese, to bacteriophages from local cheese plants in order to choose resistant strains that might be used as components of multiple starter cultures to produce Palmita-type cheese from pasteurized milk.

MATERIALS AND METHODS

Bacterial Strains

Enterococci and lactobacilli strains used in this study are members of starter cultures [16] used in the elaboration of pasteurized Palmita-type cheese on a pilot plant [6, 29]. The bacteria were isolated from commercial, non-pasteurized Palmita-type cheese [16] and belong to the culture collection of the University of Zulia Science Faculty Food Laboratory, Maracaibo, Venezuela. TABLE I shows the strains used in this study.

Maintenance

Cultures were maintained by weekly transfers of 2% inoculum into M17 broth [38] and 10% reconstituted skim milk (RSM, sterilized at 112°C during 12 min) for enterococci and into MRS broth (Merck, Rahjay, NJ) and 10% reconstituted skim milk for lactobacilli. Cultures incubation was performed at 35°C during 18 h, followed by storage at 4°C, with monthly transfers. Bacterial strains used as host indicators, TABLE II, were grown and maintained on M17 (streptococci), MRS (lactobacilli) and BHI (coliforms) broths.

Enrichment and Isolation of Phages

Samples. Ten fresh cheese whey samples from local plants were analyzed for the presence of phages.

Pretreatment of whey. Whey samples were pretreated and enriched as described by Bhimani and Freitas [5]. Samples were centrifuged at 4,000 x g for 10 min in a refrigerated Sorvall centrifuge. The supernatant was adjusted to pH 6.5 and passed through a 0.22 µm filter (Millipore Corp., Bedford, MA).

Enrichment. Filtered supernatants were inoculated (5%) into young host cultures (4-6 h), incubated at 35°C for 18 h and centrifuged at 4,000x g for 10 min. The supernatant was passed through a 0.22 µm filter and kept at 4°C until use.

Phage detection. Filtrates were tested for the presence of phages as described by Terzaghi and Sandine [38]. Young sensitive hosts were used. Lytic activity was determined on the filtrates positive to phages [9].

Phage purification. Phages were purified as described by Accolas and Spillmann [1, 2] on MRS and M17 agar plates. Samples from well-isolated plaques were transferred to test tubes containing 10 mL of MRS, M17 or BHI broth (according to the species) supplemented with calcium (0.1 mL of sterile 0.2 M $CaCl_2$). The broths were inoculated with sensitive bacterial hosts and incubated at 30°C for 3 h for phage replication.

TABLE I
BACTERIAL STRAINS USED IN THIS STUDY

Strains	Sources
Enterococcus faecalis E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, Ef11	L. Cabrera, University of Zulia, Maracaibo, Venezuela
Lactobacillus casei L1, L2, L3, L4, L5, L6, L7	D. Raffe and L. Cabrera, University of Zulia, Maracaibo, Venezuela

TABLE II
HOST INDICATOR STRAINS USED

Strains	Susceptibility to Bacteriophages	Sources
Lactococcus lactis	-	Chr. Hansen's Laboratorium A/S
Streptococcus thermophilus	+	Chr. Hansen's Laboratorium A/S
Lactobacillus bulgaricus	+	Chr. Hansen's Laboratorium A/S
Lactobacillus helveticus	-	Chr. Hansen's Laboratorium A/S
Lactobacillus casei LC 12	-	Y. Basanta. University of Zulia, Maracaibo, Venezuela
Lactobacillus casei LC 5	+	Y. Basanta. University of Zulia, Maracaibo, Venezuela
Escherichia coli Cp19	-	G. Colina. University of Zulia, Maracaibo, Venezuela
Enterobacter aerogenes Ea2, Ea6	-	L. Cabrera and D. Raffe. University of Zulia, Maracaibo, Venezuela

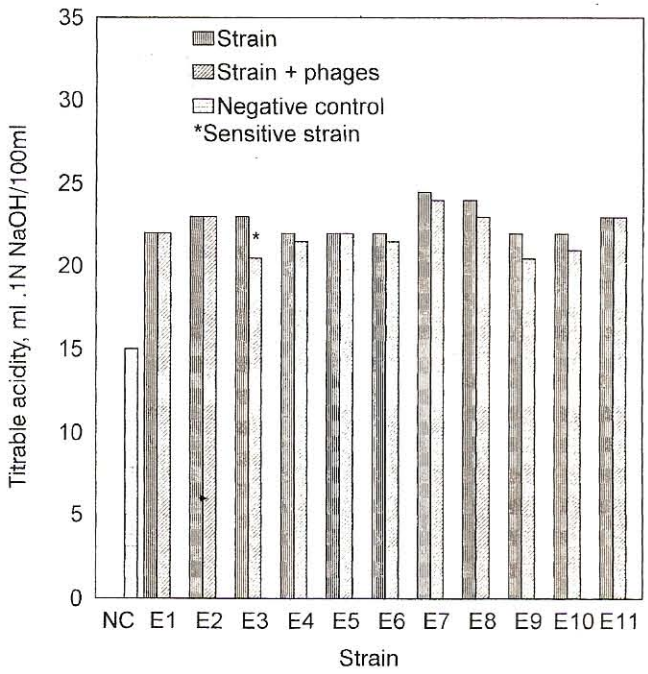

FIGURE 1. THE BACTERIOPHAGES EFFECTS ON THE FERMENTATIVE ACTIVITY OF *E. FAECALIS* SINGLE CULTURES.

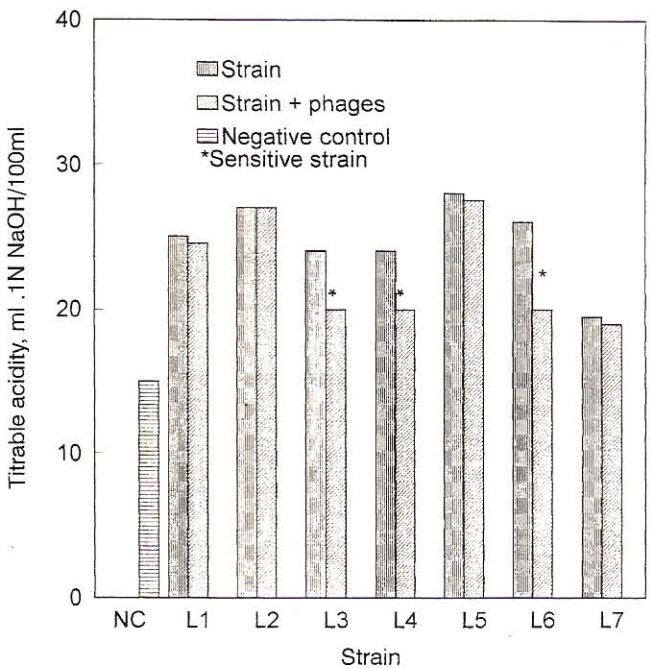

FIGURE 2. THE BACTERIOPHAGES EFFECTS ON THE FERMENTATIVE ACTIVITY OF *L. CASEI* SINGLE CULTURES.

These cultures were then centrifuged and filtered in order to obtain phage solutions.

Maintenance of phage stocks

Phage stock solutions were kept at 4 and - 20°C on both M17 and MRS broths. A pool was made with 1 mL each of the phage solutions diluted to 5 mL.

Effect of phages on the fermentative activity of starter cultures

One milliliter of enterococci and lactobacilli strains single culture was inoculated in two separate 250 mL flasks containing 100 mL of sterile RSM. One milliliter of the phage pool solution was added to one flask; 1 mL of diluent (M17 or MRS) was added to the other flask (control). The flasks were incubated at 35°C for 6 h under aerobic conditions in a water bath. Later, the cultures were tested for the development of titratable acidity. A titratable acidity value 10% lower than the value of the control was considered presumptive for inhibition of the fermentative activity of the culture by phages [20]. The experiments were done in duplicate.

Titratable acidity

The titratable acidity was determined (in duplicate) by titration of 10 mL of culture in RSM with 0.1*N* NaOH and 1 mL 0.5% phenolphthalein indicator. The result was reported as mL of 0.1*N* NaOH per 100 mL of culture [20].

RESULTS

Isolation of phages

Four of the ten whey samples obtained from cheese plants were positive for phages. All the phages formed clear, circular plaques, 1.5 to 2 mm in diameter around the wells on M17 and MRS agar plates. The phage titers obtained after the enrichment step were less than 10^4 pfu/mL. Phages were more specific against *Lactobacillus* and *Streptococcus* strains.

Effect of phages on fermentative activity of cultures

The bacteriophages effects on the fermentative activity of *E. faecalis* single cultures is shown in FIG.1. Most cultures containing phages had less acid production compared to the control. However, only E3 was sensitive according to the criterion established by Harrigan and McCance [20]. On the other hand, four cultures (E1, E2, E5, E11) were not affected by the presence of phages.

The bacteriophages effects on the fermentative activity of *L. casei* single cultures are presented in FIG. 2. Three strains of *L. casei*, L3, L4, L6 were sensitive to phage activity.

As it can be seen in FIGS. 1 and 2, the bacteriophages effects on the culture fermentative activities were less pronounced for *E. faecalis* than for *L. casei* strains.

DISCUSSION

The presence of phages in the cheese industry environment, reported in various studies [1, 36, 39, 40], was confirmed in this work since 40% of the whey samples had bacteriophages specific to lactic acid bacteria currently used as start-

ers in the manufacture of dairy products [1, 2, 4, 6, 22]. Nowadays, the lytic activity of phages on indicator bacteria grown in agar plates is commonly used for the detection of sensitive strains [26]. In this work, the size of the inhibition plaques was within the size reported in the literature for lactobacilli [1] and enterococci [24]. The plaques were clear, indicating the presence of virulent phages as suggested by Séchaud *et al.* [36]. With respect to the media used, Terzaghi and Sandini [38] have reported the M17 medium as favorable for the growth of host bacteria due to the presence of β-disodium glycerophosphate, and Bhimani and Freitas [5] has indicated that the buffer capacity of this medium favors the growth of the bacteria. On the other hand, Accolas and Spillmann [1] and Séchaud *et al.* [36] obtained good isolation, multiplication and purification of bacteriophages using MRS medium. In this work, M17 and MRS media were appropriate for the visualization of the plaques.

A total of 91% of the *E. faecalis* strains were resistant to phages, FIG. 1. Little information is available about the resistance of enterococci to bacteriophages. Nevertheless, it has been reported that lactic acid bacteria when used as starters even in the presence of bacteriophages, maintain the homogeneity of the products [22, 23, 31]. The resistance of enterococci to phages might be related to the resistance that enterococci have against adverse pH and temperature conditions [22]. *E. faecalis* has been reported as an essential constituent of starters for the manufacture of yogurt [14, 15], fermented products from India [21], and cheeses such as Palmita [6, 7, 8], Manchego [32] and Cheddar [23], based on its ability for producing acid and flavor related compounds [15]. Therefore, any delay in the onset or a complete inhibition of acid development will cause undesirable consequences in the quality of the products. According to Allison and Klaenhamer [3], it is remarkable in lactococci and *Streptococcus thermophilus* some mechanism of natural resistance either to the interference in phages absorption or to the phages inhibition of the DNA injection.

Only 57% of the *L casei* strains were resistant to phages, FIG. 2, showing higher susceptibility to bacteriophages than the enterococci, which was suggested by Lawrence [25]. The effect of a great reduction in the acid production might be due to higher specificity of the phages for lactobacilli strains. *L. casei* strains have been reported to be sensitive to bacteriophage attack and lose their ability to produce acid [1, 33, 34, 37]. In this study, similar results were observed with bacteriophages isolated from cheese plants. Variability in the resistance to phages between the lactobacilli strains was found. It has been suggested that phage resistance varies within strains [26] and that it might be due to changes in the ability of the bacterial cell to absorb phages [27]. Such variability has been observed in lactic streptococci [11, 35]. *L. casei* is a common microorganism in cheese and is currently used as a constituent of a starter for the manufacture of Palmita-type cheese [6] in which it is largely responsible for acid and flavor development.

Multiple starters have been used in the industry to minimize failures of the acid development in cheese. The substitution of sensitive strains to phages for resistant strains has also been useful [21, 25] since phage contamination is unavoidable in a milk vat [25]. The strains resistant to phages found in this work will be used as members of a multiple starter for the manufacture of Palmita-type cheese from pasteurized milk to guarantee the fermentative activity of the cultures if phages are present.

CONCLUSION

The lactic acid bacteria studied in this paper were affected in their fermentative metabolism due to the presence of bacteriophages. The strains of *L. casei* (43%) were more susceptible than the strains of *E. faecalis* (9%).

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