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Partition of *Lactococcus lactis* bacteriophage during the concentration of micellar casein by tangential 0.1 μm pore size microfiltration

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Summary — Retention behaviour of two lactococcal phages added to raw milk submitted to cross flow microfiltration with a 0.1 μm pore size membrane was studied. The microfiltrate, 'milk without casein', contains 0.4–0.14% of the phage particles added to the milk whatever the phage type and the level of inoculation. Such a retention cannot be explained by electrostatic interactions between phage particle and casein micelle or microfiltration membrane but is more probably due to a reduction of membrane pore size resulting from a concentration polarization phenomenon.

phage / microfiltration / milk / *Lactococcus* / virus

Résumé — Rétention de bactériophages de *Lactococcus lactis* au cours de la concentration de micelles de caséine par microfiltration tangentielle. *La rétention de 2 bactériophages de lactocoques, ajoutés à 2 niveaux différents d'inoculation à du lait cru, par une membrane de microfiltration ayant un diamètre moyen de pores de 0,1 μm a été étudiée. Le microfiltrat obtenu «lait dépourvu de caséine», contient entre 0,4% et 0,14% des particules phagiques ajoutées au lait, quels que soient la morphologie du phage et le niveau d'inoculation. Cette rétention ne peut être expliquée par des interactions électrostatiques entre les particules phagiques d'une part, la membrane ou son support, ou encore les micelles de caséine d'autre part. Elle résulte probablement d'une diminution du diamètre apparent des pores de la membrane de microfiltration, liée au phénomène de polarisation-concentration.*

bactériophage / microfiltration / lait /* Lactococcus *virus

INTRODUCTION

Separation of phage particles after propagation on their homologous hosts with adequate culture broth medium is usually carried out by means of dead-end microfiltration (MF) with 0.45 μm pore diameter membranes (Whitman and Marshall, 1969; Hull, 1977; Accolas and Chopin, 1980) but use of 0.2 μm pore diameter membranes does not affect phage permeation (Chopin, personal communication). On the other hand, it was demonstrated by Whitman and Marshall (1969) and Erskine (1970) that phage particle titration was affected by the pH value of milk. These authors have hypothesized an electrochemical interaction between phage particles and casein micelles.

Cross flow membrane microfiltration (CFMF) with the carrying out of a 0.1 μm pore size membrane was recently proposed for separating native micellar casein from skimmilk (Fauquant *et al*, 1988; Pierre *et al*, 1992). The proposed technology is currently scaled-up for both casein enrichment of cheese milk and the production of a new dairy product: native micellar casein which shows interesting functional properties (Maubois, 1991; Pierre *et al*, 1992).

The microfiltrate resulting from this separation technique contains no residual fat, no casein fragments, and is crystal clear in spite of the fact that it holds most of the whey proteins. Its bacterial count is very low, close to the sterility. It is the starting material for both preparation of high purity whey protein concentrates and extraction of highly valuable milk proteins such as α-lactalbumin, β-lactoglobulin, lactoferrin, lactoperoxidase, etc, which are used as nutritional ingredients in the dietetics and the infant food industries. Such an utilization evidently requires knowledge of a possible viral contamination.

This paper reports our studies on retention of two morphologically different bacteriophages of *Lactococcus lactis* added at various levels to raw skimmilk which was then cross flow microfiltered with a 0.1 μm pore diameter membrane.

MATERIALS AND METHODS

Bacteriophages

Phage 42 and 119 were chosen from our lab collection. Phage 119 is an isometric headed phage with a 52 nm head size and a 170 nm tail length; phage 42 is prolate with a 32 x 50 nm head size and a 86 nm tail length (Chopin and Rousseau, personal communication). Both give clear plaques. They belong to different lytic groups (Chopin *et al*, 1976).

Phage suspensions were prepared as described by Terzaghi and Sandine (1975) using *L lactis* IL737 for phage 119 and *L lactis* IL414 for phage 42. Suspensions containing 10^{10} pfu/ml were obtained after filtration through 0.45 μm pore size Millipore (St Quentin-en-Yvelines, France) membrane.

Phage enumerations in skimmilk, MF retentates and microfiltrates were made according to Terzaghi and Sandine (1975). All samples were 10-fold diluted with Ringer's solution and then filtrated through a 0.45 μm pore size Millipore (St Quentin-en-Yvelines, France) membrane.

The absence of sensitive hosts for phage 42 and 119 in the experimental milks was verified in order to avoid phage multiplication. The absence of phages active against host strains of phages 42 and 119 was also checked.

Mesophilic total counts in milk, retentates and microfiltrates were determined on plate count agar (Difco) at 30°C for 72 h.

Microfiltration experiments

CFMF equipment utilized was the MFS 1 Alfa Laval containing 1P19 membrane cartridge (SCT, Tarbes, France) previously described by Madec *et al* (1992). The milk used was industrial bulk raw milk, separated at 45°C, cooled and stored overnight at 4°C.

500 l were first treated at 47°C by CFMF with a 1.4 µm pore diameter membrane in order to reduce the contaminant flora as previously described by Trouvé *et al* (1991). The resulting milk (total bacterial count lower than 1000 cfu/ml) was cooled at 37°C and divided in two portions of 90 l. One aliquot was inoculated at 10^3 pfu/ml of phages 42 and 119 and the other at 10^6 pfu/ml with the same phages. Microfiltrations of both milks containing phages were successively performed at 37°C in the MFS 1 rig equipped with a 0.1 µm pore diameter Membralox cartridge. Extraction fluxes were maintained at 70 l. h^{-1} m^{-2} for the microfiltrate and the retentate, that allows a volume concentration of the MF retentate by 2. Microfiltrate and retentate were continuously recycled. Duration of each assay was 40 min.

50 ml samples were aseptically taken out through septa inserted in the feed and the two recirculation loops, after 3 min and then every 10 min. All samples were immediately cooled to 4°C and enumerated the same day as described.

After each assay, milks containing phages and rinsing solutions were heated at 90°C for 20 min to inactivate phages. MF equipment and all phage containing vessels were treated with a 1000 ppm hypochlorite solution at 25°C for 30 min.

RESULTS AND DISCUSSION

Table I shows average phage titrations and total count enumerations observed in the milks, retentates and microfiltrates; each value is the logarithmic mean of 6 enumerations. Table II indicates the calculated yields (Y) and decimal reductions (DR). Y and DR values were obtained as described by Trouvé *et al* (1991).

It appears that recoveries of phage particles from retentates and microfiltrates when compared to initial counts of the corresponding milks are much better than what is observed for the bacterial cells. Average Y value is 1.08 for phage counts against 0.77 for total bacterial counts (this study) and 0.62 (Trouvé *et al*, 1991). Such results suggest that either phage count is more accurate than bacterial count or that phage particles have much less interactions with MF membrane components.

High phage retention was observed with the 0.1 µm membrane for both phages 42 and 119. The percentage of retention was 99.6–99.86% and appears independent of the initial phage level. Such a result cannot be explained through a screen effect as in the case of UF membranes (Konowalchuck and Speirs, 1973; Nupen *et al*, 1980; Mistry and Kosikowski, 1986; Zottola *et al*, 1987). The pore size of the MF membrane is twice as large as the head size of the tested phages. Several hypotheses can be envisaged for explaining the observed phage retention.

The first one is an electrochemical interaction between the phage particles and the

Table I. Phage particle and bacterial enumerations in log.
Dénombrement (log) des bactériophages et des bactéries.

	Feed milk			*Retentate*			*Microfiltrate*		
Phage level	*Phage 42*	*Phage 119*	*Total count*	*Phage 42*	*Phage 119*	*Total count*	*Phage 42*	*Phage 119*	*Total count*
0.1 µm membrane assays	2.63	3.31	2.96	2.95	3.56	3.11	0.12	0.45	0.09
	5.80	6.14	2.85	6.11	6.40	3.07	3.38	3.62	0.00

Table II. Yield and decimal reductions.
Rendement et réductions décimales.

	Phage 42		*Phage 119*		*Total count*	
Phage level	10^3	10^6	10^3	10^6	10^3	10^6
Yield	1.05	1.35	1.02	0.91	0.71	0.83
DR	2.51	2.86	2.42	2.53	2.87	2.85

Yield, Retentate enumeration + Microfiltrate enumeration)/2 x milk enumeration. DR, decimal reduction = log milk enumeration – log microfiltrate enumeration.
Yield: Dénombrement rétentat + dénombrement microfiltrat)/2 x lait dénombrement. DR: Réduction décimale = log dénombrement lait – log dénombrement microfiltrat.

casein micelles as proposed by Whitman and Marshall (1969) and Erskine (1970). At the normal pH of milk (6.7), casein micelles are negatively charged whereas phage coat proteins would all be positively charged. Consequently, retention of casein micelles by MF membrane would also allow retention of the phages complexed by the casein micelles. Several different assays in duplicate were carried out for checking this hypothesis. Aliquots of 100 ml reconstituted low bacteria low heat skimmilk powder (Schuck *et al*, 1994) inoculated with 10^4 pfu/ml of phage 42 and 10^5 pfu/ml of phage 119 were enumerated by the double layer method of Terzaghi and Sandine (1975): a) directly; b) after a 1:10 dilution in Ringer's solution; c) after dead-end filtration with 0.45 µm pore size membrane followed by either a 1:10 dilution in reconstituted milk or a 1:10 dilution in Ringer's solution; and d) after a 1:10 in Ringer's solution followed by successively a dead-end filtration with 0.45 µm membrane and a 1:10 dilution in Ringer's solution. Phage titrations were determined immediately after phage inoculations to milk and after 1 h of incubation at 30°C. All enumerations were the same, *ie* 10^4 pfu/ml for phage 42 and 10^5 pfu/ml for phage 119. It can be concluded that: i) there is no electrochemical adsorption of both phages 42 and 119 with casein micelles at normal milk pH; and ii) there is also no adsorption of both phages with the 0.45 µm MF membrane.

Another hypothesis which comes to mind for explaining phage retention by the 0.1 µm pore size membrane is a membrane pore size reduction due to the development of the concentration polarization layer at the beginning of MF treatment; such a pore size reduction would exist for the majority of membrane pores but not for the larger one's which would allow a small proportion of phage particles to go through the MF membrane as it was hypothesized for bacterial cells partition in Bactocatch process (Marshall *et al*, 1993).

Other hypotheses related to either possible electrostatic repulsions between MF membrane and phage particles or possible aggregation of phage particles together which would increase their apparent volume in milk do not agree with the observed small and constant permeation rate of phage particles in microfiltrate.

Taking into account the phage content of raw milk is low, 11 to 195 pfu/ml according to Tsaneva (1973) apart from accidental contamination (utilization of a not carefully disinfected tank), it can be concluded that

dairy products manufactured from the microfiltrate obtained by the treatment of milk with a 0.1 μm MF membrane will have a very low phage content. They will be almost phage free. Further work is evidently needed to determine if the results observed with lactococcal phage can be extrapolated to other types of phages or virus. Nevertheless, purified whey proteins made from 0.1 μm milk microfiltrate appear to offer a higher degree of hygienic safety to the end users than the same products made from cheese or casein wheys.

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