Journal of Virological Methods 168 (2010) 103–107

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocols

Impact of milk components in recovery of the MS2 bacteriophage as an indicator of enteric viruses

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Article history:
Received 15 October 2009
Received in revised form 21 April 2010
Accepted 27 April 2010
Available online 4 May 2010

Keywords:
Viruses
Raw milk
Milk components
Recovery efficiency

A B S T R A C T

The objective of this study was to characterize the role of milk components in the recovery of viral particles from raw milk. For such characterization, four model milk formulations (A–D) were constituted by mixing different combinations of lactose, whey protein, casein, and fat into water. Each model formulation was spiked with six concentrations of bacteriophage MS2. The soluble and insoluble components of each model milk formulation were separated by centrifugation at 40,000 × *g* and viruses were enumerated in each supernatant fluid and pellet by the double agar layer (DAL) method. When samples were spiked with MS2 at concentrations lower than 4.8×10^5 pfu/ml, milk components did not significantly impact the overall recovery. However, the impact of milk components was measurable at higher concentrations. In general, higher numbers of MS2 were recovered from supernatant fluids of model milk formulations containing no fat. The highest number of viral particles were recovered from the pellet of model C (lactose + whey protein + casein). The recovery efficiency of MS2 was correlated with the dry matter contents of each model milk formulation and the initial spiking concentration of coliphage using response surface modeling.

1. Introduction

Enteric viruses are responsible for a significant portion of food-borne diseases through-out the world. Recently, an increased incidence of food-borne diseases caused by enteric viruses has been reported and raw milk has been identified as an important source for transmitting enteric viruses (Mortazavi et al., 2008; Raska et al., 1966). High loads of fecal coliform bacteria in raw milk indicate an increased probability of contamination with pathogens (Mortazavi et al., 2008). However, no direct relation between the levels of fecal coliforms and enteric viruses in raw milk have been reported. Enteric viruses also have been reported in pasteurized milk and other dairy products such as yogurt and cheese. Under refrigerated conditions, in pasteurized milk, poliovirus can survive for 90 days (Tivon, 1992). Under similar conditions, echoviruses can survive for 120 days in raw milk (Tivon, 1992). Due to their nature and size, contaminating enteric viruses can be found in different components of milk. Therefore, it is essential to characterize the role of various milk components in the recovery of viruses from raw milk. Different viral surrogates have been used extensively in the development of this process (Dubois et al., 2006; Leclerc et al., 2000). For example, male-specific F-RNA coliphages have been reported as good surrogates for human enteric viruses due to their similarities in structure, composition, and morphology (Grabow, 2001). MS2, which is a member of F-RNA coliphage serogroup 1, has been used extensively as a potential indicator for the presence of enteric viruses (Gerba et al., 2003). In this study a male-specific coliphage MS2 was used as a potential indicator for enteric viruses for the characterization of milk components for the recovery efficiency of viral particles in raw milk. A new elution procedure and a method for detection of viruses in different components of raw milk are described.

2. Materials and methods

2.1. Preparation of model formulations of milk

Milk formulations were prepared by mixing the natural components of raw milk in deionized water (Millipore, Direct-QTM, France). The amount of each component was added to the mixture based on average values of dry matter in different qualities of cow's milk. The separation of raw milk starts with removing fat and then casein and whey proteins, resulting in a lactose solution. Model milk, however, is constructed in the reverse order first making a

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doi:10.1016/j.jviromet.2010.04.028

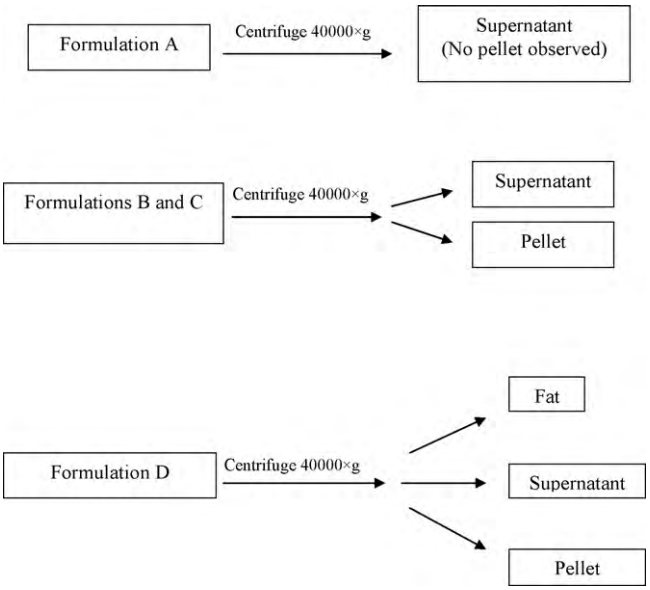

Fig. 1. Flow chart of the experimental design.

lactose solution and then adding other components to the mixture (Swaisgood, 1996). The solutions were homogenized using an IKA homogenizer (Ultraturrax, T25, Freiburg, Germany). The four model solutions were prepared in 100 ml and are described below:

Model A: lactose (L): 4.8% (w/v) (α lactose, Supelco, U47287, Bellefonte, USA).
Model B: lactose + whey protein (L + W): 4.8% (w/v) + 0.7% (w/v) (whey from bovine milk, Sigma,W1500, St. Louis, USA).
Model C: lactose + whey protein + casein (L + W + C): 4.8% (w/v) + 0.7% (w/v) + 2.75% (w/v) (sodium caseinat from bovine milk, Sigma, C8654, St. Louis, USA).
Model D: lactose + whey protein + casein + fat (L + W + C + F): 4.8% (w/v) + 0.7% (w/v) + 2.75% (w/v) + 3.5% (w/v) (triglyceride butter fat, Fulka, BCR519, St. Louis, USA).

2.2. Preparation of male-specific coliphage (MS2) and bacterial host

Male-specific bacteriophage MS2 (ATCC#15597-B1) and its host bacterium *Escherichia coli* F_{amp} (ATCC#700891), were propagated as described in USEPA Method 1601 (EPA, 2001).

2.3. Preparation of spiked model formulations

MS2 stocks were titered using the double agar layer (DAL) method as described in USEPA Method 1601. Viral stock was diluted in buffered phosphate solution (pH 7.2) to achieve the following concentrations: 48, 4.8×10^2, 4.8×10^3, 4.8×10^4, 4.8×10^5 and 4.8×10^6 pfu/ml.

In order to examine the impact of milk components on virus recovery, model milk formulations were spiked with different concentrations of MS2 and then stored overnight at 4 °C. The following day the solutions were centrifuged at $40{,}000 \times g$ for 1 h at 4 °C using a Beckman (G2-21M, Palo Alto, USA) centrifuge (Fig. 1). For precipitating whey proteins (soluble proteins) for model milk formulations or from raw milk, a higher g force is required (Shimazaki and Sukegawa, 1982). Supernatant fluid and pellet of each sample were recovered and analyzed for MS2 (ISO1075).

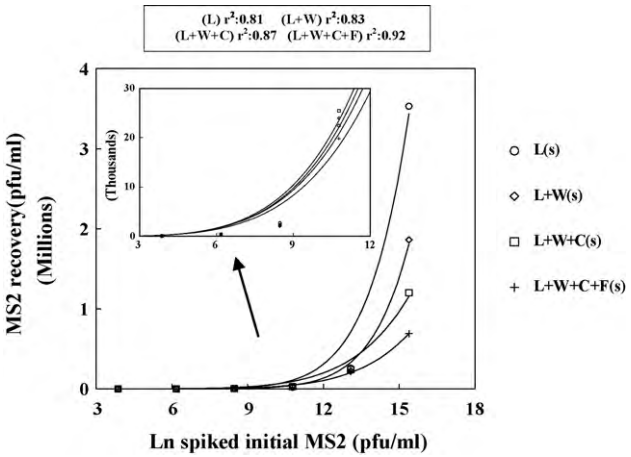

Fig. 2. Comparison of MS2 recovery from supernatant of model milk formulations.

2.4. Statistical design

Data was analyzed using Sigma Stat software (version 2.0, Jandel Corporation, San Rafael, CA, USA) for approaching the best functions, and Slide Write software (plus 2.0, Landbouw University, Wageningen, the Netherlands) for exhibiting the functions (e.g. exponential and & response surface). In addition, Slide Write software was used for predicting virus recovery efficiency using a counter isoline technique.

3. Results

3.1. Recovery efficiency in all model formulations

Recovery efficiency data for different fractions of each model milk formulation studied are shown in Table 1. At all intial spiked concentrations of MS2, a non-predictable trend in the recoveries from supernatant fluids and pellets from model formulations A and B were noted, whereas in milk formulations C and D, recovery efficiencies were inversely related to the initial spiking concentration.

3.2. Comparison of model formulations in enumerating viruses

Based on the accumulation of components in each model milk formulation (A–D), respectively, for every phase, the recovery behavior followed an exponential function ($Y = ax^b$) (Figs. 2 and 3). It was determined that in samples spiked with MS2 at concentra-

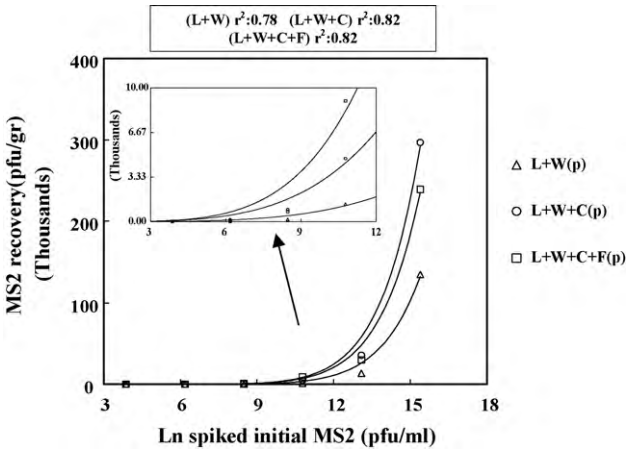

Fig. 3. Comparison of MS2 recovery from pellets of model milk formulations.

Table 1
Recovery efficiency (%) of viruses in supernatants and pellets of model milk formulations.

	Supernatant					Pellet					
Spiked initial MS2 (pfu/ml)	4.8×10^6	4.8×10^5	4.8×10^4	4.8×10^3	4.8×10^2	$4.8.\times10^1$	4.8×10^6	4.8×10^5	4.8×10^4	4.8×10^3	4.8×10^2	$4.8.\times10^1$
Spiked initial MS2(Ln)	15.38	13.08	10.77	8.47	6.17	3.87	15.38	13.08	10.77	8.47	6.17	3.87
Model milk formulation												
Lactose (A)	73.6	53.9	46.7	56.2	69.1	68.8	–	–	–	–	–	–
Lactose + whey protein (B)	38.7	46.1	50	43.3	48.3	72.9	2.8	2.8	2.7	3.3	3.7	2.1
Lactose + whey protein + casein (C)	25	49.8	53.0	47.5	65.3	67.3	6.2	7.5	9.8	18.8	31.2	31.2
Lactose + whey protein + casein + fat (D)	14.3	44.9	41.3	49.2	50.7	50	4.9	6.3	18.8	16.0	13.7	18.7
Lactose + whey protein + casein + fat (cream)	0.5	0.7	1.0	2.6	2.9	16.7	–	–	–	–	–	–

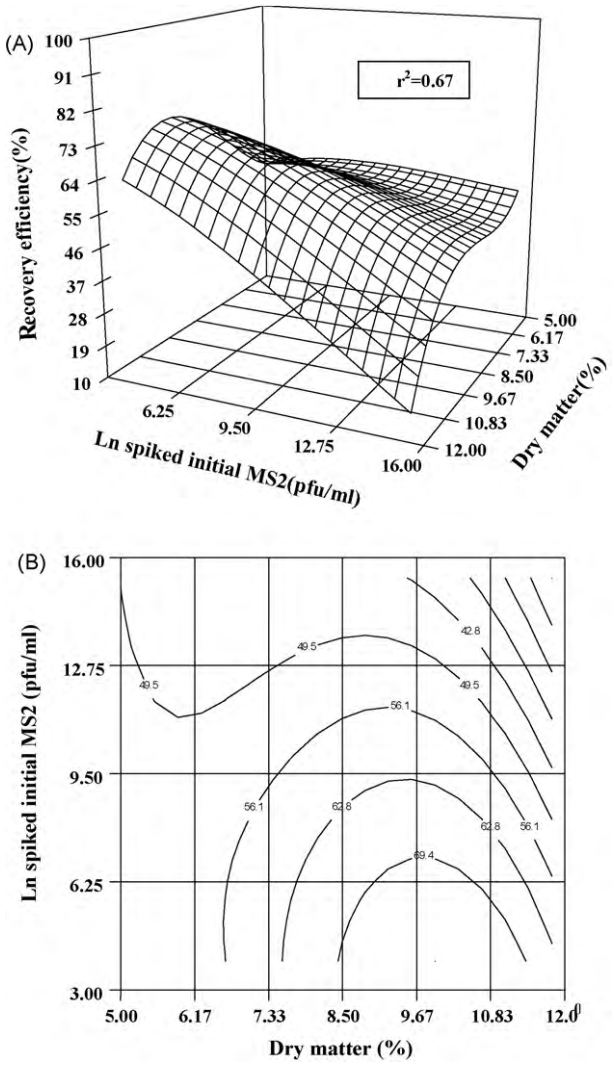

Fig. 4. (A) Impact of dry matter contents on recovery efficiency from supernatant; (B) prediction of recovery efficiency based on dry matter contents.

tions between 48 and 4.8×10^4 pfu/ml the milk components did not have a significant effect on the coliphage recoveries, but when the model formulations were spiked with MS2 at higher concentrations (4.8×10^5 to 4.8×10^6 pfu/ml) the impact of the milk components was measurable (Figs. 2 and 3).

3.3. Comparison of every milk component in recovery efficiency

In every model milk formulation, higher coliphage recoveries were achieved as the amount of the pellet increased. However, the presence of fat in the solution decreased the recovery efficiency of viral particles in both supernatant fluids and pellets (Figs. 4 and 5).

3.4. Evaluation of the amount of dry matter in the viral recovery efficiency

Based on the response surface curves in Figs. 4a and 5a, it can be concluded that increasing the nonfat dry matter contents in all of the formulations resulted in an increase in recovery efficiency from both supernatant fluids and pellets. But addition of the fat component to the dry matter (more than 8.3% of the dry matter) caused a decline in recovery efficiency for both phases (Figs. 4a and 5a).

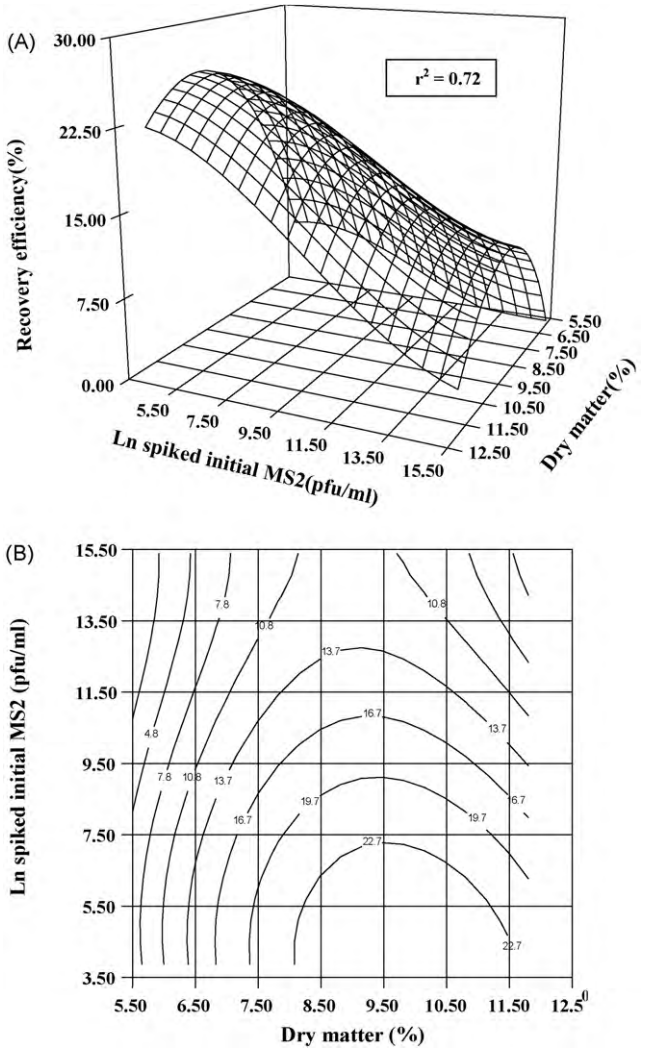

Fig. 5. (A) Impact of dry matter contents on recovery efficiency from pellet; (B) prediction of recovery efficiency based on dry matter contents.

3.5. Capability of recovery efficiency prediction according to dry matter

In this study, recovery efficiency of MS2 from raw milk was estimated based on the amount of dry matter using counter isoline curves (Figs. 4b and 5b).

4. Discussion

The results showed that the recovery of MS2 from model milk formulations can be influenced by various factors. The insoluble components had a greater impact on the coliphage recovery efficiency than the soluble components. In this study, milk formulations were prepared by maintaining the functional and hydrodynamic properties of each component. After spiking, the components were separated using centrifugation. Previous studies have shown that this technique guarantees the least loss of protein fractions (Bohren and Wenner, 1960; Lebowtiz et al., 2002; Morr et al., 1972).

High variation in recovery efficiency in the supernatant fluids of all the formulations can be related to individual milk components (Table 1 and Figs. 4 and 5). Non-predictable increases in the coliphage recovery efficiency in the supernatant fluids of model milk formulations B and C were observed, whereas a predictable increase in the recovery from the pellet was noted for model milk formulation C (Figs. 4 and 5). In model milk formulations, protein components and their structure, molecular weight, charge, and size can be used in the coliphage recovery predictive model (Swaisgood, 1996).

One of the reasons for better coliphage recovery in the supernatant fluid of formulation C compared to formulation B could be the similarity in the surface charge of MS2 proteins and milk's proteins (whey protein and casein). At near neutral pH, MS2 is predominantly negatively charged (Pham et al., 2009). The charge for casein is between −2 and −20 and for whey protein between −2 and −10 (Swaisgood, 1982; Swaisgood, 1992). This similarity in charge on MS2 and the milk components can create repulsion, resulting in disassociation of viral particles from the components, and increase the possibility of MS2 remaining in the supernatant fluid during centrifugation of the formulation.

Also increased retention of MS2 in the pellet of formulation C in comparison to formulation B is probably related to the differences in structure, molecular weight, and size between whey protein and casein.

Casein micelles (composed of submicelles) are 20–600 nm, whereas whey protein molecules are approximately 4 nm. In addition, the molecular weights of casein and whey proteins are 250,000–2,000,000 Da and 14,000–36,000 Da, respectively. According to the Svedberg equation, casein micelles can precipitate faster than whey proteins (Lebowtiz et al., 2002; Swaisgood, 1996). Moreover, casein is a porous, spongy structure with a large voluminosity (~4 ml/g) and exceptional hydration of 3.7 g H_2O/g casein. This hydration is an order of magnitude larger than that of typical globular proteins. Hence, large molecules, even proteins and coliphages have access to and can become trapped in these micelle structures (Rollema, 1992; Swaisgood, 1996). Viruses that cause foot and mouth disease can also become trapped within casein micelles (Blackwell et al., 1982). The casein colloidal suspension without any fat globules in the serum phase of milk (observed at about 50,000× magnification) can associate with MS2. This may explain the increase in MS2 concentration in the pellet of formulation C. Furthermore, whey immunoglobulins such as IgM can attract up to10 external particles at the same time. This fraction can be a critical factor in entrapping MS2 coliphage, especially in raw milk (Swaisgood, 1982).

But with the presence of fat globules in the components of model milk formulation D, MS2 concentration decreased in the pellet so that the sum of the MS2 concentrations in both phases (pellet and cream) of formulation D was lower than in corresponding phases of formulation C (Table 1 and Figs. 4 and 5). This phenomenon can be explained by the "emulsion of milk fat globules hypothesis" (observed at about 500× magnification) (Brunner, 1965; Timmen and Patton, 1989). Hence, upward movement of fat globules during centrifugation can interfere with the sedimentation of casein micelles, and the micelle network may be weakened. This phenomenon explains a lower concentration of MS2 in casein-containing fractions, because of a decline in the network firmness of casein precipitation (Lawrence et al., 1993). Lower MS2 concentrations in formulation D can also be explained based on the density, size, and number of particles in milk (specifically casein and fat globules). Each milliliter of milk contains 10^{14} casein micelles ranging 50–300 nm in diameter, and 10^{10} fat globules ranging 2000–6000 nm in diameter (Corbin and Whittier, 1965; Swaisgood, 1996). This size and number differentiation together with a huge difference in density (0.92 g/ml for fat globules and 1.11 g/ml for casein micelles) may have resulted in a significant decline in MS2 concentration in the supernatant fluid and pellet of formulation D during centrifugation.

Results of this study also point out the utility of counter isolines for predicting MS2 recovery in model milk formulations. Using this

strategy, the concentration of enteric viruses in a single component of raw milk can be used to predict the total viral load in whole raw milk.

5. Conclusions

1. The milk components did not have a significant impact on the recovery of coliphages at concentrations between 48 and 4.8×10^4 pfu/ml.
2. The presence of casein micelles was the most important factor in pelleting coliphages in model milk formulations.
3. Elimination of fat globules and separation of casein micelles from other components in raw milk is the best strategy for recovery of viruses.
4. Viral recovery efficiencies from milk formulations C and D were inversely related to the initial spiking concentration.
5. Counter isolines (response surface modeling) can be applied for determining the concentration of enteric viruses in different domains of raw milk.

Acknowledgments

The authors wish to thank the Ministry of Industries and Mines as well as Ozone Ab Corporation for their financial support of this project.

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