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Survival of enteric viruses during yoghurt making process using male-specific coliphage

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Abstract

The effects of yoghurt-making process on the survival of male specific coliphage (MS2) in raw milk
with different fat content, as well as the survival of MS2 during the storage of yoghurt were eval-
uated. All samples were analyzed for pH, acidity, and virus count. The results showed that the
heat treatment of milk (85 °C, 30 min) decreased the recovery of coliphage MS2 from approxi-
mately 7 to 0.97 and 1.49 $\log_{10}$ (pfu/ml) in the milk samples with 2.5% and 3% fat, respectively.
No MS2 could be detected in the heat-treated milk sample with 1.5% fat. The recovery of MS2
during incubation was decreased as the acidity increased particularly in milk with 1.5% fat. More-
over, during the storage time (10 days), yoghurt samples with 1.5% and 3% fat had the lowest and
the highest recovery for coliphage MS2, respectively.

Practical applications

Enteric viruses have been reported in dairy products such as pasteurized milk, cheese, and
yoghurt. Bacteriophages are viruses that infect bacteria, and exist in ecosystems in which bacteria
have been located. A case in point is the vats that are used for the fermentation of yoghurt and
other dairy products. This phenomenon renders bacteriophages suitable as surrogates for the eval-
uation of the survival of enteric viruses in foodstuffs, as is outlined in the present work and similar
studies. Due to the health concerns caused by enteric viruses, as well as an increase in the con-
sumption of dairy products, a considerable need for further investigation of their survival is
recognized. In order to achieve this aim, researchers can take advantage of the procedure applied
here, which has proven reliable and practical for the aforementioned purpose.

KEYWORDS

coliphage MS2, milk fat, recovery, storage time, survival, thermal processing, yoghurt

1 | INTRODUCTION

Viruses are small, infectious microorganisms, the size of which ranges
from 15 to 400 nm. They cause many diseases in humans, plants and
animals. Each group of viruses has its own cell preference (called tro-
pism) and typical host range. Furthermore, there are a lot of ways for
virus transmission; for instance, from stool of a person infected with an
intestinal virus which are contaminated, by droplets caused when an
infected person coughs, by contact with zoonotic viruses in infected
animals, by sexual intercourse, by contact with blood from infected
persons with bloodborne viruses and by vectors, such as ticks or mos-
quitoes for arthropod-borne (arbo-) viruses (Koopmans & Duizer,

2004). Viruses can also be transmitted via foods, and are of concern to
human health. In other words, they are shed in the feces of infected
humans and transmitted through the fecal-oral route (Sair, D'souza, &
Jaykus, 2002).

Foodborne outbreaks cause gastroenteritis deaths worldwide
and are increasingly recognized as having stemmed from viral ori-
gins. Moreover, hepatitis A virus (HAV), the human noroviruses
(NoV), Aichi viruses, adenoviruses, rotaviruses, astroviruses, human
enteroviruses such as coxsackieviruses and echoviruses, parvovi-
ruses, and other small round viruses are some of the epidemiologi-
cally significant human enteric viruses of public health concern. Also,
due to the properties of human enteric viruses, they are completely

different from the common bacterial agents of foodborne disease (Sair et al., 2002).

Enteric viruses are known as the main agents of foodborne illness in the world and could be transmitted through raw milk (Mortazavi, Habibi Najafi, Yavarmanesh, & Barouei, 2008; Raska et al., 1966). The probability of raw milk contamination with enteric viruses due to the presence of high loads of fecal coliforms has been speculated (Mortazavi et al., 2008). However, there is no evidence of a direct relation between the levels of fecal coliforms and enteric viruses in raw milk. Enteric viruses have been reported in dairy products such as pasteurized milk, cheese and yoghurt. For example, poliovirus and echovirus may survive in pasteurized milk (Under refrigerated conditions) for 90 and 120 days, respectively, (Tiron, 1992). In addition, enteric viruses may be distributed in different components of milk because of their size and nature. Therefore, the role of different milk components in the recovery of viruses from raw milk is very important (Yavarmanesh et al., 2010).

Furthermore, it is well known that food components have strongly affected the viral sensitivity to heat treatment. The heat tolerance and survival of HAV (Hepatitis A) was reported in different food matrices such as shellfish, fruit–based and dairy products (Deboosere et al., 2010). Murine norovirus (MNV-1) and Feline calicivirus (FCV-F9) are used as surrogates to better understand human noroviruses survival, transmission and infectivity since they are similar to human noroviruses (Radford, Coyne, Dawson, Porter, & Gaskell, 2007). Anyway, FCV is neither acid resistant nor heat stable and therefore cannot be used as a suitable surrogate for norovirus (NV) (Hewitt, Rivera-Aban, & Greening, 2009).

In general, a good indicator must comply with the following requirements; it must exist in a higher number than the pathogen, be absent in unpolluted areas, be associated with the source of the pathogen, not be pathogenic, be detectable by rapid, easy and inexpensive procedures, not multiply out of the host, and be resistant to artificial and natural inactivation in a manner comparable to viral pathogens (Bosch, 1998). Currently, male-specific F^+RNA bacteriophages have received the most attention as surrogates and models for enteric viruses in several researches because of their similarity to mammalian viral pathogens in shape, size, morphology, isoelectric point, resistance to environmental stress and transport characteristics (Grabow, 2004). These coliphages infect gram negative bacterial cells that produce F or sex–pili (i.e., *Ecoli K12 WG49*) and unlike other viral and bacterial indicators, working with them in laboratory is easy, inexpensive and also has little risk to human health. male-specific coliphage (MS2) has also been applied as a model for norovirus since MS2 is similar in symmetry and size, and temperature stability as well as acid resistance to norovirus (Dawson, Paish, Staffell, Seymour, & Appleton, 2005; Feng, Ong, Hu, Tan, & Ng, 2003; Gerba, Riley, Nwachuku, Ryu, & Abbaszadegan, 2003).

The objective of this study was to evaluate the survival of coliphage MS2 as a potential surrogate for enteric viruses in the process of yoghurt production.

2 | MATERIALS AND METHODS

2.1 | Preparation of host bacteria stock cultures (*Escherichia coli* F_{amp})

Preparation of stock culture *Escherichia coli* F_{amp} (ATCC#700891), the host of Male-specific bacteriophage MS2 (ATCC#15597-B1), was performed according to the method 1601 (EPA, 2001). For attaining isolated colonies, pure frozen stock cultures were activated by streaking host bacteria onto 1.5% Trypticase soy agar (TSA) plates with appropriate antibiotics (streptomycin/ampicillin). Inoculated plates were incubated for 24 hrs (overnight) at 36 °C ± 1.0 °C. An individual colony was then picked and inoculated into tryptic soy broth (TSB) with appropriate antibiotics (streptomycin/ampicillin) and grown to log phase. Sterile glycerol and TSB with log-phase host bacteria in a ratio of 1:4 were mixed and host bacteria stock cultures were kept frozen at −70 °C.

### 2.2 | Preparation of overnight host bacteria stock cultures (*Escherichia coli* F_{amp})

Twenty-five milliliters of TSB with streptomycin/ampicillin was dispensed into a sterile 125-ml flask. The flask was then inoculated with a loopful of *E. coli* F_{amp} from the frozen stock culture. Furthermore, it was incubated at 36 °C ± 1.0 °C while being shaked at 100 rpm for 18 to 20 hrs (overnight). The preparation was stored at 4 °C ± 1 °C to be used on the same day (EPA, 2001).

2.3 | Preparation of log-phase host bacteria stock cultures

0.1 to 1.0 ml of overnight activated *E. coli* F_{amp} host bacteria stock culture was added to a 125 ml flask containing 25 ml of TSB with streptomycin/ampicillin. The flask was then incubated at 36 °C ± 1.0 °C while being shaked at 100 to 150 rpm for about 4 hrs or until cultures were visibly turbid (cloudy), indicating log-phase growth. One milliliter of culture was then aseptically removed from the flask, dispensed into a cuvette, and the absorbance was read at 520 nm. Absorbance between 0.1 and 0.5 (OD) is an indication of log-phase growth. Otherwise, cultures would be returned to shaker incubator until proper OD was reached (EPA, 2001).

2.4 | Preparation of male-specific coliphage

Male-specific coliphage MS2 (ATCC#15597-B1) was propagated as described in method 1601 (EPA, 2001). In brief, for the preparation of coliphage MS2, distilled water was added to the vial containing coliphage MS2 stock. Then, 30 ml of TSB with *Escherichia coli* F_{amp} was incubated at 36/5 °C ± 2.0 °C. After that, 1 ml of coliphage MS2 was added to culture media and further incubated at 36/5 °C ± 2.0 °C for 4 hrs. It was then filtered and stored in a sterile tube. The tube was labeled with source, date, and initials, and stored at 4 °C ± 1 °C until the day of experiment.
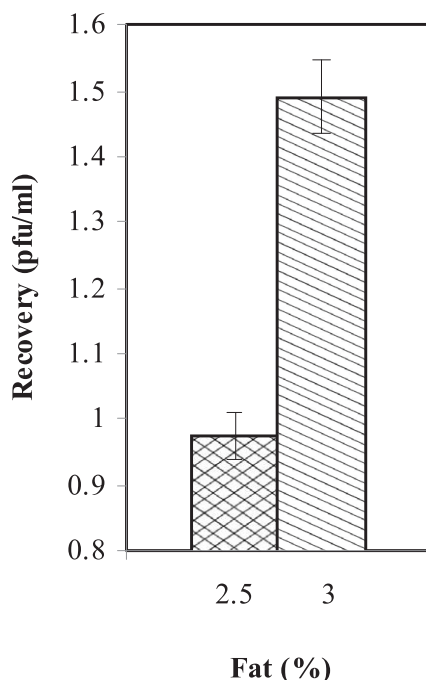
FIGURE 1 The effect of thermal processing used in yoghurt production on recovery of coliphage MS2

2.5 | Preparation of MS2 stock dilutions

Stock MS2 coliphages were titered using the double agar layer (DAL) method according to US-EPA Method 1601. Viral stock was diluted in buffer phosphate solution (pH 7.2) to obtain the following concentrations: $10^3, 10^5,$ and 10^7 pfu/ml.

2.6 | Yoghurt preparation and storage

Milk samples with different fat content (1.5, 2.5, and 3%) were obtained from a local supermarket. Commercial lyophilized yoghurt culture CH-1 (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) was provided from Chr-Hansen Co. (Hørsholm, Danmark). Each culture was propagated according to the manufacturer's instruction. The experimental cultures were added in a freeze-dried Direct Vet Set (DVS) form in the amount of 50 units of activity to 560 liters of processed milk, which corresponded to 1% addition of activated working starter.

The proper amount of bacteriophage MS2 (10^3, 10^5, and 10^7 pfu/ml) was aseptically added to milk samples (fat: 1.5, 2.5, 3%). All milk samples were then heated at 85°C for 30 min (Liu, 1997). The recovery of bacteriophage MS2 was measured after heat treatment to determine the effect of this process on survival of MS2. Moreover, yoghurt samples were prepared by different milk samples (fat: 1.5, 2.5, 3%) after heat treatment were heated at 85 °C for 30 min, followed by cooling (42 °C) and aseptically inoculating with 1% of starter culture (Biliaderis, Khan, & Blank, 1992; Lucey, 2004) and MS2 (10^3, 10^5, 10^7 pfu/ml). All treatments were incubated at 42 °C until the required pH of 4.6 was obtained (the termination of yoghurt fermentation). Yoghurt

samples were immediately cooled after fermentation and stored at 4 °C (Tamime & Robinson, 1985) for 10 days (Spreer, 1995). The acidity (% lactic acid) (AOAC, 1990), pH changes (pH meter model 691; Metrohm, Switzerland) and the recovery of coliphage MS2 in all yoghurt samples were measured during incubation time at one-hour intervals and once a day during the storage period.

2.7 | Statistical analysis

A Completely randomized factorial design was used for data analysis. Statistical analysis was conducted using Minitab version 14 (Minitab Inc., State College, PA) and Excel (Microsoft Excel version 2010). Analysis of variance and LSD test were used where applicable to determine statistically significant differences at $p < .05$.

3 | RESULTS

3.1 | Effect of thermal processing on recovery of coliphage MS2

Thermal processing of milk samples used in yoghurt preparation (85 °C, 30 min) caused significant decrease in the recovery of coliphage MS2. No coliphage MS2 could be detected in the samples with 1.5% milk fat while coliphage MS2 in milk samples with 2.5% and 3% milk fat reduced from approximately 7 $\log_{10}$ (pfu/ml) to 0.97 and 1.49 $\log_{10}$ (pfu/ml), respectively. (Figure 1).

3.2 | Effect of pH and acidity on recovery of coliphage MS2 during incubation and storage time of yoghurt

Generally, the initial pH of milk prepared for yoghurt production is directly related to fat content. Milk with higher fat content causes the initial pH of the yoghurt to increase and the rate of pH development declines during the incubation of high fat yoghurt samples (Shaker, Jumah, & Abu-Jdayil, 2000). In our study, the pH of yoghurt samples with 3% milk fat was higher than that of samples with 1.5% milk fat. pH development adversely affected the recovery of coliphage MS2 during incubation time (Figure 2A–C). In addition, yoghurt samples with 1.5% milk fat had a lower recovery of coliphage MS2, the decrease in recovery was shown to be significant. Moreover, during the storage time (after 10 days) yoghurt samples with 1.5% and 3% milk fat had the lowest and the highest recovery for coliphage MS2, respectively, (Figure 2D–F).

3.3 | Interaction effect of fat and spiked coliphage on recovery of coliphage MS2 during storage time

The effect of yoghurt fat content and spiked coliphage on recovery of coliphage MS2 was significant ($p < .05$) (Table 1). During storage time, yoghurt samples with 3% and 1.5% fat had the highest and the lowest recovery of coliphage MS2, respectively. Also, each factor alone (fat
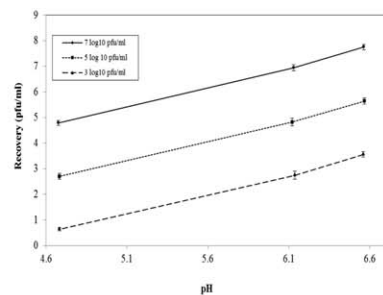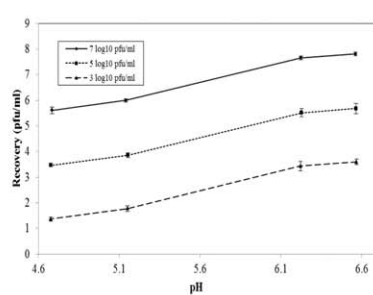
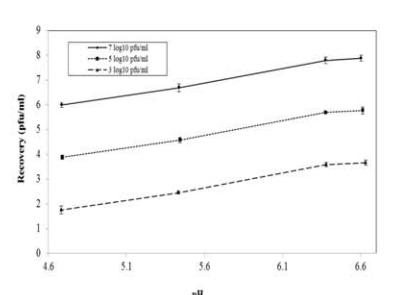Figure 2 A Moradi Moghadam et al

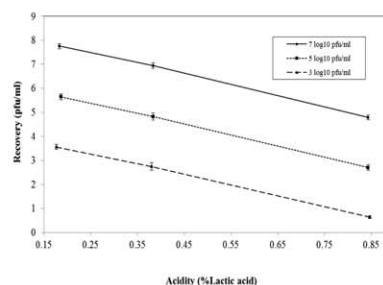Figure 2 B Moradi Moghadam et al

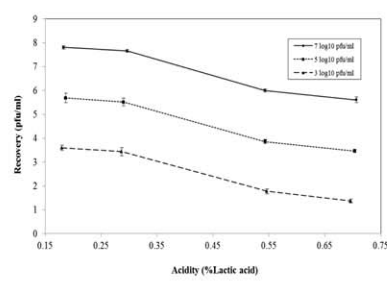Figure 2 C Moradi Moghadam et al

Figure 2 D Moradi Moghadam et al

Figure 2 E Moradi Moghadam et al

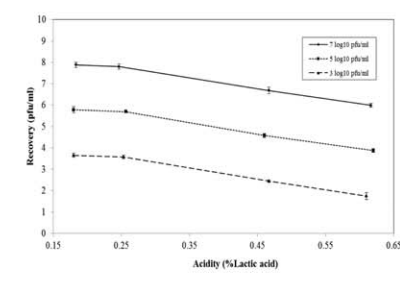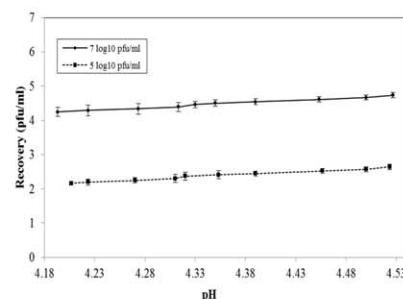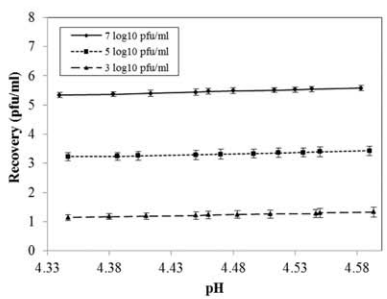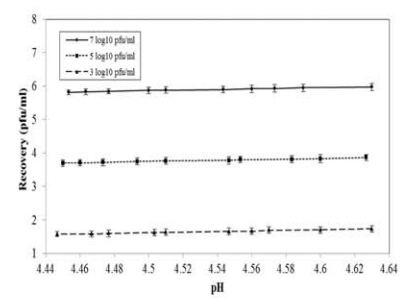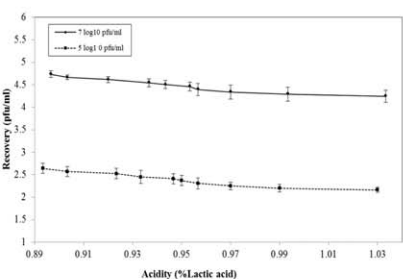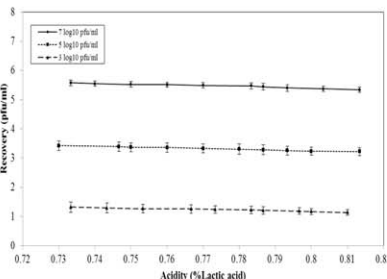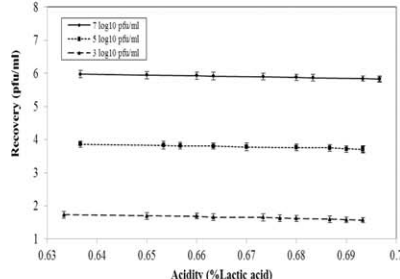Figure 2 F Moradi Moghadam et al

FIGURE 2 (A) Effect of pH and acidity on recovery of coliphage MS2 in 1.5% fat yoghurt during incubation. (B) Effect of pH and acidity on recovery of coliphage MS2 in 2.5% fat yoghurt during incubation. (C) Effect of pH and acidity on recovery of coliphage MS2 in 3% fat yoghurt during incubation. (D) Effect of pH and acidity on recovery of coliphage MS2 in 1.5% fat yoghurt during storage time. (E) Effect of pH and acidity on recovery of coliphage MS2 in 2.5% fat yoghurt during storage time. (F) Effect of pH and acidity on recovery of coliphage MS2 in 3% fat yoghurt during storage time

content or spiked coliphage) had a significant effect ($p < .05$) on recovery of coliphage MS2 (Figures 3 and 4).

3.4 | Effect of storage time on recovery of coliphage MS2

Storage time of yoghurt had a significant effect on the recovery of coliphage MS2 ($p < .05$) (Figure 5).

4 | DISCUSSION

4.1 | Effect of thermal processing on recovery of coliphage MS2

Generally, in liquid food samples, large volume is one of the main controversial factors in short-time pasteurization in which the interior areas of liquid food sample cannot be heated sufficiently (Parry & Mortimer, 1984). Strazynski, Kramer, and Becker (2002) showed

FIGURE 3 Effect of yoghurt fat content on recovery of coliphage MS2 during storage time

TABLE 1 Effect of yoghurt fat content and spiked coliphage on recovery of coliphage MS2 during storage time

Fat(%)[a]	7log$_{10}$ (pfu/ml)	5log$_{10}$ (pfu/ml)	3log$_{10}$ pfu/ml
1.5	4.47 ± 0.016[C]	2.38 ± 0.021[F]	0.00 ± 0.000[I]
2.5	5.46 ± 0.022[B]	3.31 ± 0.017[E]	1.23 ± 0.019[H]
3	5.89 ± 0.023[A]	3.77 ± 0.018[D]	1.64 ± 0.020[G]

[a]$p < .05$.
Different letters indicate significant differences.

4.2 | Effect of milk fat amount on pH and acidity of yoghurt

The effect of milk fat on rheological properties of yoghurt and acid development during fermentation was demonstrated by Shaker et al. (2000). In addition, increasing milk fat content can affect the activity and growth of starter culture (Mahdian & Mazaheri Tehrani, 2007). Increasing the milk fat content causes the initial pH of the yoghurt sample to increase and the rate of pH development declines during incubation of high fat yoghurt (Shaker et al., 2000).

4.3 | Effect of pH and acidity on coliphage MS2

Coliphage MS2 is a nonenveloped RNA virus with an icosahedral capsid measuring 22–29 nm in diameter (Blanch et al., 2002; Golmohammadi, Valegard, Fridborg, & Liljas, 1993). Also, the isoelectric point of coliphage MS2 is 3.9 (Overby, Barlow, Doi, Jacob, & Spiegelman, 1966). Viruses will lose their infectious property if their environmental pH reach equal or below their isoelectric point (pI). For instance, pI of coliphage MS2 is acidic pH (Langlet, Gaboriaud, & Gantzer, 2007), while for other viruses it can be near neutral pH (5.8 for HAV, and 6.6 for *Poliovirus* 1) (Kukavica-Ibrulj, Darveau, Jean, & Fliss, 2004; Zerda & Gerba, 1984). Studies showed that the titer of Poliovirus 1 declined 2 log$_{10}$ when stored in 1 mol/l KCl at pH 3 (Salo & Cliver, 1976). Also, pfu decrease was monitored in KCl 0.4 mol/l containing 10^6 pfu/ml MS2 phages after 4 hrs at pH 6.7, 3.9, and 2.5. No decrease was observed at pH 6.7 while at pH 3.9 and 2.5, 1.11 and 3.00 log$_{10}$ (pfu/ml) decreases were observed, respectively, (Langlet et al., 2007). Thus, one of the main reasons for decreased recovery of MS2 in yoghurt samples is correlated to the reduction of their pH toward the pI of coliphage MS2.

that polioviruses present in milk, yoghurt and water were inactivated by long-time pasteurization, high-temperature heating and short-time pasteurization for 30 s. However, short-time pasteurization for 15 s could not lead to complete inactivation of polioviruses. Moreover, the resulting loss of infection in water was higher than in milk. On the other hand, there are thermal protective substances in milk such as proteins, fats, sugars and ions to prevent thermal penetration (Abad, Pinto, Diez, & Bosch, 1994; Cliver, 1990; ICMSF, 1996). The effect of milk proteins and high concentrations of lactose in milk on thermal resistance of hepatitis A virus was demonstrated (Cliver & Salo, 1978; Parry & Mortimer, 1984). In another study, it was found that high fat content in ice-cream and ice-cream products increases thermal resistance of entroviruses so that enteroviruses can survive under pasteurization at 71.7 °C for 30 s (Cliver & Salo, 1978). In addition, application of high titers of virus (>10^5 pfu/ml) protect it against thermal processes (Cliver, 1973). These is an array of published reports in agreement with our findings.
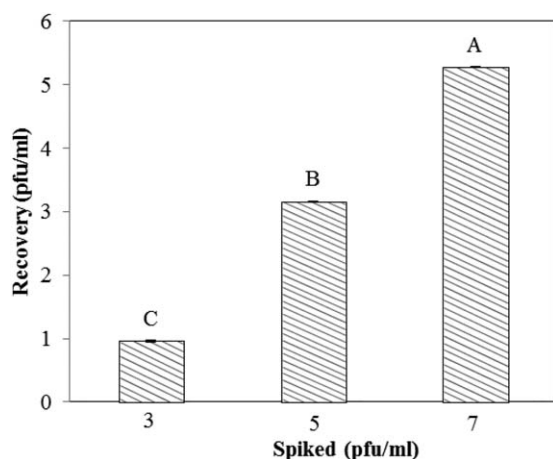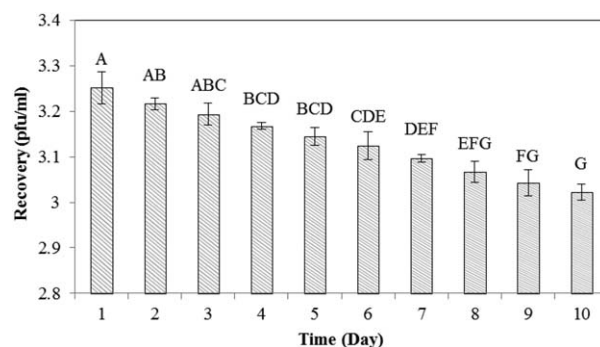

FIGURE 4 Effect of spiked coliphage on recovery of coliphage MS2 during storage time

FIGURE 5 Effect of storage time on recovery of coliphage MS2

Low pH causes the destruction of the viral capsid proteins as well as exposed nucleic acid (Olson, Axler, & Hicks, 2004). The chemical ionization may be affected by pH. There are high concentrations of hydrogen and hydroxyl ions in water at greatest pH values, therefore viral inactivation mechanisms will be dominated. Moreover, hydroxyl and superoxyl ions are highly reactive radicals in the water environment and because of their long lifetime, they may oxidize materials in the water environment. Therefore, the coat protein of viruses may have been affected by deformation, removal or denaturation at neutral and slightly acidic or alkaline pH values. On the other hand, RNA hydrolysis may occur inside or outside the virus particle when the protein coat is damaged (Feng et al., 2003). As a result, dissociation of the capsid would occur in extreme pH values and virus surfaces (capsid, tail fibers, etc.) can be attacked by the mechanism of direct oxidation (Boeye & Elsen, 1967; Katagiri, Aikawa, & Hinuma, 1971; Maizel, Phillips, & Summers, 1967). Due to its similarity in structural properties and size to many of the human enteric viruses, MS2 is used for modeling viral behavior (Calender, 1988).

High recovery of coliphage MS2 in yoghurt samples with high fat content (3%) in this study is most likely due to low acid development (Shaker et al., 2000). Low acid production causes less damage in coliphage MS2 and as a result, the recovery of coliphage MS2 will increase.

4.4 | Effect of pH and acidity on surface potential (zeta potential) of coliphage MS2

MS2 is generally negatively charged at near neutral pH (Pham, Mintz, & Nguyen, 2009). The charges of whey protein and casein are -2 to -10 and -2 to -20, respectively, (Swaisgood 1982, 1992). There is a repulsion due to similarity in charge on the milk components and MS2, which leads to the separation of viral particles from the components, and an increase in the probability of coliphage MS2 remaining in the supernatant fluid (Yavarmanesh et al., 2010). The negative charge of casein decreases in isoelectric pH during fermentation of yoghurt (Lucey, 2004). Furthermore, casein is a soft and porous structure with a large voluminosity ($\sim$4 ml/g) in which coliphages and proteins can be trapped in these micelle structures. Viruses can be trapped within casein micelles. This phenomenon has been proved for foot and mouth viruses (Blackwell, Mckercher, Kosikowski, Carmichal, & Gorewit, 1983; Rollema, 1992; Swaisgood, 1996). During the fermentation time, lactic acid development renders casein micelles unstable and whey protein denatured (Tamime & Robinson, 1985). The denaturation of casein causes the attraction and trapping of coliphage MS2 to decrease and as a result, coliphage MS2 is exposed to the acid of yoghourt starter. Hence, this is the main reason for decreased recovery of MS2 during fermentation time.

5 | CONCLUSIONS

Thermal processing of yoghurt influenced the recovery of coliphage MS2 in which no coliphage could be detected in the samples with 1.5% milk fat. However, the recovery of coliphage MS2 decreased from approximately 7 to 0.97 and 1.49 $\log_{10}$ (pfu/ml) in the milk samples with 2.5% and 3% fat, respectively. In addition, pH and acidity influenced the recovery of coliphage MS2 during fermentation and storage time. In the yoghurt samples with 1.5% milk fat, decrease in recovery was significant during fermentation time and storage time (after 10 days). The yoghurt samples with 1.5% and 3% milk fat had the lowest and highest recovery for coliphage MS2, respectively.

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