ORIGINAL ARTICLE

Stability of severe acute respiratory syndrome coronavirus 2 in dairy products

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Abstract

The present investigation was performed to determine the stability of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) under several industrial processing situations in dairies, including pasteurization, freezing, and storage in acidic conditions. Ten treatments were selected, including high-temperature short-time (HTST)-pasteurized low-fat milk, low-temperature long-time-pasteurized low-fat milk, extended shelf life (ESL)-pasteurized low-fat milk, HTST-pasteurized full-fat milk, LTLT-pasteurized full-fat milk, ESL-pasteurized full-fat milk, pasteurized cream, ice cream frozen and stored at -20 or $-80°C$, and Doogh (as a fermented milk drink with initial pH < 3.5) refrigerated for 28 days. The viral particles were quantified by RT-PCR methodology. Besides, the virus infectivity was assessed through fifty-percent tissue culture infective dose (TCID$_{50}$) assay. These products were seeded with a viral load of 5.65 log TCID$_{50}$/mL as a simulated cross-contamination condition. Pasteurization techniques were sufficient for complete inactivation of the SARS-CoV-2 in the most dairy products, and 1.85 log TCID$_{50}$/mL virus reduction in full-fat milk (fat content = 3.22%). Freezing (either $-20°C$ or $-80°C$) did not result in a virally safe product within 60 days of storage. Storage at high acidic conditions (initial pH < 3.5) completely hampered the viral load at the end of 28 days of refrigerated storage. This research represents an important practical achievement that the routine HTST pasteurization in dairies was inadequate to completely inactivate the viral load in full-fat milk, probably due to the protective effect of fat content. Furthermore, freezing retain the virus infectivity in food products, and therefore, relevant contaminated foods may act as carriers for SARS-CoV-2.

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which belongs to the family *Coronaviridae*, and *betacoronavirus* genera, has been caused the most disastrous pandemic disease of the 21st century, known as coronavirus disease 2019 (COVID-19), in the globe since late December 2019. This virus is considered the third zoonotic *betacoronaviruses* that have been identified in the present century. The Middle East respiratory syndrome-related coronavirus (MERS-CoV) and SARS-CoV, both are Beta-CoVs, resulted in significant outbreaks throughout the world. It is observed that there is ~89% resemblance between SARS-CoV-2 and SARS-CoV regarding phylogenesis (Jalava, 2020; Pressman, Naidu, & Clemens, 2020). Camels and civets were reported to be the intermediate host of MERS and SARS, respectively; however, bats were considered the source for both. Intermediate hosts are referred to as animals that carry the virus and finally transfer it to humans. Generally, CoVs required intermediate hosts before infecting people (Zhao, Cui, & Tian, 2020).

Several pieces of evidence suggest bats as a probably principal source of the COVID-19 pandemic, but its intermediatory host is unknown (Dhama et al., 2020; Rodriguez-Morales et al., 2020). COVID-19 infection can appear without symptoms or moderate to severe lung congestion (Kannan, Ali, Sheeza, & Hemalatha, 2020). The signs related to the COVID-19 infection may manifest about 5.2 days after infection (Li et al., 2020). Fever, coughing, sore throat, muscle aches, and losing smell or taste are considered some signs of COVID-19 (Rizou, Galanakis, Aldawoud, & Galanakis, 2020).

At present, evidence suggests SARS-CoV-2 can be transmitted through droplets that are formed during coughing and sneezing, close contact with spatters of patients, and aerosol droplets. Moreover, detection of the viral particles in feces, digestive system, saliva, and urine proposed the gastrointestinal tract as possibly another transmission route of this outbreak (Dhama et al., 2020; Pressman et al., 2020; Wang et al., 2020). For instance, Xiao et al. (2020) reported that viral RNA was observed in stool samples of about 39 out of 73 COVID-19 positive patients. They explained a direct correlation between ACE-2 protein expression in rectal epithelia, duodenal, and gastric cells, and the presence of nucleocapsid protein of SARS-CoV-2, suggesting the involvement of those parts in viral infection. Interestingly, the viral nucleocapsid was not detected esophageal epithelium where ACE-2 protein genes were scarcely expressed. To the best of our knowledge, ACE-2 protein receptors are responsible for SARS-CoV-2 attachment to the host cells and frequently are observed in epithelial cells of lung. In another study, Uno (2020) stated that the risk of gastrointestinal tract infection caused by SARS-CoV-2 is significant in people who have higher pH in their stomach, especially those who are suffering from intestinal metaplasia and atrophic gastritis. Therefore, the detection of viral RNA in patients' feces indicates the feasibility of the fecal–oral transmission path (Dhama et al., 2020; Yekta, Vahid-Dastjerdi, Norouzbeigi, & Mortazavian, 2020; Zhang, Wang, & Xue, 2020). As yet, there is no proof of COVID-19 transmission through breast milk (Centeno-Tablante et al., 2020). Owing to the possibility of cross-contamination by infected foodstuffs during the outbreak, the ingestion of unheated milk should be avoided. Beyond that, not only thermal processing at each temperature–time combination may not be adequate for the inactivation of viral particles, but also due to the stability of SARS-CoV-2 at -20°C for up to 2 years, some frozen milk products like ice cream may consider as a carrier for the novel coronavirus pandemic (Yekta et al., 2020).

The probable fecal–oral transmission route should not be ruled out because of the probability of food contamination by either carry-over or carry through paths. The recognition of the novel coronavirus on the frozen chicken wing from Brazil has propounded the first discovery of SARS-CoV-2 on real foods (Han, Zhang, He, & Jia, 2020). Recently, we published a review article focusing on the risk of disparate foods being vehicles of the novel coronavirus (Yekta et al., 2020). Accordingly, and considering that no report was present regarding the effects of food processing on the durability of SARS-CoV-2 in food matrices, this research was designed to evaluate the stability of SARS-CoV-2 under several industrial processing situations in dairies, including pasteurization, freezing, frozen storage, and acidic conditions (low pH and high titratable acidity).

2 | MATERIAL AND METHODS

2.1 | Dairy product treatments

Five industrial dairy products were obtained from local commercial sources, including raw low-fat milk (fat content of 1.14 ± 0.21%), raw full-fat milk (fat content of 3.22 ± 0.16%), cream (fat content of 33.76 ± 0.15%), ice cream (fat content of 10.19 ± 0.07%), and Doogh (a typical Iranian fermented milk drink with initial pH 3.48 ± 0.02 and titratable acidity of 141.56 ± 1.03°D). These products were seeded with SARS-CoV-2 at a virus titer of ~6 log $TCID_{50}$/mL and 3.5×10^6 RNA copy numbers to simulate the cross-contamination condition. Then, each product was subjected to special processing as following:

1. The low-fat milk was pasteurized by HTST (high-temperature short time) method (72°C, 15 s).
2. The low-fat milk was pasteurized by LTLT (low-temperature long time) method (63°C, 30 min).
3. The low-fat milk was pasteurized by ESL (extending shelf life) method (80°C, 15 s).
4. The full-fat milk was pasteurized by HTST (high-temperature short time) method (72°C, 15 s).
5. The full-fat milk was pasteurized by LTLT (low-temperature long time) method (63°C, 30 min).
6. The full-fat milk was pasteurized by ESL (extending shelf life) method (80°C, 15 s).
7. The cream was pasteurized (90°C, 1 min).
8. The ice cream was stored at two frozen temperatures, that is, -20°C or -80°C.
9. The Doogh was stored for 28 days at a refrigerated temperature of 5°C.

2.2 | Chemical analysis of dairy products

The samples were assessed for total protein content by the macro-Kjeldahl procedure (AOAC, 2000). The chemical analyses on samples were performed in parallel with virus seeding. Fat contents were measured by the Mojonnier method (Patel, Baer, & Acharya, 2006). The pH was determined at ambient temperature using a pH meter (Metrohm, Switzerland). Titratable acidity (TA), reported as Dornic degrees, was calculated by adding 0.1N NaOH to the pink endpoint using phenolphthalein as an indicator (Mortazavian, Khosrokhavar, Rastegar, & Mortazaei, 2010). Table 1 shows the pH, acidity, protein, and fat contents of dairy products.

2.3 | Culturing of Vero cell and SARS-CoV-2

2.3.1 | Vero cell preparation

A T-175 flask of Vero cell line culture prepared from Reference Keyvan Laboratory and trypsinize, centrifuged, and resuspended in 10% fetal bovine serum (Gibco) and 90% media composed of DMEM (Dulbecco's minimum essential medium). Also, cells subcultured in T25 flasks and incubated at 37°C and 5% CO_2 until 80% confluency. These cells were used for virus culture and micro-titration tests.

2.3.2 | Virus isolation and culturing

Previously, samples isolated from the nasopharyngeal cavity by swabs were placed in viral transportation medium (VTM), which supplied by Pasteur Institute (Tehran, Iran). COVID-19 positive diagnosed patients according to their real-time PCR analysis (cycle thresholds under [CT] values 15) used for the present study. We called the isolated virus stain K1 isolate. All investigations on the virus were performed in a biosafety level 3 laboratory. Virus quantification was performed after K1 isolate SARS-CoV-2 propagations and real-time PCR and fifty-percent tissue culture infective dose ($TCID_{50}$) assay for food seeding performed in 96-well plate and measured by the Reed–Muench method. K1 isolate with log $TCID_{50}$/mL (virus titer) ~7 and CT value 10 selected for seeding into food.

2.3.3 | Artificial contamination of various kinds of milk products

The treatments were contaminated under conditions presented in Table 2.

2.3.4 | Virus concentration and evaluation its infectivity (virus titer) by $TCID_{50}$ assay

In this study, polyethylene glycol (PEG) precipitation was used to concentrate the virus and remove cytotoxic agents and/or PCR inhibitors from food samples. 10 mL of each sample (9 mL sample + 1 mL virus) was mixed with 1.5 mL of the PEG 6000 stock solutions. The suspensions were agitated on a shaking incubator at 150 rpm for 8 hr at 4°C, and the supernatant was transferred into the centrifuge tube. Then, they were centrifuged at 3,635g for 50 min. The PEG-containing supernatants were removed, and the resulting pellet was dissolved in 1 mL phosphate-buffered saline (PBS) and recentrifuged at 4,173g for 40 min. The resulting supernatant was passage through a 0.2 μm sterile membrane filter and added to the both 96-well plates and cell culture flasks for each test group. The virus was titrated in serial 1 log dilutions (from 1 log to 10 log) to obtain a 50% tissue culture infective dose (TCID50/mL) on 96-well culture plates of Vero cell line. The plates were observed every 24 hr for a total of 6 days for the presence of CPEs (cytopathic effects) employing an inverted optical microscope. The end-point titers were calculated according to the Reed–Muench method (Reed & Muench, 1938) based on four replicates for each food titration. In parallel in the cultured flasks, after seeding selected dilution of virus (base on TCID50 in micro-titration) they were slowly agitated on a shaking incubator at 37°C for adsorbtion. Finally, the upper phase was discarded, and the medium culture with a little FBS (%1) was added. All the cultured flasks similar to micro-titration were monitored every 24 hr for CPE (cytopathic effect) until 6 days.

2.3.5 | Extraction of viral RNA by RT-PCR

All real-time RT-PCR reactions were performed in the Rotor-Gene-Q 6000 thermocycler (Corbett, Australia) based on the recommended

TABLE 1 The pH, titratable acidity, protein, and fat contents of different treatments contaminated with the virus

Dairy products	Parameters			
	Protein content (%)	Fat content (%)	pH	Titratable acidity (°D)
Low-fat milk	3.36 ± 0.12	1.14 ± 0.21	6.35 ± 0.01	19.71 ± 0.44
Full-fat milk	3.29 ± 0.23	3.22 ± 0.16	6.39 ± 0.02	18.11 ± 0.53
Doogh	1.26 ± 0.13	0.61 ± 0.08	Initial: 3.48 ± 0.02 Final: 3.41 ± 0.01	141.56 ± 2.03
Cream		33.76 ± 0.15	6.39 ± 0.02	12.77 ± 0.29
Ice cream	3.47 ± 0.28	10.19 ± 0.07	6.62 ± 0.02	16.82 ± 0.26

Treatments	Sample size	SARS-CoV-2 addition dose	Storage condition (°C)
Raw milk	9 mL	~6 $TCID_{50}$/mL	4
Cream	9 mL	~6 $TCID_{50}$/mL	4
Doogh	9 mL	~6 $TCID_{50}$/mL	5
Ice cream	9 g	~6 $TCID_{50}$/g	−20 or −80

TABLE 2 Contamination conditions of various kinds of dairy products

Abbreviation: $TCID_{50}$, fifty-percent tissue culture infected dose.

Product	Pasteurization temperature (°C)	Pasteurization time	Initial virus titer (log $TCID_{50}$/mL)	Post pasteurization virus titer (log $TCID_{50}$/mL)
Low-fat milk	63	30 min	5.65 ± 0.06	ND
Low-fat milk	72	15 s	5.65 ± 0.05	ND
Low-fat milk	80	15 s	5.65 ± 0.07	ND
Full fat milk	63	30 min	5.65 ± 0.06	ND
Full fat milk	72	15 s	5.65 ± 0.04	3.8 ± 0.03
Full fat milk	80	15 s	5.65 ± 0.05	ND
Cream	90	1 min	5.65 ± 0.04	ND

TABLE 3 The effect of different types of pasteurization on the stability of SARS-CoV-2 in dairy products

Abbreviations: LoD, limit of detection = 38 copies/mL; ND, not detected; $TCID_{50}$, fifty-percent tissue culture infected dose.

protocol. Concisely, LightMix SarbecoV E-gene plus EAV control was used (Tabibzadeh et al., 2020) (Roche, Germany). The following ingredients were utilized: 10 μL extracted RNA or blank, 0.1 μL RT enzyme, 4 μL Roche MasterMix, 0.5 μL primer-probe mix, and 5.4 μL deionized RNase DNase free water. The heating program was 3 s at 55°C, and 30 s at 95°C (1 cycle); 3 s at 95°C, and 12 s at 60°C (45 cycles); and 10 s at 40°C (1 cycle).

2.4 | Statistical analysis

The experiments as well as measurements accomplished in triplicate. Data were analyzed using SPSS software version 26 with a confidence level of 95% to recognize any significance between treatments. Statistics paired t tests were used to evaluate differences in viral titer between the initial count and after the pasteurization process. A repeated measure ANOVA was used to analyze the data obtained from shelf life study.

3 | RESULTS AND DISCUSSION

3.1 | Effect of the pasteurization process

Table 3 illustrates the influence of disparate types of pasteurization on the survival of SARS-CoV-2 in dairy products. According to this table, heating at 63°C and 80°C for 30 min and 15 s, respectively, led

to the destruction of viral particles, which inhibited infectivity. Although heating to 72°C for 15 s (HTST method) was adequate for the inactivation of SARS-CoV-2 in the low-fat milk, exposure to the latter treatment was not effective in eradicating the virus in the full-fat one. More to the point, there was only ~34.5% (from 5.8 to 3.8 log $TCID_{50}$/mL) reduction in the initial viral load of the full-fat milk after heat treatment. This could be attributed to the higher fat content of full-fat milk that presumably protected SARSCoV-2 towards heat, similar to the results from the heat inactivation of hepatitis A (HAV) virus in different dairy products (Bidawid, Farber, Sattar, & Hayward, 2000). Consequently, the routine pasteurization process (HTST) was insufficient to inactivate the viral load in the full-fat milk fully. In contrast, in the cream sample, the complete inactivation of SARS-CoV-2 (initially 5.6 log $TCID_{50}$/mL present) was achieved at 90°C for 1 min, and the virus could no longer be detected.

Infectivity of viruses depends on both capsid proteins and nucleic acids. Processing may cause virus inactivation via the destruction of capsids or by the destruction of the genomes. Occurring alteration in capsids may influence the virus's stability or viral attachment to the host cell receptors. Demolition of the genome hinders replication in host cells. Generally, the inactivation of viruses by heat can be due to the alterations in their capsids (Hirneisen et al., 2010). Not only the membrane protein (M protein) of SARS-CoV is aggregated by heat processing, but also at 35°C, the nucleocapsid protein of SARS-CoV begins to unfold and is denatured entirely at 55°C (Kampf, Voss, & Scheithauer, 2020; Wang et al., 2004). In a study performed to determine the durability of SARS-CoV-2 at various temperatures, the

results indicated that as temperature increased, the time for virus inactivation decreased. The virus remained detectable for 14, 7, and 1 day in the medium culture at 4, 22, and 37°C, respectively. When the incubation temperature increases to 56 and 70°C, the virus becomes undetectable after 30 and 5 min, respectively (Chin et al., 2020).

Bidawid et al. (2000) investigated the thermal inactivation of HAV in cream, homogenized milk, and low-fat milk, which contained 18, 3.5, and 1% fat, respectively. They demonstrated that the higher the fat content, the more increased heat resistance of HAV, possibly due to the protective functions of fat. In other words, at any particular temperature, higher exposure times were required for dairies with more fat contents to obtain a specific decrement in HAV load. In a study performed to determine the inactivation of SARS-CoV-2 in breast milk through pasteurization, the results indicated that the exposure to whether 63 or 56°C for 30 min caused complete inactivation of SARS-CoV-2 in both human milk and the control medium (Walker et al., 2020).

3.2 | Combined effects of low pH and high titratable acidity

Figure 1 shows the alterations in viral titer during 4 weeks of refrigerated storage of Doogh. SARS-CoV-2 in unheated Doogh with pH = 3.48 lost its infectivity after 28 days during refrigerated storage (4°C). Initial 5.7 log $TCID_{50}$/mL virus titer reached 2.6 and 2.2 log $TCID_{50}$/mL, respectively, in 7, 14, and then gradually diminished to under the detection level at Day 28. Additionally, the decrement of pH value (from 3.48 ± 0.02 to 3.41 ± 0.01), as well as the increment of titratable acidity (from 141.56 ± 1.03 to 148.02 ± 1.22), during 28 days of refrigerated storage may also affect the degradation rate of viral particles.

In an experiment that was performed by Weismiller, Sturman, Buchmeier, Fleming, and Holmes (1990), their data suggested that mildly alkaline conditions (pH 8) induce a conformational alteration in
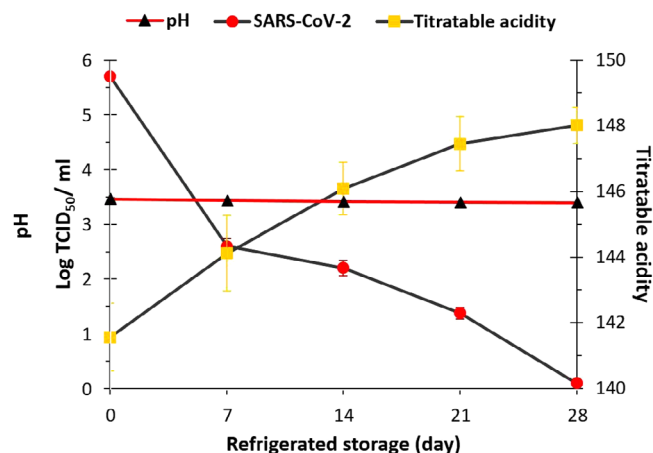
the spike (S) protein of the CoV, mouse hepatitis virus (MHV), that may be associated with the initiation of fusion and infectivity of the viral particle with the host cell. In other research, Xiao, Chakraborti, Dimitrov, Gramatikoff, and Dimitrov (2003) reported that the spike glycoprotein of SARS-CoV fused with the host cell at a neutral pH. However, Darnell, Subbarao, Feinstone, and Taylor (2004) determined the impact of various pH exposures on SARS-CoV infectivity. The virus was fully inactivated either by alkaline (pH > 12) conditions at 4, 25, and 37°C after 1 hr, or acidic (pH < 3) conditions at 25 and 37°C after 1 hr exposure time. At 4°C, pH = 3 did not completely inactivate the virus. While the virus retained its infectivity at moderate variations of pH conditions. Their results illustrate that SARS-CoV infectivity is susceptible to extreme pH conditions. In another work, Wang et al. (2004) demonstrated that the nucleocapsid protein (N protein) of SARS-CoV at a pH near 5 starts to unfold and is completely denatured at a pH near 2.7 acid unfolding process is reversible. Compared to other coronaviruses, SARS-CoV-2 could maintain its durability in a broad spectrum of pH values (pH 3–10) after 1 hr exposure time at room temperature (Chin et al., 2020). It could concisely elucidate the rapid prevalence of SARS-CoV-2 compared to SARS and MERS (Aboubakr, Sharafeldin, & Goyal, 2020). In one research, the high titer of SARS-CoV-2 remained stable under an acidic situation (pH 2.2), which imitating the gastric condition at room temperature for 30s, 30 min, or 60 min (Sun et al., 2020).

3.3 | Effect of freezing and frozen storage

The results of the log $TCID_{50}$/g of ice cream treatments were demonstrated in Figure 2. Under freezing temperatures at either −20 or −80°C, SARS-CoV-2 remained infectious in ice cream samples during 60 days study period. During the first 2 weeks of frozen storage, the virus infectivity did not considerably decline for both treatments. However, the infectivity of ice cream maintained at −20°C was significantly ($p ≤ .05$) higher than that of −80°C (~5.6 vs. 5 log $TCID_{50}$/g for samples stored at −20 and −80°C in the second week after virus

FIGURE 1 Alterations in viral titer during refrigerated storage of Doogh (5°C)
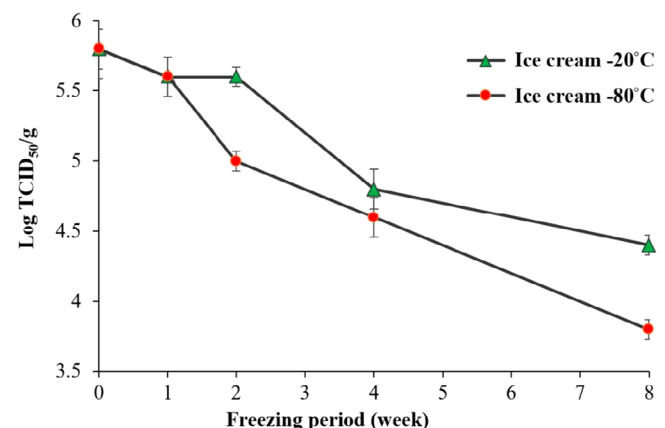
FIGURE 2 Alterations in viral titer of ice cream in frozen storage at −20 or −80°C

seeding, respectively). After Week 2, the infectivity loss was more considerable so that it finally reached ~4.4 and 3.8 after 60 days of storage at −20 and −80°C.

Freezing is considered a conventional food preservation technique to prolong the food's longevity by prohibiting the growth of deteriorative and pathogenic microorganisms. However, a multitude of viruses are stable at the freezing process, and some outbreaks of foodborne disease have been associated with the frozen foods (Amit, Uddin, Rahman, Islam, & Khan, 2017; Thippareddi, Balamurugan, Patel, Singh, & Brassard, 2020). In research performed by Daniel and Talbot (1987), the titer of MHV A59 remained stable after 15 freeze–thaw cycles. Similarly, Lamarre and Talbot (1989) demonstrated that human coronavirus 229E could withstand 25 repeated freeze (−70)/thaw cycles. According to the WHO's declaration, SARS-CoV-2 can maintain survival until 2 years in frozen storage at −20°C (WHO, 2020). At the beginning of July-mid-August 2020 in China, at least nine incidences of contamination of foods by SARS-CoV-2 were reported on imported frozen foods to encompass packaging materials and storage surroundings; in consequence, contaminated cold-storage foods may be a serious risk for the spread of SARS-CoV-2 among different areas (Han et al., 2020).

4 | CONCLUSIONS

In the current research, we have studied the impact of several industrial processing conditions (pasteurization, freezing, pH, and titratable acidity) and the storage time on SARS-CoV-2 stability in some dairy products. The results suggest that thermal processing is the most effective approach to eliminate SARS-CoV-2. Interestingly, SARS-CoV-2 survived HTST pasteurization (72°C, 15 s) in full-fat milk. Therefore, the food compositions, especially fat, possess conspicuous protective effects on the virus's survival. However, the viral infection was hampered entirely by thermal treatment at 80°C/15 s. Therefore, the products with severe heating of initial milk such as yogurt (e.g., 85°C, 30 min or 90°C, 15 min for yogurt milk) would be safe if the initial milk is contaminated. Also, other products (e.g., cheeses) with adequate pasteurization of producing milk would be safe. Raw whole milk, especially those obtained from traditional milking techniques, should be pasteurized at >80°C for 1 min to avoid viral infectivity. As was expected, the freezing and frozen storage processes were ineffective in eliminating viral load even after 2 months. The initial seeding (5.8 log $TCID_{50}$/g) decreased to ~4.4 $TCID_{50}$/g and 3.8 $TCID_{50}$/g for treatments stored at −20 and −80°C, respectively. Then, for ready-to-eat foods, for instance, frozen yogurt, ice cream, and desserts, the manufacturers should be conscious to avoid cross-contamination before the freezing and storage of such foods. Furthermore, the risk of viral contamination and retention in dairy products is significantly higher in nonindustrial (traditional) dairies because of the lower hygienic level, close contact of staff with products, and lack of adequate packaging. This study also suggests that SARS-CoV-2 can remain under acidic conditions in fermented milk products (low pH and high titratable acidity). In case, the virus was infectious for about 3 weeks in Doogh (pH ≤ 3.48 and TA ≥ 141.56°D).

Our study demonstrated that foods could be suitable carriers due to intrinsic protective factors. Further studies are required to interrogate the stability of SARS-CoV-2 in other food matrices.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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