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Inactivation of Murine Norovirus 1 and *Bacteroides fragilis* Phage B40-8 by Mesophilic and Thermophilic Anaerobic Digestion of Pig Slurry[▽]

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Mesophilic (37°C) and thermophilic (52°C) anaerobic digestion of pig slurry induced at least a 4-log decrease in murine norovirus 1, used as a surrogate virus for porcine norovirus, after 13 and 7 days, respectively. *Bacteroides fragilis* phage B40-8, employed as a universal viral model, was lowered by 2.5 log after 7 days. The viral titer declined due to temperature and matrix effects.

Surveillance data collected in 10 European countries pointed out norovirus (NoV) as a major causative agent of nonbacterial gastroenteritis outbreaks and indicated the importance of food-borne transmission (18). Human and animal NoVs might be introduced into the food chain, e.g., on crops, through contact with polluted irrigation water or organic-based fertilizer, or by infected harvesters/pickers not following good hygienic practices (23). Porcine NoVs have frequently been isolated from healthy pigs in Japan, Europe, and the United States (26, 32, 35). Additionally, human and porcine NoV strains were found to occur simultaneously in oysters (11). These porcine NoVs are classified in the same genogroup (GII) as human NoV, due to their genetic and antigenic close affinities (34, 37). Moreover, infectivity of human GII NoV was established in gnotobiotic pigs (9). These observations, and the fact that intragenogroup recombination of human NoV has been reported previously (7), raised the concern about possible recombination between human and porcine strains to new emerging strains.

Digested residue derived from a full-scale biogas plant processing animal waste, such as pig slurry, can be used as fertilizer (22, 33). Pasteurization of the digested residue for 60 min at 70°C before application on land is mandatory in the European Union (13) to prevent remaining microorganisms from being transferred to water and the food chain (10). Alternative treatments are authorized by European legislation if minimal biological risks can be ensured (13, 14). To be validated, the intended process must demonstrate a 5-log decrease in *Enterococcus faecalis* and at least a 3-log reduction in a thermoresistant virus, such as parvovirus, when it is identified as a relevant hazard (14).

In the current study, murine norovirus 1 (MNV-1) was used as a surrogate for porcine NoV to examine the stability toward mesophilic and thermophilic anaerobic digestion of pig slurry. MNV-1 is accepted as a surrogate for human NoV (4, 8). Moreover, MNV-1 is the most closely related NoV strain to human and porcine NoV strains that can be cultivated in the laboratory (32). Additionally, *Bacteroides fragilis* phage B40-8 was examined in the current study as a potential universal model for human enteric viruses. *B. fragilis* phages are present in sewage and have been reported to be stable in the environment, more resistant than F-specific RNA coliphages in surface water, and recalcitrant with respect to water treatments (12, 21, 27, 28). The resistance, the ease of cultivation, and the nonpathogenic properties make *B. fragilis* phages a potential viral model. The advantage of using *B. fragilis* phages is that they can be used, besides as a model for human enteric viruses, as potential viral indicators. Gantzer et al. (15) found a good correlation between the presence of *B. fragilis* phages and enterovirus, while somatic coliphages were poor indicators of fluctuations in enterovirus concentration.

The aim of this study was to examine the effect of mesophilic and thermophilic conditions on MNV-1 and B40-8 in raw pig slurry in order to test the necessity of further pasteurization for safe application as fertilizer. Inactivation in digested pig slurry (DP), pasteurized DP, autoclaved DP, and autoclaved tap water was investigated to assess a possible matrix effect. Inactivation in the liquid phase was compared to that in the solid phase to examine whether protection of virus particles took place due to adsorption to solid matter. Inactivation of B40-8 was examined by plaque assay to assess viability, while MNV-1 was studied using real-time reverse transcription-PCR (RT-PCR). Monpoeho et al. (19) demonstrated that inactivation treatments of wastewater sludge induced similar declines in infectious enteroviruses and genomic copies. Consequently, RT-PCR was found appropriate by Monpoeho et al. (19) to check the virological quality of sludges and was therefore applied in the current study.

Raw pig slurry was inoculated with B40-8 lysate and MNV-1 lysate to obtain a final concentration of approximately 4 to 5 log PFU/ml of both viruses. This raw pig slurry (60%) supple-

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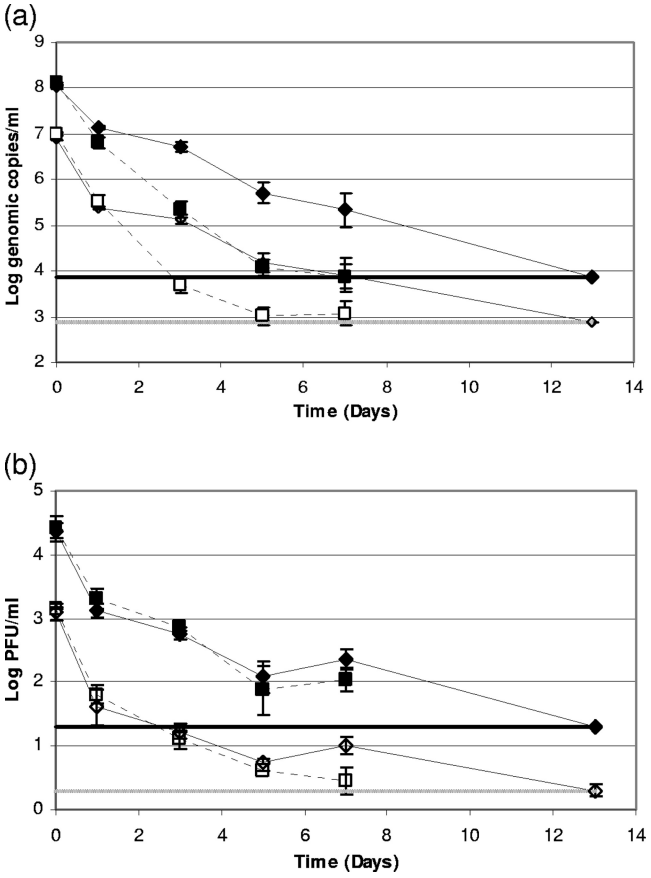

FIG. 1. Reduction in MNV-1 genomic copies/ml (a) and *B. fragilis* HSP40-infecting phage B40-8 PFU/ml (b) by mesophilic (37°C) anaerobic digestion (liquid [◆] and solid [◇] phases) and by thermophilic (52°C) anaerobic digestion (liquid [■] and solid [□] phases) of raw pig slurry. Experiments were performed in duplicate. Error bars represent standard deviations (Excel version 2003; Microsoft, Redmond, WA). Detection limits for the liquid phase and the solid phase are depicted by a solid black line and a gray line, respectively.

mented with different organic feed stocks (40%) is used as the influent in three thermophilic reactors of a full-scale biogas plant (Bio-Electric, Beernem, Belgium) performing anaerobic digestion. The B40 lysate and the MNV-1 lysate stocks were obtained as described by Baert et al. (6). After inoculation, bottles were flushed with 100% N_2 for 1 h and incubated for 13 days at 37°C or for 7 days at 52°C. Samples, taken at 0, 1, 3, 5, 7, and 13 days, were 10× diluted in phosphate-buffered saline (PBS) (Cellgro; Mediatech, Herndon, VA) and centrifuged at 855 × *g* for 20 min (Heraeus Instruments, Hanau, Germany) at room temperature. The supernatant, i.e., the liquid phase, was transferred into a new microcentrifuge tube and further used for MNV-1 detection by real-time RT-PCR (5) and B40-8 detection by double-agar-layer plaque assay (2). MNV-1 and B40-8 were extracted from the pellet, i.e., the solid phase, according to the procedure described by Arraj et al. (3), with some modifications. Briefly, 1 ml of elution buffer (3% beef extract [Sigma, St. Louis, MO], 10 mM Tris [Sigma], 50 mM glycine [Sigma], pH 9.5) was added to the solid phase. The mixtures were placed on a shaking platform (HS 260; Janke & Kunkel & Co., IKA Labortechnik, Staufen, Germany) rotating at 120 rpm for 10 min. After 0.3 ml of 1,1,2-trichlorotrifluoroethane (Janssen Chimica, Geel, Belgium) was added, the sample mixtures were vortexed for 90 s, left at room temperature for 20 min, and placed in an ultrasonic bath (Transsonic 460/H; Elma, Singen, Germany) for 2 min. Samples were centrifuged at 380 × *g* for 15 min at room temperature; MNV-1 genomic copies/ml and B40-8 PFU/ml were determined by real-time RT-PCR (5) and by double-agar-layer plaque assay (2), respectively, in the supernatant which contained the virus particles from the solid phase.

Inactivation of MNV-1 and B40-8 by mesophilic and thermophilic anaerobic digestion of raw pig slurry. Mesophilic anaerobic digestion (37°C) of raw pig slurry induced more than a 4-log decrease in MNV-1 genomic copies/ml (Fig. 1a), whereas a reduction of at least 3 log B40-8 PFU/ml (Fig. 1b) was observed after 13 days. Other studies have demonstrated that mesophilic anaerobic digestion of sludge prior to agricultural recycling did not enable more than a 1- to 2-log decrease in spiked bacterial microorganisms (16). Naturally occurring viruses were decreased by only 1 log after mesophilic anaerobic digestion of sludge (19). Thermophilic anaerobic digestion (52°C) decreased MNV-1 genomic copies by more than 4 log in raw pig slurry after 7 days (Fig. 1a). B40-8 infectious units were lowered by 2.5 log in raw pig slurry after 7 days at 52°C (Fig. 1b). *D* values at both temperatures were calculated and are presented in Table 1. It seems that MNV-1 and especially B40-8 were more persistent during thermophilic anaerobic digestion of pig slurry than rotavirus and coxsackievirus in sludge (25) and African swine fever and swine vesicular disease viruses in pig slurry (30). It should be noted that the comparison of the outcomes of the mentioned studies with that of our study is difficult due to different experimental setups and matrices.

A decline in MNV-1 genomic copies in pig slurry was observed, in contrast to previous observations in which no decrease was determined by real-time RT-PCR in cell culture medium, even after heat treatment at 80°C (5). Negative results obtained for MNV-1 by real-time RT-PCR were evaluated for the presence of possible inhibiting compounds in the RNA extracts by spiking cDNA derived from the negative sample with 10^5 copies of the plasmid p20.3 containing a full-

TABLE 1. *D* values for B40-8 and MNV-1 determined by mesophilic (37°C) and thermophilic (52°C) anaerobic digestion of pig slurry matrices

Matrix	Temp (°C)	*D* value (R^2)[a]	
		B40-8	MNV-1
Raw pig slurry	37	3.9 (0.84)	2.6 (0.98)
	52	3.0 (0.82)	1.3 (0.99)
DP	37	9.1 (0.89)	2.6 (0.99)
	52	3.2 (0.87)	0.8 (0.96)
Pasteurized DP	37	10.2 (0.89)	2.4 (0.99)
	52	2.3 (0.93)	1.3 (0.85)
Autoclaved DP	37	2.7 (0.88)	0.8 (0.88)
	52	1.0 (0.85)	ND[b]

[a] *D* values (days) were calculated from two replicates of the liquid phase of pig slurry matrices by linear regression (SigmaPlot 2000 for Windows).
[b] ND, not determined.

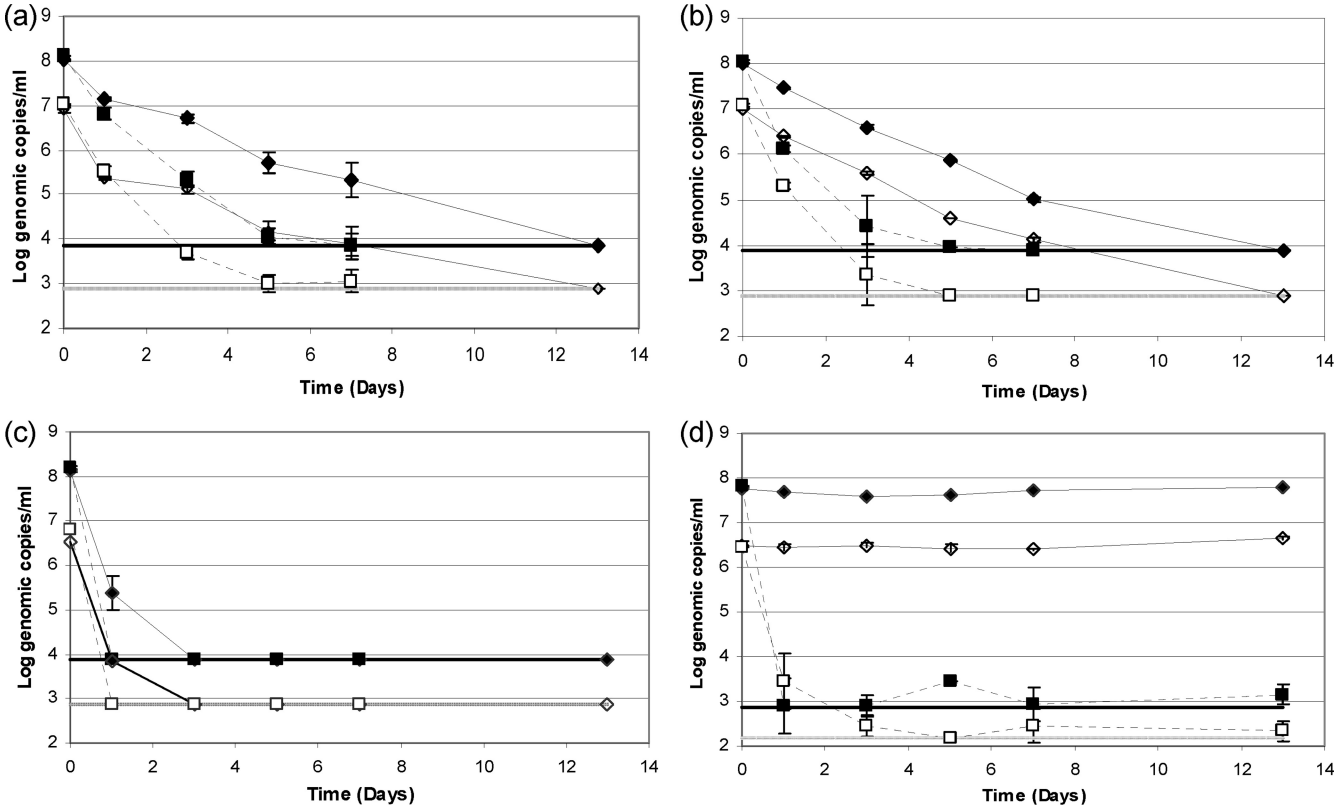

FIG. 2. Reduction in MNV-1 genomic copies/ml by mesophilic (37°C) anaerobic digestion (liquid [◆] and solid [◇] phases) and by thermophilic (52°C) anaerobic digestion (liquid [■] and solid [□] phases) of digested pig slurry (a), pasteurized digested pig slurry (b), autoclaved digested pig slurry (c), and autoclaved tap water (d). Experiments were performed in duplicate. Error bars represent standard deviations (Excel version 2003; Microsoft, Redmond, WA). Detection limits for the liquid phase and the solid phase are depicted by a solid black line and a gray line, respectively.

length cDNA clone of MNV-1.CW1 (24). None of the samples showed inhibition at any time point analyzed, because the threshold cycle (C_T) value of the spiked negative sample was similar to the C_T value of 10^5 copies of the plasmid p20 (data not shown). Real-time RT-PCR was therefore found a suitable detection method for pig slurry, in accordance with the findings of Monpoeho et al. (19). Results should be interpreted as a "worst-case" virus stability, i.e., in practice, the real decline in infectivity might be faster.

In the current study, the decay of particle-associated viruses was examined by using a virus recovery method similar to that described by Arraj et al. (3) for activated sludge. These authors found similar concentrations of hepatitis A virus and poliovirus in the liquid and solid phases but observed a higher viral log reduction in the liquid phase than in the solid phase. Yet, higher numbers of MS2 (single-stranded RNA [ssRNA] coliphage) were observed in the liquid phase than in the solid phase. In the present study, the majority of MNV-1 and B40-8 virus particles were present in the liquid phase, and inactivation rates were generally similar in the liquid and solid phases of pig slurry, indicating no additional protection due to the adsorption to solid particles.

Inactivation of MNV-1 genomic copies by a temperature effect was established, because incubation at 52°C resulted in a more rapid decrease than incubation at 37°C. In the case of B40-8, a similar decline was observed in raw pig slurry exposed to temperatures of 37°C and 52°C.

Influence of the matrix in the decline obtained by mesophilic anaerobic digestion. The effect of the pig slurry matrix during anaerobic digestion processes was investigated by studying virus inactivation in different processed pig slurries, i.e., digested pig slurry (DP), pasteurized DP, autoclaved DP, and autoclaved tap water, as the influence of the matrix has also been reported for other microorganisms (1, 17, 30). The DP (i.e., the effluent from the full-scale biogas plant [Bio-Electric, Beernem, Belgium]), pasteurized DP (70°C for 30 min), and autoclaved DP (121°C for 20 min) were inoculated similarly to the raw pig slurry. The *D* values for MNV-1 genomic copies and B40-8 infectious units in DP, pasteurized DP, and autoclaved DP are presented in Table 1. At 37°C, MNV-1 genomic copies were decreased similarly in raw pig slurry (Fig. 1a) and DP and pasteurized DP (Fig. 2a, b, and c). This means that the genomic breakdown was not affected by the matrix. Accordingly, B40-8 infectious units showed similar inactivation rates in DP and pasteurized DP (Fig. 3a and b). Yet, a greater decline in B40-8 was obtained in raw pig slurry (Fig. 1b). A pasteurization process applied to DP (30 min at 70°C) inactivates generally the most vegetative bacterial cells, except spores (22), resulting in a matrix with a microbial load lower than that of nonpasteurized DP. This observation indicated

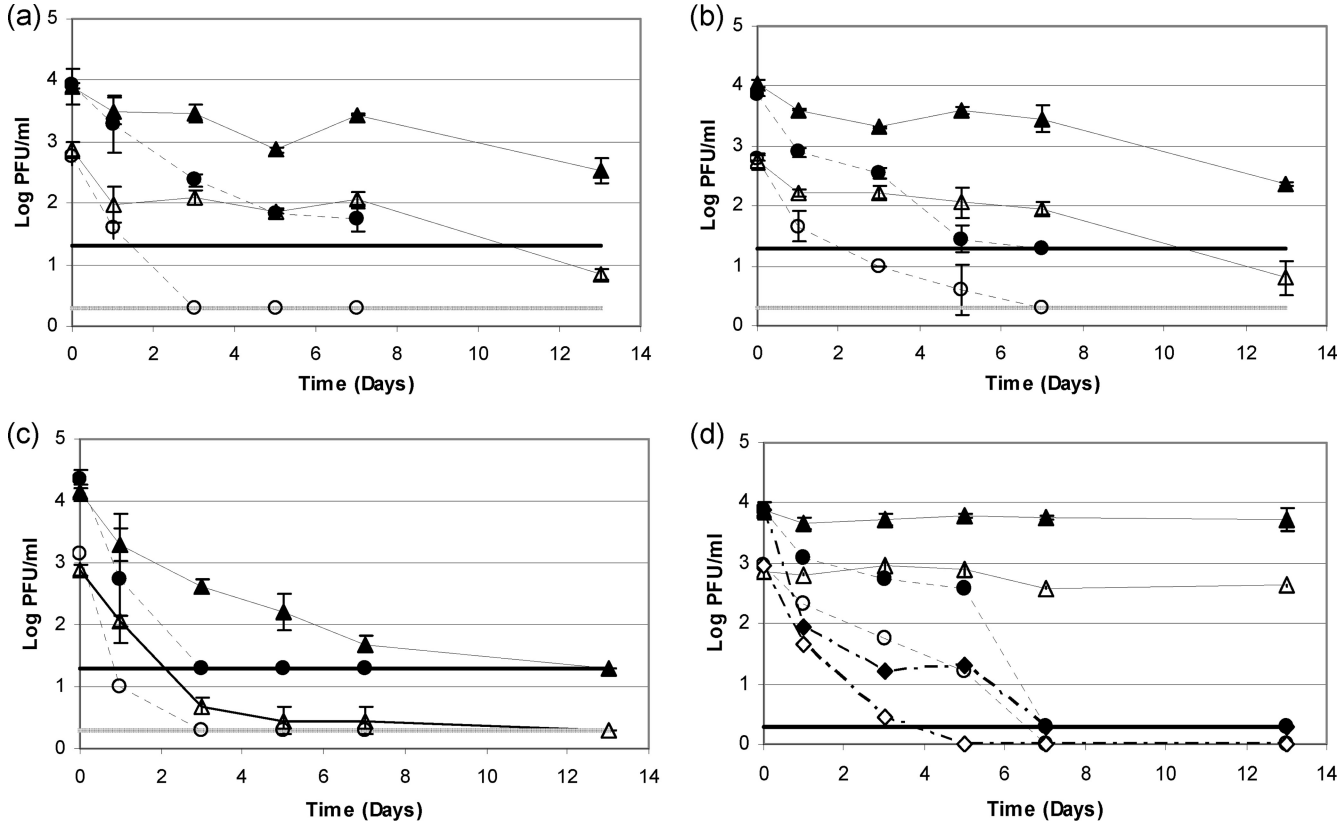

FIG. 3. Reduction in *B. fragilis* HSP40-infecting phage B40-8 PFU/ml by mesophilic (37°C) anaerobic digestion (liquid [▲] and solid [△] phases) and by thermophilic (52°C) anaerobic digestion (liquid [●] and solid [○] phases) of digested pig slurry (a), pasteurized digested pig slurry (b), autoclaved digested pig slurry (c), and autoclaved tap water (d). Experiments were performed in duplicate. Error bars represent standard deviations (Excel version 2003; Microsoft, Redmond, WA). The reduction in B40-8 PFU/ml in tap water is shown as the means from two replicate samples at 37°C (liquid [▲] and solid [△] phases), whereas the replicates at 52°C are shown separately (liquid [●] and solid [○] phases of replicate 1; liquid [◆] and solid [◇] phases of replicate 2). Detection limits for the liquid phase and the solid phase are depicted by a solid black line and a gray line, respectively.

antiviral activity exhibited by the microbial flora of the raw pig slurry at 37°C. Presumably only the capsid protein of the virus particle was affected, and therefore, no difference in MNV-1 genomic copies was observed in raw and processed pig slurry.

The fastest removal of B40-8 and MNV-1 was obtained using autoclaved DP (Table 1), which suggested the formation of products with virucidal activities during autoclaving. Thermal pretreatments above 100°C prior to anaerobic digestion indeed alter physicochemical parameters (36), and possibly some compounds that may impair the virus stability arise by subsequent heating.

Similarly to the inoculation procedure for pig slurry, autoclaved tap water (15 min, 121°C) was inoculated with MNV-1 lysate and B40-8 lysate. Water samples, without a 10-fold dilution, were treated similarly to pig slurry samples because in the autoclaved tap water, some visible precipitation due to mineral deposits was noticed. No decline in either tested viral model was observed after 13 days at 37°C (Fig. 2d and 3d) in autoclaved tap water, which might be explained by the absence of microbial activity or formed antiviral compounds. These findings indicated not only that virus inactivation in pig slurry was caused by a temperature effect but also that the matrix strongly affected the observed decline in viruses.

Influence of the matrix in the decline obtained by thermophilic anaerobic digestion. At 52°C, similar breakdowns of genomic MNV-1 RNA were observed in raw pig slurry, DP, and pasteurized DP (Table 1). Levels of inactivation of B40-8 infective units were comparable in raw pig slurry and DP (Table 1). A more rapid decline in B40-8 was achieved in heat-treated (pasteurized and autoclaved) DP than in raw pig slurry or DP. Similarly, for MNV-1, the fastest decrease was observed in autoclaved DP. The higher inactivation rate observed in autoclaved DP indicated the influence of the chemical composition of the matrix, as was observed at 37°C. Moreover, protective compounds could be present in nonheated (raw/digested) slurry matrices, resulting in the slower decay at 52°C. The presence in manure, sludge, digested waste, or water of substances that stabilize viral infectivity has also been suggested by others (20, 29, 31).

A fast decrease in MNV-1 genomic copies/ml and B40-8 PFU/ml occurred in autoclaved tap water, which strengthens the hypothesis of particles present in pig slurry matrices being protective against heating to 52°C.

Conclusions. This study demonstrates that virus inactivation was greatly influenced by both the temperature and the matrix. The matrix on the one hand may provide protective substances,

mainly of importance at 52°C, but on the other hand can enhance viral decline due to microbial activity, mainly of importance at 37°C, or formed chemical components having antiviral activity, which was shown in autoclaved DP. The decline in B40-8 and MNV-1 induced by laboratory-scale mesophilic and thermophilic anaerobic digestion processes questions the necessity for performing a pasteurization process on the digested pig slurry, currently mandated by European legislation (13). However, inactivation by the anaerobic digestion processes as such should be explored further using operational bioreactors to determine minimal retention times based on these results. The usefulness of *B. fragilis* phages as a promising viral indicator should be evaluated further by the comparison of their resistance with that of other relevant viral models.

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