applied microbiology

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Lactic Acid Fermentation Inactivates *Salmonella* Typhimurium in Contaminated Cattle Manure at Moderate and Low Temperatures

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Abstract

In the case of a notifiable animal disease like salmonellosis, manure is contaminated and must be disinfected. This can be performed using heat measures, chemical disinfectants, or long-term storage. All these measures bring along severe economic, ecological, and logistical problems. The aim of this study was to evaluate lactic acid fermentation (LAF) as an alternative disinfection method. Fermentation was started by adding a carbohydrate source to the manure and creating anaerobic conditions. For testing, cattle manure was enriched with different carbohydrate (CHO) sources and spiked with *Salmonella* Typhimurium (*S.* Ty.). The samples were incubated at 10 °C and 21 °C for 111 days (Exp1) and at 21 °C for 50 days (Exp2). The microbial shift was determined using cultural methods and MALDI-TOF. Both the change in pH and *Enterococcus* spp. were tested as suitable indicators. The results showed the different suitability of the selected CHO for hygienization by LAF. Using squeezed oat as an additive, *S.* Ty was reduced to below the detection limit under both temperature conditions within 21 days and 14 days. Additional saccharose decreased the reduction time. This study showed that LAF is a valuable alternative for disinfecting cattle manure in the case of bovine salmonellosis. Using this method, both manure and feed residues can be treated in one approach and afterwards be used as fertilizer.

Keywords: lactic acid fermentation; livestock; cattle manure; fertilizer; *Lactobacillus* spp.; *Salmonella* Typhimurium; *Enterococcus* spp.; decontamination

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Academic Editor: Margaret L. Britz

Received: 29 July 2025
Revised: 14 August 2025
Accepted: 15 August 2025
Published: 22 August 2025

Citation: Halm, H.; Woelke, S.; Lehnert, I.; Schilling, T.; Meissner, T.; Schröder, C.; Hoelzle, L.E.; Scheinemann, H.A. Lactic Acid Fermentation Inactivates *Salmonella* Typhimurium in Contaminated Cattle Manure at Moderate and Low Temperatures. *Appl. Microbiol.* **2025**, *5*, 88. https://doi.org/10.3390/applmicrobiol5030088

1. Introduction

Livestock is an important pillar of agriculture. In Germany alone, about 3.9 million dairy cows were registered in 54,300 livestock farms in 2023 [1]. The resulting amount of manure is considerable. On average, one dairy cow produces 1.5–1.8 m^3 of manure per month [2]. This equates to about 70,000 tons of manure per year for dairy cattle in Germany. Usually, manure is returned to the nutrient cycle as fertilizer. Cattle manure is an important source of carbon (C) and nitrogen (N) for agriculture. Nevertheless, there is a potential risk of spreading foodborne diseases [3,4].

Cattle excrement, and thus manure, provides a remarkable substrate for the survival and spread of a wide range of pathogenic and saprophytic bacteria [4,5]. *Salmonella* are one of the most significant bacterial pathogens in foodborne diseases, and therefore they are of public concern [6,7]. In Germany, from 2016 to 2024, between 68 and 109 proven cases per year were described in cattle livestock [8]. These Gram-negative rods survive and multiply extensively in ileal and colon fluids [4,9], and they are finally excreted in cattle feces [10]. Factors such as temperature, lack of nutrients, osmotic stress, and exposure to UV light are known to affect their survival [11–13]. *Salmonella* have high tenacity and are capable of surviving in chicken manure compost for approximately 200 days [9]. Additionally, in aquatic environments, salmonellae have a high survival rate, particularly in highly eutrophicated areas [14]. Therefore, understanding their behavior in agricultural environments is crucial for improving risk evaluations and guidelines for the safe handling of pathogen-contaminated manure, compost, and soil [9,14].

In the case of a salmonellosis outbreak, it is almost impossible to identify the source of the infection [15]. It is necessary to consider the fundamental principles of veterinary medicine, namely the separation of sick from healthy cattle, the maintenance of a high hygienic standard, the treatment and monitoring of sick animals, and the disinfection of contaminated material. During an outbreak of a notifiable animal disease in livestock farms, a large amount of contaminated manure accumulates. Hence, it is important to establish a system of monitoring and decontamination [5]. The standard decontamination methods for biomass according to disinfection guidelines are chemical disinfection, long-term storage for 3 to 6 months, aerobic thermophilic stabilization (T > 50 °C for 3 days, pH > 8.5), and, for certain diseases, the injection of liquid manure into the soil [16]. These methods require different amounts of time and technical equipment.

In this study, lactic acid fermentation (LAF) is tested as an alternative method. Lactic acid bacteria (LAB), like *Lactobacillus* or *Enterococcus*, produce lactic acid by the fermentation of organic matter like food, e.g., [17,18]. Lactic acid as a product is widely used in various industries such as food and animal feed [17–19], textiles and cosmetics [18], and pharmacy [18,19] and in the production of bioplastics [20]. LAF is also used for the production of volatile fatty acids (VFAs) [21]. Here, the initial pH and fermentation time had an important effect on the production of metabolites [21]. Cow manure contains an abundant lignocellulosic fraction, which can be valorized as the raw material for several fermentative processes, including the production of lactic acid (LA) using LAB [20,22].

To activate LAF on animal farm manure for the purpose of hygienization, anaerobic conditions and a carbon source are needed [3]. An increasing number of LAB produce lactic acid and other VFAs so that cattle manure is acidified. Depending on the pH, VFAs can be bactericidal [23–25]. This prevents oxidation and thus the growth of pathogens [26]. Earlier studies showed that pathogens were significantly reduced by LAF, and cattle manure can be returned to nutrient circulation [3]. *Enterococci* were discussed as an indicator organism for the fermentation process. Furthermore, it was shown that LAF has a good N and C outcome [3]. The mass loss during fermentation (<2.45%) is much lower compared to the mass loss through composting (15–57%), making it a cost-effective and ecologically beneficial approach [3]. Knowledge of the microbial community is necessary to improve the fermentation process [21].

The current study was conducted to determine the effect of temperature and carbon source on the inactivation of *S.* Typhimurium (*S.* Ty.) in cattle manure by LAF. The influence of outside conditions during the year were simulated by different temperatures according to the testing guidelines of the German Veterinary Medical Society [27]. Different carbohydrates (CHO) were compared, and the shift in the microbial community was investigated with both cultural methods and MALDI-TOF. The hypothesis that enterococci can be monitored as an indicator organism for the fermentation process was tested.

2. Materials and Methods

2.1. Sample Source and Preparation

The cattle manure (CM) used in this study was collected from a slurry store (cattle) from University of Hohenheim, Stuttgart, Germany. For sample preparation, hay cobs (Sniff Spezialdiäten GmbH, Soest, Germany), squeezed oat (Mühle Richter, Mockrehna, Germany), and saccharose (Raffinadezucker Diadem, Nordzucker, Braunschweig, Germany) were added to the cattle manure, according to Table 1, as sources of carbohydrates (CHO). The content of squeezed oat differs between the two setups. After mixing thoroughly, aliquots of 27 g were weighed into 50 mL tubes (Sarstedt, Nümbrecht, Germany) and frozen at −18 °C until further processing.

Table 1. Overview of kind and amount of added carbohydrates in different approaches in Exp1 (a 21 °C, b 10 °C) and Exp2. Temperatures are indicated in italics. Abrv.: CM—cattle manure; HCs—hay cobs; SO—squeezed oat; Sac—saccharose.

Approach	Abrv.	Replicates Per Approach				Liquid Manure
		Exp1 a	Exp1 b	Exp2	pH [1]	
Incubation temperature		*21 °C*	*10 °C*	*21 °C*	*21 °C*	
CM	CHO-A	7×	7×	14× [2]	3×	100%
CM + 6.2% HCs	HA	7×	7×			93.8%
CM + 10% SO	OA			7×	3×	90%
CM + 18.75% SO	OA	7×	7×			81.25%
CM + 10% Sac	SA			7×	3×	90%
CM + 10% SO + 10% Sac	OSA			7×	3×	80%

[1] parallel to Exp2; [2] 7x blank, 7x spiked with *S.* Ty.

2.2. Experimental Design

To test the influence of temperature and different CH sources, two types of setups were chosen. In Exp1 the dependence of LAF on temperature and hay cobs and squeezed oat as carbon sources was investigated. The effect of sugar and squeezed oat on the fermentation process was investigated in Exp2.

For Exp1 and Exp2, an inoculum of *Salmonella enterica* subsp. *enterica* ser. Typhimurium (*S.* Ty.) was grown in an overnight culture in tryptone soy broth (Oxoid, Wesel, Germany) at 36 °C. To determine the initial cell concentration, a dilution series to 10^{-12} was prepared and spread out on Xylose–Lysin–Desoxycholat agar plates (Roth, Karlsruhe, Germany; in the following, it is called XLD agar) [28] and *Salmonella*-*Shigella* agar (Sifin, Berlin, Germany; in the following, it is called SS agar) (Exp1) [28,29] or water-blue–metachrome-yellow lactose agar acc. to Gassner (modified, Sifin; Exp2; in the following, it is called Gassner agar) [30] in triplicate. The colonies of *S.* Ty. on XLD agar plates were characterized by a black dot in the center [31]. Unspecific colonies were not counted. After incubation at 36 °C overnight, colony-forming units (CFU) were counted, and cell concentration was calculated to $\log_{10}$ (CFU/g sample).

For every approach, seven replicates of prepared cattle manure were thawed, as shown in Table 1. Three milliliters of a cell suspension (5×10^9 CFU/mL) of *S.* Ty. was added to 27 g of each aliquot of the sample and mixed thoroughly. PBS was added to negative controls instead of the *Salmonella* suspension to achieve a comparable moisture content. For anaerobic conditions, the samples were placed into an anaerobic jar (Oxoid) with an anaerobic kit (Anaerocult A, Millipore, Merck, Darmstadt, Germany) and incubated at 21 °C or 10 °C according to Table 1.

The concentrations of the microorganisms were determined immediately after inoculation (0 d) and after 3, 7, 14, 21, 30, 50, 63, 84, and 111 days for Exp1 and after 0, 3, 7, 14, 21, 28, and 50 days of incubation for Exp2 by taking 0.5 g of each replicate and diluting it in 4.5 mL

PBS. A dilution series was prepared from each sample on a 48-well plate (Corning Inc., Corning, NY, USA). In every dilution step, 20 µL was plated in duplicate on the following agar plates: Columbia agar with 5% sheep blood (ISG Intermed, Geesthacht, Germany) for the identification of aerobic and simultaneously anaerobic organisms [32], Gassner agar for detecting Gram-negative organisms, XLD agar for the detection of *Salmonella* and *Shigella*, Citrate–azide–tween–carbonate agar (CATC, Sifin) to identify *Enterococcus* [33,34], and MRS agar (Oxoid) to screen the growth of lactic acid bacteria [35,36]. The plates were incubated under aerobic conditions, except the MRS agar plates and one of the blood agar plates, which were incubated under anaerobic conditions as described above. All agar plates were incubated for 24 h, except the CATC and MRS plates which were incubated for 48 h.

For the detection of sulfite-reducing microorganisms such as clostridia, 0.5 g of the treated sample was diluted in 4.5 mL PBS. A dilution series 10^{-2} to 10^{-7} on liquid differential reinforced clostridia medium (DRCM, Roth, Germany) was performed on a 96-well plate (U-well, Corning) and cultivated at 37 °C under anaerobic conditions [37,38]. For the estimation of sporulated sulfate reducers, the samples were heated for 10 min at 80 °C on a thermocycler (thermomixer comfort, Eppendorf, Hamburg, Germany). Samples were treated as described above. All plates were incubated for six days. The results were analyzed as described by Scheinemann et al. [3].

2.3. pH Measurement

In Exp2 for pH measurement, three sample tubes of each approach were incubated according to Table 1 without the addition of *Salmonella*. On all sampling days, pH was measured with an Orion 3 star pH Benchtop (Thermo Electron Corporated, Karlsruhe, Germany).

2.4. Salmonella Enrichment

To confirm the removal of *S.* Ty. from samples, an enrichment of *Salmonella* according to the Biowaste Ordinance was performed [39–41]. Briefly, 270 g of prepared cattle manure according to Exp1 was inoculated with *S.* Ty. and incubated at 10° or 21 °C for 80 days. Then, 50 g of each bottle was sampled for further processing. From all sample tubes of one approach from Exp2, 7 g of each aliquot was pooled to obtain about 50 g per approach. All samples were diluted in 450 mL peptone water (buffered, Roth) with novobiocin (Roth) and incubated at 37° overnight. From each sample bottle, 100 µL of suspension was transferred into ten tubes with 9.9 mL Rappaport–Vassiliadis Bouillon (Roth). Five tubes were then incubated at 36 °C and five tubes at 42 °C overnight. From each tube, 200 µL was placed on three XLD agar plates and three Gassner agar plates. Plates were incubated at 36 °C for 48 h and monitored for growth. The test was considered successful if no salmonellae were detectable in the collection samples.

2.5. MALDI-TOF

For the detection of the dominant groups of microorganisms, MALDI-TOF measurements were carried out [42]. Fourteen tubes of each of CM, CM + SO, and CM + HC were incubated, seven at 21 °C, seven at 10 °C, without the addition of *Salmonella* serovars. Samples were taken after 0, 3, 7, 14, 21, 30, 50, 63, and 100 days and treated as described above. Colonies from blood agar, CATC agar, XLD agar, Gassner agar, and MRS agar plates were picked randomly for MALDI-TOF MS analysis. Cells of each strain were aseptically transferred to microtubes with 300 µL of deionized water and mixed thoroughly. Subsequently, 900 µL of ethanol was added, mixed thoroughly, and centrifuged at the maximum speed (21,100× *g*) for two minutes. The supernatant was discarded, and the tubes were centrifuged again. The remaining ethanol was discarded by pipetting, and the pellet was air-dried for two to three minutes. Then, 70% formic acid was added and resuspended by pipetting. Pure ACN was added, softly mixed, and centrifuged at the maximum speed for two minutes. After that, 1 µL of supernatant was transferred to a MALDI target plate and air-dried. The samples were spotted with 1 µL HCCA solution within 1 h and air-dried. Each MALDI-TOF sample was spotted in triplicate to evaluate reproducibility. Samples were then analyzed in a MALDI-TOF ultraflextreme LT spectrometer (Bruker Daltonics, Bremen, Germany), using the MALDI Biotyper 3.0 automatic system. Bruker-Database with MBT-Compass 4.1-Software was used for analyses.

3. Results

3.1. Temperature Experiment

The inactivation time of *S.* Ty. depends on the temperature and the carbon source (Figure 1). At 21 °C, the process of LAF and the decrease in Salmonella were achieved much faster in all of the different test conditions (Figure 1). In the approach with squeezed oat (OA), the number CFU/g sample of *S.* Ty. dropped below the detection limit after 15 days and remained there. In the approach enriched with hay cobs (HA), the CFU/g of *S.* Ty. decreased to log 1.2 ± 2 after 30 days and was not detectable after 50 days. The numbers of *S.* Ty. fell to log 1.0 ± 1.8 after 50 days in the non-enriched approach CHO-A. The enrichment of *S.* Ty. in OA after 80 days was negative. Thus, it was shown successfully that *S.* Ty. bacterial count was reduced in the samples with OA (Table 2).

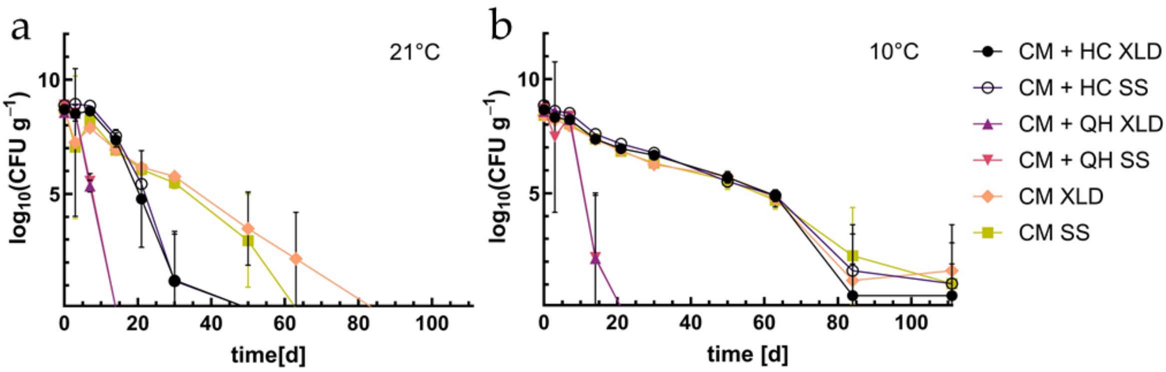

Figure 1. EXP1: Survival of *S.* Ty. in cattle manure in different CHO approaches in log10 on XLD and SS agar (**a**) at 21 °C and (**b**) at 10 °C. Abrv.: CM—cattle manure; HCs—hay cobs; SO—squeezed oat.

Table 2. Overview of results of Salmonella enrichment: (+)—detection of Salmonella in sample; (−)—negative result. Abrev. as in Figure 1.

	Temperature Experiment (EXP1)			Saccharose Experiment (EXP2)	
Incubation temperature	10 °C	10 °C	21 °C	21 °C	21 °C
day of sampling	80 d	110 d	80 d	50 d	75 d
CM + Sal	+	+	+	+	+
CM + HCs + Sal		+			
CM + SO + Sal	−	−	−	+	−
CM + Sac + Sal				−	
CM + OA + Sac + Sal				−	

At 10 °C, in approaches with hay cobs (HA) and without additional carbohydrates (CHO-A), *S.* Ty decreased from log 9 to log 5 within 60 days without significant differences between the approaches. After 111 days they were at the detection limit but still detectable on agar plates. OA decreased to log 2.1 ± 0.2 after 21 days and was undetectable afterwards. Nevertheless, the enrichment of *S.* Ty. of OA resulted in one positive sample after 80 days and showed that *S.* Ty. was still present in small numbers in the sample.

3.2. Saccharose Experiment

Changes in the microbial community depending on the carbon source (Exp2) are shown in Figure 2. Aerobically and anaerobically incubated blood agar plates provided a general overview of the microbial community and its development (Figure 2a,b). After 3 days, an increase in CFU/g was observed in all approaches, followed by a steady decrease to log 6 to log 7. The increase and decrease were visible in all approaches and under both incubation conditions.

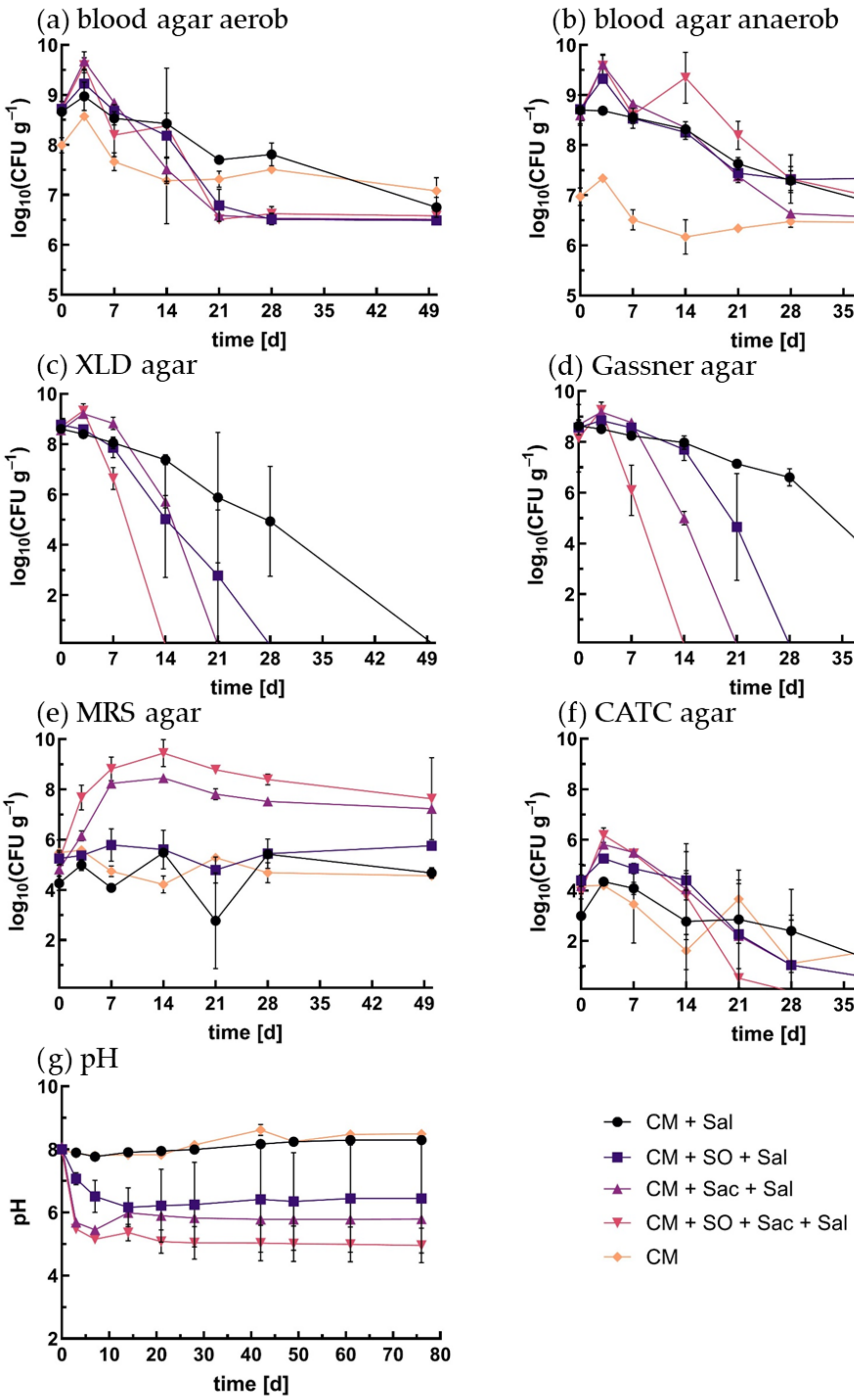

Figure 2. Results of Exp2 CFU/g of microbial community in $\log_{10}$ at 21 °C: (**a**) blood agar incubated under aerobic conditions, (**b**) blood agar incubated under anaerobic conditions, (**c**) XLD agar, (**d**) Gassner agar, (**e**) MRS agar, (**f**) CATC agar, (**g**) pH. Abrev.: CM—cattle manure; Sal—*S.* Ty.; SO—squeezed oat; Sac—saccharose.

The initial CFU/g of the non-spiked approach (NSA) of aerobic and anaerobic incubations were log 8.0 ± 0.2 and 7.0 ± 0.2, respectively, while the *S.* Ty.-enriched approaches were at log 8.7 and log 8.6 under aerobic and anaerobic conditions, respectively. The significant difference in log CFU/g at T0 was due to the addition of Salmonella. Under aerobic conditions, the CFU values of the CHO-enriched approaches dropped below those of the NSA after 14 days, while under anaerobic conditions, the CFU/g of the NSA remained the lowest.

The saccharose approach (SA) and squeezed oat–saccharose approach (OSA) reached the maximum values of the total microbial abundance up to log 9.6 ± 0.2 and 9.7 ± 0.2 CFU/g, respectively. CHO-A showed the same trends with lower values. The anaerobically incubated blood agar plates revealed the same pattern of increase and decrease in CFU/g. In contrast to the aerobic incubation, the OSA had a second peak after 14 days before a constant decrease to log 7.

In the NSA, all specific colonies of *S.* Ty. grew on both agar types. In the spiked approaches, the initial concentrations were about log 8.5 ± 0.2 (Figure 2c,d). In the OSA the number of colonies increased slightly after three days, followed by a rapid decrease, and they were not detectable after two weeks anymore. Similarly, *S.* Ty. was not detectable in SA after three weeks and in OA after four weeks. In CHO-A, the number of CFU also decreased but was still detectable until the end of sampling.

The enrichment of *S.* Ty in all approaches of Exp2 after 50 days showed negative results for SA and OA but positive results for OA and CHO-A (Table 2). The repetition on day 75 showed negative results for OA but still remained positive for CH-A.

The initial concentrations of lactobacilli were between log 4.2 and 5.2 CFU/g (Figure 2e). The maximum values of log 8.5 ± 0.1 for SA and log 9.4 ± 0.5 for the OSA were reached after 14 days. Afterwards, the number of CFU/g decreased to log 7.2 ± 0.0 and 7.3 ± 1.6 at the end of the experiment, respectively. In OA the number of CFU/g increased slightly and dropped to a minimum of log 4.8 ± 0.5 after 21 days and increased again to 5.8 ± 0.1 after 50 days. The two non-enriched approaches showed oscillating numbers of CFU/g between log 4.2 ± 0.3 and 5.6 ± 0.2 for the NSA and log 5.5 ± 0.1 and 2.8 ± 1.9 for CHO-A.

The occurrence of *Enterococcus* spp. (CATC agar) showed different numbers of CFU between the approaches at day 0 (Figure 2f). The initial CFU/g of CHO-A (log 3.0 ± 2.0) was significantly lower than the initial CFU/g of other approaches (OSA log 3.9 ± 0.3 and OA 4.4 ± 0.2). In all approaches except CHO-A, the number of CFU increased by about half a log after three days and decreased constantly. In the OSA, Enterococcus spp. were not detectable on day 28 or later. After 50 days in both approaches, enterococci were not detectable by cultivation anymore.

The non-enriched approaches (NSA and CHO-A) had a constant pH of about 8 with a minimum of pH 7.8 ± 0.1 after seven days (Figure 2g). Henceforth, pH increased constantly to 8.5 ± 0.1 after 76 days of incubation. In the enrichment approaches, pH decreased immediately. OA and SA reached a minimum value of 6.2 ± 0.6 and 5.5 ± 0.0 after 14 and 7 days, respectively. The OSA decreased to pH 5.2 ± 0.1 after seven days and decreased further to a final pH of 4.96 on day 76. Of note is the difference in the course of SA (Supplementary Figure S1). While two of the replicates decreased to pH 5.5 after 21 days and stably remained there, one approach reached a minimum after 14 days at 6.8 and then increased again to a final pH of 8.5. Obviously, fermentation did not work in this sample for reasons that were not further explored. To be sure that fermentation worked in the spiked approach samples, pH was measured after the last sampling. No such difference in pH was measurable, and therefore it can be assumed that fermentation worked in all samples.

For the enumeration of sulfite-reducing anaerobes, like *Clostridium* spp. and their spores, DRCM bouillon was used. In all approaches, the minimum was observed after seven days (Figure 3). OA showed a minimum of log 1 TCID50/mL, while in the other approach, minimum values between log 4 and 5 TCID50/mL were measured. Afterwards, the values rose to the previous level. The highest values were measured in CHO-A, whereas the other approaches showed no significant difference.

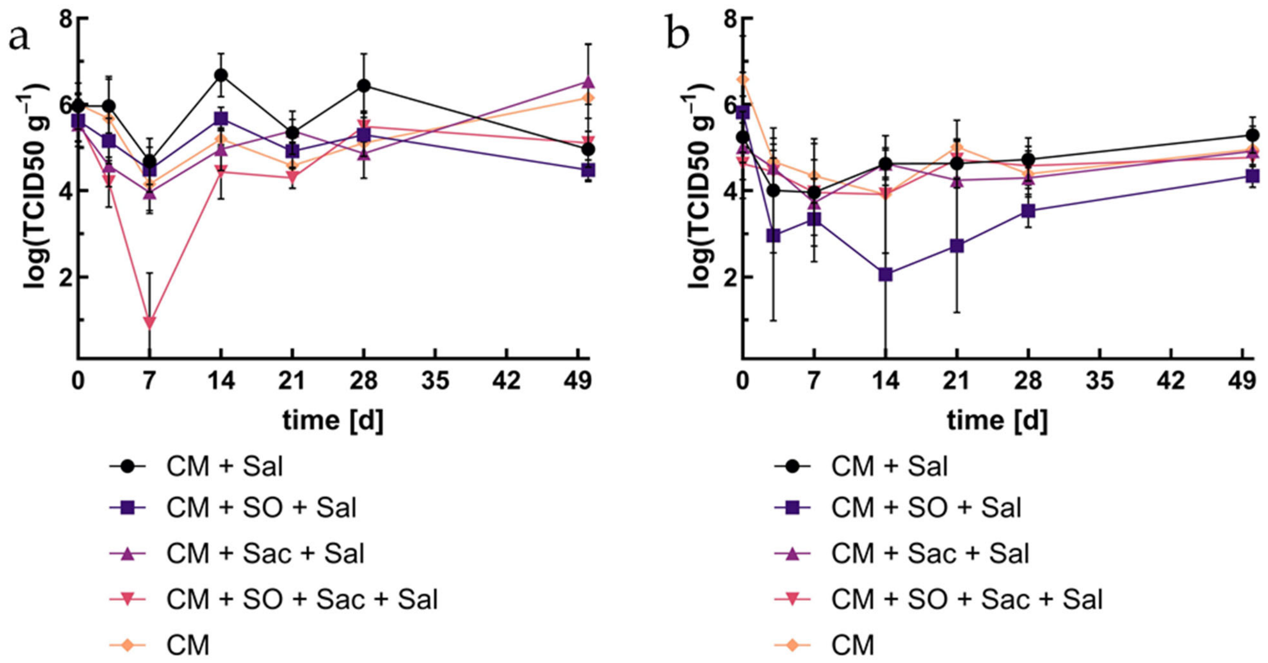

Figure 3. Occurrence of *Clostridium* spp. and other spore-forming sulfite reducers: (**a**) living cells, (**b**) after inactivation. Abrev. as in Figure 1.

For all inactivated approaches, TCID50 decreased by 2 to 3 log levels during the first three days. OA showed significantly lower values during the whole period of investigation, while no significant difference was observed for any of the other approaches.

MALDI-TOF is a qualitative method for the detection of species based on proteins. The results represent the most dominant culturable microorganisms but not the absolute abundance or completeness of the microbial community. Keeping this in mind, in Table 3, detected microorganisms are presented on the genus level with the number of detections per sampling day, approach, and incubation temperature in Exp1. The species of one genus were summarized. The full list of detections is presented in Supplementary Table S1.

At both incubation temperatures, comparable numbers of detected species were observed, 39 at 21 °C vs. 38 species at 10 °C. In CHO-A at 10 °C, the most abundant genera were *Bacillus* spp., *Clostridium* spp., and *Enterococcus* spp. In HA, most results were comparable. The most obvious difference was the detection of *Lactobacillus* spp. after 14 d until the end, while in CHO-A, it was not detected anymore after 14 days. In OA, lactobacilli were already detectable from day 3. Here, no enterococci were detected anymore after 14 days.

At 21 °C *E. coli*, *Enterococcus* spp. and *Bacillus* spp. were the most abundant microorganisms in CHO-A and HA. *Enterococcus* spp. were present all the time. Lactobacilli were detected in low numbers and without a visible trend. On the other hand, in OA, lactobacilli were detected over the whole measurement period and with five species. Bacteria of the species *Bacillus* spp. were present every day of sampling, while enterococci were not detectable after 14 days.

Table 3. Overview of results of MALDI-TOF; in column "next organism", results were summed to genus level and indicated in bold. Numbers in brackets are numbers of detected species. In column "CM", "CM + HC" and "CM + SO" numbers represent number of detected species at time of sampling. Tempera-tures are shown in italics. Abrv.: temp.—temperature; CM—cattle manure; HCs—hay cobs; SO—squeezed oat.

Temp.	**Next Organism**	**CM**									**CM + HCs**									**CM + SO**								
	day	*0*	*3*	*7*	*14*	*21*	*30*	*50*	*63*	*100*	*0*	*3*	*7*	*14*	*21*	*30*	*50*	*63*	*100*	*0*	*3*	*7*	*14*	*21*	*30*	*50*	*63*	*100*
10 °C	***Acinetobacter* sp. (2)**									**3**																		
10 °C	*Aerococcus viridans*	1		1	1							1	3	2	2						1	1						
10 °C	***Bacillus* sp.(7)**	**2**		**2**	**2**	**1**	**1**	**3**	**1**	**1**	**4**	**1**	**2**		**1**		**1**		**2**			**3**	**3**	**2**	**1**	**1**	**1**	**2**
10 °C	***Clostridium* sp.(3)**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**1**		**1**					**1**		**1**		**3**			**2**	**1**			**2**	
10 °C	***Enterococcus* sp.(9)**	**2**	**4**	**1**	**1**	**4**	**4**	**4**	**3**	**2**	**4**	**7**	**4**	**4**	**4**	**1**	**3**	**2**	**1**	**4**	**4**	**5**	**3**					
10 °C	*Escherichia coli*													1	1													
10 °C	***Lactobacillus* sp.(5)**			**1**	**1**									**1**	**1**	**1**	**1**	**1**	**2**		**1**	**4**	**4**	**3**	**3**	**2**	**2**	
10 °C	***Pseudomonas* sp.(2)**		**2**	**1**	**1**									**1**														
10 °C	***Staphylococcus* sp.(3)**		**1**						**1**					**1**														
10 °C	Others (5)										1	2						1			4		1			1		1
10 °C	Total sample number	18	15	10	8	10	11	10	9	12	17	17	12	11	13	10	7	7	8	9	15	17	15	10	7	8	7	10
21 °C	***Acinetobacter* sp.(3)**			**1**	**2**			**2**																**1**				
21 °C	*Aerococcus viridans*											1																
21 °C	***Bacillus* sp.(11)**	**6**	**3**	**3**	**1**	**3**	**1**	**5**	**3**	**1**	**4**	**1**	**1**	**1**	**3**	**3**	**2**	**2**	**3**	**7**	**1**	**2**	**1**	**3**	**2**	**2**	**4**	**5**
21 °C	***Clostridium* sp.(3)**		**2**		**1**	**1**	**2**	**1**		**2**				**1**		**1**	**3**	**1**					**1**			**1**	**1**	
21 °C	***Enterococcus* sp.(8)**	**2**	**7**	**5**	**3**	**2**	**3**	**4**	**1**	**1**	**2**	**1**	**2**	**3**	**1**	**1**	**4**	**4**	**1**	**1**	**1**	**3**	**1**					
21 °C	*Escherichia coli*		4	3	1	5	3	2	1				1	1	1													
21 °C	***Lactobacillus* (6)**					**2**	**1**			**1**		**1**				**1**	**1**	**1**			**4**	**6**	**4**	**1**	**1**	**1**	**1**	**1**
21 °C	Others (7)	**2**	**1**	**2**			**1**	**1**														**1**		**1**				
21 °C	Total sample number	14	28	16	10	14	13	15	7	8	12	9	13	9	10	9	12	10	6	9	8	12	9	8	4	6	8	8

4. Discussion

In the case of a salmonellosis outbreak, a large amount of potentially contaminated manure accumulates. To break the infection cycle, this study tested LAF as a low-tech and easy-to-use method for the hygienization of CM in low-scale experiments. The results showed that during the fermentation process, *Salmonella enterica* serovar *enterica* Typhimurium was degraded to below the detection limit.

4.1. The Role of the C Source

The enrichment of the CHO source is mandatory for the successful hygienization of contaminated cattle manure. The results showed a higher impact with the addition of squeezed oat than with that of hay cobs, but this was exceeded by saccharose. Also, the MALDI-TOF results confirm that at both temperatures, the amount and the diversity of lactobacillus detections were significantly lower in HA than in OA. Similar results were described by Scheinemann and colleagues [3].

Without treatment, *Salmonella* survived in the approach until the end of sampling at 10 °C and for at least 80 days at 21 °C. *Salmonella* species are known to survive in stored slurries and dirty waters for up to three months and for less than one month in solid manure heaps with temperatures greater than 55 °C [43]. The survival rate of *S.* Ty. depends on pH and medium composition, the chain length of the acid, and the concentration of VFAs and temperature [24].

Between the two setups, the content of OA was reduced from 18.75% in the temperature experiment to 10% in the saccharose experiment to keep enough manure material for a representative reaction when saccharose is added. As a result of reduction, the reaction time of degradation in OA was twice as long as that in Exp1. Therefore, the amount of CHO addition has an impact on the reaction speed.

With the addition of sugar, the degradation of Salmonella was enhanced considerably. The combination of both sources of CHO adds up to half of the rate of SA. Salmonella enrichments proved the complete removal of *S***.** Ty. in the CHO-enriched samples after fermentation. With the addition of sugar or squeezed oat as CHO sources, the degradation of Salmonella was reliably successful. Hence, the amount of available C sources has an impact on the speed of the fermentation reaction as well as the type. Pradhan and colleagues [44] showed comparable results with glucose and other sugars in lactic acid production. Among the carbon sources, glucose was found to be the best-performing carbon source with 2.2 times more lactic acid synthesis. Harlow and colleagues [45] compared the effect of oat, wheat, and corn with positive results on LAF and pH decrease. Therefore, it is conceivable that more grains are a suitable source of CHO with similar achievable results. More studies are necessary to improve this method.

4.2. Temperature Dependence of Salmonella Degradation During LAF

The effectiveness of LAF was thereby also temperature-dependent. The non-enriched approach showed higher survival rates of *S.* Ty at 10 °C than at 21 °C. Such temperature dependence of *S.* Ty. survival was described before. There might be several factors responsible such as the temperature-dependent activity of other microorganisms or bacteriophages [46,47].

The addition of hay cobs as a CHO source accelerated the degradation of *S.* Ty. at 21 °C compared to CHO-A, but there was no difference compared to CHO-A at 10 °C. Hay cobs contain cell wall fiber cellulose and hemicellulose, which requires the synergistic activity of multiple enzymes to degrade [48]. Ruminants have a wide range of symbiont microflora and enzymes to degrade organic matter of hay and grass [49]. In a slurry, however, microorganisms must provide these enzymes themselves. Glycolytic activity and other metabolic pathways of lactobacilli are strongly temperature-dependent [50,51]. Hay cobs were tested as a C source, because they may already be available in livestock in the case of an animal disease. Obviously, hay cobs are a less efficient source of energy for the microbial community of cattle manure at low temperatures.

Squeezed oat on the other hand is an easy-to-use substrate, so the degradation of salmonellae in OA was more efficient. *S.* Ty. were below the detection limit after two weeks at 21 °C and after three weeks at 10 °C. The results showed that LAF worked at different temperatures and may be an appropriate method for degrading *Salmonella* serovars much faster and more efficiently than without the addition of any carbon source during the winter season. Similar results were observed in large-scale experiments at summer temperatures (publ. in prep.).

4.3. Lactobacillus and pH

The shift in the microbial community was detectable in all approaches. Lactobacillae, as the main responsible organisms for LAF, showed a different growth pattern between the approaches. The addition of sugar promoted the growth of lactobacilli, which was enhanced by the addition of squeezed oat (Figure 2e). The increase in the cell numbers of lactobacillae corresponded with the decrease in the pH value. The increase in lactobacillae dependent on different grains was described before [45].

The results of MALDI-TOF revealed a high diversity in OA at 10 °C and 21 °C with the detected amount and occurrence of five out of six species (Tables S1 and 3). In a study by Hrubant [52], the peaks of different species of *Lactobacillus* were shown at different points of time during the fermentation process. Indeed, the size and shape of colonies on the MRS plates differed during the fermentation process and may be an explanation for the fluctuation. Nevertheless, the decrease to pH 6 and reduction in *Salmonella* support the assumption that LAF proceeded. Therefore, the CFU count alone is not the only measure for the activity of lactobacillae.

Salmonella spp. are known to prefer a pH above 5.5 and survive until pH 4 [53]. Therefore, the pH cannot be the only reason for the reduction in *Salmonella* by LAF. Lactobacillae are known to produce antimicrobial agents, e.g., [54]. Volatile fatty acids appear to have a toxic effect on *S.* Ty. [23]. In a study with *L. plantarum*, which is known to produce lactic and acetic acid, the antimicrobial effects of VFAs and the reduction in the pH were responsible for *Salmonella* reduction in fermented feed [55]. Therefore, it is conceivable that pathogen organisms with a known pH sensitivity like FMD virus [56,57] may be successfully inactivated by LAF as well as other bacterial pathogens.

4.4. Enterococci as Indicator Organisms

The proliferation of enterococci corresponds to the fermentation process and can be seen as a pioneer of fermentation and as a bioindicator. The MALDI-TOF results suggested the same at 10 °C. Species of *Enterococcus* are a part of common genera of LAB and often play a role in lactic acid fermentation [58,59]. The increase and following decrease went along with the start and end of the fermentation process. The genus *Enterococcus* is easy to detect and can be used to confirm the end of fermentation. Additionally, the tenacity of this genus was higher than that of *Salmonella* and is therefore a reliable indicator of the hygienisation process.. Therefore, *Enterococcus* ssp. can be approved as a suitable indicator organism for the fermentation process also at 10 °C and 21 °C as suggested earlier and approved for higher temperatures [3].

4.5. Sporulating Organisms

The sulfite-reducing and spore-forming clostridia fluctuated in all approaches but without a visible trend. In contrast to other organism groups, there was a decrease in the detection of clostridia at the beginning of fermentation. In particular, SOA dropped to the detection limit after seven days and regenerated afterwards. On the other hand, CHO-A revealed the highest detection numbers. The sporulation numbers are 1–2 log lower than those of the living cells. This corresponded with the results by Scheinemann et al. [3]. There were no differences between the results of the enriched approaches, except SOA which revealed the lowest results but without statistical significance.

It is known that *Salmonella* can coexist with *Clostridium* in guts and digesters [60]. Clostridia strains play an important role in the anaerobic degradation of dairy cattle manure during mesophilic and thermophilic digestion [48,61]. Additionally, a novel cold-tolerant *Clostridium* strain is suggested for potential applications in efficient manure fermentation [48]. However, the presence in the digestate of biogas plants can be controversial due to the potential growth of pathogenic species like *C. tyrobutyricum* and *C. butyricum* [61]. A mesophilic anaerobic digestion approach in an agricultural population reduced the clostridial population abundance and also spores significantly [61]. In contrast, our results showed that the population of *Clostridium* species remained stable throughout LAF.

4.6. LAF as Alternative

Treating pathogen-contaminated manure is essential to break the infection cycle within the agricultural cycle. Other fermentation methods were already shown to be the most efficient [5,62,63]. The simplest method is the extended storage of contaminated waste [5]. On the other hand, it was shown that without further treatment, *S. enterica* survived for about 130 d in pig manure and for over 200 d in chicken manure at 25 °C [9]. The sanitizing effect of a full-scale thermophilic high-solid anaerobic digestion process resulted in the undetectability of *Salmonella* and other pathogens after 24–48 h under anaerobic conditions [63]. Mesophilic fermentation as a hygienization method uses the microbiotic shift to inactivate pathogens such as *Salmonella* [48,62]. Also, aerated waste microcosms of manure flush lagoons resulted in a significant decrease in *Salmonella* [6].

Among this methods, LAF was already described as an efficient hygienization method [3]. The results of this study showed its efficiency at different temperatures and with different CHO sources. Fermentation degrades *S.* Ty. in cattle manure to below the detection limit within two to four weeks by agar plates or within 50 days by *Salmonella* enrichment according to Biowaste Ordinance [39]. Degradation was shown at 10 °C and 21 °C.

The same applies to other pathogens such as *E. coli*. The MALDI results showed the presence of *E. coli* mainly in manure without additives at 21 °C, while in HA, it was detected only to a small extent after 21 days and not at all in OA. An earlier study also showed that *E. coli* was inactivated by LAF within three days [3]. It can therefore be assumed that LAF is also suitable for inactivating non-sporulating pathogens such as non-O157 STEC and *E. coli* O157.

Which fermentation method is used depends on the aim of treatment and the technical possibilities on site and the availability of disinfectants. The main advantage of LAF lies in the fact that the method does not require harmful chemicals or high technical effort. One ethical objection is the use of edible food for disinfection purposes. Here, further studies can investigate the suitability of contaminated, inedible food or food waste as a CHO source.

There are more possible ways to apply LAF. First tests showed that LAF also works with pig manure (data not published). Jung and colleagues [9] showed the long survival of pathogens in pig and chicken manure, while Scheinemann and colleagues hygienized human sludge by LAF [3]. Further investigations are necessary for the transferability to other types of animal manure or sludge to establish the method.

5. Conclusions

The LAF process was shown to be able to degrade *Salmonella* Typhimurium in cattle manure. The speed and quality of the process thereby depend on the temperature and the available C source. The shift in the *Enterococcus* spp. populations confirmed that they are suitable indicator organisms for LAF. The method does not require any harmful chemicals or technical effort. In addition, disinfected manure can be used as fertilizer afterwards. Further studies should investigate alternative CHO sources and the transferability to other animal or human disease-related pathogens. This study confirms LAF as an alternative method for the decontamination of pathogen-contaminated liquid manure.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/applmicrobiol5030088/s1, Figure S1: Results of pH measurement of SA (Exp 2); Table S1: Overview of results of MALDI-TOF.

Author Contributions: Conceptualization, H.H. and H.A.S.; methodology, H.H., T.M., and H.A.S.; formal analysis and investigation, H.H. and S.W.; writing—original draft preparation, H.H.; review and editing, S.W., C.S., H.A.S., I.L., T.S. and L.E.H.; visualization, H.H.; supervision, H.A.S.; funding acquisition, H.A.S. and L.E.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Bundesministerium für Forschung, Technologie und Raumfahrt, grant number 03VP09081, and Bundeswehr Research Institute for Protective Technologies and CBRN Protection (WIS) contributed in kind.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author(s).

Acknowledgments: We thank Wiebke Lange and Yolanda Marschner from the FLI and Antje Rußmann and Kristina Sonnwald from WIS for technical support.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

C	Carbon
CATC agar	Citrate–azide–Tween®–carbonate agar
CFU	Colony-forming unit
CHO-A	Approach without carbohydrates
CM	Cattle manure
DRCM	Differential reinforced clostridial broth
HA	Hay cob approach
HCs	Hay cobs
LA	Lactic acid
LAB	Lactic acid bacteria
LAF	Lactic acid fermentation
N	Nitrogen
NSA	Non-spiked sample
OA	Squeezed oat approach
OSA	Squeezed oat and saccharose approach
SA	Saccharose approach
Sac	Saccharose
Sal	*Salmonella enterica* subsp. *enterica* ser. Typhimurium
SS agar	*Salmonella Shigella* agar
S. Thy	*Salmonella enterica* subsp. *enterica* ser. Typhimurium
SO	Squeezed oat

TCID50	Tissue culture infection dose 50
VFA	Volatile fatty acid
XLD agar	Xylose–lysine–deoxycholate agar

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