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Influence of Fermented Diets on In Vitro Survival Rate of Some Artificially Inoculated Pathogens—A Preliminary Study

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Abstract: Improving the hygienic status of feed ingredients by biotechnological processes as fermentation is of the greatest concern. This preliminary study aimed to investigate whether there are relevant effects of fermented liquid feed (FLF) on the survival of potential pathogens in vitro. The feed (fresh basis) consisted of 50% rye, 30% rapeseed extracted meal, 10% barley and 10% wheat. Glass bottles were filled about 14.1 g water (38 °C) containing the diluted starter culture and feed (8.81 g). Fermentation led to high levels of lactate (5–7% of dry matter), low pH values (<4.0) and low levels of acetic acid (<1% of dry matter) in the FLF. The survival rate of pathogens added, such as *Salmonella enterica* serovar Typhimurium, *Escherichia coli* and *Clostridium perfringens* after 6 h of controlled fermentation, was significantly reduced (<2 $\log_{10}$ CFU/g). The counts of C*andida krusei* in FLF at 3 h and 6 h post inoculation remained almost unchanged regardless of the incubation time. Even adding sodium-benzoate at a concentration of up to 0.25% in the liquid feed did not reduce the survival of *C. krusei* during fermentation. Based on this in vitro study, feeding of FLF seems a promising strategy to reduce pathogen transmission but has to be confirmed on natural feeds by pathogens for increasing the hygienic properties.

Keywords: liquid feed; in vitro fermentation; pathogens

1. Introduction

Feed cost constitutes a major part of animal production costs [1]. Optimum performance requires a high feed quality without contamination. In addition, effects on animal health as well as food safety are desirable [2]. The rapid development in microbiology, molecular biology and biotechnology has led to the performance of highly profitable industrial fermentation processes [3]. In recent decades, the fermentation process has been used to produce functional feeds to improve, for example, broiler gut health and meat production [4]. Fermentation is a dynamic process involving microorganisms and substrates to convert complex dietary compounds to simpler ones [5]. Solid material, either the complete feed or the grain, can be fermented by adding water and different microorganisms [6]. Recently, the fermented liquid feed (FLF) has been implemented as a new feeding strategy in animal nutrition and has become increasingly widespread [7]. By definition, FLF is a feed mixed with water and fermented for a period long enough to reach steady state of acidification, prepared by spontaneous or controlled fermentation with lactic acid bacteria (LAB) [8]. Studies in animals have shown that

the use of proper fermented feed is expected to have positive effects on nutritive value by increased digestibility [9].

Another interesting aspect is that it has fascinating effects on the health and well-being of animals as well as on food safety [3]. This opens the way for an important application that has not been sufficiently utilized and studied in Europe nor globally. Contamination of animal feed can be disseminated to food-producing animals and further down the food chain, causing large economic losses and most importantly can be a threat to animals and public health [10]. Adding LAB to fermented feed could produce lactic acid, which acidifies the feed to inhibit the growth of some known pathogens [11,12].

A successful fermentation is characterized by high contents of lactate formers and a low pH value in the feed [13]. According to van Winsen et al. [14], LAB contents of over 9 $\log_{10}$ colony-forming unit (CFU)/g should be achieved in the fermented diet. Furthermore, a proper fermentation is characterized by a lactate content of at least 50 g/kg dry matter, acetic acid content ≤10 g/kg dry matter and a pH value ≤4.0 [14].

However, a particular challenge in the liquid feed fermentation is the inhibition of yeast growth and acetic acid formation during the fermentation process [15]. A fermented feed could still provide a suitable habitat for C*andida krusei* despite its low pH and high lactate content [16]. Contamination of animal feeds by bacteria and molds during storage may cause spoilage, which adversely affects feed quality [4]. In the presence of metabolites, such as organic acids produced by LAB during fermentation may have preservative effects and may increase the shelf-life of fermented feeds [17].

From the point of view of public health concern and according to the World Health Organization [18], *Salmonella* is one of four key global causes of intestinal diseases in humans. *S.* Typhimurium is a ubiquitous genus of bacteria commonly found in animals, which can cause one of the most common food-borne illness in humans [19]. Additionally, *Escherichia coli* is a bacterial commensal of the intestinal microflora of a variety of animals, including humans; however, some are able to cause diseases in humans as well as in animals [20]. The pathogenic *E. coli* is implicated in diarrhea in both preweaning and postweaning piglets [21]. *Clostridium perfringens* is associated with diverse environments including soils, food and as a member of intestinal microbiota, of both diseased and non-diseased humans and animals [22]. Grahofer et al. [23] assumed that hemorrhagic bowel syndrome in fattening pigs fed liquid diet arises from overgrowth of bacteria such as *C. perfringens* or *E. coli* in the intestinal tract. These are the most common and important food-borne pathogenic bacteria, which need potential ways of being hygienized for under an One-Health approach. Therefore, the aim of the present preliminary study was to test the hygienic potential effects of fermentation liquid feed on the survival of different pathogens artificially inoculated such as *S.* Typhimurium, *E. coli*, *C. perfringens* and *C. krusei* in vitro.

2. Materials and Methods

2.1. Laboratory Fermenter

Glass bottles of 100 mL with a screw cap (Duran® Original laboratory bottle, GL 45, 100 mL, DWK Life Sciences GmbH, Wertheim, Germany) were used as laboratory fermenter (Figure 1). The glass bottles were placed on a magnetic stirring plate (MIXdrive 20, Fa. 2mag AG, Munich, Germany) in an incubator at 38 °C and stirred at 200 rpm/min to ensure an ideal environment for the starter culture.

Figure 1. Fermentation in laboratory (photo: ©Abd El-Wahab/TiHo).

2.2. Diet Composition

The fresh basis diet consisted of 50% ground rye, 30% rapeseed extracted meal, 10% barley and 10% wheat. The components of the diet (except rapeseed extracted meal) were ground by a hammer mill (Rasant-Super®, Ley, Sulingen, Germany; diameter of the sieve inset: 3 mm). Representative samples of the liquid feed were freeze-dried (freeze-drying plants Alpha 1-4-Loc-1m and Gamma 1-20-Lmc-1, Martin Christ GmbH, Osterode am Harz, Germany). For further analysis, the dry feed samples were ground with a 0.5 mm sieve (Centrifugal Mill ZM 200, Retsch GmbH & Co. KG, Haan, Germany).

2.3. Fermentation Process

The glass bottles were filled with the liquid feed to be fermented with the starter culture (ratio of water:feed = about 2:1). The starter culture composed of a bacterial concentrate (SCHAUMALAC FEED PROTECT XP concentrate, Fa H. Wilhelm Schaumann GmbH, Pinneberg, Germany) with a concentration of LAB of $2 \times l0^{10}$ CFU/g concentrate. LAB strains were included in the product: 1k2079 *Lactobacillus plantarum*, 1k2103 *Pediococcus pentosaceus* and 1k2082 *Lactococcus lactis*. To achieve the desired starter culture concentration at the start of fermentation, even with small fermenter volumes, pre-diluted starter culture was done. Briefly, about 15 g of the starter culture was added to about 985 g water (38 °C). Thereafter, about 35 g from the previous solution was added to 965 g water (38 °C). Then, about 50 g was taken from the latter solution to be added to 950 g water (38 °C). Finally, about 14.1 g water of 38 °C was mixed with feed (8.81 g ground). As agitator simulation, triangular magnetic stirring bars were inserted into the glass bottle so that by means of a magnetic stirring plate (MlXdrive 20, Fa. 2mag AG, Munich, Germany), a homogeneous stirring (200 rpm/min) of the liquid feed during the fermentation process was guaranteed. A sample weight of 37 g liquid feed was added to the glass bottle in addition to LAB at the start of fermentation of $2 \times l0^{5}$ CFU/g liquid feed. To ensure homogeneous distribution, sterile magnetic stirring bars were also placed in the glass bottles.

2.4. Measurements

2.4.1. Diet Analyses

The diets were analyzed (n = 3/diet) in accordance with the official methods of the Association of German Agricultural Analytic and Research Institutes [24]. The dry matter content was determined by drying to the weight constancy at 103 °C. The raw ash was analyzed by means of incineration in the muffle furnace at 600 °C for 6 h. Determining the crude protein content was done by analyzing the total nitrogen content using the catalytic tube combustion method (DUMAS combustion method; Vario Max®, Elementar Analysensysteme GmbH, Langenselbold, Germany). The crude fat content was determined after acid digestion in the soxhlet apparatus. The content of crude fiber was determined after washing in diluted acids and alkalis. Starch determination was carried out polarimetrically (Polatronic E, Schmidt und Haensch GmbH & Co., Berlin, Germany). The sugar content was analyzed using the Luff-Schoorl method by titration with sodium thiosulfate. Amino acids were determined

by ion-exchange chromatography (AA analyser LC 3000, Biotronik Wissenschaftliche Geräte GmbH, Maintal, Germany).

2.4.2. L- and D-Lactate

To determine the lactate, perchloric acid (1 mol/L) was first added to the sample material and centrifuged. Potassium hydroxide solution was then added to 2 mL of the supernatant until a pH range of 8–10 was achieved. After a second centrifugation, an enzymatic determination (D-/L-lactic acid UV test, Roche Diagnostics GmbH, Mannheim, Germany) of the L- and D-lactate content was performed.

2.4.3. Volatile Fatty Acids

The content of volatile fatty acids was measured by means of a gas chromatograph (610 Series, Unicam Chromatography GmbH & Co. KG, Kassel, Germany). After the sample had been mixed with an internal standard (10 mL of formic acid 89% and 0.1 mL of 4-methylvaleric acid), the mixture was centrifuged and then subjected to gas chromatography with a column temperature of 155 °C (injector: 175 °C, detector: 180 °C).

2.4.4. pH Value

The pH value measurements were carried out with a digital probe (pH 526, BlueLine electrode, Schott Instruments GmbH, Mainz, Germany) after homogenizing of the sample material.

2.5. Microorganisms Counting before and after Fermentation

A contamination of the liquid feed before fermentation was simulated as well as the liquid feed, which had already been fermented for at least 24 h, was inoculated experimentally with four potentially pathogens and their survivability in the "finished" fermented feeds was tested ($n = 3$).

The isolate of *S.* Typhimurium was isolated from the feces of fattening pigs in an accredited laboratory (LVL Lebensmittel-und Veterinärlabor GmbH, Emstek, Germany), identified and given to the institute. In contrast, *E. coli* isolate was used from the pig feces that had suffered from diarrhea before the start of the experiment and was further characterized in the Institute of Microbiology (University of Veterinary Medicine Hannover, Foundation, Germany). The *C. perfringens* isolate was culturally isolated from pigs feces in the field and was sent to the institute for further investigations. The field isolate of *C. krusei* was culturally isolated from a liquid feed for pigs at the institute and identified as *C. krusei* by mass spectrometry (Matrix-assisted Laser Desorption/Ionization Time of Flight; MALDI-TOF-MS) and ID 32 C-Test (bioMérieux SA, Marcy-l'Ètoile, France).

A separate glass bottle was used for each pathogen ($n = 3$). About 37 g of liquid feed was fermented for 24 h in the glass bottle. Thereafter, the glass bottle was removed from the incubator. Before inoculating the FLF with the respective bacteria, 1 g of fermented feed was removed from the glass bottle, a dilution series being made by spreading the feed out on selective nutrient media. In this way, it could be ensured that no significant bacterial counts of the respective bacteria were already present in the FLF before inoculation.

Salmonella detection was also carried out on Sabouraud-Glucose-Agar (SAB-Agar, PO 5096A, Thermo Fisher Scientific GmbH, Bremen, Germany). Gassner medium (Oxoid Deutschland GmbH, 46,483 Wesel, Germany) was used for *E. coli* detection, and incubation was performed anaerobically at 37 °C for 24 h. Neomycin-Polymyxin B-Cystein-Agar (NPC) agar was used as a nutrient medium for *C. perfringens*. This was produced at the Institute of Microbiology (University of Veterinary Medicine Hannover, Foundation, Germany) modified according to Gad et al. [25]. The composition of the NPC agar is described in detail by Leurs [26]. Incubation of this agar was carried out for 48 h in an anaerobic condition (model CB 160, Binder GmbH, Tuttlingen, Germany). For the detection of *C. krusei*, the inoculated SAB plates were incubated aerobically at 25 °C for 48 h.

Preparation of Bouillons

The liquid feed was inoculated with the above-mentioned microorganisms. Sterile physiological NaCl solution was used as the basis for each bouillon. Using sterile cotton swabs, colony material was removed from the sheep blood agar or Sabouraud dextrose agar (*C. krusei*) and added to the respective NaCl solution. The optical density of the bouillons was measured by means of a densitometer (DEN-1B, Biosan SIA, Riga, Latvia). Further colony material was added to the bouillon until the desired optical density was achieved regarding the germ density. A broth with the optical density for *S.* Typhimurium, *E. coli*, *C. perfringens* and *C. krusei* of 0.5, 0.5, 0.5 and 9.0, respectively with a target value (CFU/g bouillon) of 1×10^8, 1×10^8, 1×10^5 and 1×10^8, respectively was used.

After preparing of each bouillon, 36 g fermented feed was inoculated with 4 g bouillon. A separate glass bottle with 36 g of FLF was used for each bouillon and, accordingly, each pathogen. Immediately after inoculation of the FLF with the respective bacteria, the glass bottle was first placed on the magnetic stirrer for 1 min to ensure homogeneous distribution of the broth in the fermented feed. Thereafter, 1 g of the FLF was taken and the bacterial count was determined immediately after inoculation (spatula plate method). Subsequently, the glass bottles inoculated with the respective pathogen were taken from the magnetic stirrer and incubated at room temperature. Three hours after inoculation, the glass bottles were placed on the magnetic stirrer again for 1 min and again 1 g sample was taken and the bacterial count of each pathogen was determined in the liquid feed. Finally, 6 h post inoculation (*p.i.*), the bacterial count in the FLF was determined once again.

The non-fermented liquid feed (NFLF) was identical in its botanical composition to that of the FLF, with the difference that this liquid feed was not fermented, but mixed with about 16 °C warm water without the addition of a starter culture. The control preparations were inoculated with the same bouillons, stored also at room temperature and the bacterial count was determined after homogenization of the liquid feed on the magnetic stirrer, also at the three aforementioned times after inoculation.

2.6. Survival Rate of S. Typhimurium during Fermentation

Salmonella Typhimurium was cultivated aerobically in an incubator for 24 h at 37 °C on Brilliant Salmonella-Agar (BS) (Thermo Scientific™ Oxoid™ Brilliance Salmonella Agar-Basis, Oxoid Deutschland GmbH, Wesel, Germany). Before inoculating the liquid feed with *S.* Typhimurium, 1 g of the liquid feed to be tested was first taken from the glass bottle and added to 9 mL phosphate buffered saline solution, a dilution series was made and the number of CFU of *S.* Typhimurium/g liquid feed on BS agar was determined. Subsequently, the remaining 36 g liquid feed was inoculated with *S.* Typhimurium. A broth with *S.* Typhimurium was prepared and 4 g of this broth was added to the 36 g liquid feed. The optical density of the bouillon was adjusted to a value of 0.5. The liquid feed containing *S.* Typhimurium was homogenized for 1 min directly after inoculation on the magnetic stirrer. One gram of the liquid feed was taken out of the glass bottle with a pipette, a dilution series was made and spread out on BS agar to determine the bacterial count of *S.* Typhimurium/g liquid feed directly after inoculation (at the beginning of fermentation). Subsequently, the glass bottle inoculated with *S.* Typhimurium was placed on a magnetic stirring plate in the incubator at 38 °C so that the fermentation process started (experimental set-up). The same liquid feed, which was inoculated in the same way with *S.* Typhimurium, served as a control. However, in contrast to the fermented diet, the NFLF did not contain a starter culture and the incubation was performed after inoculation with *S.* Typhimurium at room temperature. At intervals of 4, 12 and 24 h *p.i.* with *S.* Typhimurium, 1 g of liquid feed was taken again from both NFLF and FLF and the CFU were determined on BS agar. To ensure homogeneity of the liquid feed at the time of sampling, the liquid feed was homogenized for 1 min on a magnetic stirrer before sampling.

2.7. Sodium-Benzoate and Survival Rate of C. krusei

In addition to the starter culture, different concentrations of sodium-benzoate (H. Wilhelm Schaumann GmbH, Pinneberg, Germany) were added to the glass bottles in three doses at the start of fermentation (0.01%, 0.05% and 0.25%, respectively to the liquid feed). To ensure high dosing accuracy even with small volumes, the sodium-benzoate was pre-diluted. A glass bottle with starter culture but without addition of sodium-benzoate was used as a control. The glass bottles were filled with 37 g of the liquid feed using a magnetic stirring rod before the start of the fermentation as described previously.

First of all, 1 g liquid feed was taken out of the glass bottles again before inoculation, a dilution series was set up and this was spread out on both SAB and de Man, Rogosa, Sharpe (MRS) agar in order to ensure that the liquid feed did not already have significant counts of yeasts and to test the counts of *Lactobacilli* directly after adding the starter culture (= start of fermentation). The remaining 36 g liquid feed was inoculated with 4 g of a bouillon of *C. krusei*. This bouillon was prepared in accordance with the procedure described above. The same isolate of *C. krusei* as described previously was used and an optical density value of 9.0 in the bouillon with *C. krusei* was set.

Immediately after inoculation of the liquid feed with *C. krusei*, 1 g of liquid feed was taken again from the respective glass bottle fermenter and the content of *C. krusei* was determined to test the "inoculation success". The inoculated glass bottles were then placed on the magnetic stirring plate in the incubator (38 °C) for 24 h. After the 24 h fermentation, 1 g of liquid feed was taken again from the glass bottles and the content of *C. krusei* and *Lactobacilli* was determined using the spatula plate method. Since the *C. krusei* isolate used showed growth not only on SAB but also on the MRS agar, which is selective for *Lactobacilli*, the colony counting on the MRS agar was followed by a precise assessment of the colony morphology, which differed between *Lactobacilli* and *C. krusei*, and by the preparation of native preparations. For this purpose, the corresponding colonies were suspended on a slide in sterile 0.9% NaCl solution and microscopically examined.

2.8. Statistical Analyses

In cooperation with the Institute for Biometry, Epidemiology and Information Processing of the University of Veterinary Medicine, Foundation, Germany the evaluation of the available data was carried out by the computer programs Statistical Analysis System for Windows, SAS® 9.4 using the Enterprise Guide Client Version 7.1 (SAS Institute Inc., Cary, NC, USA). For statistical evaluation of the data from the microbiological experiments, the germ contents were logarithmized ($\log_{10}$ CFU/g). For the analytical evaluation, the collected data were checked for normal distribution using the Shapiro-Wilk test. For normal distribution, the data were calculated by two-factor analysis of variance with the effect group (independent) and the time effect (repeated measurements) as well as a post-hoc Tukey test for multiple pairwise comparisons. The significance level alpha was set at 5 % ($p < 0.05$).

3. Results

3.1. Diet Composition

The contents of crude protein and amino acids did not differ significantly during the fermentation process, but only varied within the analytical margin (Table 1). The sugar content decreased significantly during fermentation to about 12.90 g/kg dry matter. Likewise, the starch content after fermentation was about 8.40 g/kg dry matter lower. The influence of fermentation on acidity and pH in diet is shown in Table 1. After fermentation, the L- and D-lactate and acetic acid contents in the compound diet were significantly higher. Nearly 6% of the dry matter consisted of lactic acid, with a ratio of L- to D-lactate of nearly 50:50. The butyric acid content remained unaffected during the course of fermentation. The NFLF had a significantly higher pH value in comparison to FLF (5.95 vs. 3.67).

Table 1. Chemical composition and pH value of the experimental diets; n = 3 (g/kg dry matter).

Parameter	NFLF	FLF
Dry matter, fresh basis	207.67 ± 0.577	207.67 ± 0.577
Crude ash	39.10 ± 0.550	40.84 ± 1.80
Crude protein	188.84 ± 3.29	192.76 ± 4.09
Crude fat	26.22 ± 0.816	26.82 ± 0.440
Crude fiber	85.82 ± 1.01	90.68 ± 2.24
Starch	418.78 ± 4.07	410.38 ± 1.10
Sugar	59.70 ^{a} ± 0.966	12.90 ^{b} ± 1.33
Ileucine	7.38 ± 0.268	7.39 ± 0.140
Leucine	13.23 ± 0.472	13.18 ± 0.182
Tyrosine	5.92 ± 0.682	6.00 ± 0.533
Phenylalanine	8.29 ± 0.315	8.18 ± 0.300
Lysine	9.26 ± 0.299	9.32 ± 0.204
Threonine	7.35 ± 0.254	7.45 ± 0.219
Cysteine	4.40 ± 0.396	4.06 ± 0.272
Methionine	3.83 ± 0.258	3.35 ± 0.175
L-Lactate	0.151 ^{b} ± 0.061	31.06 ^{a} ± 3.33
D-Lactate	0.113 ^{b} ± 0.023	27.98 ^{a} ± 4.80
Butyric acid	0.006 ^{a} ± 0.006	0.019 ^{a} ± 0.006
Acetic acid	0.702 ^{b} ± 0.058	7.31 ^{a} ± 1.82
pH value	5.95 ^{a} ± 0.006	3.67 ^{b} ± 0.015

^{a,b} Means within the same row with different superscripts differ significantly ($p < 0.05$). NFLF = non fermented liquid feed; FLF = fermented liquid feed.

3.2. pH Values in Liquid Feeds

The pH values of the liquid feed differed significantly directly before inoculation between NFLF and FLF regardless of the inoculation of the pathogens (Table 2). Additionally, the pH values between NFLF (all pH values above 5) and FLF (all pH values below 4) differed significantly after inoculation of potential pathogens. A slightly reduced pH value was observed in the NFLF after 6 h *p.i.*, whereas the pH value in the FLF remained at a constant low level (all values below pH 4). No significant effects of different pathogens were noted on the pH values on either NFLF or FLF.

Table 2. pH values for NFLF and FLF before and after inoculation of potential pathogen isolates (n = 3/pathogen).

Time	Diet	*S.* Typhimurium	*E. coli*	*C. perfringens*	*C. krusei*
Directly before inoculation	NFLF	5.96 ^{A} ± 0.025	5.94 ^{A} ± 0.015	5.94 ^{A} ± 0.006	5.94 ^{A} ± 0.012
	FLF	3.75 ^{B} ± 0.025	3.70 ^{B} ± 0.021	3.71 ^{B} ± 0.031	3.74 ^{B} ± 0.015
6 h *p.i.*	NFLF	5.81 ^{A} ± 0.025	5.85 ^{A} ± 0.035	5.85 ^{A} ± 0.036	5.85 ^{A} ± 0.025
	FLF	3.74 ^{B} ± 0.025	3.71 ^{B} ± 0.012	3.70 ^{B} ± 0.046	3.73 ^{B} ± 0.020

^{A,B} Means within the same column with different superscripts differ significantly ($p < 0.05$). NFLF = non fermented liquid feed; FLF = fermented liquid feed; *S.* Typhimurium = *Salmonella* Typhimurium; *E. coli* = *Escherichia coli; C. perfringens* = *Clostridium perfringens; C. krusei* = *Candida krusei*.

3.3. Survival Rate of S. Typhimurium

Salmonella could not be culturally detected either in NFLF or FLF directly before inoculation with *S.* Typhimurium (Table 3). Directly after inoculation, comparable bacterial counts of *S.* Typhimurium were detectable in both feeds (about 7 $\log_{10}$ CFU/g). In NFLF, the counts of *Salmonella* remained at the same level for 3 h and 6 h *p.i.* (6.81 and 7.13 $\log_{10}$ CFU/g, respectively). In the FLF, however, the counts of *Salmonella* decreased significantly with increasing time of incubation. After 3 h *p.i.*, the counts of *Salmonella* in the FLF were significantly reduced to about half (3.16 $\log_{10}$ CFU/g) compared to 6 h *p.i.* After a total of 6 h *p.i.*, *S.* Typhimurium could not be detected in the FLF anymore.

Table 3. Survival rate of *S.* Typhimurium ($\log_{10}$ CFU/g) in NFLF and FLF.

Time	NFLF (n = 3)	FLF (n = 3)
Directly before inoculation	<2.00 ^{a,B} ± 0.00	<2.00 ^{a,C} ± 0.00
0 h *p.i.*	7.01 ^{a,A} ± 0.059	6.95 ^{a,A} ± 0.066
3 h *p.i.*	6.81 ^{a,A} ± 0.371	3.16 ^{b,B} ± 0.656
6 h *p.i.*	7.13 ^{a,A} ± 0.028	<2.00 ^{b,C} ± 0.00

^{a,b} Means within the same row with different superscripts differ significantly ($p < 0.05$). ^{A,B,C} Means within the same column with different superscripts differ significantly ($p < 0.05$). NFLF = non fermented liquid feed; FLF = fermented liquid feed.

3.4. Survival Rate of *E. coli*

Prior to inoculation with *E. coli*, a non-specific bacterial count of 4.71 $\log_{10}$ CFU/g was culturally detected in the NFLF (Table 4), which was not the case in the FLF (<2.00 $\log_{10}$ CFU/g; method detection limit). The colonies of these bacteria differed morphologically from the *E. coli* isolate used in the experiment. In the FLF, however, no coliform bacteria could be culturally detected immediately before inoculation on Gassner medium. Immediately after inoculation, the *E. coli* counts in the NFLF and FLF were comparable. However, after inoculating the *E. coli* isolate into the FLF, a massive decrease (about 6 $\log_{10}$ CFU/g to about 4 $\log_{10}$ CFU/g) was already observed at 3 h *p.i.*. No cultural detection of the inoculated *E. coli* isolate was possible after 6 h *p.i.* of the FLF with *E. coli*. In the NFLF, the *E. coli* count increased significantly with increasing time of incubation at 6 h *p.i.* (7.14 $\log_{10}$ CFU/g).

Table 4. Survival rate of *E. coli* ($\log_{10}$ CFU/g) in non-fermented and fermented liquid diets.

Time	NFLF (n = 3)	FLF (n = 3)
Directly before inoculation	4.71 ^{a,C} ± 0.136 [1]	<2.00 ^{b,C} ± 0.00
0 h *p.i.*	6.83 ^{a,B} ± 0.042	6.05 ^{a,A} ± 0.566
3 h *p.i.*	6.61 ^{a,B} ± 0.224	3.85 ^{b,B} ± 0.599
6 h *p.i.*	7.14 ^{a,A} ± 0.029	<2.00 ^{b,C} ± 0.00

[1] The colonies differed morphologically from the *E. coli* isolate used. The yellow colonies, oxidase positive, were probably *Flavobacterium* sp. (epiphytic flora). ^{a,b} Means within the same row with different superscripts differ significantly ($p < 0.05$). ^{A,B,C} Means within the same column with different superscripts differ significantly ($p < 0.05$). NFLF = non fermented liquid feed; FLF = fermented liquid feed.

3.5. Survival Rate of *C. perfringens*

Neither in NFLF nor in FLF was a cultural detection of *Clostridium* sp. possible on NPC-agar before inoculation of *C. perfringens* (Table 5). The inoculation success was also shown in this experiment in comparably high bacterial counts ($p < 0.05$) immediately after inoculation in both feeds. Only after 6 h *p.i.* significant differences could be seen between NFLF (4.49 $\log_{10}$ CFU/g) and FLF (2.65 $\log_{10}$ CFU/g) concerning *C. perfringens* counts. However, in the NFLF, counts of *C. perfringens* at 3 h or at 6 h *p.i.* did not significantly differ from those at 0 h *p.i.* for NFLF. The counts of *C. perfringens* decreased significantly (2.65 $\log_{10}$ CFU/g) compared to directly post inoculation (4.52 $\log_{10}$ CFU/g) in FLF.

Table 5. Survival rate of *C. perfringens* ($\log_{10}$ CFU/g) in NFLF and FLF.

Time	NFLF (n = 3)	FLF (n = 3)
Directly before inoculation	<2.00 ^{a,B} ± 0.00	<2.00 ^{a,D} ± 0.00
0 h *p.i.*	4.61 ^{a,A} ± 0.057	4.52 ^{a,A} ± 0.110
3 h *p.i.*	4.32 ^{a,A} ± 0.244	4.30 ^{a,B} ± 0.109
6 h *p.i.*	4.49 ^{a,A} ± 0.086	2.65 ^{b,C} ± 0.048

^{a,b} Means within the same row with different superscripts differ significantly ($p < 0.05$). ^{A,B,C,D} Means within the same column with different superscripts differ significantly ($p < 0.05$). NFLF = non fermented liquid feed; FLF = fermented liquid feed.

3.6. Counts of S. Typhimurium during Fermentation

Prior to the targeted inoculation of the liquid feed with *S.* Typhimurium, *Salmonella* spp. could not be culturally detected in either the NFLF or FLF (Table 6). Directly after inoculation, comparable bacterial counts of *S.* Typhimurium were obtained in both NFLF and FLF cultures. In comparison to the time immediately after inoculation, the bacterial counts increased by more than one $\log_{10}$ CFU/g in NFLF and FLF for 4 h *p.i.*. Up to 12 h *p.i.*, the bacterial counts did not significantly differ between NFLF and FLF. After 24 h *p.i.*, *S.* Typhimurium counts were significantly lower in FLF compared to NFLF (<2.00 vs. 8.80 $\log_{10}$ CFU/g).

Table 6. Development of *S.* Typhimurium counts ($\log_{10}$ CFU/g) after experimental inoculation of liquid feed (n = 3).

Time	NFLF [1]	FLF
Directly before inoculation	<2.00 ^{a,C} ± 0.00	<2.00 ^{a,C} ± 0.00
0 h *p.i.*	6.95 ^{a,B} ± 0.045	6.97 ^{a,B} ± 0.014
4 h *p.i.*	8.21 ^{a,A} ± 0.445	8.34 ^{a,A} ± 0.041
12 h *p.i.*	8.35 ^{a,A} ± 0.049	8.31 ^{a,A} ± 0.236
24 h *p.i.*	8.80 ^{a,A} ± 0.055	<2.00 ^{b,C} ± 0.00

[1] Liquid feed at room temperature and without addition of starter culture. ^{a,b} Means within the same row with different superscripts differ significantly ($p < 0.05$). ^{A,B,C} Means within the same column with different superscripts differ significantly ($p < 0.05$). NFLF = non fermented liquid feed; FLF = fermented liquid feed.

Regarding the effect of inoculation time, the counts of *S.* Typhimurium after direct inoculation in NFLF and FLF were significantly higher compared to those before inoculation (Table 6). After further 4 h and 12 h *p.i.*, the counts of *S.* Typhimurium were significantly higher in NFLF and FLF compared to direct *p.i.* However, after 24 h *p.i.*, no colony of *S.* Typhimurium in FLF was detected. In the NFLF, the bacterial counts after 24 h *p.i.* were still at the same level as 4 h and 12 h *p.i.*.

3.7. Survival Rate of C. krusei

Immediately prior to inoculation, *C. krusei* could not be detected either in NFLF or FLF (Table 7). The *C. krusei* counts were significantly increased directly post inoculation in both liquid feeds compared to before inoculation. Both at 3 h and 6 h *p.i.*, the counts of *C. krusei* remained almost significantly unchanged in NFLF and FLF regardless of the incubation period or type of feed.

Table 7. Survival rate of *C. krusei* ($\log_{10}$ CFU/g) in non-fermented and fermented liquid diets.

Time	NFLF (n = 3)	FLF (n = 3)
Directly before inoculation	<2.00 ^{B} ± 0.00	<2.00 ^{B} ± 0.00
0 h *p.i.*	7.18 ^{A} ± 0.030	7.12 ^{A} ± 0.041
3 h *p.i.*	7.15 ^{A} ± 0.033	7.11 ^{A} ± 0.043
6 h *p.i.*	7.21 ^{A} ± 0.051	7.16 ^{A} ± 0.023

^{A,B} Means within the same column with different superscripts differ significantly ($p < 0.05$). NFLF = non fermented liquid feed; FLF = fermented liquid feed.

3.8. Effects of Sodium-Benzoate Addition on C. krusei

No yeast colony and certainly no *C. krusei* could be detected from any of the experimental feeds used prior to inoculation (Table 8). In all feeds, comparable counts of *C. krusei* were obtained immediately after inoculation (0 h *p.i.*). However, with a sodium-benzoate addition at concentration of 0.01%, the *C. krusei* counts at 0 h *p.i.* were slightly but statistically significantly higher (7.13 $\log_{10}$ CFU/g) than in the other feeds. The *C. krusei* counts at 0.25% sodium-benzoate after 24 h *p.i.* had significantly the lowest number (7.33 $\log_{10}$ CFU/g) in comparison to other experimental feeds. However, with the addition of sodium-benzoate, even at a concentration of 0.25%, the counts of *C. krusei* could not be

significantly reduced during fermentation. This, however, was not the case for the other experimental feeds either with or without using sodium-benzoate.

Table 8. Effect of adding sodium-benzoate to liquid diet on counts of *C. krusei*, lactate-forming bacteria (log_{10} CFU/g) and pH value before and after beginning of fermentation (n = 3).

Item	Time	Addition of Sodium-Benzoate on Fresh Basis (%)			
		-	0.01	0.05	0.25
C. krusei	Directly before inoculation	<2.00 ^{a,C} ± 0.00	<2.00 ^{a,C} ± 0.00	<2.00 ^{a,C} ± 0.00	<2.00 ^{a,B} ± 0.00
	0 h *p.i.*	7.07 ^{b,B} ± 0.029	7.13 ^{a,B} ± 0.003	7.07 ^{b,B} ± 0.024	7.04 ^{b,A} ± 0.052
	24 h *p.i.*	8.18 ^{a,A} ± 0.006	8.10 ^{a,A} ± 0.030	8.15 ^{a,A} ± 0.072	7.33 ^{b,A} ± 0.193
Lactate forming bacteria	Directly before inoculation	5.34 ^{a,B} ± 0.059	5.38 ^{a,B} ± 0.020	5.32 ^{a,B} ± 0.027	5.34 ^{a,B} ± 0.037
	24 h *p.i.*	9.08 ^{b,A} ± 0.090	9.08 ^{b,A} ± 0.072	9.36 ^{a,A} ± 0.041	9.23 ^{ab,A} ± 0.155
pH	Directly before inoculation	5.96 ^{a,A} ± 0.023	5.95 ^{a,A} ± 0.035	5.96 ^{a,A} ± 0.021	5.95 ^{a,A} ± 0.012
	24 h *p.i.*	4.18 ^{a,B} ± 0.055	4.16 ^{a,B} ± 0.040	4.14 ^{a,B} ± 0.064	4.14 ^{a,B} ± 0.081

^{a,b} Means within the same row with different superscripts differ significantly ($p < 0.05$). ^{A,B,C} Means within the same column with different superscripts differ significantly ($p < 0.05$).

In all liquid feeds, independent of the sodium-benzoate concentration, a significant increase in the bacterial counts of lactate formers was observed after 24 h *p.i.* (Table 8). Moreover, at a concentration of 0.05% sodium-benzoate the counts of *C. krusei* were significantly higher (9.36 log_{10} CFU/g) than those at 0% or at 0.01% sodium-benzoate (9.08 and 9.08 log_{10} CFU/g, respectively). No significant differences were observed for counts of *C. krusei* using sodium-benzoate at 0.05% and 0.25% concentrations. As expected, the pH values in all liquid feeds did not differ significantly (5.95–5.96) at the start of fermentation (Table 8). Similarly, the pH values (range: 4.14–4.18) in all liquid feeds did not differ significantly at the end of fermentation (24 h *p.i.*).

4. Discussion

Fermentation of feeds provides a promising way to protect against contaminating pathogens from raw materials and/or in storage [27]. Sauli et al. [28] pointed out that contaminated animal feed is a significant source of infection. Thus, in our study, the influence of liquid feed fermentation on survival rate/counts of some potential pathogens was investigated in vitro, i.e., simulation of the fermentation process. The results of the inoculation experiments are considered as preliminary investigations and still needed to be confirmed by investigations on ingredients naturally contaminated.

In the current study, the dry matter content in the liquid feed did not change significantly due to the fermentation. However, Lau et al. [29] stated that the mass losses during the fermentation cannot be excluded. Additionally, in the present study, controlled fermentation did not significantly influence the crude protein content and the amino acids pattern in liquid feed. As the amino acid contents in the liquid feed were not different before and after controlled fermentation, the decarboxylation of lysine and formation of biogenic amines during the controlled fermentation can be considered unlikely, which was confirmed by the studies of Lau et al. [29]. Since no changes in the amino acids content and NH_3 content (<0.1 g/kg dry matter) occurred in the liquid feed before and after fermentation, a degradation/conversion of amino acids by the microorganisms is unlikely. During controlled fermentation, the sugar content in the liquid feed decreased greatly (from 59.70 g/kg dry matter to 12.90 g/kg dry matter), whereas the starch content tended to be lower. Nevertheless, the differences in starch content varied within the laboratory analytical margin. A reduction in the sugar content and less of the starch content during fermentation is also described in the literature [27,30]. Thus, sugar is the main substrate of microbial metabolism during fermentation. After 24 h fermentation, there was a notable acidification, i.e., a decrease in the pH value in the liquid feed. The pH value reduction is mainly due to high contents of L- and D-lactate in the FLF. At the end of a 24 h controlled fermentation, the dry matter in the FLF consisted of almost 6% lactic acid. However, not only L-lactate was formed, but also a considerable amount of D-lactate. Besides the formation of lactic acid, the fermentation also

led to certain acetic acid contents in the liquid feed. This has already been observed by Heinze et al. [15]. The acetic acid contents differed significantly between FLF and NFLF, which was also confirmed by the investigations of Beal et al. [31]. After controlled fermentation, the acetic acid content was 7.31 g/kg of the dry matter on average, whereas in NFLF, the acetic acid content was 0.70 g/kg of the dry matter. Generally, according to Muck [32], lactic acid is the preferred end product of fermentation as lactic acid is a stronger acid (pKa 3.86) than acetic (pKa 4.76).

The butyric acid content in NFLF was approximately 0.006 g/kg dry matter. Similarly, controlled fermentation did not lead to a significant concentration of butyric acid in the liquid feed (0.019 g/kg dry matter). Naturally, many anaerobic microorganisms such as *Clostridium* sp. can produce butyric acid from sugars and other carbon sources [33]. Thus, the presence of this acid indicates metabolic activity from *Clostridium* sp. and should not be detectable in well-fermented feeds.

As the name "fermentation" already implies, this feeding method is characterized by various microbiological processes in the liquid feed. The bacterial counts of lactic acid bacteria in NFLF and FLF varied with values of 4.91 and 9.31 $\log_{10}$ CFU/g liquid feed. The liquid feed was fermented from 50% rye meal, 30% rapeseed extracted meal, 10% barley meal and 10% wheat. The higher proportion of rapeseed extracted meal in the FLF, therefore, had no negative effect on the bacterial count of LAB. The mean lactate contents and pH values in FLF (pH: <4.0; lactate >50 g/kg dry matter) were thus characteristic of a successful fermentation [15]. This is not entirely unexpected, since during the fermentation, primarily the sugar and less/hardly any starch is degraded by the LAB, and the rapeseed extracted meal usually contains little/no starch, but mostly sugar. In the small fermenters as well as in the field, the liquid feed is only stirred at intervals of a few minutes [7,34]. However, in the glass bottles, the liquid feed was continuously stirred during the fermentation process. Thus, in the glass bottles (in vitro), the air input into the liquid feed during the fermentation process was presumably higher in comparison to the small fermenters and/or in the field. Nevertheless, LAB counts of 9.31 $\log_{10}$ CFU/g and pH values <4.0 were also achieved in the liquid feed, thus fulfilling the characteristics of a successful fermentation [14]. This reduction could be explained by the low pH values and high lactic acid content in the FLF. The pH values in the FLF varied by values between 3.70 and 3.74, whereas these were significantly higher in the NFLF (5.81–5.96). The pH values in NFLF also decreased during the 6 h incubation period from the initial 5.94–5.96 to values of 5.81–5.85. This slight pH reduction is probably due to an incipient uncontrolled fermentation in the NFLF by the epiphytic flora including some LAB. Additionally, the current study stated that there was no effect of the inoculation of the pathogens on the pH values of the FLF.

The survival rate of *S.* Typhimurium was reduced in the FLF compared to the NFLF. Immediately *p.i.*, the bacterial counts of the *S.* Typhimurium in the NFLF and FLF varied at the same level, as expected. After an incubation period of 3 h, however, the bacterial counts of *S.* Typhimurium were already significantly lower in the FLF than in the NFLF. Six h *p.i.*, *S.* Typhimurium could no longer be culturally detected in the FLF. Mikkelsen et al. [35] and Koop [36] observed a close correlation between lactate content and survival rate of *S.* Typhimurium in the gastric digesta of pigs. Already in a study by Beal et al. [31], who performed similar research, a reduction in the bacterial counts of *S.* Typhimurium from about 7 $\log_{10}$ CFU/g to about 3 $\log_{10}$ CFU/g after 3 h incubation period in FLF was described, which could be confirmed in the present study. The survival rate of isolates in the liquid feed was strongly dependent on incubation time.

The survival rate of *E. coli* prior to inoculation showed significant counts of 4.71 $\log_{10}$ CFU/g in NFLF. However, immediately after inoculation, the *E. coli* counts in the NFLF and FLF were comparable (6.83 and 6.05 $\log_{10}$ CFU/g, respectively). Interestingly, in the NFLF, the *E. coli* count increased significantly with increasing time of incubation at 6 h *p.i.* (7.14 $\log_{10}$ CFU/g). Nevertheless, no cultural detection of the inoculated *E. coli* was observed after 6 h *p.i.* in the FLF. The proliferation of most pH-sensitive bacteria, such as *E. coli*, is minimized below pH 5 [37].

The bacterial counts of *C. perfringens* in the NFLF and also in the FLF did not decrease after 3 h *p.i.* *C. perfringens* could also be culturally detected for 6 h *p.i.* in NFLF and the FLF. Nonetheless, at this time,

the bacterial counts of *C. perfringens* in the FLF were nearly 2 $\log_{10}$ CFU/g, and thus, significantly lower than in the NFLF. The higher survival rate of *C. perfringens* in the FLF compared to *S.* Typhimurium and *E. coli* isolates may be due to the ability of *C. perfringens* to form spores [38]. It is also known from the feeding of ruminants that *Clostridium* sp. sometimes survive in the acidic environment of silages and can produce butyric acid during the fermentation process [39]. An antimicrobial effect of nisin against *C. perfringens* [40] in the FLF may have influenced the survival rate of *C. perfringens*. However, whether and to what extent nisin production from *Lactococcus lactis* [41], which was contained in the starter culture, occurred during fermentation, was not investigated in the present study.

The counts of *C. krusei* varied directly *p.i.* as well as 3 h and 6 h *p.i.* with values of 7 $\log_{10}$ CFU/g liquid feed vs. <2.00 $\log_{10}$ CFU/g before inoculation. A high tolerance of *C. krusei* to lactic acid has been described by Halm et al. [42]. As hypotheses for tolerance, the authors mention a low permeability of the cell wall of *C. krusei* for lactic acid, a high buffer capacity in the cytosol, a high H+-ATPase activity and/or high endogenous energy reserves of *C. krusei*. A multiplication of yeasts in liquid feed during the fermentation process has been described repeatedly in the literature [15,43]. Due to the high lactic acid tolerance of *C. krusei* [41], this yeast species was selected for the experiments. The addition of sodium-benzoate to the liquid feed at the beginning of the fermentation did not lead to a reduction in the *C. krusei* counts after 24 h of controlled fermentation. Both without the addition of sodium-benzoate, but also at a sodium-benzoate concentration of 0.01% and 0.05% in the FLF, the bacterial counts after 24 h fermentation were at a higher level than at the beginning of the fermentation. At a sodium-benzoate concentration of 0.25%, the counts of *C. krusei* at the end of the 24 h fermentation process increased only slightly compared to the time directly *p.i.*. However, even at this concentration, the aim of reducing the counts of *C. krusei* was not achieved. The pH values in the liquid feed varied on average between 4.14 and 4.18 at the end of the fermentation and were, thus, according to Heinze et al. [15], above the target pH value of <4.0 in the FLF. An inhibition of lactic acid production could have been caused by competition between *C. krusei* and the LAB for the sugar in liquid feed, a main substrate of the metabolism of bacteria and yeasts. Nevertheless, the lactic acid bacteria counts at the end of fermentation varied with 9 $\log_{10}$ CFU/g fermented feed, which corresponds to a normal bacterial count in the fermented feed [14]. Thus, *C. krusei* seems to have hardly influenced the reproduction of the LAB negatively. On the other hand, the pH-values of >4.0 in the fermentate could also be explained by a metabolization, and thus partial degradation/loss of lactic acid [44]. In addition, *C. krusei* has been described as having a high pH tolerance and affinity for habitats with high lactic acid contents [42]. Thus, fermented liquid feed could offer an ideal habitat for *C. krusei*. The enrichment of *C. krusei* during the fermentation process, thus, has a negative influence on the fermentation result (high pH value and possibly low lactic acid content). On the one hand, the addition of sodium-benzoate does not impair the proliferation of LAB during the fermentation process. On the other hand, in the three tested sodium-benzoate concentrations, no reduction in the *C. krusei* counts could be achieved at the end of the fermentation process.

5. Conclusions

Fermentation is an inexpensive mean to improve the nutritive value of some feed ingredients such as rye and rapeseed extracted meal for animals. Moreover, it could be beneficial for maintaining hygienic characteristics of the feed, owing to key properties such as low pH, high counts of lactobacilli and high lactic acid concentrations as well as low levels of potential pathogens. In this regard, reducing infection vulnerabilities in flocks may require the strategic feeding of fermented feed to animals as an effective strategy in reducing the transmission of pathogens to animals and humans. For future perspectives, studies applying similar fermentation processes on naturally contaminated feeds by pathogens for increasing the feed hygienic properties can be of special interest. This interest might go beyond animal health and could also be important with regards to pathogenic germs with zoonotic potential.

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