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Testing the aerobic stability of silage-based mixed rations using a multiple indicator approach

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Total Mixed Rations (TMR) including silages are commonly fed to cattle in many parts of the world, but they tend to deteriorate on exposure to air, especially in the warm season. The aim of the study was to develop an easy to implement protocol in order to compare the aerobic stability of different feed mixtures for lactating dairy cattle and to test the potential of TMR stabilizing products to delay spoilage. The experimental conditions were standardized to 25 °C ambient, and the sample dry matter was adjusted to 400 g kg^{-1} to challenge shelf life. Temperature rise in an insulated vessel, which is frequently used when testing silages, was shown to be one promising indicator of spoilage activity. Furthermore, determination of pH, scores for visual occurrence of yeasts and moulds and condensation from 0–4 were successfully applied at the end of the 72 h period. A dosage of 4.5 l propionic acid t^{-1} proved suitable as a positive control when compared to 1.5 and 3.0 l t^{-1}. The commercial products tested at the recommended dose had a similar efficacy. The stabilizing effect depended principally on the original hygienic condition of the ration. Visual evaluation plus the recording of pH, in addition to continuous temperature measurement facilitated this appraisal. Early signs of spoilage, in particular, can only be assessed by visual appraisal. Thus, an evaluation of a combination of indicators of aerobic stability is recommended.

Key words: TMR, aerobic deterioration, spoilage indicators, temperature rise, pH change, protocol

Introduction

Silages and total or partial mixed rations (TMR, PMR), which include ensiled forages, grains or by-products, are commonly fed to cattle on many farms around the world. These feeds are aerobically instable, i.e. they tend to deteriorate upon exposure to air. Spoilage of feed results in losses of dry matter (DM) and reduction in nutritional value and hygienic quality, which leads to depressed feed intake and ultimately impaired animal performance (Whitlock et al. 2000, Gerlach et al. 2014, Borreani et al. 2018). The investigation of the phenomenon of aerobic deterioration of silages increased in the 1970s, although in 1964, Beck and Gross (1964) had already recognized that yeasts might play an important role in the process. Many yeast species are able to oxidize lactic acid, which increases the pH of silage and leads to further changes brought about by other aerobic spoilage organisms (Middelhoven and Franzen 1986, Pahlow et al. 2003). Later, Spoelstra et al. (1988) confirmed the role of *Acetobacter spp.* especially in maize silages.

Different factors accelerate the microbial spoilage process. These include elevated temperature, humidity, exposure time and microbial load (Seppälä et al. 2013, Borreani et al. 2018). Despite increasing knowledge, there are still many gaps in the understanding of e.g. microbial interactions and apparently contradictory observations, especially in unconventional feeds such as chicory and potato by-products (Martens 2006, Avila and Carvalho 2020). A temperature rise of 2 or 3 K above ambient is internationally recognized as a reliable indicator for aerobic instability of silages (O'Kiely 1993, Cherney and Cherney 2003). However, indicators of microbial spoilage do not always match observed temperature rises. Varying observations of no or very low temperature increase, despite other signs of spoilage, are summarized in Supplementary Table 1. Therefore, not only temperature but other indicators of aerobic respiration should be monitored, for example, pH increase, visible fungal infestation and CO_2 development where feasible (Shan et al. 2021a).

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Agricultural and Food Science (2022) 31: xxx–xxx
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Currently, various products are available on the European market that aim to stabilize the moist mixed rations, which might be of interest in farm practice, especially when storing TMR in the warm season. Apart from the chemical composition of the offered products, their efficacy might also depend on other factors related to the feed and the environment, such as hygiene, humidity and temperature.

The aim of the collaborative study of four Institutes of Applied Agricultural Research was to develop a simple scheme to challenge the aerobic stability of mixed rations on a laboratory scale, in order to test the efficacy of different TMR stabilizing agents and their dosage. For this purpose, in addition to temperature, other potential indicators that could easily be carried out without special laboratory equipment were recorded and evaluated. Initially, the minimum dosage of propionic acid was determined to serve as a positive control in subsequent trials.

Materials and methods

Pre-trials served to standardize the experimental conditions in terms of duration of the test, ambient temperature, the sample moisture range, observed spoilage indicators (Martens and Steinhöfel 2019), and the method is presented here.

Fresh TMR for lactating dairy cows yielding ≥ 30 kg milk per cow d^{-1} were obtained from the experimental farm stations of the four institutes involved in the study. All of the rations contained 600–650 g silage kg^{-1} DM (maize + grass) and included cereal grains (barley, wheat or rye), rapeseed meal and minerals as common feedstuffs in varying proportions (Tables 1 & 2). The DM content was determined at 105 °C in a drying oven over night. Based on this information, another fresh TMR portion was taken for the aerobic stability test on the following day. Hence, it was possible to calculate the volume of water needed to achieve the target DM of 350–400 g kg^{-1} in order to challenge aerobic deterioration (Rinne et al. 2018, Martens and Steinhöfel 2019).

Table 1. Total Mixed Ration composition in Trials 1 and 2 (% of dry matter)

	Trial 1				Trial 2			
Component	Inst.1	Inst.2	Inst.3	Inst.4	Inst.1	Inst.2	Inst.3	Inst.4
Maize silage	34.4	24.2	31.2	29.6	34.4	28.5	27.6	29.6
Grass silage	26.0	38.5	34.0	33.2	26.0	11.5	30.6	33.2
Grass-clover silage	0	0	0	12.3	0	0	0	12.3
Crimped maize	0	0	0	7.09	0	5.11	0	0
Grain maize/sorghum	11.9	4.28	0	1.09	0	0	0	7.09
Barley/Wheat/Rye	3.17	7.13	0	2.18	11.9	7.74	4.51	1.09
Barley/Wheat straw	1.51	0	2.54	3.42	3.17	12.0	13.57	2.18
Lucerne hay	0	2.14	0	0	1.51	0	6.36	3.42
Pelleted pressed beet pulp	5.81	4.04	0	0.446	0	3.85	0	0
Rapeseed meal	6.34	7.13	12.6	7.48	5.81	3.91	0	0.446
Sunflower seed meal	7.84	0	0	0	6.34	9.67	11.34	7.48
Soybeans	0	0	0	2.33	7.84	0	0	0
Field peas & beans	0	0	0	0	0	0	0	2.33
Cattle salt	0	0	0.192	0.050	0	0	4.51	0
Glycerin	0	0.950	0	0	0	0.043	0.19	0.050
Mixture of cereal grains and minerals + trace elements	2.41	11.6	18.9	0.842	0	2.20	0	0
Urea	0.399	0	0.240	0	2.41	0.400	1.09	0.842
Vinasse from sugar beets	0.310	0	0.240	0	0.399	0	0.24	0

Inst. = institute

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

Table 2. Chemical (g kg^{-1} DM) composition and microbial counts (log cfu g^{-1} fresh matter) of the Total Mixed Rations in Trials 1 and 2

	Trial 1					Trial 2			
Parameter	Inst. 1	Inst. 2(1)	Inst. 2(2)	Inst. 3	Inst. 4	Inst. 1	Inst. 2	Inst. 3	Inst. 4
Original DM (g kg^{-1})	360	506	507	367	399	438	504	439	382
DM after remoistening (g kg^{-1})	361	359	390	347	399	393	369	362	363
Crude ash	71.0	66.0	71.0	75.0	83.0	72.0	60.0	74	70.0
Crude protein	146	154	166	141	134	157	157	164	148
Crude fibre	176	176	163	173	n.a.	174	151	187	209
Ether extract	29.0	30.0	34.0	31.0	n.a.	33.0	39.0	30.0	40
aNDFom	361	344	328	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
ADFom	194	204	196	n.a.	n.a.	n.a.	n.a.	n.a.	246
Sugar (water soluble)	57.0	62.0	61.0	59.0	63.0	53.0	20.0	40.0	26.0
Starch	242	218	215	236	158	238	249	204	212
Metabolizable Energy (MJ)	11.0	11.1	11.5	11.1	10.29	11.3	11.8	11.0	10.6
Net Energy Lactation (MJ)	6.70	6.70	7.10	6.80	6.16	6.90	7.27	6.70	6.40
pH	4.19	4.57	4.73	4.27	4.33	4.35	4.18	4.05	4.60
Lactic acid	41.0	62.6	62.9	24.3	42.0	44.7	n.a.	68.7	34.0
Acetic acid	13.1	7.12	6.99	7.65	9.00	14.9	n.a.	19.0	12.0
Propionic acid	0.19	0.13	0.13	n.d.	n.d.	<0.30	n.a.	n.d.	n.d.
Butyric acid	n.d.	0.46	n.d.	0.273	n.d.	<0.55	n.a.	0.40	n.d.
Ethanol	4.26	1.69	1.33	6.83	2.00	5.60	n.a.	18	2.00
1,2-Propanediol	5.30	0.76	0.93	3.01	n.a.	4.1	n.a.	5.90	n.a.
Undissociated VFA	10.4	4.67	3.65	5.96	6.52	10.7		16.2	7.03
Undis. VFA/(sugar + LA)	0.11	0.04	0.03	0.07	0.06	0.11		0.15	0.12
NH_3-N (g kg^{-1} N)	39.0	38.0	30.0	n.a.	n.a.	n.a.	n.a.	n.a.	1.00
Moulds	<2.00	2.40	<3.00	<2.00	2.00	3.48	3.65	<1.0	2.30
Yeasts	6.20	8.37	6.42	4.81	3.90	4.67	6.20	3.00	4.83

Inst. = Institute; DM = dry matter; ± standard deviation; aNDFom = amylase treated neutral detergent fibre exclusive of residual ash; ADFom = acid detergent fibre exclusive of residual ash; (1) = first run; (2) = second run; Undissociated VFA = sum of undissociated acetic, propionic, butyric and valeric acids; Undis. VFA/(sugar + LA) = ratio of sum of undissociated VFA to sugar + lactic acid as suggested by Gomes et al. (2021); n.a. = not analyzed; n.d. = not detectable

Trial 1: Dose-response study with propionic acid

Trial 1 was conducted in late October-early November 2020 (i.e. autumn) at the four localities in Northern (1), Eastern (1) and Southern (2) parts of Germany. Four different levels of propionic acid (n = 6): 0, 1.5, 3.0, 4.5 ml kg^{-1} original TMR were applied to determine the concentration, that would be reasonably certain to assure aerobic stability and serve as a positive control. A fifth level (6.0 ml kg^{-1}) was introduced in a second experimental run in Institute 2. They are referred to as Pr0.0, Pr1.5, Pr3.0, Pr4.5 and Pr6.0. Manufacturers of products using the same agent currently recommend 1–3 l t^{-1} to farmers. When the TMR had to be remoistened, the propionic acid (Art. No. 6026, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was mixed in the respective amount of tap water necessary to achieve at the most 400 g DM kg^{-1}. This liquid was then added to the weighed amount of TMR and mixed thoroughly. When the original TMR already had the target DM (Institute 4), the propionic acid (undiluted) was sprayed on evenly with a pump sprayer to assure a homogeneous distribution.

Institute 2 ran a second experiment with a slightly lower moisture content because of high instability observed in its first run, which had not allowed differentiation between the treatments.

Trial 2: TMR stabilizing products and both positive and negative control

Trial 2 took place between the end of November 2020 and the beginning of January 2021 at the four Institutes. In addition to the negative and positive control (no additive and 4.5 ml propionic acid kg^{-1} TMR; Pr0.0 and Pr4.5) there were four other treatments (n = 4): potassium sorbate (> 99 %) (0.4 g kg^{-1}, powder, to be dissolved) (STAB1),

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

a combination of propionic (~38 %) and formic acid (~34 %) (3.5 ml kg^{-1}, liquid) (STAB2), a combination of sodium benzoate (300 g kg^{-1}) and diacetate (20 g kg^{-1}) (3.0 ml kg^{-1}, liquid) (STAB3), and a combination of sodium formate and potassium sorbate (concentrations not disclosed by the manufacturer) (2.0 g kg^{-1}, granulate) (STAB4). The additives were applied in the same proportions as in the first trial, i.e. mixed with the tap water used for remoistening. The only exception was the granulate (STAB4), which was spread evenly on the TMR before remoistening, according to the manufacturer's instructions.

The parameters, which were determined by the treatments before the aerobic stability test were: DM, pH, yeast and mould numbers, crude ash, crude protein, ether extract, neutral detergent fibre treated with an amylase and exclusive of residual ash (aNDFom), acid detergent fibre exclusive of residual ash (ADFom), starch, water soluble carbohydrates, lactic, acetic, butyric and propionic acid, NH_3-N of total N, ethanol (VDLUFA 1976, VDLUFA 2012). The undissociated form of each volatile fatty acid in the TMR was calculated as $VFA_{undis.} = \frac{1}{1 + 10^{(pH-pka)}}$ (Henderson-Haselbalch equation) and multiplied by the respective acid concentration.

The treated material was weighed into containers based on the model of System Völkenrode in replicates (around 250 g fresh matter (FM) each), and the weight recorded (Honig 1990). A sample container consisted of 20 cm lengths of a polyvinyl chloride (PVC) drainage pipe (PVC-KG-pipe DN110, Ø 11 cm), and closed with PVC caps (DN110) at the bottom and the top. A hole of Ø 10 mm was drilled in the centre of each cap to allow air to circulate. A layer of cotton gauze was placed on the bottom of the container to avoid losses by trickling. A diagram of this system is shown in Supplementary Figure 1. When about one third of the container was filled a temperature data logger (TG 4080, Gemini Data Loggers Ltd, Chichester, UK), wrapped in a disposable polyethylene bag, was placed centrically in each tube. The loggers were programmed to record the temperature at half-hourly intervals. The filled containers were then closed and placed in a polystyrene cylinder (EPS25, 6 cm wall thickness) which provided temperature insulation. Polystyrene covers (EPS25, 6 cm thick) were placed on the top and bottom of the cylinders. They had a V-shaped notch (5 mm deep, 1 cm wide) passing straight through the middle of the cover to permit air flow.

The samples were stored at 25 (± 1) °C for 72 h. When taking them out of the polystyrene cylinder for evaluation, all PVC lids were firstly removed to evaluate the possible loss of condensed water, from both the cap and the inner walls, and the actual volume ranked between 0 and 4 (Table 3; Suppl. Fig. 2). The vessels were then weighed with their contents, but without the lids. The complete samples were examined for visible signs of yeast and mould growth (Table 4; Suppl. Figs. 3 & 4). DM and pH were also determined. FM and DM losses after the aerobic stability test (AST) were calculated as follows: FM loss (%) = 100 – net weight after AST/net weight before AST × 100, DM loss (%) = 100 – (net weight after AST × DM [%] after AST / 100)/(net weight before AST × DM [%] before AST / 100) × 100.

Table 3. Humidity score after the aerobic stability test, with immediate evaluation after opening the lids (see also Suppl. Fig. 2)

Points	Observation
0	No condensation
1	Light condensation at container wall
2	Wall continuously moist
3	Large drops at the wall (but water does not merge to a pool in the lid)
4	Wall and lid very wet

Table 4. Visual evaluation of yeast and mould growth (see also Suppl. Figs. 3 & 4) (Pahlow 1997, personal communication, DLG TestService GmbH 2018)

Points	Yeasts	Moulds
0.0	None visible	None visible
0.5	Traces of yeasts	A very small area of mould
1.0	Yeasts ~10%	-------
1.5	More yeasts	Some small mould agglomerations
2.0	Yeasts continuously present	-------
2.5	Yeasts continuously present	More mould agglomerations
3.0	Heavy presence of yeasts	Mould in every part
4.0	Completely deteriorated	Completely deteriorated

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

A demonstration of the whole procedure can be viewed in the following video:
https://lsnq.de/tmraerobicstabilitytest

Statistical analyses

Both one and two-factorial designs were used. Variance analysis using the univariate and multivariate procedures was performed for both Trials, followed posthoc by a Tukey test.

For Trial 1, the treatments were first evaluated within institute (per test run) in order to see in more detail how the different parameters would react depending on the underlying ration (Figs. 2–5):

$$Y_i = \mu + CONC_i + \varepsilon_i$$

where i = 1, 2, …, 4 (0, 1.5, 3.0 or 4.5 ml propionic acid kg^{-1} TMR) and ε = error.

Furthermore, they were evaluated across all test runs (Table 5):

$$Y_i = \mu + CONC_i + RUN_j + CONC \times RUN_{ij} + \varepsilon_{ij}$$

where i = 1, 2, …, 4 (0, 1.5, 3.0 or 4.5 ml propionic acid kg^{-1} TMR), j = 1, 2, …, 5 (test runs) and ε = error.

For Trial 2, the treatments were evaluated across all institutes (Table 6):

$$Y_i = \mu + STAB_i + INST_j + STAB \times INST_{ij} + \varepsilon_{ij}$$

where i = 1, 2, …, 6 (stabilizers), j = 1, 2, …, 4 (Institutes) and ε = error.

In some cases, the temperature did not increase by 2 K above ambient within the limited time span of the AST. In those cases, it was decided to add 0.25 d (i.e. 6 h) to the maximum evaluated time. This was done to be able to include those samples in the statistical evaluation e.g. variance analysis and the post-hoc test, and to considering the practical significance on-farm of stocking ready mixed feeds. The software IBM® SPSS® Statistics (Version 19, SPSS, Inc., IBM Company©) was used. In Trial 2 the slope of linear regression lines (extended to axes) of the temperature curves was determined (SigmaPlot 12.5, Systat Software, Inc.).

Results

Trial 1

There was a highly significant effect of the test runs at the different Institutes on the evaluated parameters. The same was true for the treatment effect except for pH. In addition, the interaction of both factors significantly influenced all parameters but FM losses and maximum temperature difference (Table 5).

Pr0.0 represents Total Mixed Ration without additives, Pr1.5, 3.0, 4.5 with 1.5, 3.0 and 4.5 l propionic acid t^{-1} Fresh Matter; different superscript letters in the same row refer to significant differences among treatments ($p < 0.05$, Tukey test). TD, temperature difference to ambient; (0–4) refers to the score given in Table 3 and 4; SEM, standard error of the mean. Tr Treatment, Run Test run.

In three out of five test runs the temperature of the control Pr0.0 rose steeply within the first 24 h (Fig. 1).

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

Table 5. Shelf life of differently treated Total Mixed Ration stored for 72 h under aerobic conditions in Trial 1. Results of test runs at four different Institutes (n = 6 per Institute).

						p-value		
Measurements	Pr0.0	Pr1.5	Pr3.0	Pr4.5	SEM	Treatment	Run	Tr x Run
h until ≥2 K TD	26.6^{d}	32.3^{c}	39.9^{b}	47.7^{a}	0.30	<0.001	<0.001	<0.001
h until ≥3 K TD	28.7^{d}	36.3^{c}	43.3^{b}	49.4^{a}	0.37	<0.001	<0.001	<0.001
Max. TD (K)	11.6^{a}	10.9^{a}	10.5^{a}	9.16^{b}	0.154	0.001	<0.001	0.075
h until max. TD	43.9^{c}	52.6^{b}	58.8^{a}	57.4^{ab}	0.71	<0.001	<0.001	<0.001
Final pH	5.79	5.75	5.67	5.61	0.053	0.563	<0.001	<0.001
Humidity (0–4)	3.55^{a}	3.39^{a}	2.57^{b}	2.04^{c}	0.046	<0.001	<0.001	<0.001
Yeasts (0–4)	2.81^{a}	2.13^{b}	1.71^{c}	0.87^{d}	0.041	<0.001	<0.001	<0.001
Moulds (0–4)	0.484^{a}	0.161^{b}	0.089^{b}	0.093^{b}	0.016	0.001	<0.001	<0.001
FM losses (%)	3.40^{a}	3.06^{ab}	2.70^{bc}	2.42^{c}	0.059	<0.001	<0.001	0.459
DM losses (%)	6.65^{a}	7.12^{a}	5.21^{ab}	4.42^{b}	0.265	0.001	<0.001*	0.004*

*Excluding Institute 1 as this parameter was not determined here.

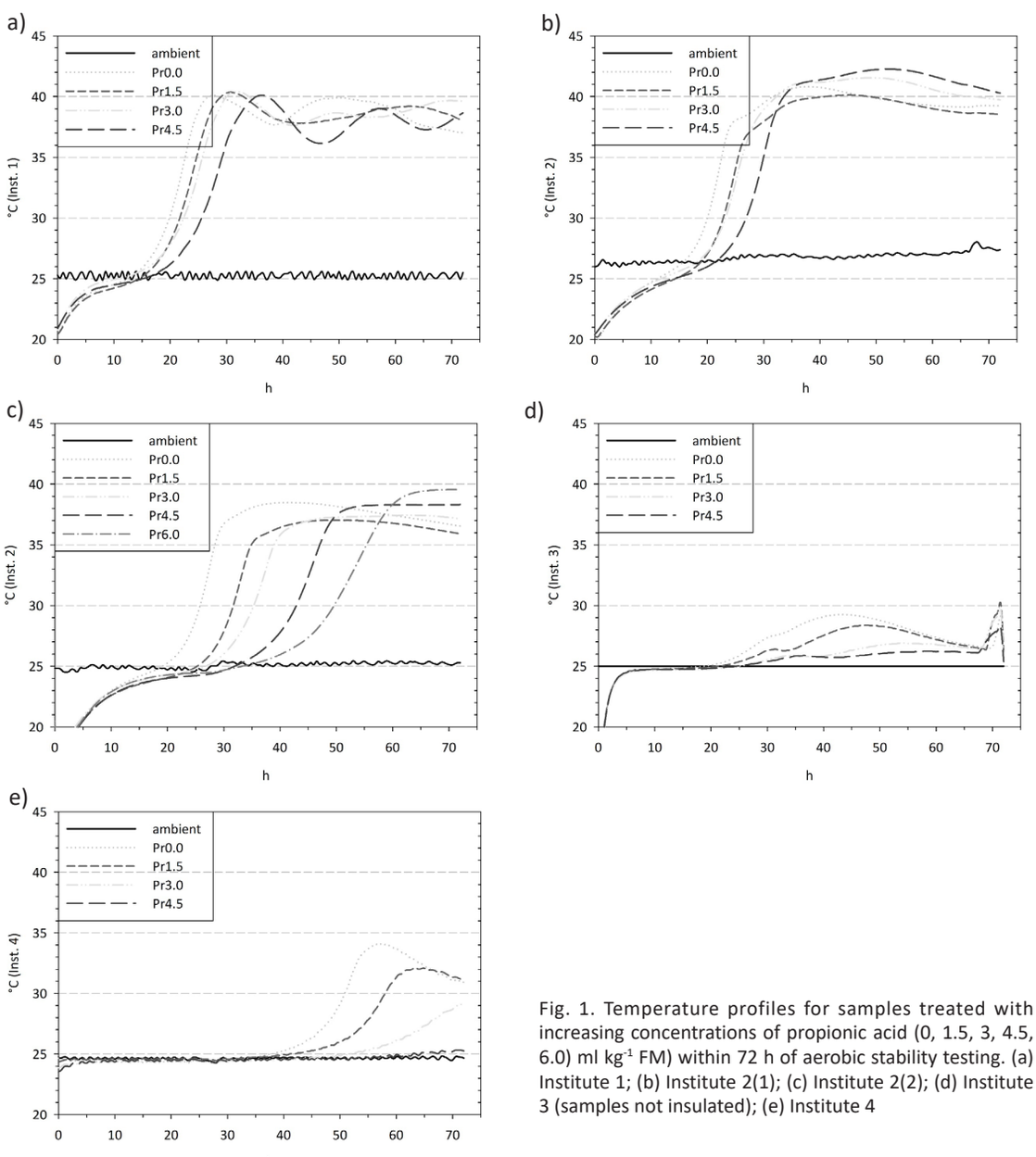

Fig. 1. Temperature profiles for samples treated with increasing concentrations of propionic acid (0, 1.5, 3, 4.5, 6.0) ml kg^{-1} FM) within 72 h of aerobic stability testing. (a) Institute 1; (b) Institute 2(1); (c) Institute 2(2); (d) Institute 3 (samples not insulated); (e) Institute 4

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

Notably, in two of those (a, b), the temperature rise of the acid treatments was only slightly delayed, and there was almost no difference between Pr1.5 and Pr3.0. They had an initial total yeast count of 6.0 (a), 8.4 (b) and 6.4 (c) $\log_{10}$ cfu g^{-1} FM (Table 2). In the case of no thermal insulation (d) there was only a slight increase, which started at the end of the first day, followed by a flattening of the curve. The initial yeast count was 4.8 $\log_{10}$ cfu g^{-1} FM. A test run (e) had shown the latest time for temperature to start to increase i.e. after 40 h, and there was almost no heating in Pr4.5 within 72 h. At the start of the experiment it had 4.2 $\log_{10}$ cfu yeasts g^{-1} FM.

Whilst the maximum temperature rise was not different among treatments during the rapidly accelerating runs (Institutes 1 & 2), lowest temperature difference was observed for Pr4.5 in the two slower runs (Fig. 2a), which was reflected in the evaluation across the test runs (Table 5). However, time to achieve the maximum temperature was more delayed at Institutes 2, 3 and 4 (Fig. 2b). Time to achieve 2 or 3 K above ambient was clearly differentiated between the treatments (Fig. 2c & d, Table 5), and was most clearly demonstrated in the slowly increasing test runs.

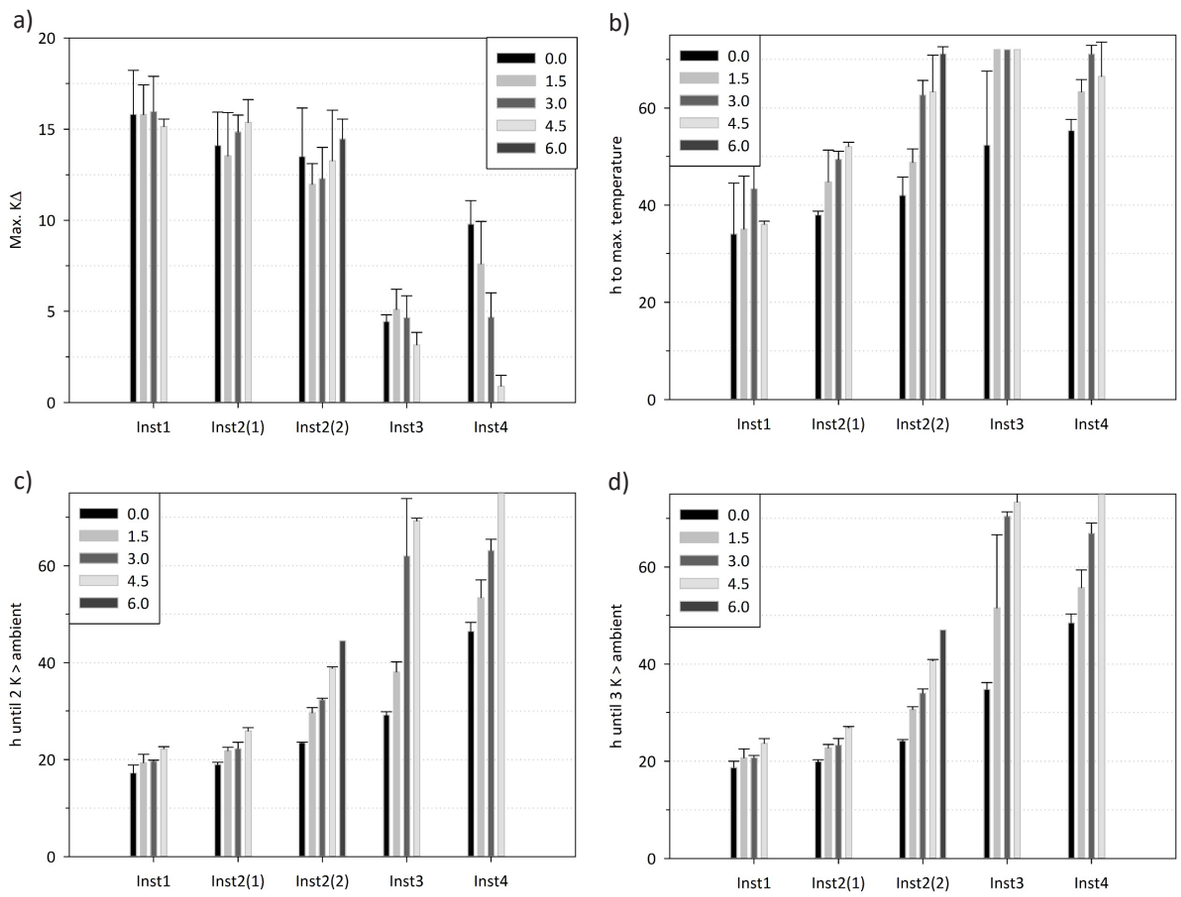

Fig. 2. (a) Maximum temperature difference; (b) hours until maximum temperature difference; (c) hours until 2 K difference above ambient; (d) hours until 3 K difference above ambient in the different treatments at the different institutions. The figures in the legend represent the applied dosage of propionic acid in L t^{-1} FM TMR. Error bars represent the standard deviation (SD). The *p*-value indicate the significance of the dosage effect within each test run.

In all of the test runs there was an increase in pH (Fig. 3). However, only in Institute 1 and 4 was there a statistically significant effect of treatment, which contrasted at both the institutes. Where the rapid temperature increase had taken place (Institute 1), the highest pH was found for the highest acid addition Pr4.5. In contrast, at Institute 4, the pH was highest in the untreated control where there was only slow heating. This contrast led to the insignificant effect of the treatment on the final pH when the statistical analysis was applied across the test runs, in contrast to the interaction with the test run (Table 5).

Agricultural and Food Science (2022) 31: xxx–xxx
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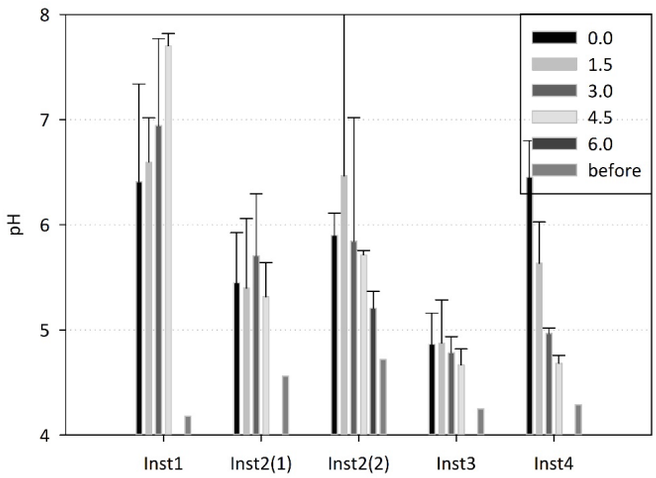

Fig. 3. Initial and final pH values for the different treatments at different Institutes. The figures in the legend represent the applied dose of propionic acid in l t^{-1} FM Total Mixed Ration. Error bars represent the standard deviation (SD). The *p*-value indicates the significance of the dosage effect within each test run.

The humidity was very high in the test runs with a rapid and steep temperature rise (Institutes 1 and 2) and only modest and small rises with the high acid treatment Pr4.5 (Fig. 4a, Table 5).

Signs of yeast growth were more varied across the institutes; the highest acid treatment usually showing the least yeast growth (Fig. 4b, Table 5). Mould growth was only observed at Institute 1 (Table 5).

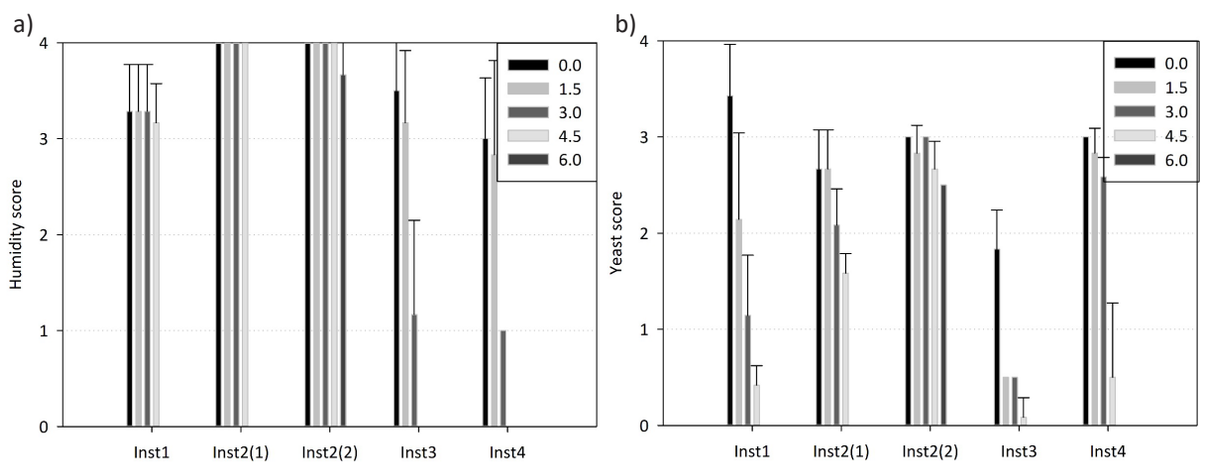

Fig. 4. (a) Score of condensed water (Table 3) at the end of the aerobic stability test at the different Institutes. (b) Score of visible yeast growth (Table 4). The figures in the legend represent the applied dosage of propionic acid in l t^{-1} FM Total Mixed Ration. Error bars represent the standard deviation (SD). The *p*-value indicates the significance of the dosage effect within each test run.

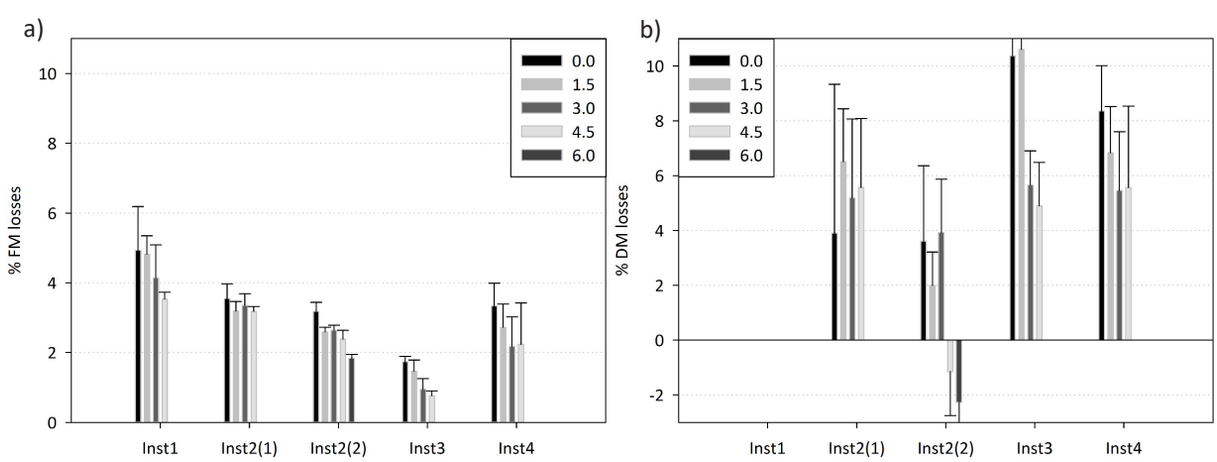

Fig. 5. (a) Fresh matter losses; (b) dry matter losses in the different treatments at the different institutions. The figures in the legend represent the applied dosage of propionic acid in l t^{-1} FM Total Mixed Ration. Error bars represent the standard deviation (SD). The *p*-value indicates the significance of the dosage effect within each test run. (n.a. = not analyzed)

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

While FM losses were consistently higher in the control (Fig. 5a) the DM losses were less consistent (Fig. 5b, Table 5).

Trial 2

As results from Trial 1 pointed to a reasonably safe effect of Pr4.5 on aerobic stability it was chosen as positive control for Trial 2. Here, the treatment had a significant effect on all parameters just as the interaction between treatment and Institute (Table 5). The same applied for the factor Institute per se except for time until maximum temperature difference.

In contrast to the first trial, the temperature only started to rise on the second day irrespective of location (Figs. 6a & b). The average slope of the linear regression line is presented in Table 5. The TMR, which showed a steep increase in temperature in the control (Fig. 6b) had an initial yeast count of 6.2 $\log_{10}$ cfu g^{-1} FM while the one with more gradual heating (Fig. 6a) had a count of 4.7 $\log_{10}$ cfu g^{-1} FM. The one with a similar yeast count (4.8 $\log_{10}$ cfu g^{-1} FM) started to increase in temperature later, but rose sharply (Fig. 6d). In one of the test runs, the temperature did not increase at all during the 72 h period (Fig. 6c), and yeasts counts were 3.2 $\log_{10}$ cfu g^{-1} FM (Table 2).

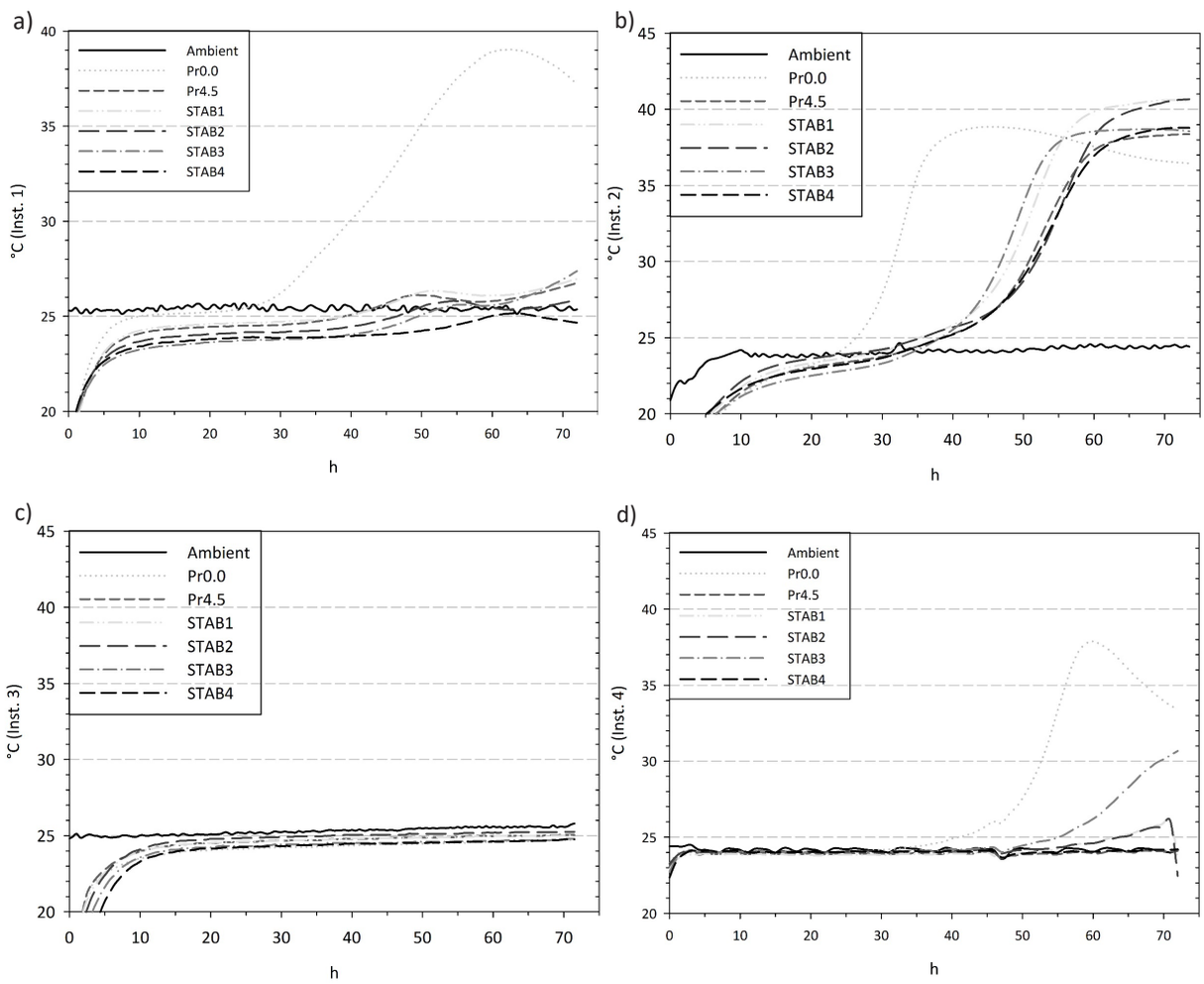

Fig. 6. Temperature development during a 72 h aerobic stability test in the second trial; (a) Institute 1, (b) Institute 2, (c) Institute 3, (d) Institute 4

The time until the temperature reached 2 or 3 K ≥ ambient was shortest for the negative control, on average for the four test runs (Table 6). The maximum temperature difference discriminated the treatments only in two out of four cases when evaluating per Institute (Fig. 7a). However, there was a highly significant effect of treatment and Institute in the overall evaluation (Table 6). When 2 K difference was used as the criterion, more samples could be evaluated properly as their temperature increased within the test time (Fig. 7c and d). Final pH, humidity, yeast score and losses were highest on average in the negative control (Fig. 9). However, in the post hoc test across institutes, only Pr4.5 and STAB4 were significantly less humid, and in STAB4 also less yeasts appeared (Table 6). Although no temperature increase was apparent for Institute 3 (Fig. 6c) first signs of yeast growth were observed there (Fig. 8c). Moulds appeared only in one out of the four test runs and in two of the treatments, the negative control and STAB3 (Fig. 8d, Table 6).

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

Table 6. Shelf life of differently treated Total Mixed Ration stored for 72 h under aerobic conditions in Trial 2. Results of test runs at four different institutes (n = 4 per Institute).

								p-value		
Items	Pr0.0	Pr4.5	STAB1	STAB2	STAB3	STAB4	SEM	Treatment	Inst.	Tr x Inst.
h until ≥2 K TD	46.6^{b}	67.7^{a}	66.2^{a}	68.0^{a}	64.3^{a}	70.0^{a}	1.63	<0.001	<0.001	<0.001
h until ≥3 K TD	48.3^{b}	70.6^{a}	69.6^{a}	69.6^{a}	66.2^{a}	70.7^{a}	1.51	<0.001	<0.001	<0.001
Max. TD [K]	10.5^{a}	3.66^{b}	4.78^{ab}	4.71^{ab}	5.65^{ab}	3.57^{b}	0.666	<0.001	<0.001	<0.001
h until max. TD	59.5^{b}	67.2^{a}	66.8^{ab}	67.6^{a}	68.7^{a}	66.7^{ab}	0.73	<0.001	0.958	<0.001
Final pH	5.82^{a}	4.55^{b}	4.63^{b}	4.75^{b}	4.72^{b}	4.69^{b}	0.067	<0.001	<0.001	<0.001
Humidity (0–4)	3.00^{a}	1.12^{b}	1.47^{ab}	1.41^{ab}	1.69^{ab}	1.18^{b}	0.161	<0.001	<0.001	<0.001
Yeasts (0–4)	2.16^{a}	0.94^{ab}	1.06^{ab}	0.91^{ab}	1.41^{ab}	0.68^{b}	0.124	<0.001	<0.001	<0.001
Moulds (0–4)	0.16	0.00	0.00	0.00	0.09	0.00	0.0180	0.004	<0.001	<0.001
FM losses (%)	2.75^{a}	1.20^{b}	1.46^{b}	1.33^{b}	1.49^{b}	1.15^{b}	0.068	<0.001	<0.001	<0.001
DM losses (%)	5.50^{a}	1.25^{b}	0.75^{b}	0.85^{b}	0.22^{b}	-0.63^{b}	0.329	<0.001	<0.001	<0.001
Slope *m*	0.266	0.120	0.140	0.125	0.154	0.111	0.0350			

Pr0.0 = Total Mixed Ration without additives; Pr4.5 = with 4.5 l propionic acid t^{-1} FM, STAB1–4 = the treatments with commercial TMR stabilizing products; different superscript letters in the same row refer to significant differences among treatments ($p < 0.05$, Tukey test). TD = temperature difference to ambient; (0–4) = the score given in Table 3 and 4; m = slope of linear regression line of temperature curve (total n = 3, Institute 3 was left out because no heating occurred); SEM = standard error of the mean; Inst. = Institute, Tr = treatment.

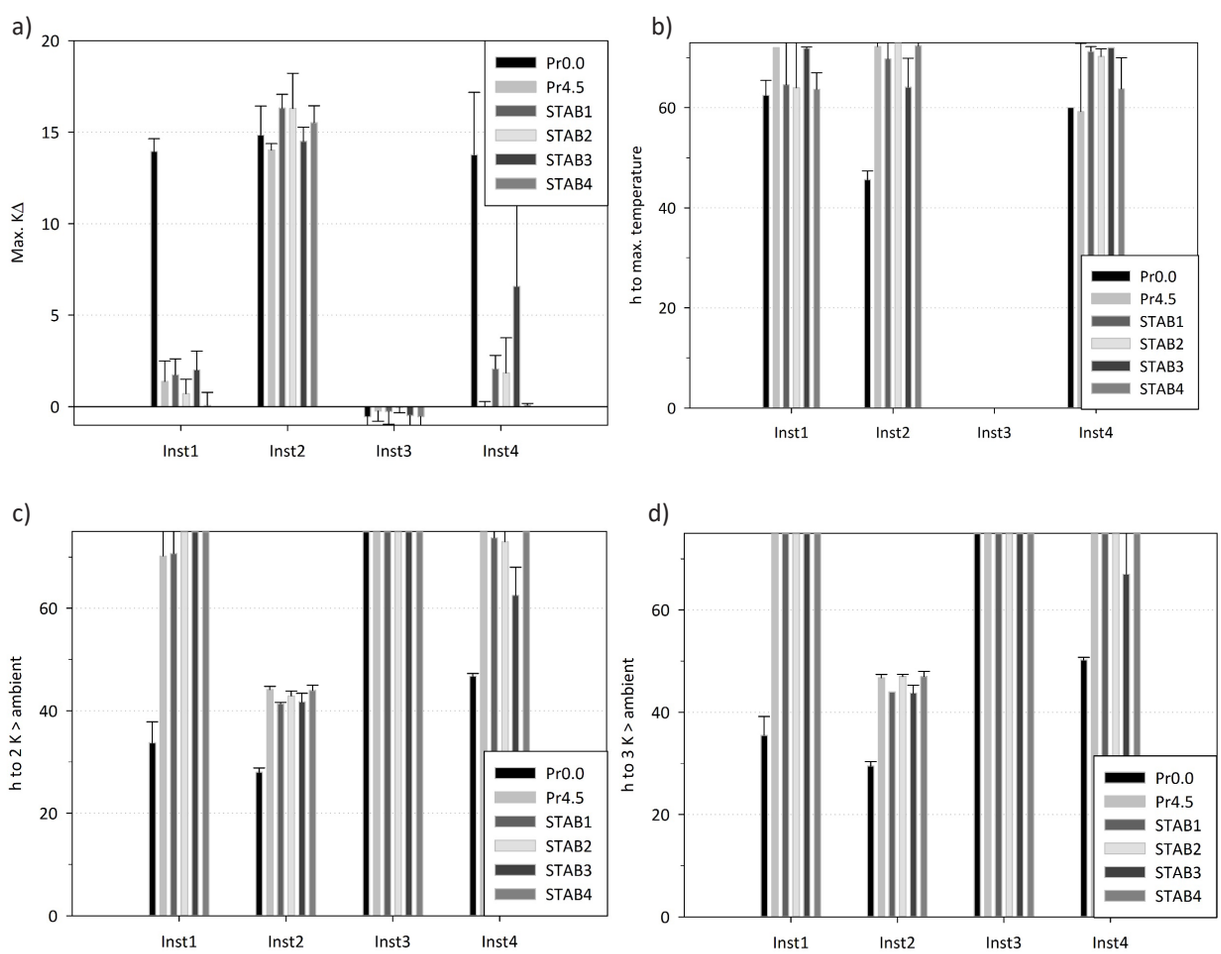

Fig. 7. (a) Maximum temperature difference to ambient; (b) hours until maximum temperature difference; (c) hours until 2 K ≥ ambient; (d) hours until 3 K ≥ ambient (Inst1–4 = Institute 1–4)

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

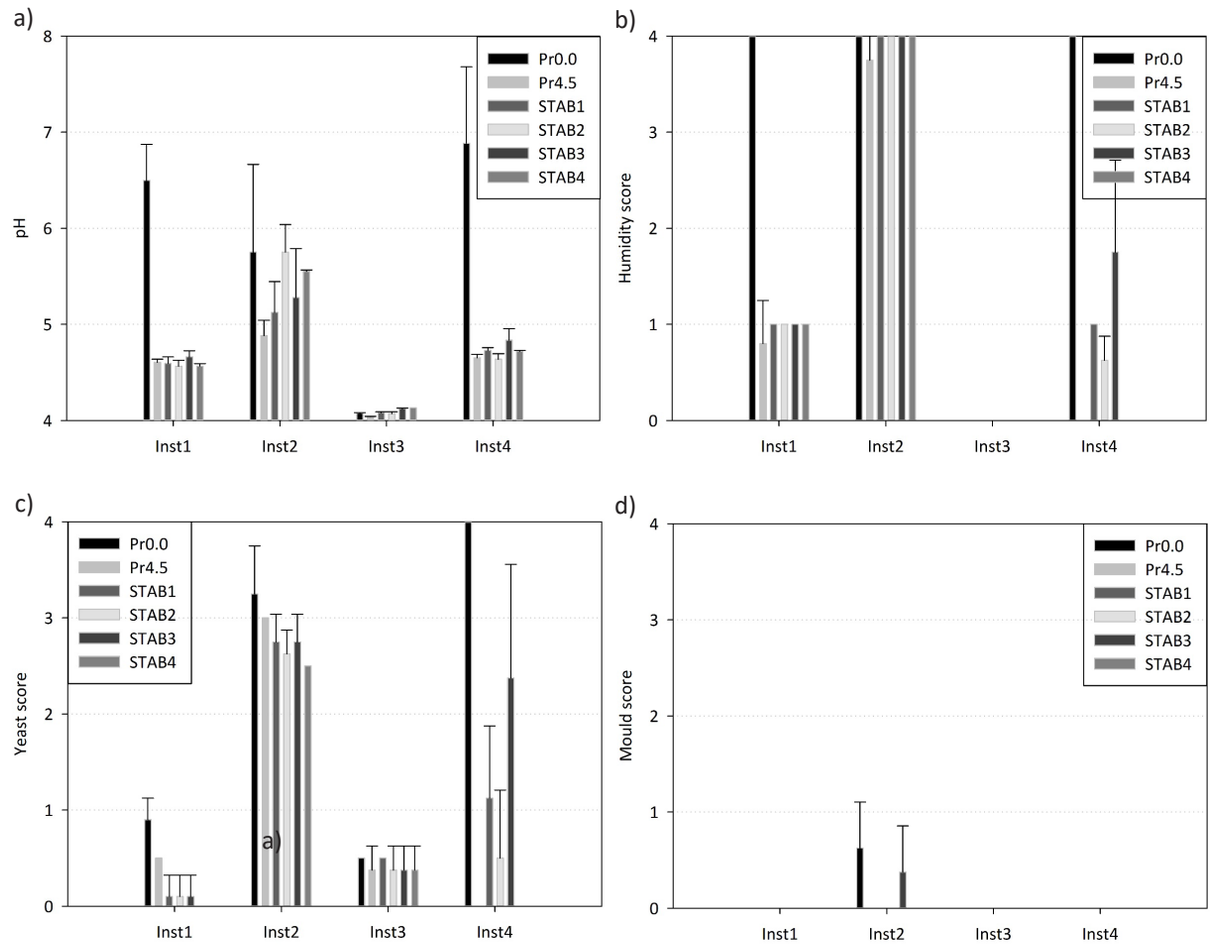

Fig. 8. (a) Final pH; (b) Humidity score; (c) Yeast score; (d) Mould score after visual evaluation (Inst1–4 = Institute 1–4)

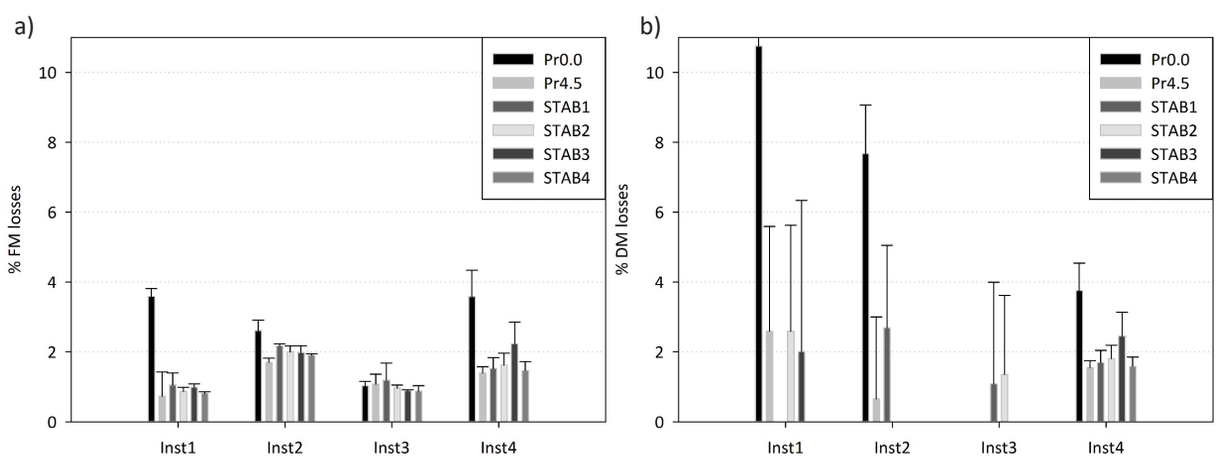

Fig. 9. (a) Fresh matter losses in %; (b) Dry matter losses in % (Inst1–4 = Institute 1–4)

Overall when evaluating all four test runs together, the time until 2 or 3 K temperature difference, pH and losses demonstrated the effectiveness of the tested products compared to the untreated control (Table 6).

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

Discussion

Temperature increase and pH changes

In general, undissociated volatile fatty acids (VFA) may exhibit fungistatic effects, and thus, Gomes et al. (2021) found that the higher the proportion of those acids in grass silage compared to the sum of soluble carbohydrates and lactic acid, the more stable the pH was on exposure to air. However, in an untreated TMR for high yielding dairy cows the amount of VFA is negligible compared to concentration of the soluble carbohydrates (Table 2). Thus, other factors may determine more clearly the course of aerobic deterioration in this kind of feed.

In terms of temperature, the results presented for Trial 1 showed higher increases occurring earlier than for Trial 2. This was reflected by the yeast counts, which could be a result of both a new batch of silage from another silo (at least at Institute 1) and/or of the oncoming cold season.

In Trial 1 at Institute 3 the samples were not insulated, which probably explains why the temperature curve flattened early, as heat diffused to the environment. For this reason the use of a thermal insulation is of paramount importance, as Honig (1990) had suggested when working with small sample sizes.

It was more helpful to evaluate the time taken for the temperature to rise ≥ 2.0 K above ambient than 3.0 K, especially in Trial 2 when samples were more stable, as it allowed at least a numerical differentiation within the specified time when looking at a particular test run. Often, this single point in time is determined when running an aerobic stability test, but it is recommended to also map the temperature development. Mathematically this can be done either by linear regression equations to show the gradient or by more sophisticated models such as the Gompertz function (Zeyner et al. 2018). In the presented case, the slope of the curve showed the changes simultaneously, at least for the speed and extent of temperature increase.

The results presented for Trial 1 also demonstrated that TMR stabilizing products are more effective when the original stability of the feed was moderate, i.e. a yeast load of < 10^5 cfu g^{-1} FM. In that situation a clear dose response is more probable. This is in accordance with the findings of Rinne et al. (2018) and Seppälä (2020). Increasing the dosage of fungistatic agents within reasonable economic constraints is unlikely to stop spoilage of feeds with a low initial hygienic quality.

The final pH allowed a reasonable differentiation of treatments overall, and in Institute 4 in the first trial and again in Trial 2 at Institutes 4 and 1. Measuring the pH after the stability test only allows a reasonable interpretation when the samples are not yet completely spoiled, as was the case in the first trial in Institutes 1 and 2(1). In this case, the results appear to be in accordance with the visual evaluation of humidity and yeasts as shown in the second trial. Otherwise, the highest level of propionic acid treatment can lead to the highest pH, as was the case for Institute 1 in Trial 1. Thus, increase in pH and visual yeast occurrence at the end of the test can tell whether spoilage has taken place, but not when it started. In an experiment with tropical grasses, pH clearly rose before temperature in several treatments (Gomes et al. 2021). It would be desirable to monitor pH over time to best make use of this parameter, which suggests that oxidation of lactic acid has taken place (Middelhoven and Franzen 1986, Pahlow et al. 2003, Martens 2006). Shan et al. (2021b) used a special pH electrode to monitor the fermentation process in a mini-bioreactor. Other researchers tried to develop a wireless pH sensor for application to feeds (Huang et al. 2012, Marsh et al. 2020). However, none of these approaches yet seems suitable or available for routine applications such as the current aerobic stability test.

Visual assessment of condensed water and fungal infestation

Humidity is a good indicator of ongoing oxidative processes. In order to minimize the subjective influence of visual inspection, different data loggers for relative humidity were tested in several preceding trials. Most of them failed in the special environment, which is why it was decided to use a visual inspection. However, in analogy with pH, its evaluation can only help to differentiate treatments when samples are not yet completely spoiled, as was the case with samples from Institute 1 in Trial 1.

Visual evaluation of signs of yeast growth allowed an early detection of spoiling processes in the case of Institute 3 in the second trial. Although no temperature increase was detected during the 72 h of the aerobic stability test, some yeast points were scored for all treatments at opening. This demonstrated that heating is a subordinate indicator in the early stage of yeast development. On the other hand, when oxidation is at an advanced stage,

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

yeasts cannot be detected easily by visual inspection. Either the sample is too wet to identify single yeast points or they are overgrown with mould, as was observed in pre-trials. This is the reason why other authors attempted to develop another method of observing fungal growth using a transparent covering, which unfortunately lacked insulation (Franco et al. 2018, Stefanski et al. 2018).

In our trials, moulds hardly ever occurred, as they usually appear after the yeast infestation (Pahlow et al. 2003). However, moulds were observed in two out of 6 treatments in Institute 2, Trial 2. This phenomenon might be a starting point for further product development work, which could include further indicators for fungal growth such as monoclonal antibodies (Le Cocq et al. 2020).

Losses

FM losses can be used, but with some reservations, as not all respired H_2O will have evaporated from the vessel. Part of it stays within the sample, another part has condensed on the wall and in the cap; this is why the latter was not included in the weighing. Such FM loss will only reflect the loss of organic matter to a certain degree and when considering the determination of the DM losses this error will multiply. Water from the wall, which has been included in the FM weight, is now multiplied with the DM concentration values of the sample. This is one reason for an apparent "gain" in DM as calculated for the second run in Institute 2, Trial 1. That is why Knicky and Spörndly (2015) introduced a correction factor of 1.44 to take the water into consideration as a respiratory loss. Another reason is the inherent error in the method. Oven drying at 100, 103 or 105 °C has become a standard for DM determination of forages in many laboratories because of ease of handling and reduced risk for health and environment compared to alternative methods. Protocols using toluene or gas chromatograph to determine water content for example or freeze drying have been compared (Minson and Lancaster 1963, Aerts et al. 1974, Huida et al. 1986, Alomar et al. 1999). An overview on methods for determining forage moisture content is given by Cherney and Cherney (2003) in their chapter on silage quality assessment. All these studies concluded that loss of volatile compounds has to be considered when oven drying. Thus, correction factors depending on analyzed volatile organic acid concentration, pH and/or ammonia have been suggested for different types of silages (Weissbach and Kuhla 1995, Porter and Murray 2001, Weissbach and Strubelt 2008). However, a correction factor for silages that have undergone aerobic spoilage has not been published, as usually the disappearance of volatile organic acids in spoiled samples is not documented analytically, and was not the subject of this investigation. Thus, in the presented case no correction factors were applied before or after the test. DM values of silages will increase by correction. Thus, the simple gravimetrical determination of mass losses is a weak tool.

Alternative indicators and evaluation options

Another way of monitoring aerobic deterioration processes is by the continuous measurement of CO_2. Honig who invented the System Völkenrode for example measured CO_2 concentrations, which he then correlated with temperature measurements with the intention of replacing the more elaborate CO_2 measurement with the latter (Honig 1990). He then calculated DM losses using the respiration equation $C_6H_{12}O_6 + O_2 \rightarrow H_2O + CO_2$. However, it is not documented how he verified his assumption. Firstly, only a minor part of silage carbohydrates consists of glucose and many intermediate steps would have to be considered. Secondly, different respiratory pathways exist with different organisms such as alternative oxidase in many fungi, and with differing extents of heat production (Joseph-Horne et al. 2001). Thirdly, carbon dioxide escaping from silage has different sources which need to be distinguished. One is the efflux of gas, which has accumulated in the pores during anaerobic storage, the other is production caused by microbial activity (Shan et al. 2019). Thus, Shan et al. (2021a) attempted to identify the proportion of aerobic microbial respiration by determining O_2, CO_2 and temperature in different layers in triticale silage.

Mapping the aerobic stability indicators presented of different feed materials in a normalized (i.e. minimum value becomes 0, maximum possible value becomes 1) radar plot can help to understand the type of microbial behaviour inherent in the feedstuff. An example where TMR was compared with pressed beet pulp silages can be found in the supplementary material (Suppl. Fig. 5). These samples were differentiated upon visible appearance of signs of yeasts and/or moulds. While moulds alone or in combination with yeasts were predominant in the spoilage process of pressed beet pulp silages, moulds did not occur solely in TMR. In that case, the combination of yeasts and moulds led to the highest temperature peaks and pH increases.

The rapid detection of biogenic amines and pathogens might also help to indicate spoilage going beyond the described and usual parameters. Respective sensors are being developed in the food sector (Park et al. 2015, Schaude et al. 2017, Müller and Schmid 2019). Such additional information might increase the practical benefit for farmers in making management decisions in the future.

Agricultural and Food Science (2022) 31: xxx–xxx
https://doi.org/10.23986/afsci.115968

Conclusions

The extended protocol described allows the evaluation of TMR stabilizing products in a specified time if the feed is of moderate hygienic quality. Highly contaminated rations will deteriorate without delay whilst hygienic rations will remain stable for a reasonably long time. This also means that the use of an antimicrobial agent cannot replace good agricultural practice in feed conservation.

In the evaluated feed materials temperature increase is usually a good indicator for spoilage processes. It is recommended that the whole temperature curve is used rather than merely a single point in time. However, early signs of respiratory activity can only be detected by visual evaluation of fungal growth. Thus, it should always accompany the appraisal of aerobic stability and equally, the determination of pH before and after the test.

Parameters such as maximum temperature difference, time to maximum temperature difference, FM losses and evaluation of condensation give an additional clue to the extent of microbial spoilage that has occurred. They cannot be evaluated in isolation.

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Suppl. Table 1. Examples where no or very slight temperature increase (< 3 K above ambient) occurred during the aerobic stability test (AST), and which however showed visible signs of deterioration after the test (values in brackets mean ranges)

Feed material	DM (g kg^{-1})	Ambient (°C)	AST (h)	Max.TD (K)	Yeasts (0–4)	Molds (0–4)	pH before	pH after AST	n	Year observed
Grass silage	428	20.6	215	2.3	3.0	3.5	4.70	8.37	1	2020 [1]
Grass silage, 2nd cut	380	24.7	310	0.7 (0–1.5)	0	2.3 (1.5–3.0)	4.19	4.36 (4.20V–4.63)	6	2020 [2]
Grass silage, 1st cut	344	22.6	336	0.4 (0–1.5)	0.9 (0.5–1.5)	0.4 (0–2.5)	4.00	4.02 (3.94–4.08)	7	2019 [2]
Grass silage, 1st cut (*L. multiflorum*)	352	24.8	333	1.4 (0.6–2.8)	0.7 (0.5–1.0)	0	3.98	4.09 (3.94–4.16)	5	2019 [2]
Lucerne silage (*M. sativa*)	465	23.7	499	0.5 (0.0–0.9)	0.8 (0.5–1.0)	2.7 (2.5–3.0)	5.09	5.58 (5.11–6.50)	3	2020 [2]
Lucerne silage	470	23.7	499	0	0	1.5	4.54	4.64 (4.60–4.66)	3	2020 [2]
Lucerne silage	387	24.1	331	0	0	1.5 (0.5–2.5)	4.44	4.44	3	2020 [2]
Pressed beet pulp silage	230	17.3	158	0.8 (02.85)	0.5 (0–1.5)	0.8 (0–2.5)	3.67 (3.53–3.85)	3.77 (3.55–4.32)	15	2017 [3]
Chicory silage (*C. intybus*)	144	23.9	192	1.5 (0–2.8)	0	2.1 (1.5–3.0)	4.29 (4.13–4.62)	4.46 (4.23–5.01)	7	2016 [3]
Maize silage	370	19	192	2.3	0	2.0	3.80	n.a.	6	2019 [4]
Guinea grass silage	226	25	150	< 2.0			4.79	5.29	4	2021 [5]
Guinea grass silage	234	25	144	< 2.0			4.44	4.94	4	2021 [5]
Fresh potato by-products	153	20	96	0	0	4.0	5.31	n.a.		2018 [6]

Max. TD = maximum temperature difference; (0–4) points: see Table 3 and Figs. 3 and 4.

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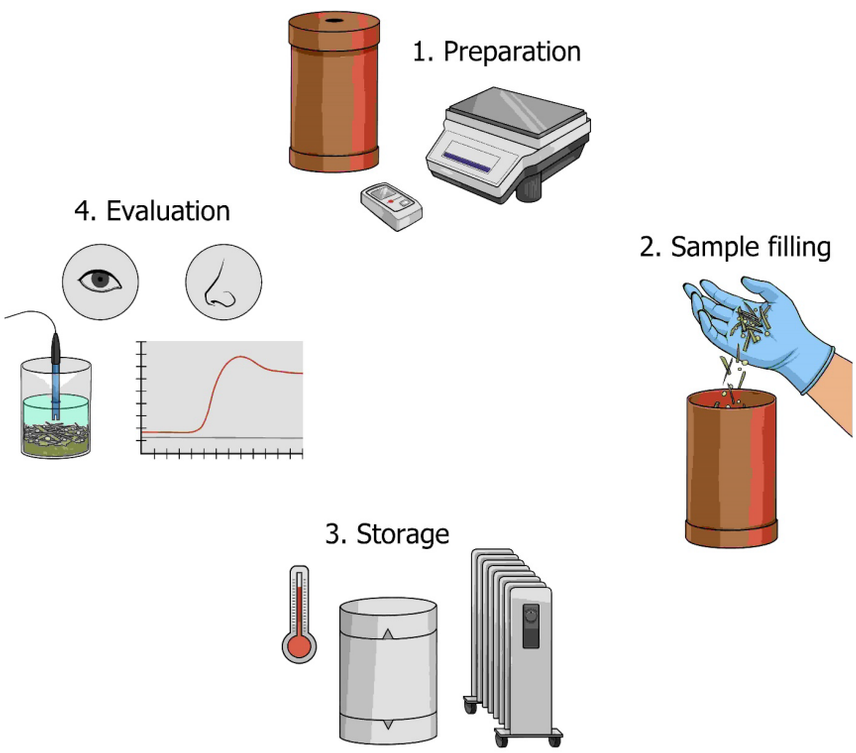
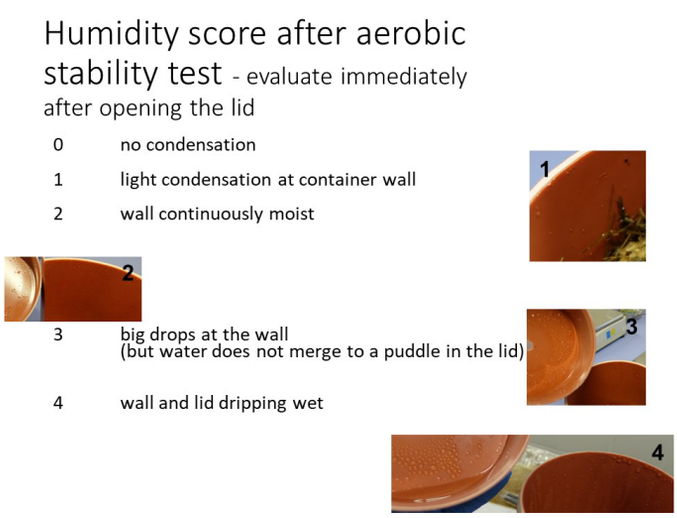


Suppl. Fig.1. Schematic of the general procedure from preparing and evaluating the aerobic stability test of fermented feeds



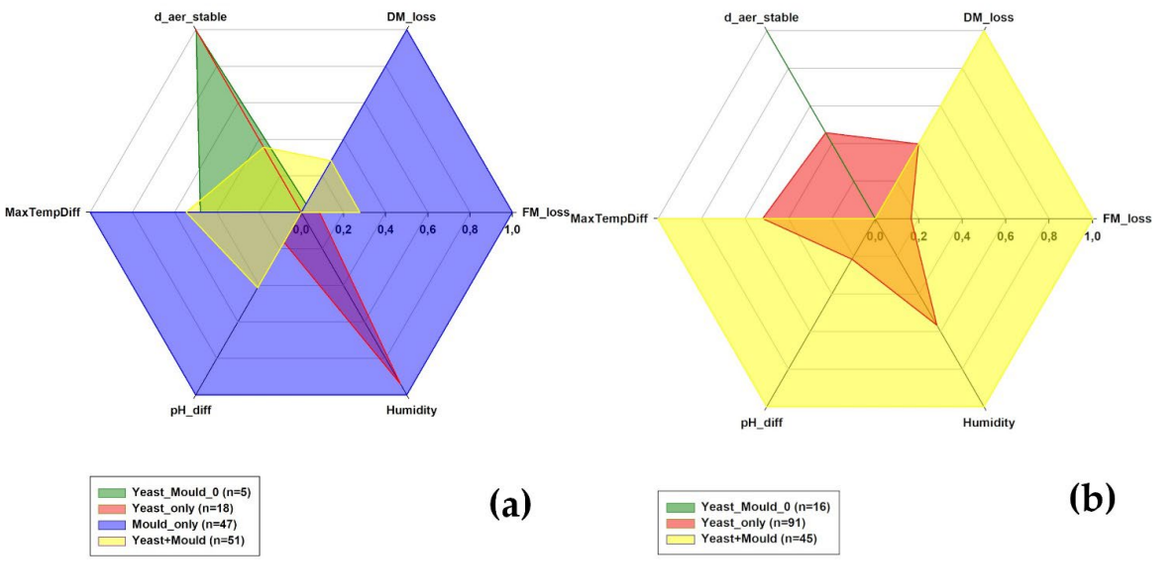
Suppl. Fig. 2. Humidity score (0–4) from visual evaluation after the aerobic stability test

Appendix

Suppl. Fig. 3. Exemplary yeast score (0–4) from visual evaluation after the aerobic stability test.

Suppl. Fig. 4. Exemplary mold score (0–4) from visual evaluation after the aerobic stability test.

Appendix



Suppl. Fig. 5. Example of normalized radar plots with aerobic stability indicators sorted by the occurrence of no yeasts + molds, only yeasts, only molds and yeasts + molds in (a) pressed beet pulp, (b) Total Mixed Rations.