The Journal of Agricultural Science

cambridge.org/ags

Fermentation quality and aerobic stability of low moisture-crimped wheat grains manipulated by organic acid-based additives

M. Franco, T. Stefanski, T. Jalava, K. Kuoppala, A. Huuskonen and M. Rinne

Natural Resources Institute Finland (Luke), Jokioinen, Finland

Animal Research Paper

Cite this article: Franco M, Stefanski T, Jalava T, Kuoppala K, Huuskonen A, Rinne M (2019). Fermentation quality and aerobic stability of low moisture-crimped wheat grains manipulated by organic acid-based additives. *The Journal of Agricultural Science* **157**, 245–253. https://doi.org/10.1017/S0021859619000546

Received: 22 March 2019
Revised: 31 May 2019
Accepted: 19 July 2019
First published online: 14 August 2019

Key words:
Carbon dioxide; cereal; gas production; spoilage; *Triticum*

Author for correspondence:
M. Franco, E-mail: marcia.franco@luke.fi

Abstract

Preservation of moist grain anaerobically by so-called crimping has many advantages. Generally, preservation has been successfully performed when grain is harvested at 30–40% moisture content (MC). However, there is a trend towards using drier than the optimal MC of the raw material. This leads to an increasing need to control aerobic spoilage of the material and also to experimental challenges in assessing the quality and stability of low-MC crimped grain. The objective of the current work was to evaluate fermentation quality, microbial composition and aerobic stability (AS) of drier than the optimal crimped grain ensiled with different additives and to use these materials to compare three different AS evaluation methods. Crimped wheat grain with 28% MC was ensiled using eight additive treatments based mainly on formic and propionic acids including a control without any additive. The low MC resulted in no lactic acid fermentation, but significant ethanol formation occurred in the control. The treatments used resulted in clear differences in microbial quality and AS of the feeds, and use of formic and propionic acid-based additives provided a clear benefit in improving the AS of crimped wheat grain. The correlation between increasing temperature and carbon dioxide (CO_2) production under aerobic conditions was very close, indicating that CO_2 produced by aerobic bacteria can be used as a method of evaluating AS. Visual inspection of mould growth resulted in somewhat different ranking of the treatments.

Introduction

Preservation of cereal grains by crimping is based on lactic acid fermentation by anaerobic lactic acid bacteria (McDonald *et al.*, 1991). The concept is the same as with grass and maize silage preservation, and the method is referred to generally as grain ensiling. If the ensiling is conducted carefully, the pH of the ensiled feed reduces to 4 or even below due to the presence of undissociated organic acids (lactic, formic, acetic, propionic and other volatile fatty acids [VFAs]), which are either added or produced by fermentation and prevent the growth of microbes. Crimping the grain breaks and flattens the whole grain, exposing the carbohydrate and protein, which can positively increase grain compaction and create a more anaerobic environment inside the silo, stimulating microbial activity due to greater surface area and substrate availability (Klemola *et al.*, 1994; Bern, 1998).

Crimping grains enables farmers to harvest, process, store and preserve moist cereals as animal feed without the use of expensive drying facilities and energy consumption for drying. Crimping grain offers an opportunity to harvest cereals earlier than conventional harvesting for dry grain (generally 15–20% moisture content [MC]) and harvesting can be carried out under more humid conditions, reducing the weather dependency of harvesting. Dry weather conditions may result in drier than optimal grains and this reduced moisture has technological advantages in harvesting and logistics but may compromise preservation due to aerobic spoilage. The unpredictable weather conditions due to climate change may alter the harvesting conditions of grain and thus flexibility of storage methods may be increasingly important in the future.

Ensiling of crimped grain has been successfully performed when the crop is harvested with 30–40% MC (Huhtanen *et al.*, 2013) and when ensiling is based on a low pH generated by fermentation and/or acid-based additives (Vanhatalo *et al.*, 1999; Jaakkola *et al.*, 2009). According to Huhtanen *et al.* (2013), extensive research in Finland in the 1970s demonstrated that ensiling of high-moisture grain is an efficient storage method as an alternative to grain drying. Nowadays the ensiling of low-MC crimped grain is common, although there is a high risk of losses when additives are not used (Olstorpe *et al.*, 2010; Pieper *et al.*, 2011). In crimped grain with MC between 20 and 30%, fermentation is restricted, so efficient protection is needed against aerobic deteriorating organisms. Harvesting should start when the grain has started ripening, since the MC required for effective lactic acid fermentation is high. However, if MC of grain is too low, water can be added to grain during the crimping to increase MC

CAMBRIDGE UNIVERSITY PRESS

(Klemola *et al.*, 1994; Palva *et al.*, 2005), although under practical conditions this may not always be feasible.

In some cases, the conventional method of evaluating crimped grain aerobic stability (AS) by a rise in temperature may not be sensitive enough. In a previous experiment (Seppälä *et al.*, 2015), rather dry (MC 19%) crimped grain did not show elevated temperatures although it was visually clearly mouldy during the measurement period. It is possible that the heat is lost to the environment from relatively dry grain so that it is not retained in the sample. Similarly, Seppälä *et al.* (2016) did not observe heating of pre-wilted grass silage (MC 460 g/kg), which may have been due to the same reason, suggesting that a more sensitive methodology might be needed for other types of feeds as well.

An alternative method to study microbial activity in a sample is to follow the formation of carbon dioxide (CO_2; Ashbell *et al.*, 1990). Further, visual inspection of moulds growing on the samples can be conducted (Seppälä *et al.*, 2015). This method gave useful results in the experiment of Rinne *et al.* (2019), when the stability of moist carrot by-products was evaluated.

The objective of the current study was to evaluate the fermentation quality, microbial composition and AS of low-MC crimped wheat grain when manipulated by the use of different organic acid-based additives. The feeds were used to compare different methods of evaluating the AS and comparing their ability in detecting differences between treatments. Three methods in evaluating the AS were compared: (1) increase in temperature, (2) production of CO_2 and (3) visual appearance of moulds.

Materials and methods

Raw materials

Autumn wheat (*Triticum aestivum*, variety Urho, Boreal Plant Breeding Ltd., Jokioinen, Finland) sown on 7 September 2016 and harvested on 25 September 2017 at the Natural Resources Institute Finland (Luke) in Jokioinen, Finland (60°48′N, 23° 29′E) was used as the raw material. The field was fertilized on 2 May 2017 (108 kg nitrogen (N)/ha, in the form of ammonium nitrate) and sprayed against weeds (Ariane S, 200 litres/ha) on 22 May 2017. After harvesting, the grain was crimped immediately without additives at the Häme University of Applied Sciences in Tammela, Finland using a farm scale crimper (MD 700 HD, Murska Ltd., Ylivieska, Finland). The material was stored in a refrigerator overnight before ensiling was conducted at the Luke Laboratory.

Treatments and experimental procedures

Eight additive treatments were used:

(1) control, without any additive;
(2) FA_1 (pure formic acid [FA] diluted to 80% [81.63 ml FA + 18.37 ml water = 100 ml 80% FA], 5 litres/tonne);
(3) FA_2 (58% FA, 20% propionic acid [PA], 2.5% potassium sorbate, 5.2% sodium formate [SF]; AIV Ässä Na, 5 litres/tonne);
(4) FA_3 (AIV Ässä Na, 7 litres/tonne);
(5) FA_4 (76% FA, 5.5% SF; AIV 2000 plus Na, 7 litres/tonne);
(6) FA_5 (37% FA, 22% SF, 18% PA, 7.3% sodium, 1% sorbic acid; GrasAAT SX, 5 litres/tonne);
(7) PA_1 (54% PA, 31.3% ammonium propionate, 5% sodium benzoate [SB], 0.1% glycerine, 0.1% propylene glycol; Eastman Stabilizer Crimp, 5 litres/tonne) and
(8) PA_2 (37% PA, 14% SB, 10% FA, 11% sodium propionate; Kofa Feed EP, 4 litres/tonne).

Additive FA_1 was sole laboratory grade formic acid from the Merck Group (Darmstadt, Germany, Emsure®, ACS, Reag. 1.00264, 98–100%). Additives FA_2, FA_3, FA_4 and PA_1 were products of the Eastman Chemical Company (Oulu, Finland) and FA_5 and PA_2 were produced by ADDCON GmbH (Bitterfeld-Wolfen, Germany). Details of the additives are presented in Table 1. Additives were dosed according to the commercial instructions, except for FA_3 which was the same product as FA_2 but used at a higher amount (5 *v.* 7 litres/tonne) to evaluate the dose response.

Glass jars of 1.5 litre volume were used to preserve the crimped grain. Three replicates were made for each additive treatment, which resulted in 24 different batches for which additives were applied separately. To have enough material, three jars were prepared per replicate. Four kilograms of crimped wheat grain were weighed into a plastic container and the additive was mixed carefully. Additives were diluted in water to have an even application so that total amount of liquid was 25 ml for one 4 kg batch. The volume of preservative solution (additive plus water) was the same for all treatments. The glass jars were filled as full as possible to minimize the volume of air in the headspace, resulting in a density of 577 kg dry matter (DM)/m^3. The top of the jar was covered with a plastic film, closed airtight and stored at room temperature (20 °C) in the dark.

The ensiling period lasted for 57 days. When opening the jars, spoiled parts were discarded and the three jars from the same replicate were combined and mixed carefully before sampling.

Preservation losses

The jars were weighed before opening. The weight losses were calculated according to Knicky and Spörndly (2015) by assuming the lost weight to be CO_2 leaving the jar during the fermentation phase. It was assumed that for each mole of CO_2, 1 mol of water (H_2O) was produced: for each gram of weight decrease due to CO_2 loss, 0.44 g of DM in the silo became water, which represents loss even if it is still inside the jar. Therefore, the DM loss was calculated as the decrease in weight of the silo multiplied by a factor of 1.44, expressed as grams per kg initial DM.

Visual inspection of mould was conducted before emptying the jars. A mould scale from 0 to 3 (0 = no mould, 1 = slight mouldiness, 2 = moderate mouldiness, 3 = severe mouldiness) was used for assessing the visual presence of moulds separately on the sides and top of the jars.

Aerobic stability measurements

The AS of the crimped grain samples after opening the jars was measured using three different methods.

Temperature measurement

As the standard method, samples of crimped grain (580 g DM) were inserted into 2.5 dm^3 polystyrene boxes in plastic bags, which were left open. Thermocouple wires were inserted into the feed material within the polystyrene boxes and connected to a data logger, which measured the temperature changes of the samples automatically at 10-min intervals. AS was defined as the time taken to increase the temperature of samples by 2 °C above the ambient temperature, which was 22 °C. The data

https://doi.org/10.1017/S0021859619000546 Published online by Cambridge University Press

Table 1. Description of additives used in the experiment

Abbreviation	Commercial name	Producer	Active substances	Dosages
Control	Control		Tap water	
FA_1	Formic acid	Merck Group	800 g Formic acid/kg	5 litres/tonne
FA_2	AIV Ässä Na	Eastman Chemical Company	580 g Formic acid/kg 200 g Propionic acid/kg 52 g Sodium formate/kg 25 g Potassium sorbate/kg	5 litres/tonne
FA_3	AIV Ässä Na	Eastman Chemical Company	See above	7 litres/tonne
FA_4	AIV 2000 plus Na	Eastman Chemical Company	760 g Formic acid/kg 55 g Sodium formate/kg	7 litres/tonne
FA_5	GrasAAT SX	Berner/ADDCON	370 g Formic acid/kg 220 g Sodium formate/kg 180 g Propionic acid/kg 73 g Sodium/kg 10 g Sorbic acid/kg	5 litres/tonne
PA_1	Eastman Stabilizer Crimp	Eastman Chemical Company	540 g Propionic acid/kg 313 g Ammonium propionate/kg 50 g Sodium benzoate/kg 1 g Glycerine/kg 1 g Propylene glycol/kg	5 litres/tonne
PA_2	Kofa Feed EP	Berner/ADDCON	370 g Propionic acid/kg 140 g Sodium benzoate/kg 110 g Sodium propionate/kg 100 g Formic acid/kg	4 litres/tonne

collection lasted for 174 h. A similar approach is commonly used in AS measurements internationally, with some modifications related to practicalities, and has been used routinely in the Luke Laboratory (e.g. Seppälä *et al*., 2016).

Increase of carbon dioxide concentration

For this method, 50 g of sample was placed in 0.5 litre glass bottles and closed, airtight, with a lid that allowed sampling of the gas from the headspace of the bottle. The large headspace volume was used intentionally so that the growth of aerobic bacteria should not be limited by availability of oxygen. The bottles were kept at room temperature (25 °C). Once daily, 200 µl of gas was withdrawn from the headspace of each bottle with a gas-tight syringe and analysed for CO_2 using a gas chromatograph (Perkin Elmer, Arnel Clarus 500, Illinois, USA, equipped with a thermal conductivity detector and a Supelco Carboxen™ 1010 PLOT fused silica capillary column [30 m × 0.53 mm]). First, gas was pumped in and out to ensure mixing of the gas. The amount of gas produced was also recorded. Thresholds of 1 and 2 mg of CO_2 per g DM were used to indicate the loss of AS.

Visual appearance of mould

A third method to evaluate AS was the visual appearance of mould. Visual inspection of the bottles used for CO_2 measurement was conducted once a day. The spoilage of the samples was evaluated visually using the following scores: 0 = no mould, 1 = slight mouldiness, 2 = moderate mouldiness, 3 = severe mouldiness. Samples were discarded when they reached score 3. The results of this test are presented as the number of days the samples remained slightly moulded (score 1) or moderately moulded (score 2) and as the average daily mouldiness scores.

Laboratory analyses

Crimped wheat grain was sampled as a raw material before applying the additive treatments and analysed for DM, ash, crude protein (CP), neutral detergent fibre (NDF), starch and microbial quality (aerobic bacteria, yeasts and moulds). Ensiled wheat grain was analysed for DM, water soluble carbohydrates (WSCs), pH, ammonia-N, ethanol, lactic acid, VFAs and microbial quality (yeasts and moulds).

Chemical analyses

Chemical analyses were carried out at the Luke Laboratory in Jokioinen. The laboratory has a quality system which follows the SFS-EN ISO/IEC 17025:2005 standards and is accredited by FINAS (the Finnish Accreditation Service) with number T024. The %DM was determined by drying at 105 °C for 16 h and corrected for volatile losses. Ash content was determined by ignition of the samples at 600 °C for 2 h (AOAC, 1990, method 942.05). Nitrogen (N) content was determined by the Dumas method (AOAC, 1990, method 968.06) using a Leco FP 428 nitrogen analyser (Leco, Saint Joseph, USA). CP content was calculated as 6.25 × N content.

Ash-free NDF on organic matter basis (aNDFom) was determined according to Van Soest *et al.* (1991) using sodium-sulphite and α-amylase. Starch was determined according to Salo and Salmi (1968). VFAs were determined according to Huhtanen *et al.* (1998), lactic acid according to Haacker *et al.* (1983), WSC according to Somogyi (1945) and ammonia according to McCullough (1967). The N content of the original material was used to express ammonia-N proportions in total N after fermentation. Ethanol was measured using an enzymatic kit (cat no. 981680, KONE Instruments Corporation, Espoo, Finland) and the selective clinical chemistry analyser Pro 981489 (KONE Instruments Corporation, Espoo, Finland) according to

https://doi.org/10.1017/S0021859619000546 Published online by Cambridge University Press

application instructions given by KONE. Ammonia and propionic acid were corrected for the amounts added in the additives. Further, a correction factor of 0.8 was used (corrected value = analysed value − 0.8 × amount added in the additive) to represent the losses of added substances based on unpublished observations to avoid overcorrection.

Microbiological analyses

The samples for microbiological analyses were mixed and 25 g was weighed in stomacher bags and mixed with 225 ml of ¼-strength Ringer solution (Merck 1.15525.0001). The samples were homogenized using the stomacher (Stomacher® 400 Circulator) for 2 min at 230 rpm. Serial decimal dilutions were prepared by mixing 1 ml of sample with 9 ml of Ringer solution.

Yeasts and moulds were determined in Dichloran Rose Bengal Chloramphenicol Agar medium (Lab M Ltd., Bury, UK, LAB217) which was supplemented with 50 µg/ml of oxytetracycline hydrochloride (AppliChem BioChemica, Billingham, UK, A5257). The Petri dishes were incubated at 25 °C. The colonies were counted after 3 and 5 days. The aerobic plate count was determined on Plate Count Agar (Lab M Ltd., Lab010) dishes incubated at 30 °C for 72 h.

Statistical analysis

The data were analysed using the MIXED procedure (SAS Inc., 2002–2012, Release 9.4; SAS Inst., Inc., Cary, NC, USA) of SAS at $P < 0.05$. Additives were considered fixed effects, while replicates were taken into account in the model as the random effect. Differences between least square means of response variables were estimated with Tukey's test. A linear effect for dose response of the additives FA_2 and FA_3 (same compound in different doses) was also evaluated. A linear correlation between AS measurement methods was evaluated using the CORR procedure of SAS.

Results

MC of wheat raw material was 19% and contained 20 g ash, 139 g CP, 670 g starch and 126 g aNDFom per kg DM. The MC of wheat grain after addition of water was 28%. Total bacteria, yeast and mould counts of the wheat raw material were 1.4×10^7, 7×10^4 and 2.3×10^5 CFU/g, respectively.

Table 2 presents the fermentation quality and microbial composition of crimped wheat grain according to additive treatments. Overall, MC was similar for all treatments ($P > 0.05$) ranging from 26.9 to 27.6%. This resulted in no production of lactic acid for the control, FA_1, FA_2, FA_3, FA_4 and FA_5 and very low amount for PA_1 and PA_2. Significant ethanol formation occurred ($P < 0.05$) in the control. Additive treatments had lower pH than the control ($P < 0.05$) and FA_3 resulted in the lowest pH value among treatments ($P < 0.05$). There was very little acetic and butyric acid in the samples, ranging from 0.55 to 1.35 g/kg and from 0.03 to 0.04 g/kg, respectively. For propionic acid, the results changed clearly after they were corrected for the amount of propionic acid delivered in the additives, and significant differences among treatments disappeared. Ammonia-N was similar for the control and additive treatments ($P > 0.05$), but higher for PA_1 ($P < 0.05$) when the correction of ammonia originating from the additive was not taken into account. However, when the correction was applied, the amount of ammonia-N was similar ($P > 0.05$) among treatments.

Numbers of yeasts were lower in all treatments compared to the raw material (Table 2). Treatments with PA_1, FA_1 and FA_4 resulted in the highest number of moulds, which were even higher than in the raw material, while the control and FA_3 showed the lowest mould numbers.

The control resulted in the lowest ($P < 0.05$) amount of WSC (Table 2), followed by PA_2, with consequently greater ($P < 0.05$) amounts of ethanol and acetic acid for those treatments compared to the others.

The average ensiling losses were rather low for all treatments as they remained below 15 g/kg of initial DM (Table 3). Among the treatments, the control had the greatest losses ($P < 0.05$) followed by PA_2 while FA_3 resulted in the lowest loss ($P < 0.05$) and the other treatments resulted in similar losses ranging from 2.9 to 4.1 g/kg of initial DM.

After 57 days of ensiling, the jars were reasonably mouldy on both the top and sides (Table 3). PA_1 had the lowest mould score ($P < 0.05$), followed by the control and FA_3. The other treatments (FA_4, FA_1 and FA_5) presented high appearance of spoiled parts ($P < 0.05$) both on the top and sides of the jars, which were in general above a score of 2.

The AS based on temperature rise was the shortest in treatments FA_1, FA_5, PA_2 and the control ($P < 0.05$; Table 4) followed by FA_4. The longest AS was achieved in FA_3 ($P < 0.05$); however, the temperature for this treatment did not even increase 2 °C above the ambient during the whole observation period of 174 h; and PA_1 with AS of 168 h. Additives were effective at increasing ($P < 0.01$) AS when compared to the control or formic acid alone (FA_1). There was a positive linear ($P < 0.01$) dose response to the same additive which was used in FA_2 and FA_3 treatments in incremental amounts.

Cumulative CO_2 production of crimped wheat grain preserved with FA_1 was the highest and fastest among the treatments (Fig. 1). Its cumulative CO_2 production decreased after 6 days of evaluation, but still remained higher than in other treatments. The control, FA_4, FA_5 and PA_2 exhibited similar patterns for cumulative CO_2, where production suddenly increased between days 3 and 5 and then reached a plateau with a flat stationary phase between days 5 and 7. Treatment FA_2 kept a low CO_2 production during the initial 4 days of evaluation and afterwards increased linearly up to 7 days. Treatment PA_1 presented a low cumulative CO_2 production throughout the evaluation period with a slight increase during the last 2 days. Cumulative CO_2 production in FA_3 remained rather close to zero throughout the whole evaluation period.

According to visual inspection of mould in crimped wheat grain after air exposure (Table 3), the mould score was greatest for FA_1, which was severely mouldy after 5 days, followed by FA_5, FA_4 and FA_2. The control and PA_2 were moderately mouldy after 1 week, followed by PA_1, which was slightly spoiled at days 6 and 7 of evaluation. FA_3 treatment did not show any spoilage during the whole evaluation period.

There was a positive correlation between AS (measured by increase in temperature) and CO_2 production (Table 4). Increase in temperature above the ambient and CO_2 production methods ranked the treatments in the same order. However, the concentrations of CO_2 at the time the treatment reached 2 °C above ambient temperature were not the same, indicating that the amount of microbial activity was not the same. The R^2 between increase in temperature and increase in CO_2 concentration using 1 mg/g DM as a threshold was 0.936 and even higher when 2 mg/g DM was used as the threshold, when R^2 reached

https://doi.org/10.1017/S0021859619000546 Published online by Cambridge University Press

Table 2. Fermentation quality and microbial composition of crimped wheat grain according to additive treatments

	Control	FA[1]	FA[2]	FA[3]	FA[4]	FA[5]	PA[1]	PA[2]	SE[a]	*P* value[b]
MC, %	27.6	27.1	27.2	26.9	27.1	27.3	27.2	27.3	0.11	0.06
pH	6.16	4.89	5.20	4.57	5.01	5.79	5.75	5.87	0.052	<0.01
In DM, g/kg										
WSCs	19	34	42	38	35	28	31	22	2.6	<0.01
Ethanol	7.8	0.2	0.3	0.3	0.2	0.6	1.3	3.3	0.12	<0.01
Lactic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.74	0.048	<0.01
Acetic acid	1.11	0.58	0.67	0.55	0.58	0.78	1.29	1.35	0.063	<0.01
Propionic acid[c]	0.1	0.2	1.2	1.7	0.8	1.0	4.3	1.8	0.15	<0.01
Propionic acid[d]	0.1	0.2	0.2	0.4	0.8	0.2	0.8	0.1	0.14	0.07
Butyric acid	0.04	0.03	0.03	0.03	0.04	0.03	0.04	0.04	0.003	0.08
Ammonia N, g/kg N[c]	5.9	4.7	5.0	4.7	5.4	4.4	16.9	4.4	0.42	<0.01
Ammonia N, g/kg N[d]	5.9	4.7	5.0	4.7	5.4	4.4	4.3	4.4	0.42	0.15
Yeasts, CFU[e]/g	2.7×10^3	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	$<1 \times 10^1$	1.5×10^3	4.2×10^3	4.1×10^4	–	–
Moulds, CFU/g	9×10^2	4.3×10^5	1.2×10^5	1.7×10^2	3.7×10^5	6.1×10^4	7.2×10^5	5.5×10^4	–	–

FA_1, formic acid; FA_2, AIV Ässä Na; FA_3, AIV Ässä Na; FA_4, AIV 2000 plus Na; FA_5, GrasAAT SX; PA_1, Eastman Stabilizer Crimp; PA_2, Kofa Feed EP; SEM, standard error of the mean; N, nitrogen; CFU, colony forming unit.
Values with the same letter in a row are not significantly different ($P > 0.05$) based on Tukey's test. For additive treatments, see Table 1.
[a]Standard error.
[b]Effect of treatment.
[c]Not corrected for the amount delivered in the additive.
[d]Corrected for the amount delivered in the additive.
[e]Colony forming unit.

Table 3. Ensiling losses and spoilage score on top and sides of jars prior opening and mould score according to days after air exposure of crimped wheat grain treated with additive

	Control	FA[1]	FA[2]	FA[3]	FA[4]	FA[5]	PA[1]	PA[2]	SE[a]	*P* value[b]
Losses, g/kg of initial DM[c]	12.2	2.9	3.3	1.7	3.1	4.1	3.3	6.6	0.32	<0.01
Mould, top	1.7	2.1	1.8	1.1	1.9	2.8	0.2	2.0	0.17	<0.01
Mould, sides	0.0	2.6	2.6	1.3	2.9	2.6	1.0	0.8	0.19	<0.01
Mould during aerobic phase	0.9	2.0	1.1	0.0	1.5	1.7	0.3	0.6	0.21	<0.01

FA_1, formic acid; FA_2, AIV Ässä Na; FA_3, AIV Ässä Na; FA_4, AIV 2000 plus Na; FA_5, GrasAAT SX; PA_1, Eastman Stabilizer Crimp; PA_2, Kofa Feed EP; SEM, standard error of the mean; DM, dry matter. Values with the same letter in a row are not significantly different ($P > 0.05$) based on Tukey's test. For additive treatments, see Table 1.
[a]Standard error.
[b]Effect of treatment.
[c]Corrected values according to Knicky and Spörndly (2015).

Table 4. AS (h) of crimped wheat grain according to additive treatments measured through standard and alternative methods, and correlation between increase in temperature and the alternative methods

	Control	FA[1]	FA[2]	FA[3]	FA[4]	FA[5]	PA[1]	PA[2]	SE[a]	*P* value[b]	R^{2c}
Increase in °C[d]	80	72	137	174[e]	108	87	168	82	12.5	<0.01	
Carbon dioxide[f]	55	54	108	168[e]	82	71	127	64	6.0	<0.01	0.936
Carbon dioxide[f]	80	64	126	168[e]	99	95	164	79	6.9	<0.01	0.979
Score[g]	112	48	114	168[e]	68	86	168[e]	132	9.9	<0.01	0.567
Score[g]	128	62	127	168[e]	92	97	168[e]	144	7.1	<0.01	0.528

FA_1, formic acid; FA_2, AIV Ässä Na; FA_3, AIV Ässä Na; FA_4, AIV 2000 plus Na; FA_5, GrasAAT SX; PA_1, Eastman Stabilizer Crimp; PA_2, Kofa Feed EP; SEM, standard error of the mean. Values with the same letter in a row are not significantly different ($P > 0.05$) based on Tukey's test. For additive treatments, see Table 1.
[a]Standard error.
[b]Effect of treatment.
[c]Correlation coefficients between increase in temperature[d] and the alternative methods to evaluate AS.
[d]Time taken to increase 2 °C above ambient temperature.
[e]Treatment did not reach the threshold during the evaluation period.
[f]Time taken to increase the carbon dioxide concentration up to 1 and 2 mg/g DM, respectively.
[g]Time taken to sample reach mould score 1 and mould score 2, respectively.

0.979. Visual mould score also showed a positive correlation with an increase in temperature, but with lower R^2 values (0.567 and 0.528 for samples reaching mould scores 1 and 2, respectively).

Discussion

Manipulation of crimped grain quality by additives

The MC of wheat grain at harvest was relatively low but still higher than the default value of 14% for dry cereal grains, while ash, CP, starch and aNDFom amounts were typical, corresponding to average values in the Finnish Feed Tables (Luke, 2019). Water addition was based on microwave DM determination, which slightly underestimated MC so the crimped wheat grain MC was 28% at ensiling, which was slightly higher than the target but still lower than recommended for crimping. According to Wilkinson and Davies (2013), low counts of yeasts and moulds at harvest are primordial factors to increase the AS of silage. Low contamination of the raw material was achieved in the current experiment according to Kristensen *et al.* (2010), which indicated that at the time of harvest, counts of yeasts and moulds above 6.36 and 5.64 $\log_{10}$/g, respectively, are correlated with low stability in maize.

The low amounts of lactic acid and VFA are due to the low MC (Kung, 2010), which inhibits fermentation. As a result of low organic acid production, the grain is more prone to aerobic spoilage as undissociated organic acids are strong inhibitors for aerobic microbes. These results are also supported by Seppälä *et al.* (2012), who studied the effect of propionic and formic acid-based additives on crimped barley grain. Under these conditions, low-MC grains need to be artificially preserved with the use of additives. The absence of lactic acid also increases the pH of crimped grain to a level that allows opportunistic bacteria and moulds to grow and further reduce silage quality (McDonald *et al.*, 1991).

The proportion of ammonia N in total N is generally considered as a good indicator of silage fermentation quality (McDonald *et al.*, 1991), but if additives that include ammonia have been used, it is necessary to correct for the amount delivered in the additives. Ammonia-N was similar for the control and other additive treatments except for PA_1, which was treated with an additive containing ammonium propionate. Correction of ammonia-N provided by the additive resulted in a similar ammonia-N amount in PA_1 as in the other treatments. According to Wilkinson (1990), grass silage with ammonia N in a range of 50–100 g/kg total N is regarded as well fermented, while grass silage having ammonium-N below 50 g/kg total N is very well fermented. In the current work, all samples remained far below this value, but it is noteworthy that guidelines for crimped grain have not been established.

The readily available sugar, presented herein as WSC, was almost totally consumed in the control treatment, followed by PA_2, which was responsible for greater amounts of ethanol and acetic acid in both treatments compared to the other treatments.

https://doi.org/10.1017/S0021859619000546 Published online by Cambridge University Press

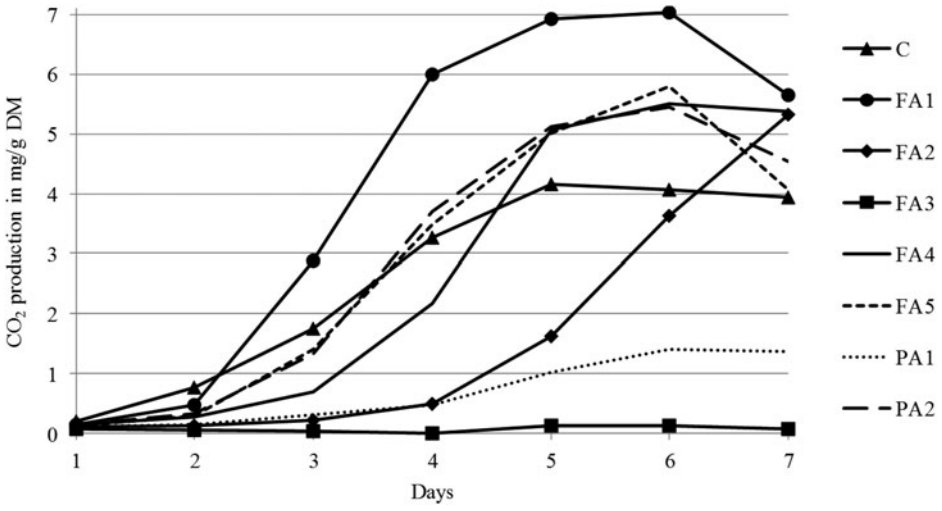

Fig. 1. Cumulative carbon dioxide (CO_2) production of ensiled crimped wheat grain according to days after air exposure and additive treatments. C: control; FA_1: formic acid, 800 g/kg, 5 litres/tonne; FA_2: formic acid, 580 g/kg, 5 litres/tonne; FA_3: formic acid, 580 g/kg, 7 litres/tonne; FA_4: formic acid, 760 g/kg, 7 litres/tonne; FA_5: formic acid, 370 g/kg, 5 litres/tonne; PA_1: propionic acid, 540 g/kg, 5 litres/tonne; PA_2: propionic acid, 370 g/kg, 4 litres/tonne.

In other treatments, WSC remained high due to restricted fermentation, which may represent a potential source of readily available substrate for the growth of aerobic microorganisms when the silos are opened and exposed to air (Wilkinson and Davies, 2013). However, this concept is not well established, since other researchers (Ohyama *et al.*, 1980; Pahlow *et al.*, 2005; Wróbel *et al.*, 2008) have identified no direct relationship between AS and residual WSC amount, and in the current data those treatments did not show poor AS.

In general, the majority of DM losses are due to aerobic deterioration occurring during storage and even short-term exposure to air results in losses (Kung, 2010). Furthermore, much of the lost DM is of high quality, e.g. in terms of digestibility. The DM losses of additive treated grain remained rather low when compared to the control, indicating the great efficiency of organic acid-based additives to prevent preservation losses.

The visual appearance of mould reflects an advanced stage of spoilage and extensive aerobic deterioration, but the beginning of spoilage is associated with the development of yeasts and aerobic acetic acid bacteria, which are not visually detectable (McDonald *et al.*, 1991; Wilkinson and Davies, 2013). In addition to gaseous losses and the need to discard feed, there is potential for mycotoxins to be produced in feed and they are known to pose serious health and production risks to animals (da Rocha *et al.*, 2014); however, mycotoxins were not analysed in the current study.

A relatively large amount of crimped grain was discarded from each jar to avoid sampling in the spoiled area. As moulds are aerobic microorganisms, their appearance shows that there was oxygen in the jars either from the beginning of ensiling or the lids were not properly airtight. Moulds are able to grow in lower moisture compared to yeasts, as indicated by the results of microbiological analysis. This reveals how challenging this moisture area is for ensiling. In the current experiment, yeast counts for all treatments remained below the established level of 10^5 CFU/g (McDonald *et al.*, 1991; Borreani and Tabacco, 2010) set as a threshold ensuring no reduction in grain AS. In practice, mouldy spots or mouldy layers are the problem detected when ensiling low moisture-crimped grains. Kung (2010) reported that management factors in controlling microorganism development are more important and efficient at the time of ensiling than afterwards, during feed out or total mixed ration preparation.

The control treatment, although without additive, resulted in rather low presence of mould spots. This was most likely due to high fermentation during the ensiling phase, converting WSC into ethanol and acetic acid, which inhibited microbial activity during the aerobic phase. The rapid reduction in pH of the grain silage is a direct effect of incremental concentrations of lactic acid; however, it has poor antifungal features. According to Woolford (1975), acetic and propionic acids have a good effect on controlling microbial growth in grain silage, which could be clearly seen in the control and both propionic acid-based additive treatments in the current study.

The FA_2, FA_3 and PA_1 additives tested in the current experiment were efficient at increasing the AS of wheat crimped grain. According to Wilkinson and Davies (2013), evaluation of AS is a primordial factor in ensuring that the feed is well-preserved and safe in terms of minimal presence of moulds before providing it to ruminants. Fermentation acids start to oxidize as soon as the silo is opened and exposed to air and lactic acid is a substrate for growth of aerobic microbes (Pahlow *et al.*, 2003). Formic acid-based additives, except for pure formic acid (80%), and the propionic acid and ammonium propionate-based additive (PA_1), proved to be efficient at prolonging the AS of crimped wheat grain. According to commercial instructions, PA_2 should not be mixed with water, which was done for all additives to promote even distribution in the raw material. This practice may have resulted in crystallization of benzoic acid and subsequently reduced the efficiency of PA_2 in the current study.

Comparison of methods to evaluate aerobic stability

The microbial oxidation of organic acids and WSC leads to an increase in temperature of silages above ambient during the aerobic phase, converting those compounds into carbon dioxide and water (Ranjit and Kung, 2000). This effect is easily observed in grass silages with higher %DM, for example, 300–500 g DM/kg fresh weight (Wilkinson and Davies, 2013). According to McDonald *et al.* (1991), a greater amount of heat is needed to increase the temperature of high-MC feed, but on the other hand, low-MC grains dissipate the heat produced to the environment rather than accumulate it in the feed, although signs of spoilage are spotted (Seppälä *et al.*, 2015). More accurate methods are thus required to evaluate AS of low-MC materials.

As described previously, the additive treatments used in the current experiment resulted in different ASs that could be detected with different methods, which allows a comparison of

https://doi.org/10.1017/S0021859619000546 Published online by Cambridge University Press

the methods. The correlation between CO_2 production (Ashbell *et al*., 1990) and increasing temperature was very close, indicating that CO_2 produced by aerobic bacteria can be used as a method to evaluate AS. The labour and analytical costs of the CO_2 production method were higher than that of the temperature method and only a single measurement point per day is realistic, at least if the CO_2 measurement is carried out manually. However, a smaller sample size can be used and it may be useful also in the cases of drier grain samples that may not accumulate heat. An ideal situation would be where CO_2 measurements can be automated and it could be used when a sensitive method is needed to follow-up aerobic spoilage.

Theoretically, the reason for the reduction of CO_2 during the later phases of the follow-up could be carbon absorption by the grains through the homoacetogenic acetyl-CoA (Wood-Ljungdahl) pathway performed by bacteria (Schmidt *et al*., 2018). However, this pathway has not been described for silages or any other feed during the aerobic phase. According to Wood and Ljungdahl (1991), anaerobic bacteria are able to reduce CO_2 into acetate, which is a well-established pathway. Although still not proven in the feed production system, this approach demonstrates a potential way to improve fixation of CO_2 into silage, which might even be used to mitigate greenhouse gas emissions. In the current work, a longer follow-up of CO_2 production would have been needed to confirm the trend.

The visual evaluation of mould did not rank the treatments in exactly the same order as the production of heat and CO_2. However, this methodology is practical, easy, efficient, low cost and yet provides useful results to compare efficacy of treatments. The visual inspection method has been used successfully in evaluating the stability of e.g. moist vegetable by-products (Franco *et al*., 2018).

Conclusions

The current results show significant potential to modify drier than optimal crimped grain preservation and AS by using different organic acid-based additives. There were clear differences in the efficacy of additives in improving the AS of relatively low moisture-crimped wheat grains, with FA_3 (high dose of formic acid-based additive including several ingredients) being the most efficient followed by PA_1 (propionic acid-based additive), while sole formic acid had the lowest performance among the treatments.

It is possible to use different methods to measure AS, since they all provided results applicable to the identification of spoilage signs of the feed. The correlation between temperature rise and CO_2 production was very high, indicating that CO_2 produced by aerobic bacteria can be used as a method to evaluate AS and it may be more sensitive than the method based on temperature rise for relatively dry feed materials. Visual appearance of mould ranked the additives somewhat differently, but could still provide useful information on the efficacy of treatments.

Financial support. This work was carried out as part of Rehvi project funded by the Centre for Economic Development, Transport and the Environment for Northern Ostrobothnia, Finland (Grant number 42237/58904). Eastman Chemical Company and Berner Ltd. provided the additives used and partial funding for the project.

Conflict of interest. The authors have declared that there is no conflict of interest.

Ethical standards. Not applicable.

References

AOAC (1990) *Official Methods of Analysis*. Arlington, VA, USA: Association of Official Analytical Chemists, Inc.

Ashbell G, Weinberg ZG, Azieli A, Hen Y and Horev B (1990) A simple system to study the aerobic determination of silages. *Canadian Agricultural Engineering* **33**, 391–393.

Bern CJ (1998) Preserving the Iowa corn crop: energy use and CO_2 release. *Applied Engineering in Agriculture* **14**, 293–299.

Borreani G and Tabacco E (2010) The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. *Journal of Dairy Science* **93**, 2620–2629.

da Rocha MEB, Freire FCO, Maia FEF, Guedes MIF and Rondina D (2014) Mycotoxins and their effects on human and animal health. *Food Control* **36**, 159–165.

Franco M, Jalava T, Kahala M, Järvenpää E, Lehto M and Rinne M (2018) Preservatives can improve aerobic stability of potato by-product. In Udén P, Eriksson T, Spörndly R, Rustas BO and Liljeholm M (eds), *Proceedings of the 9th Nordic Feed Science Conference*. Report 298. Uppsala, Sweden: Swedish University of Agricultural Sciences, pp. 143–148. Available at https://pub.epsilon.slu.se/15870/7/__ad.slu.se_common_bibul_slub_AVD_Vet_Kom_Publicering_epsilon_oppetarkiv_uden_p_et_al_190128.pdf (Accessed 4 June 2019).

Haacker K, Block HJ and Weissbach F (1983) Zur kolorimetrischen Milchsäurebestimmung in Silagen mit p-Hydroxydiphenyl. [On the colorimetric determination of lactic acid in silages with p-hydroxydiphenyl]. *Archiv für Tierernährung* **33**, 505–512.

Huhtanen PJ, Blauwiekel R and Saastamoinen I (1998) Effects of intraruminal infusions of propionate and butyrate with two different protein supplements on milk production and blood metabolites in dairy cows receiving grass silage based diet. *Journal of the Science of Food and Agriculture* **77**, 213–222.

Huhtanen P, Jaakkola S and Nousiainen J (2013) An overview of silage research in Finland: from ensiling innovation to advances in dairy cow feeding. *Agricultural and Food Science* **22**, 35–56.

Jaakkola S, Saarisalo E and Heikkilä T (2009) Formic acid treated whole crop barley and wheat silages in dairy cow diets: effects of crop maturity, proportion in the diet, and level and type of concentrate supplementation. *Agricultural and Food Science* **18**, 234–256.

Klemola E, Järvenpää M and Peltola A (1994) Viljansäilöntäopas (Grain preservation guide). *Työtehoseuran Maataloustiedote* **4**, 1–15.

Knicky M and Spörndly R (2015) Short communication: use of a mixture of sodium nitrite, sodium benzoate, and potassium sorbate in aerobically challenged silages. *Journal of Dairy Science* **98**, 5729–5734.

Kristensen NB, Sloth KH, Højberg O, Spliid NH, Jensen C and Thøgersen R (2010) Effects of microbial inoculants on corn silage fermentation, microbial contents, aerobic stability, and milk production under field conditions. *Journal of Dairy Science* **93**, 3764–3774.

Kung Jr L (2010) *Proceedings of the 2010 California Alfalfa & Forage Symposium and Corn/Cereal Silage Conference, Visalia, CA, 1–2 December, 2010*. Davis, CA, USA: UC Davis, pp. 1–14. Available at https://alfalfa.ucdavis.edu/.

Luke (2019) *Feed Table and Nutrient Requirements*. Helsinki, Finland: Natural Resources Institute Finland (Luke). Available at https://portal.mtt.fi/portal/page/portal/Rehutaulukot/feed_tables_english (Accessed 4 June 2019).

McCullough H (1967) The determination of ammonia in whole blood by direct colorimetric method. *Clinica Chimica Acta* **17**, 297–304.

McDonald P, Henderson AR and Heron SJE (1991) *The Biochemistry of Silage*, 2nd Edn. Marlow, UK: Chalcombe Publications.

Ohyama Y, Hara S and Masaki S (1980) Analysis of the factors affecting aerobic deterioration of grass silages. In Thomas C (ed.), *Forage Conservation in the 80s*. BGS Occasional Symposium No. 11. Reading, UK: British Grassland Society, pp. 257–261.

Olstorpe M, Schnürer J and Passoth V (2010) Microbial changes during storage of moist crimped cereal barley grain under Swedish farm conditions. *Animal Feed Science and Technology* **156**, 37–46.

Pahlow G, Muck RE, Driehuis F, Oude Elferink SJWH and Spoelstra SF (2003) Microbiology of ensiling. In Buxton DR, Muck RE and

https://doi.org/10.1017/S0021859619000546 Published online by Cambridge University Press

Harrison JH (eds), *Silage Science and Technology*. Agronomy Publication No. 42. Madison, WI, USA: American Society of Agronomy, pp. 31–93.

Pahlow G, Merry RJ, O'Kiely P, Pauly T and Greet JM (2005) Effect of residual sugar in high-sugar grass silages on aerobic stability. In Park RS and Stronge MD (eds), *Silage Production and Utilisation. Proceedings of the XIVth International Silage Conference, a Satellite Workshop of the XXth International Grassland Congress, July 2005, Belfast, Northern Ireland*. Wageningen, The Netherlands: Wageningen Academic Publishers, p. 224.

Palva R, Jaakkola S, Siljander-Rasi H, Valaja J, Root T and Peltonen S (2005) Viljan tuoresäilöntä (Moist grain preservation). In Palva R, Kirkkari AM and Teräväinen H (eds), *Viljasadon käsittely ja käyttö (Handling and Using Grain Yield)*. Tieto Tuottamaan 108. Helsinki, Finland: ProAgria Maaseutukeskusten Liitto, pp. 55–66.

Pieper R, Hackl W, Korn U, Zeyner A, Souffrant WB and Pieper B (2011) Effect of ensiling triticale, barley and wheat grains at different moisture content and addition of *Lactobacillus plantarum* (DSMZ 8866 and 8862) on fermentation characteristics and nutrient digestibility in pigs. *Animal Feed Science and Technology* **164**, 96–105.

Ranjit NK and Kung Jr L (2000) The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum* or a chemical preservative on the fermentation and aerobic stability of corn silage. *Journal of Dairy Science* **83**, 526–535.

Rinne M, Franco M, Jalava T, Järvenpää E, Kahala M, Blasco L, Siljander-Rasi H and Kuoppala K (2019) Carrot by-product fermentation quality and aerobic spoilage could be modified with silage additives. *Agricultural and Food Science* **28**, 59–69.

Salo M-L and Salmi M (1968) Determination of starch by the amyloglucosidase method. *Journal of the Scientific Agricultural Society of Finland* **40**, 38–45.

Schmidt P, Novinski CO and Zopollatto M (2018) Carbon absorption in silages: a novel approach in silage microbiology. In Gerlach K and Südekum K-H (eds), *Proceedings of the XVIII International Silage Conference*. Bonn, Germany: The University of Bonn, pp. 20–21.

Seppälä A, Nysand M, Mäki M, Miettinen H and Rinne M (2012) Ensiling crimped barley grain at farm scale in plastic tube bag with formic and propionic acid based additives. In Kuoppala K, Rinne M and Vanhatalo A (eds), *Proceedings of the XVI International Silage Conference, Hämeenlinna, Finland, 2–4 July 2012*. Helsinki, Finland: MTT Agrifood Research Finland and University of Helsinki, pp. 436–437.

Seppälä A, Mäki M, Orkola S and Rinne M (2015) Aerobic stability of crimped barley ensiled with organic acids. In Udén P (ed.), *Proceedings of the 6th Nordic Feed Science Conference, Uppsala, Sweden*. Report 291. Uppsala, Sweden: Swedish University of Agricultural Sciences, pp. 71–76. Available at https://www.slu.se/globalassets/ew/org/inst/huv/foder/nfsc-2016/nfsc-2015-proceedings-final.pdf (Accessed 4 June 2019).

Seppälä A, Heikkilä T, Mäki M and Rinne M (2016) Effects of additives on the fermentation and aerobic stability of grass silages and total mixed rations. *Grass and Forage Science* **71**, 458–471.

Somogyi M (1945) A new reagent for the determination of sugars. *Journal of Biological Chemistry* **160**, 61–68.

Vanhatalo A, Jaakkola S, Rauramaa A, Nousiainen J and Tommila A (1999) Additives in ensiling whole crop barley. In *Proceedings of the XIIth International Silage Conference*. Uppsala, Sweden: Swedish University of Agricultural Sciences, pp. 121–122.

Van Soest PJ, Robertson JB and Lewis BA (1991) Methods for dietary fibre, neutral detergent fibre and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* **74**, 3583–3597.

Wilkinson JM (1990) *Silage UK*, 6th Edn. Marlow, UK: Chalcombe Publications.

Wilkinson JM and Davies DR (2013) The aerobic stability of silage: key findings and recent developments. *Grass and Forage Science* **68**, 1–19.

Wood HG and Ljungdahl LG (1991) Autotrophic character of the acetogenic bacteria. In Shively JM and Barton LL (eds), *Variations in Autotrophic Life*. San Diego, USA: Academic Press, pp. 201–250.

Woolford MK (1975) Microbiological screening of the straight chain fatty acids (C_1–C_{12}) as potential silage additives. *Journal of the Science of Food and Agriculture* **26**, 219–228.

Wróbel B, Zielinska AK and Suterska A (2008) Evaluation of quality and aerobic stability of grass silage treated with bacterial inoculants containing *Lactobacillus buchneri*. In *Proceedings. 13th International Conference on Forage Conservation*. Nitra, Slovak Republic: Slovak Agricultural Research Centre, Research Institute of Animal Production, pp. 122–123.

https://doi.org/10.1017/S0021859619000546 Published online by Cambridge University Press