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Fermentation quality of ensiled crimped faba beans using different additives with special attention to changes in bioactive compounds

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Abstract

Faba bean (*Vicia faba* L.) is a grain legume that provides possibilities to increase local protein self-sufficiency both as feed and food in areas where possibilities for cultivation of other protein crops are limited. However, full ripening may not be achieved and storage of the moist beans by crimping and ensiling provides an economic alternative to drying, which is well suited for animal feeding. The objectives of the current experiment were to evaluate the effects of moisture content and various silage additives on the fermentation quality, aerobic stability and fate of bioactive compounds of ensiled crimped faba beans in a pilot scale ensiling experiment. Faba beans were combine harvested at moisture level of 443 g/kg and crimped prior to ensiling. The following additive treatments were applied using three replicates: 1) control (CON), 2) two commercial lactic acid bacteria inoculants as mixture (LABmix), 3) In-house LAB inoculant (LABLuke), 4) Formic acid based product (FA) and 5) Salt based product (Salt). Further, two of the additive treatments were prepared with additional moisture: 6) Control with tap water addition (CON + w) and 7) LABmix with tap water addition (LABmix + w). The beans ensiled without additive showed elevated pH, butyric acid and ethanol concentrations and proportion of ammonia N in total N. Addition of water further decreased the fermentation quality. The LAB inoculants improved the fermentation quality of the beans compared to CON. The fermentation was effectively restricted when FA was used. Both LAB and chemical additives improved aerobic stability compared to CON and Salt was especially effective in improving aerobic stability. Vicine disappeared totally during the ensiling period and also convicine was effectively degraded. It seems that promoting microbial activity in the silo either by LAB inoculation or increased moisture promoted the degradation of convicine, while chemical additives decreased the degradation of it. Total and particularly soluble proanthocyanidins were also degraded during the ensiling period. Crimping and ensiling is an alternative preservation method for faba beans which eliminates the energy consumption and investments required for drying. In addition, benefits regarding the concentrations of bioactive compounds may be expected.

Abbreviations: AA, acetic acid; ANF, antinutritional factors; CON, control without additive; CON+w, control without additive and water added; DM, dry matter; FA, commercial formic acid based product; FM, fresh matter; LA, lactic acid; LAB, lactic acid bacteria; LABLuke, non-commercial lactic acid bacteria inoculant; LABmix, commercial lactic acid bacteria inoculant mixture; LABmix+w, lactic acid bacteria inoculant and water added; MC, moisture concentration; mDP, mean degree of polymerization; PA, proanthocyanidin; PC, procyanidin; PD, prodelphinidin; Salt, commercial salt based product; TMR, total mixed ration; V+C, vicin and convicin

Keywords: Aerobic stability; Convicine; Fermentation; Silage additive; Tannin; Vicine

1 Introduction

There is a growing interest to improve the self-sufficiency of protein feeds and reduce the dependency of imported soya bean in Europe (European Parliament, 2011). Faba bean (*Vicia faba* L.) is a promising candidate for producing grain legumes in Europe including the Northern parts of it (Peltonen-Sainio et al., 2013) and it can successfully be used for both monogastric and ruminant livestock. The nutritional quality of faba beans is good, and faba beans contain 250–330 g/kg DM crude protein (CP) and 400–480 g/kg DM starch according to Feedipedia (2017) the corresponding values being 300 g/kg DM CP and 380 g/kg DM starch according to Luke (2020). Crépon et al. (2010) indicated that the CP content of faba beans is known to vary greatly among cultivars and due to growing conditions, ranging from 270 to 320 g/kg DM. Faba beans contain considerably less protein than soya beans but more than peas (see Feedipedia, 2017; Luke, 2020).

Faba beans have successfully been used as protein and energy supplements for dairy cows (Puhakka et al., 2016), but their use to monogastric animals is restricted to some extent due to antinutritional factors (ANF; Jezierny et al., 2010; Koivunen, 2016). The various ANF present in faba beans include (see e.g. Makkar et al., 1997; Gulewicz et al., 2014) vicine and convicine (V + C), trypsin and chymotrypsin inhibitors, protease inhibitors, lectins, saponins, flatulent oligosaccharides (such as raffinose, stachyose and verbascose) and phytates. Among the ANF present in faba beans, the most harmful for monogastric animals are trypsin and protease inhibitors, and particularly V + C (Crépon et al., 2010). Also soya bean contains ANF (lectins, saponins, and protease and trypsin inhibitors; Jezierny et al., 2010) and heat treatments are regularly used to decrease their effects on livestock. The ANF may impair growth performance, fertility and the health status of livestock due to a variety of underlying mechanisms (Jezierny et al., 2010).

Faba beans contain also condensed tannins in the form of proanthocyanidins (PA) and their role in animal nutrition has not yet been thoroughly examined. The most common structural units in PA are procyanidins (PC) and prodelphinidins (PD), and mean degree of polymerization (mDP) is commonly used as a measure of the average length of PA polymers. While some PA may be harmful for monogastric animals (Crépon et al., 2010; Jezierny et al., 2010), many types of PA have been shown to have positive effects on ruminants. For example, they have been shown to significantly decrease both methane emissions and nematode infections in ruminants due to anthelmintic activity (Novobilský et al., 2013; Hatew et al., 2015, 2016; Jayanegara et al., 2015).

The optimal PA profile needed to achieve the highest ruminant-related bioactivities is not yet known, but some major patterns are arising: e.g. larger mDP and lower PC/PD ratio seem to be effective against gastrointestinal nematodes (Quijada et al., 2015; Williams et al., 2014). The PA can also reduce ammonia levels in the rumen fluid and urea level in the milk and blood of ruminants, and they can also increase the amount of long chain n-3 fatty acids in milk and meat (Girard et al., 2016). The structure of PA, properties and concentrations may change during ensiling of the foragefeed and for that reason it is critical to know the PA composition of the foragefeed both before and after ensiling so that its net benefits could be revealed also tannin-wise.

In Northern Europe, the growing season may often be too short for full ripening which is accentuated by the continuous growth habit of seed formation in faba beans. Further, the weather conditions during autumn are often humid and as a consequence, the harvested beans may have a relatively high moisture content (MC). Drying moist beans is technically challenging, energy demanding and expensive so that other storage methods would be needed. Ensiling crimped high moist grains is a method that has been developed to reduce storage costs and expand the harvesting window of grain in humid areas, and it may also be used for beans or peas (Vanhatalo et al., 2004; O'Kiely et al., 2014). Typically, moist grain (MC 300–450 g/kg) is crimped (slightly crushed or rolled) and ensiled airtight in silos or plastic tubes, where lactic acid fermentation takes place. Silage additives can be included to improve the fermentation quality and aerobic stability (Seppälä et al., 2012, 2016; Franco et al., 2019).

An additional benefit of ensiling moist faba beans instead of drying them could be the modifications of the bioactive compounds due to the fermentation process during storage. Gefrom et al. (2013) reported that the concentrations of tannins and oligosaccharides in faba beans were substantially reduced during ensiling either without any additive or with a strain of *Lactobacillus plantarum*. Coda et al. (2015) reported reductions in V + C, trypsin inhibitor and tannin contents after a 48 h fermentation of faba bean flour. The effects of lactic acid bacteria (LAB) on bioactive compounds of faba beans has been studied to some extent (e.g. Gefrom et al., 2013; Coda et al., 2015; Pulkkinen et al., 2019), but to our knowledge the effects of chemical additives on them has not previously been evaluated.

The main objective of the present study was to compare the effects of different additives on the fermentation quality and aerobic stability of ensiled crimped high moisture faba beans. We used different types of additives which either promoted or restricted the fermentation (Muck et al., 2018). Additionally, the effects of fermentation type induced by additive treatments on the concentrations of ANF and PA in faba beans were evaluated. As the MC is an important factor affecting the type and extent of fermentation, potentially also the modifications of bioactive compounds, an additional factor of added moisture was included. A preliminary report of the current material has been published by Rinne et al. (2018).

2 Material and methods

2.1 Faba bean cultivation and harvesting

The experimental faba beans were sown on 2 June 2015 in Tammela, Finland (60°48'N, 023°45'E). The variety used was Kontu, which is an early ripening cultivar developed by Boreal Plant Breeding Ltd. (Jokioinen, Finland). The field was fertilized using a commercial inorganic fertilizer (YaraMila Pellon Y 3, Yara Finland, Helsinki, Finland) at levels of 25 kg N, 3 kg P and 9 kg K per hectare prior to sowing. The soil type was sandy clay with a pH of 6.3 and 8 mg P/l and 380 mg K/l. No chemical or physical plant protection actions were taken during the growing period.


The beans were combine harvested on 7 October 2015. The effective temperature sum achieved was 1227 degree days. The pods were already black as well as most of the canopy, but the beans were still partly green so that they were not fully ripened. Immediately after harvesting, the beans were crimped using a farm scale crimper (MD 700 HD, Murska Ltd., Ylivieska, Finland) without any additive. The crimped beans were kept refrigerated at +2 °C overnight and the ensiling was conducted on the following day.

2.2 Ensiling of crimped faba bean at pilot scale

The ensiling treatments with their abbreviations are presented in Table 1. All additives were diluted with water so that the amount of liquid added was the same for all treatments. Further, the amount of water added to CON + w and LABmix + w was chosen to increase the MC content by ca. 50 g/kg above the other treatments.

alt-text: Table 1

Table 1

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Treatments used in ensiling crimped faba beans.

Treatment	Abbreviation	Application level/kg
Control without additive	CON	Water 12.5 mL
Control without additive and water added	CON + w	Water 112.5 mL
Commercial lactic acid bacteria inoculant mixture [LABmix; Josilac® Classic (Josera, Kleinheubach, Germany; <i>Lactobacillus plantarum</i> and <i>Pediococcus acidilactici</i> 1.0 × 10 ¹¹ colony forming units (CFU)/g, xylanase from <i>Trichoderma longibractiatum</i>) and Bonsilage (Schaumann, Pinneberg, Germany; <i>L. plantarum</i> , <i>L. paracasei</i> , <i>L. buchneri</i> and <i>L. lactis</i> 1.25 × 10 ¹¹ CFU/g)] both used at half of their recommended dose to result in similar amount of bacteria applied as commercial recommendations.	LABmix	1.25 × 10 ⁶ CFU ¹ per g in 12.5 mL water
LABmix and water added	LABmix + w	1.25 × 10 ⁶ CFU ¹ per g in 112.5 mL water
In-house lactic acid bacteria inoculant containing four different LAB isolates (<i>L. plantarum</i> , <i>P. pentosaceus</i> , <i>Le. citreum</i> , <i>L. casei</i>) from plant based materials (LABLuke; Natural Resources Institute Finland, Jokioinen, Finland).	LABLuke	10 ⁶ CFU per g in 12.5 mL water
Commercial formic acid based product (AIV2 Plus, Eastman, Oulu, Finland; main components formic acid 760 g/l and ammonium formate 55 g/l)	FA	6 L/ton ¹ in 12.5 mL water
Commercial salt based product (SafeSil, Salinity AB, Göteborg, Sweden; main components sodium benzoate 50–500 g/l, potassium sorbate 50–350 g/l and sodium nitrite <50 g/l)	Salt	4 L/ton ¹ in 12.5 mL water

Table Footnotes

¹ Commercially recommended dose.

Equal batches (6 kg each) were weighed for each experimental replicate and randomized to the additive treatments (7 treatments with 3 replicates each). During weighing, the raw material was representatively sampled for analysis of its composition. The individual batches for each replicate were treated with additives, thoroughly mixed, and a 5.5 L cylindrical plastic silo was filled for each replicate. Silos were compacted manually to a density of 0.84 kg/l, weighed, and closed immediately after filling. The silos were stored at room temperature protected from light until opened.

After 106 days of ensiling the silos were weighed, opened and sampled for analyses. The clearly spoiled feed on top and bottom of the silos where oxygen leakage had taken place was discarded and not used in further analyses. The weight losses representing the fermentation losses during the anaerobic phase were calculated for fresh matter and dry matter (DM) by subtracting the weight at the end of the ensiling period from the weight at the beginning of it.

Aerobic stability was determined from each replicate by monitoring the temperature changes of the feeds when exposed to air. The beans were monitored as such, and as part of a total mixed ration (TMR) which contained on DM basis 0.49 grass silage, 0.24 ground barley grain and 0.27 ensiled beans. The grass silage used was harvested from 1st cut of a timothy-meadow fescue sward, slightly prewilted and ensiled with a formic acid based additive (AIV2 Plus, Eastman, Oulu, Finland) at target application rate of 5 L/ton. The DM concentration of the silage was 242 g/kg and that of barley grain 857 g/kg. For aerobic stability measurements, 870 g of the beans and the TMRs were placed in 2500 cm³ polystyrene containers in open plastic bags. Temperature was automatically recorded at 10 min intervals for 210 h using a thermocouple wire connected to a data logger. Aerobic stability was defined as the time (h) taken before the temperature raised by 2.0 °C above the ambient temperature.

2.3 Chemical and microbiological analyses

The samples were freeze dried (Christ Gamma 2–20 with controller LMC-2, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany, using a drying period 3–4 days beginning with -25 °C, 0.370 mbar) for analyses, and also the DM concentration was calculated from the weight loss during freeze drying. The MC was calculated as 1000 – DM (g/kg). Ash content was determined after ignition in a muffle furnace at 510 °C for 16 h (AOAC, 1990; method 942.05). Nitrogen content was analysed using the Dumas method (AOAC, 1990; method 968.06) using Leco FP428 analyzer (Leco Corp., St Joseph, MI, USA) and CP content was calculated as 6.25 × nitrogen content. Concentration of neutral detergent fibre was determined using filter bag technique (for A200 and A200I) and 25 µm nylon bags (F57, ANKOM Technology) by ANKOM 220 Fiber Analyzer (ANKOM Technology, Macedon NY, USA). The detergent solution was made according to Van Soest et al. (1991) using sodium-sulphite and amylase and results presented ash free. Starch was analysed by amyloglucosidase method according to Salo and Salmi (1968) and buffering capacity according to Weissbach et al. (1974). Digestibility of grass used in the total mixed ration in aerobic stability test was calculated based on *in vitro* cellulase solubility as described by Huhtanen et al. (2006).

The ensiled beans were analysed for lactic acid according to Haacker et al. (1983), volatile fatty acids (VFA) according to Huhtanen et al. (1998), and NH₃-N according to McCullough (1967). Ethanol was analysed spectrophotometrically using a commercial kit (Cat. No. 10 176 290 035, Boehringer Mannheim GmbH, Mannheim, Germany) according to instructions given by the manufacturer (ethanol, UV-method).

The samples for microbiological analyses were mixed and 25 g were weighed in stomacher bags and mixed with 225 mL of ¼-strength Ringer solution (Merck 1.15525.0001; Merck & Co Inc., Kenilworth, NJ, USA). The samples were homogenised with stomacher (Stomacher® 400 Circulator; Seward Ltd., Worthing, UK) 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 on Dichloran Rose Bengal Chloramphenicol Agar medium (DRBC, Difco 258710; Difco Laboratories Inc., Franklin Lakes, NJ, USA) which was supplemented with

50 µg ml⁻¹ of oxytetracycline hydrochloride (AppliChem BioChemica A5257; AppliChem Ltd., Darmstadt, Germany). The petri dishes were incubated at 25 ± 1 °C. The colonies were counted after 3 and 5 days. The LAB were determined on MRS Agar (Lactobacilli MRS Broth, Difco 288130, Bacto Agar 15 g/l, Difco 214010) dishes incubated anaerobically at 30 °C for 3 days.

The V + C were determined using high performance liquid chromatography with a diode array detection (HPLC-DAD). The method was based on [Quemener \(1988\)](#) with some modifications. For total PA, the degree of polymerization, and proportions of PD and PC were analysed according to a modified version of the thiolysis method of [Hellström and Mattila \(2008\)](#). Soluble PA including quantitative and qualitative tannin fingerprints were detected by UPLC-MS/MS. Prior to analysis, the samples were weighed, extracted and lyophilized. First, 20 mg of each sample was weighed into 2 mL Eppendorf tube. The used extraction protocol was similar to [Malisch et al. \(2015\)](#). A first 1400 µl batch of extraction solvent (4/1 acetone/water, v/v) was added to the tubes and the samples were shaken in a vortex mixer for 15 min. The samples were macerated at 4 °C overnight after which they were extracted in a planar shaker for 3 h at the speed of 280/min. Samples were centrifuged and supernatants were decanted. A new 1400 µl batch of extraction solvent was added to the samples and the extraction steps were repeated. Both extracts were combined and concentrated into water phase. Samples were then frozen and freeze-dried.

The freeze-dried extracts were diluted into 1 mL of ultrapure water, filtered with 0.2 µm PTFE syringe filters, and analysed by UPLC-MS/MS ([Engström et al., 2014](#); 2015). Similar UPLC-MS/MS instrument and MS parameters were used as described by [Engström et al. \(2014\)](#). In the UPLC system, acetonitrile (A) and 0.1 % formic acid (B) were used as eluents and flow rate was set to 0.5 mL/min. The following elution profile was used: 0–0.5 min, 0.1 % A in B (isocratic); 0.5–5.0 min, 0.1–30 % A in B (linear gradient); 5.0–8.0 min, 30–45 % A in B (linear gradient); 8.0–11.5 min, column wash and stabilization. The UV and MS data was recorded at 0–8 min. Extension and terminal units of both PC and PD were detected by the multiple reaction monitoring (MRM) ([Engström et al., 2014](#)). The quantification of samples and the utilization of PC and PD calibration curve data was carried out as described by [Malisch et al. \(2015\)](#). The ionization stability of triple quadrupole mass spectrometer was checked with catechin solution (1 µg/mL, acetonitrile/0.1 % formic acid, v/v), which was analysed five times after a batch of 10 samples. The peaks were integrated with Target Lynx software (V4.1 SCN876 SCN 917 © 2012 Waters Inc.). The PC and PD traces were quantified against calibration curves prepared separately for PC, PD and mDP. The calibration curves were previously prepared from PA rich Sephadex LH-20 fractions which were calibrated against thiolysis results ([Engström et al., 2014](#)).

2.4 Statistical analyses

The data were subjected to analysis of variance using the GLM-procedure of SAS (SAS Inst. Inc., Cary, NC, USA). The model was $Y_i = \mu + T_i + \varepsilon_i$, in which Y_{ij} = observation, μ = the general mean, T_i = the effect of the treatment ($i = 1, \dots, 7$), and ε_i = the experimental error term. Treatment effects were separated using orthogonal contrasts to test for 1) the effect of water (CON and LABmix vs. CON + w and LABmix + w), 2) the effect of LAB (CON vs. LABmix and LABLuke) 3) the effect of chemicals (CON vs. FA and Salt), and 4) LAB vs. chemicals (LABmix and LABLuke vs. FA and Salt). Further, differences between least square means of response variables were estimated with Tukey's test and significance was declared at $P \leq 0.05$.


3 Results

3.1 Preservation quality of ensiled faba beans

The composition of the crimped faba beans prior to ensiling is presented in [Table 2](#). Due to unfavourable weather conditions during spring resulting in late sowing and humid weather prior to the harvest in the autumn, the beans were relatively immature and moist at harvest. This was somewhat unexpected because the temperature sum achieved (1227 degree days) exceeded the requirement given by plant breeding company for ripening of the faba bean variety Kontu (1150–1180 degree days).

alt-text: Table 2

Table 2

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Characteristics of crimped faba beans prior to ensiling.

	Faba beans
Dry matter (DM), g/kg	557
pH	6.89
Buffering capacity, g lactic acid/100 g	4.8
Chemical composition, g/kg DM	
Ash	41
Crude protein	294
Starch	352
Neutral detergent fibre	189
Vicine	9.76
Convicine	5.69
Total proanthocyanidins ¹	10.9

mDP ²	7.36
Proportion of PD ³ , g/g	0.696
Soluble proanthocyanidins ⁴	3.1
mDP ²	9.29
Proportion of PD ³ , g/g	0.649
Microbial quality, log CFU/g	
Yeasts	5.3
Moulds	4.8
Total bacteria	7.0


Table Footnotes

- ¹ Total amount of proanthocyanidins (PA) as detected by thiolysis.
- ² mDP = mean degree of polymerization.
- ³ PD = prodelphinidin.
- ⁴ The amount of soluble proanthocyanidins (PA) as detected by UPLC-MS/MS.

The beans ensiled without additive showed elevated pH, ethanol concentrations and NH₃-N proportion (Table 3) and can be described as poorly preserved. Addition of water into CON decreased the fermentation quality even further as indicated by very high NH₃-N and butyric acid concentrations (P < 0.05). When LAB was used, the effect of water addition was not as detrimental although NH₃-N proportion increased (P < 0.05). The LAB inoculants improved the fermentation quality of the beans compared to CON indicated by lower pH, proportion of NH₃-N as well as lower ethanol concentration (P < 0.001). The preservation quality was further improved when chemical additives were used. For example, proportion of NH₃-N was reduced from 117 g/kg N in CON to 34 g/kg N in FA and 61 g/kg N in Salt. Concentration of lactic acid was higher in LAB inoculated samples compared to CON (P < 0.001), but FA effectively restricted fermentation resulting in low lactic acid and total fermentation product concentrations. LABLuke was somewhat more effective in improving the fermentation quality as it resulted in lower proportion of NH₃-N and higher LA concentration than LABmix (P < 0.05).

alt-text: Table 3

Table 3

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Fermentation quality of crimped faba beans ensiled with different additives.

	Additive treatment ¹							SEM ²	Statistical significance of contrasts ³			
	CON	CON + w	LAB mix	LAB mix + w	LAB Luke	FA	Salt		Water	CON vs LAB	CON vs chem	LAB vs chem
Dry matter (DM), g/kg	552 ^c	505 ^e	563 ^b	512 ^d	560 ^b	572 ^a	564 ^b	1.0	<0.001	<0.001	<0.001	<0.001
pH	5.38 ^a	5.10 ^b	4.29 ^d	4.27 ^d	4.29 ^d	4.92 ^b	4.61 ^c	0.048	0.008	<0.001	<0.001	<0.001
NH ₃ -N, g/kg N	117 ^b	154 ^a	80 ^d	100 ^c	67 ^e	34 ^f	61 ^e	1.7	<0.001	<0.001	<0.001	<0.001
In DM, g/kg												
Ethanol	18.2 ^a	15.3 ^a	8.0 ^{bc}	7.4 ^{bc}	5.9 ^{cd}	3.3 ^d	9.1 ^b	0.6	0.011	<0.001	<0.001	0.228
Lactic acid (LA)	27.5 ^c	35.1 ^c	48.3 ^b	53.8 ^{ab}	57.3 ^a	7.3 ^d	33.8 ^c	1.65	0.001	<0.001	0.004	<0.001
Acetic acid (AA)	7.3 ^b	10.1 ^a	7.4 ^b	6.9 ^b	5.7 ^c	1.8 ^d	7.8 ^b	0.25	<0.001	<0.038	<0.001	<0.001
Propionic acid	0.16 ^c	0.24 ^b	0.12 ^c	0.14 ^c	0.13 ^c	0.45 ^a	0.12 ^c	0.008	<0.001	0.012	<0.001	<0.001
Butyric acid	0.34 ^b	1.48 ^a	0.07 ^b	0.07 ^b	0.07 ^b	0.09 ^b	0.09 ^b	0.119	<0.001	0.084	0.106	0.871
Isobutyric acid	0 ^a	0 ^a	0.01 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0.004	0.207	0.298	1.0	0.207
Valeric acid	0.07 ^{ab}	0.11 ^a	0.04 ^{bc}	0 ^c	0 ^c	0.06 ^b	0.05 ^b	0.001	0.827	<0.001	0.116	0.003
Isovaleric acid	0.09 ^a	0.10 ^a	0.09 ^a	0.10 ^a	0.09 ^a	0.07 ^b	0.09 ^a	0.003	0.026	0.597	0.001	<0.001
Caproic acid	0 ^b	0 ^b	0.05 ^a	0 ^b	0 ^b	0 ^b	0 ^b	0.004	<0.001	<0.001	1.0	<0.001
Total fermentation acids (TA), g/kg DM	35.4 ^c	47.2 ^b	56.2 ^a	61.0 ^a	63.3 ^a	9.86 ^d	41.9 ^{cb}	1.700	<0.001	<0.001	<0.001	<0.001
Total fermentation products, g/kg DM	53.7 ^c	62.5 ^b	64.2 ^{ab}	68.4 ^{ab}	69.3 ^a	13.2 ^d	51.0 ^c	1.27	<0.001	<0.001	<0.001	<0.001
LA/TA	0.777 ^{cd}	0.741 ^d	0.861 ^{ab}	0.881 ^a	0.906 ^a	0.740 ^d	0.804 ^{bc}	0.0121	0.555	<0.001	0.767	<0.001

AA/LA	0.264 ^a	0.292 ^a	0.153 ^b	0.129 ^b	0.099 ^b	0.258 ^a	0.233 ^a	0.0140	0.878	<0.001	0.314	<0.001
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Values with the same letter in a row are not significantly different ($P > 0.05$) based on Tukey's test.

Table Footnotes

¹ For treatment abbreviations, please refer to [Table 1](#).


² SEM = Standard error of the mean.

³ Description of contrasts: Water: CON and LABmix vs. CON + w and LABmix + water; CON vs LAB: CON vs LABmix and LABLuke; CON vs chem: CON vs FA and Salt; LAB vs chem: LABmix and LABLuke vs FA and Salt.

Fresh matter (FM) losses during the anaerobic storage phase were on average 14 g/kg ([Table 4](#)). Use of LAB decreased FM losses slightly compared to CON while FA was more effective ($P < 0.05$). Similar trends were found for DM losses although in many cases the losses were negative (the weight of the silos increased during the storage) which must be a methodological artefact related e.g. to underestimation of the DM concentration of the raw material. Individual DM values for each silo were used for the silages while a single DM value within moisture level was used for the material prior to ensiling.

alt-text: Table 4

Table 4

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Preservation losses, microbial quality and aerobic stability of crimped faba beans ensiled with different additives.

	Additive treatment ¹							SEM ²	Statistical significance of contrasts ³			
	C	C + w	LAB mix	LAB mix + w	LAB Luke	FA	Salt		Water	CON vs LAB	CON vs chem	LAB vs chem
Preservation losses, g/kg												
Fresh matter	20.5 ^a	19.0 ^a	13.7 ^b	11.8 ^{bc}	11.8 ^{bc}	9.3 ^c	11.9 ^{bc}	0.61	0.014	<0.001	<0.001	0.003
Dry matter	19.6 ^a	9.4 ^b	-5.2 ^c	-12.8 ^c	-3.4 ^c	-26.9 ^d	-9.1 ^c	2.03	<0.001	<0.001	<0.001	<0.001
Microbial quality ⁴ , log CFU/g												
Yeasts	4.6-6.1	<2.0-4.8	<2.6-2.9	3.1-3.9	4.4-4.8	5.4-5.9	4.9-5.5					
Moulds	<2.0-<2.6	3.0-3.3	2.4-<3.6	<2.0-<2.6	2.4-<2.6	<2.0-<2.6	2.5-2.7					
Aerobic stability, h												
Beans	61 ^b	66 ^b	79 ^b	65 ^b	100 ^b	83 ^b	210 ^{a5}	8.8	0.594	0.021	<0.001	<0.001
Total mixed ration	123 ^a	124 ^a	146 ^a	109 ^a	138 ^a	107 ^a	159 ^a	11.7	0.137	0.205	0.500	0.448

Values with the same letter in a row are not significantly different ($P > 0.05$) based on Tukey's test.

Table Footnotes

¹ For treatment abbreviations, please refer to [Table 1](#).

² SEM = Standard error of the mean.

³ Description of contrasts: Water: CON and LABmix vs. CON + w and LABmix + water; CON vs LAB: CON vs LABmix and LABLuke; CON vs chem: CON vs FA and Salt; LAB vs chem: LABmix and LABLuke vs FA and Salt.

⁴ In many cases, the results of the microbial counts were lower than the detection limit and conducting statistical analyses was not possible.

⁵ Difference between ambient and sample temperature did not reach 2 degrees during the measuring period of 210 h.

The yeast and mould counts of the ensiled faba beans are shown in [Table 4](#). The counts were low, and all samples were observed to have less than 3.3 log CFU/g of moulds and less than 6.1 log CFU/g of yeasts. Actually, in many cases, the results of the microbial counts were lower than the detection limit of the plate count method (< 2 log CFU/g). Consequently, exact numbers could not be defined for all the samples and presenting the results as means or conducting statistical analyses was not possible.

The aerobic stability of sole beans was numerically shorter than that of TMR which included good quality grass silage ensiled with a formic acid based additive and barley grain (on average 95 and 129 h for beans and TMRs, respectively; [Table 4](#)). For sole beans, use of both LAB and Salt extended aerobic stability ($P < 0.05$). The differences between treatments were smaller and experimental error somewhat larger when TMR was used so that less statistically significant differences could be detected. Salt was especially effective in improving aerobic stability resulting in highest numerical values both in sole beans and as a TMR component. The sole beans treated with Salt did not heat over the total observation period of 210 h.

3.2 Vicin and convicine in faba beans

The concentrations of V + C in the beans prior to ensiling are presented in [Table 2](#) and those after ensiling in [Table 5](#). Vicine could not be found in the ensiled beans but on average 0.11 of the original convicine in the raw material was present after preservation. There were differences between treatments in the degradation of convicine during the in-silo fermentation. It seems that increased MC promoted the degradation of convicine, while chemical additives decreased the degradation.

alt-text: Table 5

i The table layout displayed in this section is not how it will appear in the final version. The representation below is solely purposed for providing corrections to the table. To preview the actual presentation of the table, please view the Proof.

Bioactive compounds in crimped faba beans ensiled with different additives.

	Additive treatment ¹							SEM ²	Statistical significance of contrasts ³			
	CON	CON + w	LAB mix	LAB mix + w	LAB Luke	FA	Salt		Water	CON vs LAB	CON vs chem	LAB vs chem
Convicine												
Concentration, g/kg DM	0.97 ^c	0 ^e	1.00 ^{cb}	0.40 ^d	1.25 ^{cb}	2.32 ^a	1.33 ^b	0.070	<0.001	0.089	<0.001	<0.001
Remaining ⁴ , g/g	0.10 ^c	0 ^e	0.10 ^{cb}	0.04 ^d	0.13 ^{cb}	0.24 ^a	0.14 ^b	0.007	<0.001	0.089	<0.001	<0.001
Total PA ⁵												
Concentration, g/kg DM	7.63 ^{bc}	10.3 ^a	7.13 ^c	9.38 ^{ab}	9.33 ^{ab}	7.22 ^c	3.55 ^d	0.363	<0.001	0.197	<0.001	<0.001
Remaining, g/g	0.70 ^{bc}	0.94 ^a	0.65 ^c	0.86 ^{ab}	0.86 ^{ab}	0.66 ^c	0.33 ^d	0.033	<0.001	0.197	<0.001	<0.001
mDP ⁶	6.63 ^b	8.37 ^a	6.47 ^b	7.33 ^{ab}	7.25 ^{ab}	7.60 ^{ab}	6.87 ^b	0.232	<0.001	0.462	0.065	0.174
Proportion of PD ⁷ , g/g	0.689 ^a	0.730 ^a	0.683 ^{ab}	0.700 ^a	0.712 ^a	0.696 ^a	0.635 ^b	0.0091	0.030	0.500	0.623	0.014
Soluble PA ⁸												
Concentration, g/kg DM	0.336 ^b	0.399 ^{ab}	0.295 ^b	0.481 ^{ab}	0.540 ^a	0.369 ^{ab}	0 ^c	0.0390	0.007	0.110	0.007	<0.001
Remaining, g/g	0.11 ^b	0.13 ^{ab}	0.09 ^b	0.15 ^{ab}	0.17 ^a	0.12 ^{ab}	0 ^c	0.013	0.007	0.110	0.007	<0.001
mDP	6.04 ^a	6.12 ^a	5.98 ^a	6.47 ^a	6.76 ^a	6.29 ^a	0 ^b	2.241	0.2256	0.258	<0.001	<0.001
Proportion of PD, g/g	0.579 ^b	0.592 ^{ab}	0.573 ^b	0.597 ^{ab}	0.613 ^a	0.594 ^{ab}	0 ^c	0.0069	0.016	0.116	<0.001	<0.001

Values with the same letter in a row are not significantly different ($P > 0.05$) based on Tukey's test.

Table Footnotes

¹ For treatment abbreviations, please refer to Table 1.

² SEM = Standard error of the mean.

³ Description of contrasts: Water: CON and LABmix vs. CON + w and LABmix + water; CON vs LAB: CON vs LABmix and LABLuke; CON vs chem: CON vs FA and Salt; LAB vs chem: LABmix and LABLuke vs FA and Salt.

⁴ (Amount in raw material - amount after preservation) / amount in the raw material.

⁵ Total amount of proanthocyanidins (PA) as detected by thiolysis.

⁶ mDP = Mean degree of polymerization.

⁷ PD = Prodelphinidins.

⁸ The amount of soluble proanthocyanidins (PA) as detected by UPLC-MS/MS.

3.3 Proanthocyanidins in faba beans

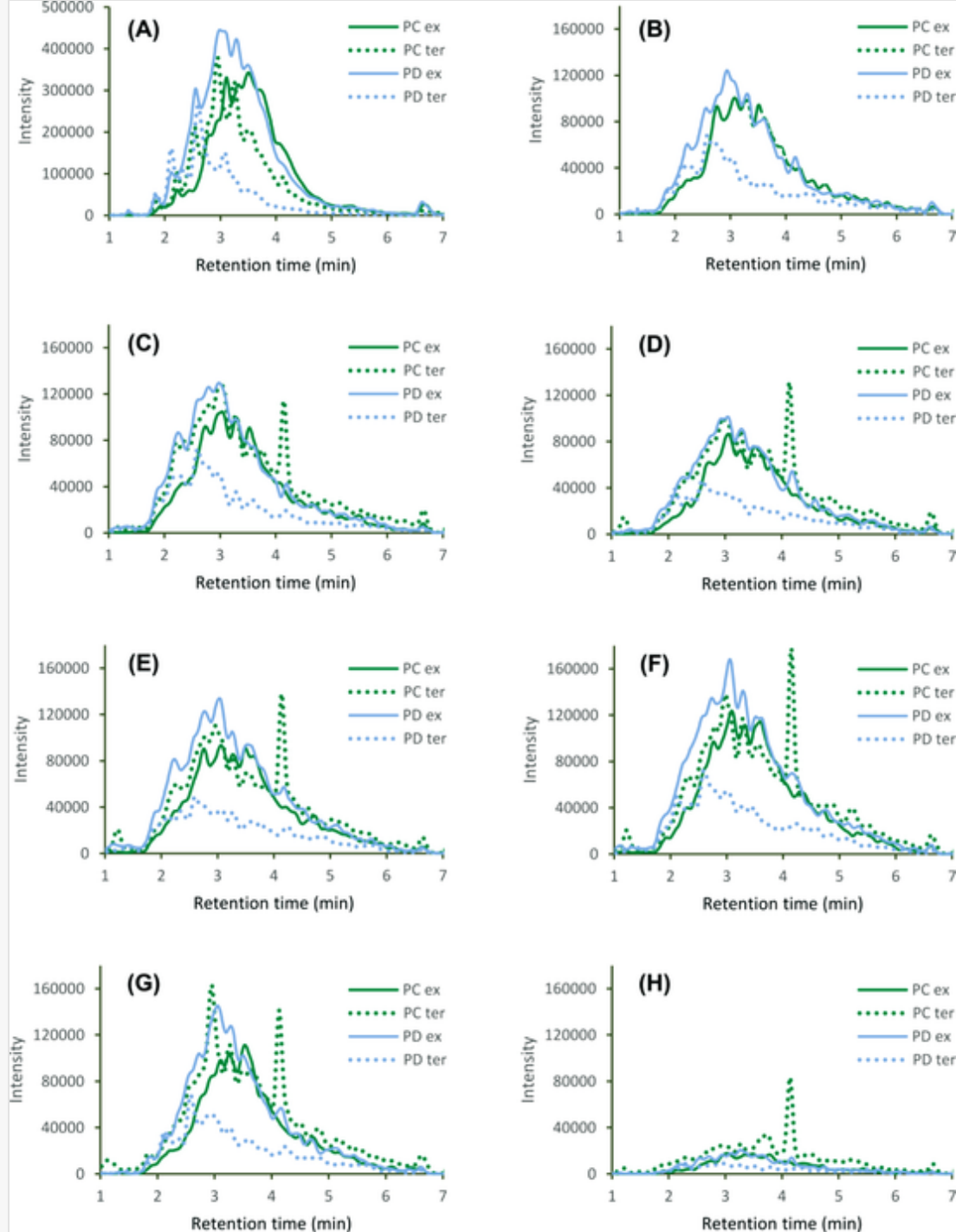
The PA composition of faba beans prior to ensiling is presented in Table 2 and the values post-ensiling in Table 5. Both total and soluble PA were reduced during the ensiling process. The average reduction of total PA was from 10.9 to 7.8 g/kg DM and for soluble PA from 3.12 to 0.35 g/kg DM indicating that insoluble PA are more resistant to reduction than soluble PA. The reduction was highest in the Salt treated faba bean where 0.323 of the original total PA were left whereas no traces of soluble PA were found after ensiling. In other treatments, the remaining amount of total PA ranged from 0.945 to 0.65, and for soluble PA from 0.09 to 0.17 in comparison to the control treatment.

The proportion of PD in PA was greater in total PA than in soluble PA (0.69 vs. 0.59 (excluding Salt with no observations), respectively). For total PA, the proportion of PD in total PA remained the same as in the fresh beans while for soluble PA, the proportion of PD decreased from 0.65 to 0.59. The effects of ensiling on tannin properties were minor except for Salt, which resulted in higher proportion of PD than other ensiled treatments and also the raw material hinting that proportionally more PC were degraded or modified into insoluble form in Salt treatment during the ensiling period.

The profiles of the tannin fingerprints (Fig. 1 A–H) were fairly similar in all analysed samples. The PC terminal unit traces showed a peak in ensiled samples (Fig. 1 B–H), which indicates that small PA oligomers might be more resistant to reduction, or they are degradation products of higher PA oligomers. The most significant changes were observed, when original faba bean (Fig. 1 A) was compared to the rest of the samples (Fig. 1 B–H). A clear reduction was observed in the amount of soluble PA. In addition, a significant reduction in PA quantity was observed when Salt treated faba bean was compared to the rest of the ensiled faba bean samples.

alt-text: Fig. 1

Fig. 1



MRM chromatograms of faba bean samples (A) before ensiling, (B) Control, (C) Control + water, (D) LABmix, (E) LABmix + water, (F) LABLuke, (G) Formic acid and (H) Salt. The abbreviations are as follows **PC ex**: procyanidin extension unit, **PC ter**: procyanidin terminal unit, **PD ex**: prodelfphinidin extension unit and **PD ter**: prodelfphinidin terminal unit (m/z 305 \rightarrow 125).

4 Discussion

4.1 Preservation quality of ensiled faba beans

Advisory guidelines recommend to ensile crimped grain at MC ranging from 300 to 450 g/kg. The MC content (438 g/kg) of ensiled beans was at the upper limit and the treatments that received additional water (492 g/kg) clearly exceeded it. The high MC resulted in intensive fermentation as water activity is a critical factor affecting it (McDonald et al., 1991). The effect of moisture on the intensity of fermentation of ensiled crimped barley grains was also demonstrated in a collection of farm samples (Olstorpe et al., 2010). They reported an increased microbial quality when MC increased from a relatively dry to a moderate level while excessively high moisture content compromised fermentation quality similarly as in the present data. This indicates a curvilinear effect of MC on crimped grain quality so that poor fermentation is the risk of low DM feeds while aerobic spoilage may occur in a relatively dry material. Practical experiences show that the most challenging MC for moulding of crimped grain is 230–250 g/kg. In the current study, increased moisture of CON was associated with numerically lower yeast counts of the ensiled beans, which is likely to be linked with the poor fermentation quality, e.g. higher concentrations of VFA and $\text{NH}_3\text{-N}$. As the MC obtained in the current study represented only a limited range of MC with high and very high values, we cannot determine the optimal MC based on the treatments currently used, but at least the higher MC was inferior to the lower MC used. A larger range in MC levels would be needed to define the optimum MC level.

All additive treatments improved the fermentation quality of faba beans compared with non-additive treated materials, which can be considered badly preserved based on several criteria. Elevated pH, $\text{NH}_3\text{-N}$ proportion, and ethanol and butyric acid concentrations confirm it, and indicate that using additives for this kind of material is recommended. Based on criteria developed for grass silages (Kaiser et al., 2004), only FA could be classified as “excellent” ($\text{NH}_3\text{-N} < 50$ g/kg N), while Salt, LABLuke and LABMix fall within “good”, LABmix + w and CON are “moderate” and CON + w “poor” ($\text{NH}_3\text{-N} > 150$ g/kg N).

With such a poor control treatment, responses to additive treatments are relatively easy to detect. The improvements with LAB were larger than detected in earlier experiments with crimped grain (Seppälä et al., 2015a) or whole-crop legumes (Seppälä et al., 2019) as raw material. Also, the concentrations of LAB were lower in the original faba beans compared to e.g. crimped barley in Seppälä et al. (2015b). The beans are covered by the pod while growing in the field and were exposed to environment just prior to ensiling during combine harvesting and crimping. This may have decreased the establishment of epiphytic LAB flora, which could explain the clearly improved preservation results obtained by LAB inoculation.

Aerobic stability is an essential quality criterion of ensiled feed materials. It is a result of the amount of aerobic microbes (typically yeasts and moulds) present in the material and presence of substances restricting their growth, either formed during the fermentation process or added in the form of additives. Aerobic stability will further be modified by substrate availability in the feed and environmental factors such as oxygen availability and temperature (Kung, 2005). The ability of heterofermentative LAB in improving the aerobic stability is based on acetic acid, which they produce during ensiling (Driehuis et al., 1999). In our case, the two

LAB inoculants resulted in similar acetic acid concentration, which is also in line with their similar aerobic stability. Aerobic stability can also be increased by silage additives containing e.g. propionic, acetic, benzoic or sorbic acids (Muck et al., 2018).

Sometimes badly preserved silages with high VFA and NH₃-N concentrations, which are strong antifungal agents, result in good aerobic stability of ensiled materials (Seppälä et al., 2016), but in the current data, all additive treatments improved the aerobic stability. Salt resulted in longest stability for both beans and TMR in this experiment similarly as in several trials using grass silage (Knicky and Spörndly, 2011; Seppälä et al., 2016).

We evaluated the aerobic stability of the sole beans and their role on the stability as a TMR component, as they may not be directly comparable (Seppälä et al., 2016). In this case, the aerobic stability of the beans was shorter than that of the TMRs with the exception of Salt. For other treatments, the TMR measurements were more obscure and ranking of the treatments varied between the two matrices. The higher stability of TMRs was probably related to the good hygienic quality and use of a formic acid based additive in the silage, which has been shown to increase the aerobic stability (Seppälä et al., 2016).

4.2 Vicin + convicin in ensiled faba beans

The V + C content of faba beans prior to ensiling, 15.5 g/kg DM, was high considering that Duc et al. (1999) reported the mean V + C content for the high V + C genotype ranging from 6 to 14 g/kg DM. The V + C contents in other Finnish materials reported by Koivunen (2016) were 9.2–10.6 g/kg DM and those found by Pulkkinen (2019) 7.3–11.2 g/kg DM. It remains unclear why the V + C content was higher in the current study. It can be however concluded that the variety used (Kontu) is a high V + C genotype.

The compounds V + C are heat stable glykopyranosides present throughout the seed so that they cannot easily be removed (Crépon et al., 2010). They are located in the cotyledons and are thermostable so they have proven difficult to be removed through technological processes, even high temperatures (Crépon et al., 2010). The means to manage with V + C include restrictions in the amounts of faba beans used in rations, and potential to breed faba bean varieties with low concentrations of V + C (Jezierny et al., 2010).

Fermentation may provide a method to decrease V + C concentrations. Short-term fermentations intended for human food preparation decreased V + C concentrations in faba beans (Coda et al., 2015; Pulkkinen et al., 2019), which is in line with our findings where vicin was totally degraded and only 0.11 of convicine remained after ensiling. In the experiment of Coda et al. (2015), 0.06 of the original vicine and 0.09 of convicine were detected after a 48 h fermentation, while Pulkkinen et al. (2019) reported residual proportions of 0.14 for vicine and 0.53 for convicine after a 24 h fermentation.

4.3 Proanthocyanidins in faba beans

We used the coloured-flowering faba bean variety Kontu, which is known to have a relatively high PA content. The analysed amount was 10.9 g PA/kg DM, which is in line with the PA content of 13.7 g/kg DM found by Koivunen (2016) for the same variety. The PA content of tannin containing cultivars (coloured-flowering cultivars) typically ranges from 5 to 10 g/kg DM (Duc et al., 1999). Crépon et al. (2010) reviewed that the mean PA content of tannin-free cultivars (white flowers) is as low as 0.1 g/kg DM. The amount of soluble PA in the faba bean seeds was 3.1 g/kg DM. Hence, majority of PA in faba bean seeds were in the insoluble form.

Gefrom et al. (2013) used a similar pilot scale ensiling approach as the current experiment. They preserved the faba beans without any additive or with a LAB inoculant, and detected reductions on PA and oligosaccharide contents during ensiling. The PA were degraded more than in the current experiment with LAB treatment. Only 0.63 of total phenols and 0.21 of PA relative to the original amount could be found in ensiled beans in their experiment while in our case, 0.72 of total PA and 0.11 of soluble PA remained in the ensiled material. It was noteworthy, that one of the additives, the Salt based product, was particularly effective in degrading both insoluble and soluble PA. The degradation of PA in the presence of salt was observed also by Kosińska et al. (2019), who showed that the stability of monomeric subunits of PA and small oligomers such as Procyanidin B2 decreased in saline solution.

In addition to degradation, the PA can also transform into insoluble form via complex formation with proteins and fibre during the ensiling process (Girard et al., 2018). The amount of soluble PD in the samples decreased slightly more than corresponding amount of PC. This suggests that PD were more prone to binding to proteins or fibre or alternatively degraded completely. The additional hydroxyl group of PD unit in comparison to PC unit might be the key structural feature from the activity point of view.

5 Conclusions

Ensiling very moist crimped faba beans without additives resulted in poor preservation quality but by using additives it could be improved. The LAB inoculants increased the lactic acid production and decreased pH of the beans, while the chemical additives, particularly the one based on formic acid, efficiently restricted fermentation. The formic acid based additive was most effective in decreasing the fermentation losses and protein degradation during the anaerobic phase, while Salt most effectively improved the aerobic stability.

The special interest in the current study was to find out how ensiling different types of additives affect the concentrations of bioactive compounds. Vicine could not be detected from ensiled beans and ensiling markedly reduced convicine content. This indicates that ensiling is a promising way to increase the use of faba beans in animal diets, in particularly for monogastric farm animals which are more susceptible to them than ruminants. There were also differences between the ensiling treatments in the convicine content. The LAB treated samples had lower levels of convicine compared to FA treated samples indicating that the greater microbial activity may be linked with the degradation of convicine. In addition, total and soluble proanthocyanids were reduced during ensiling in all treatments and particularly when using a salt based additive compared to the raw material.

Crimping and ensiling is an alternative preservation method for faba beans which eliminates the energy consumption and investments required for drying. In addition, benefits regarding the concentrations of bioactive compounds may be expected.

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Marketta Rinne: Conceptualization, Methodology, Investigation, Resources, Writing - original draft, Supervision, Project administration. **Milla M. Leppä:** Methodology, Investigation, Writing - original draft. **Kaisa Kuoppala:** Investigation, Writing - review & editing. **Erja Koivunen:** Investigation, Writing - review & editing. **Minna Kahala:** Investigation, Writing - review & editing. **Taina Jalava:** Methodology, Investigation, Writing - review & editing. **Juha-Pekka Salminen:** Conceptualization, Resources, Writing - review & editing, Supervision. **Katariina Manni:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Project administration.


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Appendix A Supplementary data [Instruction: No supplementary data was provided so this section should be deleted.]

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.anifeedsci.2020.114497>.

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 The corrections made in this section will be reviewed and approved by a journal production editor. The newly added/removed references and its citations will be reordered and rearranged by the production team.

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Highlights

- Fermentation quality of moist crimped faba beans improved using silage additives.
- Silage additives also improved the aerobic stability of crimped faba beans.
- Antinutritional vicin and convicin concentrations decreased during ensiling.
- Total and particularly soluble proanthocyanids also decreased during fermentation.

Appendix A Supplementary data

The following is Supplementary data to this article:

[Multimedia Component 1](#)

Queries and Answers

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