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Fermentation profile and dynamics of bacterial communities in vetch-oat ensiled with a novel spray-dried inoculant

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Abstract

This study aimed to examine and compare the effectiveness of a novel spray-dried inoculant and a commercial freeze-dried additive on the fermentation quality, aerobic stability and bacterial population of vetch-oat silage. An entirely random design used a 3 × 4 factorial arrangement of treatments, with and without lactic acid bacteria (LAB) inoculants and four fermentation periods. Physicochemical parameters, microbiological counts and 16S rRNA gene sequencing analysis on Nanopore MinION were conducted to characterize the ensiling process. Both LAB inoculants increased dry matter, crude protein, lactic, acetic and propionic acid contents, while reducing pH, neutral detergent fibre, ammonia nitrogen/total nitrogen and ethanol concentrations compared to the control group. Overall, the native inoculant decreased the cell load of coliforms, yeasts and moulds. In addition, bio-inoculants enhanced the aerobic stability of vetch-oat intercrops. After ensiling, bacterial alpha diversity decreased noticeably; inoculation reduced the number of observed operational taxonomic units and the Shannon and Simpson indices. Notably, the relative abundance of *Lactobacillus* in the control group was lower than in treated silages, while the relative values of *Staphylococcus* increased sharply in the uninoculated group. In conclusion, the native strains showed promise for usage as a bio-inoculant in the ensiling of vetch-oat at a mixture rate of 1:1, producing an immediate impact as well as a favourable effect on the post-opening phase. This represents the first report on 16S rRNA gene-based nanopore metagenomics applied to the bacterial analysis of vetch-oat silage, providing a microbiological insight where native and commercial strains dominate the natural epiphytic community.

Introduction

Forage preservation as silage is essential for the sustainable development of livestock systems. Certain annual legumes and winter grain cereals have historically been combined to promote crop productivity and optimize resource utilization (Kchaou *et al.*, 2022). As legume herbage, vetch species have a rich nutritional profile, a versatile growth habit and environmental benefits (Copani *et al.*, 2016). Yet, due to their high buffering capacity and low concentration of water-soluble carbohydrates (WSC), legumes can be difficult to ensile (McDonald *et al.*, 1991). In fact, Markovíc *et al.* (2022) stated that pure stands of vetch had a sugar content of about 47 g/kg DM and a buffer capacity value of 100 mEq/100 g DM. Moreover, vetch monocultures often yield poorly and hinder harvest because they normally sprawl on the soil surface (Wang *et al.*, 2021a). Caballero *et al.* (1995) reported that vetch-oat mixtures outyielded the vetch monocrop by more than 30% and attributed this improvement to enhanced resource utilization and improved soil fertility derived from the biological nitrogen fixation by the legume (Rivest *et al.*, 2013). On the other hand, small grain cereals, such as oats, provide both high yields and high WSC levels but produce forage crops with relatively low protein content. Hence, simultaneous cultivation of cereal forages with crops presents a viable strategy for enhancing the protein content of the overall ration. In particular, Lithourgidis *et al.* (2006) found that oat monocultures had a crude protein concentration around 80 g/kg DM, whereas vetch-oat mixtures exhibited a crude protein content between 90 and 120 g/kg DM. Based on the aforementioned advantages, the practice of intercropping oat with vetch emerges as a suitable and beneficial approach not only in Argentina but also in other countries.

The demand for silage additives, integral to sustainable agriculture, is on the rise due to the increasing consumption of silage across the globe. According to research and analysis by Mordor Intelligence, the European silage inoculant market is expected to attain a compound annual growth rate (CAGR) of 3.5% during the period 2023–2028, while the North American silage inoculant market is anticipated to achieve a CAGR of 4.8% during the same period

(Mordor Intelligence Report, 2023). This market is characterized by a high concentration, with a few major firms holding a significant market share, particularly in Argentina, where foreign-manufactured products dominate. In this context, most formulations are supplied as freeze-dried cultures or as water-soluble concentrates, while spray drying represents a profitable alternative for producing large amounts of dried cells in a continuous process (Wang *et al*., 2022a). The development of innovative products based on spray-dried native strains, better suited to the fermentative process of local forages, is expected to be a key factor in promoting the adoption of silage additives, potentially contributing to the enhancement of our economy.

Among fermentation stimulants, the addition of homofermentative lactic acid bacteria (LAB) is aimed at ensuring a rapid decrease in pH and lactic acid accumulation in the early stages of ensiling. This improves DM recovery, reduces proteolysis, and inhibits the proliferation of unwanted microorganisms such as the Enterobactericeae family, acetic acid bacteria, spore-forming organisms (*Bacillus* and *Clostridium*) and *Listeria* (Pahlow *et al*., 2003; Muck, 2013). Contrarily, heterofermentative LAB can produce more acetic and propionic acids, which inhibit the growth of yeasts and moulds, increasing the aerobic stability of the silage (Gomes *et al*., 2019).

Though previous studies have explored the impact of additives on the ensiling characteristics and aerobic stability of vetch-oat mixed silage (Chen *et al*., 2016; Markovíc *et al*., 2018), insufficient data are available on the dynamics of microbial populations and their interactions. Monitoring changes in bacterial communities and their progression in vetch-oat silages treated with LAB would be useful to thoroughly understand and improve the ensiling process. Metagenomic approaches involving direct DNA extraction and subsequent analyses are imperative for elucidating microbial communities and discerning functional changes associated with ensiling (Hisham *et al*., 2022). In this sense, targeted amplification of specific regions of the 16S rRNA gene (abbreviated 16S) followed by next-generation sequencing (NGS) offers the opportunity to better understand the processes underlying silage formation and estimate the effects of applied starter cultures on microbial population shifts. Unlike many NGS platforms that require substantial capital investments, extensive protocols and stationary equipment, newer sequencing platforms such as the MinION (Oxford Nanopore Technologies, Oxford, UK; ONT), constitute a simple, affordable and portable third-generation sequencing device with the capacity for generating long reads and data analyses in real time (Leggett and Clark, 2017).

In the current work, it is hypothesized that the addition of an autochthonous spray-dried LAB inoculant could enhance the physicochemical parameters, microbiological profile and aerobic stability of vetch-oat silages. Consequently, our objective was two-fold: (1) to elucidate and compare the impact of the native micro-encapsulated inoculant with a commercially available additive on the fermentation process and after silo opening, and (2) to acquire comprehensive insights into the bacterial communities within vetch-oat silages by utilizing 16S gene data.

Materials and methods

Native bacterial inoculant

Native strains *Lactiplantibacillus plantarum* Hv75 (formerly known as *Lactobacillus plantarum), Pediococcus acidilactici* 3903 *and Lentilactobacillus buchneri* B463 (formerly known as *Lactobacillus buchneri)* had previously been isolated and characterized *in vitro* as potential silage inoculants (Cristiani, 2015; Burns *et al*., 2018). These bacteria were stored at −70°C and kept in the culture collection of the National Institute of Agricultural Technology (INTA, Rafaela, Santa Fe, Argentina). When required, fresh overnight cultures were obtained by incubating the strains for 18 h at 37°C in an aerobic environment in MRS broth (Neogen, Lansing, MI, USA) after two successive transfers from frozen stocks.

Bacterial biomass was produced in a batch fermentation process and subsequently microencapsulated by spray-drying, as previously described by Blajman *et al*. (2022). Cell viability was assessed using plate counts (incubation for 72 h at 37°C, under aerobic conditions on MRS agar) and expressed as $\log_{10}$ cfu/ml before and after the spray-drying. Residual moisture and production yield were evaluated for the spray-dried powders. Residual moisture (% w/w) was determined in triplicate at 101 ± 1°C (FIL-IDF, 1993). Yield was expressed as the percentage of mass recovered after drying in relation to total solids before drying (Blajman *et al*., 2020a).

LAB-scale silage preparation and treatments

Hairy vetch (*Vicia villosa* var. Itacuá) and oat (*Avena strigosa* Schreb. var. Massa) were intercropped at a seeding rate of 20 kg/ha for each individual species within the mixture, in the experimental field of INTA (Lat.: 31°10′S; Long.: 61°32′W; Height above sea level: 100 m). The crops were sown on 29 March 2021, and harvested on 22 July 2021. Intercrops were harvested by cutting them at a height of approximately 5 cm above the ground, and field-wilted for 3 days to attain a target DM level of ∼496 g/kg. Then, the vetch-oat mixture underwent chopping into particles of approximately 20 mm in length using a precision forage harvester (Class Jaguar 950, Claas, Harsewinkel, Germany) and samples were taken to analyse its chemical composition. The proportion of vetch and oat in the intercrops, on a fresh matter (FM) basis, was 1:1, with oat at the soft-dough stage and vetch at the vegetative stage. A factorial design comprising a 3 × 4 arrangement of treatments was used, involving bacterial inoculants and four fermentation periods (0, 3, 30, 60 days), in an entirely randomized design, with three replicates assigned each treatment and sampling date. Vetch-oat forage was ensiled without microbial inoculant as control; with a native inoculant containing *L. plantarum* Hv75, *P. acidilactici* 3903, and *L. buchneri* B463; or with a commercial inoculant containing *Enterococcus faecium* M74, *L. plantarum* CH6072, and *Lactococcus lactis* SR3.54 (SiloSolve® MC, Chr. Hansen A/S, Hørsholm, Denmark). The plant material was ensiled in 36 polyethylene containers (10 l capacity) and compacted with 278 kg DM/m^3. Before undergoing ensiling, triplicate samples representing the fresh material were randomly obtained for subsequent chemical analyses.

Spray-dried *L. plantarum* Hv75, *P. acidilactici* 3903, and *L. buchneri* B463 were applied in the same proportion at a theoretical rate of 2×10^6 cfu/g of cropped forage. The LAB were resuspended in double distilled water and applied uniformly onto the mixture, using a hand sprayer (1.5 l) at the rate of 4 ml/kg of forage. The commercial inoculant was used according to manufacturer's recommendations; freeze-dried powders were diluted in double distilled water and applied at 2 ml/kg of fresh forage using a sprayer, resulting in a final expected dose of approximately 1.5×10^5 cfu/g of cropped forage. An equal amount of double distilled water was evenly sprayed onto the vetch-oat forage without

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microbial inoculant. Bucket silos were compacted, immediately sealed with fitted lids equipped with rubber O-rings, and stored at room temperature (24 ± 3°C) for 60 days. Triplicate micro-silos were opened at each time point during the anaerobic fermentation process and subsamples were collected for analyses of fermentation profile and microbiological counts.

Microbiological and physicochemical analyses

Microbiological analyses and pH determinations were conducted at 0, 3, 30 and 60 days of storage. For microbial population analysis, eight grams of each sample were mixed with 72 ml of 2% sodium citrate and homogenized for 5 min using a stomacher (Seward Stomacher 80, Lab system, London, UK). The suspension was serially diluted (10^{-2} to 10^{-7}), and 100-ml aliquots of each dilution were spread on agar media. Coliforms were counted by pour plating on Violet Red Bile Agar (Neogen, Lansing, MI, USA) after aerobic incubation at 35°C for 48 h on a heating incubator (DHP-9052 Arcano, Shanghai, China). Total LAB were counted on MRS agar (37°C, 72 h, aerobiosis), while yeasts and moulds were enumerated on Sabouraud Dextrose agar (Neogen, Lansing, MI, USA) supplemented with Chloramphenicol at 100 µg/ml (Genbiotech, Buenos Aires, Argentina) (25°C, 72 and 120 h, respectively, under aerobiosis). These chemical parameters were assessed for each replicate silo, according to standardized protocols (Jaurena, 2009): DM at 65°C, not corrected for volatile compounds loss (g/kg DM; Association of Official Analytical Chemists 1990, method numbers 130.15 and 167.03), crude protein (= Total Nitrogen × 6.25) determined by Kjeldahl (g/kg CP; AOAC 1998, method number 976.05), neutral detergent fibre analysed with added heat stable α-amylase and sodium sulphite (g/kg NDF; AOAC 1990, method number 942.05) by using an ANKOM fibre analyser (Model 220, Ankom Corp., Macedon, NY, USA), acid detergent fibre performed in ANKOM Fiber Analyzer 220 (g/kg ADF; AOAC 1990, method number 973.18), acid detergent lignin using sulphuric acid and ANKOM Fiber Analyzer 220 (g/kg ADL; AOAC 1990, method number 973.18), ether extract using the Soxhlet extraction method with petroleum-ether (g/kg EE; AOAC 1999, method number 920.39) and ammonia nitrogen/total nitrogen (NH_3-N/NT; Blain and Urtinette, 1954). The quantification of organic acids, carbohydrates and ethanol was conducted by HPLC at the same time points. Eight grams of each sample were mixed with 72 ml of sterile double distilled water, homogenized for 10 min in a stomacher (Seward Stomacher 80, Lab system, London, UK) and centrifuged (Sorvall RC-5C, Du Pont Company, Sorval Instruments, Wilmington, DE, USA) at 6000 × ***g*** and 4°C for 10 min. Chromatographic separation was carried out following the methodology described by Vénica *et al.* (2014).

Aerobic stability

To assess the strains' ability to control aerobic deterioration, silages underwent an aerobic stability test at room temperature during 4 days at the conclusion of the ensiling period. Two kilograms of thoroughly mixed silage from the central portion of each bucket silo (devoid of observable spoilage) were placed into clean plastic bags (inside an expanded polystyrene box covered with 2 layers of cheesecloth to prevent drying). Temperature sensors (Thermobutton, Akribis S.R.L., Buenos Aires, Argentina) were positioned at the centre of each silage mass, and temperature data were recorded every 30 min for 4 days (He *et al.*, 2020). Two supplementary sensors were positioned in the room to record ambient temperature. Aerobic stability was characterized as the duration (in hours) the silage remained stable before the temperature exceeded the ambient temperature by more than 2°C (Reich and Kung, 2010).

Metaprofiling of the bacterial community by nanopore sequencing

Bacteria DNA extraction

DNA isolation from silage samples was conducted in duplicate for the raw forage (day 0), after 30, and 60 days of fermentation, and for vetch-oat samples subjected to aerobic stability evaluation. Ten grams of each sample were placed into a sterile plastic bag followed by homogenization with 100 ml of PBS (pH 7.4) for 10 min using a stomacher (Seward Stomacher 80, Lab system, London, UK). The mixture underwent filtration using four layers of cheesecloth, and the resulting liquid was then centrifuged (5000 rpm, 20 min, 4°C) (Sorvall Legend X1R, ThermoFisher Scientific, Waltham, MA, USA). The precipitate was resuspended in 1 ml of sterile PBS and centrifuged (12 000 rpm, 10 min, 4°C) (Sorvall Legend X1R, ThermoFisher Scientific, Waltham, MA, USA) to collect the microbial pellet (Yang *et al.*, 2021). Total DNA was extracted using a ZymoBIOMICS™ DNA Miniprep kit (catalogue no. ZR D4300, Zymo Research Corp., Irvine, CA, USA) following the manufacturer's instructions. DNA integrity was confirmed through 1% agarose gel electrophoresis at constant voltage (100 V) for 1.5 h. DNA quantification was performed using a NanoDrop Lite spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a DeNovix-QFX fluorometer (DeNovix, Wilmington, DE, USA) with the DeNovix dsDNA Broad Range Assay kit (DeNovix, Wilmington, DE, USA). Total DNA samples were frozen at −20°C until further use.

16S rRNA gene sequencing

The PCR was performed using the 16S barcoding kit 1–24 (SQK-16S024; Oxford Nanopore Technologies, Oxford, UK). Each reaction was conducted in a final volume of 50 µl, including 12.5 µl of barcoded primers and 30 to 180 ng mould DNA. The Q5 Hot Start High-Fidelity 2 × Master Mix (New England Biolabs Inc., Ipswich, MA, USA) was used. Amplification was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with the following programme: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and elongation at 72°C for 90 s. A final extension was performed at 72°C for 2 min. All reactions were verified for successful amplification and contamination using gel electrophoresis (1% agarose gels stained with gel red, 100 V for 1.5 h). The PCR products were purified using the AMPure XP PCR Purification Kit (Beckman Coulter Genomics, Danvers, MA, USA) and quantified with a DeNovix-QFX fluorometer (DeNovix, Wilmington, DE, USA) using the Qubit 1 × dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Barcoded fragments were combined in equimolar amounts, totalling 200 ng, and rapid adaptors were added for library preparation. The mixture was placed onto R9.4 flow cells (FLO-MIN106; Oxford Nanopore Technologies, Oxford, UK; ONT) following the manufacturer's specifications. Sequencing was carried out using the MinION Mk1C instrument (Oxford Nanopore Technologies, Oxford, UK; ONT) and data acquisition was conducted using MINKNOW version 22.10.7.

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Bioinformatics analysis

All reads were subjected to basecalling using Guppy (v 6.1.5) with the super accurate model. The resulting reads were filtered, wherein reads with an average quality score over 12 and length between 1400 and 1700 bp were retained. Reads were subsequently clustered together using isONClust (Sahlin and Medvedev, 2020), with a minimum mapped fraction of read set at 0.9 and a minimum fraction of shared minimizers at 0.9. Also, singletons were discarded to prevent the presence of spurious operational taxonomic units (OTUs). A consensus sequence for each cluster was generated using the single-project optimization approach (SPOA v 4.0.9; Vaser *et al.*, 2017) and refined with Medaka (v 1.6.1) utilizing the super accurate model. Taxonomic classification of the resulting OTUs was achieved through the utilization of Kraken2 (v 2.0.8; Wood *et al.*, 2019) against the SILVA database (release 138.1; Quast *et al.*, 2012).

All 16S rRNA amplicon gene libraries from the current study have been deposited in the National Centre for Biotechnology Information Sequence Read Archive, under the BioProject accession number PRJNA1012711.

Statistical analysis

Silage fermentation was analysed to investigate the primary effects of days, inoculation, and their interactions in a fully randomized design model. Data processing was conducted using the statistical software InfoStat (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Córdoba, Argentina). The homogeneity of variance was assessed through the Levene Test, and normality was confirmed using the Kolmogorov–Smirnov Test. For certain variables that deviated from a normal distribution and/or homoscedasticity, the analysis was performed using the Generalized Linear Model (GLM) approach with a gamma distribution and a log link function. For variables exhibiting normal distribution, a two-way analysis of variance was used, followed by the Tukey post hoc test. Aerobic stability was evaluated using Kaplan–Meier analysis, which calculated time required to surpass the 2°C threshold above the ambient temperature.

R software v 4.2.2 (R Foundation, Vienna, Austria) was employed to assess bacterial diversity and composition through the treatments. Rarefaction curves were created with the 'rarecurve' function in the Vegan package in R. Alpha and beta diversity indices were computed using the Vegan (Oksanen *et al.*, 2012) and phyloseq (McMurdie and Holmes, 2013) packages. Beta diversity comparisons were determined through a permutational analysis of variance with the 'adonis' function (Martinez Arbizu, 2020). Differences in alpha diversity and bacterial composition were also estimated using a two-way analysis of variance or GLM for gamma distribution and a log link function.

Statistical significance was established at $P \leq 0.05$. Results are expressed as mean ± standard error of the mean (S.E.M.).

Results

Native bacterial inoculant

The biomass production of the multispecies inoculant was satisfactory, reaching 9.83 $\log_{10}$ cfu/ml during the stationary phase. The cell count of the suspension before spray-drying was 9.93 $\log_{10}$ cfu/ml, while the count after the dehydration process in the reconstituted powder was 9.09 $\log_{10}$ cfu/ml. Dehydration at these spray-drying conditions allowed to obtain a moisture content below <4% and a drying yield >70%.

Microbiological and physicochemical analyses

The nutrient composition of wilted substrates prior to ensiling is presented in Table 1. During fermentation, silage pH was influenced by inoculation and ensiling time, and there was an inoculation × time interaction ($P < 0.001$). The rate of pH decline was faster in the silage treated with the novel LAB and in the silage with the commercial inoculant than in the control silage. At day 30, pH values were 5.01, 4.06 and 4.37 for control, native and commercial inoculant bucket silos, respectively ($P < 0.001$). Meanwhile, at day 60, the pH levels recorded were 4.65, 4.09 and 4.22, respectively ($P < 0.001$). An interaction between inoculation and the day of conservation was observed for DM content, which was lower in the silage without additives at the end of the fermentation period ($P = 0.008$). Specifically, at day 60, DM levels dropped to 50.28, 47.04 and 44.34 g/kg for the commercial inoculant, the novel inoculant, and the control, respectively ($P < 0.05$). The application of additives determined higher amounts of CP and lower concentrations of NDF than the control silage ($P < 0.05$). Regarding NH_3-N, an inoculation × time of storage interaction existed because its content markedly increased with time in untreated vetch-oat silage ($P < 0.001$). The highest concentration of NH_3-N, 64.6 g/kg of total N, was observed at day 60 for the control group. In the silage containing the novel inoculant, the concentration was 23 g/kg of total N. Meanwhile, the content for the silage inoculated with the commercial bacteria was 42.9 g/kg of total N ($P < 0.001$). The variables ADF, ADL and EE were unaffected by the inoculants ($P > 0.05$). Although an effect inoculation × time was found for ADF and EE ($P < 0.05$), all groups behaved similarly over time, and the interactions observed were of no practical significance (Table 2).

Inoculation, ensiling time, and their interaction significantly influenced total LAB, yeasts, and moulds populations ($P < 0.05$). The count of LAB was notably greater ($P < 0.05$) in inoculated samples compared to uninoculated ones at day 30 of fermentation, but there was a reduction in total LAB counts with prolonged storage time. On the other hand, after 60 days, there was a sustained lower cell load of yeasts and moulds in native inoculant micro-silos compared to control and commercial inoculant samples ($P < 0.001$). The coliform counts were lower in the native inoculant forage ($P = 0.011$) (Table 2).

Ethanol, lactic, acetic and propionic acids were influenced by inoculation, ensiling time and their interaction ($P < 0.05$). The

Table 1. Chemical composition of vetch-oat forage before ensiling (g/kg of DM ± S.E.M.)

Item	Vetch-oat
Dry matter (g/kg)	497 ± 24.9
Crude protein	106 ± 7.7
Ash	91.7 ± 1.62
Neutral detergent fibre	483 ± 22.3
Acid detergent fibre	321 ± 9.59
Acid detergent lignin	435 ± 1.48
Ether extract	19.7 ± 0.61

Note: Values are means (±S.E.M.) of three repetitions.

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Table 2. Effect of time and inoculation on fermentation parameters and microbiological composition of vetch-oat silage (g/kg of DM ± S.E.M., unless otherwise stated)

	Time (days)				Inoculation treatment				*P*		
Item	0	3	30	60	Control	Native inoculant	Commercial inoculant	S.E.M.	*T*	*I*	*T* × *I*
pH	6.48	5.82	4.46	4.31	5.54	4.89	5.17	0.012	<0.001	<0.001	<0.001
Dry matter (g/kg)	496	487	477	472	463	487	498	2.5	<0.001	<0.001	0.008
Crude protein	103	101	103	105	100	105	103	0.2	0.215	0.023	0.444
Neutral detergent fibre	505	509	518	522	518	509	513	0.8	<0.001	0.041	0.113
Acid detergent fibre	336	316	336	330	334	326	328	3.2	0.002	0.273	<0.001
Acid detergent lignin	44.2	40.9	47.1	44.9	43.9	44.7	44.3	0.15	0.018	0.859	0.155
Ether extract	20.4	20.7	25.8	31.2	23.7	26	22.9	0.43	<0.001	0.257	0.006
Ammonia nitrogen (g/kg TN)	19.8	27.3	34.8	39.9	37.2	23.5	29.2	0.06	<0.001	<0.001	<0.001
Lactic acid	0.10	7.76	66.8	77.2	4.61	12.5	8.74	1.43	<0.001	<0.001	<0.001
Acetic acid	0.74	2.31	5.97	6.13	2.27	3.4	2.88	0.008	<0.001	<0.001	<0.001
Propionic acid	0.10	0.29	0.69	1.14	0.36	0.44	0.37	0.001	<0.001	0.006	0.004
Butyric acid	ND	ND	ND	ND	ND	ND	ND	–	–	–	–
Ethanol	1.10	2.84	5.71	3.92	3.35	2.96	2.45	0.019	<0.001	0.02	<0.001
Glucose	63.1	63.4	43.6	38.2	51.3	50	51.1	2.6	<0.001	0.892	0.130
Fructose	71.8	76.9	66.7	58.6	68.2	67.1	69.1	3.63	<0.001	0.847	0.154
Lactic acid bacteria (log_{10} cfu/g)	5.71	8.7	8.61	7.24	7.61	7.74	7.62	0.116	<0.001	<0.001	<0.001
Coliform (log_{10} cfu/g)	6.69	6.68	4.23	2.81	5.3	4.61	5.41	1.428	<0.001	0.011	0.068
Yeast and mould (log_{10} cfu/g)	6.07	6.09	5.67	4.86	5.78	5.33	5.92	0.404	<0.001	0.022	0.006

Abbreviations: TN, total nitrogen; cfu, colony-forming units; S.E.M., standard error of means; *T*, effect of time; *I*, effect of inoculation; *T* × *I*, interaction effect of time and inoculation; ND, not detected.
Note: Values are means (±S.E.M.) of three repetitions.

highest lactic acid concentration was observed at day 60, reaching 104.5 and 76.5 g/kg of DM for the silages inoculated with the novel and the commercial bacteria, respectively. In contrast, the lactic acid concentration for the control silage was 57.6 g/kg of DM at day 60 ($P < 0.05$). At day 30, the concentration of acetic acid was higher in the native and commercial inoculated silages (6.55 and 6.76 g/kg of DM) compared to the control (4.81 g/kg of DM) ($P < 0.05$). However, there was a subsequent decrease in acetic acid concentration in the silage treated with the commercial inoculant at the end of the fermentation period (5.34 g/kg of DM). The concentration of propionic acid was higher in the silage with the novel inoculant, followed by the silage with the commercial inoculant, reaching 1.57 and 1.07 g/kg of DM at day 60, respectively. Also, the concentration detected in the control silage was 0.871 g/kg of DM ($P < 0.05$). A higher ethanol content (7.82 g/kg of DM) was observed in the control compared to the treated groups at day 60 ($P < 0.001$). The concentration of ethanol in native and commercial inoculated silages increased from day 3 to a maximum value of 5.41 and 4.56 g/kg of DM at day 30, eventually decreasing to a concentration of 3.28 and 2.36 g/kg of DM at day 60, respectively. Butyric acid was not found in any of the samples (Table 2).

It should be noted that in the chromatograms with IR detector, two peaks with retention times similar to those of glucose and fructose were observed. It is crucial to emphasize that sucrose undergoes hydrolysis during the analysis because of the chromatographic conditions used, which involve high temperature and low pH. Consequently, the results for glucose and fructose are derived from the hydrolysis of sucrose. In the present study, no significant differences among groups in the level of glucose and fructose ($P > 0.05$) were observed (Table 2).

Figure 1 illustrates the temperatures in the silages during the aerobic stability test. The control silage remained stable for approximately 47 h, whereas the silages with the commercial and novel inoculants exhibited stability for at least 4 days, at which point the experiment was concluded. The highest temperature recorded in the control silage was 33.5°C. Towards the end of the assessment period, the temperature in the uninoculated silage had risen to 10°C above the ambient temperature.

Bacterial community analysis

The complete 16S was PCR amplified, sequenced, and analysed to trace the dynamic changes in the bacterial community composition of vetch-oat silages. The sequencing analysis on Nanopore MinION generated a total of 36 95 255 raw reads after basecalling, with an average of 153 969 reads per sample. After quality and length filtering, 21 60 827 sequence reads (58.04%) were retained with an average of 90 034 sequence reads per sample. High-quality reads were clustered into 174 OTUs. The rarefaction curves reached or nearly reached a plateau for most samples, indicating that the sequencing depth was sufficient to describe bacterial profiles during plant ensiling (figure not shown).

For beta diversity analysis, UniFrac matrices (both weighted and unweighted) were constructed. Non-metrix Multidimensional Scaling (NMDS) plots were used to visualize bacterial beta diversity patterns (Fig. 2). The main factor influencing the differentiation of the groups was time ($P < 0.001$) for both weighted and unweighted UniFrac measurements. Conversely, NMDS displayed no clustering of samples based on LAB inoculation ($P = 0.320$ for weighted, and $P = 0.103$ for unweighted distances). Interestingly, we observed two major clusters based on the days that had elapsed: bacterial communities of day 0 were clustered into one distinct group, whereas bacterial communities of day 30, day 60, and from the aerobic stability test were clustered into another group, in both naturally fermented and inoculated silages. The former cluster consisted of control and inoculated samples before fermentation.

The bacterial alpha diversity of vetch-oat silages is shown in Table 3. The number of OTUs detected ranged from 34 to 133. The Shannon index varied from 1.21 to 3.23, and the Simpson index ranged from 0.61 to 0.92. Alpha diversity indices were

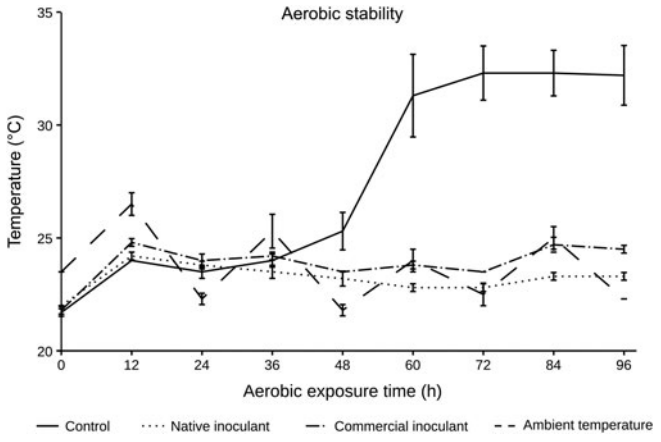

Figure 1. Effects of inoculants application on temperature of vetch-oat silage during aerobic exposure.

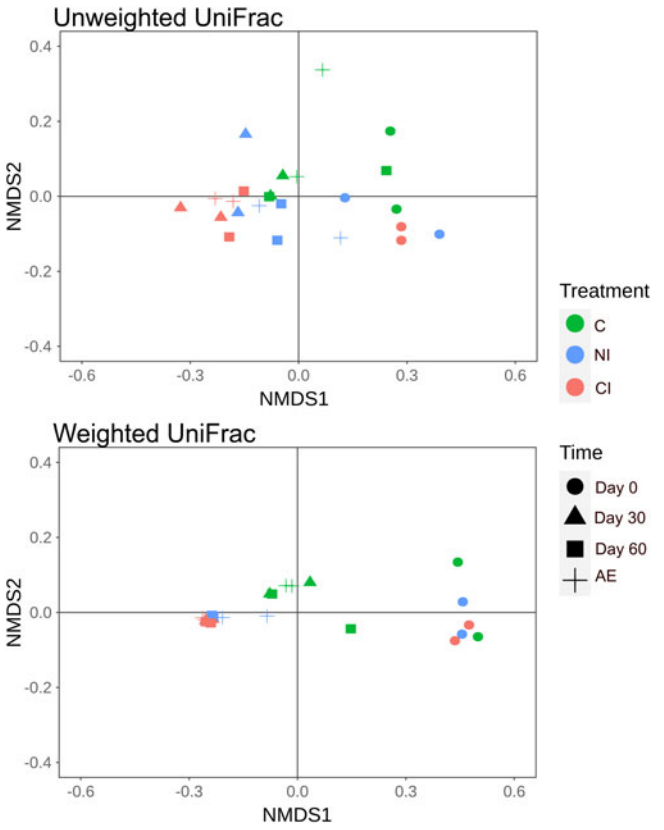

Figure 2. Beta diversity NMDS plots of Unifrac distances. The NMDS plots report the distances between different vetch-oat silages using unweighted and weighted Unifrac. Symbols represent different sampling times: day 0 (circles), day 30 (triangles), day 60 (squares) and aerobic exposure (AE, plus signs). Colours represent different treatments: control (C, green), native inoculant (NI, light blue) and commercial inoculant (CI, pink).

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Table 3. Alpha diversity analysis of vetch-oat silages bacterial communities: observed operational taxonomic units (OTUs), Shannon and Simpson indices

		Inoculation treatment						*P*		
		Control		Native inoculant		Commercial inoculant				
Item	Time	R1	R2	R1	R2	R1	R2	*T*	*I*	*T* × *I*
OTU	0	121	111	133	97	123	121	<0.001	0.018	0.312
	30	73	73	60	54	50	34			
	60	114	71	67	71	61	53			
	Aerobic exposure	90	78	99	66	51	45			
Shannon index	0	2.49	2.02	3.17	2.54	3.23	2.34	<0.001	<0.001	<0.001
	30	2.34	1.97	1.34	1.27	1.29	1.28			
	60	2.96	2.01	1.39	1.37	1.33	1.23			
	Aerobic exposure	2.31	2.25	1.88	1.35	1.28	1.21			
Simpson index	0	0.81	0.77	0.92	0.85	0.92	0.79	<0.001	<0.001	<0.001
	30	0.83	0.76	0.63	0.62	0.63	0.62			
	60	0.89	0.76	0.65	0.64	0.63	0.61			
	Aerobic exposure	0.82	0.81	0.71	0.63	0.63	0.61			

Abbreviations: R, biological replicate; OTUs, operational taxonomic units; *T*, effect of time; *I*, effect of inoculation; *T* × *I*, interaction effect of time and inoculation.

significantly higher at day 0 compared with other ensiling times and after aerobic exposure ($P < 0.001$). Besides, inoculation significantly reduced the number of observed OTUs, as well as the Shannon and Simpson indices ($P < 0.05$). In terms of the Shannon and Simpson diversity metrics, an inoculation × time interaction was identified ($P < 0.001$). The Shannon diversity index behaved in a similar manner in both supplemented silages until aerobic exposure, where a lower community diversity was identified in the silage treated with the commercial inoculant ($P = 0.012$). Conversely, the Simpson diversity parameter decreased up to day 30 and then remained relatively stable in the native and commercial inoculated samples ($P > 0.05$).

Figure 3 illustrates the dynamic changes in the bacterial community composition at the genus level, according to the

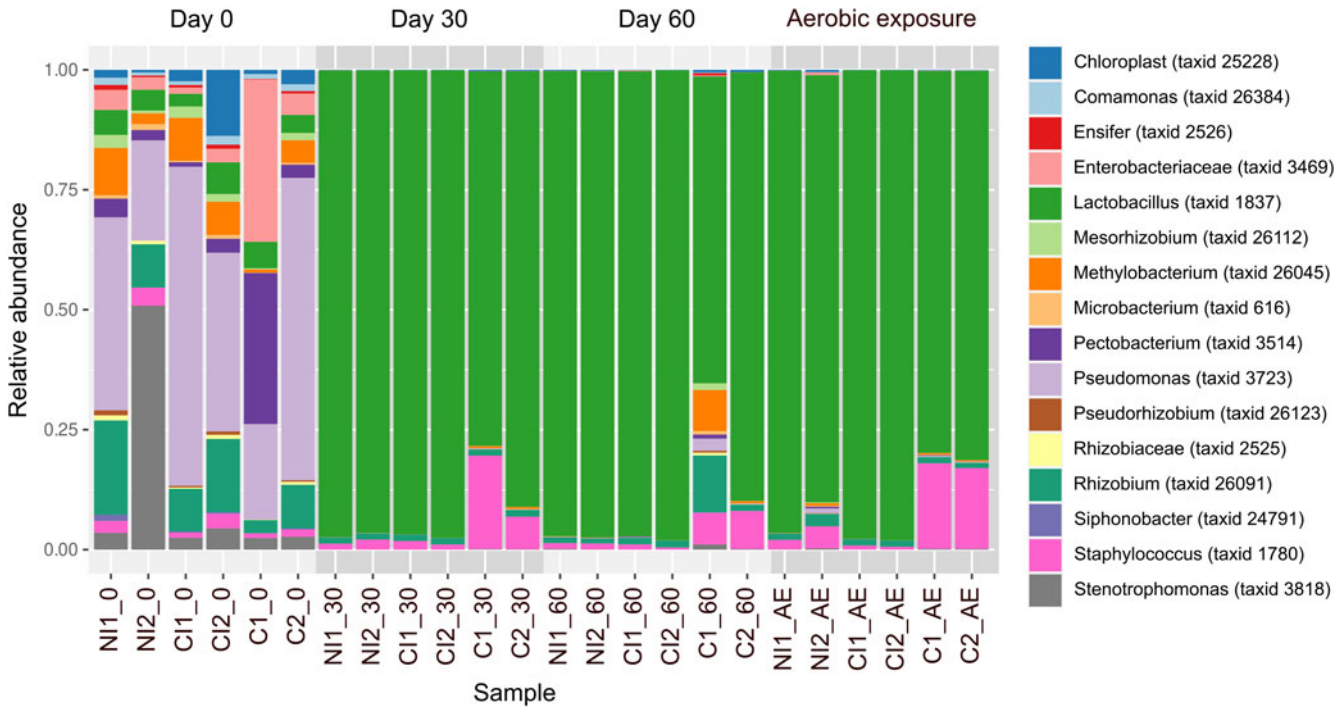

Figure 3. Effects of microbial inoculants on the relative abundance of bacterial community at the genus level in vetch-oat silages. The *x* – axis represents the name of the sample, the *y* – axis represents the abundance of the species. Coloured columns indicate different genera, and the length of the columns represents the proportion of the genera. C, control; NI, native inoculant; CI, commercial inoculant; AE, aerobic exposure.

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distribution of OTUs. In the fresh materials, the predominant genera present were *Pseudomonas* (40.6 ± 3.46%), followed by *Stenotrophomonas* (10.9 ± 7.62%), *Rhizobium* (10.6 ± 0.67%), *Enterobacteriaceae* (8.1 ± 4.59%) and *Pectobacterium* (7.3 ± 4.44%). The microbial community at day 0 also included *Methylobacterium*, *Lactobacillus*, *Chloroplast*, *Staphylococcus*, *Mesorhizobium* and *Comamonas* (1–5%). A smaller number of OTUs (<1% abundance) were associated with the genera *Ensifer*, *Microbacterium*, *Pseudorhizobium*, *Siphonobacter* and the *Rhizobiaceae* family (Fig. 3).

After 30 days of fermentation, the abundance of *Pseudomonas* decreased in all cases when compared to fresh forage ($P < 0.001$), whereas *Lactobacillus* became the predominant genus in silages ($P < 0.001$). Moreover, significant differences were observed between inoculated and control silages ($P < 0.001$), with the relative values of *Lactobacillus* estimated at 84.2 ± 0.06% in control samples, 96.8 ± 0.44% in native inoculant forage, and 97.1 ± 0.36% in commercial inoculant samples. The relative abundance of *Staphylococcus* for the novel and commercial inoculant (1.7 ± 0.42% and 1.4 ± 0.35%, respectively) was significantly lower than in the control sample (13.1 ± 6.31%) ($P < 0.001$). As shown in Fig. 3, at this sampling time, the bacterial community revealed the presence of *Rhizobium*, with no differences between groups ($P > 0.05$) and an average content of 1.3 ± 0.03%. In the control, the differentiated bacteria included the genus *Methylobacterium* (0.41 ± 0.00%), *Chloroplast* (0.26 ± 0.00%), and *Pseudomonas* (0.13 ± 0.00%) ($P < 0.05$), but at a low frequency (Fig. 3).

At day 60, the abundance of *Lactobacillus* in native and commercial inoculant silages (97.0 ± 0.11% and 97.5 ± 0.53%, respectively) consistently exceeded that in the control silage (76.1 ± 13.03%) ($P < 0.001$). The difference between control and inoculated groups was also statistically significant for *Staphylococcus* ($P < 0.001$). This genus comprised 7.2 ± 0.70% in untreated silages, 1.2 ± 0.01% in the native inoculant forage, and 0.8 ± 0.32% in commercial inoculant samples. A small number of OTUs were associated with genus *Rhizobium*, totalling 6.5 ± 5.17%, 1.1 ± 0.09% and 1.4 ± 0.04% of all OTUs, respectively. As observed, the amount was significantly lower in treated silages than in the control silage ($P < 0.001$). The control silage displayed a more complex bacterial community composition: *Methylobacterium* accounted for 4.4 ± 3.98%, and *Pseudomonas* for 1.3 ± 1.20% of the genera present, with $P < 0.05$ when the relative abundances were compared to inoculated silages. Furthermore, *Stenotrophomonas*, *Chloroplast*, *Microbacterium*, *Rhizobiaceae* and *Ensifer* had a minor but significant representation in the control group (0.4–1.4%) ($P < 0.05$). The genera *Mesorhizobium*, *Pectobacterium* and *Pseudorhizobium* were also detected, with no significant differences among groups ($P > 0.05$) (Fig. 3).

After 4 days of aerobic exposure, *Lactobacillus* predominated in all samples, covering 92.1 ± 4.17% and 97.8 ± 0.16% of the total sequences in the native and commercial inoculant forage, respectively. It should be noticed that the relative abundance of *Lactobacillus* in the control (80.0 ± 0.01%) was lower than that in the treated silages ($P < 0.001$), whereas the relative values of *Staphylococcus* increased sharply in the uninoculated group when exposed to air (17.2 ± 0.48%) ($P < 0.001$). The genus *Rhizobium* remained comparatively stable in all groups, accounted for an average of 1.5 ± 0.27% of the microbiota ($P > 0.05$). The genera *Pseudomonas* and *Methylobacterium* were significantly lower in the commercial treated silage ($P < 0.05$) (Fig. 3).

Discussion

Most researchers agree that intercrops use natural resources more efficiently than pure stands (Erol *et al.*, 2009; Baxevanos *et al.*, 2020). In mixed crops, companion cereals serve as structural support for vetch growth, increase light interception, and simplify mechanical harvesting, while the vetch component is intended to improve yield and protein content of the final forage (Wang *et al.*, 2021a). Further advantages gained include increase in soil organic matter, leading to better crops and soil nutrient status, improved water conservation, and reduced soil erosion (Kumar *et al.*, 2022). In this study, intercropped vetch-oat silages were inoculated with either a native or a commercial inoculant. Both LAB inoculants influenced ensiling characteristics, aerobic stability, and bacterial community composition.

Silage pH is one key aspect that affects the end-point of fermentation and the quality of ensiled forage. In this experiment, the low final pH in the additives-treated silages indicated good fermentation attributes (Chen *et al.*, 2016). After 60 days, the DM content was higher for both inoculated micro-silos, suggesting fewer nutrient losses caused by secondary fermentation. The feeding value regarding CP concentration improved with the addition of microbial inoculants. Similarly, both additives limited the formation of NH_3-N/NT compared to the control. This may be due to the addition of LAB inoculants during ensiling, which ensured faster acidification, consequently restricting protein degradation and deamination processes caused by plant enzymes or proteolytic microorganisms (Wróbel *et al.*, 2023). In high forage diets, the content of NDF has a major impact on DM intake and ration digestibility. As the NDF content in rations increases, the consumption of lactating dairy cattle and beef cattle decreases. In the current research, the NDF levels of the treated silages were lower than those in the untreated silage, potentially due to increased acid hydrolysis of hemicellulose. Conversely, the levels of ADF, ADL and EE in the inoculated silages were similar to the control. However, it is important to note that these findings are specific to silos that were opened within a 60-day fermentation period.

As expected, silages inoculated with LAB demonstrated higher fermentation efficiency in converting WSC into lactic acid compared to those relying on epiphytic LAB. This positive effect of additives on increasing lactic acid content was in line with the observed decline in pH. Conversely, in the control group, spontaneous fermentation generated more ethanol. The rise in ethanol levels was probably counterbalanced by the application of these bio-inoculants (Gallo *et al.*, 2021; Blajman *et al.*, 2022). In this research, ethanol concentrations decreased by day 60 in all groups. A plausible explanation is that certain microorganisms could utilize ethanol as carbon source. Schweiger *et al.* (2007) described that some acetic acid bacteria in silages could oxidize ethanol to acetic acid using membrane-bound dehydrogenases. Additionally, the possible volatilization of ethanol is another factor that may hinder its detection (Ávila *et al.*, 2009). Even in carefully sealed silos, there is some mass exchange between the silage and the environment. Moreover, some ethanol probably evaporated when silage samples were collected for analysis. This finding is in line with Pedroso *et al.* (2005), who noted that ethanol concentration suffered 50% reduction from the 120th to the 180th day in sugar cane silages, presumably due to the evaporation of alcohols. Both inoculated silages exhibited higher ratios of lactic /acetic acid compared to the control, indicating a shift towards homolactic fermentation. However, the addition of obligate

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heterofermentative *L. buchneri* B463 and facultative heterofermentative *L. plantarum* Hv75 and *P. acidilactici* 3903 resulted in higher levels of acetic and propionic acids in the silage containing the novel inoculant. This could be due to *L. buchneri's* capability to degrade lactic acid into acetic acid and 1,2-propanediol, subsequently generating propionic acid (Oude Elferink *et al*., 2001; Drouin *et al*., 2021). Moreover, facultative heterofermentative LAB has both aldolase and phosphoketolase enzymes. Species in this group produce equimolar amounts of lactic and acetic acids when the substrate is a pentose, and lactic acid, ethanol, and CO_2 with hexose substrates (Pessione, 2012). The main WSC in most silage fermentations are the hexoses fructose and glucose; however, alternative sugars for the silage microbiota may be pentoses, which are produced when matrix polysaccharides are hydrolysed (Rooke and Hatfield, 2003). Despite not being quantified in this study, xylose and arabinose can serve as important substrates for LAB in both forages (Wang *et al*., 2018; Liu *et al*., 2023). Regardless of the differences in the concentration of acetic and propionic acids between the treated and control samples, they fell within the expected values for vetch-oat silage (Chen *et al*., 2016; Ju *et al*., 2016).

Remarkably, increased total LAB numbers were observed in inoculated samples after 30 days of fermentation. This confirmed that the bio-inoculants established a beneficial symbiotic relationship with the host (Wang *et al*., 2018). By contrast, the count of coliforms, yeasts and moulds was significantly lower in samples with the autochthonous spray-dried inoculant. Reduced deleterious microbial growth in silage could be a consequence of successful *L. buchneri* development, leading to an increased production of acetic and propionic acids. These compounds exert inhibitory action against yeasts and moulds by penetrating their membrane in their undissociated form at the low pH of the silage, releasing hydrogen ions into the cytosol, causing the organism to expend ATP to maintain cellular homoeostasis, ultimately disrupting cellular metabolism (Romero *et al*., 2017). On the other hand, the decrease in coliform counts observed with our native inoculant may be attributed to the inhibitory pH of the medium, the enhanced antimicrobial activities of organic acids at low pH, or both (Queiroz *et al*., 2018).

It is important to emphasize that both biological inoculants provided substantial benefits by enhancing the aerobic stability of vetch-oat intercrops. The treated silages exhibited aerobic stability, as indicated by a temperature increase of no more than 2°C above the ambient temperature for 96 h. Regarding the native inoculant, enhanced aerobic stability can be elucidated by a reduced growth rate of undesirable microorganisms that initiate the deterioration process. Yeasts and moulds have long been recognized as responsible for the aerobic deterioration of silages, and it has been shown that silages containing more than 1×10^5 cfu/g of yeasts spoil as soon as oxygen becomes available (Mcdonald *et al*., 1991). In the case of the commercial formulation, the ability of the strains to compete and reduce harmful bacteria within the silage could be associated with prolonged stability. Even though yeasts, moulds and acetic acid bacteria are commonly associated with aerobic spoilage, other bacterial populations like enterobacteria, *Listeria* and *Bacillus* spp. can also proliferate in silages exposed to air (Li and Nishino, 2013; Queiroz *et al*., 2018).

The fermentation of silages occurs through the activity of a complex microbial community, influenced by environmental factors and management practices, including the application of inoculants (Borreani *et al*., 2018; Blajman *et al*., 2020b). In this study, MinION nanopore sequencing was used to metabarcode silage bacterial communities in vetch-oat silages. This approach allowed an in-depth description of bacterial diversity at a reasonable cost.

The phylogenetic composition, as assessed by the weighted and unweighted Unifrac metrics, indicated substantial temporal changes during the fermentation, but no clear differences within treatments. Conventionally, anaerobic fermentation begins with the activity of unwanted microorganisms present in raw materials, gradually being replaced by LAB. Therefore, the fresh material remained distinctly separated from the cluster of silages, consistent with similar studies (Franco *et al*., 2022). Alpha diversity estimates for both the fresh material and experimental silages indicated higher bacterial community diversity at day 0 for all evaluated indices. This aligns with findings reported by Xiao *et al*. (2022), who, when investigating oat silage, observed increased bacterial diversity in raw material compared to terminal silage. The higher diversity at the beginning may be explained by the inability of some epiphytic microorganisms to thrive in anaerobic and acidic silage environments (Franco *et al*., 2022). On the other hand, inoculation let to an abrupt reduction in the alpha diversity of the bacterial community compared to the control. This reduction is consistent with the results by Chen *et al*. (2020), who reported decreased bacterial diversity indices in oat silage inoculated with LAB. The decline in diversity could be attributed to the high abundance of *Lactobacillus* species, as a more dominant bacterial genus tends to result in a less diverse microbial community (Ogunade *et al*., 2017).

The analysis of bacterial abundance revealed that *Pseudomonas* was the predominant genus in fresh samples, followed by *Stenotrophomonas*, *Rhizobium*, *Enterobacteriaceae* and *Pectobacterium*. *Pseudomonas* and *Enterobacteriaceae* are considered undesirable in silage, as they may be linked to the production of biogenic amines, which can reduce the protein content and nutritional value (Dunière *et al*., 2013; Queiroz *et al*., 2018). *Stenotrophomonas*, in close association with plants, can utilize a variety of sugars and produce acid (Palleroni and Bradbury, 1993). These genera were also present in previous reports on microbial community composition of corn stover and corn-alfalfa mixtures at early stages of ensiling (Xu *et al*., 2017; Wang *et al*., 2021b). On the other hand, *Rhizobium* plays a beneficial role in nitrogen fixation for legumes, while *Pectobacterium* encompasses necrotrophic bacterial plant pathogens responsible for various diseases (Li *et al*., 2018). The functions of these genera during ensiling are not well-documented in the literature, and further research is needed to understand their roles (Zeng *et al*., 2020; Wang *et al*., 2023).

At day 30 and 60, the bacterial community changed and was heavily dominated by *Lactobacillus*. The dominance of LAB after ensiling aligns with previous research findings (Chen *et al*., 2020; Wang *et al*., 2022b; Xiao *et al*., 2022). Nevertheless, the abundance of this genus in untreated silage was lower than that in inoculated samples. These results suggested that both inoculants had the ability to affect the microbial ecology throughout the ensiling process. Uninoculated silage was characterized by the emergence of *Staphylococcus*. This observation is consistent with the results of Li and Nishino (2013), who detected *Staphylococcus sciuri* when Italian ryegrass silage spoiled, indicating that *Staphylococcus* can be detrimental to silage. Also, it is noteworthy that the genus composition of the control group exhibited diversity, with several taxa present at low relative abundances after 30 days of ensiling. These included *Chloroplast*, *Methylobacterium*

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and *Pseudomonas* -related OTUs. *Methylobacterium*, a strictly aerobic and neutrophilic genus, has been identified in studies of alfalfa (Ogunade *et al.*, 2018) and the fermentation of fresh tea leaves (Lin *et al.*, 2021). Its abundance has been positively correlated with pH value and reduced in silages treated with microbial additives (Ogunade *et al.*, 2018). By day 60, *Rhizobium*, *Methylobacterium*, *Pseudomonas* and *Stenotrophomonas* abundances were higher in the control samples. Moreover, the proportions of *Rhizobiaceae*, *Ensifer*, *Microbacterium* and *Chloroplast* became more prominent in the control group compared to the treated groups. The role of these genera in silage fermentation has not been extensively studied (Wang *et al.*, 2020; da Silva *et al.*, 2022; Feng *et al.*, 2022). In agricultural settings, most of the biologically fixed nitrogen derives from symbioses involving leguminous plants and bacteria from the *Rhizobiaceae* family. Within this family, *Ensifer* contains ecologically important nitrogen-fixing symbionts as well as nonsymbiotic species (Fagorzi *et al.*, 2020). Additionally, *Ensifer* has been demonstrated to enhance photosynthesis and the growth activity of plants (Toyama *et al.*, 2022). Regarding *Microbacterium*, members of this genus have been associated with an increase in nitrogen uptake, nitrogen accumulation and oat aerial biomass (Cerecetto *et al.*, 2023). Finally, the detected *Chloroplast* presumably belonged to the plant sample fraction, since plant chloroplast 16S rRNA genes exhibit similarities to bacterial 16S rRNA genes (Sun *et al.*, 2008), and certain methodologies have even been designed to prevent their amplification (Hanshew *et al.*, 2013).

Following a four-day period of exposure to aerobic conditions, the presence of *Lactobacillus* remained prominent in all groups. However, the inoculation of LAB resulted in a higher relative abundance of this genus compared to the control samples. Specific patterns were visible between treatments, with the most noticeable difference being the increased abundance of *Staphylococcus* in the untreated group. Infections due to *Staphylococcus* are of major importance to veterinary and human medicine (Bennett *et al.*, 2013). Contaminated silage can serve as a reservoir for pathogenic bacteria, posing risks to dairy cattle health, dairy product safety and quality, as well as overall animal and human well-being (Queiroz *et al.*, 2018).

Conclusions

Both the spray-dried native inoculant and the lyophilized commercial additive showed positive effects on the fermentation process and the post-opening phase of vetch-oat silages compared to the control. In this work, we introduced competitive strains that optimized the pH decline and lactate production, controlled the development of undesirable microorganisms, improved some nutritional and chemical silage quality parameters, and enhanced aerobic stability compared to the control group. Through metaprofiling, we observed that the native bio-inoculant contributed to the dominance of *Lactobacillus* over other bacteria and reduced the risk of potential pathogens from the *Staphylococcus* genus entering the food chain. In conclusion, the findings of this study support and promote the use of these novel strains in the ensiling of vetch-oat at a 1:1 mixture rate. Further research is required to assess their performance when inoculated in other forage crops.

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Authors' contributions. JEB, MCG and AFA formulated and designed the study. JEB, GMS, MFE and GHP conducted sample analysis and data gathering. JMI performed statistical analyses. MSL contributed to interpretation of the data. JEB wrote the article. MCG and AFA critically reviewed and revised the article.

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Competing interests. The authors state that they do not have any competing financial interests or personal relationships that might have influenced the work reported in this paper.

Ethical standards. Not applicable.

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