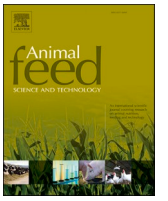
Animal Feed Science and Technology 304 (2023) 115748

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



Short communication

Short communication: Metabolization of benzoxazinoids during silage fermentation of maize and their effects on silage quality

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ARTICLE INFO

Keywords:
Benzoxazinoids
Maize
Silage
Fermentation
Metabolization

ABSTRACT

The metabolization of phytochemicals like benzoxazinoids (BXs) during ensiling and storage are largely unknown. By comparing wild-type and BX-deficient maize lines, the present study investigated the turnover of BXs during silage formation and their effects on silage. The fermentation pattern of maize silage was primarily related to the chemical composition of chopped forage and the mode of preservation. The presence of BXs in the plants affected dry matter loss and fermentation products. Concentrations of benzoxazinone glucosides declined within the first 3 days of ensiling, whereas respective benzoxazinone aglycons accumulated until 4 weeks of ensiling. Under long-term anaerobic conditions, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) represented the major fractions of BXs in maize silage, with 6-methoxy-1,3-benzoxazol-2-one (MBOA) and 1,3-benzoxazol-2-one (BOA) becoming the long-term end products of BX turnover in silage. Because benzoxazinoid derivatives are highly bioactive in other systems, they warrant more in-depth research for eventual effects on silage-feeding animals.

1. Introduction

Benzoxazinoids (BXs) represent an important group of plant chemicals present in cereals like maize, rye, and wheat, and known for their multifunctional roles in biological interactions among organisms and the environment (Wouters et al., 2016; Zhou et al., 2018). BXs are highly bioactive compounds being toxic against some insects, having allelopathic effects in plants, and anti-microbial

Abbreviations: ADF, acid detergent fiber; AAMPO, 2-acetamido-7-methoxyphenoxazin-3-one; AAPO, 2-acetamidophenoxazin-3-one; AMPO, 2-amino7-methoxyphenoxazin-3-one; APO, 2-aminophenoxazin-3-one; BOA, 1,3-benzoxazol-2-one; BX, benzoxazinoids; *bx1*, BX deficient bx1 mutant *bx1*::W22 line; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIM_2BOA, 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one; DM, dry matter; FM, fresh matter; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; HDMBOA, 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one; HDM_2BOA, 2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one; HMBOA, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HM_2BOA, 2-hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one; MBOA, 6-methoxy-1,3-benzoxazol-2-one; NIRS, near-infrared spectroscopy; NDF, neutral detergent fiber; W22, wild type W22 line; WSC, water soluble carbohydrates.

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https://doi.org/10.1016/j.anifeedsci.2023.115748
Received 5 May 2023; Received in revised form 3 August 2023; Accepted 4 August 2023
Available online 6 August 2023
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properties (Wouters et al., 2016; Zhou et al., 2018). Bioactive phytochemicals can affect the health of multiple environments and organisms, such as for instance when BXs are consumed by animals or humans (Schlaeppi et al., 2021). Putative health promoting properties of BXs such as immunoregulatory and anticancerogenic effects are suspected upon consumption in animals and humans (Adhikari et al., 2015). In a recent study, the BX profiles in maize silage were determined during aerobic exposure after opening (Gross et al., 2023).

As another step towards an understanding of phytochemical effects on animal health, the present study investigated BX metabolization during fermentation, and storage, as this remains largely unknown. Objectives aimed at understanding (i) whether BXs affect the fermentation process during silage making and in (ii) characterizing the chemical metabolization of these compounds during the silage process to determine the chemical compounds to which ultimately farm animals would be exposed to. To investigate the turnover of BXs and the effects of BXs on silage production, two maize lines, a BX-mutant line and its corresponding wild-type, were compared.

2. Material and methods

2.1. Plant resources

Two ensiling experiments were conducted immediately after maize harvest in two consecutive years (2018, 2019). For both ensiling experiments, maize (*Zea mays* L.) genotypes of the BX-producing inbreed line W22 and a Ds transposon insertion line *bx1*::W22 (referred to as *bx1*; Betsiashvili et al., 2015) were used. Although benzoxazinoneless 1 (BX1) is the main enzyme responsible for BX biosynthesis, *bx1* is not a null mutant and residual levels (< 10%) of BXs are detected because further maize enzymes can also convert indole-3-glycerolphosphate to indole, the precursor for BXs (Richter et al., 2021). Maize plants were cultivated under field conditions following conventional Swiss farming practices with usage of agrochemicals (ammonium nitrate fertilizer) and crop rotation. No herbicide treatments were applied and weed control was carried out manually and mechanically, respectively.

2.2. Experiment 1

In 2018, W22 and *bx1* plants were grown each in 10 replicate plots of 3 × 6 m (6 rows of maize, 0.5 m distance between rows) at an arable field at Agroscope in Changins, Switzerland (Plot 29 f, 2018; 46°23′56.7″ N, 6°13′58.9″ E; 430 m a.s.l., date of seeding: April 26, 2018). The average temperature between May and August 2018 was at 19.6 °C, while the concomitant average (cumulative) precipitation was 74.5 mm (298 mm). Plants were randomly harvested (cut manually 10 cm above the ground) from the individual plots on August 30, 2018 (age of plants: 126 d). The entire maize plants were immediately transported in plastic bags to the research station of Agroscope in Posieux, Switzerland, where they were chopped into pieces with a particle length of 1–2 cm with a modified forage cutter (MEX GT, Poettinger, Grieskirchen, Austria). The chopped maize material was ensiled without additives in glass containers (approximately 800 g in 1.5 L volume) and covered airtight following the DLG recommendations for laboratory silage preparation (DLG TestService GmbH, 2018). Silos were stored in the dark at room temperature (20–21 °C). Dry matter (DM) losses were determined by weighing the silos on an electronic precision balance (PG8001; Mettler Toledo, Greifensee, Switzerland) at d 0, 0.5, 1, 1.5, 2, 3, 4, 5, 7, 14, 21, 28, 60, 90, 120, 150, and 180 of ensiling. In parallel to DM loss determination at all scheduled timepoints, 2 silos per genotype were destructively opened, and individual (for pH measurements) and pooled subsamples (for BX analysis) were stored at −20 °C and −80 °C until analysis, respectively.

2.3. Experiment 2

In 2019, W22 and *bx1* plants were grown in 10 alternating blocks of 12 rows each (row distance 0.75 m) on an area of 2.53 ha at Agroscope in Posieux, Switzerland (Plot 2, 2019; 46°46′4.7″ N, 7°6′18.7″ E; 650 m a.s.l., date of seeding: May 16, 2019). The average temperature between May and October 2019 was at 15.5 °C, while the concomitant average (cumulative) precipitation was 99.3 mm (596 mm). Plants were harvested and chopped by a self-propelled forage harvester (theoretical chop length: 11 mm) on October 17, 2019 (age of plants: 154 d). Chopped maize (approximately 120–150 g) was filled without additives in 20 × 30 cm bags made out of 3 layers of polyester and polyamide (110 μm, Prima Vista, Landi Schweiz AG, Dotzigen, Switzerland) and vacuum sealed with an automatic vacuum system (CASO FastVac 3000, Arnsberg, Germany). Silage bags were stored in the dark at room temperature. Five bags per maize genotype were opened following the same timeline as experiment 1. Dry matter loss was determined at all timepoints as described above. Samples were pooled per genotype and timepoint for the determination of silage pH and fermentation products (d 0, 1, 2, 4, 7, 14, 28, 60, 90, 120, and 180 of ensiling), whereas concentrations of BX were determined in individual replicates (n = 5 per genotype) at d 0, 1, 3, 5, and 28 of ensiling. After opening of the sealed silage bags, samples were frozen at −80 °C until analysis.

2.4. Analysis of nutrient composition, silage pH and fermentation products

Nutrient composition prior to ensiling was analyzed at the feed laboratory of Agroscope Posieux, Switzerland. Measurements were carried out in triplicates. Dry matter content was determined in chopped forage samples dried at 60 °C for 20 h and at 105 °C for additional 3 h. Dried subsamples were milled to pass a 1 mm sieve (Brabender, Duisburg, Germany). Contents of crude protein, acid detergent fiber (ADF), and neutral detergent fiber (NDF) were determined by NIRS as described by Ampuero Kragten and Wyss (2014). The contents of ash, starch and water soluble carbohydrates (WSC) were analyzed as described earlier (Heublein et al., 2017).

Silage pH in experiment 1 was determined following the description provided by Grosse Brinkhaus et al. (2017) with an electrode (No. 6.0202.110; Metrohm Schweiz AG, Zofingen, Switzerland) connected to an ion meter (pH/ionmeter 692; Metrohm Schweiz AG). The pH of silages in experiment 2 was measured using a standard pH-meter (model pH 7310 with pH-electrode Sentix 21, Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany, DIN EN 12176) in the laboratory of the ISF Schaumann Forschung GmbH (Wahlstedt, Germany). Fermentation products (lactic acid, acetic acid, ethanol) in experiment 2 were quantified by high performance liquid chromatography (HPLC) at ISF Schaumann Forschung GmbH (Wahlstedt, Germany) using respective internal

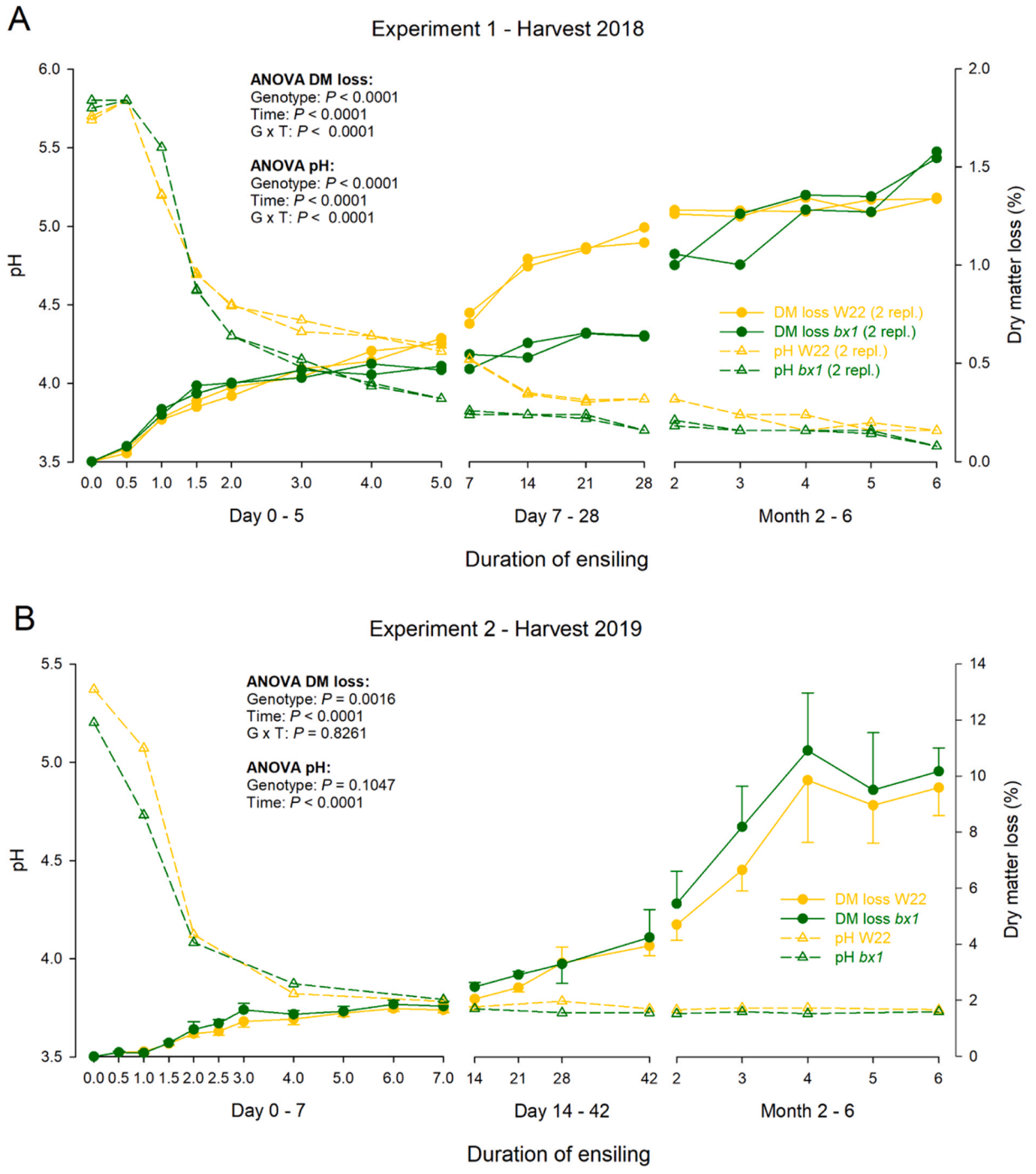

Fig. 1. Dry matter (DM) loss and silage pH during ensiling of two maize genotypes (W22: wild type W22 line, *bx1*: BX deficient mutant *bx1*::W22) in experiment 1 (panel A; maize harvest 2018) and experiment 2 (panel B; maize harvest 2019). Data are presented as individual replicates (A) and as mean values ± SEM (n = 5 per genotype; (B)).

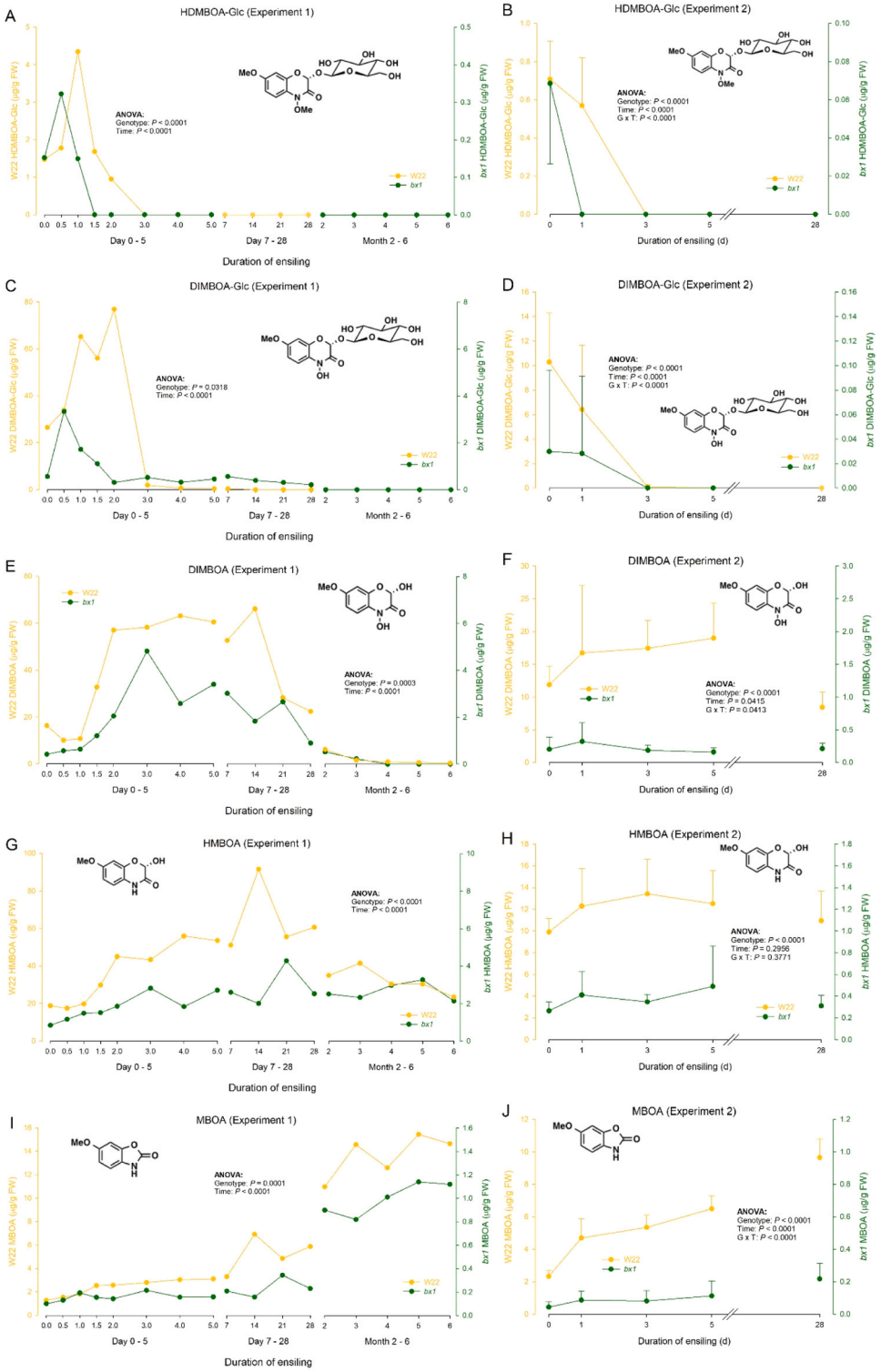

Fig. 2. Changes of benzoxazinoids during ensiling of two maize genotypes (W22: wild type W22 line, *bx1*: BX deficient bx1 mutant *bx1*::W22) in experiment 1 (panels A, C, E, G, I; maize harvest 2018) and experiment 2 (panels B, D, F, H, J; maize harvest 2019). Data are presented as mean values of pooled samples (experiment 1) and as mean values ± SEM (n = 5 per genotype; experiment 2).

standards. The HPLC system was equipped with a UV detector (Smartline 2500), RI detector (Smartline 2300), column thermostat (model Jetstream 2, all Bio Rad Laboratories, California, USA), and Aminex HPX-87H-column (300 × 7.8 mm, Bio Rad Laboratories, California, USA). Mobile phase was sulfuric acid 0.02 N with a flow rate of 0.6 mL/min.

2.5. BX extraction and analysis

After extraction of samples (100 ± 2.5 mg) at low temperature (MeOH, HPLC grade, 70% in MilliQ water + 0.1% Formic Acid, Optima grade), BX were analyzed by UHPLC coupled to a G2-XS QTOF mass spectrometer. Details on the extraction protocol, UHPLC analysis and standards are available in the supplementary material (Supplement Material and Methods).

2.6. Statistical analysis

Data presented in figures and tables are mean values ± SEM, except where denoted as SD. Data were checked for normal distribution and log-transformed in cases where normal distribution was not given. Statistical analyses were carried out using mixed models with the Tukey-Kramer post-hoc test adjusting for multiple comparisons in SAS (version 9.4; SAS Institute Inc., Cary, NC). Maize genotype, time (i.e., duration of ensiling), and the genotype × time interaction were used as fixed factors, while the individual replicates within each genotype were considered random. For the evaluation of chemical composition data, a mixed model with genotype, year, and the genotype × year interaction as fixed factors was used. Significance was assumed at $P < 0.05$.

3. Results and discussion

3.1. Chemical composition and fermentation pattern

The chemical composition of the two maize genotypes varied among years within and between genotypes (Supplementary Table S1). Maize harvested in experiment 1 had a greater DM content compared to maize harvested in experiment 2. In experiment 1, contents of ADF ($P < 0.001$), NDF ($P = 0.037$), and WSC ($P = 0.007$) were higher in *bx1* compared to W22, while contents of starch was lower in *bx1* compared to W22 ($P < 0.0001$). In experiment 2, the chemical composition of *bx1* and W22 was similar, i.e. no significant differences detected.

In both experiments, silage pH dropped rapidly within the first two days of ensiling and remained largely stable at pH-values < 4 until the end of ensiling (Fig. 1). In experiment 1, pH in *bx1* decreased more rapidly and were consistently lower compared to W22, which appears counterintuitive as BXs are weak acids (Cotton et al., 2019). However, the more rapid pH decline could be attributed to the greater WSC content in the bx1 silage providing more substrate for the lactic acid fermentation., whereas pH in experiment 2 did not differ between *bx1* and WT. Throughout all phases of silage fermentation and storage, DM losses occur that reduce both quantity and quality of the finally fed silage (Borreani et al., 2018). Up to 4% of total DM losses can be attributed to carbon dioxide production and is unavoidable by the nature of silage fermentation. Maize genotype was associated with DM loss in both experiments (experiment 1: *bx1* < WT; experiment 2: *bx1* > WT), but a greater DM loss was observed in experiment 2 ($P = 0.0016$; Fig. 1), which confirms the observations of Pitt et al. (1985), who showed that respiration rates are greater in silages with a lower DM content. In addition, differences in DM loss of experiments 1 and 2 are likely due to the differences in conservation methods and associated consequences for compaction (glass vs. vacuum bags). Packing density is much higher in glass containers at laboratory scale compared to vacuum sealed bags, where compaction of the ensiled crop diminishes with inflating bags due to gas production. Though, plastic films of PE coextruded with polyamides as used in the present study can be considered as suitable sealing methods as they show a rather high impermeability to oxygen and carbon dioxide (Borreani et al., 2018).

Fermentation products could be only determined in experiment 2. The similar pH development was reflected by similar concentrations of lactic acid in the silage of the two maize genotypes (Supplementary Table S2). Interestingly, ethanol levels were higher in *bx1* silage, while respective contents of acetic acid were lower in *bx1* compared to W22.

3.2. BX profiles during fermentation and storage

As expected, concentrations of BXs in both experiments were consistently more than 10 fold lower or even below the detection limits in the *bx1* mutant compared to W22 (Fig. 2; Supplementary Table S3). Despite the higher amounts of BXs in experiment 1, the time course of the BX transformation pattern during the ensiling of maize determined in experiment 1 could be confirmed in experiment 2. Within the first 3 days of ensiling, concentrations of the benzoxazinone glucosides (HDMBOA-Glc, DIMBOA-Glc, HMBOA-Glc, HM_2BOA-Glc, DIM_2BOA-Glc, and HDM_2BOA-Glc) decreased to concentrations close to or below the detection limit (Supplementary Fig. S3). After destruction of plant cells the stable BX glucosides, stored in the vacuole, are consequently hydrolyzed by β-glucosidases to reactive BX aglycons (Wouters et al., 2016). Concomitantly with the decline of the glucosides the aglycons (DIMBOA, and HMBOA) increased after approximately 1 day of ensiling, reaching a plateau between days 2 and 21 of ensiling, and declined thereafter.

In nature, herbivorous insects initiate this metabolic cascade and the bioactive BX function as part of the plant's defense. During ensiling however, the release and transformation of BX from stable glucosides to their aglycon forms is mostly due to the mechanical disruption of maize during harvest (e.g., chopping, compaction). Furthermore, the fermentation process causes a degradation of cell structures and a decline of the pH-value. As changes of BX during fermentation of maize silage were not yet described, we compare our findings with observations derived from published research in sourdough fermentation of cereals such as rye. Similar to the processes

during ensiling, sourdough fermentation takes place under anaerobic conditions involving lactic acid bacteria at a low pH of 3.5–4.5, but unlike to maize silage, additionally yeasts are involved too (Vogelmann and Hertel, 2011; Koistinen and Hanhineva, 2017). Once the pH-value in the present silage experiments remained at a constant low level, BX glucoside contents diminished and concomitantly the aglycon levels increased. With increasing length of the silage storage period under anaerobic conditions, MBOA and BOA accumulated, probably being the stable end products of BX turnover. Similarly, DIBOA glycoside levels decrease, while BOA accumulate during the fermentation process of sourdough (Beckmann et al., 2013; Savolainen et al., 2015). In soils, metabolization of MBOA and BOA to azobenzenes and aminophenoxazinones (e.g., APO, AAPO, AMPO, and AAMPO) occurs (Macías et al., 2005). Additional BXs, BX derivatives, and known BX degradation compounds (MBOA-Glc, HBOA-Glc. DIBOA-Glc, APO, AAPO, AMPO, and AAMPO) were not detected in the present silage experiments. In soil, it is assumed that bacteria or fungi, capable of cleaving the oxazolone ring of BOA, and oxic conditions are responsible for further BOA transformation (Fomsgaard et al., 2004). In contrast to soil, the silage microbiota is obviously not capable to further degrade BOA or MBOA under the prevailing environmental conditions (absence of oxygen, low pH-value).

4. Conclusions

This study showed that HMBOA and DIMBOA presented the major fractions of BX in maize silage, with MBOA and BOA becoming the end products of BX metabolization in silage. As some fermentation parameters differed between the two maize genotypes, BXs may additionally affect the fermentation characteristics of maize silage. Because both MBOA and BOA function as toxins against microbes and affect composition of microbiomes, it appears important to investigate these highly bioactive compounds more closely for their effects on the feeding animals and their gut microbiome.

Funding

The authors thank the Interfaculty Research Cooperation 'One Health' of the University of Bern for funding (www.onehealth.unibe.ch). The analytical support for the silage samples by ISF GmbH (Pinneberg, Germany) is highly appreciated. The work of CAMR was supported by the Swiss National Science Foundation (Grant no. 189071) and by the European Research Council (Grant no. 949595).

CRediT authorship contribution statement

Josef J. Gross: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Pierre Mateo, Klaus Schlaeppi, Ueli Wyss, Ewald Kramer, Dietmar Ramhold:** Investigation, Resources, Writing – review & editing. **Matthias Erb, Christelle A.M. Robert:** Resources, Writing – review & editing, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use any generative AI and AI-assisted technologies in the writing process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are very grateful to the Agroscope research stations Changins and Posieux for taking care of the field experiments and their help at maize harvest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2023.115748.

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