Animal Feed Science and Technology 199 (2015) 20–28

Animal Feed Science and Technology

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

Effect of invasive slug populations (*Arion vulgaris*) on grass silage. II: Microbiological quality and feed safety

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ARTICLE INFO

Keywords:
Slug invasion
Feed safety
Silage microbiology
Silage additives
Arion vulgaris
Arion lusitanicus

ABSTRACT

This study aimed to explore how invasive slug populations of *Arion vulgaris* influenced the microbiological quality and animal feed safety of grass silage, and the efficiency of silage additives and wilting to control the microbiology of slug contaminated crops. The effect of four slug contamination levels, including control, of a grass crop harvested for silage production, was evaluated in laboratory scale. The crop was wilted to two dry matter (DM) levels: low (253 g DM/kg) and high (372 g DM/kg). Adult slugs were applied to the low DM crop corresponding to 5 (low level), 10 (medium) and 20 (high level) seven-gram sized *Arion vulgaris* per m^2 in an assumed harvested regrowth yield of 2.5 ton DM/ha. For the high DM crop, slug weights corresponding to 6 (low level), 12 (medium) and 24 (high level) slugs per m^2 were applied. At low DM level, the effect of four additive treatments; control (C), inoculation with *Lactobacillus plantarum* (LP), a formic, propionic and benzoic acid mixture (ACID) and a chemical additive containing benzoic acid, $NaNO_2$, hexamethylenetetramine and propionic acid (CHEM) were tested. Slugs, slug feces, grass, soil and silages were analyzed for lactic acid bacteria (LAB), *Enterobacteriaceae*, *Listeria monocytogenes*, *Clostridium tyrobutyricum*, molds and yeasts by cultivation methods and *Clostridium botulinum* type C by real-time PCR analysis.

Increasing slug contamination reduced the microbial quality of silages by increasing *C. tyrobutyricum* levels at both silage DM levels. Only silages without slugs and silages treated with the nitrite containing additive CHEM had non-detectable mean levels of *C. tyrobutyricum*. Increasing slug contamination increased LAB enumerations in silages. No microbes of risk to human or animal health were detected in anaerobic silages even at the highest slug contamination.

DOI of the original article:http://dx.doi.org/10.1016/j.anifeedsci.2014.09.026.

Abbreviations: ACID, formic, propionic and benzoic acid mixture; C, control; CHEM, chemical additive with benzoic acid, $NaNO_2$, hexamethylenetetramine and propionic acid; DM, dry matter; LAB, lactic acid bacteria; LP, *Lactobacillus plantarum* inoculation.

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http://dx.doi.org/10.1016/j.anifeedsci.2014.09.024

1. Introduction

Slug invasions by *Arion vulgaris* (in name confusions also referred to as *Arion lusitanicus*) onto farmland including meadows for silage production, have recently become a concern in northern Europe. During wet summers, high densities of slugs are reported harvested intermixed with grass crops. Unspecified animal health disturbances together with unwillingness by cattle to consume silage are reported by Swedish and Norwegian farmers (Spörndly and Haaga, 2010; Tellevik H., Norwegian Agricultural Extension Service, pers. commun.). Populations of more than 50 slugs per m^2 have been reported from wildflower strips and meadows (Briner and Frank, 1998b). In Norway, high densities of *A. vulgaris* are mostly reported from the southern and western costal parts, but further spread is expected (Hatteland et al., 2013). Climatic changes with elevated winter temperatures and more rainfall can increase the survival rates (Slotsbo, 2012).

High quality silage depends on development of a favorable microflora under anaerobic conditions. Lactic acid bacteria (LAB) are desired microorganisms in silage and contribute to a rapidly declining pH, resulting in silage of high hygienic quality (Driehuis and Oude Elferink, 2000). A pH below 4.2 is considered an important key factor for inhibiting growth of contaminating microorganisms that may pose a risk to animal health or the human food chain (Eurofins, 2010; Driehuis, 2013). Soil and manure residues in harvested crop are well-known sources of microbial contamination. Invasive *A. vulgaris* slugs would be expected to pose the same risk, due to their close contact with soil and wide diet including all sorts of decaying organic matter as well as animal feces (Kozlowski, 2007). Slugs and snails are known to have a rich bacterial flora, both in numbers and species diversity (Elliott, 1970; Charrier et al., 2006; Wilkinson, 2011). In addition, carcass contamination of silage is known to increase the risk of botulism, a serious disease caused by the neurotoxin producing *Clostridium botulinum* (Lindström et al., 2010).

The non-pathogenic *Clostridium tyrobutyricum* is highly associated with poorly fermented silage. Due to tolerance of pH down to 4.2, and the ability of using lactic acid as a growth substrate, these butyric acid producing bacteria reduce both nutritional value and palatability of silages (Driehuis, 2013). Moreover, silage is shown to be the most important source for contamination of milk with spore-forming butyric acid producing bacteria. *C. tyrobutyricum* spores are not killed by pasteurization and cause late-blowing in semi-hard cheese, spoiling the product with excessive gas formation and off-flavors (Vissers et al., 2006).

Spörndly and Haaga (2010) studied slug contaminated silage and concluded that silage quality was not severely affected, possibly due to high levels of LAB detected in the slugs. They used juvenile slugs of about 0.64 g in the primary growth, in contamination levels relevant for early summer. Slugs increase their weight considerably from early to late summer, weighing around 10 g in August, with variation from 3 to 27 g within and between locations (Briner and Frank, 1998a). The second cut grass is reported to often have a slower fermentation rate, resulting in a higher silage pH (Fenlon, 1988). It was therefore important to investigate slug contaminated silage also from the regrowth harvest. The aim was to explore how invasive slug populations of *A. vulgaris* influenced the microbiological quality of grass silage, and whether this could cause a threat to animal feed safety. The efficiency of silage additives and wilting to control the microbiology of grass silages produced from highly slug contaminated crops was also investigated. Chemical composition of the same silages is reported and discussed elsewhere (Randby et al., 2014).

2. Materials and methods

2.1. Experimental design

The effect on silage quality of four levels, including control, of a slug contaminated grass crop, was evaluated in a laboratory scale ensiling study. The crop used was wilted to two different dry matter levels: low (253 g DM/kg) and high (372 g DM/kg). At low DM level, the effect of four additive treatments, including control, was tested in a 4×4 factorial arrangement with slug contamination using 3 replicates (altogether 48 silages). At high DM level, the four levels of slug contamination were tested with 3 replicates without any additive treatment (12 silages).

2.2. Crop for ensiling

The first regrowth of a fifth year organic meadow at the Norwegian University of Life Sciences, Ås, Norway was harvested. The sward was initially sown with timothy (*Phleum pratense*) and red clover (*Trifolium pratense*) and details in fertilization and harvesting conditions are described in Randby et al. (2014). The grass crop was ensiled on 14 August (low DM) and 15 August (high DM), and was not naturally contaminated by slugs.

2.3. Slugs for ensiling

A. vulgaris were collected late evenings and by night at about 15 °C on four locations in South-East Norway (Horten municipality). The locations consisted of woodlands, footpaths, roads and ditches, close to agriculture crops and meadows. A total of 672 slugs used for low DM silages, were collected on 12–13 and 13–14 of August and 343 slugs used for high DM silages on 14–15 August. For all slug handling, sterile gloves or tweezers were used. The slugs were kept in plastic boxes (36 cm × 27 cm × 17 cm) with lids containing vent holes, 53–61 slugs from the same location in each box. Average

weight of the slugs per box was calculated, and overall mean weight for slugs collected was 7.0 g (low DM) and 6.1 g (high DM silages). To prevent the slugs from emptying their intestine, they were offered fresh plant leaves (*Taraxacum officinale*, *Dactylis glomerata*, *Rumex longifolius*, *Trifolium repens* and *Trifolium pratense*) from the locations they were collected. Slugs were kept at 18–19 °C until ensiled.

For each replicate of silages, slugs were added evenly from the storage boxes and into a sterile bucket. Half of the slugs were cut by scissors, to simulate the harvesting process where slugs partly get mashed or cut. The slug mass was mixed by hand wearing sterile gloves. The amounts of slugs applied to the low DM crop were 138, 277 and 553 g fresh slug weight per kg crop DM corresponding to 5 (low level), 10 (medium) and 20 (high level) seven-gram sized mature *A. vulgaris* per m^2 in a harvested yield of 2.5 ton DM/ha. For the high DM crop, the applied slug weights were 167, 333 and 669 g/kg crop DM corresponding to 6 (low level), 12 (medium) and 24 (high level) seven-gram sized slugs per m^2 with the same harvested yield. The higher slug levels added to high than to low DM crop, was due to slightly lower DM level in wilted crop than assumed during preparation of the silages.

2.4. Silage additives

Four additive treatments, applied at 4 ml/kg of the fresh crop and slug mixture, were used for low DM silage: 1. Control (C; no additive applied). 2. Inoculation with two strains of *Lactobacillus plantarum* (LP; Kofasil Life, Addcon Europe GmbH, Bonn, Germany). The freeze-dried pre mixture of LP plus growth medium was activated with water at 20 °C for 48 h and applied giving, according to manufacturer, a minimum of 4×10^5 CFU/g of the fresh crop and slug mixture. 3. Acid-treatment (ACID); 57.8% formic acid, 12.0% propionic acid and 1.5% benzoic acid (GrasAAT Plus, Addcon Nordic AS, Porsgrunn, Norway) 4. Chemical treatment (CHEM); 11.0% benzoic acid, 10.4% $NaNO_2$, 6.9% hexamethylenetetramine and 3.3% propionic acid (Kofasil Ultra, Addcon Europe GmbH, Bonn, Germany).

2.5. Ensiling

Portions of crops for silages were weighed (1000 g for each portion of low DM silage and 900 g for each portion of high DM silage) and placed on plastic sheets where slugs and additives were added and mixed into each crop portion. After thoroughly mixing by hand, each portion was filled into plastic bags and sealed. Further details are given by Randby et al. (2014). Silages were stored at 15–20 °C.

2.6. Samples of soil, fresh crop, slugs and slug feces

A soil sample for microbiological analysis was withdrawn while the crop was wilted in the swath. Samples of crop and slug mass from each replicate, duplicate samples for low DM because of more silages, were representatively withdrawn during ensiling. Slug feces were sampled from the storage boxes of slugs for low DM silages, one sample from each collection night. All samples were kept in plastic bags with zip lock (emptied for air by hand) at 4–6 °C up to 24 h before analysis.

2.7. Samples of silages at opening and after aerobic exposure

Silages were opened and sampled in November after 84, 90 and 97 days of storage for replicate 1, 2 and 3 of low DM silage, respectively, and after 103 days for high DM silage. Samples were kept at 4–6 °C in up to 24 h followed by microbiological analysis. For unknown reasons, five low DM silages and two high DM silages were observed with slight damage of the plastic, which had caused small spots of molds in the silage. These silages were omitted from statistical analyses. No molds were observed in silages with undamaged plastic.

After opening, a portion of all silages was placed in perforated plastic bags in perforated polystyrene boxes at 20 °C for testing of aerobic stability during 2 weeks, further described in Randby et al. (2014). The second and third replicate of low DM silages without slugs (7 silages after omitting one silage) and with a high level of slugs (7 silages after omitting one silage), were sampled and analyzed for molds and yeasts after the 2 weeks of aerobic exposure.

2.8. Preparation of samples for bacteriology and pH measurement

From each sample, 25 g (or 1 g from slug feces) was diluted 1:10 in saline peptone water (1 g/l peptone, 8.5 g/l sodium chloride) in BagFilter®'s (Interscience, St Nom la Bretêche, France). The slug mass was finely chopped by scalpel prior to dilution. Diluted samples were homogenized by hand for 2–3 min before and after the samples were left to soak for 30 min at 300 rpm on an Orbital shaker (KL-2, Edmund Bühler, 7400 Tübingen, Germany). A 10-fold serial dilution was made from each sample and used for quantitative microbiological examinations.

The pH of soil, fresh crop, slugs and slug feces was measured using pH indicator strips (Merck KGaA, Darmstadt, Germany). For slugs, pH strips were dipped directly into the slug mass, while for the other dry samples 2 g were transferred to a Falcon tube, added 10 ml distilled water and kept on an orbital shaker for 30 min before measuring. The pH of silages was analyzed using a Termo Orion 420 A+ pH-meter with Orion 9107BN electrode (Thermo Scientific, Beverly, MA, USA).

2.9. Enumeration of lactic acid bacteria (LAB)

The NMKL culture method No.140 was followed (NMKL, 2007). Serial diluted samples of 0.1 ml were spread on MRS-aB agar (de Man Rogosa and Sharpe agar, Oxoid, Basingstoke, UK, with Amphotericin B, Sigma–Aldrich Inc., St. Louis, USA). Typical colonies were enumerated after 5 days of incubation at 25 ± 1 °C under anaerobic conditions.

2.10. Enumeration of Enterobacteriaceae

The NMKL culture method No. 144 was followed (NMKL, 2005a). One milliliter of serial diluted samples was transferred to Petri dishes and carefully mixed with 15 ml violet red-bile-glucose agar (Becton, Dickinson and company (BD), Sparks, USA), adjusted to 45 °C. The agar plates were left to solidify, followed by another thin layer of the same agar on top. After solidification of the top layer, plates were incubated at 37 ± 1 °C for 24 ± 2 h. Red to pink colonies were enumerated. Up to 5 colonies/sample were subcultivated on blood agar, incubated at 37 ± 1 °C for 24 ± 3 h and confirmed to be oxidase negative by test strips (BD). The method detected *Enterobacteriaceae* down to 1 $\log_{10}$ CFU/g.

2.11. Enumeration and detection of Listeria monocytogenes

The NMKL culture method No. 136 was followed (NMKL, 2010). For enumeration (only performed on silage samples), 0.1 ml of each 10-fold dilution was spread on the growth media of ALOA (Agar Listeria according to Ottaviani and Agosti, AES Chemunex, Bruze, France). In addition, and to improve the enumeration limit to 1 $\log_{10}$ CFU/g, 0.5 ml of the homogenized suspension was spread on each of two parallel plates of ALOA. All plates were incubated at 37 ± 1 °C for 24 and 48 ± 3 h.

For detection, 25 g (from soil, grass, slug mass and silage samples) or 1 g (slug feces) from each sample was added to Half-Fraser broth with additives (Oxoid), dilution rate 1:10, followed by incubation at 30 ± 1 °C for 24 ± 3 h. A second step of enrichment, transferring 0.1 ml of the primary enrichment culture to 10 ml Fraser broth (Oxoid), was performed. Both primary and secondary enrichment cultures were parallel plated on ALOA and RAPID' *L. mono* (Biorad, Hercules, USA) for 24 and 48 ± 3 h of incubation at 37 ± 1 °C.

For confirmation, up to five typical colonies were streaked out on blood agar and incubated at 37 ± 1 °C for 24 ± 3 h. Typical colonies showing haemolysis were confirmed as *L. monocytogenes* by test for catalase production and rhamnose but not xylose fermentation. Check for motility, CAMP reaction and Gram staining was performed on a selection of isolates.

2.12. Enumeration of C. tyrobutyricum

The culture method of Jonsson (1990) enumerating *C. tyrobutyricum* spores was followed. Five milliliter of the diluted and homogenized samples was heat treated for 10 min in water bath at 80 °C followed by a 10-fold serial dilution in saline peptone water. An amount of 0.1 ml of each dilution was spread onto Reinforced Clostrial Agar (RCA, Oxoid) added 0.005% neutral red and 200 ppm D-cycloserine (Sigma–Aldrich Inc.). To improve the enumeration limit, 0.5 ml of the heat treated sample was spread on RCA with additives. Plates were incubated for 7 days at 37 ± 1 °C under anaerobic conditions. Yellow colonies typical for *C. tyrobutyricum* were enumerated and subcultivated on RCA for 2–3 days under the same conditions. Colonies were tested for lactate dehydrogenase (LDH) production within 30 min of opening the anaerobic box. A bacterial density of 6 McFarland into a microtiter plate with reagents as described by Jonsson (1990), were used for the LDH test. A color change to dark red within 15–20 min at 37 ± 1 °C was regarded as a positive reaction as long as the negative control (*Clostridium bifermentans*) was clearly negative compared to the positive control of *C. tyrobutyricum* (ATCC-25755).

Catalase negative strains with lactate dehydrogenase production showing typical morphology on RCA with additives and blood agar were considered *C. tyrobutyricum*. Gram staining, aerobe incubation on blood agar, and confirmation by molecular methods was performed on one isolate from each positive sample. Molecular confirmations included a real-time PCR targeting the phosphotransacetylase gene for *C. tyrobutyricum* (Bassi et al., 2013), a nested PCR detecting *Clostridium* spp. commonly found in cheese by temporal temperature gradient gel electrophoresis (Le Bourhis et al., 2005) and a complete sequencing of the 16S rRNA gene.

2.13. Enumeration of molds and yeasts

The NMKL culture method No. 98 was followed (NMKL, 2005b). From each sample (except slug feces not analyzed), 40 g was diluted in saline peptone water (dilution rate 1:10) followed by 30 min soaking on an orbital shaker (Whirlmixer, VWR 1719). The diluted samples were homogenized for 2 min (Stomacher Lab-Blender 400, Seward Medical, West Sussex, UK) before a 10-fold serial dilution was made. Sample dilutions of 0.1 ml were spread on each of two parallel dichloran glycerol agar plates (DG18, Oxoid) followed by incubation in ventilated plastic bags at 25 ± 1 °C for 5–7 days. The method detected molds and yeasts down to 1.7 $\log_{10}$ CFU/g. Molds and yeasts were separately enumerated, using a binocular magnifier or microscope when needed.

2.14. Detection of C. botulinum type C by real-time PCR

One gram of each sample was added to falcon tubes with 9 ml of freshly made tryptose-peptone-glucose-yeast extract (TPGY) broth; 5% tryptone, BD, 0.5% proteose peptone, BD, 0.4% glucose, Merck, 2% yeast extract, BD, 0.1% starch, Merck, 0.1% L-cysteine-HCl and 0.14% $NaHCO_3$ (Skarin et al., 2010). The tubes were heated in water bath for 10 min at 60 °C prior to incubation at 37 ± 1 °C in 72 ± 3 h. One milliliter of the lower parts of the cultures was transferred to 9 ml freshly made TPGY broth and incubated overnight at 37 ± 1 °C. The cultures were frozen (−70 °C) in parallel Eppendorf tubes until a real-time PCR detecting the BoNTC gene covering a chimeric C/D sequence (Takeda et al., 2005) was performed (Lindberg et al., 2010). DNA was extracted using Qiagen BioSprint 15 Blood kit (Qiagen, Hilden, Germany) and KingFisher mL for magnetic based separation (Thermo, Helsinki, Finland) as previously described (Gismervik et al., 2014). The extracted DNA was analyzed with CFX96™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, USA) using the PCR mixture described in Gismervik et al. (2014). All samples and positive and negative controls were run in duplicate and both Cq values had to be below 40 to be considered as positive.

2.15. Statistical analyses

The microbiological composition of low DM silages were analyzed using the GLM procedure of SAS (release 9.3, 2002–2010; SAS Institute inc., Cary, NC, USA), by the following model: $Y_{ijk} = \mu + A_i + S_j + A_i \times S_j + R_k + e_{ijk}$, where μ = general mean, A_i = effect of additive treatment i, S_j = effect of slug level j, $A_i \times S_j$ = effect of interaction between additive i and slug level j, R_k = effect of replicate k and e_{ijk} is the random residual error. For high DM silages no additives were applied, so the A_i and $A_i \times S_j$ components were omitted from the model. During statistics, negative results were defined as half of the detection limit for the tests, due to the log value of zero is not definable. Mean values below the detection limit of the analyses are not numerically presented. The non-parametric two-sample Wilcoxon rank-sum (Mann–Whitney) test was used to compare microbe enumeration in grass and slugs (Stata version 12, StataCorp LP, Texas, USA). Results are reported as least square (LS) means with corresponding standard error of the mean (SEM) or as means with corresponding standard deviation (SD).

3. Results

3.1. Microbiology of fresh crop, slugs, slug feces and soil

Grass and slugs contained a high and equivalent bacterial load of LAB and *Enterobacteriacea*. *L. monocytogenes* was detected from all samples except soil (Table 1). Slugs contained a lower number of molds and yeasts (P<0.001) compared to grass. *C. botulinum* type C was not detected from any material tested (included silages).

3.2. Microbiology of silages

Low DM silages showed increasing *C. tyrobutyricum* with increasing slug contamination for C-, LP- and ACID-treated silages (Table 2). Silages treated with CHEM, and silages without slugs had *C. tyrobutyricum* means below the detection limit. The interaction between additive treatment and slug level for *C. tyrobutyricum* was significant, with CHEM as the additive treatment that inhibited *C. tyrobutyricum* (Table 2). *L. monocytogenes* was not detected in low DM silages, while *Enterobacteriaceae* and molds were below the detection limit for all samples except one (Table 2). An increase in LAB with increasing slug contamination was detected, and ACID-treated silages showed lower LAB compared to the other treatments (Table 2).

As for low DM silages, high DM silages also showed increase in *C. tyrobutyricum* and LAB with increasing slug levels (Table 3). High DM silages showed no or very low levels of *Enterobacteriaceae*, *L. monocytogenes* and molds.

Table 1
Microbial enumerations and pH in grass, slugs and soil by cultivation methods.

Material	*N*	Mean (±SD) $\log_{10}$ CFU/g						pH
		LAB	*Enterobacteriaceae*	*C. tyrobutyricum*	*L. monocytogenes*	Molds	Yeasts	
Low DM grass	6	8.7 (±0.1)	7.6 (±0.3)	<1.3	D	>5.1	6.7 (±0.0)	5.0
High DM grass	3	8.8 (±0.1)	7.3 (±0.3)	<1.3[a]	D	>5.1	6.1 (±0.1)[b]	5.0
Slugs (*A. vulgaris*)	9	8.6 (±0.1)	7.6 (±0.2)	<1.3	D	3.2 (±0.5)	3.5 (±0.8)	7.0
Slug feces	2	9.0 (±0.2)	8.8 (±0.4)	<1.3	D	NA	NA	NA
Soil	1	5.0	3.8	<1.3	ND	4.9	4.6	5.0

CFU, colony-forming units; SD, standard deviation; LAB, lactic acid bacteria; D/ND, detected/not detected in 25 g (or 1 g for slug feces); NA, not analyzed.
[a] One of the replicates was positive with a low value, but the mean value was below the detection limit of the test.
[b] *N* = 2 due to one replicate with overgrowth.

Table 2
Effects of additive treatment and level of slug contamination on microbiological composition of low DM grass silages.

Additive	Slug level	*N*	Mean $\log_{10}$ CFU/g						pH
			LAB	*Enterobacteriaceae*	*C. tyrobutyricum*	*L. monocytogenes*	Molds	Yeast	
C	None	3	7.4	<1	<1.3	ND	<1.7[e]	2.5	4.07
	Low	3	7.3	<1	2.2[f]	ND	<1.7[e]	2.5	4.19
	Med.	2	7.8	<1	3.2	ND	<1.7	1.7	4.44
	High	2	8.0	<1	4.5	ND	2.2 (±1.9)[h]	2.7	4.74
LP	None	2	7.2	<1	<1.3	ND	<1.7	3.9	4.03
	Low	2	7.5	<1	2.6	ND	<1.7	2.4	4.15
	Med.	3	7.4	<1	3.6[f]	ND	<1.7	2.3	4.29
	High	3	7.7	<1	4.6	ND	<1.7	<1.7	4.57
ACID	None	3	6.7	<1[e]	<1.3[f]	ND	<1.7	3.5	4.08
	Low	3	6.9	<1	2.4	ND	<1.7	<1.7[e]	4.11
	Med.	3	7.1	<1	<1.3[e,f]	ND	<1.7	<1.7	4.19
	High	3	7.5	<1	4.0[g]	ND	<1.7	<1.7[e]	4.28
CHEM	None	3	7.0	<1	<1.3	ND	<1.7[e]	2.6	4.20
	Low	2	7.5	<1	<1.3	ND	<1.7	<1.7	4.25
	Med.	3	7.7	<1	<1.3[f]	ND	<1.7	<1.7	4.28
	High	3	7.7	<1	<1.3[e]	ND	<1.7	<1.7	4.37
	SEM[i]		0.201		0.501			0.852	0.04
C		10[j]	7.6[a]		2.6[a]			2.4	4.36[a]
LP		10[j]	7.5[a]		2.8[a]			2.3	4.26[b]
ACID		12[j]	7.0[b]		2.0[a]			1.9	4.17[c]
CHEM		11[j]	7.5[a]		<1.3[b]			<1.7	4.28[b]
SEM			0.1		0.3			0.5	0.02
	None	11[j]	7.1[a]		<1.3[a]			3.1[a]	4.09[a]
	Low	10[j]	7.3[ab]		1.9[b]			1.8[ab]	4.17[b]
	Med.	11[j]	7.5[bc]		2.0[b]			<1.7[b]	4.30[c]
	High	11[j]	7.7[c]		3.6[c]			<1.7[b]	4.49[d]
	SEM		0.1		0.3			0.5	0.02
P additive			0.004		<0.001			0.31	<0.001
P slugs			0.002		<0.001			0.04	<0.001
P A × S			0.833		0.037			0.821	<0.001

C, Control; LP, Two strains of *Lactobacillus plantarum*; ACID, Formic, propionic, benzoic acid; CHEM, benzoic acid, $NaNO_2$, hexamethylenetetramine and propionic acid; CFU, colony forming units; LAB, lactic acid bacteria; ND, Not detected in 25 g.
[a, b, c, d] Means with different letters (a–d) in columns differ at P<0.05.
[e] One of the replicates was positive with a low value, but the mean value was below the detection limit.
[f] *N* = 2 (or 1[g]) due to agar plates with overgrowth.
[g] SEM is 0.9.
[h] Mean value (± standard deviation) where one of the two replicates were positive.
[i] SEM must be multiplied by 1.225 when *N* = 2.
[j] Due to agar plates with overgrowth, N for *C. tyrobutyricum* were, for additives: 9 (C and LP), 8 (ACID), 10 (CHEM), and for slug levels: 10 (None), 9 (Low), 8 (Med.) and 9 (High).

Table 3
Effect of level of slug contamination on the microbiology of high DM grass silages.

Slug level	*N*	Mean $\log_{10}$ CFU/g						pH
		LAB	*Enterobacteriaceae*	*C. tyrobutyricum*	*L. monocytogenes*	Molds	Yeasts	
None	3	7.0[a]	<1	<1.3[a]	ND	<1.7	3.2	4.20[a]
Low	3	7.3[b]	<1	1.4[a]	ND	<1.7[d]	<1.7[d]	4.36[ab]
Medium	2	7.4[b]	<1[d]	3.2[b]	ND	<1.7[d]	<1.7	4.42[b]
High	2	7.8[c]	<1	4.2[b]	ND	<1.7	2.4	4.81[c]
SEM[e]		0.04		0.045			0.60	0.048
P		<0.001		0.009			0.126	0.001

CFU, colony forming units; LAB, lactic acid bacteria; ND, Not detected in 25 g.
[a, b, c] Means with different letters (a–c) in columns differ at sign. level P<0.05.
[d] One of the replicates was positive with a low value, but the LS mean value was below the detection limit of the test.
[e] SEM must be multiplied by 1.225 for rows where *N* = 2.

3.3. Microbiology after aerobic exposure

Analysis of the second and third replicate of aerated low DM silages (14 silages) showed no significant difference in mean yeast levels for the slug contaminated silages (mean 6.8 ± SD 1.9 $\log_{10}$ CFU/g) compared to the uncontaminated (5.5 ± SD 2.5 $\log_{10}$ CFU/g). However, less molds were detected in silages contaminated with a high slug level (mean 4.3 $\log_{10}$ CFU/g)

compared to the silages without slug contamination (mean 7.9 $\log_{10}$ CFU/g), P=0.009, 2 weeks after opening. The CHEM-treated silages with high slug level showed a higher mold level (7.6 ± SD 0.47 $\log_{10}$ CFU/g) than the C- and LP-treated silages of high slug level (2.2 ± SD 0.34 $\log_{10}$ CFU/g).

Five low DM silages and two high DM silages were observed with slight damage of the plastic during ensiling. These silages had all high molds. The two silages with a high slug level, both without additives and one from each DM, showed the highest pH, mold levels and were the only ones containing high levels of *L. monocytogenes*, *C. tyrobutyricum* and *Enterobacteriaceae* (data not shown). All silages from damaged bags were omitted during statistical analyses.

4. Discussion

4.1. Microbiology of fresh crop versus slugs

Both invasive *A. vulgaris* slugs and grass used for silage production contained *L. monocytogenes* and high numbers of *Enterobacteriaceae* and LAB (Table 1). Spörndly and Haaga (2010), adding juvenile *A. vulgaris* to a primary growth harvest in May, detected more LAB in slugs (5.5 log CFU/g) than crop (2.8 log CFU/g) and used this as an explanation of slug- addition resembling the effect of an inoculant. The LAB levels were higher in the present study performed in August, which can be explained by the known LAB increase on grass during summer months (Henderson, 1993). The Swedish study showed lower microbial levels in the slugs (present study in parentheses), given in $\log_{10}$ CFU/g; *Enterobacteriaceae* 6.0 (7.6), molds <1.5 (3.2) and yeasts 2.7 (3.5). Adult slugs may contain a general higher level of bacteria due to a diet containing higher bacterial load in late summer.

4.2. Microbiological quality of slug contaminated silage

Slug contamination of crops gave a severe negative effect on the microbiological quality of silage by increasing *C. tyrobutyricum* known to decrease nutritional value and palatability (Driehuis, 2013). Although no pathogens were detected after strictly anaerobic fermentation, the pH was above the critical level of microbial inhibition (pH 4.2) with increasing slug level for both low and high DM silages (Tables 2 and 3). Application of neither LP nor ACID prevented sufficiently the increasing *C. tyrobutyricum* levels with increasing slug contamination (Table 2). Neither the use of high DM (372 g/kg) crop, that was expected to limit some growth of clostridia (McDonald et al., 1991; Driehuis and Oude Elferink, 2000), worked when crops were contaminated by slugs. Slugs contributed with a higher protein and water content compared to crop (Randby et al., 2014), which may have stimulated microbial growth. In the high DM silage, slugs may have formed micro-niches favoring *C. tyrobutyricum* enrichment. Only silages without slugs and silages treated with CHEM had mean *C. tyrobutyricum* levels below the limit of detection. Chemical additives containing nitrite, including combinations with hexamine, are known for efficient inhibition of clostridia (Woolford, 1975; Spoelstra, 1985; Knický and Lingvall, 2001).

Spörndly and Haaga (2010) detected no rise in *C. tyrobutyricum* in slug contaminated silages. Although twice as many slugs were added in the Swedish study, the present study had slug contamination levels up to five times as high on a basis of g slug per kg crop DM, since each slug was approximately ten times bigger. The Swedish study showed an increased lactic acid concentration and reduced pH in slug contaminated silage compared to controls. Opposite effects were observed in the present study, where pH increased and quality declined with increasing slug contamination. Butyric acid and ammonia nitrogen concentrations were above accepted levels for all C- and LP-treated silages with slug contamination, and ACID-treated silage with high slug level, which indicates that these silages had been subject to microbial deterioration (Randby et al., 2014). The increase of *C. tyrobutyricum* in C-, LP- and ACID-treated silages with increasing slug levels, may explain the lower levels of lactic acid together with the increasing butyric acid observed (Randby et al., 2014) because *C. tyrobutyricum* uses lactic acid as a substrate (Elferink et al., 2000). Also LAB numbers increased consistently with increasing slug level, and this could be due to higher LAB activity at higher pH or stimulating effects from slugs' moisture content (Tables 2 and 3). Yeast numbers were lower in slug contaminated compared to uncontaminated silages of low DM, although all numbers were low or moderate (Table 2). This can be explained by the considerably higher number of yeasts detected in grass compared to slugs (Table 1), giving an initial higher contamination level per gram in uncontaminated silage. Because yeasts are highly acid tolerant (McDonald et al., 1991), they might also have had a competitive advantage in non-contaminated silages with a lower pH compared to the contaminated silage.

4.3. C. tyrobutyricum levels and risk of milk contamination

By following the method of Jonsson (1990) there may have been some underestimation of *C. tyrobutyricum* due to swarming and overgrowth of other clostridia and bacilli at the lowest dilutions of these samples. In addition some *C. tyrobutyricum* strains are reported to show a weak LDH reaction (Jonsson, 1990), with the risk of misclassifying. However, the more relevant higher levels of *C. tyrobutyricum* were easier to enumerate due to less competitive flora at high dilutions. The specificity of the method was good, since all isolates tested were confirmed as *C. tyrobutyricum* by molecular methods.

Silage is considered the main source of butyric acid bacteria (BAB) contamination of milk, where even low levels of BAB are sufficient to spoil semi-hard cheeses (Vissers et al., 2006; Driehuis, 2013). Contamination levels of silage below 3 $\log_{10}$ BAB/g would provide high quality milk, given a basic pretreatment of the udder teats before milking. Levels of 3–5 $\log_{10}$ BAB/g silage

would need additional measures, while silage above 5 $\log_{10}$ BAB/g were not recommended to be fed milked cows (Vissers et al., 2006). According to these recommendations, slug contaminations of medium or high levels as in the present study will require extra efforts and economic consequences for all silages except the CHEM-treated, without considering the presence of other BAB than *C. tyrobutyricum*. An increased level of anaerobic *C. tyrobutyricum* can also be expected during the aerobic phase after opening of the silage, due to anaerobic niches (Jonsson, 1991; Driehuis, 2013), but this was not further investigated in the present study.

4.4. Microbiology after aerobic exposure

The mold and yeast analyses of 14 low DM silages exposed to air for 2 weeks showed less molds in highly slug contaminated silages compared to the silages without slugs. There was no significant difference in yeast levels, but all were high as expected after aerobic exposure (Henderson, 1993). Extensively butyric acid fermented silages with limited water soluble carbohydrates are known to have a higher aerobic stability than well fermented silage (McDonald et al., 1991). This may partly be due to the antimicrobial effect of butyric acid and other fatty acids produced in the silage. The highly slug contaminated silages were already spoiled at opening, and less molds compared to the non-contaminated silages during the aerobic phase would have little practical impact.

Anaerobic conditions are the keys to successful ensiling, but damage to the silage covering or the fact that plastic used in practice is not completely airtight are well-known problems (Driehuis and Oude Elferink, 2000). Results from the seven silages with damaged plastic covers indicated that the unwanted microbial growth during aerobic conditions was worsened when slugs were present, possibly because of the higher pH. Growth of *L. monocytogenes* in aerobically deteriorated silage, especially big bale silage, is associated with outbreaks and sporadic cases of listeriosis in cattle and sheep (Fenlon, 1988; Driehuis, 2013).

4.5. Animal health hazard by slug contaminated silage

The strictly anaerobic ensiling process managed to control growth of *Enterobacteriaceae*, *L. monocytogenes* and molds, also in silage with the highest level of slug contamination. *C. botulinum* type C was not detected in any silages, not surprisingly since the pH of all silages was below the minimum growth level of pH 5.1–5.4 (Lindström et al., 2010). A controlled laboratory study where *A. vulgaris* were fed with spores concluded that neither dead nor live slugs were shown to enrich *C. botulinum* type C, suggesting that slugs themselves are not a favorable growth substrate for *C. botulinum* type C (Gismervik et al., 2014). Based on these facts and given anaerobic ensiling conditions, there is so far little evidence of slugs constituting an animal health hazard due to infections or intoxication by botulinum C neurotoxin. Still, the high protein content of slugs, and high *C. tyrobutyricum* and ammonia-N levels detected in slug-contaminated silage, may imply risks of extensive protein breakdown producing biogenic amines. In addition to reducing palatability, amines may affect animal health and reduce animal performance (McDonald et al., 1991; Duniere et al., 2013). Farmers have experienced the massive slug invasions of meadows during rainy weather (Spörndly and Haaga, 2010). Such crop and unfavorable harvesting conditions could worsen the effects as here reported of slug contamination. The use of additives based on acids and chemicals may improve the feed safety.

5. Conclusion

Slug invasion by *A. vulgaris* into regrowth silage crops may give a severe negative effect on the microbiological quality of silages. Both in low and high DM silages spores of *C. tyrobutyricum* may increase to levels where milk may be contaminated and made unsuitable for cheese production. Only CHEM-treated silages were unaffected by the slugs, presumably due to its nitrite content that efficiently inhibited *C. tyrobutyricum* even at a high level of slug contamination. Provided anaerobic conditions, no microbial growth that can constitute a health risk for humans or animals was demonstrated in slug contaminated silage. However, there might be risks of extensive protein breakdown producing biogenic amines.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The Research Council of Norway, Foundation for Research Levy on Agricultural Products and Agricultural Agreement Research Fund and Tine are acknowledged for funding the project 199401 and Ph.D. studentships of K.G. The authors would like to thank Hallvard Gjøstein, Norwegian University of Life Sciences, for help with silage production, Rune Pedersen, Norwegian Veterinary Institute, for assistance during the late hours of bacterial analysis and Addcon for providing silage additives.

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