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EVALUATION OF SOME EGGSHELL PARAMETERS DURING THE EMBRYOGENESIS IN TURKEYS

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ABSTRACT

The aim of the experiment was to investigate the traits of eggshells of incubated turkey eggs, produced by turkeys at 34 and 46 weeks of age and to evaluate their effect on the development of embryos. This study was performed in the turkey farm of the Poultry and Rabbit Selection, Population Genetics and Technology unit at the Agricultural Institute – Stara Zagora in 2016. The eggs from turkeys of the North-Caucasian Bronze (NCB) breed were investigated. One hundred and twenty eggs were randomly collected from 34 and 46-week-old turkeys. The eggs were examined by the 9th day of incubation and before the transfer to the hatcher. The numbers of dead embryos and their eggshell parameters were registered. The shells of eggs of viable hatchlings were also analysed. Egg weight, shell weight, shell thickness (at sharp end, blunt end and equator), number of pores, egg surface area and shell density of eggs with embryos dead and hatched from 34 and 46-week-old turkeys were investigated. At 34 and 46 weeks of age, the weight of eggshells of eggs with early dead and late dead embryos was significantly higher (8.35 g at $p < 0.001$ and 8.14 g at $p < 0.01$) compared to the eggshell weight of hatched eggs - 7.27 g. There were no differences in the thickness of shells of dead and hatched eggs, laid by 34- and 46-week-old turkeys. The total number of pores on the shell surface of early dead eggs was lower when compared to the parameter of late dead eggs and hatched eggs from turkeys at 34 and 46 weeks of age. Eggs with dead embryos had thicker shells than hatched eggs in turkeys at the two studied ages (34 and 46 weeks of age). This requires further studies on the influence of the quality of the egg shell on the development of embryos.

Key words: turkey egg; shell; porosity; thickness; incubation

INTRODUCTION

The thickness and porosity of eggshells are among the most important factors influencing the hatchability of eggs (Tsarenko and Kurova, 1989; Narushin's and Romanov, 2002). For successful embryonic development and hatching, an optimum number of pores distributed properly on the eggshell surface, of specific length with regard to the adequate water and gas exchange, is necessary (Burton and Tullett, 1983; Christensen, 1983; Burton and Tullett, 1985).

The eggshell thickness and the amount of pores differ among the bird species. In general, the optimum number of pores per cm^2 of turkey eggs is 51-59 $n.cm^{-2}$, and shell thickness is about 0.37 mm. Pore diameter varies from 0.01 to 0.04 mm, with smaller base and larger surface opening (Dyadichkina *et al.*, 2014)

For chicken eggs, the total number of pores is about 8000 vs. 5000 for turkey eggs (Burtov *et al.*,1990). Water and gas exchange is directly related to egg porosity, so the latter is closely related to the intensity of the embryonic development. The hatchability of eggs with both low and high porosity is low, and viability of hatchlings is decreased (Chistyakova, 1988).

In a research on the quality of eggs from meat type chickens, Gafarova and Nuriev (2014) established that eggshells of studied eggs was 0.35 mm thick, the number of pores on the sharp end of eggs was from 6300 to 7800, whereas on the blunt end - from 11200 to 12500.

Christensen (1983) demonstrated that the age of layer had a substantial effect on eggshell porosity. Pores were more numerous in eggs laid during the first production week as compared to those laid during the 10th week and by the end of the production cycle.

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The author found a relationship between the spatial distribution of pores on eggshell surface and the good embryonic development of chicks and hatching, but not with the amount of pores. Epimahova (2010) also found a correlation between the number of pores on eggshells with the age of layers. In the beginning of lay, the number of pores in turkey eggs was by 25 to 50 per 1 cm^2 lower than those during peak production.

Besides the number of pores, thickness and weight are other eggshell features. Peeblesand Brake (1985) reported that broiler chicken eggs were the thickest also with greatest embryonic death rates. Gerzilov (2011) detected differences in eggshell thickness in the studied chicken genotypes. Sharlanov *et al.* (1988) reported increased hatchability from 67 to 85 % parallel to increase in turkey eggshell thickness from 0.44 to 0.50 mm. On the contrary, Andrews (1972) observed higher hatchability of turkey poults from eggs with thinner shells. Numerous authors (Kostova, 1974; Shatokhina, 1975; Kurova, 1986) reported higher embryonic death rate in eggs with relatively thick and thin shells compared to embryonic death rates of eggs with medium thickness. However, Malik *et al.* (2015) did not observe any statistically significant effect of eggshell thickness on the hatchability traits of broiler chicken eggs.

Eggshell quality is influenced by the age of the laying hens (Zabudskii, 2016). In turkey eggs (Ghane *et al.*, 2015), quail eggs (Genchev, 2014) and chicken eggs (Petrov *et al.*, 2011), the eggshell percentages were reported to decrease as the age of layers increased. The thickness of eggshells together with the shell membrane, as found out by Hristakieva *et al.* (2009), was 0.44 mm in eggs laid by 32-week-old turkeys and 0.43 mm in eggs from 44-week-old turkeys. Mróz *et al.* (2014) also noticed lower eggshell thicknesses as turkeys became older. Sharipkulova *et al.* (2012) reported higher eggshell thickness and density (from 1.0700 ± 0.0009 to 1.078 ± 0.2200) in 80-week-old Lohmann White layers compared to that of layers at 26 weeks of age.

The purpose of the present experiment was to investigate the traits of eggshells of incubated turkey eggs, produced by turkeys at 34 and 46 weeks of age and to evaluate their effects on the development of embryos.

MATERIAL AND METHODS

The experiments were performed in the stud turkey farm of the Poultry and Rabbit Selection, Population Genetics and Technologies unitat the Agricultural Institute – Stara Zagora in 2016.

The eggs from turkeys of the North-Caucasian Bronze (NCB) breed were investigated. The birds were reared in the stud farm on deep permanent litter at a density of 3 $birds.m^{-2}$. They were fed standard ration for turkey layers containing metabolizable energy - 2987.17 kcal, crude protein 18.10 %, calcium 2.8 7 %, available phosphorus 0.49 %. Average daily feed intake was 300 g.

One hundred and twenty eggs were randomly collected from 34-week-old turkeys. Every egg was numbered and weighed before the incubation, which took place in Optima incubators. The eggs were examined by the 9^{th} day of incubation and before the transfer to the hatcher. The numbers of dead embryos and their eggshell parameters were registered.

The shells of eggs of viable hatchlings were also analyzed. Similarly, eggs from turkeys at 46 weeks of age were studied.

The measurement of egg and shell weights was done with a precision of 0.01 g on a balance. The shell thickness was determined with a micrometer. The number of pores was evaluated with methylene blue staining (0.5 g 89 % dye in 1 L of 70 % ethanol) pipetted on shell surface, left to impregnate the pores for better visibility and staining (Board and Halls, 1973). The number of pores was counted under a 2.5 × magnifying glass in four 0.25 cm^2 squares, in each studied zone (sharp end, blunt end, equator). The average density of pores per 1 cm^2 was determined as mean arithmetic of four measurements per zone (Peebles and Brake, 1985).

The total number of pores on eggshell surface was calculated by multiplication of the average number from the three studied zones (sharp end, equator and blunt end) to the eggshell surface area.

The egg surface area (cm^2) - SA was calculated by the formula (Carter, 1975):

$SA\ (cm^2) = 3.9782 \times EW^{0.7056}$,

where, EW - egg weight (g)

The shelldensity ($mg.cm^{-3}$) SD was calculated by the formula (Curtis *et al*., 1985):

SD ($g.cm^{-3}$) - Shell weight (g) / [(surface area, cm^2) × (shellthickness, cm)]

Data were analysed using descriptive Statistics, t-Test: two-sample as suming equal variances using Excel 2003-ANOVA (Zhelyazkov and Tsvetanova, 2002).

RESULTS AND DISCUSSION

The incubation traits of eggs from turkeys at studied ages (Fig. 1) demonstrated lower fertility of eggs laid by 34-week-old birds (by 7.5 % compared to 46-week-olds). During the embryogenesis of eggs of older turkeys, the percentages of early dead and late dead embryos were higher (7.2 and 9.90 %, respectively). The hatchability of set eggs and fertile eggs was greater in eggs from younger turkeys (81.67 % and 66.67 % vs. 85 % and

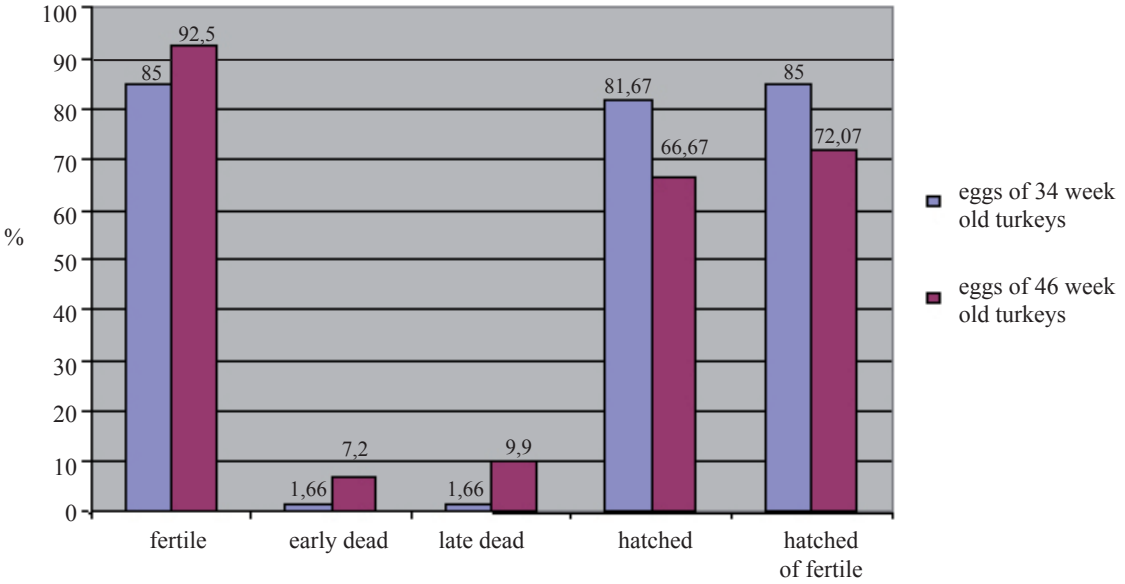

Fig. 1: Incubation traits of eggs from turkey hens at 34 and 46 weeks of age

72.07 %, resp. in eggs from 34- or 46-week-old hens).

Table 1 presents the values of studied shell parameters of incubated eggs from 34-week-old turkeys. The egg weights were the highest in eggs with embryos dead between incubation days 1 and 9 (88.23 g). At later incubation stages, there were eggs with lower weight observed (79.01 g). The eggs, from which viable poults were hatched, have average weight of 81.69 g. Eggshell weights in eggs with embryos dead during incubation were significantly higher ($p < 0.001$) than in eggs that hatched: 9.86 g in eggs with early dead embryos, 8.69 g in eggs with late dead embryos and 6.94 g in hatched eggs.

There were not significant differences in eggshell thickness between early dead and late dead eggs, as well as in hatched eggs, except for thickness measured in the equator of late dead eggs, which turned out to be significantly higher ($p < 0.01$): 0.40 mm compared to early dead and hatched eggs (0.36 and 0.36 mm,

Table 1: Eggshell parameters of incubated eggs from turkeys at 34 weeks of age (mean ± SD)

Parameters	Egg type		
	Early dead	Late dead	Hatched
Egg weight (g)	$88.23 \pm 1.400^{a***}$	79.01 ± 1.540^{b}	81.69 ± 0.390^{b}
Shell weight (g)	$9.86 \pm 0.510^{a***}$	$8.69 \pm 0.330^{a***}$	6.94 ± 0.050^{b}
Shell thickness (mm)			
Sharp end (SE)	0.36 ± 0.030	0.38 ± 0.020	0.39 ± 0.010
Equator (E)	0.36 ± 0.050^{a}	$0.40 \pm 0^{b**}$	0.36 ± 0.010^{a}
Blunt end (BE)	0.39 ± 0.050	0.37 ± 0.010	0.37 ± 0.010
Average shell thickness (mm)	0.37 ± 0.010	0.38 ± 0.003	0.37 ± 0.008
Number of pores SE ($n.cm^{-2}$)	23.50 ± 2.000	23.00 ± 7.000	31.15 ± 4.070
Number of pores E ($n.cm^{-2}$)	33.00 ± 5.000^{a}	42.50 ± 15.500^{ab}	$58.38 \pm 5.910^{b**}$
Number of pores BE ($n.cm^{-2}$)	30.50 ± 3.500^{a}	39.50 ± 2.500^{ab}	$53.61 \pm 2.450^{b*}$
Total number of pores (n)	2739.66 ± 389.540^{a}	3051.83 ± 647.750^{ab}	$4242.79 \pm 292.650^{b**}$
Egg surface area (cm^2) - SA	$93.80 \pm 0.560^{a***}$	86.82 ± 1.960^{bc}	89.15 ± 0.840^{c}
Shell density ($g.cm^{-3}$) - SD	$2.88 \pm 0.090^{a***}$	2.60 ± 0.360^{ab}	2.10 ± 0.020^{b}

Different letters (a, b, c) within a row indicate statistically significant differences: *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$

respectively). Higher embryonic death rates during the early embryogenesis were observed by Peebles and Brake (1985) in eggs with thick shells. The higher thickness of shells reduced its permeability; hen embryonic death could be anticipated due to the unfavourable effect of the two factors.

The numbers of pores in the three studied shell zones (sharp end, equator, blunt end) were significantly ($p < 0.05$) higher in hatched eggs compared to those in eggs with dead embryos. The average number of pores in hatched eggs was 31.15 n.cm^{-2} at the sharp end, 58.38 n.cm^{-2} at the equator and 53.61 n.cm^{-2} at the blunt end.

The same tendency was remained for the total amount of pores on egg surface; it was the highest in hatched eggs (4242.79), significantly ($p < 0.01$) lower in early dead eggs (2739.66) and late dead eggs (3051.83). Similar data were reported by Burtov *et al.* (1990).

The surface area of eggs was the highest in early dead eggs (93.80 cm^2, $p < 0.001$) compared to both late dead and hatched (86.82 cm^2 and 86.15 cm^2, respectively). This is attributed to the higher weight of such eggs, as surface areas is closely related to egg weight.

In the present study, there was positive correlation between eggshell density and eggshell weight, thickness and egg surface area. The highest SD values were observed in early dead eggs (2.88 g.cm^{-3}), which had also higher eggshell weight (9.86 g). Lower eggshell density was exhibited by eggs with late embryonic death (after the 10th day of incubation) and in normally hatched eggs: 2.60 g.cm^{-3} and 2.10 g.cm^{-3} respectively ($p < 0.001$).

The results of incubated eggs from turkeys at 46 weeks of age are presentedin Table 2. During that part of the production cycle, not significant differences were found in the weight of eggs between dead and viable embryos. The eggshell weight was significantly higher in early and late dead eggs ($p < 0.001$ and $p < 0.01$, respectively) compared to that of hatched eggs.

In this study, the eggshell thickness values were not substantially different in dead and viable eggs, while a number of other researchers (Kostova, 1974; Shatokhina, 1975; Kurova, 1986) demonstrated lower embryonic death rates in eggs with intermediate eggshell thickness compared to thicker or thinner eggshells.

Pores of shells were the most numerous in the equator region of hatched eggs (59.73; $p < 0.001$) compared to those in early and late dead eggs. The results of present study are not consistent with those of the study of Peebles and Brake (1985), as authors reported higher density of pores in the blunt end of hatched eggs.

In this study, the total number of pores on the surface of eggs with dead embryos in the early incubation period was the lowest (4133.43) followed by hatched eggs (4871.12) and eggs with embryos dead between the 9th and 25th days of incubation (5101.91). The eggs with extreme porosity, either very low or very high,were with poor hatchability, and hatchlings – with reduced viability (Chistyakova, 1988).

The surface area of hatched eggs was 90.87 cm^2, i.e. significantly ($p < 0.05$) lower than that of late dead embryos (91.87 cm^2) and slightly lower than that of early dead embryos (91.48 cm^2).

The shell density varied. In early dead eggs it was 2.31 mg.cm^{-3}, vs. 2.24 mg.cm^{-3} in late dead eggs and 2.10 mg.cm^{-3} in hatched eggs.

Table 2: Eggshell parameters of incubated eggs from turkeys at 46 weeks of age (mean ± SD)

Parameters	Egg type		
	Early dead	Late dead	Hatched
Egg weight (g)	85.11 ± 1.250	85.61 ± 1.770	84.30 ± 0.790
Shell weight (g)	8.35 ± 0.210^{a***}	8.14 ± 0.320^{a**}	7.27 ± 0.080^{b}
Shellt hickness (mm)			
Sharp end (SE)	0.40 ± 0.010	0.39 ± 0.010	0.39 ± 0.006
Equator (E)	0.39 ± 0.008	0.39 ± 0.012	0.38 ± 0.005
Blunt end (BE)	0.38 ± 0.010	0.39 ± 0.012	0.37 ± 0.005
Average shell thickness (mm)	0.39 ± 0.006	0.39 ± 0.009	0.38 ± 0.005
Number of pores SE (n.cm^{-2})	36.42 ± 3.400^{a***}	55.66 ± 4.580^{b}	40.80 ± 2.500^{a**}
Number of pores E (n.cm^{-2})	42.83 ± 4.840^{a}	51.83 ± 8.640^{ab}	59.73 ± 3.970^{b***}
Number of pores BE (n.cm^{-2})	55.17 ± 6.730	59 ± 5.390	56.53 ± 3.250
Total number of pores (n)	4133.43 ± 364.920	5101.91 ± 348.830	4871.12 ± 209.470
Egg surface area (cm^2) - SA	91.48 ± 0.940^{ab}	91.86 ± 0.310^{ab*}	90.87 ± 0.370^{a}
Shell density (g.cm^{-3}) - SD	2.31 ± 0.040	2.24 ± 0.090	2.10 ± 0.030

Different letters (a, b) within a row indicate statistically significant differences: *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$

Figure 2 depicts the total number of eggshell pores of early dead, late dead and hatched eggs from turkey hens at 34 and 46 weeks of age. The number of pores of shells of older turkey hens was higher both in dead and hatched eggs. This is in line with the data reported by Gupalo (2014), but disagrees with the result of Kontecka *et al.* (2012), who did not observe any significant differences in the porosity of shells of eggs laid by hens at a various age. Szczerbińska (1997) reported lower number of pores on shells of eggs from chickens in peak production.

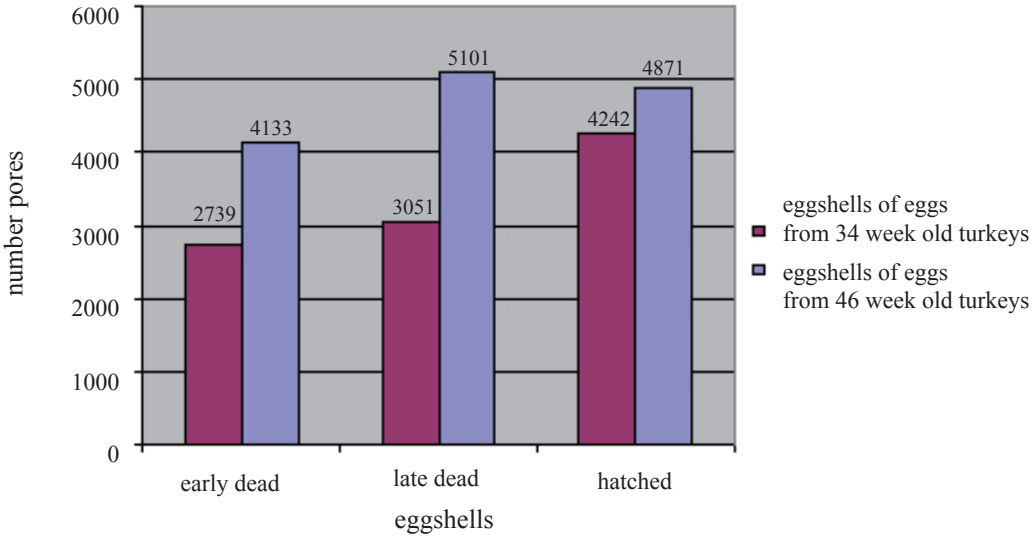

Fig. 2: Total number of eggshell pores of eggs from turkey hens at 34 and 46 weeks of age

CONCLUSION

At 34 and 46 weeks of age, the weight of eggshells of eggs with early dead and late dead embryos was statistically significantly higher ($p < 0.001$ and $p < 0.01$) compared to the eggshell weight of hatched eggs. There were no differences in the thickness of shells of dead and hatched eggs, laid by 34- and 46-week-old turkeys. The total number of pores on the shell surface of early dead eggs was lower when compared to the parameter of late dead eggs and hatched eggs from turkeys at 34 and 46 weeks of age. The eggs with dead embryos had thicker shells than hatched eggs in turkeys of the two studied ages (34 and 46 weeks of age). This requires further studies on the influence of the quality of the egg shell on the development of embryos.

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CONTENT OF MAJOR AND TRACE ELEMENTS IN RAW EWES' MILK USED FOR PRODUCTION OF TRADITIONAL WHITE BRINED CHEESE

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ABSTRACT

The content of minerals (major and trace elements) in raw ewes' milk produced in traditional way in different regions in Macedonia is the subject of this study. The households from where the milk samples were collected are exposed to different levels of anthropogenic pressure. The concentration of 17 elements (Ag, Al, As, Ba, Ca, Cd, Co, Cu, Fe, Mg, Mn, Na, Ni, P, Pb, Sr, and Zn) was analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) after performing microwave digestion. The analyses of ewes' milk did not show any significant variation in the levels of major elements. The concentration of Ca, Mg, Na and P were in the range of 1131-2070 mg.kg^{-1}, 98.3-183 mg.kg^{-1}, 223-400 mg.kg^{-1} and 569-1080 mg.kg^{-1}, respectively. The levels of Ag, As, Cd, Co, Ni and Pb in all the analyzed milk samples were below the detection limit although some households were located in areas exposed to environmental contamination with heavy metals (Cd, Pb and Zn). The raw ewes' milk contains Cu in the range of 0.66-1.47 mg.kg^{-1}, Fe of 1.42-3.82 mg.kg^{-1}, Mn of 0.04-0.16 mg.kg^{-1} and Zn of 2.90-6.27 mg.kg^{-1}. The soil composition, the traditional utensils and containers used for milk storage correlated with higher concentration of trace elements (Al, Ba, Cu, Fe, Mn, Ni, Sr, Zn) in some of the analyzed milk samples. The obtained results point out that ewes' milk produced in households and used for manufacturing of traditional dairy products is safe for consumption.

Key words: mineral elements; trace elements; ewes' milk

INTRODUCTION

Milk and dairy products are important sources of nutrients because they are rich in proteins, fats, hydrocarbons, vitamins and minerals. Minerals are also important for the human diet because of their structural, biochemical and nutritional functions (Zamaberlin *et al.*, 2012). All essential minerals can be found in milk and dairy products where major elements (Na, K, Ca, Mg, Cl and P) are present with higher concentration than the trace elements (Fe, Cu, Zn, Mn, Cr, Se). The mineral composition of milk depends on the genetic characteristics, stage of lactation, environmental conditions, type of pasture, soil contamination and the health conditions of the animals (Borys *et al*., 2006; Park *et al*., 2007; Gonzalez-Martin *et al*., 2009; Aly *et al*., 2010).

The presence of major and trace elements in milk can indicate whether the product meets the needs of particular elements in human nutrition and may also indicate possible environment contamination (Borys *et al*., 2006).

High concentration of trace elements (as well as heavy metals) in milk and cheese is the result of their growing concentration in the environment due to increase of urban, agricultural and industrial emissions (Caggiano

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et al., 2005). Animals intake heavy metals through food chain, air and water. It is considered that ingestion of contaminated fodder is the main source of metal residue in the secreted milk (Elbarbary and Hamouda, 2013). The increasing industrialization and environmental pollution with heavy metals and their negative impact on human health (Perween, 2015) has raised the public interest in metal contamination of food stuffs. The processing equipment, reagents and accidental contamination during storage may also affect the dairy products (Elbarbary and Hamouda, 2013). That is why recently a lot of studies have been carried out on mineral and heavy metal content in milk and cheese (Dobrzański *et al*., 2005; Anastasio *et al*., 2006; Vural *et al*., 2008; Aly *et al*., 2010; Kodrik *et al*., 2011; Lukáčová *et al*., 2012) and their variation during ripening and seasonal changes (Cichoscki *et al*., 2002; González-Martín *et al*.; 2009; Kirdar *et al*., 2013).

Unprocessed ewes' milk is a basic raw material for production of white brined cheese and other dairy products in traditional way in almost all regions in Macedonia, particularly the mountain areas. However, there are few studies regarding the mineral elements and heavy metals content in milk. Ivanova *et al*. (2011) analyzed the mineral content of milk from dairy sheep breeds. The mineral content of milk from three types of dairy sheep populations were compared, namely: the Bulgarian Dairy Synthetic population bred in Bulgarian, East-Friesian and the Awassi breeds bred in Macedonia. The results show a slight variation in the content of Ca and P among the studied breeds, and also that the content of trace elements in the milk is lower than the maximum permissible levels for the raw ewes' milk.

The aim of this study is to assess the concentration of mineral and trace elements in ewes' milk used for production of traditional dairy products, collected from different regions in Macedonia. It is also important to assess whether the trace elements in the collected milk samples did not exceed the allowed amounts as the households were located in areas exposed to different levels of anthropogenic pressure.

MATERIALS AND METHODS

Sampling sites

The study was conducted during May 2014 and the samples were collected form 12 households located in different regions in Macedonia exposed to different anthropogenic pressure. Three households (in the eastern region) were located near the "Sasa" lead and zinc mine, two households (in the central region) near the abandoned lead and zinc smelter in Veles and one household (near Skopje) near the oil refinery. The households from Sveti Nikole (eastern region), Bistra Mountain (western region) and Mariovo (south-western region) were located in areas without any intensive pollution.

The sheep flocks included mainly Merinolandschaft crossbreeds. During the whole year the flocks fed on pastures with the exception of winter periods when the flocks fed with grazing and hand feeding (with hay and barley or concentrates).

Sample collection

Total of 24 samples were taken from raw bulk milk collected in traditional vessels or plastic containers. Two milk samples from each household were taken in plastic laboratory bottles and brought to the laboratory in refrigerated condition. $K_2Cr_2O_7$ was added to the milk samples in order to prevent curdling. Until the analyses were performed the samples were kept frozen (-18 ºC).

Sample analyses

The milk samples were analyzed for content of major elements (Ca, Mg, Na and P) and trace elements (Ag, Al, Ba, Cu, Fe, Mn, Sr, Zn, Ni As, Pb, Cd, Co).

A microwave system was used for acid digestion of all of the samples. The milk samples were first dried at 70 ºC and then grated. To each dried sample (0.5 g), 7 ml HNO_3 (trace pure) and 2 ml H_2O_2 were added. Then the same were left overnight to react and burn the organic material. The vessels with the material were then put in microwave digestion system ($Mars_x$, CEM) for total digestion (15 min at 180 ºC). After the digestion was complete the samples were filtered and poured in 25 ml bottles. After that the samples were measured. The concentration of minerals was expressed in $mg.kg^{-1}$ wet weight.

The content of elements in samples was determined using inductively coupled plasma - atomic emission spectrometry ICP-AES, (715-ES, Varian, USA) applying ultrasonic nebulizer CETAC (ICP/U-5000AT$^+$) for better sensitivity.

Statistical analyses were conducted using F-test between the results gained from milk samples. The analyses were performed using the software package Statgraph 3.0 (Statistical Graphics, Warrenton, Virginia, USA).

RESULTS AND DISCUSSION

The results obtained from the determination of major elements (Ca, Mg, Na and P) in milk are shown in Table 1. The concentration of K in milk was not analyzed due to $K_2Cr_2O_7$ utilization for milk curdling prevention. The results show variations in the content of major elements in raw milk samples collected from different regions, but they are not significant ($p < 0.05$). Regarding Ca concentration, the obtained results were

Table 1: Content of major minerals in ewes' milk (mg.kg^{-1} wet weight)

Region	Household	Ca	Mg	Na	P
Sveti Nikole	1	1236	98.3	223	569
	2	1645	129	335	754
	3	1579	130	241	781
Av ± Sd		1487 ± 219	119.0 ± 18.0	266 ± 60	701 ± 115
Sasa	4	1131	108	236	576
	5	1885	174	339	1051
	6	1444	132	241	738
Av ± Sd		1487 ± 379	138.3 ± 34	272 ± 58	788 ± 242
Veles	7	2070	183	400	1080
	8	1508	134	252	721
Av ± Sd		1789 ± 861	158.7 ± 76	326 ± 172	901 ± 421
Mariovo	9	1558	126	281	774
	10	1314	115	284	643
Av ± Sd		1436 ± 354	120.5 ± 26	282 ± 64	709 ± 179
Skopje	11	1300	103	282	618
Bistra	12	1295	129	275	606

Average ± standard deviation

similar to the study of Ivanova *et al.* (2011). In view of Ca and P concentration, the levels were lower compared to the results of Polychroniadou and Vafopoulou (1985); Şahan *et al.* (2005); Boris *et al.* (2006); Ivanova *et al.* (2011) and Yabrir *et al.* (2014).

Variations were also observed regarding the presence of Mg and Na. The values obtained for Mg in this study were similar to the tests conducted by Şahan *et al.* (2005), but were lower compared to the results of Moreno-Rojas *et al.* (1993); Şahan *et al.* (2005); Boris *et al.* (2006); Güler (2007) and Yabrir *et al.* (2014). The lower values of major minerals in this study were probably affected by the manner of breeding and feeding the sheep, which throughout the year mainly feed on pasture vegetation, and barley, silage or concentrate are occasionally introduced in their nutrition. A diet with pasture vegetation means both unbalanced and lower minerals intake which later affects the mineral content of the milk (Ivanova *et al.*, 2011; Ahmad *et al.*, 2013). Physiological changes in the mammary gland, as well as variations in the composition of milk proteins and fats, may influence the values of mineral substances in the milk (Güler, 2007).

Besides major elements, trace elements were noted in the milk composition, whose concentrations were variable depending on the region from where the milk samples were collected (Table 2). The presence of certain trace elements (Zn, Mn, Fe) in smaller amounts can cause certain health problems since they can be found in the structure of a large part of the enzyme systems or are present in the internal organs such as liver, kidneys, muscles, bones, or participate in the metabolism of carbohydrates, fats and proteins (Perween, 2015; Ivanova *et al.*, 2011). Also, the presence of certain trace elements (Pb, Cd, Cr) in the organism in higher concentration can be toxic and can have adverse effects on the human health causing serious diseases and damages to the nervous and reproductive systems, and can also contribute to the occurrence of malignancies (Enb *et al*., 2009; Elbarbary and Hamouda, 2013; Perween, 2015).

In all the examined milk samples the concentration of Ag remained below the limit of detection (< 0.1 mg.kg^{-1} wet weight) with the exception of the milk samples taken from the farms in the Sveti Nikole and Bistra regions where the concentration of Ag was 0.12 mg.kg^{-1} and 0.32 mg.kg^{-1}, respectively. The presence of Ag in the milk samples taken from the farm in Sv. Nikole may be due to the higher concentration of silver in the soil of that region (Stafilov *et al*., 2014), but the presence of this element in the other milk samples collected from the Bistra region indicates possible contamination of the tools used during milking or milk collection. The processing equipment and milk storage containers can be a source of trace elements in milk also (Yabrir *et al*., 2014).

The concentration of Al in the milk is also variable, with highest measured concentration observed in the milk samples collected from the Bistra region (2.26 mg.kg^{-1}). Compared to the milk samples from

Table 2: Content of trace elements in raw ewes' milk (mg.kg^{-1} wet weight)

Region	House hold	Ag	Al	As	Ba	Cd	Co	Cu	Fe	Mn	Ni	Pb	Sr	Zn
Sv. Nikole	1	< 0.1	1.43	< 0.1	0.162	< 0.01	< 0.1	1.18	1.59	0.04	< 0.1	< 0.1	0.72	2.90
	2	0.12	0.93	< 0.1	0.251	< 0.01	< 0.1	1.35	2.02	0.05	0.13	< 0.1	0.95	3.99
	3	< 0.1	1.01	< 0.1	0.220	< 0.01	< 0.1	1.47	1.98	0.05	< 0.1	< 0.1	1.10	4.10
	Av ± Sd	nd	1.12 ± 0.27^{b}	nd	0.21 ± 0.04^{ac}	nd	nd	1.33 ± 0.14^{ac}	1.86 ± 0.24^{ac}	0.05 ± 0.01^{b}	nd	nd	0.92 ± 0.19^{acd}	3.67 ± 0.66
Sasa	4	< 0.1	1.07	< 0.1	0.345	< 0.01	< 0.1	0.81	1.42	0.05	< 0.1	< 0.1	0.46	3.35
	5	< 0.1	1.89	< 0.1	1.559	< 0.01	< 0.1	1.32	3.82	0.13	< 0.1	< 0.1	0.87	6.27
	6	< 0.1	0.93	< 0.1	0.837	< 0.01	1.68	0.88	1.70	0.08	< 0.1	< 0.1	0.82	3.71
	Av ± Sd	nd	1.30 ± 0.52^{b}	nd	0.91 ± 0.61^{b}	nd	nd	1.00 ± 0.27^{bc}	2.31 ± 1.31^{abc}	0.09 ± 0.04^{b}	nd	nd	0.72 ± 0.22^{cd}	4.44 ± 1.59
Veles	7	< 0.1	1.90	< 0.1	0.229	< 0.01	< 0.1	1.17	2.63	0.08	< 0.1	< 0.1	0.65	6.09
	8	< 0.1	1.26	< 0.1	0.183	< 0.01	< 0.1	0.98	3.07	0.05	< 0.1	< 0.1	0.44	3.89
	Av ± Sd	nd	1.58 ± 0.45^{ab}	nd	0.21 ± 0.03^{c}	nd	nd	1.07 ± 0.13^{ac}	2.85 ± 0.31^{abc}	0.06 ± 0.02^{b}	nd	nd	0.54 ± 0.15^{bcd}	4.99 ± 1.56
Mariovo	9	< 0.1	1.00	< 0.1	0.406	< 0.01	< 0.1	0.83	1.52	0.06	< 0.1	< 0.1	0.81	4.40
	10	< 0.1	1.22	< 0.1	0.338	< 0.01	< 0.1	0.68	2.36	0.07	< 0.1	< 0.1	0.63	3.12
	Av ± Sd	nd	1.11 ± 0.16^{b}	nd	0.37 ± 0.05^{abc}	nd	nd	0.76 ± 0.11^{bc}	1.94 ± 0.59^{abc}	0.07 ± 0.01^{b}	nd	nd	0.72 ± 0.13^{d}	3.76 ± 0.91
Skopje	11	< 0.1	0.86^{b}	< 0.1	0.158^{c}	< 0.01	< 0.1	0.66^{b}	1.70^{c}	0.05^{b}	< 0.1	< 0.1	0.37^{bd}	3.22
Bistra	12	0.32	2.26^{a}	< 0.1	0.382^{abc}	< 0.01	0.57	0.93^{bc}	3.38^{b}	0.16^{a}	0.18	< 0.1	0.26^{b}	3.89

Av ± Sd - average ± standard deviation, nd - not detected, a, b, c - values in the same column with different letters differ significantly ($p < 0.05$).

the Sv. Nikole, Sasa, Mariovo and Skopje regions this value is significantly higher at the level of $p < 0.05$. The concentration of Al measured in milk samples taken from all the regions showed a significantly greater value compared to the study of Elbarbary and Hamouda (2013), who reported that the raw milk contained 0.561 $mg.kg^{-1}$ of Al. The results of this study are in accordance with the results of Güler (2007) and Park *et al.* (2007) according to which the concentration of Al in goat and sheep milk is 3.76 ppm and 0.05-0.18 $mg.100\ g^{-1}$. Most likely the containers and utensils with which the milk comes in contact with are the cause of higher concentration of Al in the milk, also indicated by Coni *et al.* (1996).

The highest concentration of barium was observed in the milk samples collected from the farms in the surroundings of the Sasa mine. These values are significantly higher at level $p < 0.05$ compared to the values measured in the milk samples collected from the Sveti Nikole, Veles and Skopje regions. The results obtained from this study are similar to the results of Ivanova (2011), but the values are lower compared to the results of Güler (2007) according to which the value of Ba in goat milk is 0.99 ppm. The higher presence of this element in the milk samples obtained from the farms in the vicinity of the Sasa mine may be due to introduction of this element through nutrition or through pasture vegetation. Namely, the mine tailings site is located along the road leading to it, and it is possible that the Ba reaches the vegetation by the wind dust from the tailings. Also, its concentration in the soil is higher compared to the content of Ba in the soil from the other locations (Stafilov *et al*., 2014).

The results of the Cu, Fe, Mn and Zn concentration measurements are shown in Table 2 with certain variations of these elements in the milk samples collected from different regions. Higher concentration of Cu are observed in the milk samples collected from the farms in the Sveti Nikole, Sasa, Veles and Bistra regions. The content of Cu in the milk samples collected from the farms in the Sveti Nikole region showed significantly higher values ($p < 0.05$) compared to the milk samples taken from the farms in the Sasa, Mariovo, Skopje and Bistra regions. The comparison of Cu concentration in the milk samples taken from the Skopje and Veles regions indicates that the milk sample from the region of Veles contained significantly higher concentration. The results obtained with this study show higher values compared to the results of Borys *et al*. (2006); Park *et al*. (2007); Güler (2007); Yabrir *et al.* (2014) who observed presence of Cu in the amounts of 0.090-0.098 $mg.kg^{-1}$, 0.04 $mg.100\ g^{-1}$, 0.48 ppm, 0.42-0.47 ppm in ewe and goat milk. The results of this study are similar or the values are slightly higher when compared to the results of Ivanova *et al*. (2011), and lower compared to the results of Elbarbary and Hamouda (2013). The higher values of Cu in the tested milk are probably due to its naturally higher presence in the soil in the Sv. Nikole region (Stafilov *et al.*, 2014), while the Sasa and Veles regions are affected by the activities of the Pb-Zn Sasa mine (Stafilov *et al*., 2014; Balabanova *et al.*, 2015) and the smelter (Stafilov *et al*., 2010), respectively. Besides nutrition, the higher concentration may be influenced by the milk treatment (Enb *et al.*, 2009; Zamberlin *et al*., 2012).

Iron is a component of the hemoglobin, myoglobin, and other proteins and also a cofactor for many enzymes. Its presence in ewe milk according to Zamberlin *et al*. (2012) is in the range of 62-100 $\mu g.100\ g^{-1}$, while in buffalo or cow milk it is in the range of 0.786-1.242 $mg.kg^{-1}$ and 0.607-0.794 $mg.kg^{-1}$, respectively (Enb *et al.*, 2009). The concentration of Fe in the tested ewes' milk is in the range of 1.42-3.82 $mg.kg^{-1}$, while the highest concentration was observed in the milk samples collected from the farms in the Sasa and Bistra regions. Statistical analysis of data demonstrated significant differences in the content of Fe at the level $p < 0.05$, which was observed in the milk samples taken from the farms in the Sveti Nikole and Bistra, and Skopje and Bistra regions. The results of this study show similarities with those of Güler (2007) and Ivanova *et al.* (2011), but have higher values compared to the investigations of De la Fuente *et al.* (1997); Borys *et al.* (2006); Park *et al.* (2007) and Yabrir *et al.* (2014).

The region of Bistra is considered as a region that is not under high anthropogenic influence and there are no urban areas as well as industrial facilities nearby. The high concentration of Fe in the milk samples collected from this region is probably a result of the containers used for milk collection (Güler, 2007). The high concentration of Fe in the milk samples collected from farms in the Sasa region (3.82 $mg.kg^{-1}$) and Veles (3.07 $mg.kg^{-1}$) region are most likely influenced by the activity of the Sasa mine and the Veles smelting plant. Although the smelter has not been active in the recent years, the soil in its environment has accumulated Fe in higher concentration (Stafilov *et al.*, 2010, 2014; Balabanova *et al*., 2015).

The content of Mn in milk samples showed the highest value in the milk from Bistra which is significant at level of $p < 0.05$, compared to the milk samples from all other surveyed regions. When comparing the results of the tests with those of De la Fuente *et al.* (1997); Park *et al.* (2007); Ivanova *et al.* (2011); Zamberlin *et al.* (2012) and Yabrir *et al.* (2014) some similarities can be observed. Variations in the concentration of mineral components are very characteristic for ewe milk compared to cow milk (Park *et al.*, 2007). The higher concentration of Mn in the milk is affected by the metabolism of metals in sheep (Caggiano *et al*., 2005), its concentration in the nutrition and the additional intake of minerals from

the metal containers and tools used for milking and storage of milk (Güler, 2007).

The examination of Ni concentration in milk indicates values below the limit of detection (< 0.1 mg.kg^{-1}) in almost all tested milk samples. The samples taken from a farm in the Sveti Nikole region and the farm region of Bistra where the concentration of Ni was 0.13 mg.kg^{-1} and 0.18 mg.kg^{-1} are exceptions. The occurrence of Ni in these two milk samples probably originates from the containers in which the milk was collected, i.e. kept. According to the study of Lukáčová *et al.* (2012) Ni is present in the raw cow milk at a concentration of 0.84 mg.kg^{-1}, while according to Enb *et al*. (2009) cow and buffalo milk contains Ni at concentrations of 0.004 mg.kg^{-1} and 0.006 mg.kg^{-1}. In goat milk Güler (2007) found a Ni content of 1.38 ppm, and Zamberlin *et al*. (2012) provided data that the Ni content in sheep milk is 5.4 μg.100 g^{-1}.

Unlike Ni, strontium was detected in all milk samples taken for analysis. The lowest concentration of Sr was measured in the milk samples collected from the Bistra region which shows significantly lower value ($p < 0.05$) compared to the samples taken from the Sveti Nikole, Sasa and Mariovo regions. The concentration of Sr in the milk from Sveti Nikole was significantly higher than the one observed in the milk samples from Skopje and Veles, as well as the milk samples from the Sasa area in relation to Skopje. The concentration of Sr in milk also depends on its concentration in the pastures' vegetation and the ability of the plants to absorb it. The results obtained from this study indicate lower to similar values compared to the results of Güler (2007) who examined goat milk. The higher content of Sr in milk observed in the course of this study could be generally explained with its lithogenic origin in the soil in the Republic of Macedonia, which has relatively high content of Sr (Stafilov *et al*., 2014).

The concentration of Zn in the tested samples of milk was in the range of 2.90-6.27 mg.kg^{-1}. From the results, it is evident that the highest concentration of Zn was measured in the milk samples from the farms in the Sasa and Veles regions. The higher level of Zn concentration in the soil is the result of anthropogenic influence (Sasa Pb-Zn mine and Pb-Zn smelter in Veles), since it contributes to increased concentration in the vegetation that serves as fodder for the sheep (Stafilov *et al*., 2010, 2014; Balabanova *et al.*, 2015). However, statistical analysis of the data showed no significant differences in the concentration of Zn in milk samples from different regions. Elsayed *et al*. (2011) observed higher levels of Zn in the cow milk samples from the industrial regions, but the values are lower than those obtained with this study which is understandable because ewes' milk contains higher concentration of trace elements. The values in this study are lower than the results of Borys *et al.* (2006); Yabrir *et al.* (2014) but are similar to those of Güler (2007) and Park *et al.* (2007). The values for Zn in cow milk, recommended by the IDF Standard (1977), are lower in relation to the obtained results shown in Table 2. According to the Standard, MRL for Zn in milk is 0.328 mg.kg^{-1}.

As for the Co and toxic elements such as As, Cd and Pb, tests showed that in all analyzed milk samples their concentration remained below the limit of detection, namely the concentration of As is < 0.1 mg.kg^{-1}, of Cd < 0.01 mg.kg^{-1}, of Pb < 0.1 mg.kg^{-1}. The concentration of Co was < 0.1 mg.kg^{-1}, with the exception of two farms in the Sasa and Bistra regions where the concentrations were 1.68 mg.kg^{-1} and 0.57 mg.kg^{-1}, respectively. The concentration of Cr in the milk was not measured because of the added $K_2Cr_2O_7$. The higher concentration of Co measured in the milk samples from the two stated farms is probably the result of additional contamination from the dairy equipment because higher concentration of the same elements are not observed neither in the curd nor in the cheese made from the tested milk (Levkov unpublished data). According to Elsayed *et al.* (2011) higher concentration of Pb and Cd in cow milk are observed in the industrial regions. According to the IDF Standard (1977) MRL of Cd in milk from uncontaminated areas is 0.006 mg.kg^{-1} while from contaminated areas it is 0.015 mg.kg^{-1}. Güler (2007) found a concentration of Pb in goat milk in the amount of 0.06 mg.kg^{-1}, Cd 0.63 mg.kg^{-1}, Co 0.89 mg.kg^{-1} while As was not detected at all. The concentration of Pb of 0.20 μg.g^{-1} dry weight in ewe's milk was observed by Caggiano *et al.* (2005) while the concentration of Cd amounted to 0.06 μg.g^{-1} dry weight. Elbarbary and Hamouda (2013) measured Pb concentration in raw milk in the amount of 0.27 mg.kg^{-1} and Cd 0.053 mg.kg^{-1}. Higher concentration of Cd (0.27 mg.kg^{-1}) in raw cow milk was found by Lukáčová *et al*. (2012). According to Caggiano *et al.*, (2005) kidneys and liver are considered bio-accumulators of metals, which are introduced in the bodies of animals through food, so that the same are excreted in the milk in small concentration.

CONCLUSION

The results of this study showed variations in the concentration of mineral and trace elements in the raw ewes' milk used for production of traditional dairy products. For the purpose of this study, milk samples were collected from households located in different geographic regions characterized by specific soil composition and pasture vegetation. The concentration of trace elements in the soil, the mines and the industrial activities, as well as the utensils and containers used for milk storage influenced the higher concentration

of trace elements (Ag, Al, Ba, Cu, Fe, Mn, Ni, Sr, Zn) in some of the analyzed samples. Milk samples taken from the regions exposed to higher levels of pollution showed that the concentrations of toxic elements were below the limit of detection. The obtained results point out that the ewes' milk produced in households and used for production of traditional dairy products is safe for consumption. In the Republic of Macedonia there is little published data related to this issue of research, therefore it is advisable to conduct more detailed analyses of other types of milk (cow, goat) produced in other geographical regions as well as the fodder that animals consume.

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RECTAL TEMPERATURE, HEART RATE, PACKED CELL VOLUME AND DIFFERENTIAL WHITE BLOOD CELL COUNT OF LAYING PULLETS TO HONEY SUPPLEMENTED WATER DURING HOT – DRY SEASON

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ABSTRACT

Honey has medicinal properties which may help ameliorate adverse effects of heat stress in laying chickens. It has antimicrobial, antibacterial and anti-oxidant properties. This study investigated the use of honey in drinking water as a natural alternative to help alleviate heat stress in laying pullets during hot-dry season (December – April) in the tropics. One hundred and twenty *Isa Brown* layers at 28 weeks old weighing 1.5 ± 0.1 kg were used in a 16 week experiment. The birds were randomly allotted to 3 treatments of 4 replicates containing 10 birds each. The treatments were 0 ml honey per 1 L of water (0 H), 10 ml honey per 1 L of water (10 H) and 20 ml honey per 1 L of water (20 H). Data on heart rate, rectal temperature and haematological parameters were collected and subjected to one way analysis of variance. Rectal temperature was significantly ($p < 0.05$) higher in honey treated groups compared to control group. However, heart rate was significantly ($p < 0.05$) lower in 20 H birds (300.9 ± 1.70 bpm) compared to 0 H birds (313 ± 1.70 bpm). Honey supplementation did not significantly ($p > 0.05$) affect packed cell volume, heterophil, monocyte, eosinophil and heterophil-lymphocyte ratio. Birds given 20 H had a significantly ($p < 0.05$) higher lymphocytes count (50.6 ± 0.79 %) while birds that received 10 H had a significantly ($p < 0.05$) lower basophil count (3.1 ± 0.39 %). These findings indicate that the use of honey supplemented in drinking water reduced HR and basophil count.

Key words: heat stress; honey; layers; temperature; haematology

INTRODUCTION

Hot-dry season in the tropical environment is characterized by high environmental temperature, which sometimes exceeds 30 °C (Abioja *et al.*, 2011). Exposure of laying birds to high ambient temperature in poultry houses in the tropical zones elicits a series of responses in laying chickens which is generally termed heat stress (Ayo *et al*., 2011). It is caused by high ambient temperatures that exceed the thermoneutral zones of poultry species and when coupled with high humidity has a detrimental effect on commercial broiler and layers (Nienaber and Hahn, 2007). Heat stress occurs mainly in the hot – dry season and during this period, birds have limited physical resource (nutrient) for growth and reproduction in response to environmental change and voluntary feed consumption is drastically reduced (Khan *et al*., 2011). The adaptation to this new challenge requires redistribution of body reserve of energy and protein to thermoregulation at the cost of decreased growth and reproductive efficiency (Puron *et al*., 1994; Kadim *et al*., 2008).

Most poultry farmers make use of commercial and synthetic anti-stress and anti-oxidants to help chickens cope with heat stress. Alternatives to the use of chemicals such as anti-stress, anti-oxidants and antibiotics lie in discovery and proper utilization of natural plant materials and extracts that have the necessary properties needed

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(Bedford, 2000; Wenk, 2003; Vidanarachchi *et al.*, 2005; Ramnath *et al.*, 2008; Zhang *et al*., 2009; Ali *et al*., 2010). Various efforts have been geared towards exploration of these materials and one promising source of natural anti-stress/anti-oxidant is honey (Estevinho *et al.*, 2008; Mohamed *et al*., 2002; Gheldof *et al*., 2003; Aljadi and Kamaruddin, 2004; Wasagu *et al.*, 2013).

MATERIALS AND METHODS

Experimental location and meteorological observations

This experiment was carried out at Aiyedoto Farm Settlement, Ojo Lagos, Nigeria (latitude 6°27'25"N, longitude 3°12'21"E and altitude of 36 meter above sea level). The climate of the experimental site is humid, located in the rain forest vegetation zone of western Nigeria. Wet-and dry-bulb temperatures and relative humidity at the level of the birds in the pen at 8:00 and 16:00 h were monitored throughout the experimental period. The temperature-humidity index was calculated from relative humidity and wet- and dry-bulb temperature data.

Experimental animals and management

ISA Brown layer chickens (n = 120; aged 28 weeks) were used for the experiment for a period of 16 weeks. The birds were randomly allocated to three (3) treatments consisting 4 replicates and 10 layer birds per replicate. Standard ration was given *ad libitum* to the birds. The birds were offered honey supplemented water throughout the experimental period.

Data collection

Rectal temperature and Heart rate measurements

The rectal temperature (RT) of 3 birds randomly picked from each replicate was measured using digital thermometer (0.1 °C accuracy). It was inserted into the rectum and held until it beeped and the RT was read off on the visual display unit as described by Yahav and McMurtry (2001). The monitoring of RT started from week 7 to the end of the experiment due to unforeseen circumstances. The use of a stethoscope placed on the chest region of the birds was used to monitor the heart rate (HR) per minute.

Haematological parameters

Packed Cell Volume determination

Blood sample was collected from one randomly picked bird from each replicate at the end of the experimental period via brachial vein using 2 ml sterilized syringes (23 G) and needles into EDTA bottles. Haematocrit value was measured by using micro-haematocrit method according to Crawford *et al*. (1987). Blood samples were drawn into micro-haematocrit tube and one end of the tube was blocked with paraffin wax. The tubes were arranged in a micro-haematocrit centrifuge and centrifuged at 4000 rpm for five minutes. The proportion of packed blood cells was read off on micro-haematocrit reader.

Differential count determination

For differential leucocyte counts, blood smears were made on glass slides. The smears were stained with Wright stain for 15 minutes. Leucocytes differentials (heterophils, lymphocytes, eosinophils, monocytes and basophils) were counted from each smear with the aid of a microscope. The heterophil-lymphocyte ratio (H:L) was calculated by dividing the number of heterophils by the number of lymphocytes.

Statistical analysis

Data collected were subjected to one way analysis of variance (ANOVA) using SYSTAT (1992) using the model: $Y_{ij} = \mu + T_i + \sum_{ij}$; where Y_{ij} = dependent variables, μ = population mean, T_i = i^{th} effect due to addition of honey to drinking water (i = 1, 2 ,3), and $\sum_{ij}$ = residual error. Means that are statistically ($P < 0.05$) different were separated with Duncan multiple range test (DMRT).

RESULTS AND DISCUSSION

Table 1 shows the meteorological variables obtained in this study. The values were above the thermal comfort zone for egg laying chickens which is 16 - 25 °C (Sahin *et al.*, 2006) in the tropical regions. The average temperature recorded during this experiment was 31.5 °C, about 6.5 °C above the upper critical limit.

Table 1: Average daily values for meteorological parameters observed during the experimental period

	8:00 h	16:00 h	Average
Dry-bulb temperature (°C)	30.4	32.5	31.5
Wet-bulb temperature (°C)	27.8	28.8	28.0
Relative humidity (%)	82.3	76.7	79.5
THI	83.8	86.0	84.9

The combination of average ambient temperature and average relative humidity of 79.50 % gave an average temperature-humidity index of 84.92 which is higher than the THI threshold of 70 established for poultry (Tao and Xin, 2003; Karama *et al.*, 2007) which is a clear indication that the laying chickens were heat stressed.

It could be observed that layers given 20 H had lower rectal temperature than 0 H layers in the early stages of honey administration and later a reverse case as the 0 H layers had lower RT (Table 2). Layers that received 10 H had higher RT (41.13 ± 0.033 °C) than the control group (40.68 ± 0.032 °C). This is in contrast to Osakwe and Igwe (2015) who reported a mean lower RT with the administration of honey in the drinking water of the laying birds. This shows that the antioxidant effects of honey helped relieve the imbalance caused by oxidative stress due to the lack of antioxidant capacity that could be caused by abundant ROS from an environmental or behaviour stressor (Tatli *et al*., 2012) which in this case is heat stress for a certain period of time before causing a negative effect to the laying birds. The increase in temperature could be as a result of increased feeding which increases body temperature via digestion. Njoku and Nwazota (1989) demonstrated that adding ascorbic acid in the feed formula improved feed intake and feed utilisation.

Table 2: Effect of honey supplementation on rectal temperature (°C) of laying chicken during hot-dry season

Week	0 H	10 H	20 H	P-value
7	40.93 ± 0.073^{ab}	40.95 ± 0.073^{a}	40.57 ± 0.073^{b}	0.001
8	40.83 ± 0.093	41.12 ± 0.093	40.97 ± 0.093	0.093
9	41.01 ± 0.085	40.91 ± 0.085	40.85 ± 0.085	0.406
10	40.95 ± 0.118	41.09 ± 0.118	41.02 ± 0.118	0.729
11	41.17 ± 0.094^{a}	40.80 ± 0.094^{b}	41.09 ± 0.094^{ab}	0.019
12	40.73 ± 0.087^{b}	41.37 ± 0.087^{a}	41.22 ± 0.087^{ab}	0.000
13	40.68 ± 0.094^{b}	41.31 ± 0.094^{a}	41.26 ± 0.094^{ab}	0.000
14	40.72 ± 0.06^{b}	41.23 ± 0.096^{ab}	41.32 ± 0.108^{a}	0.000
15	40.85 ± 0.092^{b}	41.36 ± 0.095^{a}	41.28 ± 0.107^{ab}	0.001
16	40.71 ± 0.090^{b}	41.23 ± 0.093^{ab}	41.26 ± 0.105^{a}	0.000
Total	40.68 ± 0.032^{b}	41.13 ± 0.033^{a}	41.07 ± 0.034^{ab}	0.000

^{a,b}Means with different superscripts in the same row differ significantly (P < 0.05).

Effect of honey supplemented in drinking water on heart rate of laying chickens during the hot-dry season is presented in Table 3. It was observed that control group had a significantly lower ($p < 0.05$) HR compared to honey treatment groups in week 1. However, opposite is the case in weeks 8 – 10, 12 and 13 where HR was significantly high ($p < 0.05$) in control group than the honey treated groups. There were no significant difference in week 2 – 7, 11 and 14 – 16. The highest overall HR was recorded in 0H group (313 ± 1.70 bpm) while the overall lowest was in 20H layers (300.9 ± 1.70 bpm). The control birds had the mean highest HR in all the treatments which shows a positive impact of honey addition to laying chickens during the hot-dry season. This is in agreement with Osakwe and Igwe (2015) who also reported a lower HR in laying birds given 20 H of honey during the hot-dry season. Reduction in HR could mean that the heart is faced with less stress and is not pumping blood at a higher force as found in heat-stressed birds. The use of vitamin C has been known to decrease the amount of corticosteroids released during stress (Sahin *et al.*, 2003), making it play an important role in response to stress.

The response of the packed cell volume (PCV), heterophils - lymphocytes ratio and differential count are presented in Table 4. Lymphocytes and basophils were significantly affected ($p > 0.05$) by honey supplemented water in the laying chickens during hot-dry season. Lymphocytes count was significantly higher ($p < 0.05$) in 20 H birds (50.6 ± 0.79 %) while basophils count was significantly higher in 10 H birds (3.1 ± 0.39 %). However there was no significant ($P > 0.05$) effect of honey on PCV, heterophils, monocytes, eosinophils and H:L.

During heat stress, reduced lymphocyte count is observed (Altan *et al*., 1999). In this study, lymphocytes count increased with increasing levels of honey which could be due to the presence of foreign substance in the blood system in response to the immune system. Higher values in basophils, heterophils and H:L ratio in the 0 H

Table 3: Effect of honey supplementation on heart rate (beats per minutes) of laying chicken during hot-dry season

Week	0 H	10 H	20 H	P-value
1	255.20 ± 2.915^{b}	266.80 ± 2.915^{ab}	283.3 ± 2.915^{a}	0.000
2	285.07 ± 0.588	279.20 ± 0.588	282.40 ± 0.588	0.458
3	293.65 ± 1.343	305.56 ± 1.343	301.88 ± 1.343	0.787
4	317.87 ± 5.577	308.13 ± 5.577	322.13 ± 5.577	0.203
5	277.87 ± 5.372	288.80 ± 5.372	280.40 ± 5.372	0.331
6	323.47 ± 6.256	306.40 ± 6.256	312.53 ± 6.256	0.161
7	305.07 ± 3.970	292.53 ± 3.970	293.20 ± 3.970	0.053
8	321.73 ± 5.116^{a}	305.87 ± 5.116^{ab}	302.40 ± 5.116^{b}	0.024
9	325.60 ± 4.884^{a}	309.33 ± 4.884^{ab}	303.20 ± 4.884^{b}	0.007
10	329.07 ± 4.535^{a}	312.00 ± 4.535^{b}	311.73 ± 4.535^{ab}	0.013
11	324.80 ± 5.628	312.53 ± 5.628	305.07 ± 5.628	0.054
12	328.27 ± 4.618^{a}	313.07 ± 4.618^{ab}	309.87 ± 4.618^{b}	0.017
13	322.40 ± 5.324^{a}	311.20 ± 5.324^{ab}	300.00 ± 5.324^{b}	0.018
14	338.40 ± 4.611	304.00 ± 4.611	299.33 ± 5.155	0.000
15	322.93 ± 6.369	308.00 ± 6.593	305.09 ± 7.438	0.141
16	319.20 ± 4.616	306.86 ± 4.779	304.00 ± 5.391	0.074
Average	313.13 ± 1.660^{a}	301.60 ± 1.667^{b}	300.87 ± 1.702^{ab}	0.000

^{a,b}Means with different superscripts in the same row differ significantly ($P < 0.05$).

Table 4: Haematological indices of Layers fed different levels of Honey

Parameter	0 H	10 H	20 H	P-value
PCV	24.0 ± 1.74	24.0 ± 1.74	23.8 ± 1.74	0.993
Lymphocytes	41.3 ± 0.79^{b}	49.5 ± 0.79^{ab}	50.8 ± 0.79^{a}	0.017
Heterophils	38.5 ± 1.12	32.5 ± 1.12	30.8 ± 1.12	0.158
Basophils	8.3 ± 0.39^{a}	3.3 ± 0.39^{b}	5.0 ± 0.39^{ab}	0.017
Monocytes	10.0 ± 0.72	12.5 ± 0.72	9.5 ± 0.72	0.463
Eosinophils	2.5 ± 0.43	2.5 ± 0.43	4.5 ± 0.43	0.405
H:L	0.9 ± 0.09	0.7 ± 0.09	0.6 ± 0.09	0.058

^{a,b}Means with different superscripts in the same row differ significantly ($P < 0.05$), PCV = Packed Cell Volume, H:L = Heterophil-Lymphocyte ratio.

layers showed that the hens were heat stressed (Maxwell *et al*., 1992). 10 H layers had the lowest basophil and H:L ratios which implies that honey administered to laying chicken during hot-dry season helped reduce the effect of heat stress. This could be as a result of the phytochemicals present in honey. A slight difference in eosinophil count was observed in all treatments. Flavonoids present in honey helps increase immunity of body of laying hens by decreasing their stress (Frankic *et al*., 2009).

CONCLUSION

The use of 20 ml honey per 1 L of water reduced heart rate of laying chicken in the hot – dry season. However, honey supplemented in water did not improve the rectal temperature of the layers. Basophils count was reduced in honey treatment layers while lymphocytes count was increased. Further research should be carried out on the use of smaller quantities of honey on pullets.

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NITROUS OXIDE PRODUCTION FROM SOIL AND MANURE APPLICATION: A REVIEW

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ABSTRACT

The aim of this review is to summarize the current knowledge about the nitrous oxide (N_2O) production by soils and highlighting future research needs for emission abatement. The article investigates a scientific literature regarding N_2O emissions from the soil according to different factors, such as condition and soil type. Temporal variations can also be explained by the soil temperature and water content. The main review part is focused on solid manure and effluent application to soil. Nitrous oxide gas is formed in soils through the microbiological processes of nitrification and denitrification, and emissions are mainly released after land spreading. Emissions of N_2O are strongly affected by the timing of manure application, reflecting the effects of weather conditions. Key factors for N_2O emissions are the soil moisture, redox potential, available carbon and microbial processes. Recent studies on the effects of seasonal environmental temperature are discussed. Finally, emission factors from land application of solid or effluent manure are listed in table.

Key words: nitrous oxide; soil; manure; slurry; field application

INTRODUCTION

Agriculture is a major contributor of nitrous oxide (N_2O) emissions to the atmosphere, one of the more powerful greenhouse gases (Paustian *et al*., 2004). According to Crosson *et al.* (2011) the main components of agricultural emissions are N_2O released from soils related to application of nitrogen (N) fertilizers (38 %), N_2O from manure management (38 %), N_2O from burning of savannah, forest and agricultural residues (13 %). Animal production systems are a significant contributor to N_2O emissions from soil, it represents 30 % of agricultural emissions (Kelly *et al*., 2008; Adler *et al.*, 2015). However, the N_2O output from the agriculture is underestimated.

N_2O emissions occur both directly on agricultural lands and from N transported to non-agricultural lands. Nitrogen is used inefficiently in most cropping systems: only half of N inputs are captured in crop biomass and the remainder is lost from the system through leaching and/or through gaseous losses of N_2, N_2O, NO_x, or NH_3 from agricultural soils. A portion of the N that is subjected to microbial transformations, including oxidative pathways (nitrification) and reductive pathways (denitrification), involving mineral N compounds, both of which can form N_2O as a by-product (Paustian *et al.*, 2004). In livestock farming, N_2O production takes place in soils and manures. Typically, 70 % to 90 % of the N ingested by herbivores is excreted, either during grazing or via application of manure collected outside grazing periods (Schils *et al*., 2013). Castillo *et al*. (2001) showed that about 72 % of consumed nitrogen is excreted with faeces and urine in dairy cows. N_2O emissions can be direct emissions from organic manures or inorganic fertilizers applied to soil or direct N deposition by grazing animals (Crosson *et al*., 2011).

Microbial N_2O production and consumption processes depend on several interacting environmental controls such as N supply, soil temperature, soil moisture, oxidative reduction potential, the availability of labile organic compounds, soil type, soil pH and climate (Monaghan and Barraclough, 1993; Bouwman

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et al., 2013; Butterbach-Bahl *et al*., 2013; Marsden *et al.*, 2016). No significant correlations were observed between N_2O fluxes and environmental factors, such as rainfall and soil mineral-N (Yamulki *et al*., 1998). However, in the study of Bell *et al*. (2015a), emissions were related to fertiliser N rate; although the trend was non-linear. N_2O emissions increased linearly with increasing fertilizer N rates and NO_3 concentration and amounted to 1.0 to 1.6 % of fertilizer N applied (MacKenzie *et al*., 1997; Bhandral *et al.* (2003a). According to Whalen (2000), N_2O emission from fertilization was directly related to the level of fertilization to 150 kg $N.ha^{-1}$. Emission rates of up to 1590 μg $N_2O\text{-}N.h^{-1}m^{-2}$ occurred in the field, while small rates of deposition to the soil were occasionally observed (Allen *et al*., 1996). Nitrous oxide emission increased with increased NO_3 concentration and fertilizer N rates (MacKenzie *et al*., 1997).

N_2O is produced as a result of two microbial processes operating in the soil profile, whereby it is a by-product of the reduction of nitrate to nitrogen gas (N_2) (denitrification), the ammonification of nitrate and the oxidation of ammonium (NH_4^+) to NO_3^- (nitrification) (Casey *et al.*, 2006; Klein and Eckard, 2008; Saggar *et al*., 2013; Boon *et al.*, 2014). These processes are affected by a number of soil factors, such as soil oxygen and moisture contents, temperature, mineral N content, available soil carbon and pH. Weather conditions, such as rainfall, can affect soil moisture and oxygen contents, and consequently affect N_2O production (Luo *et al*., 2008).

Most of the N_2O originates from microbiological transformations of N in the animal excrements, urine and dung during storage and management and following application or deposition to land (Paustian *et al*., 2004; Oenema *et al.*, 2005; Ross *et al.*, 2014). The major contributor is normally the denitrification process under anaerobic conditions, but nitrification under aerobic conditions may also contribute (Crosson *et al*., 2011; Saggar *et al.*, 2013; Li *et al*., 2015). Denitrification rates in agricultural grassland systems are variable, mainly due to the soil type, management and weather conditions (Klein and van Logtestijn, 1996). Results of Boon *et al.* (2014) indicate that denitrification is the key driver for N_2O release in peatlands. The results of Ball *et al.* (1997) suggested that denitrification was the main N_2O production process at the grassland site, but nitrification may have been equally important at the drier site.

However, according to Koops *et al.* (1997), nitrification is the main N_2O producing process. Nitrous oxide production through denitrification was only of significance when denitrification activity was high. In another field study, the amount of N lost as N_2O through denitrification was negligible, and all N_2O produced must have thus originated from nitrification (Saggar *et* al., 2004b).

Soil

Soils contribute to about 65 % of the total N_2O produced by terrestrial ecosystems (Saggar *et al*., 2004a; Borhan *et al*., 2012). Although, it is well established that soils are the dominating source for atmospheric nitrous oxide (N_2O), we are still struggling to fully understand the complexity of the underlying microbial production and consumption processes.

Microbial production in soils is the dominant nitrous oxide source; the emissions are increased with use of nitrogen fertilizers (Davidson, 2009; Butterbach-Bahl *et al.*, 2013), from manure and urine excreta applied and aerobic and anaerobic degradation of livestock waste in the lagoons and dry manure piles (Crosson *et al*., 2011; Borhan *et al*., 2012; Regaert *et al*., 2015).

The rate of formation and emission of N_2O varies through time with changes in the soil conditions, denitrification rates, type of crop, porosity, moisture content, temperature, redox potential, available carbon, microbial processes, N content of the soil, nitrogen fertilizer type, crop management and soil texture (Carran *et al*., 1995; Bouwman, 1996; Klein and van Logtestijn, 1996; MacKenzie *et al*., 1997; Saggar *et al.*, 2004b; Chianese *et al*., 2009). Also, soils are key sites for denitrification and are much more important than groundwater (Bouwman *et al*., 2013).

While rates of emissions from soil vary considerably due to a number of factors, many studies show a rough proportionality between the total N entering the soil from anthropogenic inputs and the amount lost as N_2O. Bouwman (1996) analyzed measurements of N_2O emission from fertilized and unfertilized fields. N_2O losses from anhydrous ammonia and organic N fertilizers or combinations of organic and synthetic N fertilizers were higher than those for other types of N fertilizer. The excreta from grazing animals adds high amount of substrate in soil for N_2O to be produced by the microorganisms (Bhandral *et al.*, 2010). Most of the N_2O resulting from manure is produced in manure-amended soils through microbial nitrification under aerobic conditions and partial denitrification under anaerobic conditions, with denitrification generally producing the larger quantity of N_2O (Montes *et al*., 2013).

Soil moisture and temperature

The availability of N and the factors that alter the redox potential of the soil, such as changes in soil moisture conditions have major effects on the production of N_2O in soils (Bhandral *et al*., 2003a; Saggar *et al*., 2004a). MacKenzie *et al*. (1997) and Borhan *et al*. (2011b) reported that N_2O emissions increased when soil moisture increased. Authors noted that rainfall prompted anaerobic denitrification of oxidized nitrogen species in the soil environment. The heavy rainfall increased N_2O emissions (Sun *et al*., 2016). Results of Boon *et al.*

(2014) indicate that N_2O production is strongly related to rainfall events, water content movements and irrigation. According to Regaert *et al.* (2015), the high emission peaks occur due to denitrification in times of anaerobiosis, especially after a rainfall. Whalen (2000) found that simulated rainfall gave pulsed N_2O emission from denitrification of accumulated NO_3-N, indicating that further emissions will occur with an increase in soil moisture. Also, high residual soil NO_3-N can result in additional episodic N_2O efflux in response to rainfall (Whalen, 2000).

In the study of Allen *et al*. (1996), emissions were related to other factors including soil moisture, rate of plant growth and carbon availability. The corresponding data for poorly-drained soil were 0.2 mg N_2O-N.kg^{-1} of deposited dung and 148 mg N_2O-N.kg^{-1} of deposited urine (Allen *et al*., 1996). The largest N_2O fluxes occurred when water-filled pore space values were very high (70-90 %), indicating that denitrification was the major process responsible (Dobbie *et al.*, 1999). Greater fluxes were obtained from grassland soil than from arable soil at equal water-filled pore space values (Dobbie and Smith, 2001). Nett *et al.* (2015) concluded that substantial N_2O emissions can occur after high input of available organic carbon (C) and N even in a coarse-textured soil with low waterholding capacity. N_2O is produced during several microbial processes in the N cycle of terrestrial and aquatic systems (Schils *et al.*, 2013).

Also Luo *et al*. (2015) found that the greatest N_2O fluxes recorded were generally associated with rainfall events and high water-filled pore space. An increase in the water-filled pore space of the soil creates anaerobic conditions that together with high levels of N and C availability in the soil owing to the presence of the excreta would have led to a greater opportunity for N_2O production and emission (Luo *et al*., 2015). The smaller N-emissions from the grassland were attributed to its relatively dry siting on a slope of 20 % (Mogge *et al.*, 1999).

Soil temperature (15 - 20 °C) is the most significant driver of N_2O production with a 1 °C rise in the soil temperature increasing emissions in grassland fields grazed with dairy cows and with young stock (Hargreaves *et al*., 2015). With an increase in temperature from 10 to 20 °C, the denitrification rate increased about 10-fold in non-irrigated plots and three-fold in irrigated plots (Saggar *et al*., 2004b). The positive effect of temperature on denitrification rate was much more pronounced under non-irrigated than under irrigated conditions (Klein and van Logtestijn, 1996; Saggar *et al*., 2004b). Soil temperature in the field was found to limit denitrification rate in all seasons relatively to the denitrification rate measured at 25 °C in the laboratory. This temperature effect was greatest in the cool-wet season (Luo *et al*., 1999). Denitrification losses increased with temperature in pastures treated with cattle slurry, while N-losses from pastures treated with farmyard manure remained unaffected by temperature (Saggar *et al*., 2004b).

Soil condition and type

Several soil-management practices such as soil compaction, tillage and drainage affect the production and transport of N_2O. Emission release is influencing also by the soil's physical conditions, i.e. aeration and soil water content (Saggar *et al*., 2004b). Eckard *et al.* (2010) noted that high N_2O emission rates generally coincide with soil conditions that are conducive to denitrification; nitrification is often an essential prerequisite for the conversion of N fertilizer inputs into soil NO_3^-.

Compaction caused a seven-fold increase in N_2O emission. From the compacted soil, about 10 % of the total N applied as nitrate was emitted, whereas the emission was 0.5 % from the uncompacted soil (Bhandral *et al*., 2003a). In grazed pastures, animal treading is an important cause of soil compaction (Saggar *et al*., 2004). There were no significant differences in emissions among the other three sources (urine, ammonium and urea), which were about one-tenth and one-third of those from nitrate in the compacted and uncompacted soils, respectively (Bhandral *et al*., 2003a).

Porosity has substantial effects on the production of N_2O in soils (Bhandral *et al*., 2003a; Saggar *et al*., 2004a). Exponential increases in flux were occurred with increasing soil water-filled pore space and temperature; increases in soil mineral N content due to fertilizer application also stimulated emissions (Dobbie *et al*., 1999).

Emission of N_2O was higher with no till than with conventional tillage, and with corn than with soybean or alfalfa (Bhandral *et al*., 2003a). A corn system using conventional tillage, legumes in rotation and reduced N fertilizer would decrease N_2O emission from agricultural fields (MacKenzie *et al.*, 1998).

Nitrous oxide emissions from soils are highest when soil aeration is limited (e.g. under wet or compacted soil conditions) and the availability of soil mineral N is high (Klein, 2004). In intensive animal agriculture, high N_2O emission rates generally coincide with anaerobic soil conditions and high soil NO_3^-, primarily from animal urine patches (Klein and Eckard, 2008). However, no effect on N_2O was observed under the aerobic experimental conditions (Leiber-Sauheitl *et al*., 2015). Animal grazing and the use of machinery can also reduce soil aeration due to compaction and pugging, particularly under wet conditions (Klein and Eckard, 2008). Emissions continued over much longer periods (to 60 days) from sandy and stony loams than from a silty clay loam (to 30 days) (Allen *et al*., 1996). The results of Leiber-Sauheitl *et al*. (2015) indicate that sheep excreta do not significantly

increase emissions from degraded peat soils. Fluxes of N_2O between grassland and the atmosphere were measured over 1 year using three plots which have been maintained at a constant pH of 3.9, 5.9 and 7.6 over many years (Yamulki *et al*., 1997). The emissions (3 yrs) from winter wheat and spring barley were lower (0.2-0.7 kg N_2O-N.100 kg^{-1} N applied) than from cut grassland (0.3-5.8 kg N_2O-N.100 kg^{-1} N) (Dobbie *et al*., 1999).

Soil pH plays a critical role in determining the overall rate of several important processes in the agricultural nitrogen cycle. During denitrification, the activity of nitrous oxide reductase (N_2O-R) is reduced at low pH. Liming decreased N_2O production under some conditions with the greatest proportional reductions occurring in the urine amended fluvial soil (McMillan *et al.*, 2016). It was concluded that the microbial community of the soil had adjusted to the low pH and was responsible for the entire production of N_2O. Chemodenitrification is also responsible for some NO_x production, especially at low pH (Yamulki *et al*., 1997). Under laboratory conditions, similar treatments produced large emissions from loam soils having pH of 4.5–6.5 and zero emissions from a peat soil with pH of 3.8 (Allen *et al*., 1996). Mean fluxes of N_2O decreased appreciably with increasing acidity (Yamulki *et al*., 1997). Sterilization of soil cores by autoclaving reduced N_2O emissions almost to zero at all pH values, but residual production of NO_x was found even at low pH. Increasing the pH of unsterilized soil cores from pH 3.9 to 6.5 led to a reduction in NO_x and especially N_2O fluxes (Yamulki *et al*., 1997).

The study of Whalen *et al.* (2000) indicate that most of the microbial potential for nitrification and denitrification (> 90 %) was located in the upper soil horizons, in the 0- to 20cm soil zone depth. Comparisons of the ^{15}N enrichments in the soil mineral N pools and the evolved N_2O suggested that most of the N_2O was produced in the 5–8 cm zone of the soil (Monaghan and Barraclough, 1993).

Field application

N_2O emissions from soil application of animal wastes are a major contributor to total GHG emissions from agriculture (Mihina *et al.*, 2012). N_2O emissions from fertilizer use, manure application and deposition by grazing livestock were estimated at 2,482 Mt CO_2.yr^{-1} in 2010 with an expected 18 % increase by 2020 (Montes *et al.*, 2013). The fundamental problem may be that most animal excrements are being applied to small land areas close to animal confinements, resulting in environmental degradation (Sherlock *et al.*, 2002).

Although land application can supply nutrients for crop production, it leads to gaseous emissions of N_2O, which can be detrimental to the environment (Sharpe, Harper, 1997). Yamulki *et al.* (1998) noticed that the use of livestock excreta increased N_2O emissions significantly above those from the control plots. Field application is considered to be the main source of agricultural N_2O since all manure types significantly increase microbial production of N_2O from soils (Jungbluth *et al.*, 2001; Crosson *et al*., 2011).

Sources of N_2O emissions are indirect - from wastes storage and direct, associated with volatilisation of land applied manures (Ross *et al*., 2014). The fluxes of N_2O emissions vary with waste, time after application, type of application, supplemental water additions and soil type (Sommer *et al*., 1996; Saggar *et al*., 2004b). The variation in the extent of emissions from different types of manure demonstrates the effect of manure properties such as moisture content, total N and available N content on emission generation (Bell *et al*., 2016). N_2O fluxes were enhanced by the fresh dung but not by urine (Hargreaves *et al*., 2015).

Solid manure

The potential for N_2O emission after manure applications to agricultural soil is dependent on a combination of manure properties and environmental conditions (Bell *et al.*, 2016). The results of Allen *et al.* (1996) and Mogge *et al*. (1999) clearly showed that the long-term application of farmyard manure enhanced distinct C-pools in soils available for mineralization and consequently gaseous N-emissions. There were lower nitrous oxide emissions directly from mineral N-fertilizers compared to organic manures.

Mogge *et al*. (1999) measured denitrification N-losses and nitrous oxide emissions from sandy soils. They compared a field fertilized mainly with farmyard manure for 30 years (93 kg N $ha^{-1}yr^{-1}$) with a field fertilized with cattle slurry for 30 years (333 kg N ha^{-1} yr^{-1}) and with grassland (92 kg N ha^{-1} yr^{-1}). Annual gaseous N-losses from field with farmyard manure were twice of those obtained from the other sites (denitrification 4.9 kg N_2O-N. $ha^{-1}yr^{-1}$; nitrous oxide 5.3 kg N_2O-N.$ha^{-1}yr^{-1}$). The smaller N-emissions from the grassland were attributed to its relatively dry siting on a slope of 20 % (Mogge *et al.*, 1999).

Application of cattle dung increased N_2O emissions significantly in comparison to untreated field (fluxes up to 290 µg N.m^{-2} h^{-1}) for period of 15 months (Yamulki *et al.*, 1998). The emission for dung from sheep fed ryegrass during the 3 M measurement (0.13 kg N_2O-N.ha^{-1}) was lower than that from those fed forage rape (0.71 kg N_2O-N.ha^{-1}) (Luo *et al*., 2015).

Urine

According to Di and Cameron (2006), N returns to the soil in animal urine as a major source of N_2O emissions in the intensive animal agriculture. Peak emissions were observed 24-48 h after urea application, high emissions were observed immediately after

the urine application with rates reaching a peak of 89 mg $N.m^{-2}.d^{-1}$ within 6 h, with 7 % of the applied urine-N lost as N_2O over 42 days (Saggar *et al*., 2004b). Large emissions were detected immediately following cow urine application to pasture. These coincided with a rapid and large increase in water-soluble C levels in the soil, some of this increase being attributed to the solubilization of soil organic matter by high pH and ammonia concentrations (Monaghan and Barraclough, 1993). Overall, urine significantly increased N_2O emissions up to 14 days after application, with rates amounting to 6 kg N ha^{-1} d^{-1} (Saggar *et al*., 2004b). Application of urine to the soil (at a rate equivalent to 930 kg $N.ha^{-1}$) increased the amount of mineral and microbial N in the soil. This was followed by increases in emissions of N_2O (from 15 mg N_2O-$N.m^{-2}.d^{-1}$ to 330 mg N_2O-$N.m^{-2}.d^{-1}$) (Williams *et al*., 1998). The initial rate of N_2O release from the urine treatment was significantly higher than that of the other treatments. The average releases were 9.1, 6.9, 2.6 and 2.1 mg.N_2O-N for the urine, urea, ammonium sulphate and control treatments, respectively (Sherlock and Goh, 1983). A detailed study was carried out to investigate the effects of applying animal urine, fertilizer (ammonium nitrate) and fertilizer+urine on emission of NO and N_2O from soil (Williams *et al*., 1998). The fertilizer was applied at a lower rate than the urine. Nevertheless, the fertilizer still increased NO and N_2O emission with denitrification the dominant process (Williams *et al*., 1998).

Denitrification and N_2O emission rates were measured following two applications of artificial urine (40 g urine $N.m^{-2}$) to a perennial rye-grass sward on sandy soil (Klein and van Logtestijn, 1994). Urine application significantly increased denitrification and N_2O emission rates up to 14 days after application, with rates amounting to 0.9 and 0.6 g $N.m^{-2}.d^{-1}$ (9 and 6 kg $N.ha^{-1}$ day^{-1}), respectively. Total denitrification losses during the 14 day periods were 7 g $N.m^{-2}$, or 18 % of the urine-N applied. Total N_2O emission losses were 6.5 and 3 g $N.m^{-2}$, or 16 % and 8 % of the urine-N applied for the two periods. The minimum estimations of denitrification and N_2O emission losses from urine-affected soil were 45 to 55 kg $N.ha^{-1}yr^{-1}$, and 20 to 50 kg $N.ha^{-1}yr^{-1}$, respectively (Klein and van Logtestijn, 1994). The total emission for urine from sheep fed ryegrass during the 3-month measurement was higher (1.19 kg N_2O-$N.ha^{-1}$) than that from those fed forage rape (0.17 kg N_2O-$N.ha^{-1}$) (Luo *et al.*, 2015).

Klein and van Logtestijn (1994) and Koops *et al*. (1997) proposed that the initial stimulation of N_2O emission on urine application could be explained by either chemodenitrification or by anaerobiosis in microsites, as a result of CO_2 generated from rapid hydrolysis of urinary urea. The reason for the stimulated N_2O release caused by urine is unclear. One possibility is a chemical reaction (chemodenitrification) between minor urine components, especially amino acids and soil constituents.

Urine patches are considered to be important sites for N_2O production through nitrification and denitrification due to their high concentration of N. Nitrous oxide production was largest in the centre and decreased towards the edge of the patch. Maximum N_2O production was about 50 mg $N.m^{-2}.d^{-1}$ and maximum denitrification activity was 70 mg $N.m^{-2}.d^{-1}$. Total N loss through nitrification and denitrification over 31 days was 4.1 g N per patch, which was 2.2 % of the total applied urine-N (Koops *et al*., 1997). Variability in N_2O emissions from urine patches can arise due to differences in the urine composition, the amount of N excreted and the volume and frequency of urine events (Dijkstra *et al*., 2013; Marsden *et al*., 2016). Emissions from the urine patches were significantly greater than from the dung. The flux pattern showed a strong diurnal variation with maximum fluxes generally occurring in the late afternoon or early morning, and generally not in phase with the soil temperature changes (Yamulki *et al.*, 2000).

Klein *et al*. (2003) applied cow urine and synthetic urine to pastoral soils. The largest emission factor was found in a poorly drained soil, and the lowest emission factor was found in a well-drained stony soil. The N_2O emissions had not reached background levels 4 months after urine application. In the study of Lovell and Jarvis (1996) urine was added to intact turfs taken from long-term permanent pasture on clay loam and sandy loam soils. The emissions of nitrous oxide following urine application were high (0.36 μg N_2O-$N.m^{-2}$ min^{-1} and 29 μg N_2O-$N.m^{-2}$ min^{-1}) but short-living (< 40 days).

Urine-treated plots (50 g $N.m^{-2}$) were compared to control plots to which only water (12 mg $N.m^{-2}$) was applied. N_2O emission peaked at 88 mg N_2O $m^{-2}.d^{-1}$ 12 days after application. Subsurface N_2O concentrations were higher in the urine-treated plots than the controls. Subsurface N_2O peaked at 500 ppm 12 days after and 1200 ppm 56 days after application (Boon *et al*., 2014).

Cattle urine has been shown to stimulate N_2O production to a larger extent than dung due to the dual effect of a large pool of readily available N and C and increased soil water content (Boon *et al*., 2014). Application of cattle urine increased N_2O emissions significantly over that measured from control (untreated) plots and fluxes up to 192 μg N m^{-2} hr^{-1} from urine were measured over a period of 15 months (Yamulki *et al*., 1998).

Although the total average N_2O-N emissions from the dung (9.9 mg N_2O-N $patch^{-1}$) were equal to those from the urine (9.5 mg N_2O-$N.patch^{-1}$), the average loss from the urine (0.56 %) was much higher than from the dung (0.19 %) (Yamulki *et al*., 1998; Saggar *et al*., 2004b).

Slurry manure

Nitrous oxide emission from grazed dairy pasture was increased following application of slurry (farm dairy effluent). Comfort *et al*. (1990) recorded the largest emission of N_2O occurred shortly after the injection of liquid dairy cattle manure. Maximum loss occurred 5 days after injection. Higher N_2O emission was measured, when slurry was applied immediately after a grazing event. Bhandral *et al*. (2010) found that application of slurry immediately after grazing event creates conducive conditions for denitrification and also might cause priming effect on the soil. Delaying effluent irrigation after a grazing event could reduce emissions by reducing levels of surplus mineral-N. Similarly, Sharpe and Harper (1997), Whalen *et al*. (2000) and Li *et al*. (2015) suggested that emissions decreased to pre-fertilization levels within a few days. Schils *et al*. (2013) found that N_2O loss increased immediately after slurry injection and was followed by a shift in the N_2 emissions.

Slurry injections have been shown to promote conditions leading to denitrification by denitrifying bacteria, creating an anaerobic environment abundant in an inorganic N and readily oxidisable C (Comfort *et al*., 1988). In the study of Rodhe *et al.* (2006), cattle slurry was either injected below the ground or spread out on the soil surface. The injection of slurry gave rise to a broad peak of N_2O emissions during the first three weeks after application.

Lowrance *et al*. (1998) applied liquid dairy manure at four N rates (246, 427, 643, and 802 kg N ha^{-1} yr^{-1}). Denitrification rates and soil N pools increased after manure application at all rates of application. The two highest rates of manure had highest denitrification amounts. Denitrification ranged from 11 to 37 % of total N applied in the manure. Nitrous oxide evolution from the liquid dairy-manure application was often found to be greater than total denitrification from row crops on similar soils fertilised with similar rates of inorganic fertiliser (Saggar *et al*., 2004b).

Emissions of N_2O from waste storages depend on whether dung and urine are collected unmanaged in corrals and paddocks, or stored anaerobically as slurry in pits and lagoons (with or without anaerobic fermentation for biogas collection, or amended with litter in deep litter stables (Oenema *et al*., 2005). Bhandral *et al*. (2003a) and Saggar *et al.* (2004b) showed that untreated dairy-farm effluent resulted in higher emissions (0.447 kg N.ha^{-1}) than treated dairy-farm effluent (0.382 kg N.ha^{-1}) over the experimental period.

Several studies with swine effluents have shown that application of these slurries increases N_2O emissions (Cabrera *et al*., 1994; Bhandral *et al*., 2003a; Saggar *et al*., 2004b). Some unidentified component of liquid swine waste may negatively impact the microbial community, as N_2O emissions were significantly less than for soils amended at a comparable level with a liquid NH_4-N fertilizer. Nitrous oxide emission from fertilization was directly related to the level of fertilization to 150 kg N.ha^{-1} (Whalen, 2000). The direct effects of fertilizer application appear to be more immediate and short-lived for liquid swine waste (9200 μg N_2O-N.m^{-2}.h^{-1}) than for manures and slurries, which have a slower release of nitrogenous nutrients (Whalen *et al*., 2000).

Whalen *et al*. (2000) recorded 395 mg.m^{-2} N_2O-N emitted after applied 29.7 g.m^{-2} (297 kg N.ha^{-1}) of liquid lagoonal swine waste. The fractional loss of applied N to N_2O (corrected for background emission) was 1.4 %, in agreement with the mean of 1.25 % reported for mineral fertilizers (Whalen *et al*., 2000). Piggery effluent emitted the highest proportion of N_2O-N among the effluents used, with emissions of 0.585 kg N.ha^{-1} or 2.17 % of the total added effluent-N (Saggar *et al*., 2004). Increased N_2O emission from a spray field fertilized with liquid swine waste was due to the interactive effects of increases in soil moisture and N (Whalen, 2000).

Application timing

Nitrous oxide emission varies with the nature of the effluent applied. Nitrous oxide emissions from land-applied effluent are highly dynamic and affected by application time, application method and rainfall or irrigation (Li *et al*., 2015). However, the dominant environmental factors influencing N_2O losses include wind speed and temperature (Li *et al*., 2015). Following field application, infiltration of liquid is influenced by manure organic matter (Petersen and Sommer, 2011).

According to Bell *et al.* (2016), the timing of application can be critical if significant losses of N from the soil are to be avoided. Conversely, loss of N via N_2O emissions is higher when manure is applied under wet conditions as N_2O production via denitrification will occur before the crop is able to utilise the available N. A proportion of N that volatilises as NH_3 is considered to be re-emitted as N_2O upon wet or dry deposition to soils from N excretion by animals (Crosson *et al*., 2011).

A number of studies have shown that soil denitrification and N_2O emission rates are highly variable throughout the season, with high rates being associated with grazing and fertiliser application in grazed pastures (Ruz-Jerez *et al*., 1994; Williams *et al*., 1998; Luo *et al.*, 1999; Saggar *et al*., 2004a,b). The highest losses by denitrification occurred in winter when soil moisture was at or above field capacity for extended periods (Ruz-Jerez *et al.*, 1994).

Manure application timing (fall vs. spring) had no effect on N_2O emissions for the annual system. Spring application has been recommended as a mean to mitigate N_2O because it avoids the high N_2O fluxes related to spring thaw and the N is supplied to a growing crop that may reduce soil mineral N availability for nitrifiers and

Table 1: N_2O emission from land application of manure and effluent or slurry

N_2O flux, spring; fertilized grassland vs. fertilized winter wheat; spread over 2 ds, closed chambers; 0 to 134 g N_2O-N ha^{-1} d^{-1} vs. 0 to 26.4 g N_2O-N ha^{-1} d^{-1} (Ball *et al.*, 1997).

Fluxes, onion (*Allium cepa* L.) field, 100 gas samples, plastic CCH, GC; 331 µg N m^{-2} h^{-1}, CV 217 % (Yanai *et al*., 2003).

Pigs SLR, during storage and after field application (permanent grassland); 5 MS, 10 m^3 slurry tanks; twice a wk, ODC, FTIR, ds 1, 2, 4, 8, 13, 20, CCH, GC; untreated 56.2 g.m^{-3} (100.0 %), separated 41.3 g.m^{-3} (73.5 %), anaerobically digested 77.2 g.m^{-3} (137.5 %), straw covered 167.5 g.m^{-3} (298.2 %), SLR aerated 558.6 g.m^{-3} (994.5 %) (Amon *et al*., 2006a).

Dairy cattle SLR, during storage and after field application (grassland); 5 MS, 10 m^3 SLR tanks; twice a wk, OCH, FTIR, ds 1, 2, 4, 8, 13, 20, CCH, GC; untreated 23.9 g.m^{-3} (100.0 %), separated 28.6 g.m^{-3} (119.7 %), anaerobically digested 31.2 g.m^{-3} (130.3 %), straw covered 52.5 g.m^{-3} (219.7 %), SLR aerated 54.2 g.m^{-3} (226.8 %) (Amon *et al*., 2006a).

Intensively farmed pastures, silt loam; 1 h, CCH, GC; from 0 to 100 kg N.$ha^{-1}$$d^{-1}$, annual emission 3-5 kg N_2O-N.ha^{-1} (Carran *et al*., 1995).

Pigs and dairy SLR; 3 application, grassland, April, July, October (50 m^3.ha^{-1}); measured 20, 22, and 24 d after application; April, dairy SLR 1.51 kg N_2O-N.ha^{-1} vs. pig SLR 0.77 kg N_2O-N.ha^{-1}; July, dairy SLR 0.34 kg N_2O-N.ha^{-1} vs. pig SLR 0.57 kg N_2O-N.ha^{-1}; October, dairy SLR 0.15 kg N_2O-N.ha^{-1} vs. pig SLR 0.74 kg N_2O-N.ha^{-1} (Chadwick *et al*., 2000).

Pigs SLR, application, pasture, 90-d period (60 m^3 ha^{-1}, 6.1 kg total N m^3, pH of 8.14), d 25: 7.5 g N.$ha^{-1}$$h^{-1}$, d 67: 15.8 g N.$ha^{-1}$$h^{-1}$, background levels after 90 ds, total 7.6 kg N ha^{-1} (2.1 % of the N applied) (Sherlock *et al.*, 2002).

Pigs SLR, 12 ha oat field, 150,000 plants.ha^{-1}; sprinkler irrigation, 29,000 m^3 applied, 3 irrigations (ds 94, 101, and 108 of yr); TDL, from 19 g N_2O-N ha^{-1} d^{-1} to 0.25 to 0.38 kg N_2O-N ha^{-1} d^{-1} after first 2 irrigations, total 4.7 kg N_2O-N ha^{-1} (Sharpe, Harper, 1997).

Pigs SLR, 1,200 sows, farrow-to-finish swine farm; applied to soybean, pH 8.0, 3 irrigations (237, 242, 246 ds of yr), summer, total N in effluent 556 µg.g^{-1}, 537 µg.g^{-1}, 590 µg.g^{-1}, amount applied 144.6 kg N.ha^{-1}, 59.1 kg N.ha^{-1}, 70.8 kg N.ha^{-1}, total applications 274.6 kg.ha^{-1} of total N; TDL; before effluent applications 0.016 g N_2O-N.ha^{-1} d^{-1}, increased to 25 N_2O-N.ha^{-1} d^{-1} to 38 g N_2O-N.ha^{-1} d^{-1} after irrigation, total emissions 4.1 kg N_2O-N.ha^{-1} (Sharpe, Harper, 2002).

3 grassland fields (dairy cows, young stock or cut for silage); 240 N.kg.ha^{-1}, 170 N.kg.ha^{-1}, 196 N.kg.ha^{-1} during the 6 M prior study; CCH, 117.9 ng N.m^{-2}.s^{-1}, 243.5 ng N.m^{-2}.s^{-1}), 7.05 ng N.m^{-2}.s^{-1} (Hargreaves *et al*., 2015).

Sheep urine, male Romney lambs, fed ryegrass vs. brassica rape; applied (perennial ryegrass/white clover pasture, poorly drained silt-loam soil); 3 M, CCH, GC; 1.19 kg N_2O-N.ha^{-1}, 0.17 kg N_2O-N.ha^{-1} (Luo *et al*., 2015).

Sheep dung, male Romney lambs, fed ryegrass vs. brassica rape; applied (perennial ryegrass/white clover pasture, poorly drained silt-loam soil); 3 M, CCH, GC; 0.13 kg N_2O-N.ha^{-1}, 0.71 kg N_2O-N.ha^{-1} (Luo *et al.*, 2015).

Abbreviations

C = carbon
CCH = closed chamber
CO_2 = carbon dioxide
CV = coefficient of variation
d = day
ds = days
FTIR = Fourier transform infrared spectroscopy
GC = gas chromatography
h = hour
M = month
MS = manure system
N = nitrogen
NH_3 = ammonium
ODC = open dynamic chamber
SLR = slurry
TDL = Tuneable Diode Laser absorption spectrometer
yr = year
yrs = years
wk = week
wks = weeks

denitrifiers (Chadwick *et al*., 2011). Abalos *et al.* (2016) found that N_2O fluxes associated with freeze-thaw events were reduced when manure was applied in spring. However, applying manure in spring also implies higher N availability when soil temperature is rising and rainfall events are frequent, enhancing soil microbial activity. As a consequence, the highest N_2O peaks of the experimental period were measured for the spring treatment after significant rainfall events (Abalos *et al*., 2016). Bell *et al.* (2016) applied slurry and farmyard manure of cattle, broiler litter and layer manure on winter wheat. Mean annual N_2O emissions were greater in autumn (2 kg N_2O-N.ha^{-1}) than in spring (0.35 kg N_2O-N.ha^{-1}) applications, and in the spring experiment they were significantly lower from cattle slurry than from other treatments.

Allen *et al*. (1996) measured nitrous oxide (N_2O) emissions from different soils under grass after treatment with cow dung and urine in two seasons. N_2O emission rates were much higher during autumn-winter than during spring-summer, and in the case of well-drained soil were substantial for both excreta types (207 mg N_2O-N.kg^{-1} of deposited dung and 197 mg N_2O-N.kg^{-1} of urine in autumn-winter). The ratio of nitrogen released as N_2O to the amount of N excreted by the livestock varied from 0 % (summer) to 0.8-2.3 % (winter), consistent with loss rates observed for mineral fertilizers (Allen *et al*., 1996).

The evaluation models must include adequate flexibility to predict cold soil emissions as well as emissions under tropical conditions. Some recent studies indicate that many of the published direct estimates of N_2O emissions from agricultural fields may be further underestimated because they did not account for cold season N_2O emissions, which can be substantial (Mosier *et al*., 1998ab).

CONCLUSION

N_2O emissions from soils are the largest contributor to global greenhouse gas emissions. Nitrous oxide emissions are moderated by many factors, but the most important are temperature, water-filled pore space, available soil carbon, soil pH and soil nitrate. Nitrous oxide emissions from soils are highest when soil aeration is limited (wet or compacted soil conditions) and the availability of soil mineral N is high. Recent studies have shown that avoiding soil compaction or increasing soil drainage can substantially reduce N_2O emissions from soils.

Animal wastes from husbandry are an excellent nutrient source. Applying manure or effluents to agricultural fields has been shown to increase crop yields, improve the water-holding capacity of the soil and enhance soil fertility.

However, for efficient use of manure some considerations have to be made. Technology for applying manure or effluents to the land must take into account external factors when predicting N_2O releasing following manure application. Manure applied to frozen soil may be carried off to lakes and streams during thaws or during winter or early spring rains. To minimize this risk when soils are frozen, manure has to be applied only to relatively flat fields. N_2O volatilization can be reduced considerably by the use of optimum application with shallow injection compared with surface spreading. Conditions favouring N_2O losses are more often met in fall and winter than in spring and summer. Manure should be applied as soon as possible after plant cutting to reduce potential injury to the re-growth; applying when the soil is not wet, manure spreaders cause compaction on the wet soils.

An extended review revealed that more data are needed to better quantify emissions from manure management, therefore, a further research is required.

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AGRONOMIC FACTORS AFFECTING PRODUCTIVITY AND NUTRITIVE VALUE OF PERENNIAL FODDER CROPS: A REVIEW

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ABSTRACT

Perennial fodder crops such as legumes or grasses play an important role in ruminant nutrition all over the world. In the regions where area of permanent grassland is limited represent the very important protein and digestible fiber source. For successful forage production, high forage value of stand is generally required. This value consists of two parts: forage yield and quality. In spite of forage quality evaluating by a range of parameters, forage dry matter yield is only one-dimensional variable. It must be remembered yield is a key factor in forage production, especially for economic efficiency in relation to cost per hectare. Species selection, appropriate harvest frequency, level of fertilization and methods for estimate of qualitative traits were considered due to their practical impact. Changes in protein fractions and alternative forage usage were also included because they are associated with post-harvest forage utilization. It can be summarized that agronomic decisions before harvest have a high impact on both forage yield and quality.

Key words: forage quality; agronomic factors; fodder crops

INTRODUCTION

There is a range of factors influencing both forage yield and quality. The aim of this review is not list all of them but provide basic knowledge about these with the highest impact in the farming practice. It can be summarized that successful forage production of perennial fodder crops depends on achieved biomass yield and high forage quality. In this review, we would like to highlight the importance of agronomic decisions on harvested forage quality before start of conservation process. Although forage yield at the time of animal feeding is aloof interest, it contributed substantially to economic efficiency of animal production.

Suitable species/cultivars selection

Effective forage production cannot be naturally the same across different environments. In this regard, species or cultivars selection generally represents simple but highly effective tool for adaptation of feedstuff production to environment conditions. For example, in temperate zones, biennial or perennial legume or grass species are the most common whilst annual species can be in the first place in regions with intensive drought or frost period. For condition of the Europe, the most important traditional forage legume crops are lucerne (*Medicago sativa*) and red clover (*Trifolium pratense*). These two crops have complementary production responses to climatic conditions, where lucerne is high yielding in dry whilst red clover in wet conditions (Peterson *et al.*, 1992). In this zone, the important grass species are perennial and Italian ryegrass (*Lolium perenne* and *L. multiflorum*), meadow and tall fescue (*Festuca pratensis* and *F. arundinacea*), timothy (*Phleum pratense*) and orchard grass (*Dactylis glomerata*). There is a range of other grass species important for specific environments. Properties of all mentioned species are described in detail by Frame *et al.* (1997). Selection of proper species or composition of mixture of species remains the basic tool for successful start of forage production.

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Breeding process is intended to improve the properties of the selected species important for human civilization. Yield improvement in forage crops during the last century has lagged far behind that of annual grain crops (Brummer, 1999) because breeders changed rather harvest index than total biomass production, which is an explanation for low yield progress of perennial forage crops. Lamb *et al.* (2006) concluded that evidence for changes in lucerne forage yield for cultivars released between 1940 and 1995 was environmentally dependent. In environments where conditions lead to plant stand losses, recently released cultivars with multiple disease resistance had a yield advantage over older cultivars, but in environments where no differences in plant density occurred, older cultivars yielded the same as the improved new cultivars.

Except for this slow but continuous improvement of yield or quality, there were also some milestones in this process for perennial forage crops. The following is an example with high practical impact on forage production. It has long been a goal for forage breeders to combine the stress tolerant characteristics of *Festuca* species with the earliness and high nutritive value of *Lolium* species. Some breeding programmes have been designed to transfer *Festuca* genes into *Lolium*, and as a result some *Festulolium* cultivars have been developed in Europe and in the USA (Humphreys *et al*., 2003). *Festulolium* provides specialized function and novel alternatives to existing grass species/cultivars that may lack resilience against abiotic or biotic stresses.

The following example represents a case of high perspective breeding method for improving forage quality. Lignin is defined as a complex organic compound that binds cellulose fibres and hardens and strengthens the cell walls of plants. This process accelerates as plants mature and gives structural support to the plants as they become taller. Regarding to animal nutrition, lignin is well-known as highly important anti-nutritive substance which is still essential for plant functions. Due to its negative effect during digestion, experiments with genetically reduced lignin synthesis have been made in various plant species. According to Shadle *et al*. (2007), analysis of lucerne forage quality parameters showed strong reductions of neutral- and acid-detergent fibre in the down-regulated lines, in parallel with large increases (up to 20 %) in dry matter forage digestibility. Reduction of hydroxycinnamoyltransferase (HCT) enzyme activity in these lines was from at least 15–50 %. The most severely down-regulated lines exhibited significant stunting, reduction of biomass yield and delayed flowering. Vascular structure was impaired in the most strongly down-regulated lines. Although manipulation of lignin biosynthesis can greatly improve forage digestibility, accompanying effects on plant development need to be better understood. In spite of these distresses, first low lignin lucerne cultivar was released for the commercial utilization by Aflorex Seed Company. In these "Hi-Gest" cultivars, content of lignin is reduced by 7 – 10 % by natural selection without declared negative impact to agronomic traits. The grower has two general harvest options available when growing lucerne with this new technology: (1) harvesting fields on a normal ~ 28 day cutting schedule to produce a high quality forage that has increased fibre digestibility and higher animal intake; (2) extend the peak harvest date by up to 7 days to ~35 days versus 28 days. This option utilizes the low lignin trait as a means of increasing yield without sacrificing forage quality. If a field is ready to be cut but rainy weather is forecast then harvest can be delayed up to 7 days to avoid rained-on forage. This flexibility at harvest time helps the producer minimize the effect of improper weather and reduced forage quality. Synthetic cultivars harvested at the later date would have lower forage quality due to its maturity and higher lignin content.

Harvest frequency

Optimal stand utilization is very important in terms of both yield and forage quality. It is well known that higher cut frequency improves nutritive value of harvested forage because of reduced stem weight proportion and its better digestibility in relation to lower lignification. However, it must be remembered that more intensive cut regime reduces stand yield and persistence. Regarding to yield, our results show that four-cut regime obtained significantly lower yield than three-cut in Central Europe region but this reduction represented only 4 –5 % (Hakl *et al.*, 2011). For this environment, it seems that one cut over standard intensity of utilization only slightly reduces yield but provides high potential for improving forage quality. The adverse effect of intensity of lucerne harvesting on persistence and the following spring regrowth has been historically attributed to a reduction in the concentrations of organic reserves, especially total non-structural carbohydrates. For this purpose, it must be carefully distinguished between effect of number of cuts per year and their schedule over year. The regrowth interval between the last summer harvest and the autumn harvest is the major determinant of lucerne persistence and spring regrowth (Dhont *et al.*, 2004). In Central Europe, this interval was traditionally expressed as number of days which should be at least 50 days. The accumulation of growing degree days > 5° after the last summer harvest has been proposed as a criterion to estimate the duration of this interval (Bélanger *et al.*, 1992). For investigation in Europe conditions, field experiment was conducted in Central Bohemia in 2002–2004. In this experiment, the interval between summer and last autumn harvest was 40–50 days or 60–70 days, respectively. These intervals were expressed as cumulative growing degree-days (GDD)

where GDD values ranged from 540 to 905 over three years period. The plants were sampled in each autumn with four replicates for each variant; the average depth of sampling was 150 mm. The weight of roots, amount of starch, and water soluble saccharides (WSC) per m^2 was determined. The total accumulation of root reserve saccharides was determined mainly by conditions over growing period in particular year. The length of the interval or cumulative GDD influenced only variation of this basic amount. It was documented by significant differences among evaluated years in dependence on weather condition and following stand development. Table 1 shows reduction of starch concentration and amount of all reserves at early harvest interval in 2002. GDD was very high at both intervals in 2003 which resulted in no significantly different amount of root reserves between intervals. In 2004, higher GDD value was obtained at early interval in spite of lower number of days which resulted in significantly higher concentration of starch. Total amount of root and root reserves was not affected by length of the interval. In Central Bohemia condition, the GDD around 600–700 ºC was preliminarily determined for maximal accumulation of root reserve saccharides. The GDD above this level did not significantly increase the root reserve accumulation.

Fertilization

Intensive agricultural cropping system requires large quantities of plant nutrients (Lloveras *et al*., 2012) which highlight importance of suitable fertilization management. It can be simply assumed that lack of nutrients in the soil significantly reduces forage yield. The impact of forage legumes fertilization has been traditionally focused on effects of direct application of phosphorus (P) and/or potassium (K) in various combinations (Macolino *et al*., 2013) whereas direct application of nitrogen (N) is not usually effective due to N fixation by legumes. Regarding to grass fertilization, Huhtanen and Broderick (2016) concluded that this step should be optimized according to crop DM yield with no benefits from increased N fertilization in nutritive value. In spite of intensive previous research about lucerne fertilization, there is a lack of long-term studies investigating indirect effect of organic and N fertilization on yield within applied crop rotation. At present, we can investigate differences in forage yield under different combination of mineral (6 treatments) and organic (3 treatments) fertilization in long-term experiment conducted since 1955 in Ruzyně (Hakl *et al*., 2016b). Long-term absence of fertilization provided average annual dry matter yield 8.64 $t.ha^{-1}$ (Figure 1). Indirect application of mere manure or slurry significantly increased yield to 9.68 and 9.37 $t.ha^{-1}$, respectively. The highest values of dry matter yield (DMY) over 10 $t.ha^{-1}$ were observed at treatments, where organic fertilizers were applied at N3P2K2 and N4P2K2 treatment, however the same value was also observed at application of manure under N1P1K1 treatment. These results reveal that not only direct but also indirect fertilization substantially influenced lucerne DMY (Hakl *et al*., 2016b). Effect of fertilization is generally more obvious for yield than forage quality and there are only few studies about effect of fertilization on nutritive value of perennial fodder legumes. According

Table 1: Effect of length of regrowth interval between the summer and autumn harvest on concentration and amount of starch and water soluble saccharides in lucerne roots over three year period (adapted from Hakl *et al*., 2008)

	2002			2003			2004		
	early	late	P	early	late	P	early	late	P
Interval:GDD	540	850		693	905		734	621	
Interval: days	43	72		42	67		54	63	
concentration (g.kg DM^{-1})									
starch	104[a]	126[b]	0.0045	174[a]	153[b]	0.0022	199[a]	142[b]	0.0049
WSC	144	147	0.5766	193	188	0.3492	141	150	0.6663
amount (g $DM.m^{-2}$)									
starch	17[a]	33[b]	0.0004	22	22	0.9090	38	30	0.5263
WSC	23[a]	39[b]	0.0016	24	27	0.3782	27	31	0.4543
total	40[a]	72[b]	0.0008	46	49	0.5884	65	61	0.5841
root	158[a]	261[b]	0.0006	125	144	0.2686	190	212	0.6367

P = probability of F test, different letters document statistical differences in each column (Tukey HSD, $\alpha = 0.05$).
GDD - growing degree-days, WSC - water soluble saccharides, DM – dry matter

to Lissbrant *et al.* (2009), low P and K soil fertility reduced fibre concentrations in the lucerne forage. This is in line our preliminary results from long-term experiment in Ruzyně, where variable fertilization resulted in different stand structure. The highest plant density was observed in control, slurry or manure treatments. Increasing rate of N reduced plant density but maintained stem density up to N3 level. Intensive fertilization also increased stand height which was in line with lower leaf weight ratio. These investigations suggest explanation for reduced forage nutritive value under higher nutrient supply described by Lissbrant *et al.* (2009). Further research is warranted to identify the influence by which long-term fertilization management affects lucerne yield components, nutrients content and digestibility within separate lucerne leaves and stems.

Forage quality prediction

Timing of the forage harvest is critical for obtaining optimal quality for animal production. For forage crops that serves as the primary fibre source in the diet, NDF is the principal forage quality variable of concern (Parsons *et al.*, 2006a). Some predictive equations can be used to estimate the forage quality, assisting the producers in decision making at harvest time. Parsons *et al.* (2006b) described an ideal method for estimating quality in the field as a harvest decision aid must be quick, simple, inexpensive, and consistent across all harvests during the season and across a wide range of environments. The most widely used of these are the predictive equations for lucerne quality (PEAQ). This method is based on the length of the tallest stem and the stage of the most mature stem in the sample (Hintz and Albrecht, 1991). These equations have been developed for many regions of the USA. Results indicated some bias in using the equations outside the state of development; however, the prediction errors have been sufficiently low to suggest the PEAQ equations are robust over a wide range of environments (Parsons *et al.*, 2006a). GDD are a temperature-derived index representing the amount of heat to which plants are exposed. It was used similarly to assess length of interval between harvests which was mentioned above. This method has been used with mixed success with the perennial types of forage (Sulc *et al.*, 1999). In the Czech Republic, these methods have not been tested for any perennial forage crops; therefore Hakl *et al.* (2010) tested their accuracy and suitability for lucerne prediction within the first cut period in Central

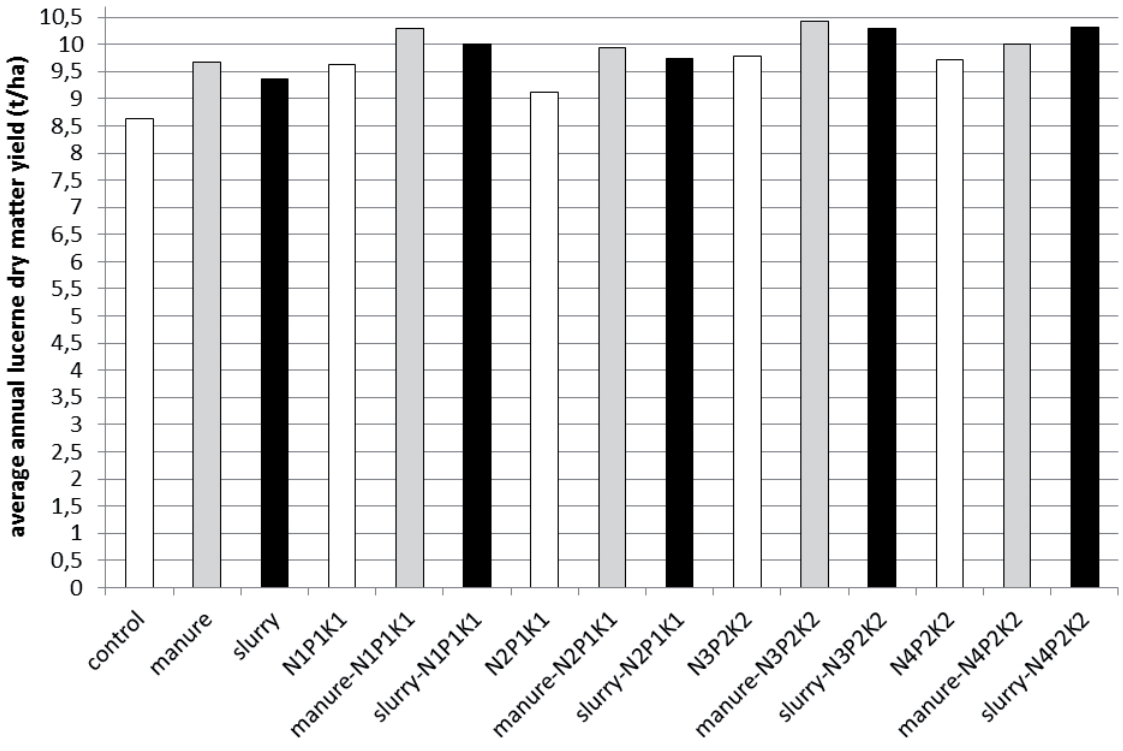

Fig. 1: Lucerne annual dry matter yield (t.ha^{-1}) after 60 years of various nutrient applications (adapted from Hakl *et al*., 2016b)

Bohemia. Their results revealed higher accuracy for PEAQ in comparison with GDD. Suitability of PEAQ method was later reported by Anderzejewska *et al.* (2013) also for northern Europe. Further research has shown that the developmental stage was not suitable indicator for forage quality in year with untypical weather condition (Figure 2). The best solution was a combination with stem length with clear relation to crude fibre content whilst a lower relation was observed to crude protein content. For optimal lucerne quality, the term of first harvest should be in a bud stage when the stem length is to 60 – 65 cm (Hakl *et al*., 2012b). Recent investigations in this research area has shown that canopy reflectance (i.e., remotely sensed) data may allow rapid assessment of nutritive values, such as total N, neutral detergent fibre (NDF), and acid detergent fibre (ADF) of lucerne. The remote sensing based prediction equations explained from 78 to 83 % of the variation in measured total N, NDF, and ADF, correctly predicted about 78 % of the measured TDN/CP ratios. This technology could help improve profit margins by timing the cutting or harvesting of lucerne, in rapid assessment of nutritive values over large areas devoted to growing lucerne, and assessing nutritive quality in real time (Starks *et al.,* 2016).

Forage conservation

Forage conservation cannot be excluded from group of highly important factors affecting quality of feed for animals; however its impact is strongly limited in terms of forage quality before conservation. In this case, we properly cannot talk about increasing of quality but only about decreasing nutrients or quality losses during conservation process. Despite this limitation, the conservation of forage crops is one of the most risk-intensive processes undertaken by farm managers. From the time of harvest until it is used as feed, it is subject to significant losses both in quantity and quality. These losses occur during harvesting and field operations, and later during storage and handling of the product. To minimize the risk associated with forage conservation it is important to understand these processes, how they interact with one another, and how their effects can be mitigated through various management practices. These management tools were summarily described for example by Moore and Peterson (1995).

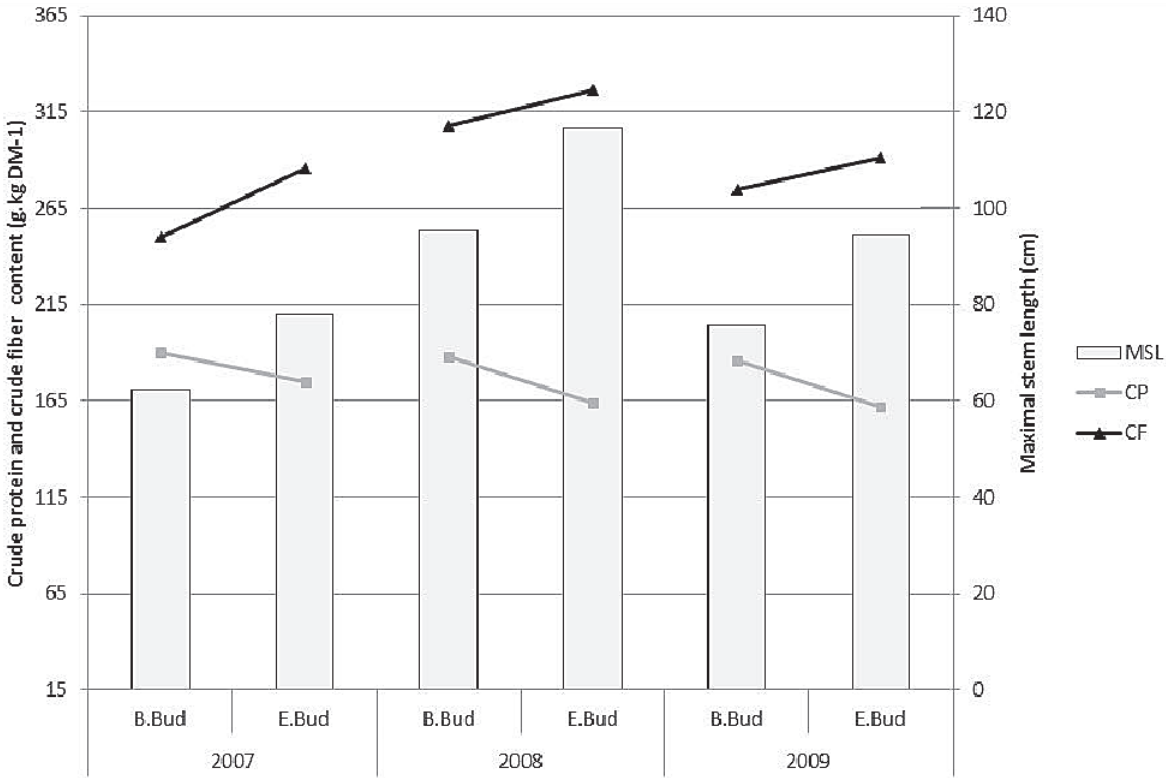

Fig. 2: Lucerne maximal stem length (cm), crude protein and fibre content (g.kg DM^{-1}) in the first cut (3-year period, site Červený Újezd, adapted from Hakl *et al*., 2012b)
MSL – Maximal stem length, CP – crude protein, CF – crude fiber

Protein utilization

Contrast to previous topics, this theme covers area about specific evaluation of legume forage quality in connection with animal utilization. According to our opinion, it is a very hot and highly important topic and therefore included in this review. Forage legumes such as lucerne or red clover represent a major protein source for ruminant nutrition in Europe (Krawutschke *et al.*, 2013). Protein degradability in forage legumes is of global importance because utilization efficiency of forage has economic and environmental consequences. Rumen protein degradation and the resulting imbalance between carbohydrate and protein supply leads to lower N-use efficiency by ruminants (Broderick, 1995). Increasing the amount of protein that escapes from the rumen could benefit ruminant nutrition and improve the economics of the dairy industry (Chen *et al.*, 2009). The most commonly studied factors affecting protein fractions include plant species and harvest maturity (Kirchhof *et al*., 2010; Krawutschke *et al*., 2013). Previous studies (e.g. Lemaire *et al.*, 2005) have shown that N concentration in forage is closely related to plant morphology in the lucerne stand. In spite of it, in almost all published studies that investigated CP fraction of legumes, information on stand traits was not presented (e.g., Kirchhof *et al*., 2010; Krawutschke *et al*., 2013). Therefore, we hypothesize that changes in plant morphology within a dense canopy could also be connected to variation in CP fractions. Within two year period, lucerne leaf and stem samples were taken in the three cuts and plant density, stem density, maximal stem length and leaf weight ratio were assessed. All dried stem and leaf samples were milled to pass through a 1 mm screen and analysed for CP fractions according to Licitra *et al.* (1996) where protein content was fractionated into A and B_1 (soluble fractions), B_2, and insoluble fractions B_3 and C. Recently published results of Hakl *et al.* (2016a) suggest that stand traits make an important contribution, accounting for about 75 % of CP fraction variability. Above all, maximal stem length is a variable that can be easily assessed for individual plants and has a strong negative correlation with leaf weight ratio, which is assessed as less easily than maximal stem length. The findings of this research indicate that plant morphology should be considered, particularly when evaluating the genetic variability of the CP fraction within legume species (Tremblay *et al*., 2003) or measuring protein composition among lucerne cultivars (Chen *et al.*, 2009).

Alternative forage utilization

Traditional utilization of forage biomass is connected with ruminant nutrition. In many European countries, decrease of number of cattle units in connection with recently low milk production profitability make an issue with utilization of produced forage (e.g. Stypinsky *et al.,* 2009). This is a key problem for permanent grassland because grassland area cannot be reduced due to environmental impact in landscape. In the arable land, perennial fodder crops simply are not included in a crop rotation. However, absence of these crops together with lower production of organic fertilizers has negative impact on soil fertility and balance of organic matter. For this reason, researchers are looking for alternative utilization of these crops for various purposes. For example, there is a tendency for utilization of forage legumes as a protein source for monogastric animals, pharmacy or human nutrition. In spite of these minor possibilities, the major activity is energy production from forage biomass because generation of energy from biomass has a key role in current EU strategies to enhance energy security. At present, biogas production from energy crops in the arable land is mainly based on the anaerobic digestion of maize. Maize achieves the highest methane yield per hectare in comparison with cereal or sunflower (Amon *et al.,* 2007). On the other hand, it must be noted that maize cultivation is limited in some areas and can have some negative impact on environment as higher pesticide and fertilizers are required. Maize fields are, in general, relatively vulnerable to both water and wind erosion (Graebig *et al*., 2010).

Unlike maize, biogas production from lucerne or clover forage is not a common practice. Legume crops could also be a suitable source for biogas production and it is generally accepted that their cultivation significantly improves soil fertility in contrast to maize cultivation. According to Walla and Schneeberger (2006), lucerne grass mixture is a more efficient energy crop than silage maize on organic farms. Forage legume stands seem to be a suitable biomass source because of its persistency, high productivity, self-sufficiency of N_2 and positive impact on soil fertility. According to Amon *et al.* (2007), specific harvest and processing technologies are required when crops are used as a renewable energy source compared to growing them as a forage source for ruminants. The traditional harvest management for livestock feed recommend the cut term in the bud stage in relation to high quality of forage (Hakl *et al.,* 2010). In contrast, the suitable harvest management of lucerne in a biogas production system is unknown. It must be taken into account that a two cut management system produced more total forage than a three- or four- cut management system harvested at early bud (Lamb *et al.,* 2003). The impact of changes in lucerne biomass quantity and quality under different harvest management could be different for biogas production in comparison with animal utilization.

For clarifying these relationships, biogas production from lucerne biomass was tested over two years in a field plot experiment (Hakl *et al.*, 2012). Biomass was tested in 120 ml bottles in five replications

for each variant. After basic homogenization and grinding of fresh matter, two grams of tested biomass and 80 ml of inoculum were dosed into fermentors. Active mesophile anaerobic sediment from biogas plant was used as the inoculum. Cultivation took place in thermo box at 40 °C for a period of 40 days. Production of biogas in laboratory tests of biomass was evaluated once a day, using gas-metric burette. In figure 3, values of substrate biogas yield were in wide range of 423 to 648 L.kg^{-1} DM. When 10 % as average ash content in lucerne forage and 60 % methane content in biogas is considered, methane yield from 280 to 430 L CH_4.kg$^{-1}_{OM}$ could be obtained. This range corresponds with results published by Amon *et al*. (2007) about methane yield from other energy crops. The average methane yield 398 L CH_4.kg$^{-1}_{OM}$ was obtained from maize silage whilst from wheat it ranged between 140 and 343, from sunflower between 154 and 454, and from grassland between 128 and 392 L CH_4.kg$^{-1}_{OM}$. As was noted by Prochnow *et al.* (2009), the aim of energy crop for biogas production is to achieve the highest possible methane yield per hectare. Results show that area biogas yield from lucerne forage could be significantly increased by change in harvest management towards delayed cuts. It is in accordance with Lamb *et al*. (2003), that harvesting twice per season at a later maturity stage would be an effective management strategy for maximizing yield in a lucerne biomass energy production system. In our study with biogas production, the average increase of yield in late flower stage was relatively stable across the year and achieved approximately 50 and 35 % in the first and second cut, respectively. In spite of substrate biogas yield higher than 25 % in the bud stage in 2009, the higher area biogas yield was produced in late bloom stage. This results in increasing area biogas yield in spite of decrease in substrate biogas yield support idea, and requirements on the biomass quality are different when crops are anaerobically digested in biogas plants compared to being fed to cattle. The reason could be that the digester at the biogas plant offers more time to degrade the organic substance than the rumen does. Another important point could be a different micro-organism population in the digester (Amon *et al.,* 2007) or the fact that higher proportion of NDF in the forage does not result in lower dry matter intake in the case of biogas plant. In this experiment, lucerne reached lower methane yield per hectare in comparison with maize and probably would not play a dominant role in biogas production from crops growing on arable land. Nevertheless, the methane yield of lucerne seems to be higher or comparable with other crops as cereal or sunflower and lucerne cultivation could be a suitable supplement for biogas production due to lucerne's non-productive function with positive impact on soil fertility and reduction of soil erosion.

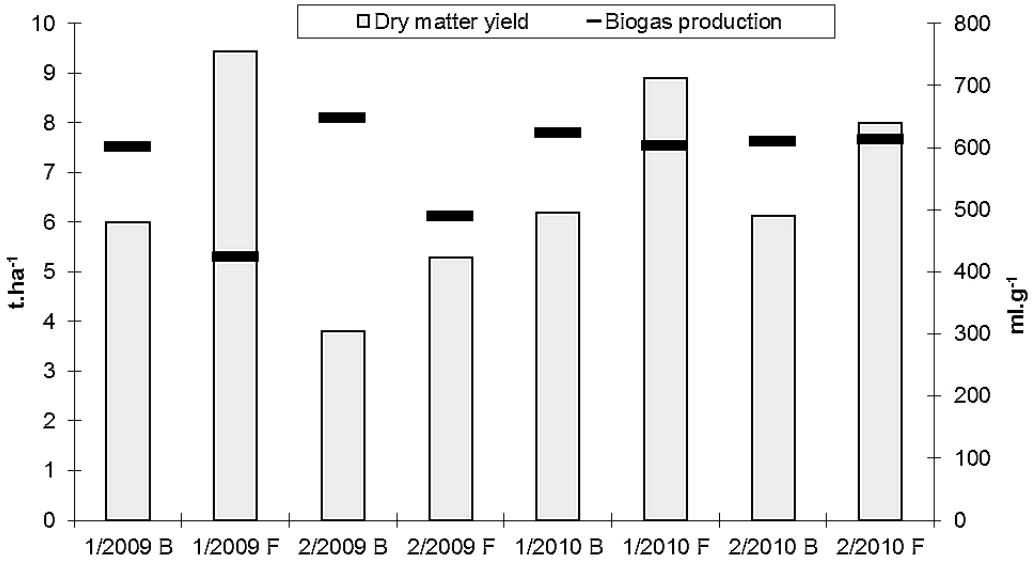

Fig. 3: Lucerne dry matter yield (t.ha^{-1}) and substrate biogas production (ml/g) in first (1) and second (2) cut at bud (B) and flower (F) stages in 2009 – 2010 (adapted from Hakl *et al*., 2012a)

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ADDITIVES FOR GRAIN SILAGES: A REVIEW

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ABSTRACT

Microbial inoculants have been used as a tool to improve the fermentation and aerobic stability (AS) of high moisture grain silages. To access the effects of additives in high moisture corn silages (HMCS), thirty-five scientific papers were reviewed. Other six scientific papers were used to investigate changes in winter cereal grain silages (HMWCS). Application of chemical additives in HMCS preserved WSC due to inhibition of fermentation. Yeast growth was efficiently controlled, reducing ethanol production and linearly increasing AS. The HMWCS treated with chemicals showed a marked reduction in fungal growth and in ethanol formation, and a higher AS. The inoculation of HMCS with homolactic bacteria decreased silage pH by 0.26 unit and decreased proteolysis, but did not promote AS. The HMCS inoculated with heterofermentative strains had lower WSC and higher content of weak acids with antifungal properties, reducing mold and yeast counts and increasing AS. Maximum improvement in AS was achieved when heterofermentative bacteria were applied at 4.67×10^5 cfu.g^{-1} ($P < 0.01$, $R^2 = 0.50$). The combination of homo and heterofermentative bacteria in HMCS ensured a lower pH and decreased yeast counts and ethanol production, whereas AS was not changed. Since fermentative losses were usually low, we conclude that the use of chemical additives and heterofermentative bacteria are justified to improve AS of high moisture grain silages.

Key words: high moisture corn; winter cereals; microbial additives; chemical additives; aerobic stability

INTRODUCTION

Ensiling is an efficient strategy for grain storing and processing. Improvements on the nutritive value and the lower costs compared to other processing methods has stimulated the use of high moisture grain silages (HMGS). The typical lower field/harvesting losses accompanied by early harvesting are considered side advantages, which may increase farming efficiency. Insect and rodent damages typically observed in dry grains are also reduced by adopting HMGS. Additionally, HMGS allows the use of homegrown, traceable (source-verified) feedstuffs instead of purchased concentrates. However, it will constraint the cash crop at farm level because of the wet storage.

To exploit the benefits of HMGS, a proper management is mandatory to minimize fermentative losses and prevent the aerobic deterioration. A number of studies have accessed the effects of silage additives on HMGS, however, to our knowledge, a systematic analysis of these data has not been conducted.

This review is focused on high moisture corn (HMCS) and winter cereals (HMWCS; barley, wheat and triticale) silages. The objective of this meta-analysis was to address the effect of chemical and microbial additives on the conservation of HMCS and HMWCS.

MATERIAL AND METHODS

Two data sets based on a literature review were compiled from scientific papers that reported treatment means. Silages made from whole or processed grains were considered and the minimal storage period adopted was 30 days. To analyze the effect of applying additives on winter cereal silages (barley, wheat and triticale),

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the data set included 3 referred journal articles (Adesogan *et al*., 2003; Mathison *et al*., 1989; Pieper *et al.*, 2011) and 3 abstracts (Davies *et al*., 2009; Seppala *et al*., 2015; Stacey *et al*., 2009). The small number of publications found was due to the rare use of grain silages when compared to the production of whole plant silages.

The data set used to study high moisture corn silages was composed of 24 referred journal articles (Bíro *et al*., 2006; 2009; Canibe *et al*., 2013; Da Silva, T. *et al*., 2015; Dawson *et al.*, 1998; Doležal and Zeman, 2005; Dutton and Otterby, 1971; Ferrareto *et al*., 2015; Flores-Galarza *et al*., 1985; Gálik *et al*., 2007; 2008; Ítavo *et al*., 2006; 2009; Jobim *et al*., 2008; Kung *et al*., 2004; 2007; Loučka, 2010; Morais *et al*., 2012; Prigge *et al*., 1976; Pys *et al*., 2009; Reis *et al*., 2008; Taylor and Kung, 2002; Wardynski *et al*., 1993); one technical note (Basso *et al*., 2012) and 10 abstracts published in international scientific meetings on forage conservation (Auerbach *et al*., 2015; Coudure *et al*., 2012; da Silva N. *et al*, 2015; Davies *et al*., 2009; Doležal *et al*., 2014; Gallo *et al*., 2015; Mlynar *et al*., 2006; Pys and Kowalski, 2014; Pys *et al*., 2010; Revello-Chion *et al*., 2012).

Data sets of corn and winter cereals were analyzed separately. A minimal of four treatment means from at least two articles was the prerequisite for keep the dependent variable into the data set. Data were analyzed using the mixed procedure of SAS (Littell *et al*., 1996). The model included a fixed effect of treatment (control or additive) and random effect of experiment, due to the variations across experimental protocols that would contribute to study effects in these comparisons (St-Pierre, 2001).

Because the current knowledge indicates divergent responses for types of silage additives, they were sorted into different classes: "Homolactic" (homolactic bacteria), "Hetero" (heterofermentative bacteria), "Combo" (Homolactic plus heterofermentative bacteria) and "Chemical" (chemical additives). Despite of the scarcity of data for wheat, barley and triticale silages, only the consequences of heterofermentative bacteria and chemical additives in winter cereals silages were presented.

RESULTS AND DISCUSSION

Untreated HMGS

Survey data from untreated HMCS showed a wide variation in moisture content (range of 232 $g.kg^{-1}$ to 397 $g.kg^{-1}$) and an average storage time of 91 ± 54 d. Benton *et al.* (2005) reported increases in both total *in situ* dry matter digestibility (ISDMD) and degraded intake protein (DIP) when moisture level was increased in both high moisture corn (240 or 300 $g.kg^{-1}$) and reconstituted corn (280 or 350 $g.kg^{-1}$), with major variations in ISDMD and DIP occurring during the first 28 d after ensiling. Taylor and Kung (2002) showed fermentative changes in HMCS over the storage period. The control silages showed most WSC consumption during the first 14 d, culminating in pH values below 4.0. At 49 d, the highest $N\text{-}NH_3$ value was recorded, which would be an indication of proteolysis caused by amino acid deamination (Oshima and McDonald, 1978). Specifically, Hoffman *et al*. (2011) working with different corn hybrids, found marked production of lactic acid and quick pH drop in the first 15 d of fermentation of a given hybrid without additives; however, for a second untreated hybrid, lactate quantification was possible only at day 30, besides the slow and gradual pH drop. For both hybrids, $N\text{-}NH_3$ and soluble protein levels increased with time for up to 240 d.

Untreated HMCS can be characterized by a moderate fermentation when compared to whole plant corn silage. In this review, the average production of acids (lactic acid + acetic acid) was 20.0 ± 11.8 $g.kg\ DM^{-1}$, with pH values varying between 3.73 and 4.95. These silages pointed a significant ethanol production (until 28.4 $g.kg\ DM^{-1}$), which would be an indication of the yeast metabolism (counts from 3.22 to 6.7 $cfu.g\ FM^{-1}$).

A wide range of moisture content was obtained in HMWCS (range of 256 to 461 $g.kg^{-1}$) and silages were stored for 88 ± 30 d. Previous reports relate the importance of moisture in silages as described by Pieper *et al.* (2011), regarding the fermentation profile of triticale, barley and wheat silages at 250 $g.kg^{-1}$ or 350 $g.kg^{-1}$ of moisture. In silages with higher moisture levels, pH declined within 3 days regardless *L. plantarum* inoculation. Treated low moisture silages had a pH decline after 10 d of storage, however pH of untreated grains remained unchanged. Lactic acid, propionic acid, acetic acid and NH_3 concentrations were also influenced ($P < 0.01$) by moisture content, showing a better pattern in silages with higher levels of moisture.

Winter cereals have a higher concentration of soluble carbohydrates in their composition, compared to corn. This greater availability of fermentable substrates may affect the effectiveness of additives during fermentation and feed-out phases. In this survey the average content of residual WSC observed in HMWCS without additives was 46.5 ± 28.0 $g.kg\ DM^{-1}$ and the pH values ranged from 3.85 to 5.90. The average concentration of lactic acid plus acetic acid was 21.1 ± 13.2 $g.kg\ DM^{-1}$, and the ethanol content ranged from 3.2 to 19.4 $g.kg\ DM^{-1}$.

Additives to HMCS

Chemical additives

Chemical additives reported in the reviewed articles included in the final data set are shown

in Table 1, with the respective application rates as reported by the authors. While some compounds were used alone (pure compounds or solutions), most treatments were based on mixtures of chemical substances.

High moisture grain silages with or without chemical additives displayed a large variation in chemical composition, DM loss, microbial counts and aerobic stability, indicating that the data set was broad, representative and covered a large part of the practically wide range of HMCS (Table 2). As expected, HMCS treated with chemical additives revealed a significant fermentation inhibition as indicated by the higher content of WSC and lower content of fermentation end-products, especially lactic acid.

Chemical additives were also effective in preventing DM losses, which explains the higher DM content of silages containing chemicals. Changes in variables such as ash, CP, $N\text{-}NH_3$ and pH reflect the formulations of chemicals added to the silages. The presence of minerals in the chemicals altered the ash content of silages and the presence of nitrogenous compounds affected CP and $N\text{-}NH_3$ concentration, in addition to the inhibitory effects on the microorganisms, preventing the pH drop.

A noticeable response achieved with chemical additives was the higher aerobic stability of silages, since spoiling microorganisms such as yeasts were markedly decreased. Higher stability associated with lower nutrient oxidation upon air exposure is a reasonable justification to recommend chemical additives for HMCS.

Table 1: Description of chemical additives used in the meta-analysis

Additive	Application rate
Ammonia	1.1 % to 2.3 %
Ammonium isobutyrate	2 %
Diammonium phosphate	4.6 %
Formic acid	3 $L.t^{-1}$ to 4 $L.t^{-1}$
Sulfur dioxide	1.3 % to 1.7 %
Urea	0.4 to 2 %
Urea solution	50 $L.t^{-1}$
Acetic acid, isobutyric acid	8 $L.t^{-1}$
Ammonium formate, propionate, ethyl benzoate and benzoate	4 $L.t^{-1}$
Ammonium propionate, sodium propionate, acetic acid, benzoic acid and sorbic acid	0.1 % to 0.2 %
Formic acid, ammonium formate, propionic acid, benzoic acid	4 $L.t^{-1}$
Formic acid, ammonium formate, propionic acid, benzoic acid and ethylbenzoate	6 $L.t^{-1}$
Formic acid (42.5 %), formic ammonia (30.3 %) and propionic acid (10 %)	6 $L.t^{-1}$
Formic acid (55 %), propionic acid (20 %), ammonium formate (4.3 %) and potassium sorbate (2.5 %)	4 $L.t^{-1}$
Formic acid (55 %), propionic acid (5 %) and ammonium formate (24 %)	4 $L.t^{-1}$ to 4.5 $L.t^{-1}$
Formic acid (55 %), propionic acid (5 %) and ammonium formate (24 %) plus ammonium Propionate	4.5 $L.t^{-1}$
Propionic acid (80 %) and acetic acid (20 %)	1.5 %
Propionic acid and formic acid	3.5 $kg.t^{-1}$
Propionic acid-based additive: ammonium and sodium propionate, ethoxyquin, BHA, and BHT	0.1 to 0.2 %
Propionic acid (50 %) and formic acid (50 %)	3 $L.t^{-1}$
Propionic acid (90 %), ammonium propionate (4 %) and 1,2-propandiol (4 %)	3 $L.t^{-1}$
Propionic acid, ammonium propionate, sodium benzoate, potassium sorbate	1.5 to 3 $L.t^{-1}$
Propionic, acetic, benzoic and sorbic acids, sodium and ammonium hydroxide, methylparaben and propylparaben (Liquid mold inhibitor, 82 % acid content)	0.1 %
Propionic acid, formic acid, benzoic acid and calcium formate	3.4 $kg.t^{-1}$
Propionic acid (37 %), sodium benzoate (14 %) and sodium propionate (11 %)	5 $L.t^{-1}$
Sodium benzoate (22.9 %) and sodium propionate (8.3%)	3 $L.t^{-1}$ to 6 $L.t^{-1}$
Sodium benzoate (5 to 50 %), potassium sorbate (5 to 35 %) and sodium nitrite < 5 %	2 $L.t^{-1}$ to 6 $L.t^{-1}$
Sodium benzoate, sodium azide and calcium formate	3.5 $kg.t^{-1}$
Potassium sorbate, sodium benzoate, ammonium propionate	1 $L.t^{-1}$ to 2 $L.t^{-1}$

Table 2: Data set of high moisture corn silages treated without or with chemical additives and their effects on silage quality

Item	Data set					Treatment effect		SEM	*P*
	n[1]	Mean	SD	Minimum	Maximum	Control	Chemical		
DM, g.kg^{-1}	62	661.8	38.2	598.0	748.0	657.8	665.8	6.88	0.02
Ash, g.kg DM^{-1}	26	15.2	1.49	13.3	19.1	14.6	15.8	0.38	0.01
NDF, g.kg DM^{-1}	24	99.7	6.79	79.2	107.0	99.5	99.9	2.00	0.59
ADF, g.kg DM^{-1}	24	39.7	12.3	22.8	57.4	39.8	39.7	3.64	0.85
Hemicellulose, g.kg DM^{-1}	24	59.9	11.9	42.4	76.2	59.7	60.2	3.50	0.35
Starch, g.kg DM^{-1}	40	712.9	51.8	593.0	796.7	711.5	714.3	11.7	0.44
Ether extract, g.kg DM^{-1}	14	43.3	8.33	34.8	66.2	45.5	41.1	3.15	0.36
CP, g.kg DM^{-1}	36	95.8	29.9	57.3	202.0	88.9	102.8	6.95	0.04
N-NH_3, g.kg DM^{-1}	40	0.54	1.07	0.00	5.00	0.33	0.76	0.24	0.21
Soluble protein, g.kg CP^{-1}	6	520.1	21.7	476.7	531.8	531.8	508.3	11.3	0.28
WSC, g.kg DM^{-1}	20	9.84	3.97	1.00	15.2	8.34	11.3	1.19	< 0.01
pH	74	4.41	0.72	3.70	8.30	4.31	4.51	0.12	0.08
Lactic acid, g.kg DM^{-1}	78	12.4	7.09	0.20	26.5	14.0	10.8	1.11	< 0.01
Acetic acid, g.kg DM^{-1}	78	4.89	2.84	0.00	16.0	5.20	4.57	0.46	0.15
Propionic acid, g.kg DM^{-1}	64	1.02	2.52	0.00	18.3	0.16	1.89	0.42	< 0.01
Butyric acid, g.kg DM^{-1}	24	0.23	0.26	0.00	0.70	0.27	0.18	0.07	0.23
Ethanol, g.kg DM^{-1}	58	6.91	9.05	0.00	44.0	9.50	4.32	1.62	< 0.01
Lactic:Acetic ratio	78	3.79	3.33	0.00	11.3	3.82	3.77	0.58	0.88
LAB, log cfu.g^{-1}	6	2.25	0.23	2.00	2.45	2.45	2.04	0.03	0.01
Yeasts, log cfu.g^{-1}	20	3.35	1.19	0.57	4.69	4.06	2.65	0.31	< 0.01
DM losses[2], g.kg^{-1}	10	14.2	11.9	5.8	41.0	16.7	11.8	5.50	0.11
Aerobic stability, h	52	125	112	21	500	59	190	18	< 0.01

[1]Number of means, [2]Fermentative losses.

Microbial additives

Nowadays, there is enough knowledge indicating divergent responses for homolactic and heterolactic microbial inoculants (Kung *et al*., 2003). Thus, homolactic, heterofermentative (including species of *Propionibacteria*) and combinations of homolactic and heterofermentative bacteria were evaluated separately. The microbial species used as silage inoculants are described in Table 3.

Homolactic bacteria are recognized for their efficiency in producing lactic acid, which is a strong acid (pKa = 3.86) capable to quickly drop the pH decreasing fermentative losses. On the other hand, heterofermentative bacteria are skilled in ferment sugars (pentoses and hexoses) into other products besides lactic acid, for instance acetic and propionic acids. These weak acids are good antifungal agents able to promote aerobic stability in silages (Moon, 1983).

Homolactic bacteria

Chemical composition, DM loss, microbial counts and aerobic stability of HMCS with or without homolactic inoculants are shown in Table 4. Nutrient compositions of HMCS were quite similar. Silages treated with the homolactic inoculants showed higher protein content and reduced ammonia content mainly due to the inhibition of proteolysis.

Although the database did not provide quantification of LAB, there was a trend towards greater use of soluble carbohydrates in the inoculated silages. As a consequence of the typical metabolism of added bacteria, the lactic acid content was higher in silages inoculated with homolactic bacteria, and this difference promotes significant changes in pH. The DM losses have been numerically lower in inoculated silages, however, both control and treated silages had shown low fermentative losses. Low concentrations of other organic acids indicated that fermentation profile was generally shortly interrupted.

Table 3: Microorganisms used as silage inoculants in the current meta-analysis

Bacterium	Inoculation rate (cfu.g^{-1} as fed)
Lactobacillus buchneri	5×10^4 to 5×10^6
Lactobacillus fermentum	1×10^5
Lactobacillus plantarum	5×10^4 to 1×10^7
Leuconostoc mesenteroides	1×10^5
Propionibacterium acidipropionici	1×10^7
Propionibacterium freudenreichii	1×10^7
L. buchneri and *L. plantarum*	2.5×10^5 to 6×10^5
L. buchneri and *P. pentosaceus*	7.5×10^5 to 9×10^5
L. plantarum and *P. acidipropionici*	1.5×10^5 to 3×10^5
L. plantarum and *P. freudenreichii*	1×10^5 to 1×10^7
L. rhamnosus and *E. faecium*	1×10^5 to 5×10^5
P. pentosaceus and *P. freudenreichii*	1.2×10^5 to 2.4×10^5
L. buchneri, *L. plantarum* and *E. faecium*	5×10^6
L. plantarum, *E. faecium*, and *P. acidilactici*	1.5×10^5 to 2×10^6
L. plantarum, *P. pentosaceus* and *P. acidipropionici*	1.5×10^5
L. plantarum, *L. bulgaricus* and *L. acidophilus*	1×10^5
L. plantarum, *L. casei*, *E. faecium* and *P. pentosaceus*	5×10^4
L. buchneri, *L. plantarum*, *E. faecium*, *L. casei*, and *P. pentosaceus*	1.5×10^5
L. buchneri, *L. plantarum*, *L. brevis*, *L. rhamnosus* and *P. pentosaceus*	2.5×10^5

Table 4: Data set of high moisture corn silages treated without or with homolactic inoculants and their effects on silage quality

	Data set					Treatment effect		SEM	*P*
Item	n[1]	Mean	SD	Minimum	Maximum	Control	Homolactic		
DM, g.kg^{-1}	28	669.3	54.9	595.0	768.0	665.9	672.5	14.9	0.13
Ether extract, g.kg DM^{-1}	6	34.5	3.37	29.3	38.4	32.3	36.7	1.54	0.18
CP, g.kg DM^{-1}	20	85.7	11.1	66.0	97.6	84.5	86.9	3.57	0.03
N-NH_3, g.kg DM^{-1}	18	0.26	0.17	0.10	0.70	0.20	0.19	0.04	0.06
Soluble protein, g.kg CP^{-1}	4	296.3	119.1	225.0	473.0	225.0	367.5	74.6	0.41
WSC, g.kg DM^{-1}	8	26.5	10.8	12.3	37.4	29.1	23.9	5.62	0.12
pH	24	4.29	0.48	3.88	5.65	4.42	4.16	0.14	0.02
Lactic acid, g.kg DM^{-1}	16	24.5	17.4	8.80	69.3	21.4	27.6	6.25	0.10
Acetic acid, g.kg DM^{-1}	16	9.13	9.14	1.10	28.7	9.85	8.41	3.33	0.40
Propionic acid, g.kg DM^{-1}	10	0.77	0.61	0.00	1.50	1.00	0.54	0.27	0.27
Ethanol, g.kg DM^{-1}	12	7.85	9.66	2.70	28.5	8.23	7.47	4.13	0.22
Lactic:Acetic ratio	16	3.96	2.42	1.64	9.74	3.02	4.89	0.81	0.12
Yeasts, log cfu.g^{-1}	6	4.77	0.73	3.92	5.67	4.76	4.78	0.47	0.95
DM losses[2], g.kg^{-1}	10	17.4	24.2	4.60	68.0	18.3	16.5	11.5	0.42
Aerobic stability, h	4	118	27	96	156	138	98	13	0.27

[1]Number of means, [2]Fermentative losses.

Unsurprisingly, homolactic inoculants were less effective in controlling aerobic deterioration, since lactic acid has a typical weak antifungal property (Moon, 1983). The influx of air into the silage mass has negative effects on silage quality, especially in HMGS due to its high content of nutrients, low moisture, and because it ferments more slowly and less extensively compared to typical forage crop silages (Taylor and Kung, 2002). Nutrient losses and excessive production of heat by microbial spoliation result in lower feed quality and

may result in poor animal performance (Hoffman and Ocker, 1997; Salvo *et al.*, 2015). This makes the use of exclusively homolactic microorganisms inappropriate for HMCS.

Heterofermentative bacteria

The characteristics of HMCS treated or not with heterofermentative inoculants are presented in Table 5. Overall quality of HMCS was typical for well-preserved silages, although DM and WSC contents, which are key factors for silage fermentation, showed a wide range.

Indeed, the production of antifungal compounds (e.g., acetic and propionic acids) by heterofermentative bacteria was an effective way for decreasing yeast and fungi population (Honing and Woolford, 1980) and largely improved the aerobic stability of HMCS. Silages inoculated with heterofermentative strains had lower WSC, indicating higher fermentative activity. *Lactobacillus buchneri*, a typical heterofermentative bacteria, has a predominant metabolic pathway leading to accumulation of acetic acid, whereas lactic acid concentration and pH, in general, remains similar to control silages. Furthermore, heterofermentative strains increase propionic acid as well, which might be produced either by the addition of *Propionibacterium* spp or by the degradation of 1,2-propanediol (Krooneman *et al*., 2002) resulted from *L. buchneri* metabolism.

Table 5: Data set of high moisture corn silages treated without or with heterofermentative inoculants and their effects on silage quality

Item	Data set					Treatment effect		SEM	*P*
	n[1]	Mean	SD	Minimum	Maximum	Control	Hetero		
DM, g.kg^{-1}	74	698.9	37.4	629.5	738.0	700.7	697.1	6.19	0.01
Ash, g.kg DM^{-1}	10	14.0	0.92	12.7	15.6	14.0	13.9	0.44	0.73
NDF, g.kg DM^{-1}	26	78.2	17.3	55.3	106.5	78.3	78.1	4.89	0.85
ADF, g.kg DM^{-1}	26	26.9	13.7	10.4	56.8	26.9	26.9	3.87	0.92
Hemicellulose, g.kg DM^{-1}	26	51.3	10.7	38.6	74.3	51.5	51.1	3.02	0.78
Starch, g.kg DM^{-1}	12	740.2	38.0	687.0	794.0	739.8	740.6	16.3	0.85
CP, g.kg DM^{-1}	32	94.9	11.6	72.3	109.6	95.3	94.5	2.95	0.48
N-NH_3, g.kg DM^{-1}	64	0.24	0.21	0.03	0.81	0.23	0.26	0.04	0.02
WSC, g.kg DM^{-1}	38	1.89	2.51	0.10	10.8	2.38	1.41	0.57	< 0.01
pH	66	4.22	0.43	3.73	5.65	4.24	4.20	0.07	0.39
Lactic acid, g.kg DM^{-1}	66	13.5	10.6	1.40	39.0	13.8	13.2	1.87	0.17
Acetic acid, g.kg DM^{-1}	66	5.75	4.21	0.40	27.1	4.06	7.44	0.68	< 0.01
Propionic acid, g.kg DM^{-1}	36	0.37	0.67	0.00	3.50	0.10	0.65	0.14	0.01
Butyric acid, g.kg DM^{-1}	8	0.16	0.24	0.00	0.60	0.23	0.10	0.12	0.19
Ethanol, g.kg DM^{-1}	46	5.87	4.92	1.20	18.0	6.07	5.67	1.04	0.25
Lactic:Acetic ratio	66	3.11	2.95	0.48	10.9	3.71	2.51	0.51	< 0.01
LAB, log cfu.g^{-1}	6	7.88	0.54	7.11	8.46	7.63	8.13	0.30	0.34
Yeasts, log cfu.g^{-1}	46	4.24	1.34	1.34	6.70	4.83	3.65	0.24	< 0.01
Molds, log cfu.g^{-1}	30	3.65	1.98	1.10	7.29	3.95	3.36	0.51	< 0.01
DM losses[2], g.kg^{-1}	16	30.1	10.4	7.50	41.0	27.2	33.0	3.65	0.07
Aerobic stability, h	72	129.0	114	20.0	450	70	188	16	< 0.01

[1]Number of means, [2]Fermentative losses.

Combination of homo- and hetero-fermentative bacteria

Chemical composition and fermentative characteristics of silages treated with both homo- and heterofermentative inoculants are presented in Table 6. Combo inoculants led to silages with higher protein content, whereas ammonia concentration tended (P = 0.11) to be lower, as observed for homolactic inoculants. According to Ferraretto *et al*. (2015), inoculation had no effect on CP of silages added or not with homolactic or heterofermentative bacteria; instead, CP values remained similar even with protease addition. In this review changes in the protein fraction were observed suggesting the importance of further studies aiming to investigate the influence of different microbial strains as their fermentative routes on silage proteolysis.

Silages treated with combo inoculants showed lower NDF content, which can be attributed to a higher hemicellulose disappearance (P = 0.10). It should be also noted that in the current meta-analysis, the inoculation with heterofermentative bacteria did not change the fibrous components of HMCS, most probably because none of the strains tested provided ferulic acid esterase activity.

The fermentation profile observed in silages treated with combo inoculants blended features from both homo- and hetero-fermentative bacteria. Inoculated silages had a greater consumption of soluble carbohydrates. In despite of the lower pH value attributed to the action of homolactic bacteria, treated silages had similar concentrations of lactic acid and higher levels of acetic and propionic acids than control silages. In turn, the presence of weak acids with antifungal properties reduced yeast counts and ethanol concentrations. Silages included in this data set generally

Table 6: Data set of high moisture corn silages treated without or with combinations of homo- and hetero-fermentative bacteria and their effects on silage quality

	Data set					Treatment effect		SEM	*P*
Item	n[1]	Mean	SD	Minimum	Maximum	Control	Combo		
DM, g.kg^{-1}	52	685.3	47.8	596.3	739.0	686.2	684.3	9.46	0.17
Ash, g.kg DM^{-1}	28	14.0	0.71	12.7	15.5	13.8	14.1	0.19	0.12
NDF, g.kg DM^{-1}	8	100.1	5.3	91.1	107.0	101.6	98.5	2.72	0.02
ADF, g.kg DM^{-1}	8	40.9	13.9	27.3	56.8	41.2	40.6	7.51	0.18
Hemicellulose, g.kg DM^{-1}	8	59.2	14.8	39.7	74.3	60.4	57.9	7.95	0.10
Starch, g.kg DM^{-1}	30	718.7	31.7	679.0	794.0	719.6	717.8	8.32	0.50
Ether extract, g.kg DM^{-1}	8	38.2	3.83	33.9	44.2	38.2	38.2	2.07	1.00
CP, g.kg DM^{-1}	36	82.4	8.98	72.3	97.2	81.8	83.1	2.14	0.05
N-NH_3, g.kg DM^{-1}	44	0.20	0.21	0.00	0.80	0.23	0.18	0.04	0.11
WSC, g.kg DM^{-1}	10	4.05	3.94	0.10	10.8	4.86	3.24	1.83	0.04
pH	52	4.21	0.41	3.73	5.65	4.29	4.13	0.08	0.03
Lactic acid, g.kg DM^{-1}	44	15.3	6.81	3.90	25.1	15.3	15.4	1.47	0.90
Acetic acid, g.kg DM^{-1}	44	5.77	3.14	1.50	14.2	5.08	6.47	0.66	0.03
Propionic acid, g.kg DM^{-1}	18	0.37	0.47	0.00	1.38	0.16	0.59	0.14	0.04
Butyric acid, g.kg DM^{-1}	14	0.19	0.19	0.00	0.62	0.23	0.16	0.07	0.47
Ethanol, g.kg DM^{-1}	42	3.52	3.02	0.90	15.6	4.06	2.98	0.66	<0.01
Lactic:Acetic ratio	42	3.18	2.10	0.14	8.63	3.11	3.25	0.46	0.78
Yeasts, log cfu.g^{-1}	10	3.61	1.13	2.00	5.67	4.37	2.84	0.37	0.03
Molds, log cfu.g^{-1}	18	1.83	0.58	1.09	2.90	1.82	1.83	0.20	0.94
DM losses[2], g.kg^{-1}	24	27.8	9.96	4.00	42.2	27.3	28.3	2.94	0.56
Aerobic stability, h	28	216	117	35	427	194	237	31	0.22

[1]Number of means, [2]Fermentative losses.

were stored for longer than 60 days (85 % of evaluated averages). Therefore, it is likely that assimilation of lactic acid by *L. buchneri* strains occurred throughout the fermentation process, giving rise to organic acids derived from the heterofermentative pathways. Puzzling, fermentative losses and aerobic stability were not altered.

Optimal dose of additives for improving aerobic stability to HMCS

In the current data set, aerobic stability was the most important response improved by additive utilization. Heterofermentative bacteria and chemical additives successfully enhanced aerobic stability of HMCS. For recommending an optimal application rate, a broken-line regression model was fitted to the data set.

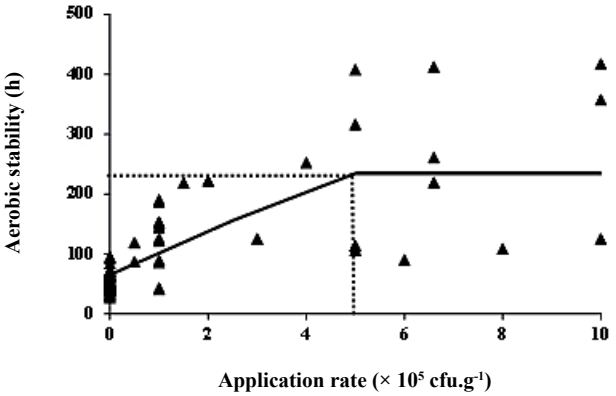

Fig. 1: Aerobic stability of HMCS according to inoculation rate of heterofermentative bacteria. If Dose ≤ 4.67 × 10^5 cfu.g^{-1}, aerobic stability = 64.4 + 28.3 × Dose (g.kg^{-1}); otherwise, aerobic stability = 235 h. *P* < 0.01, R^2 = 0.50, RMSE = 47.88.

For heterolactic bacteria, treatment effectiveness was achieved when bacteria was applied up to the optimal dose of 4.67 × 10^5 cfu.g FM^{-1} (Figure 1). It is important to highlight the inoculation rates of microbial inoculants. In the study reported by Taylor and Kung (2002), the inoculation of HMCS stored for 92 d with a low dose of *L. buchneri* (1 × 10^5 cfu.g^{-1}) did not enhance the aerobic stability. In contrast, application rates ≥ 5 × 10^5 cfu.g^{-1} improved the aerobic stability more than six-fold compared with untreated HMCS stored for the same period. However, in silages stored for 166 d, *L. buchneri* improved the aerobic stability even at 1 × 10^5 cfu.g^{-1}. Additionally to the inoculation rate, extending the length of storage is a potential practice to improve the aerobic stability and nutritive value of HMGS (Taylor and Kung, 2002; Hoffman *et al*., 2011; Der Bedrosian *et al*., 2012).

For chemical additives, the aerobic stability increased linearly within the studied range of application rates (Figure 2). Inhibition of spoiling microorganisms (e.g., yeasts and molds) requires a minimum acid concentration in silage aqueous fraction. Organic acid concentrations between 12.5 and 30 g.kg^{-1} of water may be required to control spoiling microorganisms in feedstuffs with high DM content (Collins, 1995).

In the present data set, 1.0 to 4.0 g.kg^{-1} are the most frequent range of application rate of chemicals. Probably, the cost:benefit ratio issue and negative effect on animal responses might be plausible justifications for these lower application rates. The lack of data for higher dosages focusing on aerobic stability in HMCS also contributes to this trend. Extrapolations should be avoided for silages with high moisture content (i.e., whole plant silages) because they typically contain higher levels of fermentation end-products.

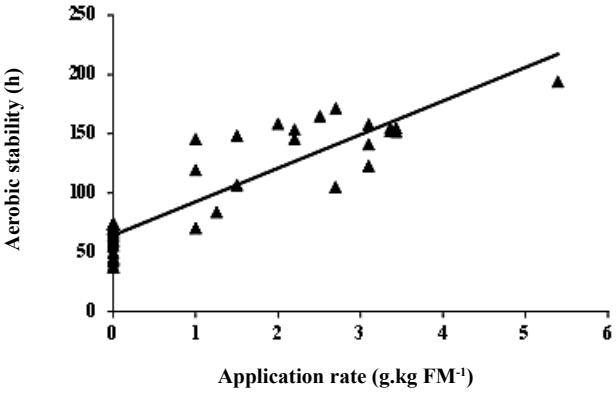

Fig. 2: Aerobic stability of HMGS according to chemical additive dosage. Aerobic stability = 64.4 + 28.3 × Dose (g.kg^{-1}). *P* < 0.01, R^2 = 0.81, RMSE = 20.14.

Additives to HMWCS

Chemical additives

The changes imposed on HMWCS by adding chemical additives are shown in Table 7. The starch disappearance in silages treated with additives may have been favored by chemical solubilization of the protein matrix. However, the agents promoting such breakage are not easily identifiable, since the chemical compositions are diversified.

Crude protein, N-NH_3 and pH were directly influenced by applying chemical additives and their composition. Nitrogenous compounds added to silages certainly contributed to increase CP and N-NH_3 concentrations. The pH was further increased (5.59) when silages were treated with chemical additives. This response is relevant because a proper acidification is mandatory to control pathogenic microorganisms. If silage does not reach low and stable pH (< 4.6), clostridial activity can be encouraged (Pitt *et al*., 1990). However, this review does not allow further conclusions about clostridium growth risk, since the concentration of butyric acid and spore counts were not accessed.

The lower concentration of lactic acid in the treated silages suggests that the fermentation process was inhibited. The chemicals were efficient in controlling molds and yeasts growth, reducing the formation of ethanol. The antifungal activity has also been proven effective at the feedout phase, increasing the aerobic stability of the grains.

Table 7: Data set of high moisture winter cereal silages treated without or with chemical additives and their effects on silage quality

Item	Data set					Treatment effect		SEM	*P*
	n[1]	Mean	SD	Minimum	Maximum	Control	Chemical		
DM, g.kg^{-1}	52	641.6	67.4	527.0	756.0	639.6	643.6	13.3	0.35
ADF, g.kg DM^{-1}	10	63.8	11.9	46.3	82.0	62.3	65.3	5.59	0.61
Starch, g.kg DM^{-1}	40	608.7	34.1	532.0	679.8	614.3	603.1	7.62	0.07
CP, g.kg DM^{-1}	48	118.8	29.8	94.6	221.0	111.7	125.9	5.96	0.02
N-NH_3, g.kg DM^{-1}	12	0.325	0.23	0.20	0.90	0.28	0.37	0.09	0.04
WSC, g.kg DM^{-1}	42	45.5	25.3	16.8	100.0	43.9	47.1	5.57	0.57
pH	52	5.16	1.44	3.80	9.20	4.73	5.59	0.27	0.01
Lactic acid, g.kg DM^{-1}	52	11.0	10.5	0.88	40.0	12.6	9.41	2.05	0.07
Acetic acid, g.kg DM^{-1}	52	3.55	3.70	0.10	21.7	3.16	3.95	0.73	0.35
Ethanol, g.kg DM^{-1}	50	8.70	5.91	0.02	20.8	12.0	5.45	0.99	< 0.01
Lactic:Acetic ratio	52	4.59	4.15	0.37	21.6	4.50	4.68	0.82	0.86
Yeasts, log cfu.g^{-1}	10	4.88	1.95	1.50	6.80	6.44	3.32	0.50	0.01
Molds, log cfu.g^{-1}	10	4.40	2.34	1.70	6.90	5.86	2.94	0.84	0.03
DM losses[2], g.kg^{-1}	4	31.5	15.2	16.0	45.0	33.0	30.0	13.0	0.37
Aerobic stability, h	6	223	78	87	301	165	281	29	0.10

[1]Number of means, [2]Fermentative losses.

Heterofermentative bacteria

The chemical composition, pH and fermentation end products of HMWCS treated with heterofermentative bacteria inoculants are shown in Table 8. The lower DM content of treated silages may be associated with the heterofermentative pattern, evidenced by the increase in acetic acid production. Despite losses have not been measured, the metabolic pathway of acetic acid production leads to carbon losses, which may explain the lower DM content of treated silages. Numerically, residual WSC content was lower in inoculated silages, corroborating with higher microbial activity in these silages.

Even with an increase in acetic acid levels, ethanol production was similar among silages. The final content of lactic acid was similar, but the pH of the inoculated silages was lower, highligthing that winter cereals have enough substrate for an efficient acidification of the mass. Anaerobic assimilation of lactic acid performed by *L. buchneri* can also explain the similar content of this acid in the silages (Oude-Elferink *et al*., 2001).

Table 8: Data set of high moisture winter cereal silages treated without or with heterofermentative inoculants and their effects on silage quality

Item	Data set					Treatment effect		SEM	*P*
	n[1]	Mean	SD	Minimum	Maximum	Control	Hetero		
DM, g.kg^{-1}	18	584.2	66.5	499.0	744.0	618.6	549.8	19.4	0.02
Starch, g.kg DM^{-1}	18	623.2	43.7	537.0	680.8	631.4	615.0	14.7	0.22
CP, g.kg DM^{-1}	18	101.2	4.8	93.8	110.0	100.1	102.4	1.59	0.08
WSC, g.kg DM^{-1}	18	46.6	27.9	15.0	100.0	53.6	39.5	9.26	0.17
pH	18	4.37	0.49	4.00	5.90	4.57	4.17	0.15	0.06
Lactic acid, g.kg DM^{-1}	18	13.4	5.86	1.80	19.7	12.7	14.1	2.00	0.50
Acetic acid, g.kg DM^{-1}	18	9.10	10.2	1.00	33.6	3.66	14.5	2.93	0.02
Ethanol, g.kg DM^{-1}	18	10.4	4.48	5.29	19.4	11.3	9.55	1.51	0.27
Lactic:Acetic ratio	18	2.21	1.48	0.35	6.00	3.06	1.36	0.41	0.02

[1]Number of means.

CONCLUSION

Control of fermentative losses is not a concern in properly made high moisture grain silages. Therefore, use of additives is justified if aerobic stability is improved. Additives based on chemical or heterofermentative bacteria proven to be effective in preventing aerobic deterioration at the same magnitude. The aerobic stability of high moisture corn silages was linearly increased with the application rate of chemical additives, whereas the optimal dose of heterofermentative bacteria was 4.67×10^5 cfu.g FM^{-1}.

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VOLATILE ORGANIC COMPOUNDS IN SILAGES – EFFECTS OF MANAGEMENT FACTORS ON THEIR FORMATION: A REVIEW

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ABSTRACT

Based on empirical observations from commercial farms that well preserved, but odd-smelling maize silages may cause problems regarding feed intake and milk yield by dairy cows, volatile organic compounds (VOC) were analyzed in recent studies. The aim of this paper was to summarize the results concerning the occurrence of VOCs in silages and the effects of silage additives on their formation. Elevated levels of ethanol, ethyl acetate (EA) and ethyl lactate (EL) as well as propanol and propyl acetate (PA) occurred in maize and whole crop silages, grass/ grass-legume-mixtures silages, furthermore in sugar cane silages. Ester and ethanol levels were highest in silages stored under strict anaerobic conditions. In conclusion it can be stated that the ester concentrations were strongly correlated with the ethanol concentration and the silage pH. Results from ensiling experiments on the effects of silage additives on ester formation in different ensiling materials clearly indicated that chemical products containing active ingredients with specific antifungal effects can significantly reduce ethanol and ester concentration. Salts of sorbic, benzoic or propionic acids or mixtures are effective treatment for reducing VOC production. Buffered formic acid-containing products stimulated it due to an increase in ethanol content. A survey was carried out to investigate the incidence of VOCs in maize silages from German dairy farms and to monitor the concentrations of ethanol, n-propanol and the corresponding esters ethyl acetate, ethyl lactate and propyl acetate. With increasing compaction the contents of VOCs increased and their concentration depends on the sampling site in the silo.

Key words: volatile organic compounds; management factors; silages

INTRODUCTION

Based on empirical observations from commercial farms that well preserved, but odd-smelling maize silages may cause problems regarding feed intake and milk yield by dairy cows, volatile organic compounds (VOC) were analyzed in recent studies (Weiss *et al*., 2009a, b). Ethyl and propyl esters of lactate and acetate have been found in farm silages (Weiss, 2009a, Weiss *et al*. 2015a). Researchers have correlated feed intake negatively with concentrations of some of the VOCs (Kriszan *et al*., 2007, Raun and Kristensen, 2010, Gerlach *et al.*, 2013). The knowledge on the effects of specific VOCs on feed intake by ruminants is still very limited. In addition, those substances have been discussed in relation to climate-damaging ozone formation, and it was reported that silages on dairy farms may be a significant source of VOC emission (Mitloehner *et al*., 2009).

Correlations were found among maize silages between ensiling conditions, type of silage additive as well as ethanol content and the concentrations of the ethyl esters – ethyl acetate (EA) and ethyl lactate (EL) (Weiss *et al.*, 2016). Ester and ethanol levels were highest in silages stored under strict anaerobic conditions. Elevated levels of ethanol and the corresponding esters EA and EL were not only detected in maize silages, but also in silages from grass, grass-legume-mixtures, legumes, whole-crop cereals and sorghum (Weiss and Auerbach, 2012a,b; Weiss and Kalzendorf, 2016). Regardless of silage type, silage additive and ensiling conditions, in the most cases a strong correlation was found between ethanol and ester concentrations, highlighting

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the prominent role of alcohol in ester formation. Therefore, any measure that reduces ethanol will restrict ester content.

OCCURRENCE OF VOLATILE ORGANIC COMPOUNDS IN SILAGES

VOCs in maize silages

On the basis of the results from investigations in farm silages with well preserved, but odd-smelling maize silages the first laboratory scale ensiling experiments were carried out with maize (Table 1). Elevated levels of ethanol, EA and EL were detected in maize silages, but also in silages from whole-crop wheat and high-moisture corn (Table 2). Ester and ethanol levels were the highest in silages stored under strict anaerobic conditions. It was also shown that esters remain detectable in silages for a few days after opening of the silos under aerobic conditions (Weiss *et al*., 2011). Data from farm silages presented in Table 2 (whole-crop maize 6 and 7) also showed high ethanol and ester concentrations. These silages were well fermented and highly compacted. In 7 out of 14 silages biological additives were used.

Results of ensiling experiments concerning effect of storage period on fermentation pattern indicates that concentration of ethanol strongly effected formation of esters during fermentation process. Weiss *et al*. (2009b) found increasing contents of ethanol and especially lactic acid over 90 days, whereas the corresponding ethyl esters increased during the first 30 days. These findings are in accordance with results from Gerlach *et al.* (2015) who investigated the effect of storage length of different maize silage varieties.

Table 1: Characterization of the data set of maize silages (ensiling experiments, n = 439)

Type of Silage	DM g.kg^{-1}	n	Storage length (days)	Silage additives
Laboratory scale ensiling experiments				
Whole-crop maize 1	310	60	60, 90	biological, chemical (Weiss *et al.*, 2009a)
Whole-crop maize 2	316	30	2,14, 28, 49, 90	biological (Weiss *et al*., 2009b)
Whole-crop maize 3	349	180	2,14, 28, 49, 90	biological, chemical*)
Whole-crop maize 4	332	12	90	chemical (Weiss and Auerbach, 2012b)
Whole-crop maize 5	315 – 513	79	112 anaerobic, 0 – 8 aerobic	without (Gerlach *et al*., 2013)
Whole-crop wheat	276	34	60, 90	biological*)
High-moisture corn	635	30	97	biological, chemical (Auerbach and Weiss, 2011)
Commercial farm silages				
Whole-crop maize 6	254 – 322	3	approx. 90	without (Weiss *et al*., 2009a)
Whole-crop maize 7	299 – 403	11	approx. 90 – 180	biological , Weiss *et al*., 2016)

*(Weiss and Auerbach, unpublished)

Table 2: Contents of volatile organic compounds (VOCs), especially esters and their correlation, in different maize silages (Weiss and Auerbach, 2012a)

Type of Silage	Lactic acid	Acetic acid	Ethanol	Ethyl acetate (EA)	Ethyl lactate (EL)	Regression EA+EL(y), Ethanol (x)	
	g.kg DM^{-1}	g.kg DM^{-1}	g.kg DM^{-1}	mg.kg DM^{-1}	mg.kg DM^{-1}	y = ax + b	R^2
Whole-crop maize 1	6.9 – 74.8	5.8 – 79.4	0.9 – 51.7	12 – 284	16 – 379	12.50x + 91.2	0.70
Whole-crop maize 2	32.5 – 119.8	8.6 – 25.8	3.2 – 28.3	55 – 343	30 – 683	26.47x + 121.5	0.65
Whole-crop maize 3	13.7 – 67.4	0.5 – 26.7	3.3 – 20.1	38 – 639	0 – 224	18.10x + 91.7	0.20
Whole-crop maize 4	73.8 – 124.6	5.3 – 29.2	6.2 – 50.8	116 – 262	156 – 661	11.55x + 266.0	0.93
Whole-crop maize 5	0 – 75.5	0 – 36.6	0 – 36.9	0 – 1109	0 – 986	52.51x + 0.2	0.88
Whole-crop wheat	20.7 – 99.9	9.1 – 42.4	21.9 – 121.8	84 – 951	309 – 1277	6.76x + 684.0	0.24
High-moisture corn	6.1 – 20.7	1.0 – 14.5	0.2 – 7.6	0 – 107	0 – 47	17.62x + 0.3	0.78
Whole-crop maize 6	11.3 – 70.8	25.8 – 48.7	21.0 – 64.0	357 – 789	118 – 1263		
Whole-crop maize 7	37.2 – 86.9	10.4 – 28.3	1.1 – 24.1	12 – 64	47 – 1305		

VOCs in grass silages

Extensive literature search yielded one study only by Krizsan *et al*. (2007), who detected variable concentrations of esters in grass silage, but the mean content never exceeded 30 mg.kg DM^{-1}. Therefore, the aim of investigations with grass silages (Table 3) was to determine the incidence of VOCs in grass silages, particularly ethanol and the ethyl esters of lactic and acetic acids.

Grass silages contained high ethanol and ester concentrations, particularly in those from trials 1 and 2 (Table 4). This may be attributed to the lower storage temperature, which promotes ester formation. Weiss *et al.* (2009a) observed that maize silages stored at 20 °C had higher ester contents than were detected at 35 °C. The correlation coefficients presented in Table 4 varied widely between 0.35 and 0.85, depending on the trial.

Table 3: Characterization of the data set from grass silages (laboratory scale ensiling experiments, n = 620)

Trial	Silage DM ($g.kg^{-1}$)	n	Storage length (days)	Silage additive type used in experiment
1	211 - 438	213	252 - 266	biological, chemical, molasses (Lengyel *et al*., 2012)
2	191 - 464	209	252 - 266	biological, chemical, molasses (Lengyel, unpublished data)
3	230 - 318	49	81	biological, chemical (Nadeau, unpublished data)
4	318 - 383	12	91	biological (Nadeau, unpublished data)
5	223 - 299	45	90	biological, chemical (Kalzendorf, unpublished data)
6	274 - 357	17	142	biological (Nadeau, unpublished data)
7	283 - 373	12	270	chemical (Nadeau, unpublished data)
8	202 - 219	21	131	biological, chemical (Kalzendorf, unpublished data)
9	223 - 240	21	121	biological, chemical (Kalzendorf, unpublished data)
10	243 - 268	21	139	biological, chemical (Kalzendorf, unpublished data)

Table 4: Fermentation products, pH and ester concentrations in grass silages (n = 620) (Weiss and Auerbach, 2013)

Trial	pH	Lactic acid ($g.kg\ DM^{-1}$)	Acetic acid ($g.kg\ DM^{-1}$)	Ethanol ($g.kg\ DM^{-1}$)	Total esters* ($mg.kg\ DM^{-1}$)	Correlation**	
						r_s	*P* value
1	3.7 - 6.7	0 - 99.5	1.5 - 62.8	0.7 - 39.6	0 - 3540	0.35	< 0.001
2	3.6 - 5.8	0 - 89.8	2.0 - 46.7	0 - 35.3	0 - 3995	0.37	< 0.001
3	4.0 - 4.5	60.6 - 117.5	11.1 - 36.5	2.2 - 18.7	0 - 359	0.91	< 0.001
4	3.8 - 4.2	42.7 - 81.8	13.2 - 35.4	6.7 - 12.0	216 - 455	0.52	ns
5	3.8 - 4.5	32.3 - 89.2	14.2 - 76.7	1.6 - 13.1	73 - 378	0.64	< 0.001
6	4.2 - 4.9	30.0 - 116.7	19.7 - 49.3	2.4 - 7.8	0		-
7	4.3 - 4.7	36.6 - 86.5	7.5 - 13.3	2.1 - 19.9	0 - 161	0.65	< 0.05
8	3.8 - 4.2	42.6 - 105.1	8.4 - 19.9	0.9 - 15.1	0 - 378	0.84	< 0.001
9	3.9 - 4.3	49.9 - 110.6	1.6 - 13.9	1.0 - 14.1	0 - 189	0.85	< 0.001
10	4.0 - 4.7	24.0 - 76.2	14.0 - 31.5	3.9 - 12.3	62 - 272	0.85	< 0.001

*sum of ethyl acetate and ethyl lactate, **correlation between ethanol and total ester concentrations
r_s - Spearman rank correlation coefficient, ns - not significant

The pH of the silages had a pronounced effect on ester levels (Table 5). Strong relationships ($r_s > 0.50$) were mostly observed when the pH of the silages did not exceed the value of 4.25. This is in line with observations by Hangx *et al*. (2001) who found ester reactions be stimulated by low pH in the environment.

The allocation of the grass silages to different ethanol classes was done as described by Weiss and Auerbach (2012a) and showed clear effects of ethanol content on the relationship between pH and total ester concentration (Table 6). Within each ethanol class, a great variation in ester concentration was observed. The detected ester levels in grass silages were extremely high compared with those reported by Weiss and Auerbach (2012b) for maize silages.

Table 5: Relationship between ethanol and ester contents in grass silages (n = 620) as affected by pH (Weiss and Auerbach, 2013)

pH class	n	Total esters* (mg.kg DM^{-1})	Ethanol (g.kg DM^{-1})	Correlation** r_s	*P* value
> 3.50 - 3.75	19	482 - 3995	0 - 35	0.60	< 0.01
> 3.75 - 4.00	126	0 -1856	1 - 40	0.72	< 0.001
> 4.00 - 4.25	176	0 - 920	1 - 25	0.55	< 0.001
> 4.25 - 4.50	131	0 - 762	1 - 18	0.21	< 0.05
> 4.50 - 4.75	81	0 - 550	1 - 24	0.26	< 0.05
> 4.75 - 5.00	42	0 - 384	0 - 38	0.49	< 0.001
> 5.00 - 5.25	26	0 - 255	1 - 37	0.49	< 0.05
> 5.25 - 5.50	10	63 - 211	4 - 28	-0.35	ns
> 5.50	9	0 - 171	3 - 24	0.25	ns

*sum of ethyl acetate and ethyl lactate, **correlation between ethanol and total ester concentrations
r_s - Spearman rank correlation coefficient, ns - not significant

Table 6: Relationship between pH and ester contents in grass silages (n = 620) as affected by ethanol (Weiss and Auerbach, 2013)

Ethanol class (g.kg DM^{-1})	n	Total esters* (mg.kg DM^{-1})	pH range	Correlation** r_s	*P* value
≤ 5	257	0 - 1180	3.7 - 5.8	-0.12	ns
> 5 - 10	181	0 - 1856	3.8 - 6.7	-0.46	< 0.001
> 10 - 15	100	0 - 1147	3.7 - 5.7	-0.66	< 0.001
> 15 - 20	39	87 - 3116	3.7 - 5.7	-0.88	< 0.001
> 20 - 25	21	0 - 3540	3.6 - 6.1	-0.93	< 0.001
> 25 - 30	12	63 - 3589	3.7 - 5.3	-0.83	< 0.001
> 30 - 35	5	274 - 2054	3.8 - 4.8	-0.60	ns
> 35 - 40	5	182 - 3995	3.7 - 5.2	-0.90	< 0.05

*sum of ethyl acetate and ethyl lactate, **correlation between ph and total ester concentrations
r_s - Spearman rank correlation coefficient, ns - not significant

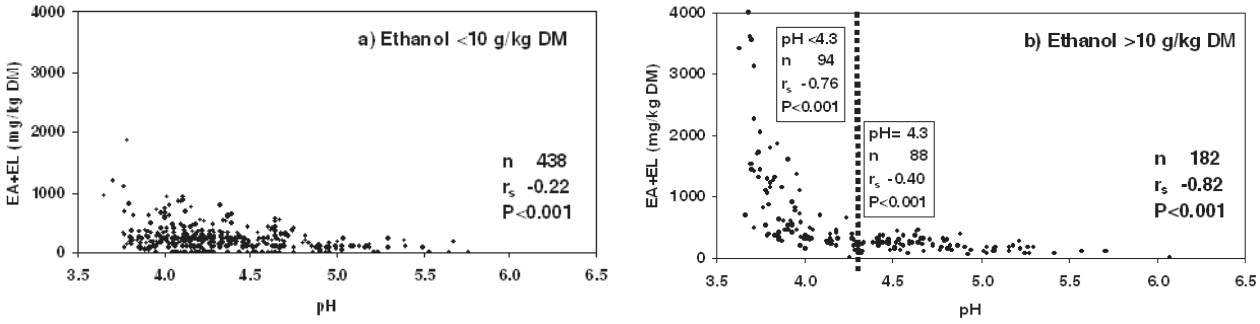

Fig. 1: Total ester concentrations as affected by ethanol class a) ≤ 10 g.kg DM^{-1}, b) >10 g.kg DM^{-1} (Weiss and Auerbach, 2013)

As shown in figure 1a, the correlation between total ester content and pH in grass silages was very weak (r_S = -0.22; $P < 0.001$) up to an ethanol content of 10 g.kg DM^{-1}, whereas a very strong negative relationship was found (r_S = -0.82; $P < 0.001$) at higher ethanol levels (Figure 1b). The least correlation existed if silage pH exceeded the threshold value of pH 4.3.

In summary it can be stated that grass silages may also contain ethyl esters. However, the relationship between ethanol and ethyl esters in grass silages seems to be not as close as that for maize silages. This can be explained by the fact that the intensity of ester reactions is affected by the pH of the silage and grass silages often have pH values above 4.0. As a consequence,

Table 7: Concentrations of fermentation products in sugarcane silages (n = 33), Daniel *et al.* (2013a)

Common name	Mean	SD[a]	Min.	Max.
	g.kg^{-1}			
DM oven[b]	28.3	4.0	22.2	34.9
DM corr[c]	31.1	3.1	26.7	36.5
	g.kg DM^{-1}			
Ethanol	54.2	48.1	5.0	154.5
Acetic acid	32.8	11.5	14.3	53.5
Lactic acid	26.0	20.9	6.5	60.4
	mg.kg DM^{-1} [d]			
Propane-1,2-diol	1532	2348	< 100	12186
Ethyl lactate	697	799	132	2401
Acetone	573	527	< 5	2072
Butane-2,3-diol	358	250	< 100	905
Propionic acid	284	350	< 100	1107
n-Butyric acid	273	369	< 100	1383
Ethyl acetate	167	174	< 5	597
2-Butanol	135	194	< 5	538
Methanol	133	359	< 100	1555
Propanol	123	81	< 5	290
iso-Butyric acid	< 100	55	< 100	274

[a]Standard deviation.
[b]Dry matter determined by oven drying (predrying at 55 °C for 72 h followed by drying at 105 °C for 12 h).
[c]Dry matter corrected for volatile compounds (Weissbach, 2009).
[d]iso-valeric acid, n-valeric acid, and caproic acid were below the limit of detection of 100 mg.kg DM^{-1}; 1-butanol was below the limit of detection of 5 mg.kg DM^{-1}.

the correlation coefficients decrease with increasing pH. In conclusion it can be stated that the ester concentrations are strongly correlated with the ethanol concentration and the silage pH.

VOCs in sugar cane silages

In tropical areas ensiled sugarcane is important forage with more than 400 g.kg DM^{-1} water soluble carbohydrates which act as substrates for intensive fermentation (Daniel *et al.*, 2013a). Ethanol is the main fermentation end product in sugar cane silages (Kung and Stanley, 1982). Concerning feed intake Daniel *et al*. (2013b,c) reported no difference when fresh sugar cane silage was compared with oven-dried material resulting in the loss of volatiles, which was reconstituted with water before feeding. On the one hand ethanol has been correlated with esters and other volatile organic compounds (Weiss *et al*., 2009a) and, on the other, Kriszan *et al*. (2007) and Gerlach *et al*. (2013) observed negative correlations between some VOCs and feed intake.

Daniel *et al*. (2013a) found that the VOCs comprised up to 22 % of the sugarcane dry matter. Table 7 contains data concerning the occurrence of VOCs in sugarcane silages, without additives, with sodium benzoate, and with *Lactobacillus buchneri*. In addition to high contents of ethanol, acetic acid, and lactic acid, 1,2-propanediol, ethyl lactate, acetone, 2,3-butanediol, propionic acid, n-butyric acid, ethyl acetate, 2-butanol, methanol, propanol, and iso-butyric acid were also found (Daniel *et al*., 2013a).

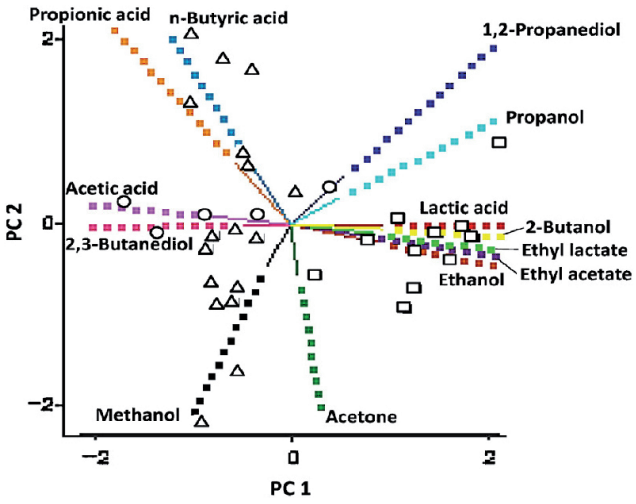

Fig. 2: Principal component analysis of volatile organic compounds in sugarcane silages. PC 1, first principal component (0.48); PC 2, second principal component (0.15). Silages were untreated (□), treated with sodium benzoate (Δ) and inoculated with *Lactobacillus buchneri* (O) (Daniel *et al.*, 2013a)

Daniel *et al*. (2013a) performed a statistical calculation with principal component analysis using PRINCOMP procedure of SAS (Figure 2). They postulated some functional relationships among the fermentation end-products in sugarcane silages. Ethanol was negatively associated with acetic acid and 2,3-butanediol, but positively correlated with lactic acid and esters.

EFFECTS OF SILAGE ADDITIVES ON FORMATION OF VOCs

Whole crop maize silage

The findings that VOCs are frequently found in silages and may detrimentally affect feed intake by dairy cattle (Weiss *et al.*, 2009a; Weiss and Auerbach, 2012a) have initiated more research with focus on the use of silage additives to reduce ethanol and ester formation. It is well known that silage additives can alter ethanol contents thereby exerting an effect on ethyl ester production. Weiss and Auerbach (2012b) tested the effects of chemical silage additives on fermentation pattern, production of VOCs and aerobic stability of maize silage. They found that treatment had significant effects on all parameters tested, except pH, which was very low in all silages (Table 8). DM losses were highest in acid treatments, whereas a significant reduction in DM loss was found by liquid mixture of sodium benzoate and potassium sorbate (SBPS). These observations can be explained by differences in ethanol concentrations, whose formation always results in CO_2 release, which escapes from the silo. The most significant reduction in ethanol was caused by the chemical additive SBPS, whereas acid additives (FAPA, FAPAP) stimulated ethanol production.

The concentration of ethyl esters in this study were also clearly affected by the concentration of ethanol and the respective organic acids. In general, lactate content was high, and SBPS increased the concentration of this fermentation acid over that of silages of all other treatments. Hafner *et al.* (2014, 2015) confirm these findings. They postulated that especially potassium sorbate is an effective additive for reducing production of ethanol and ethyl esters in corn silage. Acetic acid concentration was reduced by all used additives. As contents of lactic acid were higher than those of acetic acid, the formation of ethyl lactate was also more pronounced than that of ethyl acetate. SBPS decreased contents of ethanol, ethyl lactate (EL) and ethyl acetate (EA). FAPA and FAPAP stimulated the production of ethanol and EL, whereas no effect was found on EA. By using all experimental data from all individual silages of all treatments (n = 12), a very high correlation was found between ethanol and total ester concentrations (R^2 = 0.985). Elevated ethanol production in anaerobic conditions can be attributed to the activity of yeasts, which may have been present at

Table 8: Effects of silage additives on DM losses, fermentation pattern, VOCs and aerobic stability of whole-crop maize silage (DM 332 g.kg^{-1}); Weiss and Auerbach (2012b)

Parameter	Treatment				SED	Significance
	CON	SBPS[5]	FAPA[6]	FAPAP[7]		
DM loss (%)	6.5^{b}	4.3^{a}	7.5^{c}	7.5^{c}	0.31	***
WSC[1,2]	13.9^{a}	17.3^{ab}	20.5^{b}	19.2^{b}	1.28	**
pH	3.65	3.53	3.63	3.63	0.05	*
NH_3-N (g.kg^{-1} total N)	108^{a}	106^{a}	90^{b}	87^{b}	3.28	***
Lactic acid[2]	86.5^{a}	118.8^{b}	84.6^{a}	78.2^{a}	7.31	**
Acetic acid[2]	22.2^{b}	13.1^{a}	5.7^{a}	5.7^{a}	2.53	***
Propionic acid[2]	0.5^{a}	0.3^{a}	1.2^{b}	1.7^{c}	0.08	***
Ethanol[2]	23.2^{b}	6.5^{a}	49.1^{c}	46.5^{c}	1.57	***
1,2-propanediol[2]	0.3^{b}	0.4^{b}	0^{a}	0^{a}	0.08	***
Ethyl lactate[3]	398^{b}	166^{a}	617^{c}	612^{c}	39.3	***
Ethyl acetate[3]	223^{b}	123^{a}	189^{b}	184^{b}	16.1	**
Total ethyl esters[3]	621^{b}	289^{a}	806^{c}	795^{c}	31.3	***
ASTA[4] (days)	5.9^{a}	12.7^{b}	14.0^{b}	14.0^{b}	1.17	***

[1]water-soluble carbohydrates; [2]g.kg DM^{-1}; [3]mg.kg DM^{-1}; [4]aerobic stability; means in columns with unlike superscripts differ significantly at $P < 0.05$ (Tukey test); [5]liquid mixture of 21.9 % sodium benzoate and 13.2 % potassium sorbate, 2 l/t; [6]liquid mixture of 35 % formic and 12 % propionic acids, 25.5 % sodium formate, 1.5 % sodium benzoate, 4 L.t^{-1}; [7]liquid mixture of 48.8 % formic acid/ formate, 18.4 % propionic acid/propionate, 6.1 % sodium, 4 L.t^{-1}.

high numbers during the initial stages of fermentation but died off during later storage.

Further investigations by Weiss *et al*. (2015b, 2016) with maize confirmed that silage additives containing sodium benzoate, calcium propionate and potassium sorbate were superior to other treatments regarding suppression of ethanol and ester formation as well as improvement of aerobic stability, with and without air ingress.

Sorghum silages

A study of Auerbach and Weiss (2012) with sorghum silages aimed at testing the effects of different silage additives on dry matter (DM) losses, fermentation pattern, VOCs production and aerobic stability of this type of silages. Sorghum was chosen as silage type because it represents an important forage source for ruminants in semi-arid regions, and its production often bears the risk of excessive ethanol fermentation so that high concentrations of VOCs are to be expected.

Lactic and acetic acids were affected by variety and treatment, and an interaction was determined between the two factors for lactic acid (Table 9). Ethanol was reduced by *Lactobacillus buchneri* (LB) at all inoculation rates, and the lowest levels were consistently found if a mixture of sodium benzoate and potassium sorbate (BS) were used. The use of *Lactobacillus plantarum* (LP) alone or in combination with LB1 did not affect ethanol production when compared with control silages. The concentrations of reaction products of ethanol and organic acids – ethyl lactate and ethyl acetate – were affected by variety and treatment. Application of BS and LB (regardless of inoculation rate) caused the lowest ester contents, and no differences between CON, LP and LP+LB1 were found.

Grass silages

However, the knowledge of the formation of VOCs in grass silages and the effects of additives thereon is also still very limited. Weiss and Auerbach (2015) carried out a laboratory ensiling experiment with fourth-cut natural grassland, wilted overnight to 26.8 % DM. Forages received the treatment with 21 commercial additives (Table 10) which were obtained from the German marketplace and used according to the instructions of the manufacturers.

Grass silages were well fermented as reflected by low pH (Table 10) and no butyric acid was found (data not given). The production of lactic acid was stimulated by some additives of the types Ho, HoHe and HoCh whereas the pure He inoculant as well as two chemicals reduced it. The treatment with homofermentative LAB, either applied alone or in combination with antimycotic chemicals, always resulted in lower acetate levels. The lowest ethanol and ethyl ester contents were detected in silages that had received

Table 9: Effects of silage additives on volatile organic compounds of sorghum silages (Auerbach and Weiss, 2012)

Treatment	Lactic acid (g.kg DM^{-1})		Acetic acid (g.kg DM^{-1})		Ethanol (g.kg DM^{-1})		Ethyl esters[1] (mg.kg DM^{-1})	
Variety	Goliath	Maya	Goliath	Maya	Goliath	Maya	Goliath	Maya
CON[3]	92.0^{bA}	40.3^{cB}	24.9^{abA}	27.3^{bcA}	31.7^{cA}	34.2^{cdA}	381^{dA}	587^{dB}
LP[4]	90.2^{bA}	38.3^{bcB}	20.2^{aA}	22.0^{aA}	34.9^{cA}	28.8^{cA}	506^{dA}	586^{cdA}
LB[5]1	24.3^{aA}	22.8^{bA}	47.4^{bcB}	37.0^{abdA}	19.9^{bA}	19.5^{bA}	251^{cA}	414^{bcB}
LB[5]2	22.3^{aA}	24.6^{bA}	51.6^{cB}	45.5^{cdA}	18.9^{bA}	18.3^{bA}	245^{bcA}	365^{abcB}
LB[5]3	19.9^{aA}	17.8^{aA}	53.5^{cA}	51.8^{dA}	17.3^{bA}	19.6^{bA}	214^{abA}	299^{aB}
LP+LB1[6]	103.6^{bA}	26.9^{abcB}	24.3^{abA}	22.8^{abA}	34.2^{cA}	39.5^{dA}	460^{dA}	559^{dB}
BS[7]	95.4^{bA}	24.4^{bB}	27.6^{abA}	27.9^{bA}	6.9^{aA}	7.7^{aA}	131^{aA}	239^{abA}
SEM	8.18	1.83	3.05	2.46	2.23	2.31	31.1	33.2
Significance level[2]								
Variety	***		*		ns		***	
Treatment	***		***		***		***	
Variety x Treatment	***		ns		ns		ns	

[1]sum of ethyl acetate and ethyl lactate; [2]means in columns with unlike superscripts and means within rows bearing unlike capital superscripts differ significantly at $P \leq 0.05$ (Tukey test); [3]Control; [4]*L. plantarum* , 1 x 10^5 cfu.g^{-1} forage; [5]*L.buchneri* (1 x 10^5 cfu.g^{-1} forage; 2 (2.5 x 10^5 cfu.g^{-1} forage), (5 x 10^5 cfu.g^{-1} forage); [6]*L. plantarum* + *L. buchneri* (2 x 10^5 cfu.g^{-1} forage); [7]500 g.t^{-1} sodium benzoate + 300 g.t^{-1} potassium sorbate (applied in 2 L.t^{-1} aqueous solution)

Table 10: Effects of additives on fermentation pattern, volatile organic compounds and aerobic stability of grass silage stored for 72 days (Weiss and Auerbach, 2015)

Treatment	pH	Lactic acid[1]	Acetic acid[1]	Ethanol[1]	EE[2,3]	Propanol[3]	Acetone[3]	Methanol[3]	2-Butanol[3]	AS[4]
Con[5]	4.0	82.0	14.1	10.2	344	236	0	697	205	7.4
Ho[6]	3.9*	85.1	7.9*	8.7	301	0*	119*	789	224	3.3*
Ho[6]	3.9*	96.0*	11.0§	9.0	316	23*	131*	844§	222	7.0
Ho[6]	3.8*	87.2	7.9*	7.5$^{\#}$	258	0*	109*	796	212	2.3*
Ho[6]	3.8*	90.9§	8.5*	8.2§	284	0*	108*	845§	128§	1.8*
Ho[6]	3.8*	91.2§	7.6*	7.5*	309	0*	99*	686	152	2.7*
Ho[6]	3.8*	88.6	8.9*	7.7$^{\#}$	261	0*	89*	694	160	4.3§
He[7]	4.1*	61.1*	22.8*	13.4*	353	1080*	100*	817	195	8.8
HoHe[8]	3.9$^{\#}$	85.2	14.0	10.5	384	44*	126*	828	208	6.3
HoHe[8]	3.9*	79.6	11.3	8.7	342	100§	76*	816	197	7.2
HoHe[8]	3.9*	86.8	11.3	8.8	411	72$^{\#}$	111*	878$^{\#}$	130§	6.4
HoHe[8]	3.9*	96.2*	10.2§	7.8$^{\#}$	329	0*	108*	853§	214	5.4
HoHe[8]	3.9*	89.0	12.3	8.8	327	78$^{\#}$	99*	786	196	6.7
HoCh[9]	3.9*	93.2§	10.3§	9.8	300	0*	18	641	155	7.4
HoCh[9]	3.9*	81.7	10.2§	8.9	292	0*	24	660	187	8.1
HoCh[9]	3.9*	85.0	9.5$^{\#}$	8.6	272	0*	69*	692	179	6.8
HoCh[9]	3.9*	87.0	7.7*	7.5*	208	0*	51*	598	202	7.3
HoCh[9]	3.9*	84.2	8.6*	7.5*	265	0*	97*	729	241	10.9§
Ch[10]	4.0$^{\#}$	72.4§	16.5	2.3*	80*	499*	0	809	161	15.0*
Ch[11]	4.0	77.9	15.6	4.2*	143*	165*	0	611	153	15.0*
Ch[12]	4.0	61.0*	11.6	4.5*	105*	0*	0	583	164	14.1*
Ch[12]	3.9*	78.1	11.5	3.2*	61*	0*	0	630	209	15.0*

Means of each additive treatment in columns bearing unlike superscripts differ compared with untreated; $^{*}P < 0.001$, $^{\#}P < 0.01$, $^{\S}P < 0.05$; [1]g.kg DM^{-1}; [2]ethyl lactate + ethyl acetate; [3]mg.kg DM^{-1}; [4]aerobic stability, days; [5]untreated; [6]homofermentative LAB; [7]heterofermentative LAB; [8]combination of homo- and heterofermentative LAB; [9]combination of homofermentative LAB and antimycotic chemical(s); [10]nitrite, hexamine, sorbate; [11]nitrite, benzoate, sorbate; [12]buffered formic and propionic acid blends.

chemical additives. There was a strong positive linear correlation between these two parameters (R^2 = 0.72, $P < 0.001$). The production of 1-propanol was the highest in silages treated with the heterofermentative inoculant.

Legume silages

Investigations concerning the effect of wilting and silage additives on silage quality of lucerne, red clover and grass mixtures (Weiss and Kalzendorf, 2016) demonstrated the occurrence of VOCs in legume silages. The DM content and silage additives affect the concentrations of alcohols, acids and esters. However, yeast counts were high and increased during wilting period. In accordance to the fact that under anaerobic conditions yeasts are responsible for ethanol formation, the ethanol content in silages without any additives was between 4.8 and 10.9 g.kg DM^{-1} with a strong negative correlation to DM content (R^2 = 0.81) and positive correlation to ester content (R^2 = 0.65). Therefore elevated levels of alcohols and esters occur in silages with low DM. The total esters ranged between 124 und 197 mg.kg DM^{-1} in untreated silages and consisted of only ethyl lactate. These ester contents are comparable with contents in grass silage (Weiß and Auerbach, 2013) considering the pH level between 4.0 and 6.3. Silage additives with LAB did not primarily affect the contents of ethanol, the same applies for the contents of esters. The additive salts containing benzoate, nitrite and hexamine strongly reduced the ethanol and ester contents. According to Woolford (1975) these substances are able to inhibit yeasts and possibly heterofermentative LAB which also produce ethanol.

Sugar cane silages

The study of Cardoso *et al*. (2016) to evaluate the chemical composition, fermentation pattern and microorganisms of sugar cane without and with chemical additives and inoculants (Figure 3) confirmed that a correlation between ethanol and ethyl esters is strong. In sugarcane silages with CaO this chemical additive inhibited ethanol and ester formation.

White lupin-wheat silages

Laboratory ensiling trials with white lupin-wheat silages (König *et al*., 2015) demonstrated the occurrence of volatile compounds, also esters, in this special ensiling material. The authors found that increased proportion of lupin increased the concentration of VOCs and confirmed the safe effect of chemical additives due to their influence on fermentation pattern.

Summary

Results from ensiling experiments on the effects of silage additives on ester formation in different ensiling materials clearly indicated that chemical products containing active ingredients with specific antifungal effects can significantly reduce ester concentration. Salts of sorbic, benzoic or propionic acids or mixtures are effective treatment for reducing VOCs production.

Buffered formic acid-containing products, which were always applied at 4 $L.t^{-1}$ stimulated it due to an increase in ethanol content (Weiss and Auerbach, 2012b; Auerbach and Weiss, 2012).

VOCs IN MAIZE SILAGES IN GERMAN DAIRY FARMS

A survey has been carried out to investigate the incidence of VOCS in maize silages from German dairy farms and to monitor the concentrations of ethanol, n-propanol and the corresponding esters ethyl acetate, ethyl lactate and propyl acetate, depending on the sampling site in the silo and the compaction of silages (Weiss *et al.*, 2015a).

The survey included a detailed examination of silages stored in bunker silos on 52 dairy farms. Most silages were produced without silage additives (n = 43), whereas 9 farms had used biological additives. The highest contents of fermentation acids (acetic, lactic and propionic acids) and alcohols (methanol, ethanol, n-propanol) in maize silages were found in the bottom, highly compacted core and to some extent in middle core samples taken from bunker silos (Table 11), which supports empirical observations by Weiss *et al*. (2009a). Ethanol was detected at up to 17.8 g.kg DM^{-1} and the highest n-propanol level was 20.2 g.kg DM^{-1} (Figure 4a). In agreement with data by Weiss *et al*. (2009a), ethyl lactate (EL) concentrations in maize silages were higher than the levels of ethyl acetate (EA) and propyl acetate (PA) (Figure 4b). The contents of total esters (up to 925 mg.kg DM^{-1}) were higher than in silages from laboratory ensiling trials (Weiss *et al*., 2009a). With increasing compaction, the concentrations of n-propanol and ethanol as well as those of the ethyl esters EA and EL (Figure 4) and aerobic stability (R^2 = 0.920, $P < 0.001$) increased (data not shown). This may be explained by the usually lower pH in the bottom, more compacted and less air-affected zones in farm silos. Esterification processes were shown to be stimulated by low pH (Weiss and Auerbach, 2013).

Table 11: Fermentation characteristics of maize silages on 52 German dairy farms in different sections of bunker silos (mean ± SEM, g.kg DM^{-1} unless otherwise stated) (Weiss *et al.*, 2015a)

Parameter	BC[4]		MC[5]		TE[6]		*P*-Value
DM (%)	34.1	± 0.5	33.4	± 0.4	34.0	± 0.5	0.950
pH	3.85^{a,b}	± 0.18	3.83^{a}	± 0.02	3.89^{b}	± 0.03	0.036
Lactic acid	49.3^{b}	± 2.6	51.4^{b}	± 1.9	41.8^{a}	± 2.1	0.001
Acetic acid	23.0^{b}	± 1.2	19.5^{a}	± 0.9	19.6^{a}	± 1.0	0.009
Prop. acid[1]	0.8^{b}	± 0.2	0.4^{a}	± 0.1	0.6^{a,b}	± 0.1	0.028
Methanol	0.3^{b}	± 0.0	0.2^{a}	± 0.0	0.3^{b}	± 0.0	0.008
Ethanol	6.9^{b}	± 0.5	5.9^{a,b}	± 0.4	5.1^{a}	± 0.4	0.001
2-Butanol	0.2^{b}	± 0.1	0.2^{a,b}	± 0.1	0.1^{a}	± 0.0	0.015
n-Propanol	4.4^{b}	± 0.7	2.7^{a}	± 0.5	2.1^{a}	± 0.4	0.001
Ethyl acetate[2]	51^{a,b}	± 4	40a	± 3	59^{b}	± 5	0.007
Ethyl lactate[2]	210^{b}	± 17	176^{a,b}	± 15	150^{a}	± 14	0.003
Propyl acetate[2]	44	± 17	30	± 7	46	± 16	0.626
Total esters[2]	305	± 24	246	± 18	255	± 24	0.080
Ammonia	1.3^{b}	± 0.0	1.1^{a}	± 0	1.1^{a}	± 0.0	< 0.001
WSC[3]	8.2^{a}	± 0.7	10.5^{b}	± 1.0	9.9^{a}	± 0.7	0.001
AS (d)	7.2	± 4.8	6.6	± 4.1	6.3	± 4.2	0.2613
Yeasts (log cfu.g FM^{-1})	4.7^{a}	± 4.6	6.2^{b}	± 5.9	6.1^{b}	± 5.8	< 0.001
Compaction (kg.m^{-3})	256	± 5.6	226	5.8	217	± 5.9	< 0.001

[1]Propionic acid; [2]mg.kg DM^{-1}, [3]water-soluble carbohydrates; [4]Bottom core; [5]Middle core; [6]Top edge; means in rows with unlike superscripts differ at $P < 0.05$ (Tukey's test).

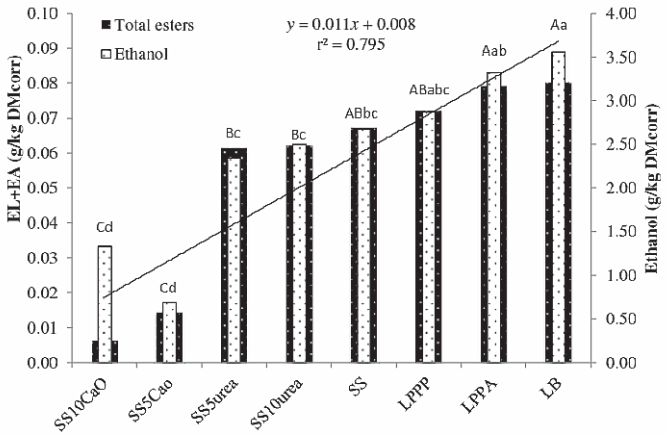

Fig. 3: Correlation between ethyl acetate and ethyl lactate (EL + EA) and ethanol contents in silage, averages in g.kg^{-1} of DMcorr. Sugarcane silage without inoculant (SS), SS with *Lactobacillus buchneri* (LB), SS with *Lactobacillus plantarum* and *Pediococcus pentosaceus* (LPPP), SS with *Lactobacillus plantarum* and *Propionibacterium acidipropionici* (LPPA), SS with 5 g.kg^{-1} CaO (SS5CaO), SS with 10 g.kg^{-1} CaO (SS10CaO), SS with 5 g.kg^{-1} urea (SS5urea), and SS with 10 g.kg^{-1} urea (SS10urea) (Cardoso *et al.*, 2016)

ESTIMATION OF ESTER CONTENT

Based on a total of 1148 data sets from grass silages (Weiss and Auerbach, 2013) as well as from silages from whole-crop maize, whole-crop wheat, sorghum, high-moisture corn (Weiss and Auerbach, 2012a), a regression model was used to describe the relationship between total ester and ethanol concentrations, which is valid for all silage types. As shown in figure 5, each incremental increase in ethanol content by 5 g.kg DM^{-1} resulted in increased total ester concentration by 114 mg.kg DM^{-1} (R^2 = 0.76). Therefore, the following equation can be applied to calculate ester concentration in silages based on their ethanol content: predicted total ester concentration [mg.kg DM^{-1}] = ethanol concentration [g.kg DM^{-1}] x 114/5. The use of this predictive model offers the possibility to avoid laborious and expensive chemical ester analyses.

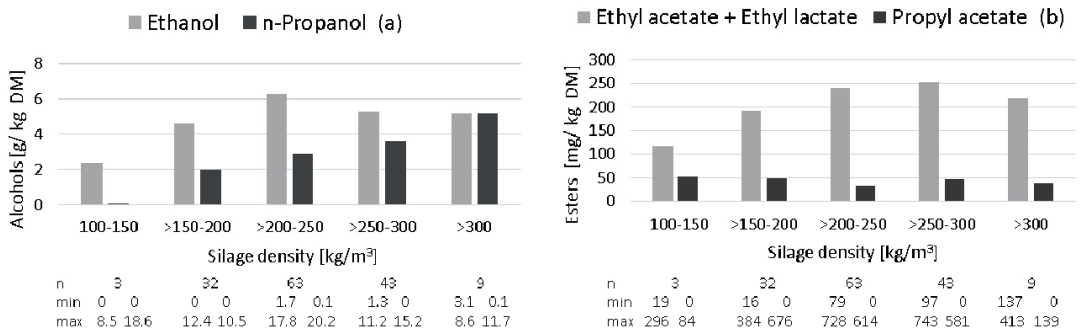

Fig. 4: Average concentrations of ethanol and n-propanol (a) and the esters ethyl acetate + ethyl lactate and propyl acetate (b) as affected by silage density (Weiss *et al.*, 2015a).

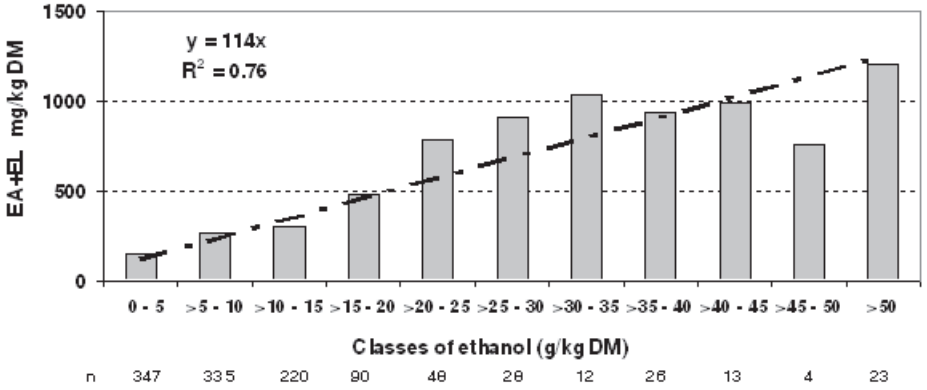

Fig. 5: Average total content of esters (ethyl acetate and ethyl lactate) in classes of ethanol in silages from whole-crop maize, whole-crop wheat, sorghum, high-moisture corn and grass (n = 1148) (Weiss and Auerbach, 2013)

CONCLUSIONS

With regard to the current body of evidence on VOCs formation in silages and their potential negative impact on feed intake in dairy cows and goat it can be stated that the reduction in ethanol production may lead to lower levels of ethyl esters. This is substantiated by data from ensiling experiments on the effects of different silage additives on ester formation in maize, grass, legume and sorghum silages. Only chemical products containing active ingredients with specific antifungal properties (sodium benzoate, potassium sorbate) consistently and significantly reduced ethyl ester concentrations.

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Erratum to "EFFECT OF *IN OVO* ADMINISTRATION OF BUTYRIC ACID INTO BROILER BREEDER EGGS ON CHICKEN SMALL INTESTINE PH AND MORPHOLOGY"

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ABSTRACT

This experiment was conducted to evaluate effects of *in ovo* butyric acid (BA) administration into broiler eggs on chicken small intestine pH and morphology. 480 fertile eggs were obtained from Ross broiler breeder (45 wk) and divided into 3 treatments with 4 replicate and 160 eggs per treatment. On the 18th day of incubation, 1 ml of *in ovo* solution was injected into amniotic fluid. Treatments were including 0.3 % BA solution, 0.9 % NaCl solution and control group. For pH and intestinal morphometric examination, 4 chicks per replicate were euthanized. The results showed that effect of BA injection on jejunum ($p < 0.01$) and ileum pH ($p < 0.05$) on hatch day was significant. Jejunum villi height increased ($p < 0.05$) on the 7th day compared with the control group. The highest ileum villi was observed following the BA injection ($p < 0.01$). It can be concluded that BA injection affects small intestine morphology and increases body weight of chicks.

Key words: *in ovo* injection, butyric acid, pH, small intestine morphology

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