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Yeast in fresh crop and silage from 15 Swedish farms and its impact on silage aerobic stability

R. Spörndly & A. Persson
Swedish University of Agricultural Sciences (SLU), Department of Animal Nutrition & Management, Feed Science Division, BOX 7024 Uppsala, Sweden.
Correspondence: Rolf.Sporndly@slu.se

Introduction

Yeast is commonly regarded as the organism initiating growth upon silo opening and responsible for silage temperature rise (Daniel *et al.*, 1970; Jonsson & Pahlow, 1984). Yeast growth on crops starts already during wilting and continues during the storage period if air infiltrates in silage (Henderson, 1993). Yeasts are able to grow and multiply within the pH range of 3 to 8 (McDonald *et al.*, 1991). Wilkinson & Davies (2013) compiled factors that may increase the risk of yeast and mould presence on crops at harvest; 1) dead plant materials at the bottom of leys, 2) damaged crops from rain and/or wind during the last days of growth, 3) crops in advanced stages of maturity, 4) crops that have begun to reach senescence just before harvest, and 5) swaths that have been wilted for more than two days especially under poor weather conditions (Wilkinson & Davies, 2013). A recent study has shown an increase in numbers of yeasts, LAB and enterobacteria on the crop if the harvest date is delayed (Schenck & Müller, 2014). Different yeast species can be found in silage depending on when in the ensiling process sampling occurs and which crops are ensiled. In one study, the non-fermentative genera *Cryptococcus, Sporobolomyces, Rhodotorula, Torulopsis* and *Aurebasidium* were found on the fresh crop prior to ensiling (Jonsson & Pahlow, 1984). Three days after sealing, these species were replaced by different species of yeasts. If the ensiling was anaerobic, the dominant species belonged to the genus *Saccharomyces spp*. These yeasts are not able to utilize lactate but ferment galactose and glucose. In aerobic silages, the composition of yeast flora was dominated by *Candida lambica* and colonies of *Wickerhamomyces anomalus* and *C. krusei* were also present from day 49 to the end of the ensiling period. During silage aerobic stability test, the yeast counts rose from log 7 to log 9 and the pH value increased from 5 to > 7 (Jonsson & Pahlow, 1984).

Since it is reasonable to assume that yeast species vary with location we found it interesting to investigate the floras existing on typical leys in Sweden. The aim of the present study was to compare green forage crops from 15 random Swedish farms as to yeast prevalence and to study if a correlation exists between silage aerobic stability and yeast species. A second aim was to describe how the forages were produced and relate aerobic stability to forage management factors.

Materials and Methods

Samples of 4-7 kg fresh crop were collected from 15 different farms in Sweden in 2014. Eight samples were from the first harvest (May 28 to June 9) and seven samples were from the second harvest (July 15 and July 19). The farms were located in the southern and middle parts of Sweden. The samples were taken outside the silos when the transport wagons or choppers arrived from the field and unloaded the harvested forages. The samples were transported to our laboratory within 30 hours while being kept at 4°C. The crops were packed in laboratory glass silos of 1649-mL with packing density being adjusted to forage dry matter (DM) contents. Densities were 144 kg DM/ kg^3 for DM content of 20%, and 245 kg DM/ m^3

for DM content of 52% (DLG, 2006). The silos were sealed with a lid fitted with water-lock and were weighed on day 0 and at opening on day 100 to determine weight loss during storage. At opening, silages were subjected to aerobic stability test. Silage samples were loosely filled into sterile PVC pipes (1320 mL) which had been fitted with a piece of geo textile (a material which air can easily pass) at the bottom. The pipes were placed in a block of Styrofoam with holes and thermocouples were placed in the middle of the silage. Samples were kept at 20˚C with relative air humidity of 80% to prevent a fast drying out. The silos were covered with Styrofoam with small holes in it to enable air penetration. The temperature was logged every second hour for ten days.

The fresh crops and the silages were analysed for DM, Ash, CP and WSC content with standard methods as described by Spörndly & Persson (2015). For microbial analyses, an amount of 30 g forage sample was placed in a stomacher bag and 270 mL of autoclaved ¼ - strength Ringer solution (Merck, Darmstadt, Germany) was added. Samples were pummelled in a laboratory stomacher (Stomacher 3500, Seward Ltd, Worthing, West Sussex, UK) for 60 seconds twice. Serial 10-fold dilution was prepared from microbial solution before plating on MEA agar fortified with streptomycin sulphate (30 mg/L) and penicillin G (30 mg/L). For samples from the second harvest, an additional set of substrates was prepared where chloramphenicol replaced streptomycin. Colonies of yeasts were counted after three days of aerobic incubation at 25°C and the average colony number was determined according to Niemelä (1983). Colonies from the fresh crop were streaked on MEA-plates to ensure pure yeast colonies. A total number of 16 colonies from the first harvest and 40 colonies from the second harvest were grown in test tubes with 3 mL Yeast Extract Peptone Dextrose (YPD)-medium (Becton Dickinson Company, Sparks, Maryland, USA). The suspensions were incubated on a shaking table at 25°C for 48 hours. The YPD-medium had a concentration of 10 g yeast extract/L, 20 g bacteriological peptone/L and 20 g glucose/L. Thereafter, 600 µL was pipetted into cryo tubes (2 ml) and mixed with approximately 600 µL glycerol and put in a freezer at -70°C. Sample preparation for DNA sequencing which includes DNA extraction, PCR, gel electrophoresis and purification of PCR-products were done by the Department of Microbiology of the Swedish University of Agricultural Sciences in Uppsala. Sequencing was carried out by Macrogen (Amsterdam, Netherlands). The sequences were compared with sequences in NCBI's (National Center for Biotechnology Information, Bethseda, Maryland, USA) database using BLAST® (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.*, 1990).

A set of forage management factors were collected at each farm and the influence of such factors on aerobic stability were tested, when applicable, by the General Linear Model (GLM) procedure in SAS. The effect was considered statistically significant if $P < 0.05$ and tendency if $0.05 \leq P \leq 0.10$. Pearson correlation coefficients were calculated between chemical variables in fresh crop and silage, yeast counts in fresh crop and silage, ensiling losses, aerobic stability and temperature increase during aerobic test as well as correlations between these variables and management factors, using the procedure CORR in SAS.

Results and Discussion

In all farms, the ley consisted of a mixture of perennial grasses and red and white clover. Six farms were fertilized with liquid manures and in four farms commercial fertilizers were used. Three farms were also fertilized with a combination of liquid manure and commercial fertilizer. The amount of liquid manures spread ranged from 20 to 30 tonnes per hectare. The

fields were fertilized before each harvest except for one farm which was fertilized only before the first harvest. The age of the swards varied between one and four years. In none of the farms herbicides were used. In all farms, white silage film was used to cover bunker silos but eight farms additionally also used micro foil.

Use of manure as fertilizer tended to result in a higher yeast count in the fresh crop (P=0.08). Silages made from the first harvest had a tendency for longer stability than silages from the second cut (11.3 vs. 8.6 days) (P=0.06). The temperature of the silages from the first harvest only rose on average 0.14°C while the temperature of the silages from the second harvest rose on average 3.3°C.

The method used for quantifying the yeast resulted in number of colonies in the fresh crop ranging from log 4.6 to log 5.9 cfu/g (Table 1).

Table 1. Content of dry matter (DM), water soluble carbohydrates (WSC), number of microbial colonies in fresh crop and after ensiling, and aerobic stability after opening the silos at 15 farms. Farm 1-8 were sampled during the first harvest and farm 9-15 during the second harvest.

Farm	DM in fresh crop (%)	WSC in fresh crop (g/kg DM)	WSC in silage (g/kg DM)	Number of microbial colonies in fresh crop (log cfu/g)	Number of microbial colonies in silage (log cfu/g)	Time (h) until temperature +2°C above ambient temperature
1	25.0	83	0.3	4.6	3.2	213.4
2	25.5	109	1.0	4.7	3.0	181.6
3	23.7	109	4.3	5.6	<1.7	>288
4	33.2	108	13.7	5.6	2.05	>288
5	22.2	81	1.3	5.9	<1.7	>288
6	23.3	70	0	5.9	1.7	>288
7	33.7	149	18.8	5.5	2.4	>288
8	24.2	68	0	5.0	<1.7	>288
9	40.2	75	5.0	5.1	<1.7	>240
10	21.5	57	0.8	5.4	<1.7	>240
11	18.8	35	1.8	5.6	<1.7	205.9
12	52.8	108	43.9	5.8	<1.7	>240
13	35.9	102	18.2	5.6	<1.7	>240
14	30.1	106	26.6	5.6	<1.7	>240
15	28.1	107	17.9	5.0	3.7	87.9

For the green forage samples, the use of penicillin, streptomycin and chloramphenicol in the growth media was not effective in preventing mould and bacterial growth. However, this was not the case for silage samples.

The fresh forage samples from the two harvests contained 15 different species of yeast (Figure 1). The two most common yeast species found were *Rhodosporidium babjeave* and *Rhodotorula glutenis* found in 6 and 5 farms, respectively.

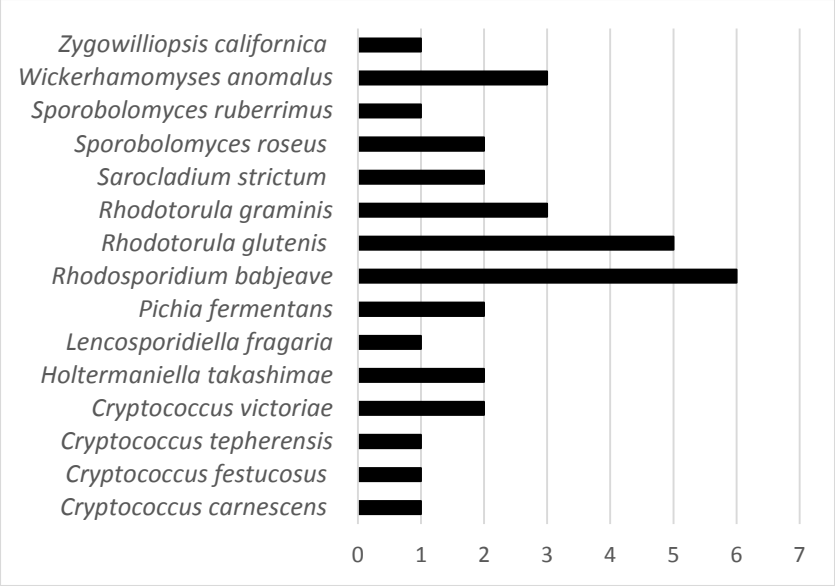

Figure 1. Number of farms per yeast species present in fresh crop from 1st and 2nd harvest at a total of 15 farms.

The correlation analyses showed higher DM losses at lower silage DM and WSC contents. The ash content was positively correlated with the yeast counts in fresh forages and was negatively correlated with the aerobic stability (Table 2). Silage yeast count was negatively correlated with storage stability.

Table 2. Correlation coefficients between yeast count in fresh crop and in silage, silage concentration of dry matter (DM), water soluble carbohydrates (WSC) and ash, and silage DM loss, aerobic stability (time lapse before 2˚C increase above the ambient temperature) and temperature increase during the aerobic phase

	Yeast count in fresh crop	**Yeast count in silage samples**	**DM loss**	**Aerobic stability**	**Temperature increase during aerobic storage**
Yeast count in fresh crop		NS	NS	0.47 P<0.07	NS
Yeast count in silage samples	NS		NS	-0.61 P<0.02	0.43 P<0.11
Silage DM	NS	NS	-0.57 P<0.03	NS	NS
Silage WSC	NS	NS	-0.65 P<0.009	NS	NS
Silage ash	0.55 P<0.03	NS	NS	-0.56 P<0.03	0.49 P<0.06

Conclusions

The method used to cultivate silage yeasts is not applicable for fresh crops as bacterial growth cannot be prevented in the growth medium by the antibiotics commonly used. The most frequently seen genera of yeasts in fresh crops were *Rhodosporidium* and *Rhodotorula*. Silage yeast counts and aerobic stability were negatively correlated.

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Sveriges Lantbruksuniversitet
Institutionen för husdjurens utfodring och vård
Box 7024
750 07 UPPSALA
Tel.018/672817

Margareta.Norinder@slu.se