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THESES OF DOCTORAL (PH.D) DISSERTATION

Investigation on the mould contamination of hays in Hungary and development of methods for its evaluation

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1. Introduction

The preparation and use of hay can be dated back to the time when farm animals have been domesticated. In some northern parts of Eurasia, the cutting, drying and storage of summer grass yields allowed the sheltering of ruminant cattle and sheep for winter, and provided horses with forages. Today hay is indispensable in the feeding of the farm animals for the following reasons:

1) In Hungary, the climatic conditions are not suitable for grazing all around the year. Animals (cattle, sheep, and goat) which need forages (fibres) feed on preserved (dried, fermented) forages in the winter period, and they also need to consume hays for the healthy functions of their rumen.
2) The high fibre content of hays meets the fibre requirements of ruminant animals in the most natural way.
3) The nutrients of hays, such as protein, high carotene and mineral content, also meet animal requirements.

To ensure the prospected production level of farm animals, it is essential to feed the animals with forages rich in nutrients in intact, undamaged microbiological condition.

Generally, the qualification of raw-materials for forages covers the following groups of characteristics:

1) Nutrient-content (can be calculated on the basis of chemical analysis: as digestible - , available - , net -, macro- and micro-components.

2) Undesirable substances (prohibited substances), toxic agents (environmental, from processing), heavy metals, toxic chemicals, mycotoxins.

3) Factors causing deterioration quality:such as microbiological conditions, mycotoxins, fat oxidation.

In the process of official Hungarian microbiological feed evaluation, parameters for examination, typical values and limit values (at the time of compiling the dissertation) are stipulated by Appendix 2. 44/2003. (IV. 26) FVM (Ministry of Agriculture and Rural Development) decree. A major shortcoming of this decree is that it does not cover mould and yeast numbers and does not set limit values. Similarly, it ignores the qualification of roughages and forages.

In Hungarian practice hay have been classified exclusively according to their nutrient values (Tabulated data in Hungarian Feed Codex II.) so far. An objective system of qualification and related classification in terms of organoleptic properties (colour, smell, stem length, consistency) and primarily of the signs of microbiological decay have not been established.

The MSz (Hungarian Standard) 17671-1988 standard, in qualifying hays according to their nutrient content, contains only a single sentence related to microbiological requirements: "hay should not be objectionable microbiologically and toxicologically". Therefore, the degree of moulding or its lack cannot be quantified objectively, so it cannot be specified still less accounted for.

However, hays are exposed to an invasion of moulds during their preparation to a greater extent than all other forage components. The reasons are the following: a) at harvest time, spore contamination from the soil and from decaying plant parts is considerable (product typical fungal contamination); b) as a result, depending on technology, the harvested hay can be exposed to water activity above a(w) = 0.75, which can provide optimal conditions for the sudden propagation of moulds besides the biological heat generation of rotting plants, the quick gradation of yeasts and aerobe sporophoric bacteria; c) moreover, moulds can propagate at relatively low water activity, where the gradation of bacteria (aerobe sporophoric) and yeast can not yet start up.

The existing lack in the specifications of legal force is that they do not set limit values and guiding figures, although the objective microbiological qualification of hay cannot work without them. If hays are exclusively qualified on the basis of their nutrient content and their microbiological properties are ignored, one can easily get misleading results in terms of their practical use.

The above mentioned situation served as the baseline for my research and objectives:

- to characterize the mould flora of Hungarian hays, to evaluate the quantified level of moulding in comparison to common sensory assessment,
- to assess the toxicological risks of moulds on hay
- to investigate the effects of certain factors in processing on the propagation of moulds, and

- to adapt and develop experimental methods, which can be adjusted to the special properties of hays.

2. Research objectives

1) **Representative, qualitative and quantitative examination of the mould flora in Hungarian hays, in comparison with sensory evaluation.**

The goal of this examination was to identify the most frequent product typical flora elements and the product typical mould count (CFU) of average quality, and also to define the average (typical) quality in microbiological terms.

2) **Evaluation of the toxicological risks of moulds on hay**

Evaluation on the occurrence of toxinogenic flora elements on the basis of the research material, experiments to detect mycotoxin contamination in highlighted cases, gathering information from the literature on the official regulation of toxic contamination in hays and on casuistic data.

3) **Experimental examination of environmental factors during processing responsible for the mass propagation (gradation) of moulds.**

These experiments identify the most responsible factors for moulding among all of the factors at harvest, i.e. the gradation of product typical mycoflora. Regarding the practical importance of haylage and the occurrence of secondary moulding after its release from storage, we examine the effects of acids and pH, which originate during fermentation, on the secondary aerobe propagation in product typical mould flora.

4) **Adaptation of methods for mould examination, suitable for hay samples**

As we have performed our examinations under objective 1) it became evident that methods of propagation specified for the examination of forages cannot be used in the case of hays, because certain expansive species living on hay spread in the cultures and prevent the evaluation of other flora elements. Therefore, a medium composition more suitable for the morphological evaluation of colonies should be experimented and complemented with an inhibitor. Besides this, we examined the suitability of *Aspergillus*-invertase test (MÁTRAI et al., 2000) having been developed in relation to our earlier studies for the detection of *Aspergillus* contamination in forages to indicate mould contamination in hays.

We attempted to shorten the time needed for mould detection so we also studied whether the extraction of mould DNS and its RT-PCR examination with AllFunTaq fungus-specific primer could provide consistent results by using the so far authentic CFU definition based on live germ cultivation in feed evaluation. The microscopic examination of a standard suspension prepared from various hay samples may also be used for the fast, informative evaluation of mould contamination in hays. Related to this, we performed experiments to work out a selective staining method with which formations of mould origin can be differentiated from other micro-formations of plant and animal origin and can be counted as well.

5) So far we have had few data from the literature on mouldy forages. Publications had to be collected from the fields of mycology, feed hygieny and laboratory practice which might provide an understanding of the problem of "hay-mycology". My objective was to collect the literature regarding mould fungi living on hays, their detection and their living conditions.

3. Research methods

3.1. Identification of product typical germ number in hays

• *Collection of representative samples*

The mould contamination of hays (mould number, CFU) was determined on representative hay samples collected from 76 sites (41 alfalfa and 35 grass) plots of various conditions in Hajdú-Bihar and Pest counties. During sampling, all kinds of qualitative preferences were set aside, users' complaints were also disregarded so that samples could be accepted as a practical average. In these examinations we have always denoted the part representing the largest quantity of portion as a sampling plot. We collected about 1 kg of sample batch into plastic bags from inside the bales after peeling off an outer layer of hay of about 10 cm from the bales. The selection of bales took place randomly, both from under the shed or from batches stored outside. We recorded their serial numbers, sampling data, the location of bales and their exposure to weather conditions, and if available, their history ("once became wet", "rain - wetted on the field" etc.)

On the day of sampling, the bags were transported into the laboratory then they were processed immediately.

• *Sensory examination*

First the samples were examined by sensory assessment. The typical botanical composition, colour, smell, porosity and signs suggesting moulding in samples were identified.

• *Mycological examination*

The standard suspension was prepared by shaking 10 g of sample, chaffed manually (3mm average chaff) with 90 ml water for 20 min. The logarithmic dilution set was inoculated into a medium specified under the MSz ISO 7954 horizontal standards. From the 10-3, 10-4 and 10-5 dilutions of the sample we pipetted 1.0-1.0 ml into Petri-dishes and then they were covered with 15 g of warm MSz ISO 7954 culture medium (5.0 g yeast extract, 20.0 g glucose, 0.01 g chloramphenicol, 12-15 g agar/1000 ml distilled water , set for pH 6.6).

The dishes were placed into a thermostate adjusted at 25°C. on day 5. CFUs were counted over those dilutions where the number of grown colonies was 100-200. According to microbiological practice, in the evaluation and presentation of CFU data, the decimal logarithms were expessed

3.2. Examination of product typical flora elements in hays and the occurrence of toxinogenic species

Following the CFU evaluation of hay samples, during a further incubation period of 1-5 days, the colonies of toxinogenic *Aspergillus*, *Penicillium* and *Fusarium* species were examined. According to the morphology of conidial formations, the colonies of these species were evaluated by microscopic identification on each plate. The occurrence of toxinogenic species was compared with results from the sensory assessment and CFU results of hays.

3.3. Examination on physical factors responsible for the mass propagation of moulds on hays

The propagation kinetics of product typical mould flora was studied in moisture chambers of constant water activity., on grass samples of different water content (17.65, 37.5, 52.64%) at different relative humidity levels (75, 84, 95, 100%) and temperatures (15, 25°C).

•*Preparing spaces of constant humidity*

The used moisture chambers providing constant humidity (ERP, expressed in %) are based on the fact that the saturated salt solutions of inorganic crystals provide strictly constant relative ERP, typical of certain salts in the space over the solution in a closed system. The forage sample placed in this space can be kept at constant humidity, and through this, its water activity can be calibrated at the selected value. A plastic box with its lid for microwave cooking was used as a moisture chamber. The saturated solution was in the bottom, and the sample was placed on its stand. To stop ventilation, the chambers were closed with plastic adhesive tapes. These chambers were easy to place in incubators providing various temperatures. For the satisfaction of the life conditions of mould fungi and for their propagation at least 0.7 a(w) is required. Therefore, three of the inorganic crystals suitable for this purpose were selected: NaCl - a(w)=0.75 with RH 75%, KCl - a(w)=0.84 with 84% and KH_2PO_4 - a(w)=0.95 with 95% RH value.

Weighed quantities of pre-wilted cut grass samples of various, 17.65%, 37.5%, 52.64% moisture (the mixture of *Lolium* 80%, *Festuca* 10%, *Bromus* 10%) were placed into calibrated moisture chambers and incubated at 15 and 25 °C.

•*Mycological examination*

Mould growth was determined on days 0., 4., 8. and 14. by counting CFU values with plate pouring according to MSZ ISO 7954.

3.4. Sensibility of mould flora of hays, the product-typical mould flora of maize and *Aspergillus parasiticus* to the presence of fermentation acids (lactate, acetate).

• *Sample preparation*

a) a standard suspension of hay was prepared from samples of average quality, containing dominantly *Lolium perenne,* in the framework of a national survey for the examination of product-typical mould flora, according to the mycological examination method described in 3.1.
b) a standard suspension was prepared for the examination of maize (grain) from the mixed meals of healthy portions from yields of 2002.
c) a conidiium-suspension with a concentration of 109/ml was prepared from the fresh culture of *Aspergillus parasiticus,* cultivated as a model organism in the strain collection of our laboratory, standardized by the Hungarian National Microbiological Strain Collection (1999).

• *Preparation of standard culture medium (control)*

Standard medium: composition under MSz ISO 7954 (20.0 g glucose, 5.0 g yeast extract, 0.01 g chloramphenicol, 12-15 g agar/1000 ml distilled water); pH 6.6.

• *Preparation of experimental culture medium variants*

1. For examining the effect of lactate: neutralized Na-lactate was added to the above mentioned medium in a concentration of 1.0 or 2.0% (w/v)
2. For examining the medium for lactic acid fermentation: a submerse culture of 109/ml *Lactobacillus plantarum (Lb. plantarum)* has been prepared on MRS medium killed off for 20 min at 80 °C to examine the supposed inhibitory effect of dead *Lactobacilli* and their metabolites.
3. For examining the effect of acetate: neutralized acetate in equimolar amount with lactate was added in a concentration of 1.5%.
4. For examining the effect of pH: lactic acid equal to 1% of lactate was added to the standard medium, setting pH. values at 6.5, 5.5 and 4.5 with NaOH.

• *Experimental culture media:*

Taking the composition of the culture medium according to MSz ISO 7954 as a basis (hereinafter related to components: abbreviated such as glu-ye-chlo etc.) the relative growth on the following experimental culture media (in Table 1). were studied:

Table 1. Composition of experimental media

Serial number of culture medium	**MSz ISO 7954 components**	**Treatment**
1.	glu-ye-chlo	control, pH 6.5
2.	glu-ye-chlo	+ 1.0 *mass/volume* % Na-lactate

3.	glu-ye-chlo	+ 2.0 mass/volume % Na-lactate
4.	0-ye-chlo	chlowithout carbon source
5.	0-ye-chlo	+ 2.0 % Na-lactate as carbon source
6.	glu-ye-chlo	mould culture medium on killed-off submers *Lactobacillus* culture
7.	glu-ye-chlo	+ 1.5% acetate
8.	glu-ye-chlo	+ 2% Na- lactate + 1.5% acetate
9.	0-ye-chlo	+ 1.5% acetate
10.	glu-ye-chlo	+ 1% Na-lactate pH 5.5
11.	glu-ye-chlo	+ 1% Na-lactate pH 4.5
12.	0-ye-chlo	+ 1% Na-lactate pH 4.5

• *Inoculation, incubation*

Plate pouring was performed with experimental and control media and inoculums into 10 cm diameter Petri-dishes according to Koch, then they were incubated in a bacteriological thermostate at 25 °C.

• *Evaluation and measurement of inhibition of mould growth*

On all culture media, evaluating the colonies:

- numerical inhibition - the percentage of CFU compared to control
- the rate of a mycelial growth - the average diameter of colonies expressed in the percentage of the control colony during inoculation of the same age.
- the start of conidial development - on incubation days and in addition
- typical culture medium colonies

3.5. Experiments to develop formulae for media suitable for the mycological examination of hays

The mould contamination of forages can be characterized by the frequent presence of *Mucor* species. Their fast growth covering the culture medium and vigorous aerial mycelia generation are typical of this species, making quantitative evaluation difficult and sometimes impossible. Our objective was to develop a formula for culture media that would inhibit *Mucor* growth without exerting inhibitory effects on other flora elements. The reference medium was standard MSz ISO 7954, suitable for the microbiological examination of grains.

In our examinations we processed mixed grass and alfalfa hay samples in terms of places of origin and quality. Hay samples were chopped into pieces of 0.5-1cm and then we weighed 10.0 grams of them for the preparation of a standard suspension.

• *Preparation of a standard suspension and dilution sets*

It was performed according to the method described in 3.1. From the members of the dilution sets (generally dilutions of 10-3, 10-4, 10-5) plate pouring was performed with the experimental culture media.

• *Composition of culture media*

MSz ISO 7954

Horizontal method for the determination of mould and yeast count. Composition of standard culture medium:

DRBC

A generally used formula, according to King et al. (1979)

MSz ISO 7954 + DC

Addition of Dichloran (2.6- Dichlor-4-nitroaniline) in the quantity applied in DRBC medium, postulating its inhibitory effect on expansive species.

1/4 N MSz ISO 7954

The nutrient level of the culture medium was reduced to one-fourth, supposing that it would inhibit the mycelial growth of expansive species relatively better than that of species of slower growth.

1/4 N DRBC

To reduce the nutrient components of DRBC formula to one-fourth, while maintaining the inhibitors.

1/4 N DC

Here the nutrient concentration was one-fourth of King's DRBC recipee, but it contained only Dichloran as an inhibitor.

DRBC + DSA

On the analogy of aromatic dinitro-derivatives (dinitro-orto-cresole, DNOC, fungicide),
in this experimental culture medium variant 2-4-dinitro-salicyl acid was supplemented, which has been buffered to neutral in DRBC composition, being more polar than DNOC.

• *Stock-solutions:*

- Rose Bengal stock solution: diluted solution of 5.0%. (0.5 ml in 1000 ml of medium)
- Dichloran stock-solution (2.6-Dichlor-4-nitroaniline (Merck): dilution with ethanol of 0.2% (1.0 ml/1000 ml of medium)

• *Plate pouring*

According to the method of plate pouring MSz ISO 7954

• *Reading CFU values*

After 24 hours, the appearance of *Mucor* growth was checked daily.

All the mould colonies have developed on the plates were counted on the different culture medium variants after a cultivation of 4-5 days, and then morphological evaluations were performed.

• *Morphological evaluation*

On the basis of the colour, surface and shape of colonies.

3.6. Experiments to develop a selective propagulum staining technique

In our experiments we attempted to find a staining agent for the microscopic examination of standard suspensions from hay, which

stains both hyphas and spores intensely but

binds to plant fibres, plant hair, microscopic animal organisms and colonies only slightly,

has excellent water solubility, which enables the differentiation of non-bound background stain from stained preparate by aequous washing.

According to this technique, the standard suspension was put into an agar-matrix, heat-treated with the staining solution and was streaked on the slide. After the solidification of agar-matrix differentiation was performed in water. It was expected that propagulums would retain the colour after washing, while the other formations lose the staining agent due to its good water solubility.

• *Preparation of standard suspension*

1.0 ml of suspension from hay, grains or exclusively hypha was added to 0.1 ml of water containing 15% of agar in a test-tube of 12 x 120 mm.

• *Staining*

Experimental staining solutions:

- 3 drops (cca. 70 µl) of Rose Bengal (RB) stock solution of 5% ($C_{20}H_2O_5I_4Cl_4Na_2$, CI: 45440)
- 3 drops of malachite green saturated diluted solution (cca. 70 µl), ($C_{23}H_{25}N_2Cl$, CI: 42000)
- 6 drops of amido black (AB) (buffered for 3.4; 0.6%) solution (cca. 140 µl), ($C_{22}H_{14}N_8Na_2O_9S_2$, CI: 20470)
- Sudan III, ($C_{22}H_{16}N_4O$, CI: 26100)

The content of test-tubes was stirred with Vortex several times, boiled three times on alcohole flame and while still hot, it was streaked on the slides in a thickness of 0.5 mm.
After solidification the agar layers, the slides were placed in distilled water bath in vertical position and were differentiated for 5, 30 and 60 minutes. In the case of Sudan III. staining, ethylene glycol was used instead of water for 10 minutes, and then it was rinsed with water.

• *Evaluation, examination*

With a light microscope, illuminated by transmission light, by a magnification of 250 x

- in a fresh layer or
- after drying (in this case, the distorting effect of dried agar layer was still not significant)

3.7. Attempt to apply the invertase test in the case of some typical genera occurring on hays

Invertase tests can be applied for the rapid indication of mould contamination of hays. In contrast with product-typical and storage moulds in grains, the product-typical flora of hays contain slower growing species except *Mucor* and *Trichoderma,* so their cultivation on agar takes much longer than the necessary incubation time for grains. In our experiments we examined the invertase production capacity of the most frequent components in mould flora of the hay to find out whether their invertase production was typical enough to indicate the extent of general mould contamination on the basis of the invertase test as rapid pre-screening method.

• *Examined mould fungus species*

Isolated and identified species in our study were: *Alternaria alternata, Cladosporium herbarum, Fusarium poae, Fusarium sporotrichoides* (identified on the basis of colony morphology) *Mucor piriformis, Nigrospora, Trichoderma viride* and *Trichotecium roseum.*

• *Aspergillus-invertase test*

It was performed according to the method developed by Mátrai et al. (2000).
Its principle: mycelium is cultivated in a sucrose-based liquid medium, and then the quantity of reducing sugar splitted as a result of invertase action indicates the presence of moulds and the extent of their contamination.

Liquid culture medium: 5 g of yeast extract, 20 g sucrose and 1.0 g chloramphenicol, ad. 1000 ml of distilled water (yeast extract has to be pre-tested whether is it free from detectable invertase activity and sucrose also has to be free of reducing sugar).

2-2ml of liquid medium was portioned into test tubes of 16 x 200 mm, the test tubes were closed and were sterilized in atmospheric steam for 60 minutes and cooled in a water bath of 37 °C.

Basal suspension of the species tested: The subcultures were one week old, on saccharose - yeast extract (MSz ISO 7954) agar cultivated By platinum loop conidia were suspended into 2.0 ml sterile physiological solution, containing 1% of Tween 80. The inoculated spore number per microlitre was counted in a Buerker chamber.

Logarithmic dilution set: From the basal suspension a decimal dilution series was prepared. From each element of the dilution set, 100 µl was inoculated into test tubes containing 2 ml of liquid medium, in three replications.

Incubation: inoculated test tubes were placed in a tilted position into an incubator of 37 °C so that the surface of the medium exposed to air should be of the length of about 6-7 cm. Thus the oxygen was enough for mycelial growth and later spore generation as well.

• *Determination of the reducing sugar splitted*

It was performed according to the method described in a publication by Mátrai et. al (2000) with 2-hydroxy-3,5-dinitrobenzoacid (dinitro-salicyl-acid , DSA) reagent.

DSA reagent: 5.0 g of 2-hidroxy-3.5-dinitro-benzoic acid was diluted in 400 ml, warm aequous solution of 2% sodium-hydroxide. Then 100 g KNa-tartrate, 1.0 g phenol and 0.25 g water-free sodium sulphate were dissolved. The solution was filled up with distilled water to 500 ml. It can be stored stable in a well closed dark bottle, in a cool place for more than one year.

In a test tube, 0.1 ml of DSA reagent was added to a 0.1 ml aliquot of the liquid culture samples having been incubated. This mixture was placed into boiling water for five minutes. Then this sample was diluted with distilled water to 2.0 ml and after stirring its absorbency was measured at 540 nm. The reference (blank) was not inoculated, but incubated. To get accurate results, a glucose standard set was prepared with a concentration of 0.0, 10, 50, 100, 500 and 1000 µg/ml, and a calibration curve was drawn. Thus absorbencies were expressed in the form of µg reducing sugar 1 ml liquid medium. Reducing sugar values above 10 µg/ml were regarded as positive reaction.

3.8. Examination of the correlation of the copy numbers obtained by DNS RT-PCR, using the ALLFun-Taq mould specific primer and the CFU numbers assessed by the (standard) method of cultivation.

The comparative examinations were performed on 8 hay samples of mixed quality (5 grass, 2 alfalfa and 1 straw).

<u>*Assessment of colony forming unit (CFU) number by plate pouring*</u>

Mould CFU was determined according to the method described in 3.1. (MSz ISO 7954 horizontal method).

<u>*Detection of mould fungi by RT-PCR reaction*</u>

• *Preparation of hay samples for DNS extraction*

In the first series of experiments hay samples were chaffed (in the same way as it was done in the determination of mould count) into a size of about 0.8 mm. The serial numbers of the samples were the following: 42, 43, 44, 45, 46, 47, 48, 49.

In the second series of experiments, during the preparation of samples No. 42, 43, 45, 47 and 48, the closing up of the cells in the samples was more intense, since the first series of experiments have suggested that the chaffing and mechanical closing up of hay samples were not satisfactory. The samples, which has been chaffed into a size of 0.8 mm were disintegrated with quartz sand in a mass ratio of 1/1 to a grain size of 0.1 mm or smaller in a “Pulverisette” 02.102-type agate mill for 120 minutes.

After the disintegration of mouldy alfalfa-hay sample No. 48, the non-disintegrated fibres were separated, and was denoted as 48R, whereas the sieved dust fraction as 48 P.

When analysing the copy number of samples 48P and 48R, the objective was to compare DNS quantity remaining in the non-disintegrated fraction with the quantity isolated from the sieved dust.

The chaffed samples and the sample powders, having been disintegrated with quartz sand additionally for more effective DNA extraction, were sent to the cooperating institute, Bundesforschungsanstalt für Ernährung und Lebensmittel (BFEL), Karlsruhe.

The AllfunTaq copy numbers of prepared samples were assessed in the cooperating laboratory in Germany.

• *DNS extraction and isolation from hay samples*

The technical steps of DNS and RNS isolation from pure cultures of different mould species and artificially contaminated food in the course of AllfunTaq examination and also development techniques for AllfunTaq primer are presented in Chapter 3.5.2.

In the first sample set (samples 42, 43, 44, 45, 46, 47, 48 and 49) DNS isolation was performed from hay samples of 0.1 g with "Dneasy Plant Mini Kit" (Quiagen, Hilden, Germany) according to the producer's instructions. The mechanical treatment of cells by rubbing in mortar with liquid nitrogen was performed in the cooperating institution. Evaluating of the copy number of the first sample set the problem of insufficient mechanical disintegration emerged.

The second sample set (42, 43, 45, 47, 48P, 48R) was disintegrated in a "Pulverisette" 02.102-type agate mill for 30 minutes and in this way a fine dust fraction of 100 micrometers and a few fibrous cell wall fractions of 0.1-0.3 mm were obtained.

In the case of the sample No. 48 they were examined separately, as sample numbers of 48R and 48P. Then the treatment with liquid nitrogen was supplemented with shaking in aluminium oxide for half an hour in the cooperating institution. After the last step of washing, DNA was eluted with the eluting buffer of 2x100 µl. 1 µl of the DNS solution obtained in this way was used in AllfunTaq Real-Time PCR reactions.

4. Main conclusions of the theses

4.1. Determination of product typical mould count in hays

Our examination using the MSz ISO 7954 standard procedure showed that the CFU number in good quality samples was 3×10^3 - 2.7×10^4 per gram whereas that of samples having been assessed to be mouldy by sensory evaluation varied between 3.2×10^4 - 3.45×10^8. Moulding assessed by organoleptic examination suggested at least 5.7×10^5 CFU, which is 1.0 log higher than Pálfy's limit value (Pálfy and Kupai, 1986) (3.0×10^4).

Out of 76 (100%) hay samples collected 70 (92.1%) proved to be above Pálfy's limit value and only 6 (7.9%) samples proved to be good on the basis of CFU examinations. On the contrary, sensory assessment regarded 58 (76.3%) samples to be good and only 18 (23.7%) samples as damaged.

Therefore the conclusion was drawn that random samples (practical average), on the basis of microbiological examinations, mostly gave a mould count above Pálfy's limit value having been recommended earlier, although sensory examination has detcted only one fourth of the samples to be of poor quality. This observation gives reasons for the necessity of microbiological examinations besides organoleptic inspection.

If the botanical composition of samples were compared with their mycological status, it can be seen that out of the examined 41 alfalfa-hay samples 39 samples (95.1%) exceeded Pálfy's limit value. On the basis of the CFU/g content of 53 grass hay samples (100%), 31 samples (88.5%) exceeded Pálfy's limit value.

In the two hay types as substrates there was no significant difference regarding mould counts

The most frequent mould count in hays was 10^5 (27.6%), 10^6 (25%) and 10^4 (23.6%) /g. while 15.7% of the samples had the magnitude of 10^7. Extremely high mould count of 10^8 occurred in 5.2%. The mathematical average of CFU counts was exactly 1.987 x 10^7, which value did not reflect the typical (most common) mould count. Therefore this mathematical average is not suitable for the definition of typical contamination in hays.

The collected hays in our study contained an average of 10^5 mould count, the magnitude of one hundred thousand.

4.2. Occurrence of product typical hay flora elements and toxic species

Mould flora was evaluated as regarding the occurrence of toxic genera in 60 samples (57 above Pálfy's limit, 3 below). Out of them, *Aspergillus* species were found in 23 (38.3%) samples. 22 samples showed CFU values exceeding Pálfy's limit of III. class.

Penicillium species were detected in from 26 samples (43.3%). 24 samples were above, 2 samples were below the above limit. *Fusarium* species were detected in 23 samples (38.3%) and all their mould numbers exceeded the limit value. Therefore, in the sample evaluated to be "good" on the basis of the CFU values *Penicillium* or/and *Aspergillus* were found.

It can be concluded that the occurrence of toxinogens was not related to total mould count above or below the limit value.

Out of the 23 *Aspergillus* samples 6 (26%) could be regarded mouldy organoleptically, while 17 could not. Out of *Penicillium* samples 3 (11.5%) were visibly mouldy, and out of the 23 *Fusarium* samples only 6 (26%) proved to be visibly mouldy, 17 not mouldy.
Therefore, the probability of the occurrence of toxinogens cannot be predicted on the basis of the organoleptic properties of samples as well.
As opposed to Hungarian examination practice in feed-microbiology, the members of *Alternaria* genus were not grouped to be potential toxinogens. Both in Hungarian and international literature no clear data suggest that alternariol-toxicosis were significant among animal species fed on hays.

Other observations:
In dry alfalfa hay, *Aspergillus* genera propagated to a great extent as a result of remoistening (storing shed leaked several times, bales wetted by rain).
The occurrence of *Fusarium* species was evidently more frequent in autumn.

On a culture medium prescribed by MSZ-ISO 7954 standard, mould flora on hay developed fast enough to satisfy the requirements of mass examination. However, the excessive growth and spreading of *Mucor* and *Trichoderma* species posed a problem, which could be solved by using a special culture medium containing inhibitors.

4.3. Effect of physical factors responsible for the mass propagation of moulds on hays
The propagation kinetics of moulds was tested in incubated grass samples in chambers of constant humidity, by monitoring the changes in CFU/g values. This growth rate can be expressed with the value of delta-logTKE/day. We found that growth rate during the first 8 days was primarily determined by the initial moisture content of the grass sample.
If the starting moisture content of the sample exceeded 40%, the growth of mould fungi was intensive, independent from initial mould contamination, humidity and temperature of the atmosphere, but if the humidity of the atmosphere was below 90%, at 15 °C the growth started only after the 4. day. Samples with a lower initial moisture content (below 40%) showed a fall in their mould count in an atmosphere of higher relative humidity. After the 8. day in samples with

moisture content lower than 40% the pace of mould growth was determined predominantly by relative humidity of the atmosphere. Depending on the moisture content of samples, at the humidity of 84% and below the growth of different genera of mould fungi did not show any significant differences. The growth of mould fungi was less by 0.1-0.21 logCFU/day at 15 °C than at 25 °C. This difference is insignificant.

It can be concluded that the growth of mould fungi on hay is determined by the moisture content at cutting and harvest during <u>the first 8 days of storage</u>. <u>After day 8.</u> the growth of mould fungi is depending on relative humidity of the atmosphere of the storage primarily.
Higher temperature does not accelerate the growth of mould fungi considerably.

4.4. Sensitivity of product typical mould flora of hay, maize grain and *Aspergillus parasiticus* to the presence of fermentation acids (lactate, acetate)

Evaluation of cultures incubated with lactate and acetate.

1. Lactate exerted a detectable inhibition on the product-typical mould flora of hay, but it did not inhibit its mycelial development (culture media 1.2. and 3.) The inhibitory effect of lactate is likely to be selective as regards the various species of hay flora.
2. The species of hay flora grew practically in 100% on carbohydrate-free substance which contained lactate, their colony development was of 100%, so they utilized lactate to the same extent as carbohydrates. Their growth on a substance which contained neither carbohydrate nor lactate reached only 55% (media 1. and 5.).
3. Hay flora growth was detectably inhibited by the medium prepared from killed off *Lactobacillus* culture both in terms of CFU and the rate of colony growth, but this inhibitory effect was not stronger than Na-lactate in itself (media 1. and 6.). Therefore, the inhibitory effect was due to the lactate content of the medium.
4. In *Asp. parasiticus* cultures, Na-lactate did not inhibit the germination of spores (numerical inhibition) in 1.0 and 2.0%, w/v, calculated on water basis, neither colony growth (mycelial inhibition) – see media 1. 2. 3. It follows from this that under aerobic circumstances fermented forages can be susceptible to the propagation of aflatoxin producing species just as well as non-fermented substances of similar water activity.

5. The germination of *Asp. parasiticus* spores was significant in carbohydrate free subtrates as well, whereas the rate of inhibition for mycelial growth is high (media 1. and 4.) When the fermentation of lactic acid has already metaabolized the carbohydrates, lactate is present in the substance. The comparison (CFU and colony diameter %) of medium compositions 1. and 5. revealed that *Asp. parasiticus* could utilize lactate as well.
6. The substance of killed-off submerse *Lactobacillus* culture does not exert special inhibitory effects on *Asp. parasiticus* (media 1. and 6.). As lactate has no inhibitory effect on this species, besides lactate, the substance of killed-off *Lactobacilli* does not produce other types of inhibition on *Asp. Parasiticus.*
7. Where as 2% lactate inhibited hay flora slightly, acetate in 1.5% concentration (equimolar with lactate) inhibited them completely. Acetate was not utilized by any element of the mould flora.
8. Acetate in 1.5% inhibited the growth *Asp. parasiticus* completely. The same was found with the equimolar combination of lactate and acetate. *Asp. parasiticus* did not seem to utilize acetate.
9. Decreasing pH (6.5; 5.5; 4.5) somewhat depressed the total growth of hay flora and selected the species composition of the flora.
10. Decreasing pH did not inhibit the growth of *Asp. parasiticus* significantly. Growth and numerical inhibition could br found in a subtrate of pH 4.5, but conidial generation started faster.
11. Lactate in concentration of 2% did not exert any inhibitory effects on product typical mould flora of maize (mostly *Aspergillus).* The flora utilized lactate almost equivalently, whereas equimolar acetate exerted complete inhibition.

4.5. Results of studies aiming to develop medium suitable for the mycological evaluation of hay.

Rose Bengal stains the the medium pink, causing difficulties in the evaluation of colours, especially pink shades of the colonies. In the same time the inhibitory effect of Rose Bengal was insignificant in itself and it was intensive only for yeasts. Earlier detection of growing colonies in initial stages, based on their pink linking capacity was not a considerable advantage.

The development of *Mucor* was retarded on a medium with reduced nutrient concentration (1/4 N); the CFU values were practically equal with values from media with full nutrient concentrations, but the colonies of various species having generally developed were smaller and their colours was less intensive.

In the case of a slight inhibitory action *Mucor* was loosing its capacity to generate intensively spreading aerial mycelia, but retained its capacity for surface spreading. It could still occupy large areas, so it was hampering the detection of CFU. In the case of more intense inhibition it was not suppressed completely, and its spreading was about equal with that of other elements of the flora.

Conclusions:

1. MSz ISO 7954 medium specified for the determination of feed mould count is not suitable for the examination of forages, as in about 50% of the cases the excessive growth of *Mucor* hampered the enumeration of CFU count and the recognition of toxinogenic colonies as well.
2. Using inhibitors or reducing the nutrient levels of media, CFU count enumerable from samples was practically equal with CFU values with standard MSz ISO 7954 medium, including the usual sources of error.
3. DRBC composition, which contained inhibitors (King et al., 1979) allowed early CFU counting (more uniform colony sizes), and at the same time it suppressed *Mucor*. However, the background colour of media made difficult to detect the colour of conidial formations especially that of toxinogenic *Fusarium.* The hypha staining property of Rose Bengal was not a considerable advantage.
4. In the current phase of our examinations MSz ISO 7954 - Dichloran combination was found to be the most suitable, since it allowed the early counting of CFU value, suppressed Mucors just like DRBC and it was colourless, so it facilitated the recognition of characteristical colony colours.
5. 2-4-dinitro-salicylic acid (DSA) did not prove to be useful as an inhibitor due to its intensive background colour and inefficient inhibiting activity.

4.6. Results of experimentation with a selective staining technique for propagulums

Staining propagulum formations in standard basal suspensions of hays and forages, no considerable selectivity could be found with malachite green (MG), amidoblack (AB) and Sudan III,.
Rose Bengal (RB) was bound by conidia (spores) well and they could be recognized on the basis of dark red coloration, while the stain got dissolved from other microformations in the smear. Species which generate pigmented (black) colonies did not get stained with RB, but they could be recognised due to their pigmentation. RB was slightly bound to starch particles during staining. In the suspension from maize meal, as a result of the boiling action during the staining, destructured starch particles could only be seen as a pink fragment- mass. RB also stained bacteria, which could be seen as faint dust particles with the recommended magnification. Plant fibres, hair, fragments and often nematodes did not bind RB, so they could be safely distinguished from formations of mould origin.

In sample suspensions AB bound to propagulums slightly, and its bluish-green colour was much less characteristical than in the case of RB staining. We examined the Sudan lipid staining regarding the apolar elements of spore shells. Its selectivity was not sufficient, it did not show septulatedness in hyphas, and it bound to starch particles as well.
Our method was suitable for making dry preparatums. Particles in the dried agaragar layer on the slide could not clog together even at the edges. Compared to water preparations, as a result of the greater refraction index of dry agaragar, the contours of phase objects were rather faint.
Staining coped with this problem, as the absorbancy of the colour practically converted the phase object into an amplitude object. Dry preparates have the advantages as they are suitable for archivation, which can facilitate the objective documentation of any evaluation. On the basis of this, with a known dilution and volume of the suspension taken for the staining, taken into account the volume of the material stained spread on the glass plate, the spore count of mould contamination could be calculated, which was closely linked with CFU and indicated the antedecents of the batch as well.

4.7. Experiments on the applicability of invertase tests in the case of some genera typical in hay

Among the examined product specific mould species, ***Nigrospora, Alternaria*** and ***Cladosporium*** species produce detectable invertase. The growth rate of *Nigrospora* reached an extent which had a value of invertase test indication as this activity was significant in 24 hours and it prevailed for 48 or 72 hours as well. *Alternaria* showed intensive production after the 24-hour lag phase in the following 24 hours, but it stopped in 48-72 hours. *Cladosporium* produced significant invertase after even a longer phase of 72 hour. Interestingly, all the three species are pigmented.
Regarding the fact that on the basis of experience "black moulds" add up to about 50% of product typical moulds, and *Cladosporium* dominates here as well, invertase tests cannot be used for the fast detection of hay mould contamination.

4.8. Examination of the correlation of the copy numbers obtained by DNS RT-PCR, using the ALLFun-Taq mould specific primer and the CFU numbbers assessed by the (standard) method of cultivation

The copy number of fungus 18S rDNS, measured with AllFunTaq primer, did not indicate the mycological status of hay samples even as in tendency, as compared to organoleptic quality and authentic CFU. Correlation between mould numbers detected with methods based on molecular biology and traditional cultivation was not found (the value of correlation was very low R=0.17).
High PCR copy numbers besides relatively low CFU could be explained by the fact that dead residues of earlier mould gradation were detected in the sample. However, these samples looked healthy by organoleptic and sensory examination. (these samples contained mostly *Alternaria*, *Cladosporium* and *Mucor* species in their product typical mould flora. Mayer (2002) proved their reactivity with AllFun Taq in her model experiments).
Copies could not be detected from some evidently mouldy samples with high CFU values. The quantification of a mass of moulds having generated in hay samples under natural circumstances with RT-PCR was highly uncertain due to the fact that copy number available from the same sample was basically influenced by the method of DNS extraction from the mycelium cells closed by chitin-walls and from cellulose-lignin structures of the matrix
Similarly, between the log - copy numbers (sample 48P: 11.721; sample 48R: 11.491) from the RT-PCR examination on sample 48P (mouldy alfalfa hay disintegrated with quartz sand, dust fractions) and 48R (the fibre fraction of the same) suggested that the mycelium mass which had

grown on fibres contained mould fungus DNA to the same extent as the dust fraction, which contained presumably conidia mostly.

The suitability of AllFunTaq RT-PCR method for detecting mould contamination will be realistic as soon as the total quantitative extraction of DNS becomes feasible from the structures desiccated with dry vegetative substances in some way, which is still stillknown. The problem could not be solved by the definition of total DNS quantity in the studied samples, since a much greater percentage of the total DNS volume is likely having been extracted from plant cell formations than from micro-formations of mould origin.

Other observations:
When comparing CFU data - meals from chaffed hays, sample set 1. and meals from sample set 2. - a mould number smaller of two logs could be detected. This decay could have been caused by heat generated during grinding, i.e. the warming up of the meal..

5. Scientific

- On the basis of processed hay samples from Hungary we determined the product typical value of mould contamination: this is 1 x 10^6. This is indispensable for classification by quality classes and for the determination of the CFU limit values for moulds.

- The occurrence of toxinogens in hay mould flora is independent from the extent of mould contamination.

- The propagation of moulds on hay depends mostly on water content at the start of storage, much less on water activity during storage and even less on temperature during storage.

- As fermentation products, lactate fails to inhibit both the propagation of product-typical hay flora and toxinogenic *Aspergillus parasiticus*, while acetate inhibits them significantly.

- ◆ MSz ISO 7954, specified for the mould count determination of feed, is not suitable for forages as standard medium, since in about 50% of the cases the overgrowth of *Mucor* disturbs both the counting of CFU values and the recognition of toxinogenic colonies. MSz ISO 7954 standard composition with Dichlorane supplementation can be recommended for the mycological examination of hays with *Mucorales* suppression, satisfactory colony growth and well observable colors of the colonies.

- ◆ Selective staining method with Rose-Bengal in agar matrix was developed for the informative microscopic evaluation of mould contamination in hays.

- ◆ The -D-fructofurazonidase (invertase) test for enzyme production, in the case of hays, shows a significant reaction in only three product-typical genera, unlike to toxinogenic genera of storage specific moulds in corns.

- ◆ No correlation has been found between the copy number obtained with RT-PCR, AllFun-Taq mould specific primer and the CFU determined by the standard method of cultivation. As yet, this method can not be recommended as routine method of evaluation of hays.

Publications

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