THE FUNGAL FLORA OF HOKKAIDO HAY

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Introduction

Hay can be defined as the forage resulting from the drying of grass crops. This process of making hay is a very old one, dating back to the time when people used hand sickles for harvesting, a process still common in many dariy farms today. This food is one of the principal feeds for live stock during that period of the year when the animals are not on pasture. Even when animals are on pasture it is sometimes used in small quantities to ward off bloat or to supplement the pasture during periods of extreme drought[1].

Whereas the identification of the microflora of grass preserved by ensilage is extensive, including the work by one of the authors[2], the identification of molds found on field-cured hay is limited. Work has been done though, on the bacterial identification on hay such as *Pseudomonas*[3], *Flavobacterium*[4], and *Erwinia*[5] on grass and that of yeasts such as *Torulopsis*, *Candida*, *Cryptococcus* and *Rhodotorula*[6,7,8].

Much work has been done in the past fifteen years in the field of mycotoxins and their effects on animals. The most famous one, aflatoxin from *Aspergillus flavus*, was isolated from poultry feed containing high protein diets of groundnut meal[9]. Since then, toxins from molds such as ochratoxin[10], patulin[11], rubratoxin[12], tremortin[13], and other substances have been identified from moldy animal feeds.

The object of this study was (A) to examine the viable counts of molds in hay made under various conditions, (B) to isolate molds from these samples, (C) to identify typical molds from these samples, (D) to see if there was any correlation between hay making conditions and the growth of molds and (E) to see if there were any mycotoxin producing molds among the ones identified.

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Materials and Methods

As shown in Table 1, eleven samples of hay were obtained from the Hokkaido National Agricultural Experimental Station. These were from four regions of Hokkaido: Shihoro, Teshio, Urahoro, and Shibecha. They were prepared in the spring of 1977 and sent to the above mentioned experimental station for quality examination that summer. They ranged in grade from excellent through poor based on color, leaf to stem ratio, and the amount of extraneous plant material.

TABLE 1. Characteristics of sampled hay*

Sample number	Grade	Place of production	Green degree	Ratio of green leaf to stems	Reference
I	Excellent	Shihoro	55	20.0	
II	3rd class	Shihoro	48	9.5	
III	Poor	Shihoro	30	23.7	Moldy, including other plants
IV	Poor	Shihoro	5	1.2	Green degree decreased by heavy rainfall
V	Poor	Shihoro	25	1.2	Moldy by heavy rainfall
VI	1st class	Teshio	45	16.6	Including other plants
VII	Poor	Teshio	20	8.8	Moldy
VIII	1st class	Teshio	40	19.3	
IX	2nd class	Urahoro	47	9.2	
X	Poor	Urahoro	44	12.6	Including other plants
XI	3rd class	Shibecha	45	4.5	

*: From Hokkaido National Agricultural Experimental Station.

The procedure used to sample the hay was as follows: First the scissor blades were swabbed with alcohol and then the hay was cut up into small pieces. Five grams were added into a sterile Erlenmeyer flask along with 100 ml of sterilized water. This was then shaken for ten minutes. One to ten sterile water dilution blanks were made up and used to dilute the solution to 10^{-6} dilution.

One ml of each dilution was then added to a sterile petri dish along with sterile warm malt extract agar and one ml of 1% lactic acid to inhibit bacterial growth. These were then incubated at 27°C until we were able to count the number of mold colonies on one of the dilution plates for each hay sample.

From these plates, representative mold colonies were isolated on malt extract and Czapek's agars and identified[15,16,17,18,19,20,21,22]. For a direct obser-

vation of the sample, scotch tape was touched to the surface of the hay and then observed under the microscope.

Results and Discussion

Table 2 shows that the molds per gram of hay varied from a low of 1.0×10^4 for sample II to a high of 2.4×10^6 for sample IX. The authors expected that the low quality moldy hays would show higher viable counts of molds, but on the contrary, they showed a lower number than the excellent quality hays. For example, in the samples from Shihoro, the excellent sample had a concentration of 1.0×10^6 while the third class sample had a low concentration of 1.0×10^4. The same is also true for the samples from Teshio and Urahoro, the lower quality hays also having the lower number of molds. This suggests that the excellent quality hays would be low in viable counts during hay making process, but the growth of xerophilic molds occurred during the course of preservation. On the other hand, the lower quality hays, produced under wet conditions, were visibly moldy but the powdery molds' spores on the hay supposedly died off during the drying process of hay making due to the spores inability to survive under low moisture conditions.

From Fig. 1, one can see that the overwhelming number of isolations are from the genus *Aspergillus*. Also in Fig. 2, in the genus *Aspergillus*, the overwhelming number are from the *Aspergillus glaucus* group. Among these molds, the dominant one is *Asp. montevidensis*, a member of the *Aspergillus glaucus* group. This group is a xerophilic one and also includes

TABLE 2. Viable counts of molds in various qualities of hay

Sample number	Grade	Place of production	Molds/gram of hay
I	Excellent	Shihoro	1.0×10^6
II	3rd class	Shihoro	1.0×10^4
III	Poor	Shihoro	8.4×10^5
IV	Poor	Shihoro	1.9×10^5
V	Poor	Shihoro	1.9×10^6
VI	1st class	Teshio	8.4×10^5
VII	Poor	Teshio	3.4×10^4
VIII	1st class	Teshio	1.6×10^6
IX	2nd class	Urahoro	2.4×10^6
X	Poor	Urahoro	6.2×10^4
XI	3rd class	Shibecha	2.0×10^5

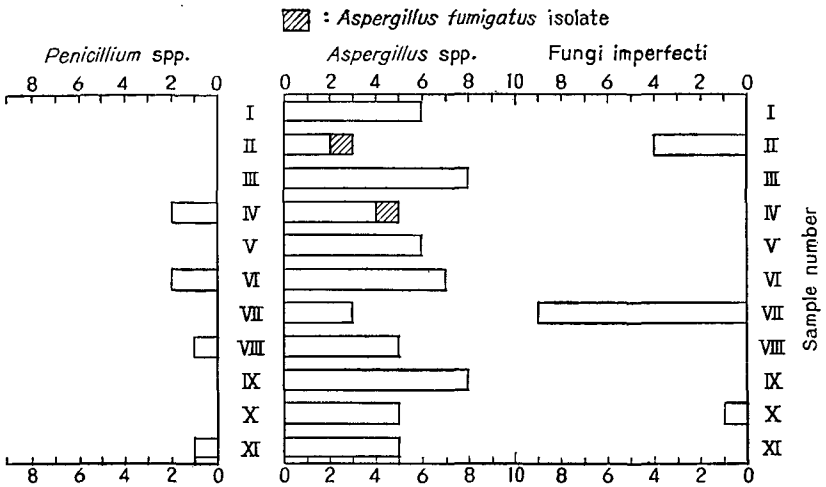

Fig. 1. Number of isolates from hay samples.

the other isolates; *Asp. amstelodami*, *Asp. repens*, *Asp. chevalieri* and *Asp. chevalieri* var. *intermedius*[21]. The results of these two figures agree with GREGORY and LACEY[23], who worked with various qualities of hay, some of which caused farmer's lung disease. This disease is possibly caused by the inhalation of dust from moldy hay. They found that among spores recovered from better grades of hay, most had a preponderence of *Aspergillus glaucus* type spores, whereas the poorer grades of hay had a preponderence of *Aspergillus fumigatus* type spores.

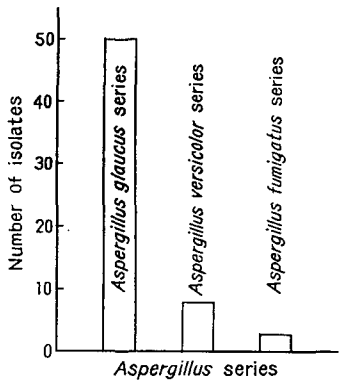

Fig. 2. Distribution of *Aspergillus* spp.

Table 3 shows that the hay of good qualities, excellent, first and second class (samples I, VI, VIII, and IX), are composed entirely of *Aspergillus glaucus* group and some *Penicillium* species. In contrast to this, *Aspergillus fumigatus* type occurs only in the poorer classifications of hay, that of sample II and IV.

Table 4 shows the results of the direct observation of hay in the first seven samples. In the hay of good quality, samples I and VI, mainly *Aspergillus* and some *Alternaria* were found. Whereas for the poor hay samples II–V and VII, several different molds from the Fungi imperfecti were identified. Since most of these genera weren't found in the dilution method

TABLE 3. Identification of molds

Species identified	Hay sample number										
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Aspergillus amstelodami	2		4		3	2	1		5	2	1
" *montevidensis*	2	1	1					1	3	1	1
" *repens*	2		2			2	1	2		2	2
" *chevalieri*						1					
" *chevalieri* var. *intermedius*			1			2		2			1
" *fumigatus*		1		2							
" *versicolor*		1		3	3		1				
Penicillium cyclopium				1							
" *implicatum*						1					1
" *thomii*						1		1			
" *expansum*				1							
Papularia sphaerosperma		3					1				
Cladosporium herbarum							2				
Mucor hiemalis							2			1	
Alternaria tenuis							1				
Phoma sp.							1				
Fusarium graminearum							1				
Epicoccum purpurascens							1				
Mycelia sterilia		1									

TABLE 4. Number of species counted by direct observation of hay

Species observed	Hay sample number						
	I	II	III	IV	V	VI	VII
Aspergillus sp.	5	1	1		1	3	1
Alternaria sp.	1	1		1	1		1
Fusarium sp.		1					
Cladosporium sp.		1	1				
Scopulariopsis sp.		1	1	1			
Helminthosporium sp.			1		1		1

technique, this might explain the reason for the low numbers of fungi observed in the poor moldy hay samples. Possibly these molds on the hay were already dead, or it was difficult to dislodge them from the hay or possibly they remained dormant on the malt extract agar.

Among the aspergilli isolated, *Aspergillus fumigatus* and *Asp. chevalieri* have been shown to produce toxic byproducts. Under experimental conditions, *Asp. fumigatus* is known to produce three antibiotics toxic to animals; fumigatin[24], helvolic acid[25], and gliotoxin[26]. *Asp. chevalieri* was isolated from bread crumb feed pellets that produced hyperkeratosis in calves. Extracts from cultures of this fungus produced hyperemia on the skin of a calf and rabbit and caused extensive visceral hemorrhages and death of calves when administered orally[27].

Among the penicillia isolated, *Penicillium cyclopium* has been reported to be toxic to animals. It reportedly produced tremorgenic toxins that were neurotoxic to sheep and horses[28]. Lastly, among the other Fungi imperfecti isolated, *Alternaria* and *Fusarium* have been shown to cause illness in animals[29,30,31]

Summary

The fungal flora of hay was determined to clarify the relationship between hay qualities and microflora. Nineteen species of molds were identified, predominant among which were members of the *Aspergillus glaucus* group. The hays of excellent quality were found to have higher viable counts than those of lower quality, presumably due to the growth of xerophilic molds during the preservation process and inhibition of powdery molds's spores during the drying process of hay making.

Among the isolates, *Asp. fumigatus* is known to be a causative mold of aspergillosis in man and almost every species of domestic animal and bird. *Asp. fumigatus*, *Asp. chevalieri*, *Pen. cyclopium*, and *Fusarium graminearum* have been shown to produce toxic substances to animals. The results show that the prevention of fungal health hazards from hay is a very important problem for farmers and domestic animals.

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