Navarro, S. and Donahaye, E. [Eds.](1993) Proc. Int. Conf. Controlled Atmosphere and Fumigation in Grain Storages, Winnipeg, Canada, June 1992, Caspit Press Ltd., Jerusalem, pp. 151-162.

ANAEROBIC GROWTH OF STORAGE YEASTS IN CONTROLLED ATMOSPHERE STORAGE OF INTERMEDIATE MOISTURE GRAINS

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ABSTRACT

In vitro experiments carried out on cereal grains at intermediate moisture contents (a_w approximately 0.90) revealed extremely low oxygen (O_2) requirements by the so-called "storage yeasts". Experiments at the farm level demonstrated that at water contents between 18 and 20%, respiration of storage yeasts is sufficient to consume the O_2 that penetrates silos filled with well-compressed ground-wheat or maize, that is protected from the ambient air by plastic sheeting only. In the outermost layers of grain in contact with the air, where temperatures for one or two days rose to 25-30°C, dry matter losses remained very low and without an evident reduction in nutritional value. The stored moist cereals appeared perfectly sound, and following their consumption, no pathogenic or toxinogenic effects were reported.

INTRODUCTION

For microbiologists, the use of naturally or artificially created modified atmospheres (MAs) in grain storage is of interest only for grains or derived products with sufficiently high moisture content (m.c.) to allow some growth, or enable metabolic activity, of microorganisms. The microorganisms that compose the microflora of cereal grains and their products are now well-known with regard to their main ecological requirements and the damage they are capable of causing during storage. The extraordinary physiological abilities of xerotolerant moulds enable them to grow under dry conditions and they invariably develop on stored grains unless additional "barriers", such as antifungal agents or modified atmospheres (MAs) are utilized. These microorganisms contaminate cereal grains world-wide, though the growth of the so-called "xerotolerant or xerophilic" species is very slow, and usually is not accompanied by mycotoxin formation or by significant deterioration of nutritional value of the infected commodities. In some countries, and mainly for economic reasons, grain is often stored at m.c.s in the order of 15 - 16 % (wet basis) that permit

storage for several months without visible mould development or other microbial damage, but enable xerophilic species to develop slowly.

Sometimes weather conditions do not permit harvesting at these m.c.s, or it is not economically feasible to dry the grains. Then, providing the relative humidity (r.h.) in equilibrium with the grain is sufficiently high, and providing O_2 is available, growth of microorganisms occurs, while the higher the humidity and temperature, the more intense and rapid the development of microorganisms.

Although storage yeasts such as *Hyphopichia burtonii*, *Candida sp.*, or *Cryptococcus sp.* have an absolute need for O_2 for ergosterol biosynthesis, this requirement is so limited that from a practical point of view they may be considered capable of growing anaerobically. This indicates that microbiological stabilization of such grains would be impossible to achieve in practice, at the farm level, where sophisticated structures for hermetic storage are not available. Consequently, the question arises as to how this natural "microbial O_2 demand in grains" due to yeasts, may be put to an advantage to protect grains of intermediate moisture destined for animal feed from mould development.

Taking into account the ability of lactic acid bacteria and of some hydrophilic field fungi, such as *Fusarium* species, to tolerate anoxia and high concentrations of carbon dioxide (CO_2), it seems evident that for the storage of very wet grains, MAs are inappropriate. By contrast, for grain at intermediate m.c.s (i.e., a_w from 0.85 - 0.90, or 18-20 % m.c.), controlled atmosphere storage appears feasible provided that O_2 levels can be reduced sufficiently to inhibit the gradual growth of moulds and yeasts. It is necessary, nevertheless, to study more carefully the growth of microfungi under reduced-O_2 atmospheres.

I. MOULD AND YEAST GROWTH UNDER LOW-O_2 CONDITIONS

The tolerance to anoxia of the main groups of microflora most frequently encountered in cereal grain and their water requirements have been reviewed recently, and data from the literature are summarized in Table 1 (Richard-Molard, 1990).

In previous experiments carried out in the laboratory using microsilos (10-liter capacity) filled with grains at 0.90 a_w and flushed every day for 2 min with "pure" nitrogen (containing only traces of O_2), it was established that the absolute O_2 requirement of moulds to continue to sporulate ranged from 35 to 60 µg O_2/day/g grain. It was also observed that the sporulation of storage yeasts such as *Candida* sp. *Cryptococcus* sp., or *H. burtonii* was not inhibited when levels of available O_2 were as low as 5 µg/day/g of grain (Diawara *et al.*, 1989). Under such conditions, the so called "storage yeasts" compete aggressively with moulds for O_2 uptake and mold growth would probably be delayed and reduced when they develop.

Table 1: Minimum water requirement and tolerance to anoxia of cereal microflora.

Average m.c. (% w.b.)	Minimum a_w for growth (at 20°C)	Microorganisms	Tolerance to anoxia
> 30	0.95	Most lactic acid bacteria	High
20	0.90	Strains of lactic acid bacteria, Most yeasts, Most field fungi	High
18	0.85	Storage yeasts Storage fungi	Medium Low
16	0.80	Xerotolerant fungi	Low

These storage yeasts, first described under different taxonomic names by Teunisson (1954a, b), and observed by Burmeister and Hartmann (1966), Nichols and Leaver (1966), and Richard-Molard *et al.* (1984), among others, are not unicellular microorganisms when cultivated in laboratory culture media, and are capable of developing in mycelial forms like fungi. Therefore, their growth cannot be estimated correctly only by colony forming unit (CFU) counting, because there is absolutely no evidence that the biomass produced would be in good correlation with CFU under the particular conditions of water activity and O_2 denial. It was, therefore, deemed necessary to study the growth of these storage yeasts in more detail.

Experimental

Experiments were performed in laboratory microsilos using grain re-moistened to 20 % m.c. (0.90 a_w) and inoculated with *H. burtonii*, probably the most typical storage species, and with *Penicillium cyclopium* for comparison. The rate of inoculation was approximately 10^4 CFU/g grain and was found to be sufficient to inhibit the natural microflora of the cleaned grains (wheat and rice) used in these experiments. Inocula of both microorganisms were obtained from pure cultures at 0.90 a_w in order to minimize the osmotic changes in spores and conidia, and were harvested from the cold water utilized for moistening the grain (15 hr of gentle mixing at 4°C). At the same a_w (0.90) and temperature (20°C), the growth was determined on grain by CFU counting on appropriate culture media (Malt Extract Agar, after 7 days of incubation at 25°C), and ergosterol measurement by HPLC, according to methods described elsewhere (Cahagnier *et al.*, 1983).

Under these experimental conditions, it was not possible to detect any microbial biomass by ergosterol determination, nor CFU produced when strict anaerobiosis was applied (i.e., silos flushed once only with "pure" nitrogen immediately after filling). Only traces of ergosterol could be detected in experiments in which extremely limited quantities of O_2 were released into the microsilos (below 60 µg /day/g grain). It was, therefore, considered of interest to examine the effect of higher rates of O_2 supply on the storage yeasts. This was done by supplying O_2 at a rate of 2.5 mg/day/g grain, as shown below.

Ergosterol content of H. burtonii biomass

The estimation of a fungal biomass by ergosterol determination assumes that the ergosterol content of the biomass remains constant irrespective of the a_w and the O_2 level. This was established previously for storage moulds (Richard-Molard *et al.*, 1985), but not for storage yeasts.

In preliminary experiments carried out in liquid culture media (1-liter flasks containing 300 ml malt extract medium + glycerol, $a_w = 0.90$, at 20°C) *H. burtonii* was cultivated under different conditions of oxygenation in order to assess the ergosterol content of the biomass produced. Flasks were inoculated with yeast at levels of approximately 10^4 CFU/ml and placed in the laboratory microsilos described previously. Growth was studied for 30 days in air (i.e., microsilo open, O_2 supply not restricted), under airtight conditions (microsilos flushed with "pure nitrogen" providing about 70 µg O_2 per day), and with mixtures containing about 1.5 mg O_2 provided every day, with or without 50% CO_2.

Results

Results obtained are shown in Fig. 1. After 5 days of growth, ergosterol levels in the biomass appeared widely dispersed without evident cause. The reason may be due to the very low biomass produced after only 5 days, that introduced a relatively high error in HPLC determination of ergosterol. After 10 days, all ergosterol levels were found to be between 1 and 2 mg/g of the biomass, and remained constant for at least one month. Biomasses produced ranged from 50-150 mg per flask after 10 days, and 300-400 after 30 days, the higher the O_2 ingress, the greater was the biomass.

It was, therefore, concluded that ergosterol measurement could be used to quantify, with a reasonable degree of precision, the growth of storage yeasts in solid media such as cereal grains. For comparison, results of other studies showed that microfungi of the storage microflora (*Penicillium* spp. and *Aspergillus* spp.) produced ergosterol levels of approximately 6-8 mg/g of the biomass.

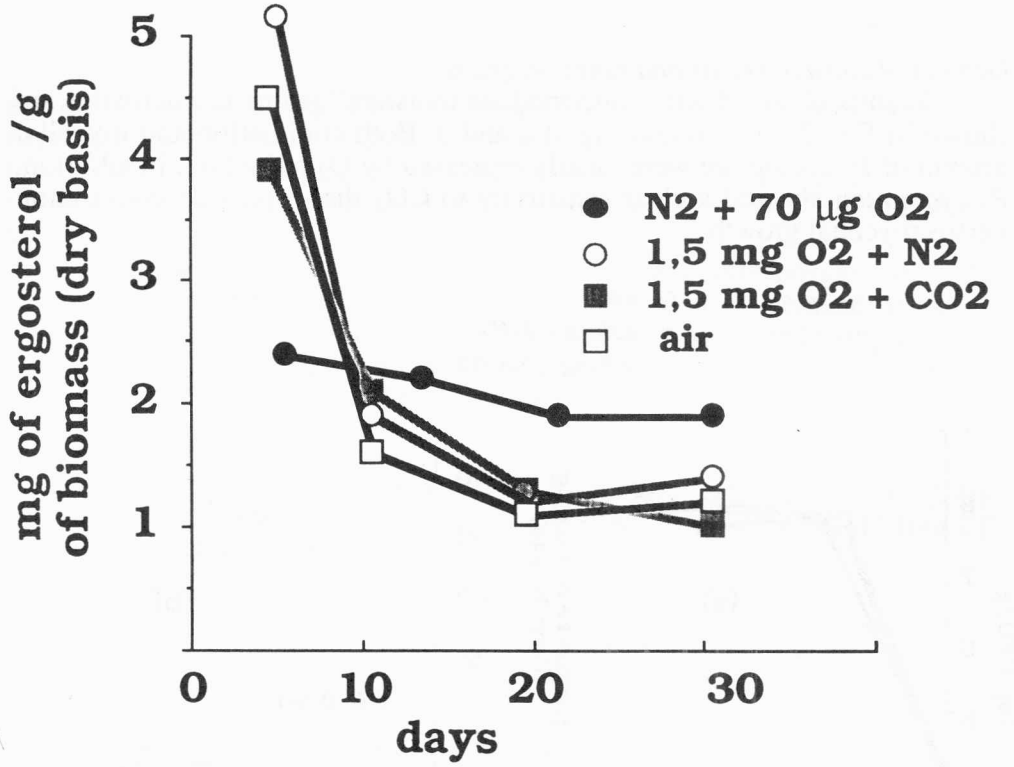

Fig. 1: Ergosterol content of the biomass of *Hyphopichia burtonii* grown under different gaseous conditions.

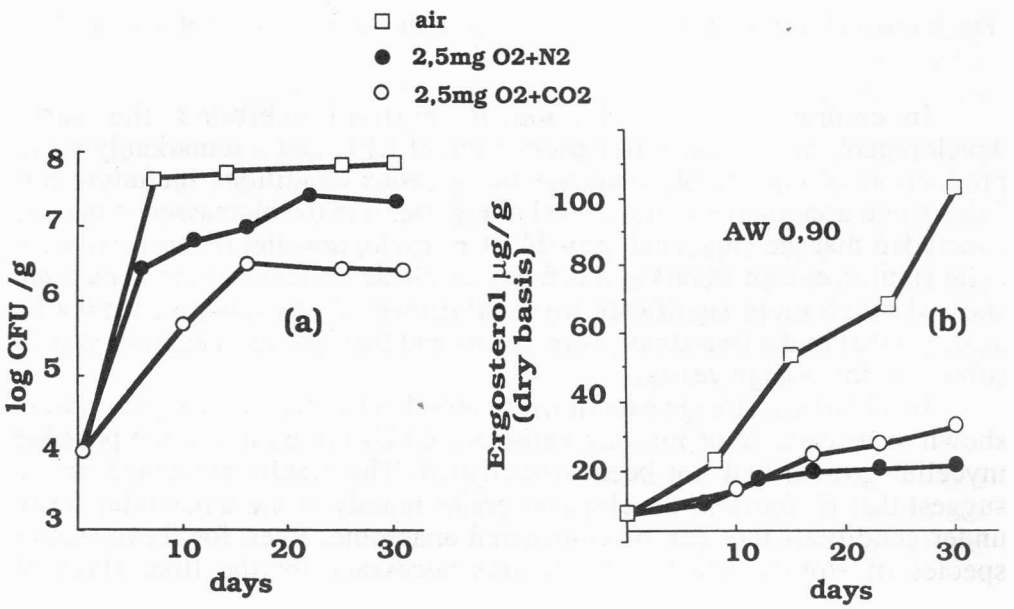

Fig. 2: Effect of controlled atmospheres on (a) conidiation and (b) growth of *Penicillium cyclopium*.

Growth of storage yeasts and fungi on grain

Results obtained with "intermediate moisture" grains in microsilos are shown in Fig. 2 a and b and Fig. 3 a and b. Both conidiation and mycelial growth of *P. cyclopium* were clearly repressed by O_2 reduction. In addition, *P. cyclopium* showed a clear sensitivity to CO_2 that repressed conidiation versus mycelial growth.

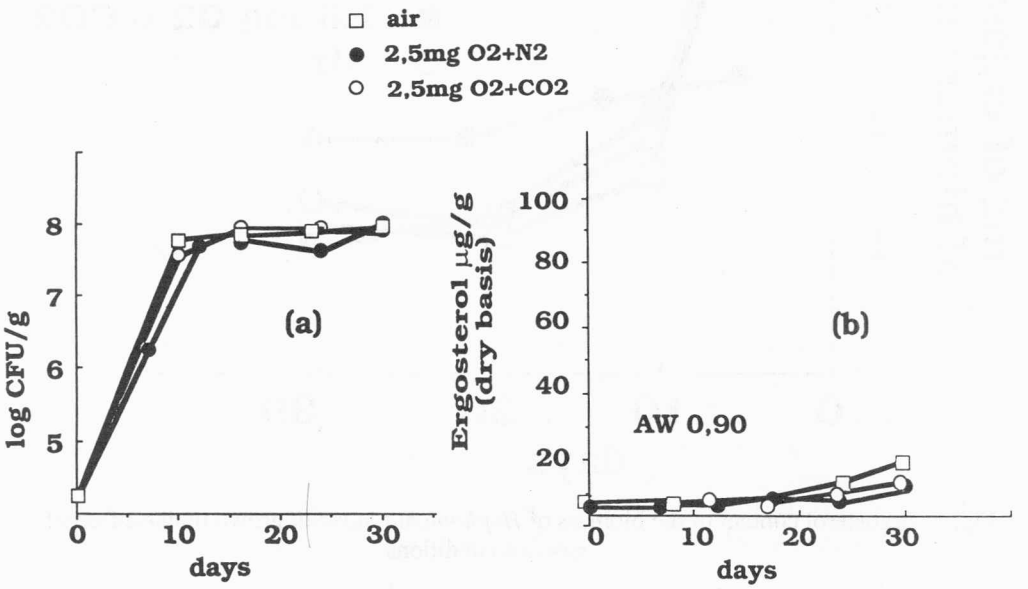

Fig. 3: Effect of controlled atmospheres on (a) sporulation and (b) growth of *Hyphopichia burtonii.*

In contrast to *P. cyclopium*, *H. burtonii* exhibited the same development, namely an active production of CFU and a remarkably weak production of ergosterol, whatever the gaseous conditions including air. Taking into account the average level of ergosterol in the biomasses, it may be concluded that the "mycelial growth" of *P. cyclopium* and *H. burtonii* were quite similar, except when O_2 was freely available. In that case, *P. cyclopium* showed much more significant mycelial growth. Consequently, it may be assumed that in the free atmosphere, grains and their products are not optimal substrates for storage yeasts.

In all airtight storage experiments carried out so far, storage yeasts were shown to be capable of forming numerous CFUs on grain, but the possible mycelial growth had not been investigated. The results presented above suggest that *H. burtonii* develops on grains mainly in the unicellular form, under conditions that can be considered anaerobic. Even for fermentative species of storage yeasts, O_2 remains necessary for the final stage of

ergosterol synthesis. When very strict anaerobiosis was applied in laboratory experiments, no growth occurred if ergosterol was not added to the culture medium, but in practical situations, the level of anaerobic atmosphere obtainable in hermetic storage facilities was never high enough to inhibit the growth.

In view of the special behaviour of these microorganisms, the possibility of taking advantage of this natural "microbial O_2 demand" of yeasts to protect grains against mould development was investigated using ground-grains for animal feed, stored at the m.c.s existing at the time of harvest, namely at intermediate m.c.s below a_w <0.90.

II. MODEL OF MICROBIAL GROWTH IN THE OUTER LAYERS OF FARM STORAGE GROUND-GRAIN AT INTERMEDIATE MOISTURE CONTENT

Experimental

Because of the cost and risks involved in large-scale experiments at the farm level, it was decided to first study the microbial evolution in a model of the outer layer of a bulk of ground-grains. This was done using a plastic cylinder (1.2-m high; 0.6-m Ø) containing ground-grains (particle size 0.5-3.5 mm) re-moistened at 0.90 a_w (representing the highest average m.c. of wheat at harvest-time in western Europe) and held at 18°C.

Several nylon-net bags containing known quantities of product were inserted at different depths, to determine the loss of dry matter due to microbial activity during the storage period (Fig. 4).

Following the common practice of French farmers whereby approximately 20-30 cm of the outer layer of the bulk is removed each day for consumption, the cylinder was emptied progressively over one month, by withdrawing 25 cm layers at regular intervals (Fig. 5.)

This procedure represented an exaggeration of the rate of utilization of the stored grain, in which the outer layer of the bulk is normally in contact with air for only one or two days, whereas in this experiment, the outer layer was exposed to the air for 5 days or more. The last sample to be withdrawn had been exposed for more than 20 days at a distance of less than 1 m from the free atmosphere, the only barrier being a progressively decreasing layer of ground-grains, and protection by the microbial consumption of O_2 by yeasts and fungi in the progressively withdrawn outer layer .

Results

In all samples no dry matter losses during the experiment were detectable. This indicates that microbial respiration or fermentation did not utilize more than 0.5% of the dry matter in about one month at the above mentioned m.c.

Evolution of CFUs is given in Fig. 5. At the beginning of the experiment, the ground-grains contained less than 10^3 CFU storage yeasts per gram. This population increased slowly but significantly with time, reaching an apparent maximum level of 10^6 CFU/g after 20 days. Storage fungi were not detected clearly after 15 days but were counted at a level of 5.10^5 CFU/g after 20 days (field fungi, unable to develop at the above m.c. were not shown in the figure).

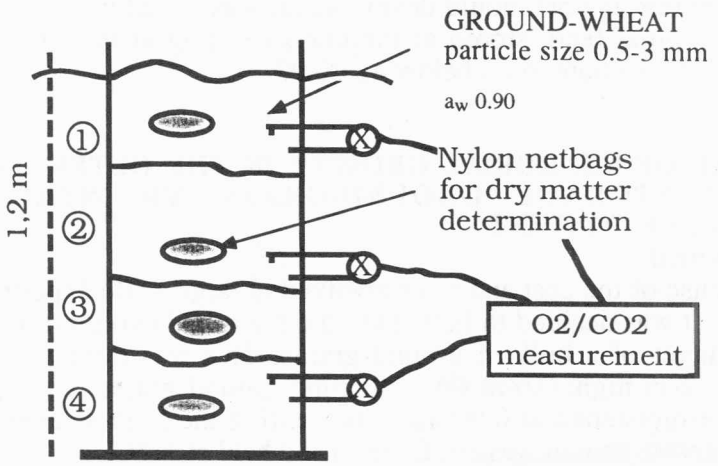

Fig. 4: Model of the outer layer of a farm silo.

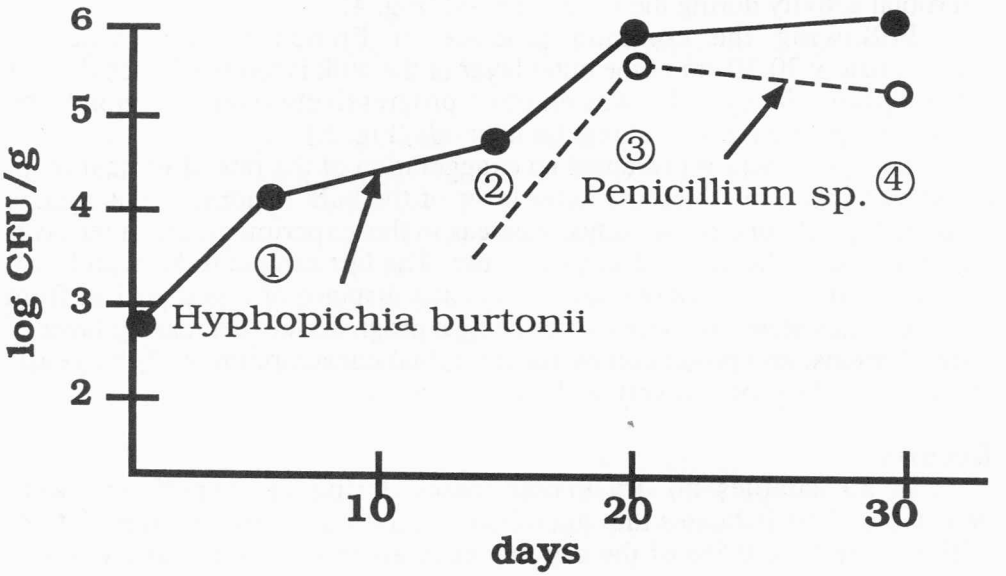

Fig. 5: Microbiological evolution of ground-wheat in the model.

As shown by these data, the yeast population acted as an active O_2 consumer, exerting aggressive competition with moulds, the growth of which was clearly repressed and delayed (comparison in Fig. 2). In consequence, it appeared interesting to carry out larger scale experiments on farms.

III. FARM TRIALS

Experimental

Storage trials were carried out at various locations in western France, using wheat at different m.c.s, ground immediately after harvest and stored between concrete walls to form a bulk of approximately 10 x 3 m, with a height of 2 m. The upper surface of the compressed ground-wheat was protected from the air by a plastic film, as shown in Fig. 6. The structures were open at one end to allow periodic withdrawal of the product. During utilization, a layer of approximately 30 cm product was withdrawn each day for animal feed (pigs and poultry), with the thickness of the layer depending on the daily feed requirement and being a function of the silo geometry.

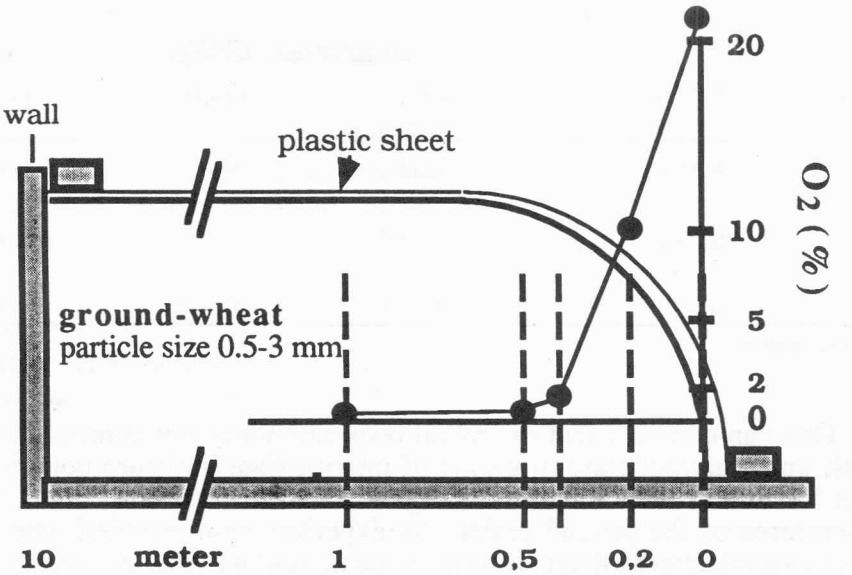

Fig. 6: Oxygen concentrations in a farm silo containing ground-wheat at 0.88 a_w during utilization.

Using small bore tubing and valves and a paramagnetic oxygen analyser, O_2 concentrations in the silo were recorded at different points in the silo during the trials. An example of data obtained with ground-grains at 18% m.c. is given in Fig. 6. Clearly, a given sample of ground-grains remains

exposed to air for not more than 1 or 2 days, which is much less than in the model presented, and discussed previously.

An experiment using ground-grain re-moistened to 29% m.c. was carried out to compare high-moisture silage of maize grains at 29% m.c. as is common practice in France, with the intermediate moisture silage of wheat that we wished to promote, for animal feed on the farm.

Results

Numerous microbiological data were obtained in these trials. Representative results obtained with ground-grain stored at three m.c.s of 15, 18, and 29% (wet basis) are presented in Table 2. Samples compared were those taken from the outermost layer of the bulk after one month of storage and normal use. Average temperatures of the bulks were approximately 20, 25, and 35°C, for grains at 15, 18, and 29% m.c., respectively. Outside temperatures fluctuated from 15-20°C.

Table 2: Microbiological evolution of stored grains in farm experiments.

		Microorganisms (CFU/g)		
Average m.c.	Dry matter losses	Lactic acid bacteria	Yeasts	Fungi
15%	N.M.*	N.M.	N.M.	$5x10^3$
18%	0.3%	$3x10^4$	$5x10^3$	N.M.
29%	9%	$8x10^6$	$2x10^8$	N.M.

*not measurable

One can consider that microbial respiration was not detectable in dry grains, and was moderate in grains of intermediate moisture content. Dry matter losses and evolution of microorganisms were in close accordance with temperatures of the ground-grains. As expected, re-moistened wheat was subject to marked growth and activity of lactic acid bacteria and yeasts, and a normal dry matter loss of about 9% was recorded after one month. Microfungi reported in dry samples (5.10^3 CFU/g) belonged to the so-called "field microflora" and were no longer found in samples at 18% m.c. after one month of "anaerobiosis". In intermediate moisture silage, dry matter losses remained below 0.5%, but lactic acid bacteria and storage yeasts showed only weak development.

No particular zootechnical assays were performed during the use of these products for pigs and poultry feeding. It should be emphasized that

absolutely no significant differences were noticed with regard to animal performance as compared with their consumption of dried grains or grains protected with propionic acid. In particular, no symptoms of any disease or animal discomfort due to storage yeasts could be observed.

CONCLUSIONS

Grain microflora include some yeasts with so high a tolerance to anoxia that they can be considered capable of growing anaerobically. In intermediate moisture grains (18-20% m.c.), quantitative determination of their growth suggested that yeasts such as *H. burtonii* are capable of producing a high number of CFU, but only a very low associated biomass, and cause extremely weak biochemic degradation of the stored-product.

Modern hermetic structures used for CA storage are still incapable of ensuring sufficient airtightness to inhibit these storage yeasts. However, due to the natural O_2 consumption they require, the yeasts contribute significantly to the inhibition and reduction in growth of storage fungi that need higher levels of O_2. This permits the safe storage of ground-grains for farm animals in very simple and inexpensive structures, without any addition of antifungal agent or rehumidification leading to lactic acid protective fermentation. Provided the farmers accept such techniques, farm-storage can be carried out with negligible dry matter losses.

Nevertheless, further studies should be undertaken to more fully demonstrate the absence of any pathogenicity or toxicity due to these yeasts, especially in the *Candida* group. However, at present, such "dry fermented grains" can be regarded as particularly convenient for animal feeding.

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