Feed improvement by energy efficient storage using *Pichia anomala* inoculated ensiled cereal grain

Jenny Borling

Uppsala BioCenter
Department of Microbiology
Faculty of Natural Resources and Agriculture Sciences
Swedish University of Agricultural Sciences

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Jenny Borling

Supervisors: Matilda Olstorpe
Volkmar Passoth

Examiner: Johan Schnürer

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ABSTRACT

Borling, J. 2010. Preservation of moist crimped cereal grain can be achieved as a result of fermentation by lactic acid bacteria. Climatic variations make it difficult under practical farm conditions to harvest the grain at a moisture content (30-45%), suitable to ensure fermentation. Therefore the yeast *Pichia anomala* J121, previously found to prevent mould spoilage and improve preservation of moist grain in malfunctioning air tight silos, was added to the storage of moist crimped cereal grain in big plastic tubes. Freshly harvested barley was rolled and inoculated with 10^5 colony-forming units (Cfu) g^{-1} *P. anomala*, strain J121. Due to the local weather conditions, harvest was delayed and moisture content in the cereal grain had decreased to only 16-18%. Inoculation of *P. anomala* was done in 48 tons of barley, packed into three plastic tubes. Three additional sets of plastic tubes were packed with cereal grain without addition of *P. anomala*. The grain tubes were left closed for 5 months, after which feeding to cattle commenced. The population diversity of lactic acid bacteria (LAB) was very high both in the *P. anomala* inoculated barley and in the non-inoculated barley. A shift in the dominant LAB over the course of storage to *Pediococcus pentosaceus* and *Lactobacillus paracasei* was discernible in inoculated and non-inoculated respectively. The yeast population in the inoculated barley was totally dominated by *P. anomala* during the entire storage period. In the non-inoculated grain, the yeast population was more diverse, displaying shifts in the dominant species during storage. *Pichia burtonii* was the dominant species at the last sampling occasion. In the inoculated grain, mould numbers were reduced by approximately two log units, and the number of *Enterobacteriaceae* was reduced beneath detection limit. Negative effects on animal health or on weight gain were not observed.
Keywords: Cereal grain storage, Microbial population, Feed hygiene, Moulds, *Pichia anomala.*

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1. INTRODUCTION

1.1 Moist crimped grain packed in plastic tubes

The most common preservation method for cereal grain in Sweden is drying. The water content of the kernels can vary highly between different years, depending on weather conditions during harvest (Ekström and Lindgren 1995). Fan drying is an energy and cost intensive method; in Sweden 60% of the total energy used for plant husbandry was spent on drying grain (Pick et al. 1989). One alternative method to drying is crimping and packing wet grain in airtight plastic tubes. Provided that the water content is sufficient, preferably 30-45%, a natural fermentation process starts that prevents growth of spoilage micro organisms. The conservation is based on the production of acids in an oxygen free environment, mainly by lactic acid bacteria (LAB). The respiration of the micro organisms and the crimped kernels uses up the remaining oxygen in the tube creating an environment with high levels of CO_2 and low levels of oxygen (Ekström and Lindgren 1995). Hygienic problems arise when the water content is too low (<30%), which leads to an insufficient fermentation process (Olstorpe *et al*. 2010) or if gas exchange with the surrounding environment enables oxygen to leak in and CO_2 to leak out (Druvefors *et al*. 2002; Ekström and Lindgren 1995; Lacey and Magan 1991). Harvesting and packing the crimped grain at optimal water content can prove difficult as the water levels can change very quickly once the kernel reaches yellow ripening (Sundberg 2007). If the grain is poorly fermented the preservative quality solely depends on the absence of oxygen in the system, with a great risk of mould growth as soon as the plastic tube is opened or if the plastic is damaged. Fungi living on grain are divided into two groups, the field and the storage fungi. After the initial domination of fungi belonging to field flora, moulds belonging to the genera *Aspergillus* and *Penicillium* will be predominant in the grain. *Penicillium roqueforti* is one of the most important spoilage fungi in airtight storage systems. It has the ability to grow in conditions of low temperature, high levels of CO_2 and low levels of oxygen (Lacey & Magan 1991). *P. roqueforti* can produce several potent mycotoxins (Samson *et al*. 2004) and is known to poison animals (Häggblom 1990).

1.2 *Pichia anomala* as biocontrol

Different yeasts are part of the naturally occurring flora on cereal grain (Fleet 1992). Yeasts are generally considered as spoilage organisms when present in fermented feeds. LAB and yeasts are assumed to compete for the same substrates, and some yeast species metabolize lactic acid resulting in an increased pH endangering the hygienic quality (Middelhoven & van Balen 1988). The presence of yeast in feed is not always deteriorative; on the contrary, the yeast *Pichia anomala* has been shown to inhibit mould growth in malfunctioning airtight storage systems. *P. anomala*, J121, thrives over a wide range of temperatures (3- 37˚C) and pH (2-12) in anaerobic environments with very low water activity (a_w 0, 85) (Fredlund *et al*. 2002). The mould restricting ability of *P. anomala* has been confirmed in studies using small to large scale silos containing moist grain (Petersson *et al*. 1999; Petersson and Schnürer 1995, 1998). Several different mechanisms of fungal inhibition have been suggested for *P. anomala*. Competition for limiting nutrients and space (Janisiewicz & Korsten 2002), production of killer toxins (Walker *et al* 1995) and production of cell wall degrading enzymes (Jijakli & Lepoivre 1998) have all been concluded not to be the main mode of action of biocontrol by *P. anomala* (Druvefors *et al*. 2005; Druvefors & Schnürer 2005). Instead formation of ethyl acetate, a product of glucose metabolism in yeast, was concluded to be a major component of the mould inhibiting activity of *P. anomala*. Ethyl acetate is a

highly volatile compound that disperse easily amongst the crimped grain and evaporates quickly once the feed is taken out of the tube (Fredlund *et al*. 2004) (Druvefors *et al*. 2005). An extra addition of yeast to fermented grain also helps consume oxygen quicker at the beginning of storage and in the event of leakage later on (Druvefors *et al*. 2002).

1.3 Nutritional benefits

An addition of yeast to feed may enhance the nutritional value of the feed due to the high content of essential amino acids, vitamins and minerals of the yeast (Bui and Galzy 1990; Ravindra 2000; Schroeder *et al*. 2004; Stringer 1982). In addition, yeasts can increase the availability of phosphorus in cereal grain by degrading inositol hexaphosphate (IP_6) (Carlson and Poulsen 2003). *P. anomala* has been found to have considerable phytase activity (Olstorpe *et al.* 2009).

1.4 Aim of study

The major practical problem with storing moist grain in plastic tubes is the difficulty to harvest when the kernel holds enough water to ensure storage stability by lactic acid fermentation. This has been shown in a study were only at one out of eight participating Swedish farms a sufficient increase in lactic acid bacteria, and following reduction of other microbial groups, was observed (Olstorpe *et al*. 2010). It is therefore necessary to design a preservative system that will ensure hygienic stability even if the lactic acid bacteria fermentation fails. The present study evaluates the hygienic quality of cereal grain inoculated with the biocontrol yeast *P. anomala* on a farm in Sweden that is using the moist grain crimping system for feed preservation. Beside the hygienic evaluation, species composition of lactic acid bacteria and yeast over the storage period was monitored and the amino acid content was measured on cereal grain inoculated and non-inoculated with *P. anomala*. Parameters deciding the feed's nutritional value for beef cattle were also determined.

2. MATERIALS AND METHODS

2.1. Experimental design

The experiment was carried out on a farm on Gotland in Sweden. Due to unsuitable weather conditions the cereal grain, barley (*Hordeum vulgare*), was harvested during six days between the 28th of July until the 2nd of August in 2007. Immediately after harvest, the grains were crimped and packed into plastic tubes with on farm existing machinery (Winlin silage bagger). Post crimping 48 tons of the grain was inoculated with the yeast *P. anomala*, the remaining 48 tons was not treated with any additives. The grain was packed in six plastic tubes; three with inoculated grain referred to as the inoculated treatment, and three with the remaining untreated grain referred to as the non-inoculated grain in the study. The non-inoculated grain was harvested, rolled and packed prior to the inoculated grain to diminish the risk of cross contamination between the treatments. The tubes were thereafter closed to exclude air from the storage system. To ensure proper fermentation of the grain, the tubes were not opened for four weeks. Samples from all tubes were taken at harvest post inoculation, and thereafter continuously every fourth week until January when feeding began. Due to an exceptionally warm winter, feeding could not start simultaneously from the triplicates as this might jeopardize feed hygiene. The inoculated cereal grain was fed to 42 bulls, beef cattle of the Charolais breed, and the non-inoculated grain to 35 bulls (Permits;

Jordbruksverket, foderkvalitetsenheten, Dnr 37-2629/07 and Djurskyddsmyndigheten, Dnr S 104-07). Weight gain of the beef cattle was measured during the feeding period.

2.2. Yeast isolate

Strain *P. anomala* J121 used during the study was originally isolated from stored grain (Björnberg and Schnürer 1993). The yeast is stored in the fungal collection of the Department of Microbiology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, in glycerol stock at -70°C.

2.3. Grain inoculation

Yeast strain J121 was grown in laboratory fermentors and freeze dried according to Melin et al. (2007) and stored at -20°C before usage. Prior to inoculation the yeast was rehydrated with 80 litres of tap water. The yeast suspension was added to the grain post rolling, with a Perstorp pump™, 7 l min^{-1} (Perstorp AB, Perstorp, Sweden), calculated to inoculate $1*10^5$ cells g^{-1} grain.

2.4. Analytical methods

Moisture content of the cereal grain was determined by drying samples at 103°C for 16 h. Grain water activity (a_w) was determined using an AquaLab CX-2 (Decagon Devices, Inc., Pullman, Washington, USA) at 22 °C.

2.5. Analysis of amino acids

Amino acid contents were analysed on samples taken at harvest and after 5 months from each triplicate from both treatments, by AnalyCen Nordic AB, Lidköping, Sweden. Amino acid measurements were done according to standard SS-EN ISO 13903:2005, except tryptophan that was analyzed according to EU standard (Eu Dir 2000/45/EG part C).

2.6 Microbial quantification

2.6.1 Sample treatment

Aliquots of 20 g of the cereal grain were diluted with 180 ml sterile physiological saline solution supplemented with 0,15 g l^{-1} Tween 80 (Kebo AB Stockholm, Sweden) and homogenized for 120 seconds at normal speed in a Stomacher 400 Laboratory blender (Seward Medical, London England). Samples were serially diluted in physiological saline solution and spread on different agar plates.

2.6.2 Different media and growth conditions

LAB were quantified on de Man Rogosa Sharp (MRS) agar (Oxoid LTD, Basingstoke, Hampshire, England) supplemented with 0,1 g l^{-1} Delvocid (Gist-brocades B. V., Delft, The Netherlands). Delvocid inhibits growth of fungi including yeasts. The plates were incubated at 30˚C for 48 h in an anaerobic environment created by using a GasPack system (Becton Dickinson; Sparks, Md., USA).Yeast strains were isolated using Malt Extract Agar (MEA) (Oxoid LTD, Basingstoke, Hampshire, England) plates supplemented with 0,1 g l^{-1} chloramphenicol (Sigma-Aldrich Inc., St Louis, USA). Chloramphenicol prevents growth of a large variety of aerobic and anaerobic bacteria. MEA plates were incubated at 25˚C for three to five days. Moulds were quantified on MEA plates with a supplement of both chloramphenicol and cykloheximide (Sigma-Aldrich Inc., St Louis, USA) to prevent growth of bacteria and yeasts, respectively. Plates were incubated at 25˚C for three to five days. In order to study fungi thriving at a

low a_w Dichloran-Glycerol Agar (DG18) (Oxoid LTD, Basingstoke, Hampshire, England) plates were used and incubated at 25˚C for three to five days. The taxonomic key of Samson et al. (2004) was used to morphologically identify moulds. Total count of aerobic bacteria was quantified in Tryptone Glucose Extract Agar (TGEA) (Oxoid LTD, Basingstoke, Hampshire, England) incubated in 30˚C for three days.
Enumeration of *Enterobacteriaceae* was made in Violet Red Bile Agar (VRBG) (Oxoid LTD, Basingstoke, Hampshire, England). Plates were incubated at 37˚C for 24 h. For all enumerations except total count of anaerobic bacteria and *Enterobacteriaceae* 100 µl of each dilution were spread on the plates. For total count of aerobic bacteria and *Enterobacteriaceae* 1 ml of sample were pour-plated and overlaid with respective medium before incubation. After incubation colonies were counted and the mean cfu g^{-1} grain was calculated.

2.7 Strain conservation of LAB and yeast

Ten LAB and ten yeast colonies were selected at random from enumeration plates from all six tubes. LAB colonies were inoculated in 10 ml MRS Broth (Oxoid LTD, Basingstoke, Hampshire, England) and incubated in 30˚C overnight. Cells were harvested by centrifugation at 4500g for three minutes and the pellet was suspended in cryo medium [K_2HPO_4 0,82 g l^{-1}, KH_2PO_4 0,18 g l^{-1}, $C_6H_5Na_3O_7*H_2O$ 0,67 g l^{-1}, $MgSO_4*7H_2O$ 0,25 g l^{-1} and glycerol to 15% of the total volume (all substances from Merck KGaA, Darmstadt, Germany)] and frozen at -70˚C.
Yeast colonies were inoculated in 4 ml Yeast Peptone D-glucose (YPD) broth [yeast extract 10g l^{-1} (Oxoid LTD, Basingstoke, Hampshire, England), bacteriological peptone 20g l^{-1} (Oxoid LTD, Basingstoke, Hampshire, England) and D glucose 20g l^{-1} (VWR international Ltd., Poole, England)] and incubated overnight at 25˚C. One ml of cell suspension was mixed with an equal volume of glycerol and frozen at -70˚C.

2.8 DNA extraction, Fingerprinting and sequencing

2.8.1 DNA extraction

LAB and yeasts were harvested from respective overnight culture by centrifugation. Lactic acid bacterial DNA was isolated by using the DNeasy tissue kit (Qiagen, Hilden, Germany). DNA from yeast was isolated by shaking the pellet in a detergent buffer (de Souza Liberal *et al*. 2005).

2.8.2 Fingerprinting

Repetitive-DNA-element PCR fingerprinting using a primer $(GTG)_5$ (purchased from MetaBion, Munich, Germany) targeting a micro satellite sequence was done to obtain genotypic differentiation (Lieckfeldt et al.). PCR samples were mixed according to the supplier's recommendations (TaKaRa Taq™, Takara Bio Inc., Shiga, Japan). Amplification was performed in a MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories Inc., USA). The reaction conditions for LAB fingerprinting started with denaturation at 95˚C for 7 minutes, followed by 29 cycles of 90˚C for 30s, 95˚C for 1minute, 40˚C for 1 min and 65˚C for 4 min, with a final extension of 65˚C for 16 min. Yeast reaction conditions for fingerprinting: Initial denaturation at 94˚C for 2 min followed by 29˚C cycles of 94˚C for 30s, 50˚C for 30s, and 72˚C for 2 min with a final extension of 72˚C for 5 min.
Amplification products were submitted to electrophoresis in 1% Agarose gel in 0,5x TBE buffer(ICN Biomedicals Inc., USA) LAB-amplificates were run at 70 V, 80 mA for 3,5 h and yeast amplificates at 100 V, 80 mA for 2 h. PCR products were visualised

by ethidium bromide staining and recorded by a digital photo documentation system; Quantitiy One, 1-D Analysis software (Bio-Rad Laboratories Inc., USA).

2.8.3 Analysis of the DNA patterns from $(GTG)_5$ primers

Fingerprints from LAB and yeast were analyzed using GelCompar II V4, 5 software (Applied maths, Kortrijk, Belgium). During gel image processing the optimisation and band position tolerance were both set to 1%. A dendrogram was constructed using the Pearson correlation (0, 0%-100%) and the unweighted pair group method with arithmetic means. LAB and yeasts were selected, at the Pearson correlation of 80% homogeneity level, from the dendrogram. The according rDNA genes of the selected LAB and yeasts were sequenced to identify the organisms and to evaluate population diversity in the cereal grains.

2.8.4 DNA sequencing

In order to identify LAB the 16SS rDNA gene was amplified using two primers specific for the domain *Bacteria*: 16SS (5´-AGAGTTTGATCCTGGCTC-3´) and 16SR (5´-GGGAACGTATTCACCG-3´) (Pedersen *et al*. 2004). Amplification of the yeast D1D2-region of the 25S rRNA-gene was carried out using two primers: NL1 (5´GCATATCAATAAGCGGAGGAAAAG3´) and NL4 (5´GGTCCGTGTTTCAAGACGG3´) (Valente *et al*. 1999). Reaction conditions for both LAB and yeast DNA had the following parameters: Initial denaturation at 94˚C for 5 minutes, followed by 29 cycles of 94˚C for 30 s, 49˚C for 30 s and 72˚C for 2 min, final extension of 72˚C for 10 min. Amplification products were submitted to electrophoresis with 110V and 80mA for approximately 1h. Resulting PCR products were purified using QIAquick gel extraction Kit (Qiagen, Hilden, Germany). Purified fragments were sequenced by McLab (San Francisco, USA) using primer 16SS for LAB and primer NL4 for yeast. Sequence comparison against the EMBL database (http://www.ebi.ac.uk) was performed using the NCBI-BLAST2 program. Sequence similarity defining a positive match to a known species was set to 98% for LAB (Amann *et al*. 1995; Kurtzman & Robnett 1998) and 99% for yeast (Kurtzman & Robnett 1998).

3 RESULTS

3.1 Water content and water activity

Weather conditions prior to harvest resulted in dry cereal grain. The water content of the kernels was lower than the recommended level for ensiling grain (30-45%) in both treatments. The non-inoculated grain had a moisture content of approximately 16%, corresponding to a_w 0.83. Due to heavy rain the inoculated grain was harvested a few days later with a moisture content of approximately 18%, a_w 0.89.

3.2 Microbial activity

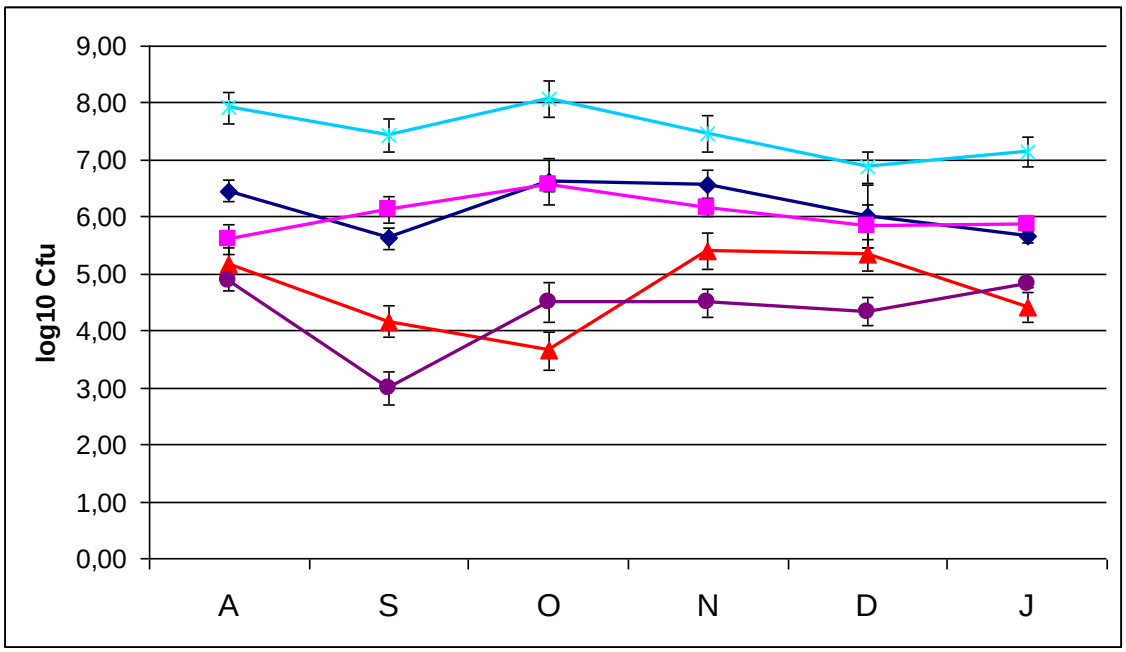
Samples from all tubes were taken at harvest post inoculation, and thereafter continuously every fourth week until January when feeding began. No *Clostridia* were detected in any samples (detection limit 100 cfu/g). Figure 1 shows the numbers of colony-forming units (Cfu) of yeast, bacteria and moulds in the storage experiments. A

drop in the Cfu counts was observed after the first month for all microorganisms except for yeasts from the inoculated treatment and LAB from the non-inoculated treatment.
In Sweden, the acceptable maximum levels of microorganisms in different feeds are only stated as recommendations (Table1; Swedish code of statutes SJVFS 2006:81, AnalyCen AB). However, these levels provide an indication of the desired hygienic status. Exceeding the value does not necessarily indicate hazardous feed, but suggests that the risk for obtaining harmful feed is increased, and a case-by-case assessment is required to evaluate hygienic quality. All levels of microorganisms, except for total aerobic bacteria, were below the recommended levels for microorganisms in dry grain and silage. From October the Cfu counts for *Enterobacteriaceae* in the inoculated treatment decreased steadily until it fell below detection level (10 cfu g^{-1}) in January; Cfu counts in the non-inoculated treatment were on average 10^4 cfu g^{-1} for *Enterobacteriaceae* (Fig. 1). After the first month of storage mould Cfu was reduced in both treatments, the reduction being greater in the inoculated treatment. In January mould Cfu counts had increased back to the same level as at harvest for the non-inoculated treatment, while the Cfu counts in the inoculated grain had stabilized at a significantly lower level. Identification of moulds showed that after the initial domination of field flora consisting mainly of *Cladosporium* and *Fusarium* species, the population shifted into a storage flora predominated by *Penicillium* species with some *Aspergillus* and *Mucor* present. The species were identified as *Penicillium verrucosum, Penicillium roqueforti, Mucor hiemalis, Aspergillus flavus* and *Aspergillus fumigatus* presented in the order of dominancy in the non-inoculated grain with highest prevalence first. In the inoculated grain, the dominancy was slightly shifted to *P. verrucosum, M. hiemalis, P. roqueforti* and *A. flavus*. There were no *A. fumigatus* detected in the inoculated cereal grain.

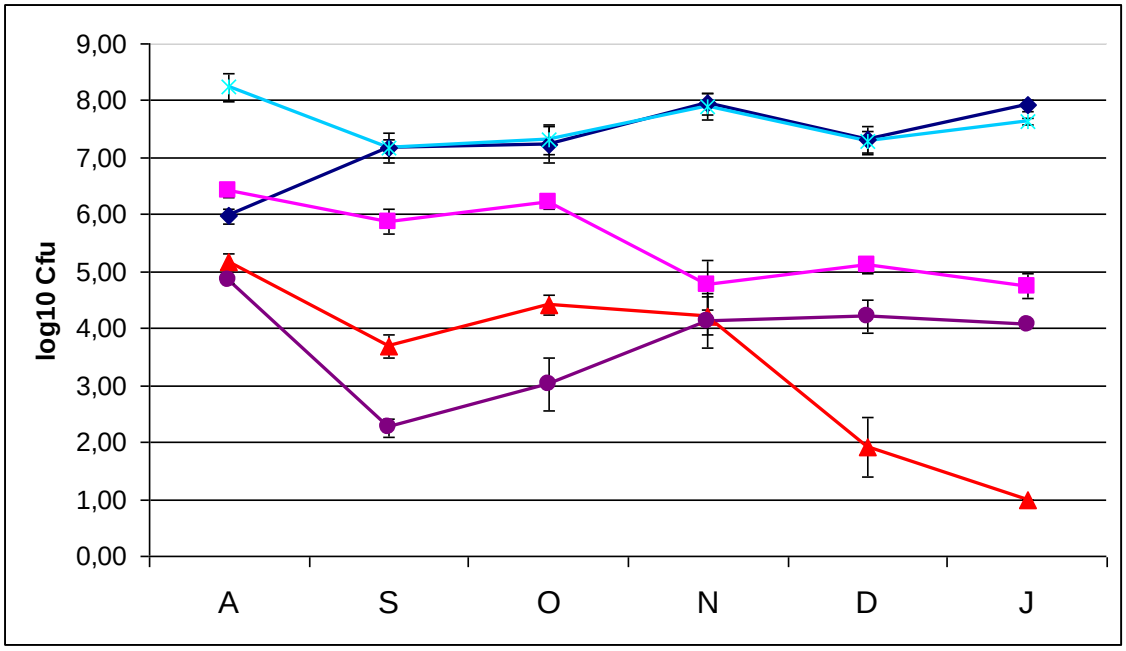
Table 1. Recommended levels of micro organisms in different feeds.

Analysis (log cfu/g feed)	**Dried cereals**[a]	**Hay/straw**[b]	**Silage**[b] (<60 %)
Total aerobic bacteria	<7,7	<8,0	
Yeast			<6,0
Total mould	<5,0	<5,5	<4,5
Enterobactericiae			<6,0

[a]Recommended levels of micro organisms in dried cereals according to Swedish code of statues SJVFS 2006:81 appendices 16.
[b]Recommended levels of micro organisms in hay/straw and silage according to AnalyCen AB (http://www.analycen.se/grovfoder_resultat_ensilage.asp; http://www.analycen.se/grovfoder_resultat_halm.asp) 2008.05.20.



a)



b)

Figure 1. **a)** log10 Cfu counts of Total aerobic bacteria *, Yeast ♦, LAB■ *Enterobacteriaceae*, ▲ and mould ●, in moist crimped barley. **b)** log10 Cfu counts of total aerobic bacteria *, Yeast ♦, LAB■ *Enterobacteriaceae*, ▲ and mould ●, in *P. anomala* inoculated moist barley from August to January.

3.3 Identification of yeast and LAB

Sequence analysis rendered the identification of yeast and LAB species possible. DNA from August, September and January's LAB and yeast samples, were isolated and fingerprinted using rep-PCR. The GelCompar II program was used to cluster fingerprints in a dendrogram. One isolate was identified from each cluster and then considered to be representative of the whole group. The LAB population was somewhat different between treatments, and the dominating species within the treatment shifted over time. In the non-inoculated grain (Table. 2) *Lactobacillus curvatus* and *Leuconostoc citreum* were the dominating species in August and September; in January species diversity was greater and the most common species were found to be *Lactobacillus paracasei* and *Lactobacillus coryniformis*. In the inoculated treatment *Leuconostoc mesenteroides* dominated the LAB flora in August, to be completely substituted by *Lactobacillus brevis* and *Weissella thailandensis* in September. The species diversity increased in January and *Pediococcus pentosaceus* that was present at harvest and after one month storage, was dominating the bacterial population after 5 months storage. The yeast population was less diverse than the LAB population and consisted of fewer species (Table. 3). In August the dominating species in the natural treatment were *Cryptococcus macerans* and *Cryptococcus victoriae*, in September it was the yeast-like fungus *Acremonium strictum*. In January *Pichia burtonii* was dominating but naturally occurring *P. anomala* was also detected. From the inoculated treatments only *Pichia anomala* was isolated during the three months.

Table 2. Bacteria species identified by 16S rRNA sequencing. Numbers show quantity out of 30 identified species per month.

Treatment		**Inoculated**			**Non-inoculated**	
Lactobacilli identified species	**August**	**September**	**January**	**August**	**September**	**January**
Lactobacillus curvatus	5	1	2	8	7	1
Lactobacillus brevis	2	9	1	2	3	3
Lactococcus lactis	1					
Lactobacillus buchneri			3			2
Lactobacillus coryniformis			5			6
Lactobacillus kefiri						1
Lactobacillus paracasei			3			7
Lactobacillus paralimentarius						3
Lactobacillus plantarum						2
Lactobacillus sakei	6	5		1	3	
Enterococcus gilvus		3				
Enterococcus malodoratus						1
Leuconostoc citreum	1			8	6	
Leuconostoc mesenteroides	9			2	5	
Leuconostoc paramesenteroides					4	
Pediococcus pentosaceus	5	4	8	3		1
Pediococcus parvulus			1			
Weissella cibaria	1		1	6		2
Weissella thailandensis		8	5		1	
Weissella paramesenteroides			1			1

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Table 3. Yeast species identified by NL4 rRNA gene sequencing. Numbers show quantity out of 30 samples taken each month.

Treatment		**Inoculated**			**Non-inoculated**	
Yeast identified species	**August**	**September**	**January**	**August**	**September**	**January**
Acremonium strictum	1			2	21	
Cryptococcus carnescens				1		
Cryptococcus macerans	3			11	2	
Cryptococcus victoriae				8	5	
Cryptococcus wieringae	1					
Cryptococcus tephrensis					2	
Pichia anomala	25	30	30	5		8
Pichia burtonii						22
Sporobolomyces roseus				3		

Table 4. Nutritional parameters and amino acid composition of barley non-inoculated and inoculated with *Pichia anomala*. Samples taken for analysis at harvest and prior to feeding (5 months). Data are given as g amino acid kg^{-1} dry matter. Values represents mean values ± standard deviation (n=3). The measuring tolerance given by the contract laboratory (Analycen AB, Lidköping, Sweden) is 8% for each amino acid.
NDF-Neutral Detergent Fibre,
AAT (Aminosyra Absorberad Tunntarm) Amino acid absorbed in Duodenum
PBV (ProteinBalans Våm) Ratio protein: carbohydrates in rumen
EPD Efficient Protein Degradation (in rumen)
* Essential amino acid.

	Non-inoculated		***P.anomala* inoculated**	
	Harvest	**5 months**	**Harvest**	**5 months**
Dry matter	80,3±0,72	81.2±0,8	79.1±0,7	76.5±0,71
Crude fat		29.7±1,2		29.0±8,0
Crude fiber		61.3±4,04		56.0±10,2
Starch	602,±39,04	609±70,5	791.3±45	591±74,5
NDF	176,7±15,95	172±7,2	178.7±17,1	213±61,5
AAT	94,7±0,58	93.3±8,3	93.6±1,53	95.3±3,2
PBV	-21,0±9,64	-24.7±8,3	-16.7±7,6	-12.3±7,8
EPD	77,±0	77±0	77±0	77±0
Energy (cattle)	13,2±0,12	13.3±0,06	13.0±0	13.4±0,3
Effective cp	99,0±1,0	98±7,0	104.3±6,1	109.7±3,1
Crude protein	129,7±2,9	127,3±9,07	135,7±7,6	142,7±4,04
Cysteine	2.4 ± 0.06	3.1 ± 0.15	2.6 ± 0.10	3.4 ± 0.15
Methionine*	1.7 ± 0.06	2.1 ± 0.10	1.8 ± 0.12	2.3 ± 0.12
Aspartic acid	5.9 ± 0.40	7.5 ± 0.59	5.97 ± 0.42	7.7 ± 0.42
Threonine*	3.4 ± 0.15	4.4 ± 0.32	3.5 ± 0.20	4.6 ± 0.2
Serine	4.5 ± 0.21	5.9 ± 0.32	4.5 ± 0.25	6.4 ± 0.32
Glutamic acid	25.1 ± 0.70	35. ± 0.96	25.7 ± 0.96	39.5 ± 2.57
Proline	11.3 ± 0.20	15.1 ± 0.70	11.9 ± 0.40	16.4 ± 0.38
Glycine	4.1 ± 0.15	5.2 ± 0.32	4.2 ± 0.25	5.5 ± 0.38
Alanine	4.2 ± 0.15	5.3 ± 0.38	4.3 ± 0.23	5.6 ± 0.31
Valine*	5.2 ± 0.12	6.2 ± 0.35	5.6 ± 0.25	6.8 ±0.46
Isoleucine*	3.9 ± 0.06	4.6 ± 0.25	4.1 ± 0.21	5.2 ± 0.23
Leucine*	6.97 ± 0.15	8.9 ± 0.47	7.4 ± 0.30	9.8 ± 0.25
Tyrosine (calculated)	3.5 ± 0.15	2.6 ±1.15	3.7 ± 0.15	3.9 ± 1.16
Phenylalanine*	5.3 ±0.06	6.4 ± 0.26	5.5 ± 0.15	7.03 ± 0.31
Histidine*	2.4 ± 0.10	2.8 ± 0.15	2.4 ± 0.15	3.03 ± 0.15
Ornitin	<0.1	<0.1	<0.1	<0.1
Arginine	4.97 ± 0.15	6.2 ± 0.36	5.1 ± 0.31	6.7 ± 0.46
Lysine*	3.5 ± 0.10	4.5 ± 0.29	3.7 ± 0.23	4.7 ± 0.4
Hydroxiproline	<0.1	<0.1	<0.1	<0.1
Total amino acids	98.3 ± 1.57	125.5 ± 4.4	101.6 ± 4.45	138.6 ± 3.95

3.4 Nutritional Evaluation

During storage of barley in plastic tubes the amino acid content increased in both treatments. However, the increase of amino acids was higher in the inoculated cereal grain than non-inoculated cereal grain. Essential amino acids were found at slightly higher levels in inoculated cereal grain (Table. 4). When feed rations are calculated for milk and beef cattle in Sweden, the feed's ratio between carbohydrates and rumen soluble proteins (PBV) are one of the factors considered. The PBV value increased considerably for the inoculated treatment (Table. 4).There was a small increase of daily weight gain of bulls fed the *P. anomala* inoculated cereal grain (1570 g day^{-1}, sd 113, n=42) compared to bulls fed non-inoculated cereal grain (1540 g day^{-1}, sd 125, n=35).

4. DISCUSSION

Harvest of cereal grain within moisture limits suitable for moist crimped cereal grain mostly provides a hygienically safe feed. Storage of dryer material could endanger the hygiene and lead to deterioration of the feed. The biocontrol yeast *P. anomala* has previously been shown to prevent growth of spoilage moulds in model systems of airtight storage of moist feed grain. In this study *P.anomala* was inoculated in moist crimped cereal grain in a trial of farm scale size. The inoculated *P.anomala* thrived and conquered out other yeast species initially present in the moist cereal grain. The mould flora in the inoculated grain including *P. roqueforti* was reduced during the storage period although the higher moisture content in this treatment should rather favour mould growth compared to the non-inoculated control. It is particularly noteworthy that the potentially pathogenous mould *A. fumigatus* was detected only in the non-inoculated cereal grain.

All levels of microorganisms, except for Total aerobic bacteria, were below recommended levels for micro organisms in dry grain and silage (Table1.). A surprising result was that in the inoculated cereal grain the number of *Enterobacteriaceae* was decreased below detection level (10 cfu g^{-1}). An inhibitory effect of *P. anomala* on *Enterobacteriaceae* in cereal grain has never been reported before. From a hygienic point of view, this finding is highly important, as it has been shown that reducing the number of *Enterobacteriaceae* in the feed also results in a reduction of the number of *Enterobacteriaceae* present later in the food chain (Brooks *et al*. 2001). Guidelines for acceptable levels of *Enterobacteriaceae* in hay and dried cereals do not exist, because it is assumed that these bacteria cannot grow at low mc (Adams and Moss 2000). In this study *Enterobacteriaceae* were initially present in both inoculated (mc 18%) and non-inoculated (mc 16%) cereal grain, not exceeding guideline values, but only reduced in inoculated cereal grain.

The lactic acid bacteria flora differed between the two treatments. In the inoculated cereal grain, the cfu of LAB was reduced compared to the non-inoculated cereal grain. Whether this effect was due to a possible competition for resources between *P. anomala* and lactic acid bacteria or due to the differences in moisture content in the feed could not be distinguished. However, if isolated strains are to be used as starter cultures they need to be able to grow in co-culture with *P. anomala*. The LAB *P. pentosaceus* was present during all sampling occasions in the inoculated cereal grain, and becoming the dominant LAB in the population after 5 months storage. *P. pentosaceus* are used in

starter cultures for sausage making and silage inoculants (Axelsson,. 1998) indicating its potential also for crimped cereal grain fermentation.

Amino acid levels increased during storage for both treatments, although to higher levels in inoculated cereal grain. This may be due to the increased yeast growth in the inoculated cereal grain compared to the non-inoculated cereal grain. The slightly higher increase of daily weight gain of bulls fed the *P. anomala* inoculated cereal grain compared to bulls fed non-inoculated cereal grain and the greater increase in PBV value for the inoculated treatment support this assumption. Yeasts are a proteinaceous feed source and are regarded as a single cell protein (SCP) supplement for live stock feeding (Ravindra 2000; Stringer 1982). Growing SCP in the form of *P. anomala* directly on the feed instead of buying it as a separately processed feed additive could prove more cost effective.

5. CONCLUSIONS

The addition of the biocontrol yeast *P. anomala* provides a hygienically sound feed during storage of moist crimped cereal grain when the moisture content is too low to initiate fermentation. The never before seen inhibition by *P. anomala* on *Enterobacteriaceae* is especially noteworthy. *P. anomala* thrives in crimped cereal grain and out conquers other yeast species, creating a predictable yeast flora. It would also contribute as a feed additive, due to increased protein levels when grown *in situ* on the selected feed components. Levels of all essential amino acids increased in the inoculated feed. Bulls fed with *P. anomala* inoculated cereal grain showed a slight weight gain compared to bulls fed non-inoculated cereal grain.

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