Sveriges lantbruksuniversitet
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**Examensarbete** / SLU, Institutionen för husdjurens utfodring och vård, **389**

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## Abstract

The study was conducted to investigate the hygienic and nutritional value of *Wickerhamomyces anomalus* inoculated barley and also in combination with lactic acid bacteria starter culture inoculated barley in fermented liquid feed for growing pigs and their effect on the fermentation process. The experiment was carried out in SLU's experiment stable. Six growing pigs Yorkshire purebred at an average initial live weight of (29±2.5) kg were surgically fitted with a PVTC – cannula. Four diets, 6 pigs and 6 periods were arranged in a change-over design. The fermented liquid diets were fermented dry (FD), fermented control (FC), fermented inoculated with *Wickerhamomyces anomalus* (FW) and fermented inoculated with *Wickerhamomyces anomalus* and Starter culture (FWS). Water was added to the feed in a 3:1 ratio. All of the treatments were fermented during an initial 7 days. When feeding commenced 1/2 of the contents were replaced with fresh feed and water once a day in the afternoon. The pH of the treatments was around 4.0. There were no *Enterobacteriaceae* found in the feed during the 6 periods. Cfu counts for yeast in all treatments slightly fluctuated around log 7.5 Cfu/ g grain during the experiment. Cfu counts for lactic acid bacteria (LAB) in *Wickerhamomyces* inoculated treatments tended to be lower than that in non-inoculated treatments. Cfu counts for mould in FW and FWS were lower (approximately log 1 for FW and log 3 for FWS) than in FC in the periods that they were detected. The apparent ileal digestibility of OM was different *($P < 0.05$)* among treatments. The total tract digestibility of OM in FWS was higher *($P < 0.01$)* than that in FC. The total tract digestibility of OM in FW was higher compared with in FC *($P < 0.05$).* There was a significant difference in the total tract digestibility of CP *($P < 0.0001$)* between treatments. The digestibility of phosphor (P) showed no significant difference *($P > 0.05$)* among treatments. In conclusion, *Wickerhamomyces anomalus* and lactic acid bacteria starter culture inoculated to moist crimped grain can give good hygiene in wet fermented feed and high total tract digestibility of organic matter and crude protein.

**Key Words:** *Wickerhamomyces anomalus*, lactic acid bacteria, fermented liquid feed, digestibility of protein, digestibility of organic matter.

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# 1. Introduction

Fermented liquid feed systems have an increasingly important role in agricultural practice. The use of fermented liquid feed is widely spread in pig production. Several studies have reported that using fermented feed benefits the gastrointestinal health of animals (Canibe and Jensen, 2003; van Winsen *et al.*, 2001). The digestibility in pig diets can be improved by using fermentation as a strategy (Lyberg *et al.*, 2006). Fermentation may improve the digestibility of phosphorus in cereal grain by activating the naturally occurring enzymes phytase (Canibe and Jensen, 2003). The quality of fermented liquid feed rely on a number of factors, such as temperature, fermentation time, ingredients, the strains of indigenous lactic acid bacteria and yeasts present and their population density in the feed (Passoth *et al.*, 2011). The result of spontaneous microbial development with poorly understood population dynamics during the fermentation process, give a product with an unpredictable final quality (Passoth *et al.*, 2011). Undesirable yeasts' presence in liquid feed may cause energy loss, off-flavors and taints that reduce the palatability of the feed (Lyberg *et al.*, 2008).

Storage of moist crimped grain is becoming a common preservation method, which is feasible via fermentation by lactic acid bacteria. It is also an efficient method to store cereal grain because it requires less energy than fan drying. However, climatic variation makes it difficult to harvest cereal grain at moisture content around 30-40 per cent, which is necessary to support optimal fermentation. Mould growth may occur in cereal grain stored when the preferred moisture content in the grain is not obtained (Olstorpe *et al.*, 2010a). Moulds, for example *Penicillium roqueforti*, can cause intoxication of animals by producing several potent mycotoxins (Olstorpe *et al.*, 2010a). Thereby, the fermented liquid feed made by moist crimped cereal grain may, in some cases, not be safe and palatable. The bio-control yeast *Wickerhamomyces anomalus (Pichia anomala)* inoculated to moist cereal grain can improve feed hygiene by reducing moulds and *Enterobacteriaceae*, and the nutritional value of the feed can be enhanced by increasing the protein content and reducing the content of phytate (Olstorpe and Passoth, 2011). The yeast *Wickerhamomyces anomalus* keeps the hygienic stability during storage in moist crimped cereal grain by inhibiting the mould growth with several mechanisms, including the production of killer toxins, production of ethyl acetate or the competition for nutrients (Furman, 2011; Olstorpe *et al.*, 2010b). *Wickerhamomyces anomalus* might also become the dominating yeast flora during the fermentation of the wet feed and hereby prevent other species of yeast to proliferate and cause spoilage. Lactic acid bacteria in fermented wet feed may have a positive effect on the lower-gut microflora (Vanbelle *et al.*, 1990). The lactic acid bacteria reduce pH in diets by producing lactic acid (Vanbelle *et al.*, 1990). High concentration of lactic acid and a low pH can prevent the proliferation of spoilage organism and food-borne pathogens (Olstorpe *et al.*, 2008). Thanks to these good effects of *Wickerhamomyces anomalus* and lactic acid bacteria, inoculation of *Wickerhamomyces anomalus* together with lactic acid bacteria culture to fermentation systems in order to control yeast flora may be a good strategy to improve the quality of wet feed.

The aim of this study was to investigate the hygienic and nutritional value of *Wickerhamomyces anomalus* inoculated barley, in combination with a lactic acid bacteria starter culture, in fermented wet feed for growing pigs.

# 2. Literature review

## 2.1. Moist crimped cereal grain

Cereal is the principal component in pig nutrition, characterized by high starch and low fiber contents. Among cereals, barley *(Horderum sativum)* is a common grain used in pig feeding. According to Swedish Board of Agriculture (Jordbruksstatistisk årsbok, 2007) around 5.5 million tons of cereal grains are produced each year, of which 60 % are used for animal feed (SJV, 2007). Drying is the most common preservation method for cereal grain (Olstorpe and Passoth, 2011). To achieve safe long-term storage, cereal grains should be dried to a water content below 13 per cent, with a water activity ($a_w$) below 0.65. Finch *et al*. (2002) reported that in Sweden, grain drying occupied approximately 60% of the energy used in the total plant husbandry operation. Because energy prices increase and climate concerns grow, alternative preservation methods are considered. Airtight storage of moist cereal grains is becoming a common preservation method because of the lower energy requirement compared to that in high temperature drying (Finch *et al.*, 2002; Olstorpe and Passoth, 2011). However, growth of harmful microorganisms, such as *Penicillium roqueforti*, during storage of moist feed may impair the feed hygiene. A perfect airtight sealing with a modified atmosphere controlling the deteriorative microbial growth is needed in order to have safe storage (Lacey and Magan, 1991; Magan *et al.*, 2003). It is not easy to maintain the modified atmosphere because unsatisfactory sealing and opening for feed outtake causes gas leakage (Druvefors *et al.*, 2002).

Fermentation in moist crimped cereal grain is caused by naturally occurring lactic acid bacteria (LAB) on the grain. Production of organic acids, hydrogen peroxide, carbon dioxide, antimicrobial substances, and lowering the pH inhibit pathogenic and spoilage organisms (Arthur and Satu, 2004; Canibe *et al.*, 2007). A successful fermentation relies on several factors, such as the strains of indigenous LAB and yeast present and their population density, cultivation, crop management, conditions of harvest and conditions of storage (Lacey and Magan, 1991). For optimal fermentation the cereal grain should have reached yellow ripeness and the kernels have a water content of 30-40 per cent. However, the preferred moisture content in the grain is not always obtained because the moisture content can change rapidly with weather conditions when the kernel reaches yellow ripening (Olstorpe *et al.*, 2010c). Most of the farm-stored crimped cereal grain in a study by Olstorpe and Passoth (2011) had lower moisture content than recommended which cause an insufficient growth of lactic acid bacteria and occurrence of undesirable microbes (Olstorpe and Passoth, 2011).

## 2.2. Fermented liquid feed

Feeding liquid feed to pigs has been applied in practice for many years (Canibe and Jensen, 2003; Scholten, 2001). Fermented liquid feed is defined as a mix of feed and water prepared at a certain temperature and time before feeding, allowing a fermentation process to take place, whereas non-fermented liquid feed is a mixture of feed and water mixed at feeding (Canibe and Jensen, 2003). Fermentation is a dynamic process produced by change of physical properties and chemical composition. Carbohydrates are transformed into lactic acid, volatile fatty acids, ethanol and $CO_2$ during fermentation. Thereby, storage time is important for the scale of changes in chemical and physical composition of liquid pig diets (Scholten, 2001). Fermentation will begin from the moment feed and water is mixed, with the initial phase having low numbers of lactic acid bacteria and yeast, with low levels of lactic acid and a high pH. A high number of *Enterobacteriaceae* is common. The initial phase of the fermentation is followed by a second

phase characterized by: a low pH, low numbers of *Enterobacteriaceae*, high numbers of lactic acid bacteria and yeast, and a high concentration of lactic acid (Canibe and Jensen, 2003; Canibe *et al.*, 2001; Lawlor *et al.*, 2002). Several studies report that compared to feeding dry feed or non-fermented liquid feed, feeding pigs with fermented liquid feed decreases clinical diseases, for example the prevalence of *Salmonella*, the incidence of dysentery with *Brachyspira hyodysenteriae*, and the levels of *Enterobacteriaceae* along gastrointestinal tract of pigs and piglets (Boesen *et al.*, 2004; Lindecrona *et al.*, 2003; Mikkelsen and Jensen, 2000). Pigs fed with fermented feed seem to have a higher growth performance than those fed with non-fermented feed (Scholten, 2001). However, it has also been reported that during the fermentation process there is a significant loss of supplemented amino acid (lysine in particular) due to decarboxylation of amino acids (Pedersen *et al.*, 2002). An increase in the risk of *Salmonella* infection has been shown in pigs eating liquid feed soaked in a trough for a few hours (Scholten *et al.*, 2002; van der Wolf *et al.*, 1999).

### 2.3. *Wickerhamomyces anomalus* and lactic acid bacteria

The yeast *Wickerhamomyces anomalus* is used in food and feed production as a production organism. It is a kind of non *saccharomyces* wine yeast, which production of volatile compounds contributes to wine aroma. *Wickerhamomyces anomalus* has the ability to grow well at low pH, low osmotic pressure and low oxygen tension in preserved food and feed environments. Since it has a strong mould inhibiting effect and anti-microbial characteristics *Wickerhamomyces anomalus* may be used as a bio-control agent (Passoth et al., 2006). Introduction of the biocontrol yeast *Wickerhamomyces anomalus* to malfunctioning airtight storage systems is a way of securing feed hygiene and quality (Olstorpe and Passoth, 2011).

Besides the hygienic benefits of *Wickerhamomyces anomalus* the nutritional value of the feed may be increased by addition of yeast because of the high content of essential amino acids, vitamins and minerals (Anupama and Ravindra, 2000; García-Garibay *et al.*, 1999; Stringer, 1982). The availability of phosphorus in cereal grain can be increased by the *inositol hexaphosphate* ($IP_6$) degrading capacity of the yeast (Carlson and Poulsen, 2003).

The lactic acid bacteria (LAB) in fermented wet feed may provide positive effects on lower gut micro-flora by their metabolic activity (Vanbelle *et al.*, 1990). The LAB can prevent potentially pathogenic microorganisms by low the pH and high concentration of lactic acid, affect on the intestinal epithelium and improve absorption capacity (Vanbelle *et al.*, 1990). Thanks to its phytase activity, the LAB may contribute to the degradation of $IP_6$ (Reale *et al.*, 2004). Additionally, lactic acid bacteria can be used as starter cultures to enhance control of fermentation conditions (Olstorpe *et al.*, 2010a).

### 2.4. Digestibility of protein

Chemically, the nitrogen content of the food is used for calculation of the protein content through the Kjeldahl technique or the Dumas method. To calculate the protein from nitrogen, two assumptions are made: the first is that all the nitrogen of the food is present as protein; the second is that all food protein contains 160 g N/kg. The nitrogen content of the food is then showed in terms of crude protein (CP) as CP (g/kg) = g N/kg * 1000/160 or CP (g/kg) = g N/kg * 6.25 (McDonald et al., 2011).

The crude protein expresses the nitrogen present in the food and gives little sign of its value to the animal (McDonald *et al.*, 2011). Protein tissue in an animal contains amino acids that are

ingested and absorbed from intestine. The digestibility of amino acid is measured instead of absorption of amino acid which is difficult to measure. Digestibility of a given amino acid (AA) or crude protein (CP) is the difference between the amount of the AA or CP ingested by the animal and the amount that is excreted in the feces or ileal digesta of the animal divided by the amount that is ingested. Then, the digestibility coefficient is calculated by multiplying the result by 100 (Stein, 2003). Digestibility of CP is often determined based on ileal digestibility rather than fecal digestibility (Fan *et al.*, 1994). Apparent ileal digestibility (AID) is the net disappearance of ingested dietary nutrients in the distal ileum (Stein *et al.*, 2007a). However, AID coefficient of CP is dependent on the dietary concentration of CP which can lead to inaccuracies (Stein *et al.*, 2007a). Standardized ileal digestibility (SID) gives more precise and greater accuracy values of ileal digestibility than apparent digestibility (Moter and Stein, 2004) because SID of CP is less influenced by CP level (Stein *et al.*, 2007b). Generally, two different approaches can be used for determination of the standardized ileal digestibility of crude protein: estimation by regression analysis (Fan *et al.*, 1995), or calculation by correcting AID for basal ileal endogenous CP losses (Jansman *et al.*, 2002). Both rely on accurate measurements of basal ileal endogenous CP losses and AID of CP which should not be confounded by different age or body weight (Eklund *et al.*, 2008; Fan *et al.*, 1994). Basal ileal endogenous crude protein may be estimated by using one of the following procedures: measuring the CP in digesta when nitrogen–free diets are consumed, measuring the CP in digesta when completely digestible protein is consumed, or measuring the recoveries of nitrogen from digesta, when graded levels of protein are consumed (Stein *et al.*, 2007a).

### 2.5. Digestibility of phosphorous

Most of the phosphorus (P), approximately 60-90 percent, in cereal grain is present in the form of phytate (Mitchell *et al.*, 1997; Olstorpe *et al.*, 2009). Phytate (known as inositol hexaphosphate (IP6) or phytic acid) is the main storage form of phosphorus in grains and oil seeds (Vohra and Satyanarayana, 2001). The utilization of the phosphorus bound in phytate in pigs is limited because they lack endogenous phytase which is a gastrointestinal tract enzyme for the dephosphorylation of the phytate (Cromwell *et al.*, 1995; Lyberg *et al.*, 2005). Therefore, pig diets are commonly added inorgainic phosphates to ensure that the P requirement is fulfilled (Lyberg, 2006; Olstorpe *et al.*, 2009). Inorgainic phosphates and phytate that is excreted from the animals may cause phosphorus pollution and the eutrophication of water environments (Olstorpe *et al.*, 2009). Addition of phytase to animal feed is considered as a solution because it makes the availability of phosphorus for the animal increase by degradation of the phytate, and the phosphorus excreted in animal manure decreases by up to 50%. However, application of phytase as feed supplement is currently restricted due to the high price of commercial phytase (Olstorpe *et al.*, 2009). A study of Lyberg *et al.* (2006) showed that phytate was degraded and ileal apparent digestibility of P was improved by fermentation. Olstorpe et al. (2009) detected a strain-specific extracellular phytase activity in *Wickerhamomyces anomalus.*

# 3. Material and methods

## 3.1. Experimental design

The experiment was carried out in SLU's experiment stable. Six growing pigs of Yorkshire purebred at an average initial live weight of 29±2.5 kg,were surgically fitted with a PVTC – cannula. The post valve "T" Caecum technique was developed by Leeuwen (van Leeuwen et al., 1991). Average final body weight was 57±4.6 kg. The pigs were stabled individually in pens (230 x 145 cm) with rubber mats without bedding in during the trial. Once a day, the pigs were allowed to run around in the aisle for about 10 to 15 minutes, for observation and animal welfare reasons. The temperature of the stable was maintained at $19.7\pm0.87^{0}$C. The pigs had free access to water which was provided by low pressure water bowls with nipple drinkers.

Four diets, six pigs and six periods were arranged in a change-over design. Each period lasted fourteen days in which the first seven days was adaptation period to the experiment diets followed by four days of feces collection, from day 8 to 11, and two days of ileal digesta collection, day 12 and 14. Collection of ileal digesta through the PVTC-cannula was carried out during 1-h intervals, on day 12 from 08:30 to 09:30, 10:30 to 11:30, 12:30 to 13:30, and 14:30 to 15:30h, and on day 14 from 09:30 to 10:30, 11:30 to 12:30, 13:30 to 14:30 and 15:30 to 16:30h. Digesta was collected in polyethylene bags (8 x 30 cm) from the pigs in the pens. The bags were emptied into a container at 10- to 15-minutes intervals, depending on how fast they filled up during the collection hour, and were immediately frozen at $-20^{0}$C. Feed was collected during the final seven days of each period, and pH was measured.

## 3.2. Experimental diets

The fermented liquid diets were fermented dry (FD), fermented control (FC), fermented inoculated with *Wickerhamomyces anomalus* (FW) and fermented inoculated with *Wickerhamomyces anomalus* and Starter culture (FWS). Water was added to the feed in a 3:1 ratio. All of the treatments were fermented initially for seven days. When feeding began 1/2 of the contents were replaced with fresh feed and water once a day in the afternoon. The fermented feed was mixed together with a premix (minerals, vitamins) by an electric mixer (Vinon tools) before being given to the pigs twice daily at eight o'clock in the morning and four o' clock in the afternoon. The total daily amount of DM feed corresponded to 4% of their body weight. Table 1 show the nutritional content of the treatments.

**Table 1**. Feed ingredients and chemical composition (g/kgDM) of experimental diets

| | FD | FC | FW | FWS |
|---|---|---|---|---|
| ***Ingredients*** | | | | |
| Barley | 978 | 978 | 978 | 978 |
| Premix + $TiO_2$* | 22 | 22 | 22 | 22 |
| ***Analyzed chemical composition*** | | | | |
| DM | 298 | 294 | 296 | 293 |
| Ca | 5.5 | 5.5 | 5.7 | 5.5 |
| P | 2.3 | 3.3 | 4.0 | 3.3 |
| Crude Protein | 136.8 | 135.0 | 133.8 | 131.4 |
| Energy (MJGE/kgDM) | 18.5 | 18.4 | 18.3 | 18.4 |

**Content per kg premix:* Vitamin: retinol 183 995IE, cholecalciferol 18 418 IE, alpha-tocopherol 2760 mg, phytylmenaquinone 92 mg, thiamine mononitrate 92 mg, riboflavin 92 mg, pyridoxine hydrochloride 138 mg, cyanocobalamin 0.92 mg, pantothenic acid 460 mg, nicotinic acid 920 mg; Mineral: Ca 297 g, Mg 4.58 g, S 3.19 g, Fe 2.84 g, Cu 0.70 g, Mn 1.18 g, Zn 3.47g, I 9.2 mg, Se 18.4 mg, $TiO_2$ 9.29 g.

### 3.3. Sample analysis

Dry matter (DM), ash of feed, digesta and feces samples were analyzed based on Association Official Analytical Chemists (AOAC, 2000). Total amount of nitrogen in samples was determined via Kjeldahl method (Nordic-Committe-on-feed-Analysis, 2003). Titanium dioxide ($TiO_2$) was used as marker and was analyzed according to Short *et al.*1996.

Ashed samples of feed, feces and digesta were analyzed for P with optimal ICP-AES (Nordic-Committe-on-feed-Analysis, 1991). Gross energy of treatment diets was analyzed using an adiabatic oxygen bomb calorimeter (model 1563, Parr instruments Manual, Parr Instruments, Moline, IL) (Fastinger and Mahan, 2006)

### 3.4. Microbial quantification

Fresh feed samples were collected from each treatment at harvest (post-inoculation), at the start of the feeding trial, and consecutively every two weeks during the trial. The feed samples, 20g, were diluted with 180 ml sterile peptone water (Bacteriological peptone 2 g $l^{-1}$, Oxoid Ltd., Basingstoke, Hampshire, England), 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). The homogenate was then serially diluted in peptone water and 100 ml portions were spread onto various solid culture media. De Man Rogosa Sharp (MRS) agar (Oxoid Ltd.) incubated anaerobically at 37 ◦C for 48 h, supplemented with 100g$ml^{-1}$ Delvocid (Gist-brocades B.V., Ma Delft, The Netherlands) was used to quantify the LAB in the experiment. An anaerobic environment was obtained by using A GasPack system (Becton Dickinson; Sparks, Md., USA). Malt Extract Agar (MEA) (Oxoid Ltd.) plates incubated at 25 ◦C for 2–4 days, supplemented with 100 g$ml^{-1}$ chloramphenicol (Sigma-

Aldrich Inc.) was used to enumerate yeasts. Moulds were quantified on MEA plates with a supplement of 100 μg $ml^{-1}$ chloramphenicol and 50 μg $ml^{-1}$ cyklohexamide (Sigma-Aldrich Inc., St Louis, USA) to prevent growth of bacteria and yeasts, respectively. *Enterobacteriaceae* were enumerated on Violet Red Bile Agar (Oxoid Ltd., Basingstoke, Hampshire, UK.) by the pour plate method and incubated at 37 ◦C for 24 hours. Fungal and bacterial counts were expressed as mean cfu $g^{-1}$ fermented feed.

### 3.5. Calculations

Apparent ileal digestibility (AID) was calculated by using the $TiO_2$ as indigestible marker in the feed and digesta via the equation: AID, SID or TTD = 100 − ([ND/NF] × [$TiO_2$F/$TiO_2$D] ×100). Where, ND is the nutrient concentration in the ileal digesta or feces, NF is the nutrient concentration in the feed, $TiO_2$F is the $TiO_2$ concentration in the feed, and $TiO_2$D is the $TiO_2$concentration in the ileal digesta or feces (Fastinger and Mahan, 2006; Stein *et al.*, 2001).

Ileal endogenous losses of AA and CP ($ILE_N$) were previously estimated for growing pigs by Presto *et al.* (2010), in pigs of the same breed and with the same life condition as the pigs in the present study.

### 3.6. Statistical analysis

The mixed procedure of Statistical Analysis Systems Institute was used to analyze all treatments. The statistical model for the digestibility values had treatment and period as fixed effects and pig as random effect. Pre-treatment were included in the model as a fixed effect. An overall significant effect of treatment and differences between treatments were tested using least square means (*t*-tests). Digestibility was also tested with estimate statements, with comparisons between treatments. Satterthwaite degree of freedom method was used in the analyses. The results are presented as least square means with standard error of means (SEM).

## 4. Results

All pigs remained healthy during the experiment and there was not any problem with the PVTC-cannulas. The pH of the treatments was 3.86 for fermented dry feed (FD), 4.08 for fermented control feed (FC), and 4.10 for both FW and FWS. No pre-treatment effects were found.

### 4.1. Microbial quantification

There were no *Enterobacteriaceae* found in the feed during the experiment. Figure 1 presents the number of colony-forming units (Cfu) of yeast and lactic acid bacteria in the experiment.

Cfu counts for yeast in all treatments fluctuated around log 7.5 during the experiment. In general, Cfu counts for lactic acid bacteria (LAB) in *Wickerhamomyces* inoculated treatments tended to be lower than that in non-inoculated treatments. Cfu counts for lactic acid bacteria in FD were higher, than that in other treatments, except for in period three and five. Mould was detected in five periods for treatment FD at levels between log 2.0-2.8 Cfu/g grain. Cfu counts for mould in FC were detected in four first periods at levels between log 4.6-5.4 Cfu/g grain which seem to be the highest levels among the treatments. Whereas, cfu counts for mould in FW was detected only in period three and four, and cfu counts for mould in FWS was detected in the period three, four and five (Figure 2).

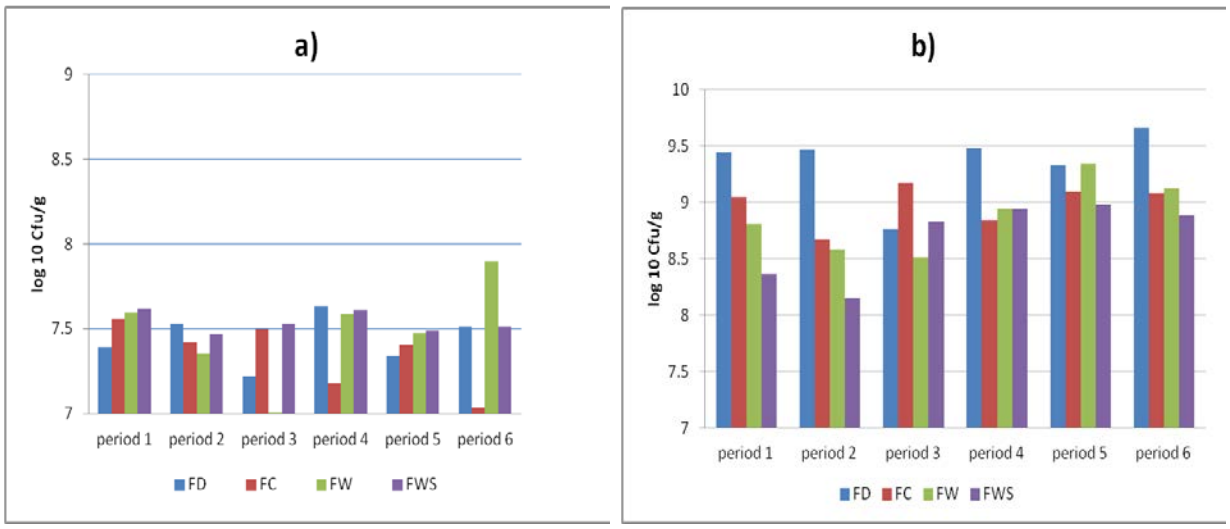


**Figure 1**. **a)** log10 Cfu/ g grain of yeast in the treatments **b)** log 10 Cfu/ g grain of lactic acid bacteria in the treatments.

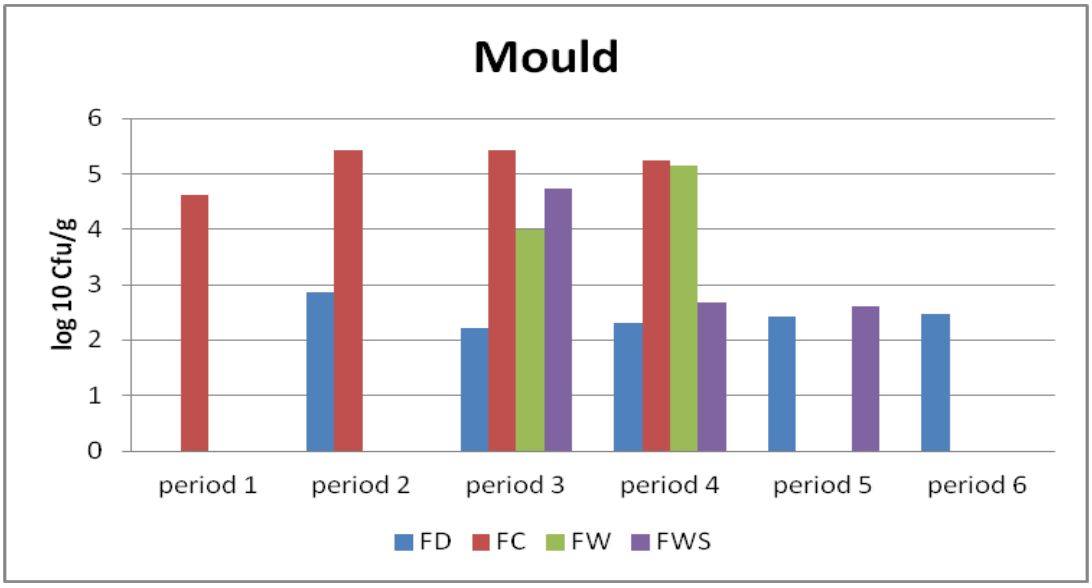


**Figure 2**. log10 Cfu/ g grain counts of mould in the treatments.

### 4.2. Digestibility of organic matter

The apparent ileal digestibility (AID) of OM was different *(P < 0.05)* among treatments. The AID of OM in fermented dry treatment (FD) was higher (*P < 0.01*) than in fermented control treatment (FC). The apparent total tract digestibility (TTD) of OM was different *(P < 0.01)* among treatments (Table 3). The TTD of OM in FWS was higher *(P < 0.01)* than that in FC. The TTD of OM in FW was higher compared with in FC *(P < 0.05).* The TTD of OM in FD was higher *(P < 0.01)* than in FC.

### 4.3. Digestibility of crude protein

The AID and SID of CP tended to be different among treatments *(P = 0.0508* and *P=0.0514,* respectively*)*. There was a difference in the TTD of CP *(P < 0.0001)* between treatments. The TTD of CP in FD was higher than in FC, FW and FWS *(P < 0.01)* (Table 2).

### 4.4. Digestibility of phosphorus

The AID and TTD of phosphor (P) showed no significant difference *(P >0.05)* among treatments (Table 2). The AID of calcium (Ca) was similar (*P > 0.05)* among treatments. The TTD of Ca was different among treatments, *(P <0.05)* (Table 2).

**Table 2**. Apparent ileal, Standardized ileal and Apparent total tract digestibility of diets.

| | | FD | FC | FW | FWS | SEM | P- value |
|---|---|---|---|---|---|---|---|
| Apparent ileal digestibility (AID) | Crude protein | 79.7 | 68.7 | 73.7 | 72.1 | 3.77 | 0.0508 |
| | Organic matter | 86.9[a] | 78.8[b] | 82.7[ab] | 82.1[ab] | 2.15 | 0.0494 |
| | Ca | 37.2 | 45.5 | 38.5 | 37.9 | 4.11 | 0.1880 |
| | P | 54.8 | 58.1 | 67.8 | 57.9 | 4.07 | 0.1016 |
| Standardized ileal digestibility (SID) | Crude protein | 80.5 | 69.9 | 74.7 | 73.2 | 3.68 | 0.0514 |
| Total tract digestibility (TTD) | Crude protein | 87.6[a] | 76.9[c] | 81.9[b] | 82.4[b] | 1.72 | <0.0001 |
| | Organic matter | 90.3[a] | 83.7[b] | 87.5[a] | 88.0[a] | 1.58 | 0.0014 |
| | Ca | 47.8[a] | 60.6[b] | 48.3[a] | 48.4[a] | 4.2 | 0.0489 |
| | P | 52.3 | 55.9 | 64.4 | 58.7 | 3.43 | 0.0792 |

[a,b] values within a row with no common superscript letters are significantly different at *P < 0.05*

## 5. Discussion

The fact that no *Enterobacteriaceae* was detected, together with the high number of lactic acid bacteria and low pH, indicate that the treatments in this study reached the second stage of fermentation (Canibe and Jensen, 2003; Canibe *et al.*, 2001; Lawlor *et al.*, 2002), and had a good microbial quality (Jørgensen *et al.*, 2010). Olstorpe and Passoth (2011) showed that the bio-control yeast *Wickerhamomyces anomalus* inoculated to cereal grain can improve the hygiene of feed by reduction of moulds and *Enterobacteriaceae*. This was supported by the present study as *Enterobacteriaceae* were not detected in inoculated treatments and moulds in *Wickerhamomyces anomalus* inoculated treatments were usually much lower than in the control treatment. However, there was a blooming of mould in one of the barrels in which we stored moist crimped barley for fermented liquid feed. This mould were present in *Wickerhamomyces anomalus* inoculated treatments in some periods of the experiment, due to the continuous supply of spores during feed replacement by the "back-slopping" technique. In this study, the highest mould counts in the treatments were log 5.4 CFU/g which was considered as an acceptable level, according to Vita Plus (forages.vitaplus.com/pdf/YeastsMo.pdf. Available from: http://www.vitaplus.com/). Feeding may not be recommended when mould counts excess 7.0 log cfu / g (100.000.000 cfu/g). Yeast counts in our study were found at normal levels for fermented feed. Cfu counts for lactic acid bacteria in *Wickerhamomyces anomalus* inoculated treatments tended to be lower than in non-inoculated treatments. This might be because lactic acid bacteria and yeast compete for space and nutrition. In addition, the yeast *Wickerhamomyces anomalus* produces killer toxins under acidic conditions against several microbial organisms (Ädel Druvefors, 2004; Furman, 2011). This might also have affected the lactic acid bacteria in *Wickerhamomyces anomalus* inoculated treatments negatively.

Pedersen and Lindberg (2003) found an improvement of organic matter digestibility in fermented liquid feed *in vitro*. Digestibility of organic matter can be improved by fermentation (Lyberg *et al.*, 2006), this was supported by the present study, the lowest appearance ileal digestibility of organic matter (OM) in our study, in FC, was higher (78.8% vs. 65%) than the AID of OM in non-fermented liquid barley in study of Sholly *et al*. (2011). Additionally, the digestibility of organic matter in yeast inoculated feed might be improved because of a more extensive degradation by the yeast, this was in agreement with our study, the higher TTD of OM in FW and FWS compared to that in FC (87.5 and 88%, respectively compared to 83.7 %), the AID of OM in FW was higher (82.7 vs, 69%) than the AID of OM in barley fermented feed in study of Sholly *et al.* (2011) and the TTD of OM in FW in this study was slightly higher (87.5 vs. 83%) than the TTD of OM in barley fermented feed in study of Sholly *et al*. (2011). The digestibility of organic matter in our *Wickerhamomyces anomalus* inoculated treatments were higher than in the study of Sholly *et al*. (2011) because their treatments were not *Wickerhamomyces anomalus* inoculated.

Pedersen and Lindberg (2003) also found an improvement of crude protein digestibility in fermented liquid feed *in vitro.* The nutritional value of the feed may be increased by addition of the yeast *Wickerhamomyces anomalus,* in accordance with an earlier study by Passoth *et al.* (2011) nutritional value of feed and food supplemented with certain *Wickerhamomyces anomalus* strains was improved because the addition of advantageous proteins, this was agreed by our study, the higher TTD of CP in FW and FWS, 81.9 and 82.4 % respectively compared to that in FC of 76.9 %).

This study showed a high protein digestibility in the fermented dry treatment compared to other treatments. Fermented dry treatment had a high number of lactic acid bacteria, contributing to a high lactic acid concentration (Lyberg *et al.*, 2008) and a low pH level (3.86) which can improve dietary protein hydrolysis (Canibe and Jensen, 2012; Lyberg *et al.*, 2006; Lyberg *et al.*, 2008). The higher protein digestibility in the fermented dry feed might result from the smaller particle size of the feed, FD was made by crimped barley of a somewhat smaller particle size compared to the other treatments, According to Kyriazakis and Whittemore (2006) protein digestion will be improved by reduction of particle size. However, studies on the effect of particle size on digestibility were carried out on dry feed unfortunately; we could not find any study which addresses the effect of particle size on protein digestibility in wet feed.

Lyberg *et al.* (2006) presented that the ileal digestibility of phosphorus in diets that included barley can be improved by fermentation, this was supported by our study. The AID and TTD of P in FW in this study were higher than the AID and TTD of P in a study of Lyberg *et al.* (2006) (67.8 compared to 48% and 64.4 compared to 48%, respectively). The AID and TTD of P in FWS in this study were also higher than the AID and TTD of P in a study of Lyberg *et al.* (2006) (57.9 compared to 48% and 58.7 compared to 48%, respectively). It might be because of benefits from *Wickerhamomyces anomalus* as a study of Reale *et al.* (2004) reported that the yeast may help in the degradation of $IP_6$ by its phytase activity.

The digestibility of Ca and P in this study showed a wide variation, this might be due to several reasons; first, pigs did not eat all of their feed, the premix was mixed together with fermented feed immediately before feeding and the major part of the premix was contained in the liquid fraction of the feed. During feeding, some liquid or feed was left which may have included some minerals, for example Ca which may have an effect on Ca digestibility. Second, some pigs were very active on the digesta collection days while others were not, which may have an effect on the variation of digestibility because movement may cause a faster rate of passage through the gastrointestinal tract, resulting in lower digestibility of nutrient (Kemme *et al.*, 1997).

## 6. Conclusion

The treatments in this study had a good microbial quality, low pH level and no *Enterobacteria* which suggest that we had good hygiene in wet fermented feed. *Wickerhamomyces anomalus* and lactic acid bacteria in fermented feed seem to have a little negative effect on each other. *Wickerhamomyces anomalus* and lactic acid bacteria starter culture inoculated fermented treatments presented a higher total tract digestibility of organic matter and crude protein compared with fermented control feed of moist cereal.

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