THE EFFECT OF ENZYME AND MILD HYDROTHERMAL TREATMENT ON THE NUTRITIVE VALUE OF BARLEY GRAIN AND CANOLA MEAL FOR DAIRY CATTLE

by

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ABSTRACT

High ingredient costs and a competitive market have promoted support for research by the feed industry to improve the utilization of protein and energy feedstuffs such as barley and canola meal. The objective of this research was to determine the feasibility of using enzyme and mild hydrothermal treatment to improve the nutritional value of barley grain and canola meal for use by dairy cattle. In experiment 1, barley which was subjected to β-glucanase, protease and xylanase enzymes and mild hydrothermal treatment, was evaluated using 48 h *in vitro* dry matter disappearance (IVDMD), *in vitro* true digestibility (IVTD), acid detergent fiber (ADF) and neutral detergent fiber (NDF) analyses. A number of interactions ($P < 0.05$) were observed for the digestibility parameters. Results of fiber analyses were inconclusive. Overall, the addition of xylanase was found to be detrimental and the mild hydrothermal treatment did not have an effect on enzyme activity. The addition of protease and/or β-glucanase to barley suggest potential application that may be further investigated. In experiment 2, canola meal was subjected to protease, xylanase enzymes and mild hydrothermal treatment. Treatments were evaluated using IVDMD, IVTD, ADF and NDF. Significant interactions ($P < 0.05$) were observed for IVDMD, IVTD, ADF and NDF. The addition of moisture significantly increased ($P < 0.05$) IVDMD and IVTD. Protease also elicited an effect ($P < 0.05$) on the NDF content. From this experiment, similar conclusions to those in experiment 1 were drawn: neither xylanase nor mild heat treatment was effective in improving digestibility. However, the addition of protease to canola meal at an intermediate

level of application improved it's digestibility significantly ($P < 0.05$). Fiber analyses resulted in a large number of interactions which produced variable responses. Experiment 3 evaluated the effects of the treatments on the fermentation characteristics of canola meal using the *in vitro* gas production technique. Rate of gas production was significantly ($P < 0.05$) affected by protease, whereas, the length of the lag phase was significantly affected by the interaction between protease and xylanase ($P < 0.05$). In general, the treatments increased rate and decreased lag time. Volatile fatty acid (VFA) analyses revealed a significant interaction ($P < 0.05$) between protease and xylanase. There was a positive linear relationship ($P < 0.05$) between the level of protease application and the ratio of acetate to propionate. Protease also had an effect ($P < 0.05$) on the ratio of butyrate to propionate. Xylanase also significantly ($P < 0.05$) both acetate and butyrate production relative to propionate. Results from experiment 3 indicated the application of protease at higher levels was beneficial in terms of improving the nutritional value of canola meal. From the work conducted in this thesis, it may be concluded, in the case of both barley and canola meal, that xylanase was detrimental to digestibility and that mild hydrothermal treatment did not improve enzyme activities. Many enzyme treatments increased the proportion of fiber, suggesting a shift in feed composition. The results of this study suggest potential applications of the treatment of barley with protease or a combination of protease and β-glucanase enzymes. Treatment of canola meal with protease between the levels of 0.01% and 0.05% (vol / wt) resulted in the greatest improvement in it's

nutritive value for dairy cattle. The *in vitro* work presented in this thesis demonstrates the potential improvements with enzyme technology in the feeding quality of barley and canola meal fed to dairy cattle.

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CHAPTER 1

1.0 INTRODUCTION

Feed companies in British Columbia (B.C.) are challenged with supplying dairy producers with affordable, high quality feedstuffs. Two major developments that have affected the feed manufacturing, dairy and other livestock industries in recent years are the North American Free Trade Agreement and the recent loss of rail subsidies. The majority of concentrates fed to ruminants in B.C. are transported from other provinces and the United States at a significant cost for use by B.C. farmers. Feed represents approximately 60% of dairy farm costs, with grain accounting for 30% of this expense in the area of the Lower Fraser Valley (Barker and Cheatley, 1992). Therefore, feed companies are constantly challenged to supply feeds which are inexpensive and efficiently used by dairy cattle.

In areas like the Lower Fraser Valley region of B.C. and Europe, where intensive monogastric animal production is prevalent, exogenous enzymes have played an increasing role in reducing the excretion of polluting nutrients and optimizing the use of feed resources. It has been reported that 80% of the world's poultry diets are now enzyme treated (Treacher and Hunt, 1996).

The feed industry has primarily focused on the use of exogenous enzymes to improve the nutritive value of ingredients in swine and poultry diets. The Canadian feed industry has applied enzymes to poultry diets since the late 1980's (Leslie, 1995). Improvements in feed utilization as a result of enzyme supplementation have

helped producers remain competitive in the marketplace. Research into the use of enzymes in ruminant diets was briefly investigated through the 1960's, however, results were too inconsistent to warrant further investigation, resulting in the lack of applications (Chesson and Austin, 1996). Major advances in enzyme technology and the increased costs of livestock production have renewed interest into the use of enzymes in ruminant diets. Two major feeds incorporated into dairy cattle diets in Western Canada are barley and canola meal, however the levels of inclusion of these feeds are limited because high fiber content decreases their energy value. The supplementation of these feedstuffs with exogenous enzymes has the potential to alter the fermentation profile of the carbohydrate fraction and improve the digestibility of these feeds.

The intent of this thesis project was to investigate the feasibility of improving the nutritional value of canola meal and barley for dairy cattle using enzyme technology and mild hydrothermal treatment.

1.1 EXOGENOUS ENZYMES

1.1.1 Sources of Enzymes

Enzymes used in the feed industry are primarily derived from sources used by the food and beverage industries (Chesson and Austin, 1996). Enzyme preparations are generally not specific but contain an array of enzyme activities (Campbell and Bedford, 1992). Although the food and beverage industries require greater specificity in their enzyme requirements, enzymes prepared for the feed industry are rarely required to be so specific in nature. The development of an enzyme complement to a specific ingredient requires a careful match between enzyme and substrate as well as consideration of storage and processing conditions in order to obtain the desired response. Sources of enzymes include those from fungi (e.g. *Trichoderma* and *Aspergillus* spp.), bacteria (e.g. *Bacillus* spp.) and yeasts (e.g. *Saccharomyces cerevisiae*).

Commercial enzyme products or "enzyme cocktails" usually contain a mixture of specific enzyme activities (Newman, 1994). Enzyme "cocktails", have been generally more successful in animal feeding systems than a single enzyme activity (Mulder et al., 1991; Chesson, 1993). Knowledge of the type of enzyme, whether applied alone or as a "cocktail", it's specificity and mode of action is crucial in order to take advantage of beneficial effects and minimize negative effects the enzyme may have on the substrate (Amado, 1993).

1.1.2 Mode of Action

Improvements observed with exogenous enzymes, with both monogastric and ruminant species, include increased feed utilization and reduced variation in the availability of nutrients in feedstuffs. It has been suggested that enzymes aid in the disruption of complex plant cell walls which causes intracellular nutrients, such as starch and protein, to be released (Gruppen, 1996; Chesson, 1993). By the same mechanism, an enzyme "cocktail" is more effective in degrading the cell matrix (Gruppen, 1996). For example, the synergistic effect of several carbohydrases was shown to enhance the rate of hydrolysis and produce the desired products when applied to wheat and sorghum flour, sunflower and palm-kernel meals (Dusterhoft et al., 1993). The improved digestion of fiber observed with some enzyme supplemented ruminant diets may also be a result of improved colonization of feed particles by rumen microbes (Cheng et al., 1995). However, results with fibrolytic enzyme supplements are inconsistent. When calves were fed an alfalfa and orchardgrass hay diet where a cellulolytic enzyme was sprayed onto the grain supplement, the animals showed an unexpected lack of response (Leatherwood et al., 1960). On the other hand, a multienzyme supplementation fed to calves improved gain and feed efficiencies (Modyanov and Zel'ner, 1983).

Graham and Pettersson (1992) found that several enzymes were required in order to obtain a production response in broiler chicks fed a barley based diet. This effect was attributed to the increased availability of intracellular nutrients. An enzyme "cocktail" consisting of β-glucanase, cellulase and xylanase also resulted in

the release of more intracellular nutrients than when β-glucanase alone was applied to barley (Mulder et al., 1991).

1.1.3 Monogastric Success

The successful application of exogenous enzymes may be illustrated by research conducted using monogastric animals. Several reviews on the use of enzymes in monogastric feeds summarize improvements in animal performance with the use of β-glucanases, pentasonases (xylanases) and other enzymes (Campbell and Bedford, 1992; Chesson, 1993; Geraert et al., 1996). Enzymes have been shown to: destroy anti-nutritional factors, assist in the breakdown of bonds not hydrolyzed by the animal's endogenous enzyme complement, further enhance the endogenous enzyme activities of the animal and thereby increase the nutrient and energy availability of alternative feedstuffs (Chesson, 1993; Close, 1996). It has been reported that enzymes reduce the variability of apparent metabolizable energy (AME) values associated with cereals (Geraert et al., 1996). Improvements in feed efficiency and growth rates have been reported with poultry and swine that were fed enzyme supplemented diets (Marquardt et al., 1987; Rotter et al., 1987). With chicks, increased rate of gain, efficiency of feed utilization, AME and the digestibility of DM, fat and protein have also been reported (Marquart, 1994). The primary goals of the feed industry involving current enzyme research are mainly directed towards monogastrics and are to remove or destroy the effects of anti-nutritional factors (ie. NSPs), increase overall digestibility of the feed and the bioavailability of specific

nutrients and decrease nutrient pollution (Newman, 1994). For example, improvements in the digestibility of the non-starch polysaccharides (NSP) in cereals have been shown to reduce gut content viscosity, thereby increasing the availability of nutrients and improving animal performance (Campbell and Bedford, 1992; Chesson, 1993).

1.1.3.1 Anti-Nutritional Properties of Cereals

The continued increase in feed prices and the demand to meet increasing nutritional requirements of production animals have resulted in larger amounts of different cereals now being included in monogastric diets. Cereals may contain a large proportion of NSPs which are polymers of simple sugars and include cellulose, hemicellulose, pectins and oligosaccharides. Monogastric animals do not posess the endogenous enzymes to break down NSPs. In the digestive tract, these compounds become soluble therefore contributing to an anti-nutritional effect. These soluble NSPs increase the viscosity of digesta which results in a reduction in the rate of feed passage, feed intake and the efficiency of feed utilization; all of which are reflected in poor animal performance (Bedford, 1993). The resulting impaired gut function reduces the availability of nutrients such as glucose, fat and protein (Fengler and Marquardt, 1988; Bedford, 1993).

Increased viscosity of the gut also causes "sticky droppings", a condition affecting poultry which can result in poor litter quality (Campbell and Bedford, 1992). Meat quality may be affected if broiler birds develop breast damage and hock

problems (Campbell and Bedford, 1992). Poultry diets that contain a large proportion of NSPs have a lower AME content which is negatively correlated with content of soluble polysaccharides (arabinoxylans) in the diet (Annison, 1991). The anti-nutritional effects of NSPs may be reduced with dietary enzyme supplementation which will be discussed further. Specific enzymes of interest for use in monogastric feeds include xylanases for wheat in addition to β-glucanases and cellulases for barley and oats (Marquardt, 1994). However, the increase in production resulting from the use of enzymes such as β-glucanase and xylanase, cannot be solely explained by the destruction of the gel forming polysaccharides (Chesson, 1993). Improvements in nutrient absorption and growth rates may also be attributed to the improved conditions within the gastrointestinal tract (Chesson, 1993).

1.1.3.2 β-glucanases

A large portion of the endosperm cell walls of cereals is composed of β-glucans and arabinoxylans (Graham et al., 1989). In barley and oats, the major cell wall polysaccharides are the β-glucans which become soluble and increase the viscosity of digesta in the gut thus affecting poultry and to a lesser degree swine (Campbell and Bedford, 1992). β-glucans are soluble because the β-(1,4) backbone is interspersed with β-(1,3) linkages between the glucose polymers preventing inter-molecular bonding. The addition of β-glucanase to poultry diets hydrolyzes the

soluble mixed linked β-glucans which minimizes their anti-nutritional effects such as causing viscous digesta in the gut. The increase in the production response of broilers fed a barley diet supplemented with β-glucanase can be explained by the increased availability of intracellular nutrients resulting from the loss of the integrity of the cell wall (Hesselman and Aman, 1986).

1.1.3.3 Xylanases

Like β-glucans, arabinoxylans are soluble and may lead to similar anti-nutritional effects as described above if present in significant amounts in poultry diets. Arabinoxylans are the major cell wall components of rye, wheat and triticale and a minor component in barley. Their effects can be eliminated by supplementing the diet with xylanase to aid in the solubilization and cleavage of the polysaccharides (Gruppen et al., 1993). However the activity of the enzyme may be limited if the xylan chain is highly substituted with glucuronic acid residues or arabinose molecules (Biely, 1985). The use of xylanases has focused on improving dietary AME values and increasing the digestibility of rye, wheat and barley in broiler diets (Annison and Choct, 1991; Kaoma et al., 1996). It is now established that xylanase applied to rye diets reduces the incidence of "sticky droppings" and improves performance in poultry (Pettersson and Aman, 1988, 1989; Teitge et al., 1991; Bedford and Classen, 1992).

1.1.3.4 Proteases

Proteases may be used to increase protein availability and eliminate protein anti-nutritional factors and potential allergenic proteins (Classen, 1996). A low digestibility of protein sources such as soybean meal and canola meal by poultry results in reduced feed utilization. The supplementation of canola meal with proteolytic enzymes has been shown to improve it's digestibility by poultry (Bedford and Morgan, 1995).

1.1.4 Ruminant Research with Enzymes

The successes observed with monogastrics have renewed interest in the applications of exogenous enzyme in ruminant diets. The use of exogenous enzymes may benefit ruminant nutrition by reducing the current limitations on the digestion processes in the rumen thereby enhancing animal performance. Although the addition of exogenous enzymes to feedstuffs has indicated that there is the potential to improve efficiencies and performance, their applications are mainly limited to the ensiling process (Modyanov and Zel'ner, 1983). Interpretation of results from enzyme research is difficult since they are inconsistent and the mode of action of the enzymes in the ruminant animal is largely unknown (Beauchemin and Rode, 1996). The potential use of alternate feedstuffs and goals that are directed towards maximizing nutrient efficiencies, justifies further investigation into the use of enzymes with ruminant feeding systems (Chesson and Austin, 1996).

1.1.4.1 Sources of Variation in Results

The variation in the responses that have been found with enzymes used in ruminant feeding systems have been attributed to a number of different factors. Sources of variation include: type and age of animal, diet composition, enzyme type, level and combination of enzymes used, enzyme stability and method of application (Beauchemin and Rode, 1996).

The response of steers fed alfalfa, timothy hay or silage based diets supplemented with cellulase and xylanase was found to be dependent on the forage source and the level of enzyme applied (Beauchemin et al., 1995). Substantial improvements in average daily gain (ADG) were found when the enzymes were added to the alfalfa and timothy hay diets, however, only a minor effect was observed when the enzyme was added to barley silage. In another study where a fibrolytic enzyme was applied to barley silage at various levels and fed to calves, the increase in final weight was linearly dependent on the level of enzyme; whereas, ADG, feed conversion ratio (FCR) and dry matter intake (DMI) all remained unaffected (Treacher et al., 1996). A later study conducted by the same researchers reported that when finishing cattle were fed a total mixed ration consisting of 70% rye grass silage and 30% barley grain which had been treated with a fibrolytic enzyme, ADG increased without a corresponding increase in intake (Treacher et al., 1996). Speculation on the mode of action by the authors resulted in suggestions that increased enzyme activity was necessary with silage diets. This theory was supported by unpublished research conducted by the same laboratory where feedlot

animals were fed a corn silage diet that was supplemented with an enzyme (Beauchemin and Rode, 1996). The increases in ADG and FCR were found to correspond with the increases in the application rate of the fibrolytic enzyme complement.

1.1.4.2 Fibrolytic Enzymes

1.1.4.2.i) Forages

The majority of enzyme research conducted in the ruminant field has concentrated on the fibrous fraction of feedstuffs which is less digestible in the rumen; with the objective being to optimize feed utilization by the entire animal. For example, the application of fibrolytic enzymes has been shown to increase the digestibilities of both dry matter (DM) and fiber (Grainger and Stroud, 1960; Van Wallegham et al., 1964). This may explain the improvements in ADG and FCR observed in steers that were fed a forage based diet supplemented with exogenous enzymes (Galiev et al., 1982; cited by Beauchemin and Rode, 1996). Increases in milk production and DMI were found when dairy cattle were fed either hay or barley with silage diets in which the forage ration was supplemented with cellulase and xylanase enzymes (Lewis et al., 1995; Stokes and Zheng, 1995). The increases in milk production and DMI, however, may not be as a result of improved DM nor neutral detergent fiber (NDF) digestibilities but may be as a result of increased rate of passage of feed (Lewis et al., 1995). Improvements in the ADG and FCR of steers were also observed when an unspecified enzyme was applied to corn silage

rations (Rovics and Ely, 1962). Increases in ruminal *in vitro* and *in situ* DM and NDF digestibilities were observed when fibrolytic enzymes were added to dry forages however, when enzymes were added to fresh or wilted forage, no effects were observed (Feng et al., 1992a). In contrast, steers fed fresh, wilted or dry grass supplemented with fibrolytic enzymes all elicited a positive growth response although increases in DMI as well as in DM and NDF total tract digestibilities were only found with dry grass supplemented with enzymes (Feng et al., 1992b).

1.1.4.2.ii) Concentrates

Grains such as barley and sorghum and oilseeds such as canola meal contain high amounts of fiber and would be expected to respond to fibrolytic enzyme supplementation however, studies in this area are limited. In a recent study the effects of xylanase on barley grain were investigated. It was reported that higher xylanase levels improved feed efficiency of steers whereas lower xylanase levels had no effect (Beauchemin et al., 1996).

1.1.4.3 Other Enzymes and Enzyme Combinations

1.1.4.3.i) Forages

An active area of research has been the investigation of the effects of a fibrolytic enzyme used in conjunction with an amylase and/or protease applied to forages in order to enhance their digestibility and improve animal performance. Early research with beef cattle fed either corn or oat silage found no changes in DMI although both gain and feed efficiencies increased with amylase, protease and cellulase enzyme supplementation (Burroughs et al., 1960). Perry et al. (1966) fed cattle a corn or corn silage diet with the same fibrolytic enzymes as used by Burroughs et al. (1960) and reported beneficial effects on digestibility. However, Perry et al. (1966) observed no response in the digestibility of the feed when proteolytic enzymes were applied to a similar diet. In contrast to both of these latter studies, increases in DM digestibilities have been reported with forages with the addition of either a fungal (Ralston et al., 1962) and bacterial protease (Rust et al., 1965).

1.1.4.3.ii) Concentrates

Improvements in gain and feed efficiencies of steers and heifers were observed when enzymes containing proteolytic, amylolytic and fibrolytic activities were added to high grain rations (Burroughs et al., 1960; Clark et al., 1961; Rovics and Ely, 1962). Similar results were produced with an unspecified enzyme supplement applied to corn grain and corn silage fed to steers (Weichenthal et al.,

1996: cited by Beauchemin and Rode, 1996). Enzyme supplementation of corn grain diets is expected to elucidate a response as the rumen microbes gain access to and hydrolyze additional substrates. Corn protein and starch granules have been shown to be surrounded by a protein matrix which lends resistance to microbial colonization and digestion (McAllister et al., 1990). However, the response to enzyme supplementation of corn based rations has been inconsistent.

The same enzyme complement and diet of similar composition to the one used by Burroughs et al. (1960), was fed to calves and did not elicit any response in feed efficiency although there were improvements in DMI and ADG when the calves were fed an enzyme supplemented high corn grain diet (Perry et al., 1966). In contrast, several studies involving feedlot steers fed corn grain supplemented with an enzyme "cocktail" of protease and amylase, failed to yield responses in ADG or feed efficiencies (Perry et al., 1960; Ward et al., 1960; Wing and Wilcox, 1960). Some studies have also yielded results from enzyme research that were inconclusive (Theurer et al., 1963, Nelson and Catron, 1960).

1.1.5 Feed Processing and Enzyme Applications

Recent research with monogastric animals has investigated the possible marriage of enzymes and feed processing where the enzymes were applied post processing. Thermal processes such as extruding, steam rolling and pelleting alone have been shown to improve feed utilization by increasing digestibility and improving production performance in poultry (Bedford, 1993). For example, subjecting

sorghum grain to dry rolling has been shown to give positive responses in terms of DMI, ADG and FCR (Boyles et al., 1992). However, heat treatment increases the solubility of NSPs in many cereals and their anti-nutritional effects may counteract any benefit of the processing treatment (Chesson and Austin, 1996). This effect can be removed by supplementing the diet with exogenous enzymes (Bedford et al., 1991). Combining processing and enzyme technologies may benefit digestion by acting synergistically in both monogastric and ruminant animals. In the latter case, results from studies investigating the combined effects of enzymes and processing treatments are more inconsistent relative to those obtained with monogastrics. The application of hydrothermal and enzyme treatments to cereal grains has been briefly investigated with varying results.

Dairy cows fed enzyme treated dry rolled or steam rolled sorghum showed improved digestibilities of dry matter (DM), organic matter (OM), crude protein (CP) and NDF fractions, without any adverse effects on milk production (Chen et al., 1995). Krause et al. (1989) reported that steers fed steam flaked sorghum that was treated with an unspecified enzyme had increased DMI and an even greater response in ADG, however, there were no improvements in feed efficiency. In the same study, when dry rolled sorghum was also treated with an unspecified enzyme and fed to steers, even greater increases were reflected in DMI and ADG with a large improvement in feed efficiency. In contrast to the above, digestibility was not improved in a study where steers were fed a processed and enzyme treated sorghum diet (Richardson et al., 1990).

The above review summarizes many of the results from research with exogenous enzymes applied to diets fed to ruminant animals. The majority of the experiments were animal studies, although a few studies involved *in vitro* analyses of enzyme treated feed. *In vitro* assays have been shown to be an inexpensive alternative to animal trials and invaluable in evaluating of a large number of samples. The work conducted for this thesis required *in vitro* analyses as a preliminary step to investigating a large number of samples and the interactive effects of enzymes and mild hydrothermal treatment of barley and canola meal.

1.2 *IN VITRO* TECHNIQUES

In vitro techniques designed for feed evaluation purposes have gained wide acceptance as cost effective alternatives to animal based research which is often expensive, labor intensive and limited in the number of samples that may be assessed at one time. The majority of *in vitro* methods that are specific to evaluating ruminant feedstuffs involve the incubation of a sample of the feed with rumen fluid inoculum. These techniques are usually based on a measure of end point digestion or the appearance of metabolic products over time. Comprehensive reviews detailing the history, development and methodology of *in vitro* ruminant feed evaluation techniques are given by Johnson (1963; 1966). One of these reviews presents the pioneering research conducted between 1882 and 1888 in the area of science which investigated the fermentation of cellulose using rumen fluid from an ox. Another significant contribution to the evolution of *in vitro* techniques was the

determination of the mineral composition of sheep saliva (McDougall's, 1948). This discovery enabled the chemical reproduction of saliva and the development of the buffer solution which is still in use today (Johnson, 1966). In 1963, Tilley and Terry developed an *in vitro* technique to evaluate forage digestibility which has become the standard.

1.2.1 Apparent Digestibility: Tilley and Terry *In Vitro* Technique

The Tilley and Terry (1963) *in vitro* technique is a two stage method that provides an estimate of the apparent digestibility of a feedstuff. The technique involves the incubation of a feedstuff with rumen fluid inoculum for 48 h, followed by a 48 h incubation in an acid pepsin solution. This procedure simulates ruminant digestion where the feed is first fermented by microbes in the rumen, referred to as the first stage, and then subjected to postruminal digestion, the second stage. An end point dry matter digestibility (IVDMD) or organic matter digestibility (OMD) measurement is obtained. The only difference between IVDMD and OMD is essentially that the latter is corrected for ash content which is assumed to be indigestible in this system. IVDMD represents the fraction of the feed that is digestible in the rumen by microbes and includes both the fibrous and cell soluble fractions. The undigested plant residue however, is contaminated with some undigested microbial material. This technique has been successful because of the high correlation between the *in vitro* results and *in vivo* apparent digestibility measurements (Van Soest, 1982). Although the method was originally developed to improve estimates of *in vivo* forage digestibility, it has also been successfully used with concentrates. For example, apparent IVDMD of wheat was found to be well correlated (R^2 = 0.83) with *in vivo* DMD measurements (Adesogan et al., 1995).

1.2.2 True Digestibility

Goering and Van Soest (1970) modified the second step of the Tilley and Terry method to arrive at an estimate of "true digestibility" (IVTD). The true digestibility technique involves incubating feed samples with rumen fluid for 48 h and then substituting the acid pepsin incubation with a neutral detergent fiber rinse. This procedure results in the removal of all cell solubles which, in essence, represents end point digestion.

1.2.3 Enzymatic Method

A digestibility measurement can also be obtained using an enzymatic method. Several enzymatic methods are available (Van Soest, 1994). For example, a procedure developed by Jones and Hayward (1975) involves incubating the feed sample in an acid pepsin solution followed by a second incubation with a fungal cellulase. There is less precision with this method because rumen microbes produce a variety of enzymes and may adapt to a variety of different substrates (Van Soest, 1982). For a review of several enzymatic methods the reader is referred to Nocek (1988). Enzymatic techniques differ from other *in vitro* techniques because they do not require the use of rumen fluid and thus the maintenance of donor animals and an anaerobic environment.

1.2.4 Rumen Fluid Inoculum

1.2.4.1 Animal Diet

Lindberg (1985) briefly discussed a number of factors which influence estimates of rumen fermentation by various *in vitro* techniques. Some of these factors include: solubility, endproduct accumulation, continuous fermentation, proteolytic enzymes, optimal pH and the basal diet fed to the animal. Fiber source in the diet has also been shown to affect the results of *in vitro* experiments (Cherney et al., 1993). The ratio of concentrate to forage affects the rumen microbial population as the various microorganisms have different affinities for substrates (Russel and Baldwin, 1979) and the products of fermentation vary accordingly (Owens and Goetsch, 1988). Rumen fluid donor animals should therefore be maintained on a balanced ration with similar dietary components as to be tested *in vitro* (Warner, 1956; Bowie, 1962). The diurnal variation in rumen fluid activity can be minimized by increasing the feeding frequency (Warner, 1965) and conducting collections at similar times. The retention of rumen digesta is decreased with increased feeding frequency which may be relevant when applying the *in situ* technique (Warner, 1981).

1.2.4.2 Rumen Fluid Collection

Some studies recommend collecting rumen fluid a few hours after feeding to ensure a mixture of bacterial species (Moore et al., 1962). It has been reported that post feeding collection of rumen fluid reduces the variation between incubating samples *in vitro* (Tilley and Terry, 1963). Other studies, including some involving the gas production system advocate collecting rumen fluid prior to feeding to ensure low rumen fluid activity in blanks (Menke et al., 1979; Menke and Steingass, 1988). Recent studies involving the *in vitro* gas production system however, have reverted to collecting the rumen fluid after feeding when bacterial activity is assumed to be at it's maximum (Pell and Schofield, 1993; Schofield and Pell, 1995).

1.2.4.3 Inoculum Preparation

The majority of rumen fluid inoculum preparations used in the first *in vitro* studies were comprised of rumen fluid which had been strained through cheesecloth and added to a buffer and mineral mixture (Quin, 1938, 1943; Hungate et al., 1955; Pearson and Smith, 1943: cited by Johnson, 1966), although a few researchers used unstrained rumen fluid (Louw et al., 1949; Burroughs et al., 1950). Adhesion of bacteria to the plant cell wall increases linearly up to 9 h (Latham et al., 1978). It has been suggested that approximately 70-80% of the rumen microbes are associated with the particulate matter in the rumen whereas strained rumen fluid alone accounts for 20-30% of microbial activity (Cheng, K.-J.; personal communication). In

many studies, the mixing of rumen fluid in a blender to dislodge microbes attached to feed particles has proven to be beneficial in obtaining reliable estimates of *in vitro* digestion coefficients. However, some research suggests that blending affects the viability of bacteria and destroys protozoa (Craig et al., 1984). In the gas production system, preliminary studies conducted by Pell and Schofield (1993) indicated that blended and unblended rumen fluid gave similar results. Unblended rumen fluid is preferred since blending has been shown to increase the error in gas production measurements by increasing the amount of small feed particles in the fluid and exposing the microbes to more oxygen (Pell and Schofield, 1993).

1.2.5 Advantages of *In Vitro* Techniques

The greatest advantages of *in vitro* techniques used to simulate animal performance is that they are less intensive and costly than animal studies. These methods have also been shown to be useful in screening a large number of samples and good correlations have been obtained when compared with *in vivo* results (Van Soest, 1982). Although chemical methods may be faster and have better replication, the *in vitro* methods more accurately represent biological reality.

1.2.6 Disadvantages of *In Vitro* Techniques

All of the above *in vitro* methods, with the exception of the enzymatic method, require the maintenance of cannulated animals to serve as rumen fluid donors. It is this latter point which creates one of the greatest challenges surrounding these types of studies, the maintenance of an anaerobic environment in the rumen fluid. It is imperative that an anaerobic environment be maintained in order to ensure success of the experiment. The determination of apparent or true digestibilities requires long periods of time and involves many preparatory steps. A major drawback to these methods is that no information on the kinetics of degradation may be obtained unless lengthy and labor intensive time course incubations are conducted.

1.2.7 Kinetic Descriptions of Feed Degradation

1.2.7.1 *In Situ* Technique

The *in situ* bag technique was first developed by Quin et al., (1938) (cited by Johnson, 1966) and allows for a thorough description of the fermentation profile of a feedstuff thereby enabling the determination of the rate and extent of disappearance of a feedstuff. Feedstuffs are contained within small nylon bags which are suspended and incubated in the rumen for various periods of time (Mehrez and Orskov, 1977; Orskov, 1982). The disappearance of material from the bag indicates apparent DMD (Mehrez and Orskov, 1977). This technique is commonly used to

determine fractional rates of protein degradation employing the equations of Orskov and McDonald (1979). Although the method most closely resembles the rumen environment (Broderick et al., 1988), the method has also been criticized for several technical problems.

The *in situ* method is intensive, requires the maintenance of cannulated animals and only a few feed samples may be incubated at one time. In addition, fine feed particles may be lost through the pores of the bags which are assumed to be part of the immediately soluble fraction but may not in fact have been digested (Broderick et al., 1988). The entrance of feed particles into the nylon bag (Nocek, 1985) and bacterial contamination of the sample (Nocek, 1987) have been shown to affect degradability values of feeds. Microbial contamination is small with protein supplements, and highly digestible forages (Mehrez and Orskov, 1977; Varvikko and Lindberg, 1985). However, with rapidly fermentable cereals such as barley and poorly digested forages, contamination could be significant (Varvikko and Lindberg, 1985). It should be noted that the presence of microbial matter will not greatly affect DM degradability measurements but will influence nitrogen studies (Varvikko and Lindberg, 1985). In evaluating the fermentation kinetics of feedstuffs the greatest disadvantage of this technique is the assumption that the soluble fraction of a feedstuff is entirely degradable (Broderick et al., 1988). The "true" fermentation profile of a feedstuff, however, can be described using the rumen *in vitro* gas production technique.

1.2.8 The Gas Production Technique

The kinetics of digestion of a feedstuff can be described with the use of the gas production technique first developed by Menke et al. (1979). The gas production technique involves the incubation of a feedstuff with rumen fluid inoculum and as fermentation proceeds, the gas produced is recorded at specific times. Gas production is related to the digestibility and may be used with other parameters to estimate the metabolizable energy value of a feedstuff fed to ruminant animals (Menke et al., 1979; Menke and Steingass, 1988). An estimate of the energy value of a feedstuff is obtained by combining the results of proximate analysis such as crude protein, crude fat, crude fiber and crude ash, with those obtained from gas production studies (Menke and Steingass, 1988).

Gas production and *in situ* degradability were both found to be well correlated with the rates of fermentation for wheat (Adesogan et al., 1995). However, results may depend on the type of feedstuff as there was no significant correlation between gas production and the *in situ* or *in vivo* degradabilities in the studies conducted by Piva et al., (1988) which involved hay, silage and grass. This latter study also determined that nitrogen free extract (NFE) and ADF had the greatest influence on feed fermentation. A strong linear relationship between NDF disappearance and gas production has been shown to exist with forages (Pell and Schofield, 1993). Blummel and Orskov (1993) found a good correlation between *in vitro* gas production and the nylon bag degradability of feed which was closely related to feed intake and growth rate in cattle.

Measurement of the gas produced from rumen microorganisms in batch cultures was first conducted by Menke and Ehrensvard (1974) (cited by Theodorou et al., 1994). Menke et al. (1979) monitored the gas accumulation of a feedstuff incubated in glass syringes with rumen fluid inoculum. As fermentation progressed, gas production caused the syringe plunger to rise and enabled a direct measurement of gas volume. This author reported a high correlation between the accumulation of gaseous products and feed degradation (Menke et al., 1979). The gas production technique was further refined by Menke and Steingass (1988).

Volumetric recordings of gas produced from microbial fermentation were first accomplished using a manual method where the gas volume was determined by visually reading the movement of the syringe plunger. Recent technological advances have allowed for the changes in gas volumes to be electronically measured by recording the changes in pressure.

Theorodou et al. (1994) measured gas production by using a light emitting diode voltmeter and electronic pressure transducers that were manually inserted into fixed volume fermentation flasks. As gas accumulated within the flasks, the pressure required to stabilize the system to atmospheric pressure was determined at specified time intervals. This technique is based on the assumption that microbial digestion would be influenced by the accumulation of gas in the system. It has been recently shown that if pressure is maintained within the range of 0 to 0.6 atmospheres, fermentation will not be affected (Pell and Schofield, 1993).

A more sophisticated computerized system to obtain gas production results was developed by Pell and Schofield (1993). In this system, electronic pressure transducers were connected to fermentation flasks and interfaced with a data logger and computer (Pell and Schofield, 1993). As gas accumulated in a fixed volume container, the resulting pressure was recorded at determined time intervals. A calibration equation is necessary in order to determine the relationship between pressure increases and voltage readings. The data can be easily transferred to a spreadsheet which enables corrections for blanks and easy manipulation of the data.

1.2.8.1 Advantages of the Gas Production Technique

The main advantage of the gas production technique is it measures the appearance of metabolic products and thus it reflects the "true" fermentation of a feedstuff. It has been shown that the observed fermentation profile is representative of the entire sample (Huntington, 1995). The gas production technique does not require separation of the digested and undigested components unlike many other *in vitro* methods (Menke et al., 1979). Furthermore, both the soluble and insoluble carbohydrate fractions produce gas as they are metabolized to volatile fatty acids (VFA) by rumen microorganisms (Huntington, 1995). The procedure also requires a small sample size which is reflected in the use of limited amounts of laboratory supplies.

1.2.8.2 Disadvantages of the Gas Production Technique

There are several shortcomings associated with the gas production technique. Firstly, the computerized technique is a costly investment because it requires highly sophisticated equipment. Secondly, a standard feedstuff must be incubated within each run in order to correct for the variation in rumen fluid activity between runs if necessary (Menke and Steingass, 1988). Thirdly, microbial cells in the rumen fluid contribute to gas production as they die and are fermented to VFAs by other microorganisms. This latter point is of particular importance when short incubations are used since it has been shown that VFAs are a poor indication of fermentation rates and extent of digestion (Van Soest, 1982). Lastly, the fermentation of readily fermentable feedstuffs such as barley grain, results in the production of high amounts of propionic acid which does not contribute to gas production, thus requiring careful interpretation of gas data.

1.2.8.3 The Fermentation Process of Carbohydrates in the Rumen.

Dietary carbohydrates include soluble sugars, starch and fiber. Soluble sugars are rapidly fermented in the rumen where the type of sugar determines the rate of fermentation. Increasing the levels of these soluble sugars in the diet results in the production of high amounts of propionic acid thus decreasing rumen pH (Hungate, 1966). The decrease in fiber digestion associated with low rumen pH may be caused by a shift in the types of microbes from cellulolytic to amylolytic populations (Mann and Orskov, 1975). Structural carbohydrates are comprised of

cellulose and hemicellulose fractions. Starch, pectins and polyuronids are more quickly digested than fiber components although more slowly fermented by rumen microorganisms than the soluble sugars (Van Soest, 1982).

Dietary carbohydrates are metabolized by rumen microorganisms to obtain a source of energy. The main end products of the anaerobic fermentation process are the short chain volatile fatty acids (VFA): acetate, propionate, and butyrate, and the gases: carbon dioxide (CO_2) and methane (CH_4) (Wolin, 1975). Ruminants utilize VFAs as a major source of energy, for example, forage diets supply 50-85% of the metabolizable energy (Owens and Goetsch, 1988), however, gas produced as a result of carbohydrate metabolism constitutes a major loss of energy (Hungate, 1966). The equations of Wolin (1960) are presented below to illustrate the production of gas during the production of VFAs (Equations 1, 2 and 3). Excess hydrogen produced during the process combines with CO_2 to produce CH_4 (Equation 4). From these equations it can be determined that in the production of VFAs, acetate and butyrate participate directly in gas production whereas propionate does not.

$C_3H_6O_3 + H_2O \Rightarrow CH_3COOH + CO_2 + 2H_2$ (acetate) (Equation 1)

$C_3H_6O_3 + H_2 \Rightarrow CH_3CH_2COOH + H_2O$ (propionate) (Equation 2)

$2C_3H_6O_3 \Rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ (butyrate) (Equation 3)

$CO_2 + 4H_2 \Rightarrow CH_4 + 2H_2O$ (Equation 4)

There is increased energy efficiency when the proportion of propionate increases relative to acetate and butyrate. This is, in part, as a result of a decrease in energy loss through decreased gas production, primarily methane (Van Soest, 1994). Methane is produced by rumen methanogenic bacteria which metabolize CO_2 and H_2 via the production of formate (Owens and Goestch, 1988). From the stoichiometric equations of Hungate (1966) and the equations of Wolin (1960), gas production can be estimated from the VFA composition of the rumen fluid.

1.2.8.4 Estimation of Gas Volume

Direct gas production can be explained by the amount and proportions of VFAs produced from fermentation (Menke and Steingass, 1988; Blummel and Orskov, 1993; Beuvink and Sploestra, 1992; Opatpatanakit et al., 1994). Each mmol of VFA produced from the fermentation process also results in the release of an indirect equivalent mmol of CO_2 from the *in vitro* buffer solution as the VFAs are neutralized (Menke and Steingass, 1988; Beuvink and Sploestra, 1992; Blummel and Orskov, 1993). Indirect gas production will occur as long as the buffering capacity of the incubation medium is not exceeded. Direct and indirect gas production resulting from each mmol of acetate, propionate and butyrate has been determined to be 4, 3 and 2 mmols CO_2, respectively (Beuvink and Sploestra, 1992). The following equation has been shown to describe the amount of gas directly produced from the various carbohydrate sources when considering total VFA content and proportions thereof (Beuvink and Sploestra, 1992):

mL gas = Mv mmol HAc + 2 Mv mmol HB + 0.87 Mv mmol total VFA (Equation 4)

where *HAc* is acetate, *HB* is butyrate and *Mv* is molar gas volume at standard temperature and pressure (24 L*mol $^{-1}$).

1.2.8.5 Use of the Gas Production Technique in the Evaluation of Concentrates.

The gas production technique has been successfully used in the evaluation of parameters estimated from the gas production of concentrates (Menke et al., 1979; Menke and Steingass, 1988, Krishnamoorthy et al., 1991, 1995; Ribeiro et al., 1990; Beuvink and Sploestra, 1992; Opatpatanakit et al., 1994, 1995). Quin (1943) (cited by Johnson, 1966) measured the production of gas from carbohydrates and found that the products of fermentation were closely related to digestion in the animal. The evaluation of concentrates *in vitro* requires careful monitoring of the pH of the system. The fermentation of concentrates results in higher amounts of acid production putting a greater load on the buffering capacity of the system. The buffering capacity of the system will be affected if the pH falls below 6.0 (Menke et al., 1979). The presence of CO_2 in the rumen system helps maintain pH balance by acting as a buffer in the form of bicarbonate (HCO_3^-). All of the VFAs contribute to the proton pool. However, as previously reported, the formation of propionate does not produce CO_2 in addition to contributing to the proton pool, both of which

contribute to the decrease in rumen pH seen in cows fed high concentrate diets (Van Soest, 1994).

The fermentation of concentrates generally results in the formation of greater amounts of propionate at the expense of acetate production resulting in less CO_2 and CH_4 production (Menke et al., 1979, Menke and Steingass, 1988). This ratio will vary depending on the content of readily fermentable carbohydrates in the sample. For this reason, interpretation of the fermentation profiles of concentrates may be more difficult than that of forages especially when comparing concentrates from different sources.

1.2.8.6 Kinetic Analysis of Gas Data

Mathematical models provide a means for describing the fermentation kinetics of various feedstuffs. Many models have been developed to reflect the relationship between bacterial numbers and substrate disappearance. Sigmoidal models used to describe the digestion of fiber in the rumen were primarily developed for use in conjunction with data obtained from the nylon bag technique (Orskov and McDonald, (1979); Broderick and Craig, (1980); and Van Soest, (1982)). The only non-sigmoidal model is the exponential model developed by Orskov and McDonald (1979). This model has come under criticism since it produces high root mean square values when fitted to gas production data because of the assumption that after a discrete lag period, feed is fermented at a maximum rate (Beuvink and Sploestra, 1992).

Schofield et al. (1994) fit the simple exponential model which incorporated lag, logistic and both the Gompertz single and dual pool models to gas data obtained from various substrates including bacterial cellulose, alpha-cellulose and mixtures of the two purified substrates of fiber digestion. These authors found that a logistic model describing bacterial growth that incorporated the different fermentation rates of the various components in their mixed-fiber substrate most accurately described gas production. Using gas production data from the incubation of grass silages with rumen fluid, Beuvink and Kogut (1993) developed a modified Gompertz equation to describe the fermentation process. France et al. (1993) developed a complex exponential model including lag to describe the fermentation of leaves from tropical forage trees. The simple exponential model with lag (Schofield et al., 1994) is a modification of the equation of Mertens and Loften (1980). Krishnamoorthy et al. (1991, 1995) found the model to accurately reflect the gas production profiles of energy supplements and tropical feedstuffs. This model was found to accurately reflect the gas production profile of canola meal in experiment 3.

1.3 OBJECTIVES OF THE THESIS

Evaluation of enzyme and mild hydrothermal treatment of barley and canola meal was conducted with a system employing the use of IVDMD and IVTD results (Tilley and Terry, 1963; Goering and Van Soest, 1970). Further evaluation of the effects of the treatments on canola meal was conducted using the computerized *in*

vitro gas production technique (Pell and Schofield, 1993). Unfortunately, due to the large number of samples and time constraints, barley could not be evaluated to the same extent. The use of the gas production system provided information on the rumen *in vitro* fermentation characteristics of canola meal when treated with enzymes and hydrothermal processing.

The objectives of this thesis project were to:

1) Determine optimal treatment combinations of the enzymes and hydrothermal treatment using IVDMD, IVTD, ADF and NDF values as indices of the nutritive value of barley for dairy cattle.

2) Determine the effects of enzyme and hydrothermal treatment using IVDMD, IVTD, ADF and NDF values as indices of the nutritive value of canola meal.

3) Further evaluate the treatment effects on the characteristics of fermentation of canola meal using as indices: rate of gas production, length of the lag phase, the production of VFAs and their proportions.

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CHAPTER 2

2.0 THE EFFECT OF ENZYME AND MILD HYDROTHERMAL TREATMENT ON THE *IN VITRO* DIGESTIBILITY OF BARLEY GRAIN.

2.1 ABSTRACT

Barley grain is a major component of lactating dairy cattle diets in the Lower Fraser Valley region of British Columbia and much of the Pacific Northwest. The inclusion of barley increases the energy density of the ration, however, it's high fiber content has a negative impact on it's content of available energy. The objective of the present study was to evaluate the feasibility of using enzymes and mild hydrothermal treatment to improve the available energy content of barley grain. The experiment consisted of a 4 x 4 x 4 x 2 x 2 multi-factorial design which included the following treatments: heat applied at 55°C for 15, 30 or 60 min periods or not applied at all; moisture applied at 0%, 10%, 20%, or 30%; β-glucanase (*Trichoderma sp.*) applied at 0%, 0.001%, 0.01% or 0.1%; protease (*Bacillus sp.*) applied at 0% or 0.0025% and xylanase (*Trichoderma sp.*) applied at 0% or 0.001%. Samples were incubated *in vitro* to determine apparent and true digestibilities and fiber content. Nutrient composition of the barley grain was 87.3% DM and 12.7% CP, 7.32% ADF and 22.8% NDF, on a DM basis. A significant third order interaction ($P < 0.01$) on IVDMD was noted for the duration of the heat treatment, and the

addition of moisture and xylanase where treatments either had no effect or significantly decreased ($P < 0.05$) IVDMD. There was also a significant interaction ($P = 0.0001$) between the enzymes: β-glucanase, protease and xylanase where both increased and decreased IVDMD values were observed ($P < 0.05$). An interaction among the enzymes was also determined to exist for IVTD values ($P = 0.0001$) where results paralleled those of IVDMD. A quadratic effect ($P < 0.01$) of moisture on IVTD was described by $Y = 87.75 + 0.071^{*}$ moisture $- 0.00136^{*}\text{moisture}^2$. *In vitro* digestibility results were inconsistent, as some treatments increased and others decreased digestibility relative to the control. Fiber analyses reflected results obtained with *in vitro* digestibilities. ADF was significantly ($P < 0.01$) affected by the significant interaction ($P < 0.05$) between β-glucanase, protease and xylanase. There was also a significant interaction ($P < 0.05$) noted for the duration of the heat treatment, and the application of β-glucanase and protease enzymes on ADF content. Two-way interactions which were found to have a significant effect ($P < 0.05$) on the NDF content of barley included: β-glucanase and protease, β-glucanase and xylanase, protease and xylanase. The responses were variable. Xylanase was detrimental on the digestibility of barley. Results indicated the addition of protease at 0.01% or a combination of β-glucanase and protease are areas worthy of further research which may improve the nutritional value of barley grain in ruminant feeding systems.

2.2 INTRODUCTION

Barley grain is the major energy source fed to dairy cattle in the Lower Fraser Valley region of British Columbia and may comprise 50-60% of the concentrate ration (Pro Form Feeds Inc., personal communication). It's lower digestible energy content compared to corn is partly a result of high levels of ADF (9% ADF) and NDF (21% NDF) which may limit it's performance in dairy cattle diets.

The barley kernel consists of a hull, endosperm and embryonic germ layer. The hull contains the majority of the fiber. The endosperm is made up of an aleurone layer, starchy endosperm and a depleted cell layer, the latter being of little nutritional value to the ruminant animal (Newman and McGuire, 1985). The aleurone layer is primarily composed arabinoxylans and β-glucans. (Newman and McGuire, 1985).

It is well established that β-glucans and arabinoxylans, when fed to monogastrics, exhibit anti-nutritional effects as these animals do not possess the enzymes to break them down (Bedford, 1993). Thus, supplementation of exogenous enzymes to monogastric diets is increasing in order to reduce the effects of these non starch polysaccharides (NSP). This has led to increased levels of inclusion of cereals, such as barley and wheat, in poultry and swine diets without comprimising animal performance. These NSPs rarely pose a problem to ruminant animals because rumen microbes possess the enzymes to assist in their digestion. β-glucans, for example, are 98% rumen degradable (Engstrom et al., 1992). However,

in high producing cows, feed intake and feed passage rate may be increased to such an extent that a reduction in the digestion of specific feed components may occur.

The successful application of feed enzymes to monogastric feeding regimes has renewed interest in the possible application of these feed additives to ruminant feeding systems. Research into the addition of exogenous enzymes to concentrates that are fed to ruminants has yielded variable results (refer to Chap. 1, sect. 1.1.4) (Beauchemin and Rode, 1996). Enzyme composites containing a spectrum of different enzymes, such as those with proteolytic, amylolytic and fibrolytic activities, elicited positive responses in terms of DMI and / or ADG and / or feed efficiencies when added to high grain rations (Burroughs et al., 1960; Clark et al., 1961; Rovics and Ely, 1962 Boyles et al., 1992; Weichenthal et al., 1996). In contrast, a lack of response in DMI, ADG or feed efficiency was reported in other studies which investigated the effects of multi-enzyme preparations added to a variety of concentrates (Ward et al., 1960; Wing and Wilcox, 1960; Richardson et al., 1990; Perry et al., 1966). Barley grain, in particular, has been the focus of several research experiments. In a study where steers were fed a 65% barley grain diet supplemented with proteolytic and amylolytic enzymes, there was a noted improvement in feed efficiency (Kercher, 1966). When steers were fed a barley grain diet supplemented with xylanase, there were improvements in DMI, ADG and feed efficiency (Beauchemin et al., 1996).

The application of exogenous enzymes to barley as a means of improving it's digestibility may impact digestion in several ways. Firstly, increasing barley digestibility may positively affect the production of microbial protein by providing greater amounts of available carbohydrates as an energy source. The dairy farmers located in the Lower Fraser Valley region of British Columbia rely on high quality grass silage as the main forage source fed to their lactating dairy herds. These silages tend to be high in soluble nitrogen (von Keyserlingk et al., 1996). This large supply of soluble nitrogen to the rumen must be balanced with a complementary supply of readily available carbohydrates. This is important in order to optimize the use of rumen degradable nitrogen and energy sources for the production of microbial protein (Krishnamoorthy et al., 1991) and to maximize the supply of microbial protein to the intestine (Stokes et al., 1991). Maintenance of this balance is also essential in order to reduce the excretion of excess nitrogen and maximize feed utilization (Huntington, 1995).

Secondly, increasing the ruminal degradability of barley may improve total tract digestibility. Although ruminal acidosis is associated with high ruminally digestible cereal grains, Lenventini et al., (1990) found that the effect of low rumen pH was minimized with the addition of a buffer to the ration. Both a low rumen pH and a shift in the rumen microbial population may contribute to reduced fiber digestion. The addition of fibrolytic enzymes to particular diets may enable this disadvantage to be overcome.

The digestibility of barley may be improved by increasing the digestibility of the structural carbohydrates, the hull fraction in particular, and by enhancing the access by rumen microbes to the starch granules that are embedded within the protein matrix. Research is warranted in order to further improve the digestibility (Hunt et al., 1995) and subsequently the digestible energy content of barley. The Alberta Barley Commission (1995) reports that for every 1% increase in improvement in the feed efficiency of barley, the livestock industry will accrue 10 million dollars in benefits. Therefore, the objective of this study was to investigate the feasibility of using exogenous enzymes applied in conjunction with mild hydrothermal treatment in order to increase the nutritive value of barley fed to dairy cattle.

2.3 MATERIALS AND METHODS

2.3.1 Feed Treatments

The experiment consisted of a completely randomized 4 x 4 x 4 x 2 x 2 multi-factorial design to evaluate the effects of enzymes and mild hydrothermal treatment on barley grain using rumen *in vitro* dry matter disappearance (IVDMD) and rumen *in vitro* true digestibility (IVTD). Barley feed treatments under investigation were the application of β-glucanase (*Trichoderma sp*) at 0%, 0.001%, 0.01% or 0.1%, protease (*Bacillus sp.*) at 0% or 0.0025%, xylanase (*Trichoderma sp.*) at 0% or 0.001%, the addition of moisture at 0%, 10%, 20% or 30% and the application of heat at 55°C for 0, 15, 30 or 60 min periods (Table 2.1). Treatment levels were

primarily based on the recommendations of the enzyme supplier, Finn Feeds Intl. (Wiltshire, U.K). Enzymes were not pure cultures and substrate activity of the enzymes may be found in Appendix 6.1.

2.3.2 Processing Samples

Barley grain, ground through a 3 mm screen was obtained from Pro Form Feeds Inc. located at Chilliwack, B.C. Feed samples were weighed and placed in a Hobart mixer (model A-200, Hobart Mfg. Co. Ltd., Don Mills, Ont., Canada) at which time moisture and / or enzymes were added during mixing as required for each treatment. Moisture was applied with a manual sprayer in order to facilitate equal distribution over the entire feed sample. Enzymes were diluted in 5 mL of distilled water and applied (% vol / wt) via syringe to the feed. Treatments were thoroughly mixed for an additional 5 min at which time a 400 g sample of the feed was placed within a 4 L glass vessel and sealed for further processing in a simulated hydrothermal cooker.

A laboratory incubator was adapted to simulate a hydrothermal cooker by fitting rollers on which the 4 L sealed glass vessels containing the treated material were placed (Figure 2.1). The rotation of the vessels was set at 15 rpm to simulate commercial hydrothermal conditions. (Pro Form Feeds Inc., personal communications). The incubator and vessels were both preheated and maintained at 57°C ± 2°C. Feed samples treated with 20% or 30% moisture levels took

approximately 7 min to attain 55°C, whereas all others required approximately 5 min.

Following removal of the samples from the cooker, individual treatments were spread out (2 cm depth) on large trays and air dried at ambient temperature for 3 h. Drying was facilitated with a large fan which simulated the forced air dryers used by feed manufacturing facilities. Samples were then sealed in plastic bags and stored in a chest freezer at -10°C for subsequent laboratory analyses.

2.3.3 Chemical Analyses

A representative sample from each feed treatment was collected and ground through a 1 mm screen for IVDMD and IVTD using the Ankom *in vitro* system (Daisy 100, Ankom co., Fairport, NY) and for chemical analyses. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined on all samples, in duplicate, using the filter bag technique (Komarek et al., 1994) and the Ankom fiber analyzer (#F200, Ankom Co., Fairport, NY). Filter bags were of synthetic non woven filter material with a pore size of 40 microns (#F57, Ankom Co., Fairport, NY). The NDF procedure included the use of a heat stable alpha-amylase (#FAA, Ankom Co., Fairport, NY) as suggested by Van Soest et al., (1991) for use in feeds containing high amounts of starch. Residual dry matter (DM) determinations were conducted by drying a representative 1 g sample at 100°C in a forced air oven until constant weight was achieved.

2.3.4 Animals and Feeding

Two Holstein cows fitted with ruminal cannula were used as donor animals for rumen fluid. The cows were fed a 60% grass hay and 40% grain diet (Appendix 6.2) twice daily at 07:30h and 16:00h. The animals were housed in free stalls for the duration of the experiment. Animals were cared for according to the guidelines set forth by the Canadian Council of Animal Care (1993).

2.3.5 Rumen Fluid Inoculum

Rumen fluid was collected 3 h post feeding from both animals and the collections pooled. Rumen fluid and solids from the fibrous mat were collected into a prewarmed and airtight container for transport to the laboratory while maintaining anaerobic conditions as much as possible. All equipment was prewarmed to 39°C. Rumen fluid was blended in a Waring blender at maximum speed for 2, 30 sec periods (Fay et al., 1980). The homogenate was then filtered through 5-8 mm of glass wool and 4 layers of cheese cloth and the filtrate used as inoculum when mixed with mineral and buffer solutions (Goering and Van Soest, 1970).

2.3.6 *In Vitro* Procedure

IVDMD was determined according to the first step of the Tilley and Terry (1963) rumen *in vitro* digestion procedure. An *in-vitro* apparatus (IV100, Daisy II, Ankom Co., Fairport, NY) was used to determine IVDMD of the samples (Ankom Co., Fairport, NY; Tilley and Terry, 1963). The system was maintained at 39.5°C and consisted of four 4 L reaction vessels which rotated at 1 rpm to simulate rumen conditions.

Quadruplicate 0.5 g samples were weighed out into filter bags (#F56, Ankom Co., Fairport, NY) and heat sealed. Sixteen samples, grouped into reaction vessels according to enzyme treatment, were placed in each run. After a 48 h incubation, samples were removed, washed in distilled H_2O and dried at 60°C until constant weight was achieved. IVDMD was obtained by difference in sample weights. Bag and samples were then subjected to NDF analysis in order to obtain true digestibility by difference from the original sample weight (Goering and Van Soest, 1970).

2.3.7 Statistical Analysis

Statistical analyses were conducted using the General Linear Model (GLM) procedure of Statistical Analysis System (SAS Institute Inc., 1990) using the program listed in Appendix 6.3, which also includes the model statement. In the present study, it was assumed the mean square error of the highest order interaction was representative of the experimental error (Cochran and Cox, 1950).

Mean separations were obtained using the least square means for IVDMD, IVTD, ADF and NDF measurements.

2.4 RESULTS AND DISCUSSION:

Dry matter (DM) content of the untreated barley grain sample was 87.3% and the level of crude protein (CP) was 12.7% of DM. Acid detergent fiber (ADF) was 7.32% of DM and neutral detergent fiber (NDF) was 22.8% as a percent of DM. The barley used in this experiment had similar nutrient composition, with the exception of NDF, to the values cited by NRC (1989) for barley grain which has a typical DM content of 88%, CP content of 13.5%, ADF and NDF content of 7% and 19%, respectively.

2.4.1 IVDMD

There was a significant three way interaction ($P < 0.05$) noted for duration of heat treatment, moisture and xylanase application (Figure 2.2). However, no treatment significantly increased IVDMD ($P < 0.05$) when compared to the untreated sample (Table 2.2). All treatment means were either similar or lower than the IVDMD value of 81.8% for the control. A previous study reported a comparable DM loss for broken barley following a 48 h ruminal nylon bag incubation of 85% (Nordin and Campling, 1976). In the present study, the lowest IVDMD value was 79.2% which was obtained with the application of 30 min of heat at 55°C and 0.001% xylanase.

From Table 2.2, it is apparent the majority of treatment combinations decreased IVDMD relative to the control. Results presented in Table 2.2 do not support the suggestion that moisture may play a role in determining the efficacy of enzymes (Beauchemin and Rode, 1996). The results of the present experiment are difficult to interpret due to the large number of interactions present.

Figure 2.3 illustrates the significant interaction ($P = 0.0001$) noted for the addition of β-glucanase, protease and xylanase. The addition of xylanase decreased IVDMD with the exception of the combination of both 0.0025% protease and 0.001% xylanase where IVDMD increased from 82.4% to 82.9%. The addition of β-glucanase alone did not significantly ($P < 0.05$) affect IVDMD results. The addition of β-glucanase in combination with protease significantly increased IVDMD relative to the untreated sample ($P < 0.05$). The addition of 0.1% β-glucanase and 0.0025% protease increased IVDMD from 82.40% to 84.42% (Table 2.3). Within each level of β-glucanase application however, the addition of protease did not further increase IVDMD ($P < 0.05$). The addition of protease alone also did not have a positive effect on IVDMD.

The addition of protease has been previously shown to increase the degradability of barley starch following a 24 h incubation (McAllister et al., 1993). These results led the authors to speculate that structural carbohydrates may also play a role in the limited digestion of barley by rumen microbes.

Starch granules are storage polysaccharides locked within a protein matrix (Rooney and Pfluglder, 1986). McAllister et al., (1993) discovered through the use of

electron microscopy that although grinding cereal grains exposed the endosperm to enzymatic attack, the starch granules remained embedded within the protein matrix. The difference in the degradability of cereals may be partly attributed to the presence of a protein matrix which surrounds the starch granules (McAllister et al., 1990a, b, c). Starch structure and composition, in addition to it's interaction with protein, is important in determining it's digestibility. The efficiency of microbial colonization has also been suggested as a factor in determining the rate and extent of degradation in the rumen (McAllister, 1990b). Enhancing the microbial colonization of feed particles has been suggested as a means of improving fiber digestibility in the rumen (Cheng et al., 1995). Unfortunately, this was not measured in the present experiment.

Waldo (1973) reported that barley starch was 94% ruminally degradable. This value is higher than that reported by Herrera-Saldana et al. (1990), who found that barley starch was 91% rumen degradable. These same authors determined that barley grain was 78% ruminally degradable, assuming a 6% h^{-1} rate of passage,. This latter value is comparable to the 81% IVDMD value obtained in the present study following a 48 h rumen *in vitro* incubation. Although barley is considered to be intermediately ranked with regards to starch and DM degradabilities when compared to other cereal grains such as oats, wheat, corn and milo; digestibility experiments often do not recognize the poor digestibility of the hull fraction (Herrera-Saldana et al., 1990). For example, only 50.4% of the barley hull disappeared following a 10 h incubation in the rumen (Aronen et al., 1991). In feeding a high producing dairy cow

it is important to consider the faster rate of rumen passage which may negatively influence digestibility.

As previously mentioned, results from the three way interactions are difficult to interpret. Although significantly lower ($P < 0.05$) IVDMD values were obtained with a variety of enzyme combinations, a clear pattern is lacking. The greatest decrease in IVDMD was obtained with the combination of 0.001% β-glucanase and 0.001% xylanase which resulted in a 6.5 digestibility unit decrease in IVDMD when compared to the control. β-glucanase applied at 0.01% alone resulted in a significant decrease of 5.0 digestibility units in IVDMD from 82.4% to 77.4%; whereas the lowest and highest level of application of β-glucanase did not affect IVDMD ($P < 0.05$) (Table 2.3).

Although the results of this study are not as definitive as the results of previous studies, it appears there may be an optimal level of protease application or yet undefined combination of β-glucanase and protease to barley grain where there should be a response in terms of digestibility. Past research has shown that negative responses are possible if the enzyme level is sub-optimal (Krause et al., 1996 cited by Beauchemin and Rode, 1996). In a study conducted with steers fed an enzyme supplemented barley ration, a quadratic response was noted in terms of ADG (Rode and Beauchemin, unpublished data; cited by Beauchemin and Rode, 1996). A second study found a linear response in ADG from steers fed a tempered rolled barley grain diet supplemented with xylanase (Iwaasa et al., unpublished

data; cited by Beauchemin and Rode, 1996). This may also be true in the present study where some enzyme treatments decreased the rumen *in vitro* degradability.

When Krause et al., (1996) (cited by Beauchemin and Rode, 1996) investigated the *in situ* degradability of barley in steers fed an enzyme supplemented barley straw or silage diet the authors reported increased degradability of barley grain only with the silage based diets. This led to speculation that enzyme-substrate binding may play a role in improving the observed digestibility in the silage diets (Beauchemin et al., 1996). Enzyme substrate binding may also play a role in the current study where results are inconsistent and trends cannot be established. A combination of a number of factors may provide an environment conducive or detrimental to enzyme action.

2.4.2 *IVTD*

A quadratic relationship ($P < 0.01$) was found between the application of moisture and the IVTD of barley grain described by the following equation: $Y = 87.75 + 0.071$*moisture level $- 0.00136$*moisture level^2 (Figure 2.4). Means from treatment with 20% and 30% moisture were significantly higher ($P < 0.01$) than the control but were not different from each other (Table 2.4). The optimal level of moisture application in order to maximize IVTD, derived from the regression equation was determined to be 26% with a resulting IVTD of 88.7%; a small increase of 0.9% relative to the control. Moisture treatment of cereal grains has generally not produced a great response when applied in the absence of heat (Tait

and Beames, 1988). However, soaking cereal has been suggested to release endogenous enzymes contained within the grain (Newman, 1994). As previously mentioned, moisture may also have an affect on enzyme activity (Beauchemin and Rode, 1996).

There was a significant three way interaction ($P = 0.0001$) noted among the addition of the enzymes: β-glucanase, protease and xylanase (Figure 2.5). These results are similar to those obtained with IVDMD determinations. None of the enzyme combinations significantly increased ($P < 0.05$) the IVTD of barley grain from the 89.4% IVTD value obtained for the control (Table 2.3). IVTD did not reflect a positive response of barley grain to the addition of either xylanase or protease. Treatment of barley grain with xylanase either decreased or had no effect on IVTD relative to the control. The lowest IVTD value (86.8%) was obtained when barley grain was treated with the combination of 0.1% β-glucanase, 0.0025% protease and 0.001% xylanase. This same treatment also resulted in one of the lowest IVDMD values. Positive results previously observed with the addition of xylanase to barley were mainly attributable to it's high fiber content (Beauchemin and Rode, 1996).

Hristov et al. (unpublished data; cited by Beauchemin and Rode, 1996) compared the application of cellulolytic and xylanolytic enzymes to barley and corn and found that reducing sugars were only released in barley. This suggests xylanase is an effective enzyme for use with barley. Further investigation into whether fibrolytic enzyme treatment of a barley or corn based ration increased the digestible energy content of the feed was conducted by Beauchemin and Rode

(1996). These authors found that again only barley responded to the enzyme supplementation. The application of high levels of xylanase increased DMI, ADG and consequently the feed conversion ratio (FCR) of steers fed a high barley diet. The positive response was suggested to occur as a result of improved total tract digestibility of barley. The lack of response of corn was attributed to its lower fiber content.

In general, IVTD values were less responsive to enzyme treatment than the IVDMD values. This is reflected by fewer differences between means in addition to the reduced spread of means and lower pooled standard error in the IVTD results. The pooled standard error (SEp) for the IVDMD means for the interaction between β-glucanase, protease and xylanase was 0.40, whereas the SEp for true digestibility was 0.26. The same third order interaction between enzymes was detected in the evaluation of IVTD, however, the spread of means was reduced from 8.3 percentage units with IVDMD and a reduction of 3.0 percentage units with IVTD. The large spread in values obtained with IVDMD results may reflect microbial contamination of the samples. Differences that are present at this stage of the digestion process may not be detected with a subsequent IVTD determination because this latter procedure eliminates all cell solubles including all undigested microbial cell components (Van Soest, 1982).

The results of the present experiment suggest that IVTD may be a better indication of the effect of enzyme action as IVDMD results may be masked by other compounding factors. Cheng et al., (1995) indicated that dietary β-glucanase

supplementation would benefit digestion by degrading the cell wall components of barley. Previous research has shown that the endosperm walls are degraded by β-glucanase however, due to it's complex nature, a multi-enzyme preparation is more effective (Chesson, 1993). This explanation however, may be offered for IVDMD and IVTD values for only a selection of enzyme treatments, most notably those in combination with 0.01% β-glucanase.

2.3.3 *ADF*

There was a significant interaction ($P < 0.05$) shown in Figure 2.6, noted for β-glucanase, protease and xylanase which was also previously determined to exist with IVDMD and IVTD determinations. Table 2.5 shows that none of the treatments significantly decreased the ADF fraction of barley ($P < 0.05$). The ADF content of the control sample was 8.2% on a DM basis. A significant increase ($P < 0.05$) in ADF was found with a number of treatments. The application of xylanase either had no effect on the ADF content or increased it's content relative to the control. The largest increase in ADF content (to 9.3 percentage units) was obtained with the application of 0.01% β-glucanase and parallels the results of a low IVDMD value observed for the same treatment. Many of the treatments with high ADF values have decreased IVDMD and IVTD. A shift towards more soluble components in the feed subjected to particular treatments, would result in higher ADF values. For example, an increase in the proportion of the soluble starch or other feed components would be reflected

in increased ADF values. The enzyme hydrolysis of high molecular weight materials usually results in an increase in the solubility of such components (Wiseman, 1975).

A significant interaction on ADF content ($P < 0.05$) was also noted for: duration of heat treatment, β-glucanase and protease (Figure 2.7). It is of interest that this tended to occur in samples which had higher ADF values compared to the control (Table 2.6). The untreated sample was not significantly different from the sample with the lowest ADF content (8.1% of DM) obtained by treating barley with heat for 30 min and applying 0.001% β-glucanase. The largest increase in ADF content was found when barley was treated with heat for 15 min, 0.1% β-glucanase and 0.0025% protease.

It is of particular interest to note that the interaction between the 3 enzymes was detected in IVDMD, IVTD and ADF measurements. In general, the ADF values increased as digestibility measurements decreased. This is in agreement with the negative association of fiber with digestibility (Van Soest, 1982). It has also been reported that enzymes may not affect the ADF fraction but may elicit their effects by altering the hemicellulose fraction, which would be reflected in the NDF residue (Gwayumba et al., 1995).

2.4.4 *NDF*

A significant interaction was found to exist ($P < 0.01$) between he enzymes β-glucanase and protease (Figure 2.8). Within this interaction, NDF content of the control was 21.8% of DM, which was the lowest of all treatment means. The following three treatment combinations significantly increased ($P < 0.05$) NDF (Table

2.7): 0.0025% protease; 0.001% β-glucanase and 0.0025% protease; and 0.01% β-glucanase.

Another significant second order interaction ($P < 0.01$) was found between β-glucanase and xylanase (Figure 2.9). Treatment means are presented in Table 2.8. The addition of 0.001% β-glucanase significantly increased ($P < 0.05$) NDF from 23.0% to 24.5% as a percentage of DM. In contrast, the application of the highest concentration of β-glucanase significantly decreased ($P < 0.05$) NDF from 23.0% to 21.8%. These results do not parallel those of IVDMD where it was determined that the addition of both the lowest and highest levels of β-glucanase did not significantly affect IVDMD ($P < 0.05$). The observed decrease in NDF content with the application of 0.1% β-glucanase may be explained by the breakdown of cell wall components which may contribute to the NDF fraction. It appears that the addition of β-glucanase may have had an affect on the hemicellulosic fraction of barley and not the ADF fraction as indicated by the interactions investigated.

There was also a significant interaction ($P = 0.01$) found between protease and xylanase which is illustrated in Figure 2.10. Within this interaction, it was determined that the treatment of barley with 0.001% xylanase significantly decreased ($P < 0.05$) the NDF content from 23.1% to 21.9% of the DM content of barley, whereas all other treatment means were similar to the control (Table 2.9). This specific result was reflected in a slight increase in IVTD but not with a higher IVDMD value.

Results from fiber analyses are difficult to interpret, however, they reflect the lack of response obtained with IVDMD and IVTD determinations. The variation in NDF was much greater than the variation observed with ADF. Consequently, fewer differences were observed between NDF determinations. It has been shown that the high amount of starch in cereal grains interferes with the NDF filtration process and may contaminate the sample (Van Soest et al., 1991). The present study incorporated the use of alpha-amylase in the NDF procedure in order to reduce the interference from starch however, it is possible that the elimination of the interference from starch was not achieved.

2.5 CONCLUSION

Inconclusive results from the present study and inconsistencies from other studies exemplify the difficulties in implementing exogenous enzyme applications in the ruminant feed industry. The present study revealed that further investigation is necessary should there be an interest pursuing the application of enzymes to barley. A number of treatments suggested areas of further research. In terms of digestibility, results of the present study indicated the application of xylanase to barley was detrimental. The application of protease, β-glucanase or a combination of these two enzymes appeared to have the greatest positive response. Fiber analyses reflected similar results that were obtained with the digestibility measurements. The economics of implementing any of these treatments was not attempted in this study. Production trials are necessary to establish a production response. The degree of

response in production will dictate further research and any potential applications of selected enzymes and their concentrations to barley.

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Table 2.1. Treatments investigated in order to determine the effects of exogenous enzyme application with and without added moisture and heat on rumen *in vitro* dry matter disappearance and *in vitro* true digestibility of barley grain.

Factor	Level of application
Heat[1]	control (0), 15, 30, 60 min
Moisture[2]	0%, 10%, 20%, 30%
β-glucanase[3, 4]	0%, 0.001%, 0.01%, 0.1%
Protease[3, 5]	0%, 0.0025%
Xylanase[3, 6]	0%, 0.001%

[1] Applied at 55°C.
[2] Applied on a % vol / wt basis.
[3] Supplied by Finn Feeds International Ltd., Wiltshire, U.K.
[4] Avizyme 1110
[5] Heptex sb-protease
[6] Avizyme 1310

Table 2.2. The effect of the duration of heat treatment and the application of moisture and xylanase on the 48 h *in vitro* dry matter disappearance of barley grain (DM basis).

	Feed Treatment		
Duration of heat treatment[1] (min)	Moisture[2] (%)	Xylanase[2] (%)	IVDMD[3] (%)
0	0	0	81.8^{cdefg}
0	0	0.001	79.3^{ab}
0	10	0	82.2^{efg}
0	10	0.001	81.8^{cdefg}
0	20	0	82.2^{efg}
0	20	0.001	80.0^{ab}
0	30	0	82.5^{fg}
0	30	0.001	80.5^{abcd}
15	0	0	82.1^{defg}
15	0	0.001	79.5^{ab}
15	10	0	82.0^{cdefg}
15	10	0.001	79.6^{ab}
15	20	0	82.1^{efg}
15	20	0.001	80.0^{ab}
15	30	0	82.3^{efg}
15	30	0.001	80.4^{abc}
30	0	0	81.8^{cdefg}
30	0	0.001	79.2^{a}
30	10	0	81.9^{cdefg}
30	10	0.001	79.7^{ab}
30	20	0	80.9^{bcdef}
30	20	0.001	80.3^{abc}
30	30	0	82.2^{efg}
30	30	0.001	80.8^{abcde}
60	0	0	79.9^{ab}
60	0	0.001	80.9^{bcdef}
60	10	0	81.5^{cdefg}
60	10	0.001	80.4^{abc}
60	20	0	82.6^{g}
60	20	0.001	79.3^{ab}
60	30	0	82.8^{g}
60	30	0.001	80.5^{abc}
S.E.M.			0.56

[1] Heat was applied at 55°C.
[2] Applied on a % vol / wt basis.
[3] IVDMD, *in vitro* dry matter disappearance.
[4] S.E.M., pooled standard error of the mean.
[a-g] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 2.3. The effect of β-glucanase, protease and xylanase application on the 48 h *in vitro* dry matter disappearance and *in vitro* true digestibility of barley grain (DM basis).

Enzyme treatment[1]				
β-Glucanase (%)	Protease (%)	Xylanase (%)	IVDMD[2] (%)	IVTD[3] (%)
0	0	0	82.4^{def}	89.4^{e}
0	0	0.001	82.6^{def}	88.6^{bcd}
0	0.0025	0	79.3^{bc}	87.2^{a}
0	0.0025	0.001	83.9^{gh}	89.0^{cde}
0.001	0	0	83.1^{efg}	89.1^{cde}
0.001	0	0.001	76.1^{a}	87.0^{a}
0.001	0.0025	0	82.7^{ef}	88.6^{bcd}
0.001	0.0025	0.001	81.5^{d}	88.3^{bc}
0.01	0	0	77.4^{b}	88.2^{b}
0.01	0	0.001	78.4b	87.0^{a}
0.01	0.0025	0.001	80.1^{c}	88.1^{b}
0.1	0	0	83.6^{fgh}	89.1^{de}
0.1	0	0.001	79.9^{c}	89.0^{cd}
0.1	0.0025	0	84.4^{h}	89.3^{de}
0.1	0.0025	0.001	78.5^{b}	86.8^{a}
S.E.M.[4]			0.40	0.26

[1] Applied on a % vol / wt basis.
[2] IVDMD, *in vitro* dry matter disappearance.
[3] IVTD, *in vitro* true digestibility.
[4] S.E.M., pooled standard error of the mean.
[a-h] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 2.4. The effect of moisture level on the 48 h *in vitro* true digestibility of barley grain (DM basis).

Moisture[1] (%)	IVTD[2] (%)
0	87.8[a]
10	88.3[ab]
20	88.6[b]
30	88.6[b]
S.E.M.[2]	0.14

[1] Applied on a % vol / wt basis.
[2] IVTD, *in vitro* true digestibility.
[3] S.E.M., pooled standard error of the mean.
[a,b] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 2.5. The effect of β-glucanase, protease and xylanase application on the ADF fraction of barley grain (DM basis).

Enzyme treatment[1]			
β-Glucanase (%)	Protease (%)	Xylanase (%)	ADF[2] (%)
0	0	0	8.2[a]
0	0	0.001	8.4[ab]
0	0.0025	0	8.4[ab]
0	0.0025	0.001	9.1[de]
0.001	0	0	8.5[abc]
0.001	0	0.001	8.2[a]
0.001	0.0025	0	8.4[ab]
0.001	0.0025	0.001	9.0[cde]
0.01	0	0	9.3[e]
0.01	0	0.001	9.1[de]
0.01	0.0025	0	8.6[abc]
0.01	0.0025	0.001	8.5[ab]
0.1	0	0	8.3[a]
0.1	0	0.001	8.8[bcd]
0.1	0.0025	0	9.1[de]
0.1	0.0025	0.001	9.0[de]
S.E.M.[3]			0.15

[1] Applied on a % vol / wt basis.
[2] ADF, acid detergent fiber.
[3] S.E.M., pooled standard error of the mean.
[a-e] Means in a column with different superscripts are significantly different *($P < 0.05$).*

Table 2.6. The effect of heat treatment and the application of β-glucanase and xylanase on the ADF fraction of barley grain (DM basis).

	Enzyme treatment[1]		
Duration of heat treatment[2] (min)	β-Glucanase (%)	Protease (%)	ADF[3] (%)
0	0	0	8.3^{abcd}
0	0	0.0025	$8.7^{bcdefghij}$
0	0.001	0	8.1^{abc}
0	0.001	0.0025	8.2^{abc}
0	0.01	0	$8.9^{efghijk}$
0	0.01	0.0025	8.4^{abcdef}
0	0.1	0	$8.7^{cdefghij}$
0	0.1	0.0025	$8.7^{abcdefghij}$
15	0	0	8.3^{abcd}
15	0	0.0025	$8.5^{abcdefghi}$
15	0.001	0	$8.4^{abcdefgh}$
15	0.001	0.0025	$8.5^{abcdefgh}$
15	0.01	0	9.2^{jkl}
15	0.01	0.0025	$8.7^{cdefghij}$
15	0.1	0	8.1^{ab}
15	0.1	0.0025	9.6^{l}
30	0	0	8.3^{abcde}
30	0	0.0025	$8.8^{defghijk}$
30	0.001	0	8.0^{a}
30	0.001	0.0025	9.1^{jkl}
30	0.01	0	9.4^{kl}
30	0.01	0.0025	8.3^{abcd}
30	0.1	0	$8.8^{defghijk}$
30	0.1	0.0025	9.1^{ijkl}
60	0	0	8.3^{abcd}
60	0	0.0025	9.0^{hijkl}
60	0.001	0	$8.8^{defghijk}$
60	0.001	0.0025	9.0^{ghijkl}
60	0.01	0	9.2^{jkl}
60	0.01	0.0025	$8.8^{cdefghij}$
60	0.1	0	$8.4^{abcdefg}$
60	0.1	0.0025	$9.0^{fghijkl}$
S.E.M.[4]			0.21

[1] Applied on a % vol / wt basis.
[2].Heat was applied at 55°C.
[3] ADF, acid detergent fiber.
[4] S.E.M., pooled standard error of the mean.
[a-l] Means in a column with different superscripts are significantly different *($P < 0.05$).*

Table 2.7. The effect of β-glucanase and protease application on the NDF content of barley grain (DM basis).

Enzyme treatment[1]		
β-Glucanase (%)	Protease (%)	NDF[2] (%)
0	0	21.8[a]
0	0.0025	23.2[bc]
0.001	0	22.8[abc]
0.001	0.0025	23.7[c]
0.01	0	23.4[c]
0.01	0.0025	22.1[ab]
0.1	0	22.0[a]
0.1	0.0025	22.1[a]
S.E.M.[3]		0.37

[1] Applied on a % vol / wt basis.
[2] NDF, neutral detergent fiber.
[3] S.E.M., pooled standard error of the mean.
[a-c] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 2.8. The effect of β-glucanase and xylanase application on the NDF fraction of barley grain (DM basis).

Enzyme treatment[1]		
β-Glucanase (%)	Xylanase (%)	NDF[2] (%)
0	0	23.0^{bc}
0	0.001	22.1^{ab}
0.001	0	24.5^{d}
0.001	0.001	22.0^{ab}
0.01	0	22.4^{abc}
0.01	0.001	23.2^{c}
0.1	0	21.8^{a}
0.1	0.001	22.2^{abc}
S.E.M.[3]		0.37

[1] Applied on a % vol / wt basis.
[2] NDF, neutral detergent fiber.
[3] S.E.M., pooled standard error of the mean.
[a-c] Means in a column with different superscripts are significantly different *($P < 0.05$).*

Table 2.9. The effect of protease and xylanase application on the NDF content of barley grain (DM basis).

Enzyme treatment[1]		
Protease (%)	Xylanase (%)	NDF[2] (%)
0	0	23.1[b]
0	0.001	21.9[a]
0.0025	0	22.7[b]
0.0025	0.001	22.9[b]
S.E.M.[3]		0.26

[1] Applied on a % vol / wt basis.
[2] NDF, neutral detergent fiber.
[3] S.E.M., pooled standard error of the mean.
[a,b] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Figure 2.1. Laboratory scale hydrothermal cooker.

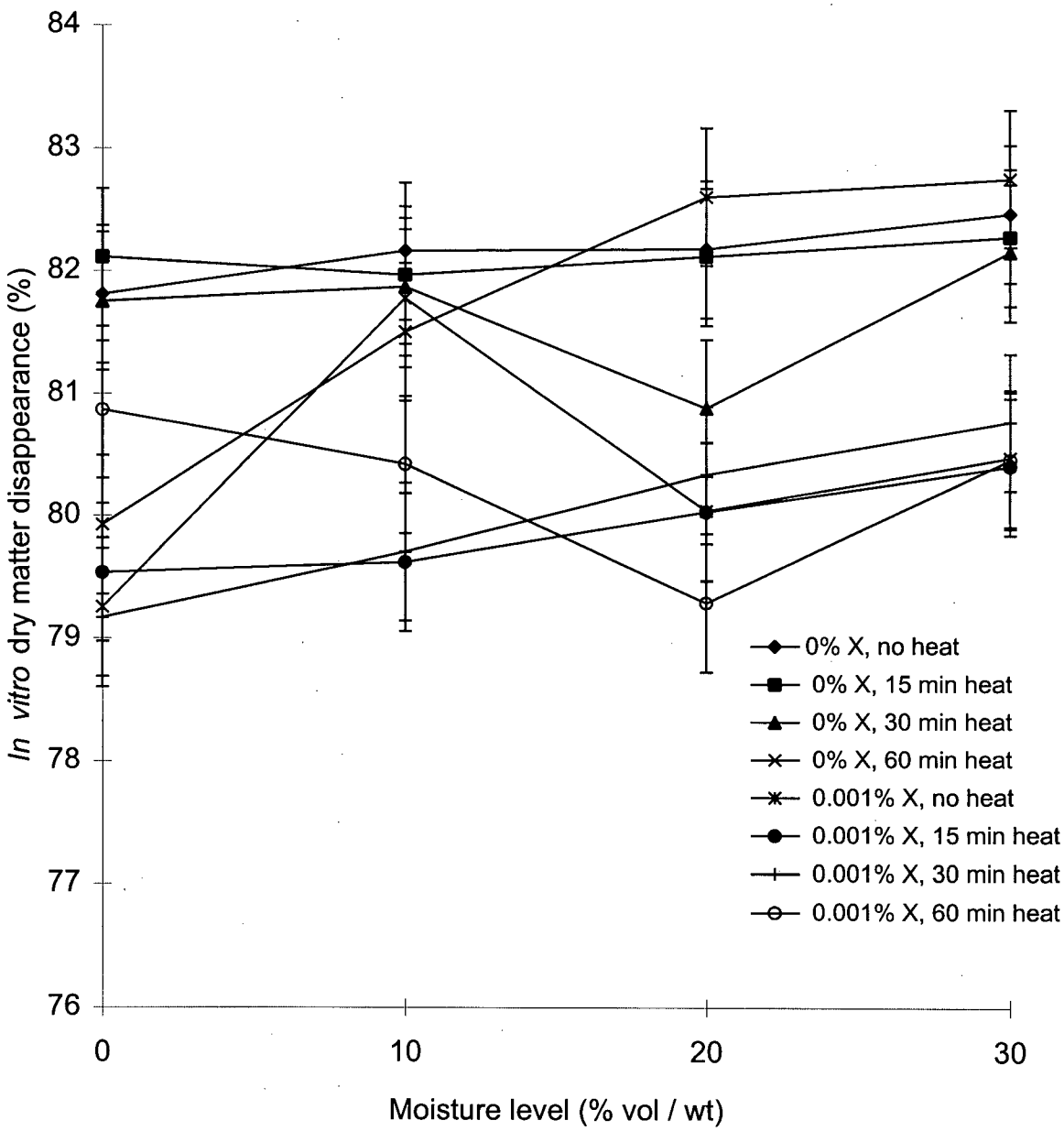

Figure 2.2. The effect of the addition of moisture, xylanase (X) (% vol / wt) and duration of the heat treatment (55°C) on the *in vitro* dry matter disappearance of barley grain.

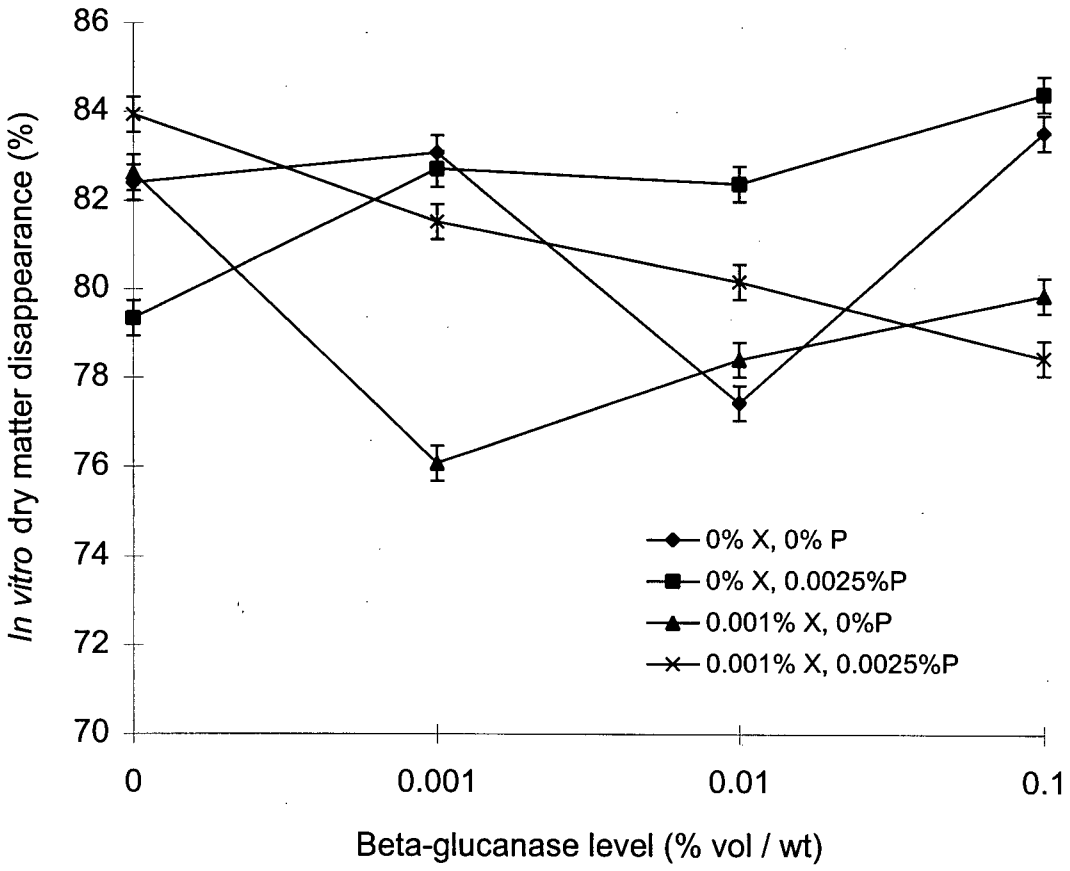

Figure 2.3. The effect of beta-glucanase, xylanase (X) and protease (P) application (% vol / wt) on the *in vitro* dry matter disappearance of barley grain.

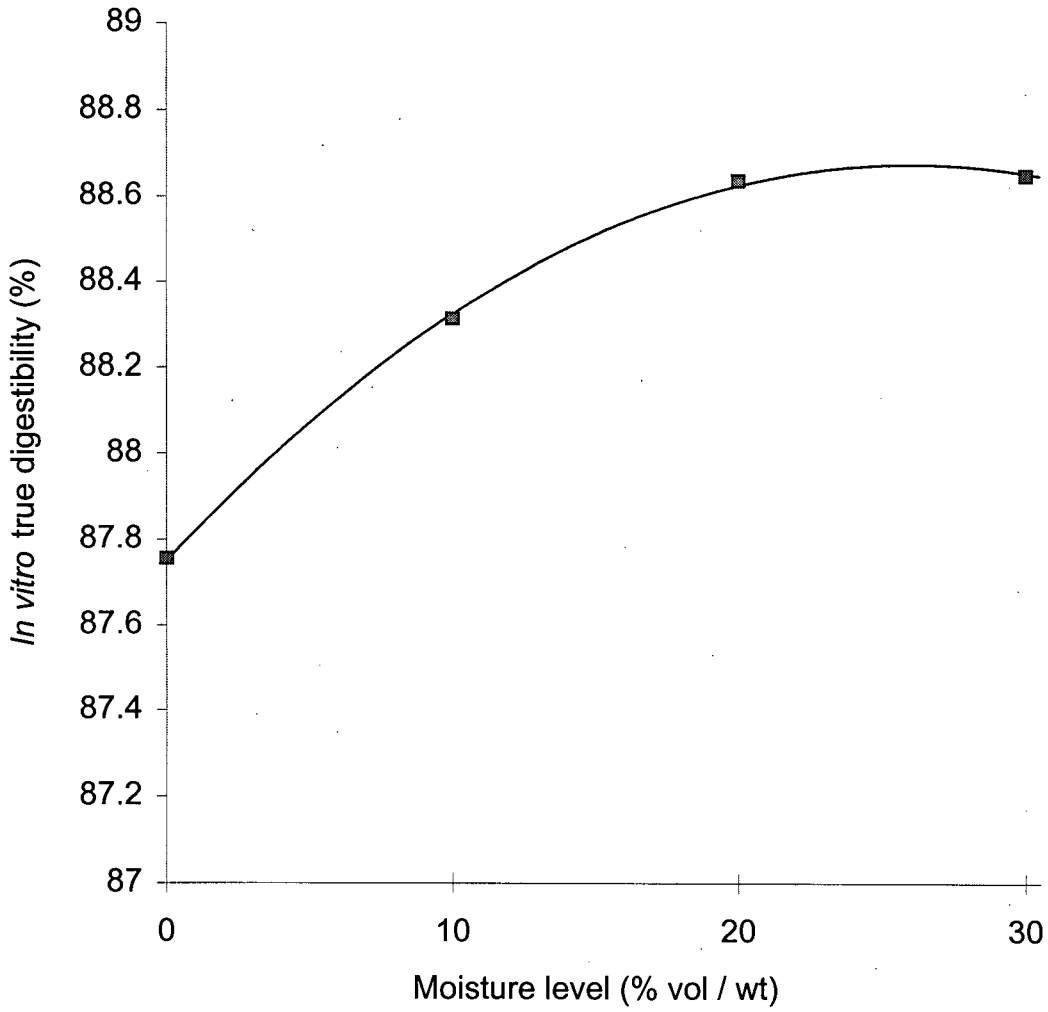

Figure 2.4. Quadratic relationship between level of moisture and *in vitro* true digestibility of barley grain (Y = 87.75 + 0.071 * moisture level - 0.0014 * moisture level ^{2}: n = 4, R^2 = 0.998, S.E. = 0.018).

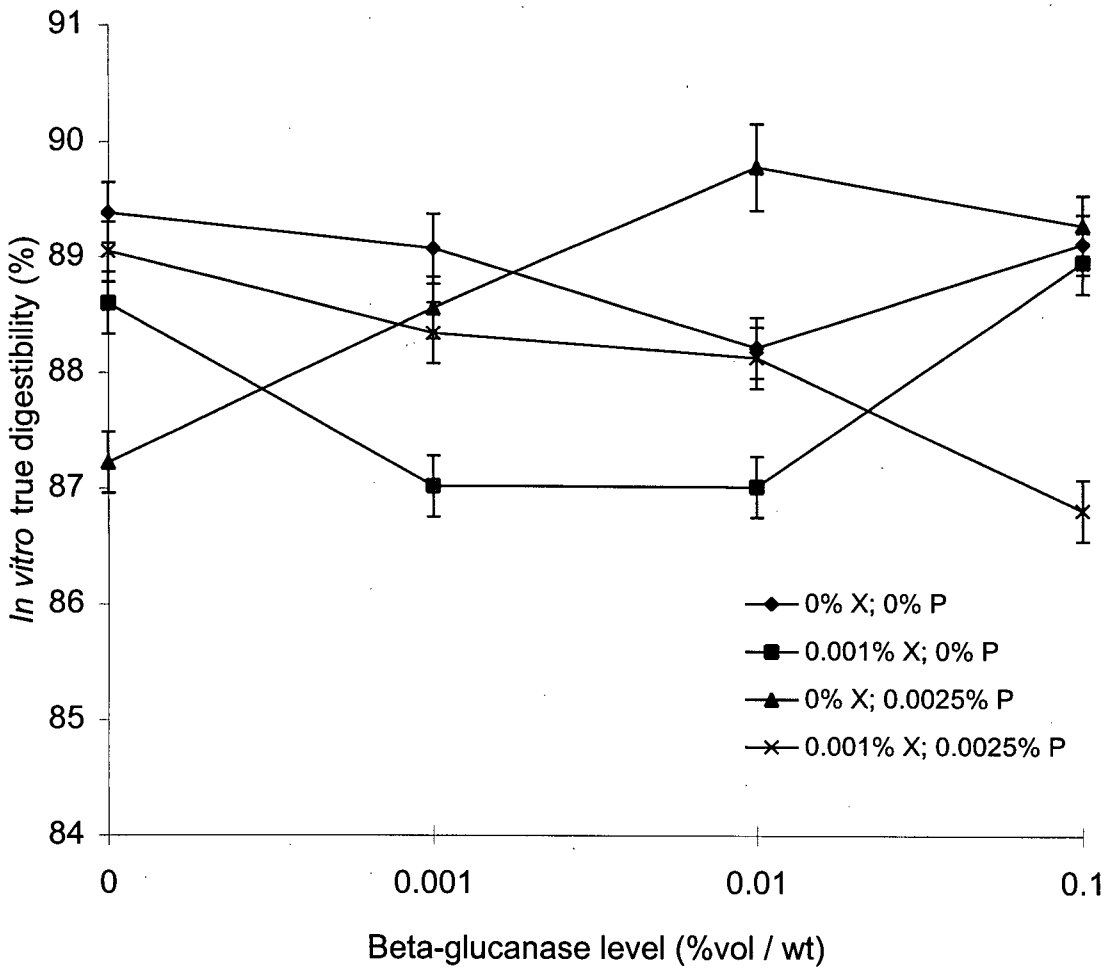

Figure 2.5. The effect of beta-glucanase, protease (P) and xylanase (X) application (% vol / wt) on the *in vitro* true digestibility of barley grain.

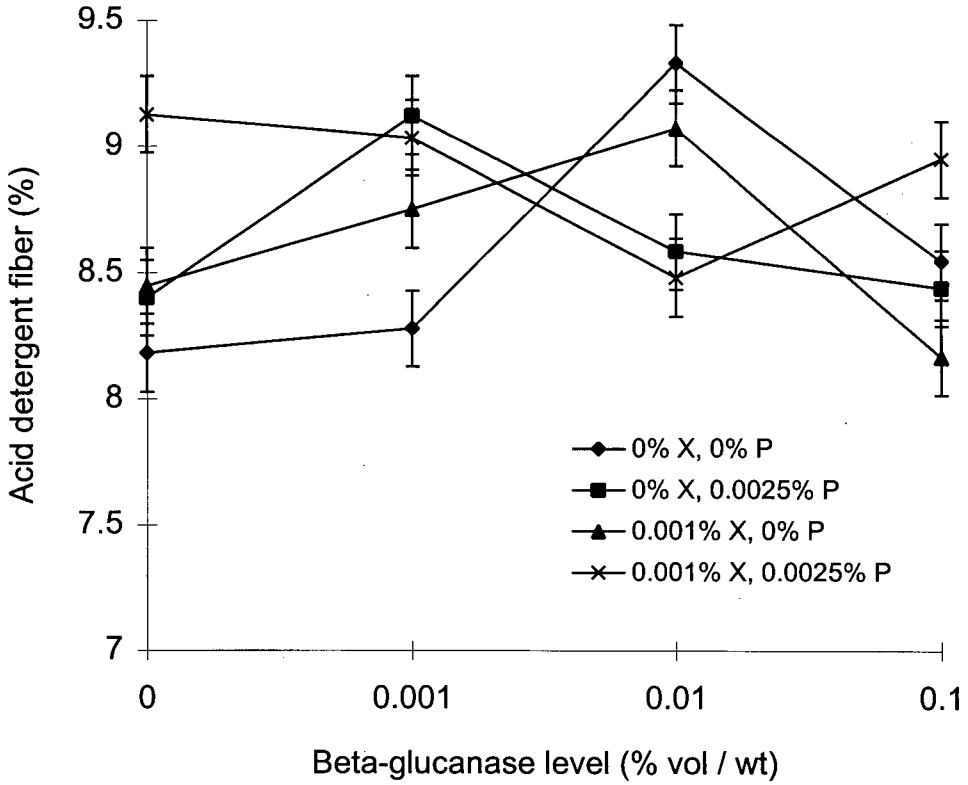

Figure 2.6. The effect of beta-glucanase, protease (P) and xylanase (X) application (% vol / wt) on the content of acid detergent fiber in barley grain.

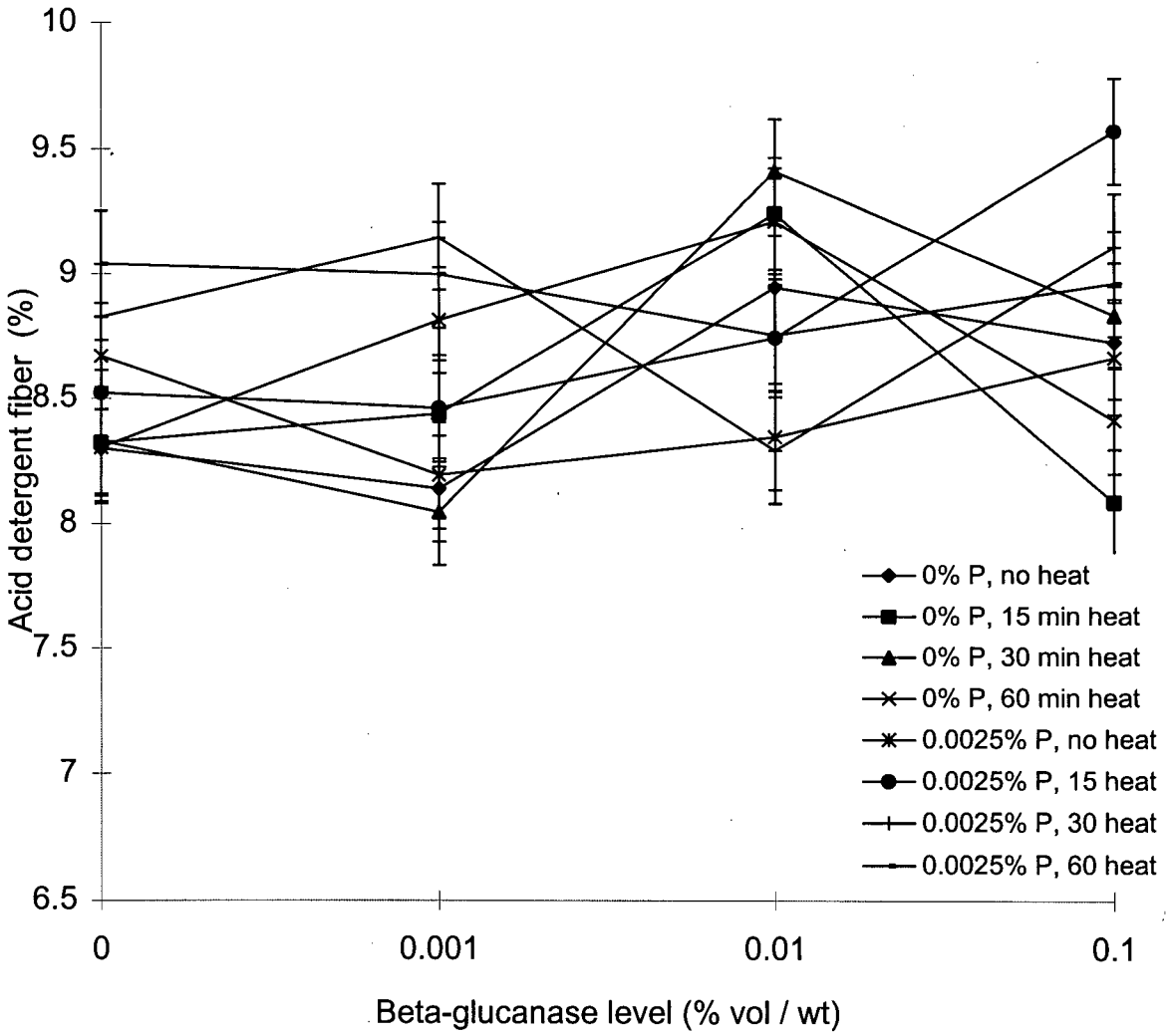

Figure 2.7. The effect of beta-glucanase and protease (P) application (% vol / wt) and duration of the heat treatment (55°C) on the content of acid detergent fiber in barley grain.

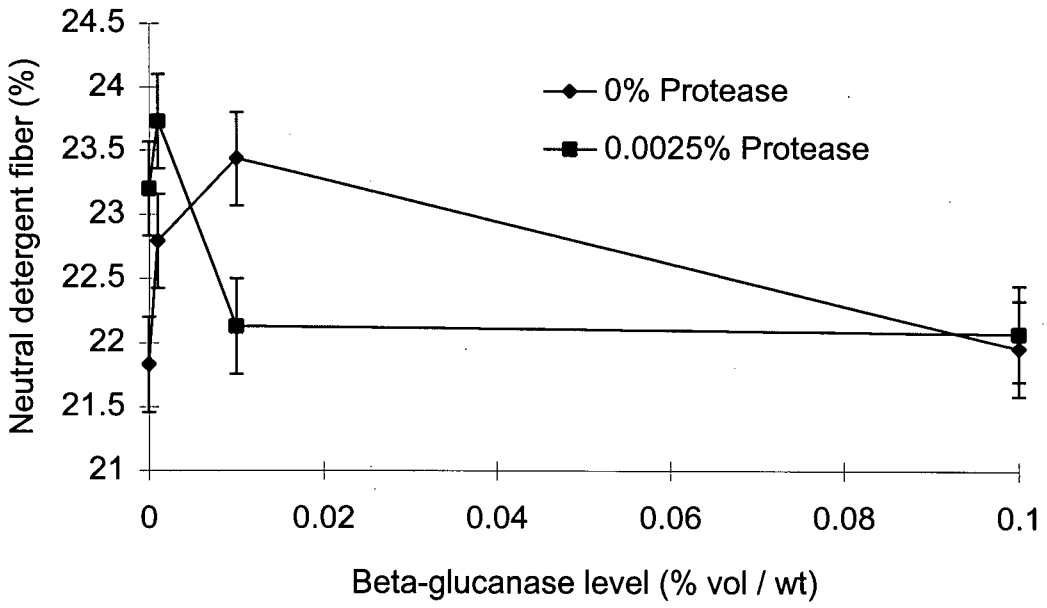

Figure 2.8. The effect of the addition of beta-glucanase and protease (% vol / wt) on the content of neutral detergent fiber in barley grain.

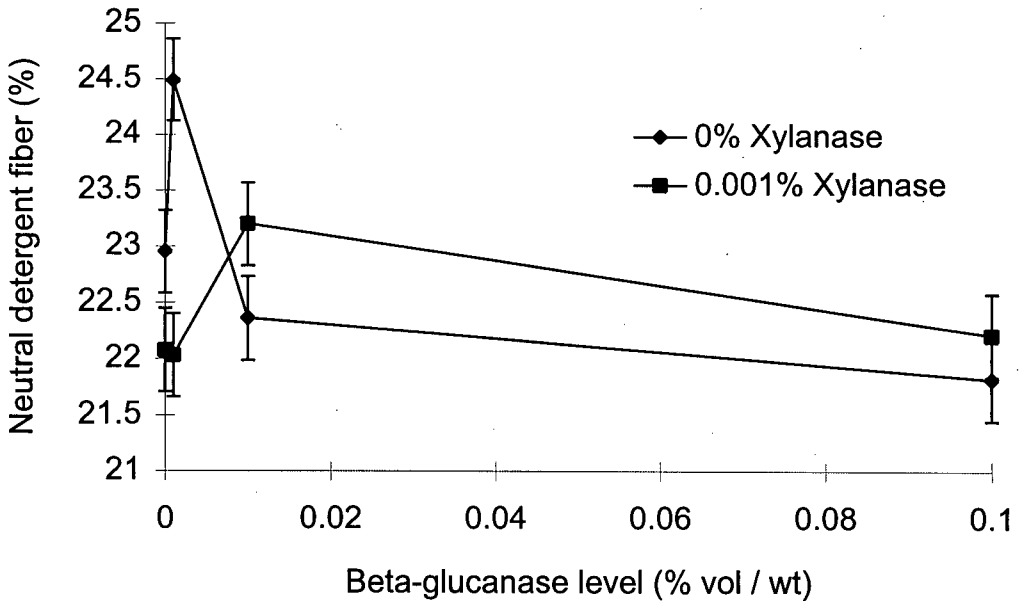

Figure 2.9. The effect of the addition of beta-glucanase and xylanase (% vol / wt) on the content of neutral detergent fiber in barley grain.

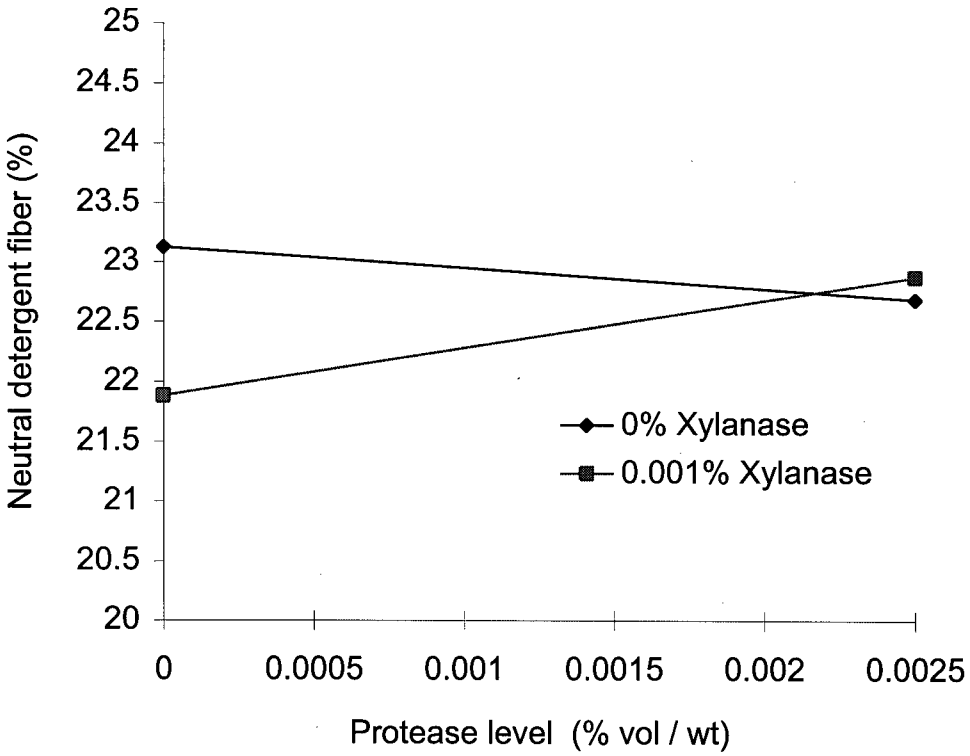

Figure 2.10 . The effect of the addition of protease and xylanase (% vol / wt) on the content of neutral detergent fiber in barley grain.

CHAPTER 3

3.0 THE EFFECT OF ENZYME AND MILD HYDROTHERMAL TREATMENT ON THE *IN VITRO* DIGESTIBILITY OF CANOLA MEAL.

3.1 ABSTRACT

Canola meal is the primary alternative protein supplement to soybean meal. Canola meal is restricted in lactating dairy cattle diets because of it's lower energy availability partly attributable to it's high fiber content. The present study investigated the feasibility of using enzyme technology in conjunction with mild hydrothermal treatment in order to improve the nutritional value of canola meal for dairy cattle. The experiment consisted of a 4 x 4 x 4 x 2 multi-factorial design in which apparent and true *in vitro* digestibilities and fiber analyses were used to evaluate treatment effects. Treatments included the application of heat at 55°C for 15, 30 or 60 min periods or not applied at all; moisture applied at 0%, 10%, 20% or 30%; protease (*Bacillus sp.*) applied at 0%, 0.005%, 0.01% or 0.05% and xylanase (*Trichoderma sp.*) applied at 0% or 0.001%. Nutrient composition of the untreated canola meal sample was 89.3% DM; 36.6% CP, 3.25% EE, 17.67% ADF and 21.83% NDF on a DM basis. There was a significant interaction (P = 0.0001) between protease and xylanase on IVDMD. The addition of protease was the major factor responsible for increased IVDMD. A highly significant quadratic relationship (P = 0.0001) between moisture and IVDMD was described by: Y = 78.62 + 0.165*moisture -

0.003*moisture^2. IVTD was affected by the significant interaction ($P < 0.01$) between protease and xylanase, where results parallel those obtained with IVDMD. A quadratic relationship ($P = 0.0001$) was also found between moisture and IVTD described by $Y = 87.25 + 0.073$*moisture $- 0.002$*moisture^2. The addition of 10% moisture was sufficient to significantly increase both IVDMD and IVTD ($P < 0.05$). However, with IVTD no further increase in digestibility was observed with a further increase in moisture level. There were several third order interactions which affected fiber content: duration of the heat treatment, and the addition of protease and xylanase ($P < 0.05$) and the addition of moisture, protease and xylanase ($P < 0.01$). NDF was significantly affected ($P < 0.05$) by the addition of protease ($P < 0.05$) and moisture ($P = 0.0001$). The mild heat treatment used in the present study did not enhance enzyme activity in most instances. Evaluation of canola meal treatments, using the selected indices, provided results from which several conclusions may be drawn. The application of xylanase was not effective, although the addition of protease in many instances, elicited a positive response. This response was similar to that obtained with the combination of protease and xylanase. Treatments which indicated a possible improvement in the digestibility of canola meal included the application of protease or moisture alone or in combination with other factors. Further investigation using other *in vitro* techniques, such as *in vitro* gas production may reveal the effects of the treatments on fermentation characteristics which was unavailable in the present study and is certainly justified in order to facilitate the selection of treatments for canola meal.

3.2 INTRODUCTION

Canola meal is the primary alternative to soybean meal for use as a protein supplement in rations for lactating dairy cows. It may comprise 10-15% or upwards to 25% of the concentrate ration depending on the cost of soybean meal and the protein content of the forage (Pro Form Feeds Inc., personal communication). Canola meal is a by-product from canola seed following the oil extraction process. The hulls from canola seed are present in the meal up to 30% by weight and are rich in fiber and lignin (Bell and Shires, 1982). The high fiber content of canola meal is the major contributing factor to it's relatively lower energy availability when compared to soybean meal (Emanuelson, 1994). Research to improve the energy content of canola meal is therefore, of major interest to the feed and dairy industries.

Increasing the nutritive value of feedstuffs for ruminants through the use of enzyme technology has received renewed interest. This is primarily a result of the recent advances in monogastric feeding regimes using exogenous enzymes (Chesson, 1993; Beauchemin and Rode, 1996). However, research into the use of enzymes as feed additives for ruminants has yielded variable results (Beauchemin and Rode, 1996). In a review of the literature, it is evident that little work has been conducted on the use of enzymes in the treatment of canola meal as a means of improving it's digestibility for ruminants. For further detail regarding the results of enzyme research with ruminants, the reader may be referred to Chapter 1, section 1.1. The use of *in vitro* fermentation techniques has been successfully applied for decades in the evaluation of forages for ruminants (Tilley and Terry, 1963; Goering

and Van Soest, 1970). The major advantage of *in vitro* techniques is that a large number of samples may be evaluated at one time. The objective of the current study was to identify treatments that would improve the digestibility of canola meal, particularly of the fiber component, in order to improve it's nutritional value for dairy cattle.

3.3 MATERIALS AND METHODS

3.3.1 Feed Treatments

The experiment consisted of a completely randomized 4 x 4 x 4 x 2 multi-factorial design to evaluate the effects of enzymes and hydrothermal treatment on canola meal using rumen *in vitro* dry matter disappearance (IVDMD) and rumen *in vitro* true digestibility (IVTD). Canola meal feed treatments under investigation included the application of protease at 0%, 0.005%, 0.01% or 0.05% (*Bacillus sp.*); xylanase at 0% or 0.001% (*Trichoderma sp.*); moisture at 0%, 10%, 20% or 30% and heat applied at 55°C for 0, 15, 30 or 60 min periods (Table 3.1). Enzymes were not pure cultures and substrate activity of the enzymes may be found in Appendix 6.1.

3.3.2 Processing Samples

Canola meal, ground through a 3 mm screen, was obtained from Pro Form Feeds Inc. Feed samples were processed according to the procedure outlined in Chapter 2, section 2.3.2.

3.3.3 Chemical Analyses

Chemical analyses were conducted on the samples according to the procedures outlined in Chapter 2, section 2.3.3.

3.3.4 Animals and Feeding

Donor animals were maintained as described in Chapter 2, section 2.3.4.

3.3.5 Rumen Fluid Inoculum

Rumen fluid was collected and prepared according to the procedure outlined in Chapter 2, section 2.3.5.

3.3.6 *In vitro* Procedure

IVDMD and IVTD analyses were determined on the feed samples according to the procedures outlined in Chapter 2, section 2.3.6.

3.3.7 Statistical Analysis

Statistical analyses were conducted using the General Linear Model (GLM) procedure of Statistical Analysis System (SAS Institute Inc., 1990) using the program listed in Appendix 6.4, which also includes the model statement. In the

present study, it was assumed that the highest order interaction did not exist, but it's mean square error was representative of the experimental error (Cochran and Cox, 1950). Statistically significant differences between means ($P < 0.05$) were determined using the program which employed the use of least square means for IVDMD, IVTD, NDF and ADF measurements. Further investigation of the relationship between a significant main effect and a measured parameter with orthogonal polynomials was conducted using the CONTRAST statement of SAS PROC GLM (SAS, 1990) and reported when the relationship could be defined.

3.4 RESULTS AND DISCUSSION

Dry matter (DM) content of the untreated canola meal sample was 89.3%. Crude protein (CP) content of the sample was 36.6% and crude fat (EE) was 3.2 %, both presented as a portion of DM. Acid detergent fiber (ADF) was 17.7% of DM and the neutral detergent fiber (NDF) was 21.8% of the DM fraction. These results are comparable with the values reported by Bell and Keith (1991) from a survey of canola meal from 7 crushing plants in Western Canada over a 4 week period. Those authors reported mean values (DM basis) of: 41.9 % CP, 3.9% EE, 19.1% ADF and 23.5% NDF.

3.4.1 IVDMD

The IVDMD of canola meal was significantly affected (P = 0.0001) by the interaction between protease and xylanase (Figure 3.1). Following a 48 h *in vitro* incubation, 79.8% of the untreated canola meal sample had disappeared (Table 3.2). This is not much higher than a value reported for *in situ* dry matter disappearance (ISDMD) of 72.1% for a ruminal incubation of 24 h (Moshtaghi and Ingalls, 1995).

The control sample was intermediately ranked with respect to the samples treated with protease and xylanase enzymes. Protease applied alone at 0.01% decreased IVDMD from 79.8% to 77.8%. The combination of 0.005% protease and 0.001% xylanase decreased IVDMD from 79.8% to 78.7%. Protease applied at 0.05% however, increased IVDMD from 79.8% to 82.4%. From Table 3.2, it is evident that there is a synergistic effect between the two enzymes when protease is applied at 0.01% or 0.05% in conjunction with xylanase. However, protease at certain levels had a positive effect when applied with xylanase. The synergy between the two enzymes is shown with the application of either 0.01% or 0.05% protease in combination with 0.001% xylanase (Table 3.2). The combination of the two higher levels of protease (0.01% and 0.05%) applied with xylanase significantly increased ($P < 0.05$) the IVDMD from 79.8% to greater than 81%. This synergistic effect would be expected to elicit an effect by degrading the protein matrix and other resistant plant cell wall compounds. The protein matrix in cereals has been shown to be responsible for dictating the rate and extent of digestion (McAllister, 1990 a, b, c).

The combination of the lowest level of protease with xylanase significantly reduced ($P < 0.05$) IVDMD relative to the control. Xylanase application did not have an effect when applied alone. Protease applied alone at an intermediate level (0.01%) produced a significantly lower ($P < 0.05$) IVDMD value than the IVDMD values obtained with the control sample and the lowest and highest levels of protease application.

The effect of moisture applied in the absence of heat on the digestibility characteristics of cereal grains, with the exception of sorghum, has been found to be minimal (Tait and Beames, 1988). Interestingly, in the present study, it was determined that moisture had a highly significant quadratic relationship ($P < 0.01$) with the IVDMD of canola meal described by $Y = 78.62 + 0.165 * \text{moisture level} - 0.003 * \text{moisture level}^2$ (Figure 3.2). From the regression analysis, it could be determined that an optimal level of 26% moisture would result in a DM degradability of 81%. Even the application of the lowest level of moisture (10%) was sufficient to elicit a significant increase ($P < 0.05$) in IVDMD from 78.6% to 79.9% (Table 3.3). The application of 20% or 30% moisture both produced IVDMD values of 80.7%, which was significantly greater than the control and than that obtained with the 10% moisture treatment. It has been suggested the beneficial effects of moisture addition may be attributed to the release of endogenous enzymes within the cereal grain (Newman, 1994). This would provide the opportunity for the cereal to be partly degraded prior to being exposed to microbial attack in the rumen. A recent study reported the 12 h ruminal *in situ* dry matter degradability of canola meal increased

from 48.9% to 55.4% with the addition of 15% moisture (Weurding, 1996). The lack of response to the mild hydrothermal conditions used in this study, may be attributed to the low temperature (55°C). Previous studies which have reported heat effects or effects from hydrothermal treatment used processing technologies including steam rolling, steam flaking, pressure cooking, exploding, extruding or pelleting which employ temperature in excess of 60°C (Tait and Beames, 1988).

3.4.2 IVTD

The results of IVTD determinations were similar to the results obtained for IVDMD. The interaction between protease and xylanase on IVTD of canola meal was highly significant ($P < 0.01$) (Figure 3.3). However, none of the treatments significantly increased IVTD ($P < 0.05$) from the value of 87.9% obtained for the control (Table 3.4). The application of 0.01% protease significantly reduced ($P < 0.05$) IVTD from 87.9% to 87.4%. Results from IVDMD for this treatment reflect a similar response: the addition of xylanase did not have an effect on IVTD which is also reflected in the results of the IVDMD determinations. Differences in the sensitivity of the results between IVDMD and IVTD may be a attributed to the components each one estimates. The major difference between the IVTD and IVDMD procedures is in the latter *in vitro* test, all cell solubles are eliminated, including undigested microbial matter (Van Soest, 1982).

There was a quadratic relationship between moisture level and IVTD ($P = 0.0001$) described by: $Y = 87.45 + 0.73 * \text{moisture level} - 0.002 * \text{moisture level}^2$

(Figure 3.4). The optimal level of moisture, determined from the regression equation, was 24% with a corresponding IVTD value of 88%. The application of the lowest moisture level (10%) was sufficient to significantly increase ($P < 0.05$) the IVTD of canola meal from 87.3% to 87.8% (Table 3.5). Interestingly, further increases in moisture level did not further improve degradability. This is in contrast to the findings of the effect of moisture on IVDMD where the application of 20% and 30% moisture both resulted in an IVTD value of 88.1%. As previously mentioned, moisture may promote the release of endogenous enzymes within the grain allowing further digestion of the feed by rumen microbes (Newman, 1994). In addition, microbial attachment may be enhanced in the moist environment through some mechanism (Cheng et al., 1995).

In this study, there was a lack of response in both IVDMD and IVTD to mild heat applied at 55°C. Recommendations from the enzyme supplier (FFI, Wiltshire, UK) suggested a possible increase in enzyme activity under high moisture and optimal heat conditions. However, the results of the present experiment suggest that no further increase in enzyme activity was attained with the application of heat or moisture. A possible explanation may be that enzyme activity is limited by other factors which could not be overcome with this mild hydrothermal treatment. These results may also be attributed to the lack of any increase in enzyme activity on the hydrothermally treated ingredient and with the concentrations and / or combinations of enzymes used in the present study.

The application of heat at 55°C for any of the selected time periods did not affect digestibility, however, moisture increased the digestibility of canola meal. In general. the response to enzyme addition was inconsistent, however, the positive effects that were observed were primarily as a result of the addition of protease.

3.4.3 ADF

A large number of interactions were found in the interpretation of fiber results which reflected treatment differences. A significant third order interaction ($P < 0.05$) was noted for duration of the heat treatment, and the application of protease and xylanase on the content of ADF (Figure 3.5). From Table 3.6, it is evident that only 3 treatments reduced the content of ADF in canola meal, although none of these treatment means were significantly different from one another ($P < 0.05$). The application of heat for 15 min reduced ADF from 20.1% to 18.9% of DM. The application of protease at 0.005% reduced ADF from 20.1% to 19.3% of the DM and the combination of 60 min of heat with 0.005% protease reduced ADF to 19.1% on a DM basis. An increase to greater than 21% of the DM in the ADF content was observed with two treatments where heat was applied for 30 or 60 min periods and the addition of 0.005% protease and 0.001% xylanase. The combination of either 0.01% or 0.05% protease, xylanase (0.001%), in addition to any level of heat, produced ADF values that were not significantly different ($P < 0.05$) from the control (Table 3.6).

produced ADF values that were not significantly different ($P < 0.05$) from the control (Table 3.6).

A significant interaction ($P < 0.01$) was also noted for the addition of moisture, protease and xylanase (Figure 3.6). The ADF content of the untreated sample was 18.3% of DM, which was significantly lower ($P < 0.05$) than any other treatment (Table 3.7). All treatments resulted in ADF means that were significantly different than the untreated sample with the exception of the application of 0.01% protease. Increasing the level of moisture applied to canola meal generally increased the ADF content with a few exceptions (Table 3.7). The addition of 20% or 30% moisture, irregardless of enzyme treatment, significantly increased ADF ($P < 0.05$) relative to the control.

An increase in the ADF content may be attributed to artifact fiber formed during the heating process. However, this explanation is unlikely as preliminary experiments evaluating ADIN content, which has been shown to be an indicator of artifact fiber, found no effect of treatments on this parameter. Another more likely explanation is that the proportion of soluble feed components decreased without a change in fiber composition. Treatments may have affected the non-fibrous components of canola meal. As fiber results are presented as a portion of the DM, this would result in proportionally greater amounts of fiber being detected, thus an increase in ADF values. This explanation would also explain the similar trend observed in digestibility results which paralleled those of the fiber results.

3.4.4 NDF

The NDF fraction of canola meal was significantly affected ($P < 0.05$) by protease treatment. Treatment of canola meal with 0.005% protease significantly ($P < 0.05$) increased the NDF content from 19.6% to 20.5% of DM (Table 3.8). However, treatment with higher levels of this enzyme did not significantly alter ($P < 0.05$) the NDF content relative to the control. It is of interest to note the response of NDF to the lowest level of enzyme was significant, whereas higher levels did not elicit a response. A similar explanation to the one offered for the increase in ADF content may be applied for the increase in the NDF content: that there was an increase in soluble feed components without a change in the fiber content. Enzyme action on feed components other than the fibrous portion would be reflected in higher fiber values compared to the control. It is unlikely there was contamination of the sample from feed entering the bag due to the small pore size.

NDF content was also affected ($P = 0.0001$) by the level of moisture applied to canola meal. The application of moisture at any level, significantly reduced ($P < 0.05$) the NDF content of canola meal (Table 3.9). The addition of 20% moisture reduced NDF from 22.0% to 20.3% of the DM. The application of either 10% or 30% moisture produced NDF values of 18.6% and 18.2% of DM, respectively which were significantly lower than the control as well as the 20% moisture treatment.

The minor differences observed in the ADF and NDF determinations among the various treatments are unlikely to have any practical significance. The differences between means are significant due to the small standard error of the

mean (S.E.M.). Bell and Keith (1991) obtained S.E.M. values for ADF and NDF of 0.24 and 0.16 for canola meal obtained from several crushing plants which are comparable to those obtained in the present study.

3.5 CONCLUSIONS

From the results of this study, it was determined that xylanase was not effective in terms of improving the digestibility of canola meal. Protease, however, showed potential improvements when applied at the highest level (0.05%). Although the combination of protease applied at 0.01% or 0.05% levels with xylanase often generated positive responses, the magnitude of the response was no greater than when protease was applied alone at the same levels. Heat treatment for any length of time was not effective in improving enzyme activity, nor did it independently elicit any response from any of the measured parameters. Moisture, on the other hand, had an effect on both the digestibility and the fibrous components of canola meal. Results which increased the *in vitro* digestibility or the fiber content may be attributable to a shift in the soluble components which needs to be further investigated.

The high number of interactions observed in the present study made interpretation of the results difficult. It is obvious the effects of enzyme treatment combinations produced both positive and negative responses. It is this inconsistency in the results from the research with enzymes with ruminant diets, combined with the high cost of enzymes that will continue to restrict enzyme

applications in this area. The present study is not conclusive but the data may be added to a growing database to narrow the field of research with ruminant enzyme research applications. Further evaluation of the effects of the treatments using other *in vitro* techniques, such as the gas production technique, will allow examination of the fermentation profiles of the canola meal samples and will provide more information on the effects of enzyme addition and mild hydrothermal treatment of canola meal.

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Table 3.1. Treatments investigated in order to determine the effects of exogenous enzyme application with and without added moisture and heat on *in vitro* dry matter disappearance and *in vitro* true digestibility of canola meal.

Factor	Level of application
Heat[1]	control (0), 15, 30, 60 min
Moisture[2]	0%, 10%, 20%, 30%
Protease[2, 3, 4]	0%, 0.005%, 0.01%, 0.05%
Xylanase[2, 3, 5]	0%, 0.001%

[1] Applied at 55°C.
[2] Applied on a % vol * wt $^{-1}$ basis.
[3] Supplied by Finn Feeds International Ltd., Wiltshire, U.K.
[4] Heptex sb-protease.
[5] Avizyme 1310.

Table 3.2. The effect of the addition of protease and xylanase to canola meal on the 48 h rumen *in vitro* dry matter disappearance of canola meal (DM basis).

Enzyme Treatment[1]		
Protease (%)	Xylanase (%)	IVDMD[2] (%)
0	0	79.8[c]
0.005	0	79.1[bc]
0.01	0	77.8[a]
0.05	0	82.4[e]
0	0.001	79.4[bc]
0.005	0.001	78.7[ab]
0.01	0.001	81.5[d]
0.05	0.001	81.1[d]
S.E.M.[3]		0.31

[1] Applied on a % vol * wt $^{-1}$ basis.
[2] IVDMD, *in vitro* dry matter disappearance.
[3] S.E.M., pooled standard error of the mean.
[a-d] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 3.3. The effect of moisture added to canola meal at the time of processing on the 48 h rumen *in vitro* dry matter digestibility of canola meal (DM basis).

Moisture[1] (%)	IVDMD[2] (%)
0	78.6[a]
10	79.9[b]
20	80.7[c]
30	80.7[c]
S.E.M.[3]	0.22

[1] Applied on a % vol * wt $^{-1}$ basis.
[2] IVDMD, *in vitro* dry matter disappearance.
[3] S.E.M., pooled standard error of the mean.
[a-c] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 3.4. The effect of the addition of protease and xylanase to canola meal on the rumen *in vitro* true digestibility of canola meal (DM basis).

Enzyme Treatment[1]		
Protease (%)	Xylanase (%)	IVTD[2] (%)
0	0	87.9[bc]
0.005	0	87.6[ab]
0.01	0	87.4[a]
0.05	0	88.2[c]
0	0.001	87.8[abc]
0.005	0.001	87.5[ab]
0.01	0.001	88.2[c]
0.05	0.001	87.9[bc]
S.E.M.		0.18

[1] Applied on a % vol * wt $^{-1}$ basis.
[2] IVTD, *in vitro* true digestibility.
[3] S.E.M., pooled standard error of the mean.
[a-c] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 3.5. The effect of moisture added to canola meal at the time of processing on the rumen *in vitro* true digestibility of canola meal (DM basis).

Moisture[1] (%)	IVTD[2] (%)
0	87.3[a]
10	87.8[b]
20	88.1[b]
30	88.1[b]
S.E.M.[3]	0.13

[1] Applied on a % vol * wt $^{-1}$ basis.
[2] IVTD, *in vitro* true digestibility.
[3] S.E.M., pooled standard error of the mean.
[a,b] Means in a column with different superscripts are significantly different *($P < 0.05$).*

Table 3.6. The effect of the duration of heat treatment and the application of protease and xylanase to canola meal on the ADF fraction of canola meal (DM basis).

	Feed Treatment		
Duration of heat treatment[1] (min)	Protease[2] (%)	Xylanase[2] (%)	ADF[3] (%)
0	0	0	20.1^{def}
15	0	0	18.9^{a}
30	0	0	20.0^{cdef}
60	0	0	19.4^{abcd}
0	0.005	0	19.3^{abc}
15	0.005	0	20.3^{efg}
30	0.005	0	19.6^{abcde}
60	0.005	0	19.1^{ab}
0	0.01	0	19.8^{bcdef}
15	0.01	0	20.0^{cdef}
30	0.01	0	20.0^{cdef}
60	0.01	0	19.8^{bcdef}
0	0.05	0	20.2^{efg}
15	0.05	0	20.1^{def}
30	0.05	0	19.6^{abcde}
60	0.05	0	20.4^{efg}
0	0	0.001	20.3^{efg}
15	0	0.001	20.2^{defg}
30	0	0.001	20.3^{efg}
60	0	0.001	20.5^{fg}
0	0.005	0.001	19.7^{abcdef}
15	0.005	0.001	20.0^{cdef}
30	0.005	0.001	21.0^{g}
60	0.005	0.001	21.2^{g}
0	0.01	0.001	20.0^{cdef}
15	0.01	0.001	19.7^{abcde}
30	0.01	0.001	19.7^{bcdef}
60	0.01	0.001	20.0^{cdef}
0	0.05	0.001	19.7^{abcdef}
15	0.05	0.001	19.9^{bcdef}
30	0.05	0.001	20.3^{efg}
60	0.05	0.001	20.5^{fg}
S.E.M.[4]			0.27

[1] Applied at 55°C.
[2] Applied on a % vol * wt $^{-1}$ basis.
[3] ADF, acid detergent fiber.
[4] S.E.M., pooled standard error of the mean.
[a-g] Means in a column with different superscripts are significantly different *(P < 0.05)*.

Table 3.7. The effect of moisture, protease and xylanase application to canola meal on the ADF fraction of canola meal (DM basis).

Feed Treatment[1]			
Moisture (%)	Protease (%)	Xylanase (%)	ADF[2] (%)
0	0	0	18.3^{ab}
10	0	0	19.3^{cd}
20	0	0	20.5^{fghij}
30	0	0	20.5^{fghij}
0	0.005	0	19.3^{cde}
10	0.005	0	19.4^{cde}
20	0.005	0	19.7^{cde}
30	0.005	0	19.9^{defghi}
0	0.01	0	18.0^{a}
10	0.01	0	19.5^{cde}
20	0.01	0	20.9^{jkl}
30	0.01	0	21.2^{jkl}
0	0.05	0	19.0^{bc}
10	0.05	0	19.8^{cdefg}
20	0.05	0	20.6^{hijk}
30	0.05	0	21.1^{jkl}
0	0	0.001	19.2^{cd}
10	0	0.001	19.6^{cde}
20	0	0.001	20.6^{ijk}
30	0	0.001	21.8^{l}
0	0.005	0.001	19.4^{cde}
10	0.005	0.001	19.9^{defghi}
20	0.005	0.001	21.2^{jkl}
30	0.005	0.001	21.4^{kl}
0	0.01	0.001	19.4^{cde}
10	0.01	0.001	19.8^{defgh}
20	0.01	0.001	19.7^{cdef}
30	0.01	0.001	20.5^{ghij}
0	0.05	0.001	19.2^{cd}
10	0.05	0.001	20.1^{efghi}
20	0.05	0.001	20.5^{ghij}
30	0.05	0.001	20.6^{hijk}
S.E.M.[3]			0.27

[1] Applied on a % vol * wt $^{-1}$ basis.
[2] ADF, acid detergent fiber.
[3] S.E.M., pooled standard error of the mean.
[a-l] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 3.8. The effect of the level of protease application to canola meal at the time of processing on the NDF fraction in canola meal (DM basis).

Protease[1] (%)	NDF[2] (%)
0	19.6[a]
0.005	20.5[b]
0.01	19.4[a]
0.05	19.4[a]
S.E.M.[3]	0.25

[1] Applied on a % vol * wt $^{-1}$ basis.
[2] NDF, neutral detergent fiber.
[3] S.E.M., pooled standard error of the mean.
[a,b] Means in a column with different superscripts are significantly different *($P < 0.05$).*

Table 3.9. The effect of moisture addition to canola meal at the time of processing on the NDF fraction in canola meal (DM basis).

Moisture[1] (%)	NDF[2] (%)
0	22.1[c]
10	18.6[a]
20	20.3[b]
30	18.2[a]
S.E.M.[3]	0.25

[1] Applied on a % vol * wt $^{-1}$ basis.
[2] NDF, neutral detergent fiber.
[3] S.E.M., pooled standard error of the mean.
[a-c] Means in a column with different superscripts are significantly different *($P < 0.05$).*

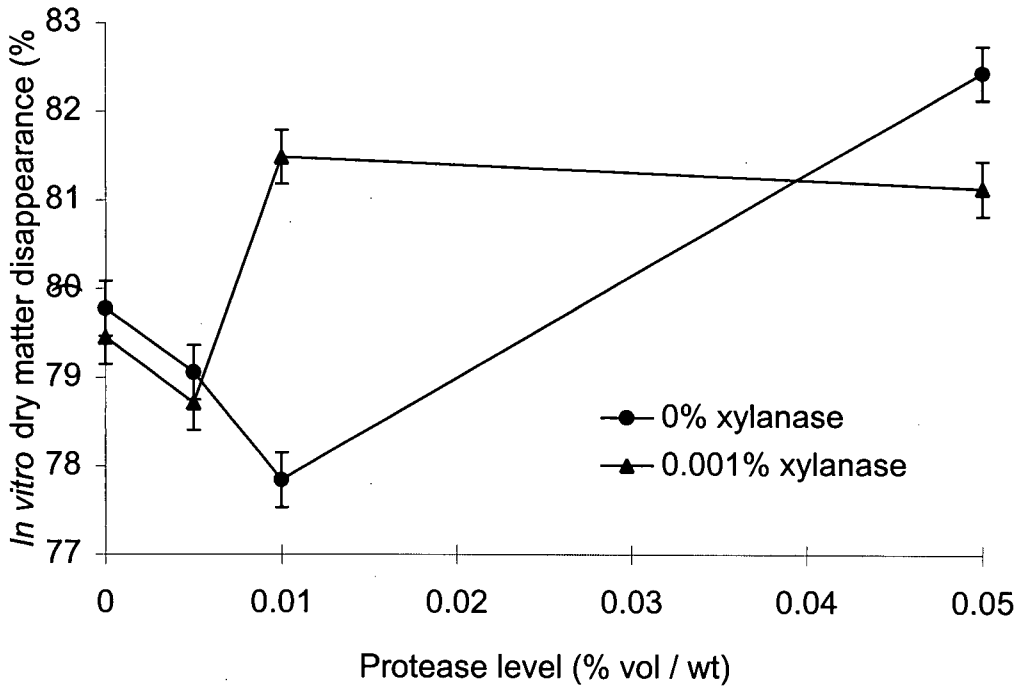

Figure 3.1. The effect of the addition of xylanase and protease (% vol / wt) on the 48 h rumen *in vitro* dry matter disappearance of canola meal.

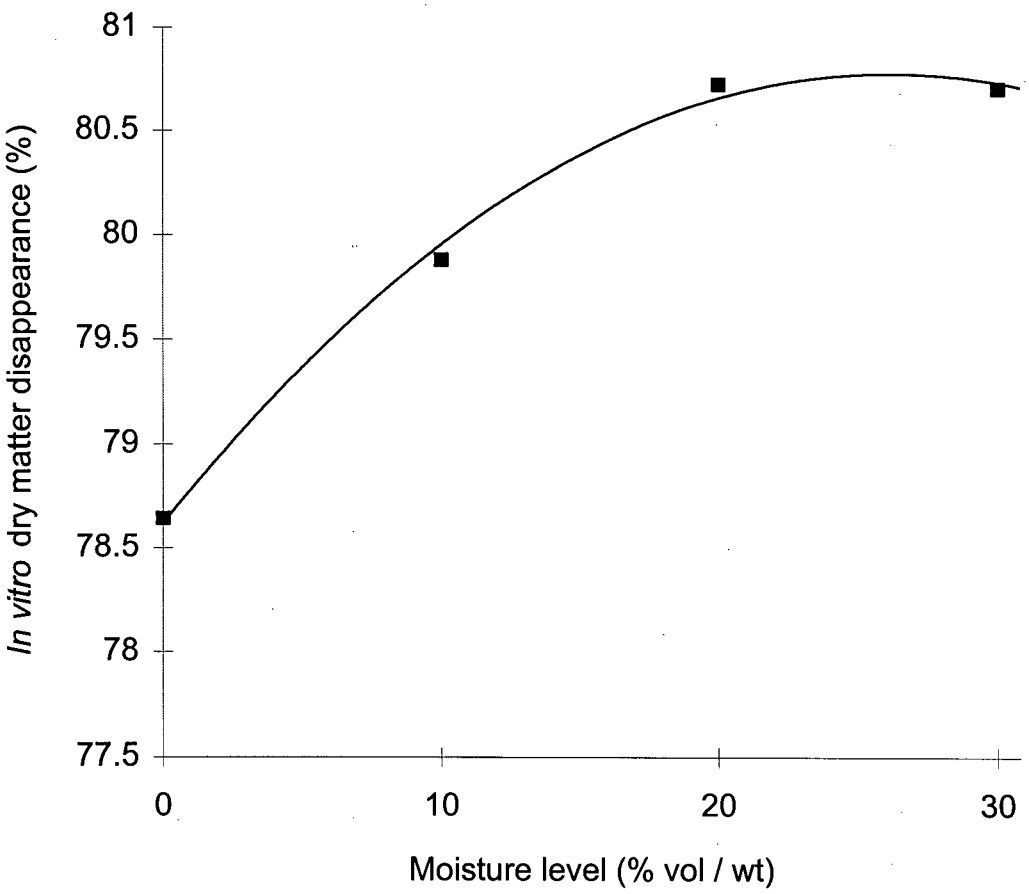

Figure 3.2. Quadratic relationship between moisture level and the 48 h *in vitro* dry matter disappearance of canola meal (Y = 78.62 + 0.165 * moisture level - 0.003 * moisture level^2, n = 4, R^2 = 0.988, S.E. = 0.105).

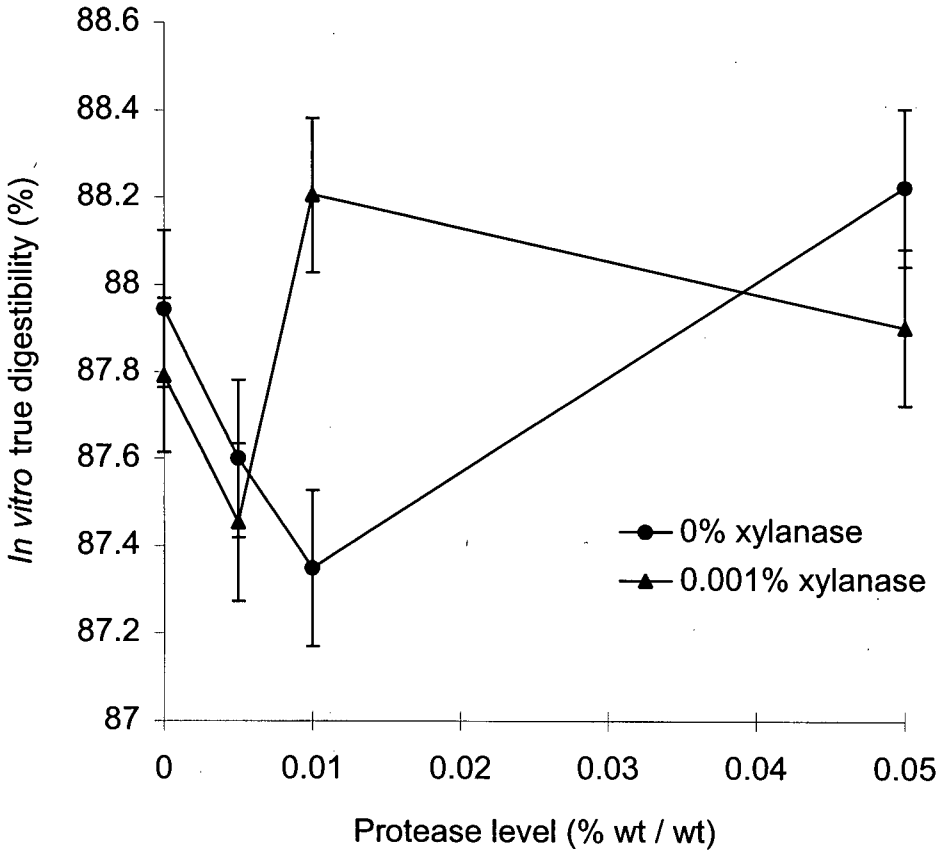

Figure 3.3. The effect of the addition of protease and xylanase (% vol / wt) on the rumen *in vitro* true digestibility of canola meal.

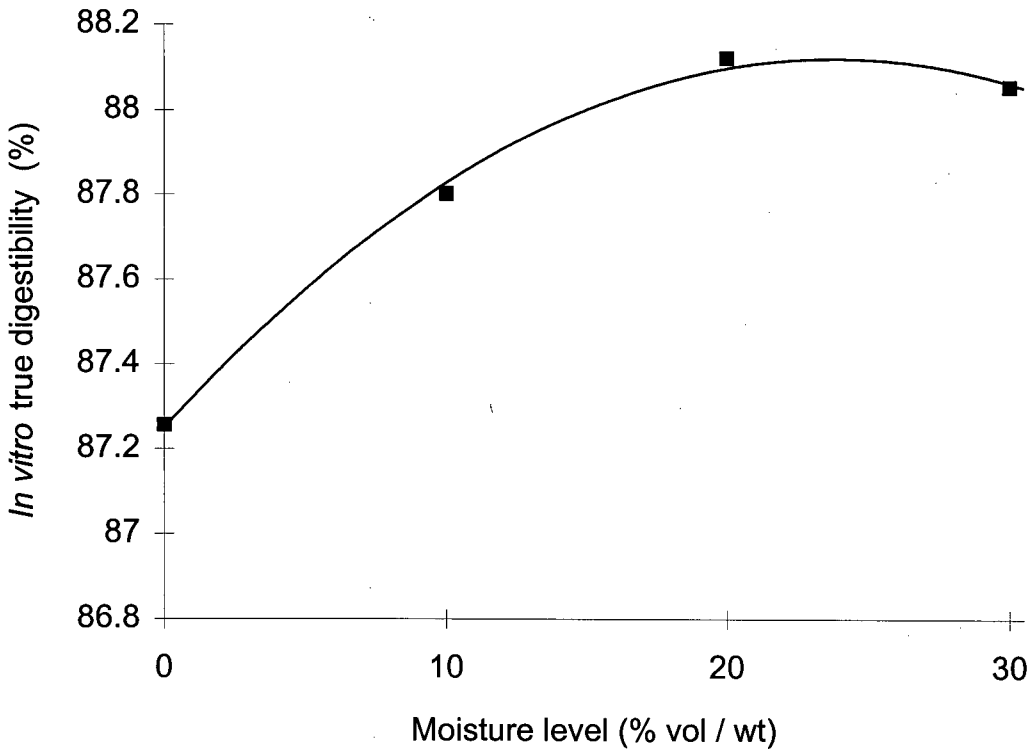

Figure 3.4. Quadratic relationship between moisture level (% vol / wt) and the rumen *in vitro* true digestibility of canola meal (Y = 87.25 + 0.073 * moisture level - 0.002 * moisture level^2: n = 4, R^2 = 0.991, S.E. = 0.0355).

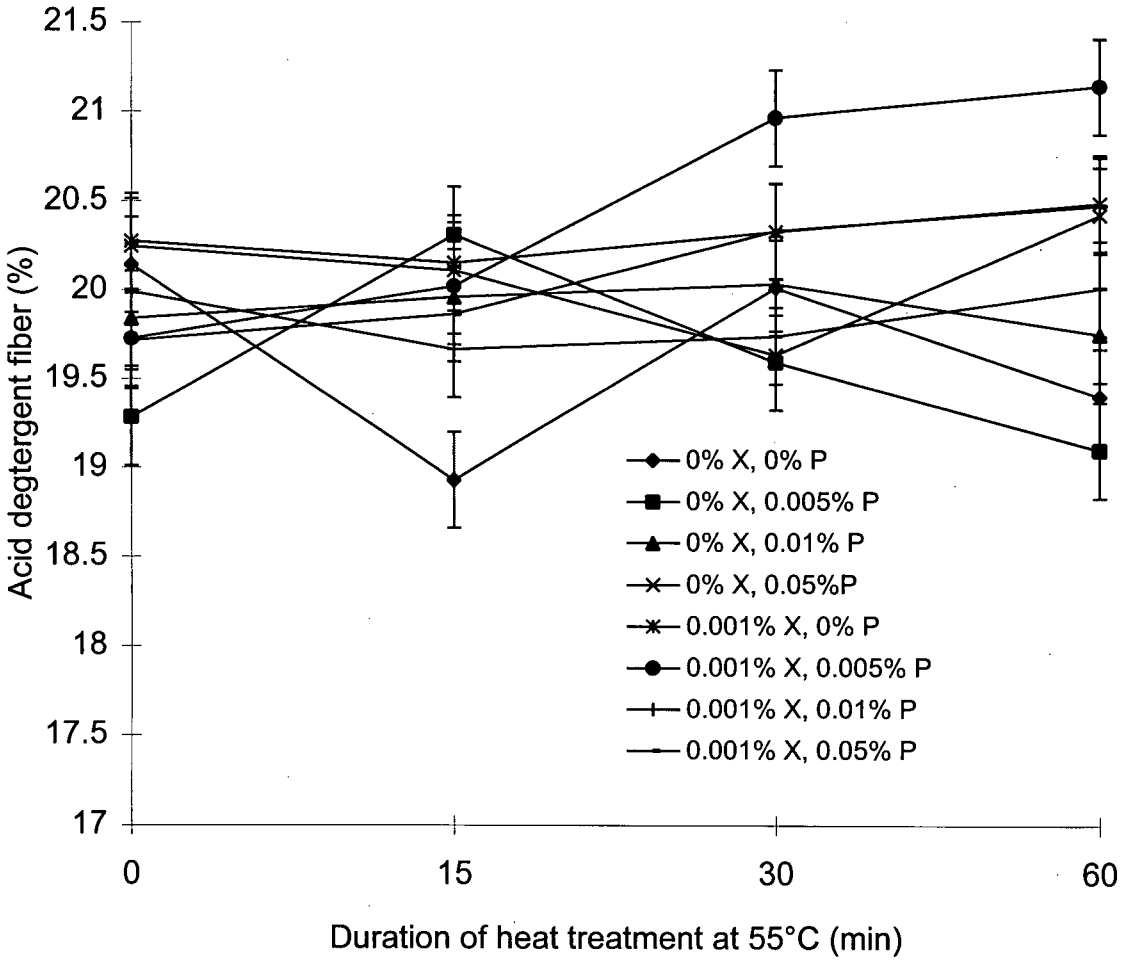

Figure 3.5. The effect of the application of protease (P), xylanase (X) (% vol / wt) and duration of heat treatment on the content of acid detergent fiber in canola meal.

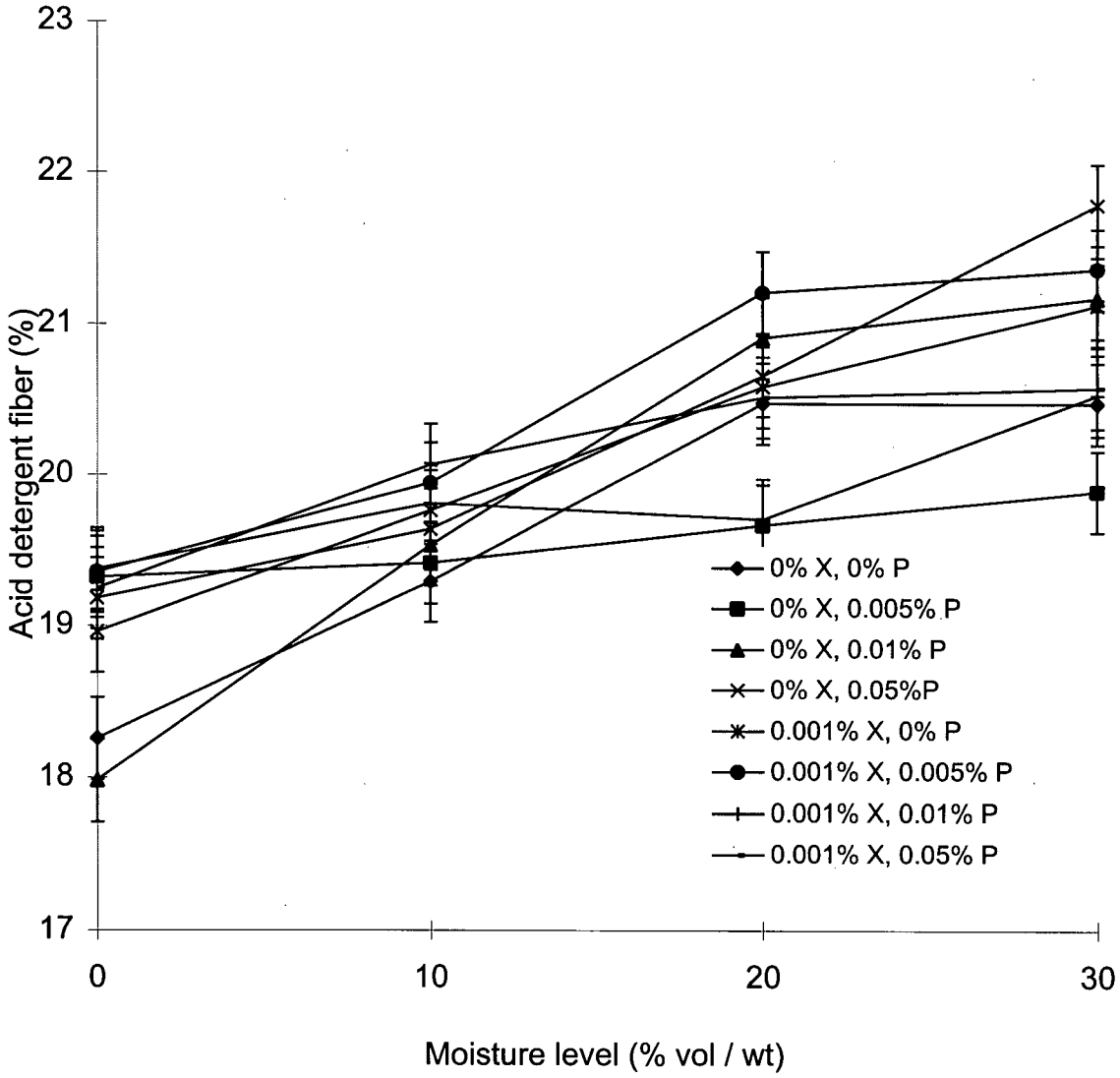

Figure 3.6. The effect of the application of moisture, protease (P) and xylanase (X) (% vol / wt) on the content of acid detergent fiber in canola meal.

CHAPTER 4

4.0 THE EFFECT OF ENZYME AND MILD HYDROTHERMAL TREATMENT ON THE *IN VITRO* GAS PRODUCTION IN CANOLA MEAL.

4.1 ABSTRACT

The high fiber content of canola meal limits it's inclusion in dairy cattle diets. Abundant in Canada and grown worldwide, canola meal is a cost effective alternative protein supplement to soybean meal. The objective of this research was to investigate the feasibility of using enzyme technology and mild hydrothermal treatment in order to improve the nutritional value of canola meal. The experiment consisted of a 4 x 4 x 4 x 2 multi-factorial design. The *in vitro* gas production technique and VFA analyses were used to assess the response of canola meal subjected to the following treatments: heat applied at 55°C for 15, 30 or 60 min periods or not applied at all; moisture applied at 0%, 10%, 20% or 30%; protease (*Bacillus sp.*) applied at 0%, 0.005%, 0.01% or 0.05%; and xylanase applied at 0% or 0.001% (*Trichoderma sp.*). Gas parameters were estimated by fitting the gas production data acquired over 18 h, to the substrate limited exponential growth model with lag ($Y = A\ (\ 1 - e^{-b(t-l)}\ )$). Total VFA production and VFA ratios were determined from one of the incubating samples for each treatment. The application of heat (55°C) did not improve enzyme activity as determined by the *in vitro* gas production technique and corresponding VFA data. The rate of gas production was

not affected by the addition of xylanase. The application of 0.01% protease significantly increased ($P = 0.0001$) the rate of gas production from 0.077 mL/h^{-1} to 0.108 mL/h^{-1}. The length of the lag phase was affected by the interaction between protease and xylanase ($P < 0.05$). There was also a significant interaction ($P < 0.01$) between protease and xylanase which increased the production VFAs. The ratio of acetate to propionate was affected by protease ($P < 0.01$) and xylanase ($P = 0.0001$) application. The ratio of butyrate to propionate was also affected by protease ($P < 0.01$) and xylanase application ($P = 0.0001$), independently. The 0.05% protease treatment significantly increased ($P < 0.05$) the proportion of acetate to propionate or butyrate being produced. Xylanase applied to canola meal at 0.001% also resulted in an increase in the proportion of acetate relative to propionate or butyrate. *In vitro* results obtained in the present experiment suggest that enzyme supplementation of canola meal may be an alternative process in improving the rate of gas and VFA production, in addition to decreasing the length of the lag phase. In particular, the results of this study indicate that the application of protease and xylanase, at optimal levels, may be used to improve the nutritional value of canola meal for dairy cattle.

4.2 INTRODUCTION

Canola meal as a feedstuff is high in protein making it the primary alternative to soybean meal in diets for lactating dairy cows. It's level in ruminant diets is limited however, due to it's high fiber content relative to that of soybean meal. The lower available energy content of canola meal is related to fiber content, composition of cell wall polysaccharides and the impact of these fractions on the utilization of both the protein and energy components of the feedstuff (Brenes et al., 1993).

Slominski and Campbell (1990) investigated the carbohydrate content of canola meal and found that 87% of the glucose released during enzymatic hydrolysis was derived from cellulose. High amounts of xylose indicated the presence of xylans and xyloglucans and large amount of uronic acid residues (30%) suggested that the polysaccharides in the cellulosic fraction also included a large amount of pectins (Slominski and Campbell, 1990). Pectins are generally regarded as readily fermentable in the rumen, however, in a high producing cow where rumen passage rate is often greater than 8% h^{-1} and the residence time of the feed in the rumen is less than 10 h, complete degradation of pectic substances is unlikely (Van Soest, 1982). For the same reasons, xylans and xyloglucans also may not be completely degraded within the short residence period in the rumen. In addition, linear xylans are easily degraded by enzymes in the rumen whereas branched and highly substituted xylans are less readily hydrolyzed (Van Soest, 1994). The fibrous carbohydrate fraction of canola meal has been determined to be the main

contributing factor to it's low energy availability in ruminant animals and is therefore an important factor in feed formulation (Bell, 1993).

Many studies have shown the benefit of adding exogenous enzymes to concentrates fed to ruminants, as well as deleterious effects. Several studies were inconclusive in their results. For a brief review of the literature the reader is referred to Chapter 1, sections 1.1.4.2.ii and 1.1.4.3.ii. A review of the literature reveals there has been limited investigation into the possible application of exogenous enzymes applied in particular to canola meal in order to improve it's nutritional value when fed to ruminants. Preliminary assessment of the effects of mild hydrothermal and enzyme treatment on the nutritional value of canola meal was conducted using *in vitro* analyses (refer to Chapter 3).

In vitro fermentation techniques have been successfully used in the evaluation of forages for ruminants for decades (Tilley and Terry, 1963; Goering and Van Soest, 1970). Gas production in particular has been shown to be indicative of the digestibility and fermentation of a variety of feedstuffs as it measures the appearance of fermentation products rather than their disappearance over time (Menke et al., 1979; Menke and Steingass, 1988; Blummel and Orskov, 1993; Opatpatanakit et al., 1994, Krishnamoorthy et al., 1995). The production of gas (CO_2 and CH_4) arises as a result of the fermentation of carbohydrates by rumen microbes to volatile fatty acids (VFA), primarily acetate, butyrate and propionate, the latter of which does not directly contribute to the production of gas. A computerized system was developed by Pell and Schofield (1993) in which changes in pressure were

indicative of the gas produced and therefore the fermentation profile of various feedstuffs. The present study investigated the use of a similar system in the evaluation of the nutritive value of canola meal.

The primary objective of this study was to use the gas production technique to investigate the feasibility of using enzymes and mild hydrothermal treatment applied to canola meal, in order to improve it's nutritional value for dairy cattle.

4.3 MATERIALS AND METHODS

4.3.1 Feed Treatments

The experiment consisted of a completely randomized 4 x 4 x 4 x 2 multi-factorial design which evaluated the effects of enzymes and hydrothermal treatment on canola meal using the *in vitro* gas production technique. Treatments under investigation are described in Chapter 3, section 3.3.1.

4.3.2 Processing of Feed Treatments

Canola meal treatments were processed according to the procedure outlined in Chapter 2, section 2.3.2.

4.3.3 Chemical Analyses

Chemical analyses were conducted on the samples according to the procedures outlined in Chapter 2, section 2.3.3.

4.3.4 Animals and Feeding

Donor animals were maintained as described in Chapter 2, section 2.3.4.

4.3.5 Rumen Fluid Collection

Rumen fluid was collected prior to the morning feeding as suggested by Menke and Steingass (1988). Following collection, rumen fluid was filtered through 4 layers of cheesecloth and a layer of glass wool into a prewarmed and airtight container for transport to the laboratory.

4.3.6 Gas Production System

Gas production measurements were made using a computerized system adapted from Pell and Schofield (1993) (Figure 4.1) with the following modifications. A laboratory incubator maintained at 39 ± 0.5°C was modified to accommodate 32 customized Erlenmeyer flasks (50 mL). A rotating bottom plate allowed for continuous mixing throughout the experiment. Each flask was fitted with a small port in which a vacutainer stopper was placed which allowed for system calibration

(Appendix 6.5), rumen fluid injection and VFA sampling. Anaerobic conditions were maintained by moistening the seal with water. Pressure transducers (PX170 series, Omega Engineering, Inc., Stamford, CT, U.S.A.) were attached through the top of silicone stoppers which contained a 0.5 cm hole. The stoppers and transducers were secured with elastic bands anchored to two small "ears" located at the neck of the flask throughout the acquisition of gas measurements (Figure 4.2).

4.3.7 Gas Measurements

Gas measurements were made using 0 to 103 kPa (0 to 15 psi) pressure transducers which measured the pressure within a flask and generated a differential output voltage proportional to that pressure. Output voltage was measured using Tempscan/1000 (Omega Engineering, Inc., Stamford, CT, U.S.A.), a high speed measurement system set to acquire data from 32 channels and equipped with a direct transfer to a computer. The channels were configured to acquire data at 5 min intervals over an 18 h incubation period. The following regression equation (r^2 = 0.994) was used to determine the gas volume from a voltage response (Appendix 6.5):

$$Y = 64.60\ mL*V^{-1}\ X - 1.27\ mL, \qquad \text{(Equation 1)}$$

where Y is the gas volume and X is voltage response.

4.3.8 Procedure for the Gas Production Technique

Samples of 0.150g were weighed in triplicate into 50 mL erlenmeyer flasks to which 12 mL of preheated phosphate-bicarbonate medium and reducing solution (Goering and Van Soest, 1970) was added. The flasks were flushed with CO_2 to establish anaerobic conditions prior to sealing. Rumen fluid (3 mL) was transferred via an 18 gauge needle through the port into the flasks to make up a 20% inoculum (Goering and Van Soest, 1970; Menke and Steingass, 1988). The solutions in the flasks were allowed to equilibrate for 5 min and the excess gas released with a needle (26.5 gauge) through the port prior to data acquisition.

Each run included three blanks (rumen fluid and buffer) which were averaged in order to correct each channel for rumen fluid activity. Standard hay and canola samples were also incubated to monitor daily variation and detect abnormal rumen fluid activity. The buffer capacity of the gas production system was monitored using pH measurements conducted on a random selection of flasks at the end of each gas run. The buffer capacity of the system was maintained within a pH range of 6.7-7.0.

4.3.9 Data Processing

All voltage measurements acquired with the data logger were recorded using TempWindows 3.1® software (Omega Engineering, Inc., Stamford, CT, U.S.A.). Determination of gas production for a specific channel required:

1) Subtraction of the baseline voltage at time zero from the observed voltage at any time.

2) Conversion of the voltage output to gas volume using the calibration correction factor (corrected to 103 kPa, 39°C), (Appendix 6.5).

3) Normalization of the gas production data to 1 g dry sample weight.

4) Use of steps 1 and 2 to calculate gas production of the blank, hay and canola standards.

4.3.10 Kinetic Analysis of Gas Data

The gas production profile of a canola meal sample is illustrated in Figure 4.3. The simple limited exponential growth (SLEG) model with lag was used to describe the gas production data. This model has been previously used to determine gas production parameters in tropical feedstuffs (Krishnamoorthy et al., 1991; Krishnamoorthy et al., 1995):

$$Y = A\,(1 - e^{-b\,(t-l)}), \quad \text{(Equation 2)}$$

where Y is the cumulative gas production at a given time t, l is the initial lag for the onset of fermentation and A is the potential cumulative gas production which may be produced at a specific rate b (mL/h^{-1}).

Rates of fermentation and lag periods were estimated using nonlinear regression and the Secant method to minimize the residual sums of squares associated with the regression model (Statistical Analysis Systems, (SAS), 1990) (Appendix 6.7). The Secant method is a linear iterative curve fitting procedure of the PROC NLIN procedures of SAS (1990).

4.3.11 VFA Sampling and Analyses:

Preliminary experiments conducted in our laboratory indicated that 6 h total gas production measurements of canola meal treatments were reflected in a 6 h sampling time for VFAs (Appendix 6.6). Preliminary investigation of the changes in the ratios of VFAs produced also indicated this was an adequate sampling time. Inherent analytic error associated with VFA analyses in this laboratory is 5%. One flask of each feed treatment was sampled and analyzed in duplicate for total and individual VFAs (acetate, propionate, isobutyrate, n-butyrate, iso-valerate and n-valerate) according to the procedure of Erwin *et al.*, (1961). A luer lock fitted with a tube (I.D. = 1.19 mm, O.D. 1.70 mm) was immersed below the rumen fluid inoculum and was used to sample from a flask using a syringe. After 6 h of incubation, a 0.5 mL representative sample was withdrawn from the fermentation flasks. Samples were centrifuged at 3000 g for 10 minutes, frozen and acidified to pH 2 with a 20%

phosphoric acid solution (v/v). The supernatant was later injected into a gas chromatograph (Model GC14A, Shimadzu Corp., Kyoto, Japan) equipped with a fused silica capillary column (30m x 0.25 I.D. Stabilwax-DA, Restek Corp., Bellefonte, PA, USA). Column temperature was set to increase at $10°min^{-1}$ from 120°C to 180°C and iso-caproic acid was used for the internal standard.

4.3.12 Statistical Analysis

Statistical analyses were conducted using the General Linear Model (GLM) procedure of Statistical Analysis System (SAS Institute Inc., 1990) using the program listed in Appendix 6.4. In the present study, it was assumed that the highest order interaction did not exist, but it's mean square error was representative of the experimental error (Cochran and Cox, 1950). Statistically significant differences ($P < 0.05$) between means were determined using the program which employed the use of least square means for rate of gas production, length of the lag phase, total VFA production, acetate to propionate ratios and butyrate to propionate ratios. Further investigation of the relationship between a significant main effect and a measured parameter with orthogonal polynomials was conducted using the CONTRAST statement of SAS PROC GLM (SAS, 1990).

4.4 RESULTS AND DISCUSSION

Nutrient analysis of the control sample can be found in Chapter 3, section 3.4.1.

4.4.1 Validation and Comparison of the Gas Production System

The results of the present study with canola meal are comparable to those obtained from other studies using various gas systems to evaluate a variety of feedstuffs. In the present study, gas production parameters were evaluated using the exponential model including lag. The rate of gas production of the control treatment was determined to be 0.077 ± 0.004 mL/h^{-1} and the length of the lag phase was determined to be 0.449 ± 0.046 h. Although selection of the appropriate model has been shown to affect the estimation of gas production parameters (Krishnamoorthy et al., 1991), rates of gas production from studies with other feeds have produced comparable results to those obtained in the present study irrespective of whether single or dual-pooled logistic models were used. Rates of gas production in a study with ryegrass were determined using a dual phase logistic model (France et al., 1993) which yielded values between 0.0074 mL/h^{-1} and 0.0192 mL/h^{-1} from data obtained with a manual system (Theorodou et al., 1994).

A review of the literature reveals that manual methods of recording gas production are inherently associated with greater experimental error than a computerized system. The present study employed the use of a computerized

system which closely resembled that developed by Schofield and Pell (1995) with the minor modifications previously noted. The computerized system has been previously used to evaluate alfalfa, timothy and corn silage and produced a standard error ranging from 0.7% to 2.6% of the estimated parameters using either single or dual phase models (Schofield and Pell, 1995). In the present study, the pooled standard error for rate was ±0.004 mL/h^{-1} with values for treatments ranging from 0.077 mL/h^{-1} to 0.108 mL/h^{-1}. The error associated with the length of the lag phase was ± 0.046 h with values for treatments ranging from 0.136 h to 0.449 h. The values for lag in the present study have a greater error associated with them than most other reported feed values with other systems. This may be attributed to variation within a feedstuff being investigatedor the large number of observations over a specific time interval which is unique to this type of system. This variation however, is not reflected in the values for rate.

The manual system used by Krishnamoorthy et al. (1991) to evaluate energy supplements, presented standard errors ranging from 1.8% to 3.4% for rate of gas production and errors ranging from 8% to 57% for lag period. In the present study, the spread of observations as a proportion of the estimated value was on average 5.2% for rate of gas production and 10.2% for length of the lag period. The larger error associated with the length of the lag phase in both methods may be attributed to a number of physiological conditions (Krishnamoorthy et al., 1991). Some factors which may require consideration are: sample size, rumen fluid activity, microbial population and chemical composition of the feedstuff.

The gas parameters estimated in the present study with canola meal, using a computerized system, are comparable to those obtained with a manual gas production system used to evaluate energy supplements (Krishnamoorthy et al., 1991). Employing the exponential model with lag, the rates of gas production for wheat, barley, maize, tapioca, oat, rye, sorghum were 0.1196, 0.1193, 0.0767, 0.1349, 0.0866, 0.0875 and 0.1101 mL/h^{-1}. The lag periods for the same feeds were 1.1, 0.5, 1.2, 0.7, -0.3, -0.2 and 1.5 h, respectively. A subsequent study evaluated rapeseed meal and determined that the rate of gas production was 0.125 mL/h^{-1} and the length of the lag phase was -0.37 h (Krishnamoorthy et al., 1995). The value for rate of gas production obtained by these authors was faster than the value of 0.077 mL/h^{-1} determined in the present study. In contrast, the value for lag obtained in the present study is much longer than the value obtained by these authors. There are a number of possible explanations for the large difference in lag times between these two studies (- 0.37 h vs. 0.45 h). The greater amounts of anti-nutritional factors present in rapeseed meal compared to canola meal, may have a detrimental effect on lag time through some action on rumen microbes. Another possible explanation may be that manual readings are subject to greater error than a computerized system. In addition, the frequency of gas measurements may impact on the estimation of parameters from the model which is dependent the accuracy of accumulated measurements. A computerized system is more likely to detect the point of maximum rate of gas production from which lag is estimated than a manual system with few measurements.

4.4.2 Rate of *In Vitro* Gas Production

Evaluation of the rates of gas production from the canola meal treatments established that the application of protease had a significant impact ($P < 0.05$). Protease applied at 0.01% significantly increased the rate of gas production ($P < 0.05$) from 0.077 mL/h^{-1} to 0.108 mL/h^{-1} (Table 4.2). However, the lowest and highest levels of protease produced rates of 0.075 mL/h^{-1} and 0.089 mL/h^{-1}, respectively which were not significantly different ($P < 0.05$) from the control. The increased rates of gas production observed with the addition of 0.01% protease may reflect the accessibility and the ease of fermentation of the carbohydrates in canola meal by rumen microbes. With forages, increased bacterial colonization density has been associated with increased rates of gas production (Hidayat et al., 1993). The improved digestibility of the fibrous carbohydrate fraction of canola meal is important in order to improve it's energy value and optimize the use of an expensive commodity.

The non linear effect of protease is illustrated in Figure 4.4. Investigation into the nature of this effect was inconclusive. Further research is warranted on the effects of canola meal treated with levels of protease between 0.01% and 0.05%. A non linear effect of enzyme treatment of a feedstuff has been previously reported. When steers were fed cubed alfalfa hay that had been sprayed with various levels of xylanase, the animals had improved DMI, ADG and feed efficiencies at 4 times the application dose than at 8 or 16 times the application dose (Beauchemin et al.,

1995). This non linear response to enzyme concentration may be attributed to the saturation of enzyme binding sites where an optimal rather than maximum dose response is found.

The lack of response of rate from xylanase treatment may be a consequence of it's low level of application. A lack of response was also observed to mild heat and moisture treatments which were applied in order to benefit enzyme activity as recommended by the enzyme supplier (FFI, personal communication). Current research of hydrothermal treatment of canola meal now focuses on moderate heat processes which employ temperatures in excess of 100°C in order to increase ruminal escape protein (Mir et al., 1984; Moshtaghi Nia and Ingalls, 1992). Therefore, the lack of response observed with the mild hydrothermal treatments was not unexpected. It may be concluded that hydrothermal treatment did not improve enzyme activity as there were no interactive effects of enzyme and hydrothermal treatments.

There are relatively few experiments which have used the gas production technique in order to evaluate the effect of enzymes on the fermentation kinetics of a feedstuff. One experiment investigated the use of cell wall degrading enzymes to improve the quality of grass silage using gas production parameters (Beuvink and Sploestra, 1994). Results obtained by Beuvink and Sploestra (1994) where rate of gas production was affected but total gas production remained the same, were attributed to a shift in the solubility of feed components as a result of enzyme action. These authors found that in grass silage that was enzyme treated, the enzymes

appeared to modify the cell wall material to more rapidly fermentable components leaving a more slowly degradable fraction as indicated by the shorter initial lag phase and lower maximum rate of gas production. In that instance, the authors speculated that changes in the chemical composition (NDF, lactic acid) of the feed were responsible for the observed changes in the rate of gas production. A similar explanation may be offered in this study where total gas production between treatments was determined to be similar however, the rates of fermentation and lag times were significantly affected by enzyme pretreatment (Appendix 6.8). Feed treatments had no effect on total gas production, this indicates the treatments did not affect the total amount of fermentable material but perhaps caused a shift towards more rapidly degradable components. Cumulative 18 h gas production for the control treatment was 201.12 mL/g DM and was not significantly different ($P < 0.05$) from any other of the 128 treatment means (Appendix 6.8). The solubility of feed components may have an effect on the rate of gas production however, a similar chemical composition would explain the lack of response from total gas production at 18 h. Cumulative gas production has been associated with the extent of digestion of a feedstuff (Beuvink and Sploestra, 1994). The extent of digestion of a forage has been shown to be determined by it's chemical composition (Mertens, 1977).

4.4.3 Lag Period

The length of the lag phase was significantly ($P < 0.05$) affected by the interactive effect of protease and xylanase which is illustrated in Figure 4.5. The length of the lag phase of the untreated sample in the present study was 0.449 ± 0.046 h. From Table 4.3 it may be determined that the control treatment had a significantly longer lag phase than any of the enzyme treatments ($P < 0.05$). In addition, any combination of enzyme treatment shortened the length of the lag phase of canola meal to less than 0.294 h.

The application of protease at 0.01% with or without 0.001% xylanase showed the greatest reduction in the length of the lag phase compared to the control. Protease applied at 0.01% alone shortened lag time from 0.449 h to 0.140 h; whereas protease applied at 0.01% in conjunction with 0.001% xylanase decreased the lag phase from 0.449 h to 0.136 h. The application of 0.001% xylanase was as effective in decreasing lag period as any treatment where an enzyme had been applied. Protease applied at 0.05% significantly increased ($P < 0.05$) the length of the lag phase to 0.273 h from a lag period of 0.140 h for the 0.01% protease treatment. This observation is however, difficult to explain since many factors have been associated with lag.

Lag time may be interpreted as the time required for: hydration of the feed particle, fermentation of soluble sugars, attachment and colonization of rumen microbes to the feed as well as the length of time required for the commencement of enzymatic degradation (Cheng et al., 1980). A shorter lag phase reflects the ease in

which these processes occur. In addition, the structure and composition of the feed, the rumen microbial population and the *in vitro* environment may also significantly impact the length of the lag phase.

Negative lag has been associated with a number of treatment means in this experiment (Appendix 6.8). Negative lag may be explained by the production of gas in the early stages of the fermentation process from rapidly fermentable solubles (Krishnamoorthy et al., 1991). Small feed particles and microbial debris present in the rumen fluid are fermented and contribute to the gas volume.

It has been shown that the rate and extent of digestion of cereal grains may be attributed to the presence of a protein matrix which renders starch and protein less readily digestible by rumen microorganisms (McAllister et al., 1990 a, b, c).The results of the present study show that rate of gas production may be increased from 0.077 mL/h^{-1} to a maximum of 0.108 mL/h^{-1} with the addition of protease. The length of the lag phase was shortened from 0.449 h to a minimum of 0.136 h with the addition of a combination of protease and xylanase. The combination of enzymes would be expected to elicit a synergistic effect by degrading the protein matrix and other resistant plant compounds.

In the present study a single enzyme was sufficient to elicit a positive effect on rate and lag values (Table 4.2 and 4.3, respectively). It appears from these results that the lag phase is more sensitive to the effects of enzyme supplementation than rates of gas production. In the case of lag, although treatment means were often lower with the application of the combination of protease and

xylanase, they were not significantly different from the means observed with either xylanase or protease applied at 0.005% or 0.01%.

Evaluation of the changes in gas production kinetics has been shown to be a useful and rapid method to determine the effects of enzyme supplementation on grass silage (Beuvink and Sploestra, 1994). These same principles may be applied to the current study where enzymes are expected to elicit their response in terms of increasing the fermentability of canola meal. Gas production parameters such as rate and lag provide information about the fermentation pattern of various feedstuffs which may be useful in formulating rations to maximize the utilization of nutrients in the rumen.

4.4.4 Volatile Fatty Acids

Information about the composition of VFAs and their amounts is especially important in the evaluation of gas production results from concentrates. The fermentation of concentrates produces a large proportion of propionic acid which does not contribute to the formation of CO_2 nor CH_4 and thus gas volume may be underestimated. Therefore, interpretation of results from gas production studies involving concentrates requires consideration of the production of total VFAs in addition to their proportions.

It has been reported that the application of exogenous enzymes to canola meal in order to degrade the cell wall results in the release of starch and protein that was previously unavailable (Slominski and Campbell, 1990). This may explain the

significant increase ($P < 0.05$) in the production of VFAs from 2.58 mmol/g DM^{-1} for the untreated sample to VFA production values ranging between 3.24 and 3.92 mmol/g DM^{-1} for samples treated with an effective combination of protease and xylanase (Table 4.4). Figure 4.6 illustrates the significant interaction between protease and xylanase ($P < 0.01$) which affected the production of VFAs. The increase in available substrates for rumen microbial fermentation may be the cause of the observed increase in VFA production. Similar to the effect of protease supplementation on the rate of gas production, the effect of enzyme addition is non linear with the combination of enzymes. A possible explanation for this was previously eluded to as enzyme binding sites on the substrate become saturated.

In the present study, treatment means for VFA production that were significantly higher than the control were, (in increasing order): 1) 0.001% xylanase, 2) 0.005% protease, 3) 0.001% xylanase and 0.01% protease and 4) 0.01% protease. Only the 0.001% xylanase treatment was significantly ($P < 0.05$) lower than the other treatment means listed.

The increase in VFA production observed with a number of the enzyme treatments may be attributed to an increase in the availability of substrates for microbial fermentation resulting from enzymatic hydrolysis of various components. Polysaccharides not included in the cellulosic fraction included a large amount of pectins, xylans and xyloglucans, which are largely insoluble in water (Slominski and Campbell, 1990). The solubility of feed components is known to be related to the digestibility of a feedstuff (Van Soest, 1994). Slominski et al., (1993) determined that

the supplementation of increasing levels of carbohydrase-like enzymes to canola meal resulted in minor increases in the soluble carbohydrate fraction. It has also been suggested that carbohydrases result in the release of short chain polysaccharides from the cell wall by systematically removing the surface components (Hotten, 1991). The increase in VFA production from 2.58 mmol / g DM^{-1} to 3.88 mmol / g DM^{-1} observed with the combination of 0.01% protease and 0.001% xylanase treatment may be attributed to increased microbial substrate availability. Proteolytic enzymes have been shown to increase the total water soluble carbohydrate content when applied to canola meal (Slominski et al., 1993). It should be noted however, that xylanase alone resulted in higher VFA than protease applied alone. The effectiveness of xylanase is evident from it's contribution to the interactions as well as it's main effects. As previously mentioned, many of the carbohydrates contained within canola meal such as the high amounts of xylans and xyloglucans (Slominski and Campbell, 1990) are susceptible to xylanolytic attack (Biely, 1985). Therefore, improvements in the fermentability of canola meal as reflected with increased VFA production would be expected with the addition of xylanase.

The heat and moisture treatments of canola meal did not significantly affect the production of VFAs nor did it improve enzyme activity. These results are comparable to those obtained with the estimated gas production parameters.

4.4.5 VFA Ratios: acetate to propionate; butyrate to propionate

Ratios were evaluated in this experiment as they were more likely to reflect differences in the proportions of VFAs being produced and therefore reflect the type of substrate being fermented. It was determined that protease elicited a highly significant linear effect (P = 0.0001) on the ratio of acetate relative to propionate production (Figure 4.7). The ratio of butyrate to propionate was also significantly affected by the protease treatment (P = 0.0001), although the nature of the effect could not be determined.

The highest level of protease significantly increased the acetate to propionate ratio from 2.79 to 3.74 whereas the lower levels of application did not significantly affect the ratio (Table 4.5). The application of protease at 0.01% did not affect the ratio of acetate to propionate nor the ratio of butyrate to propionate. In contrast, the ratio of butyrate to propionate showed the greatest increase from 0.59 to 0.68 with both the lowest and highest level of protease application (Table 4.5). The non linear effect of protease was previously observed with rate of gas production and total VFA production. Table 4.6 shows that xylanase applied at 0.001% increased the ratio of acetate to propionate production from 2.78 to 3.50, and the ratio of butyrate to propionate production from 0.57 to 0.69. Greater increases in the ratio of acetate to propionate production were obtained with the addition of 0.05% protease than with any other treatment. The greatest increase in the ratio of butyrate was obtained with the supplementation of 0.001% xylanase. The cellulosic fraction of a feedstuff is often associated with an increase in the production of acetate (Van Soest, 1994).

The increases in acetate production observed with the described enzyme treatments suggest that the enzymes elicited their actions on the structural carbohydrate components of canola meal. None of the treatments increased propionate relative to acetate nor to butyrate production. Increased propionate production would indicate better utilization of energy as acetic and butyric acids result in a higher production of gases (CO_2 and CH_4), which are a source of dietary energy lost to the animal (Van Soest, 1982). This explanation may be appropriate for describing the results of the present study where proteolytic and xylanolytic enzymes elicited their effects on the fibrolytic components of canola meal as acetate production in particular increased with specific treatments.

The mechanistic action and limitations of protease and xylanase help explain some of the observed results of the present study. The enzyme protease attacks the proteolytic bonds whereas xylanase attacks the xylan chain and both of which render more carbohydrates available for digestion by rumen microbes. Xylanase activity however, may be limited by the number of glucuronic acid residues and arabinose side chains (Biely, 1985). This may explain some of the lack of response from xylanase supplementation in the present study. The response obtained with the protease enzyme may be attributed to an increase in substrate availability for rumen microbial digestion which is reflected in a greater production of VFAs, higher rate of gas production and shorter lag phases relative to the untreated canola meal.

4.5 CONCLUSIONS

Evaluation of the fermentation kinetics of canola meal using gas production was successful in determining the effectiveness of enzyme and mild hydrothermal treatment of canola meal. The addition of 0.01% protease to canola meal increased rate of gas production, decreased lag time and increased VFA production but had no effect on the ratio of VFAs. Xylanase was only effective when used in combination with protease, however, it's effects were no greater than when protease was applied alone for both the length of the lag phase and the production of VFAs. The application of xylanase and of the highest level of protease increased the production of acetate relative to propionate. The lowest and highest level of protease application or the addition of xylanase, also increased the ratio of butyrate relative to propionate. The mild hydrothermal treatment of canola meal imposed in this experiment did not benefit enzyme activity as indicated by the lack of response to any of the measured variables. The results of the present study support further research into the effectiveness of the application of protease in order to improve the nutritional value of canola meal for dairy cattle.

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Table 4.1. Treatments investigated in order to determine the effects of exogenous enzyme application with and without added moisture and heat on *in vitro* gas production parameters and VFA analyses of canola meal.

Factor	Level of application
Heat[1]	control (0), 15, 30, 60 min
Moisture[2]	0%, 10%, 20%, 30%
Protease[2, 3, 4]	0%, 0.005%, 0.05%, 0.01%
Xylanase[2, 3, 5]	0%, 0.001%

[1] Applied at 55°C.
[2] Applied on a % vol / wt basis.
[3] Supplied by Finn Feeds International Ltd., Wiltshire, U.K.
[4] Heptex sb-protease.
[5] Avizyme 1310.

Table 4.2. The effect of the protease application on the *in vitro* rate of gas production of canola meal.

Protease[1] (%)	Rate ($mL*h^{-1}$)[2]
0	0.077^{a}
0.005	0.075^{a}
0.01	0.108^{b}
0.05	0.089^{ab}
S.E.M.[3]	0.004

[1] Applied on a % vol / wt basis.
[2] Rate of gas production (*b*) was calculated using the equation $Y = A (1 - e^{(-b(t-l))})$.
[3] S.E.M., pooled standard error of the mean.
[a-b] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 4.3. The effect of protease and xylanase application on the length of the lag phase *in vitro* of canola meal.

Enzyme treatment[1]		
Protease (%)	Xylanase (%)	Lag (h)[2]
0	0	0.449[c]
0.005	0	0.266[ab]
0.01	0	0.140[a]
0.05	0	0.273[b]
0	0.001	0.187[ab]
0.005	0.001	0.294[b]
0.01	0.001	0.136[a]
0.05	0.001	0.187[ab]
S.E.M.[3]		0.046

[1] Applied on a % vol / wt basis.
[2] Length of the lag phase (*l*) was calculated using the equation $Y = A (1 - e^{(-b(t-l))})$.
[3] S.E.M., pooled standard error of the mean.
[a-c] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 4.4. The effect of protease and xylanase application on the 6 h *in vitro* VFA production of canola meal.

Enzyme treatment[1]		
Protease (%)	Xylanase (%)	VFA (mmol / g)
0	0	2.58[a]
0.005	0	3.36[cd]
0.01	0	3.92[d]
0.05	0	3.26[ab]
0	0.001	3.24[bc]
0.005	0.001	2.34[a]
0.01	0.001	3.88[d]
0.05	0.001	2.66[ab]
S.E.M.[2]		0.208

[1] Applied on a % vol / wt basis.
[2] S.E.M., pooled standard error of the mean.
[a-d] Means in a column with different superscripts are significantly different *($P < 0.05$)*.

Table 4.5. The effect of protease application on the 6 h *in vitro* ratio of acetate to propionate and butyrate to propionate of canola meal.

Protease[1] (%)	A:P[2]	B:P[2]
0	2.789[a]	0.59[a]
0.005	3.128[a]	0.68[b]
0.01	2.890[a]	0.57[a]
0.05	3.736[b]	0.68[b]
S.E.M.[3]	0.154	0.253

[1] Applied on a % vol / wt basis.
[2] Molar ratios
[3] S.E.M., pooled standard error of the mean.
[a-b] Means in a column with different superscripts are significantly different *($P < 0.05$).*

Table 4.6. The effect of xylanase application on the 6 h *in vitro* ratio of acetate to propionate (A:P) and butyrate to propionate (B:P) of canola meal.

Xylanase[1] (%)	A:P[2]	B:P[2]
0	2.779	0.57
0.001	3.493	0.69
S.E.M.[3]	0.108	0.018

[1] Applied on a % vol / wt basis.
[2] Molar ratios
[3] S.E.M., pooled standard error of the mean.

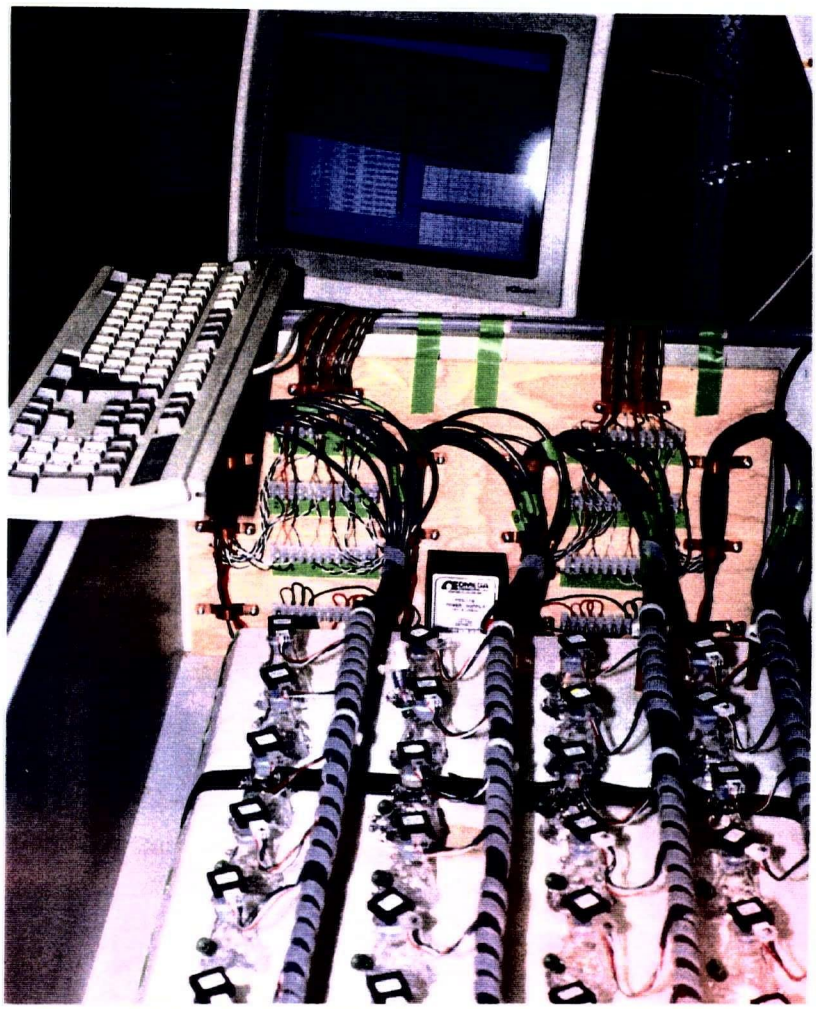


Figure 4.1. The computerized gas production system (adapted from Pell and Schofield, 1993).

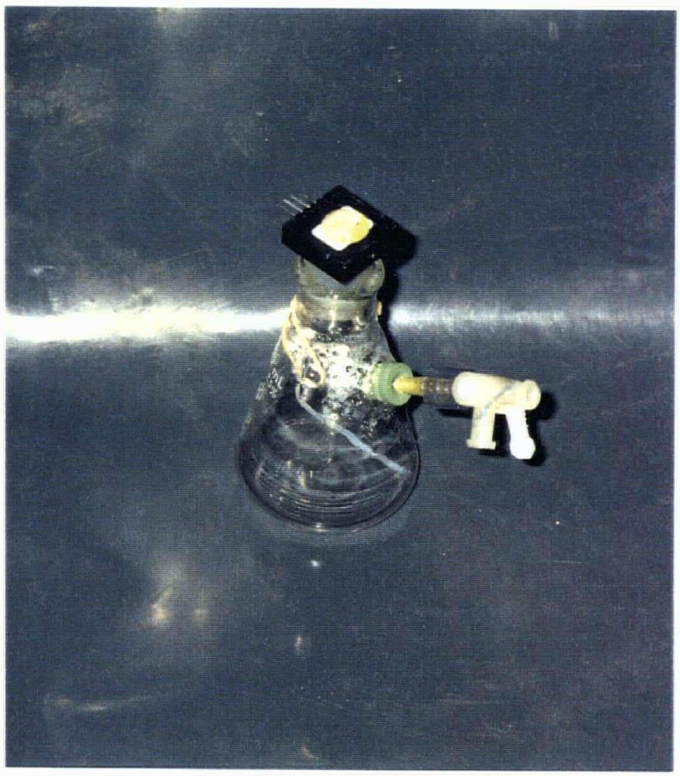


Figure 4.2. Customized Erlenmeyer flasks with sampling port containing luer lock and tubing apparatus for VFA sampling.

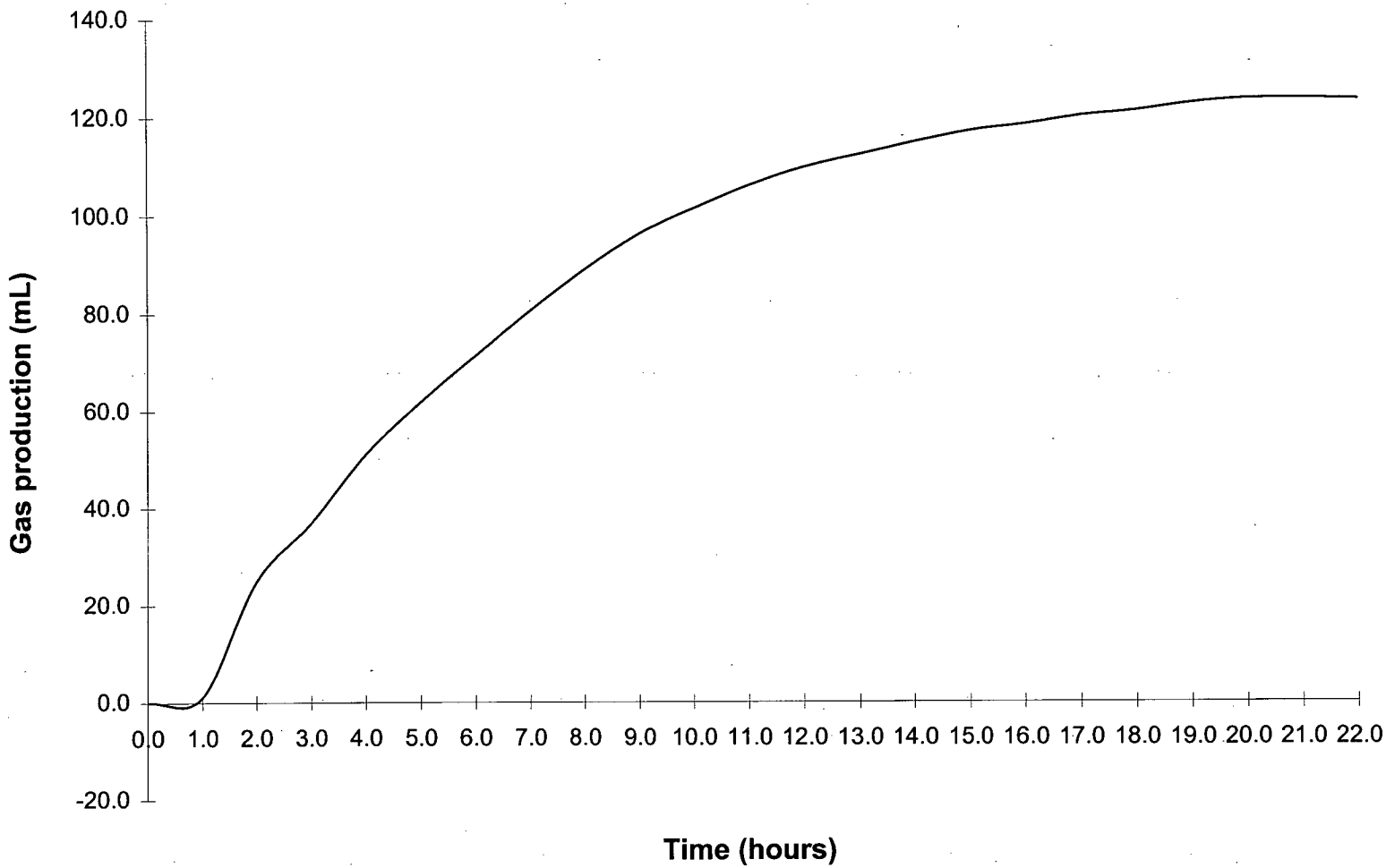


Figure 4.3. Gas production profile for a sample of canola meal over a 22 h incubation period with rumen fluid.

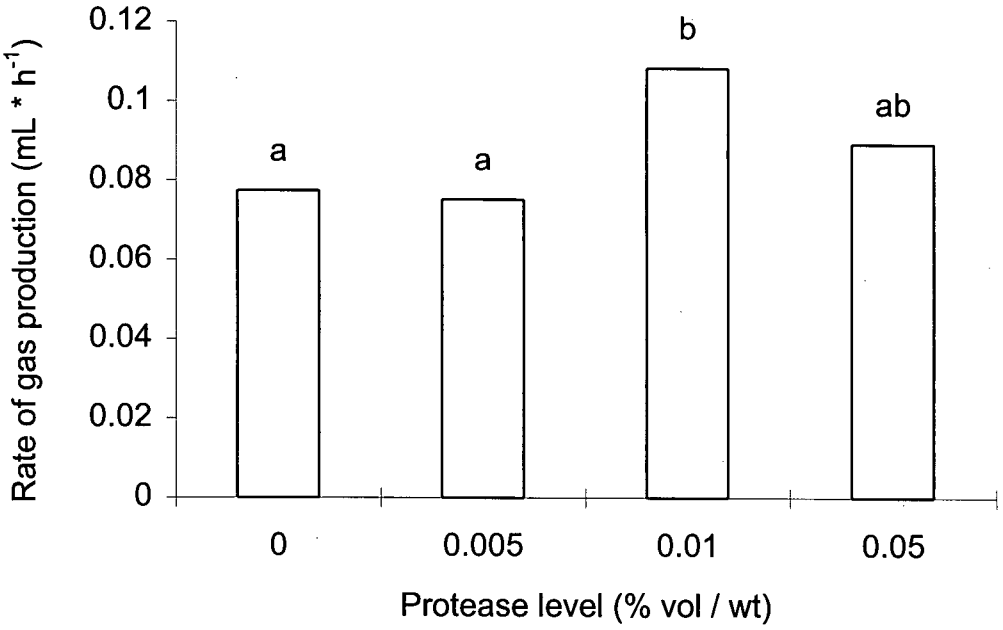


Figure 4.4. The effect of the addition of protease on the rate of rumen *in vitro* gas production of canola meal incubated with rumen fluid.

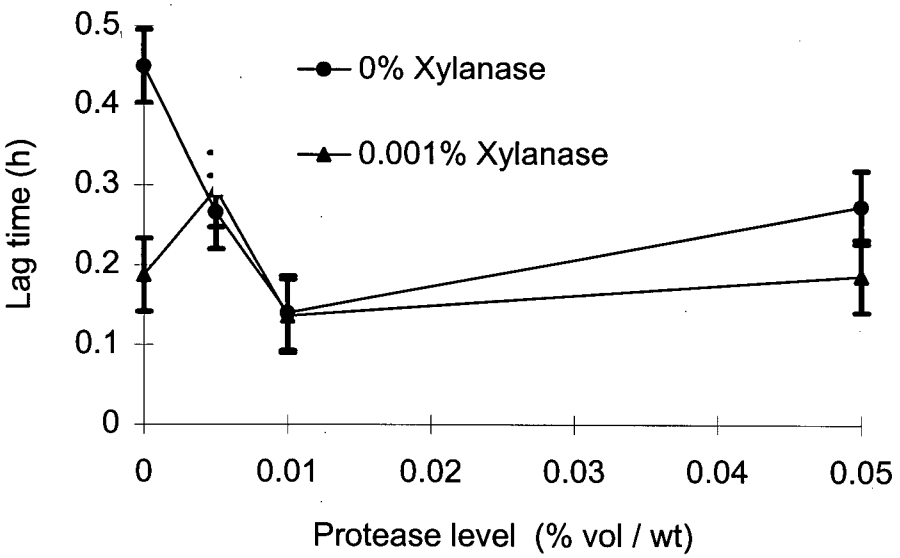


Figure 4.5. The effect of the addition of protease and xylanase (% vol / wt) on the length of the lag phase of canola meal incubated *in vitro* with rumen fluid.

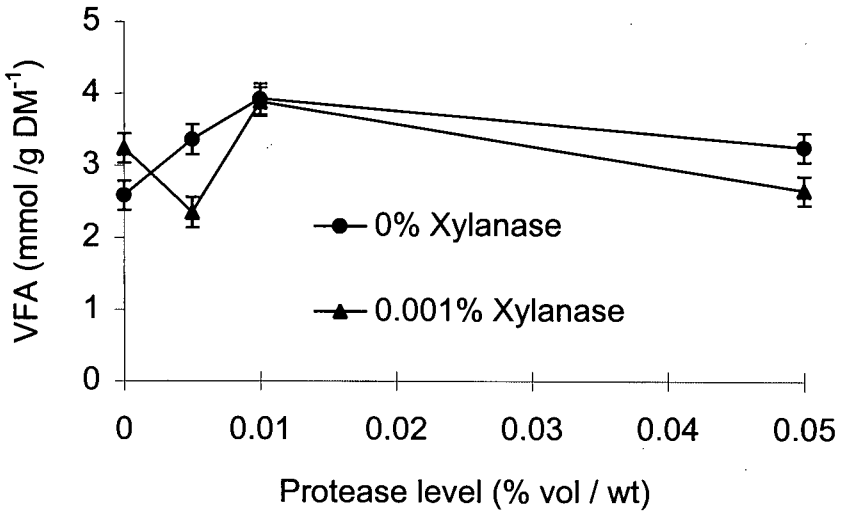


Figure 4.6. The effect of the addition of xylanase and protease (% vol / wt) on the production of VFAs in canola meal following a 6 h *in vitro* incubation with rumen fluid.

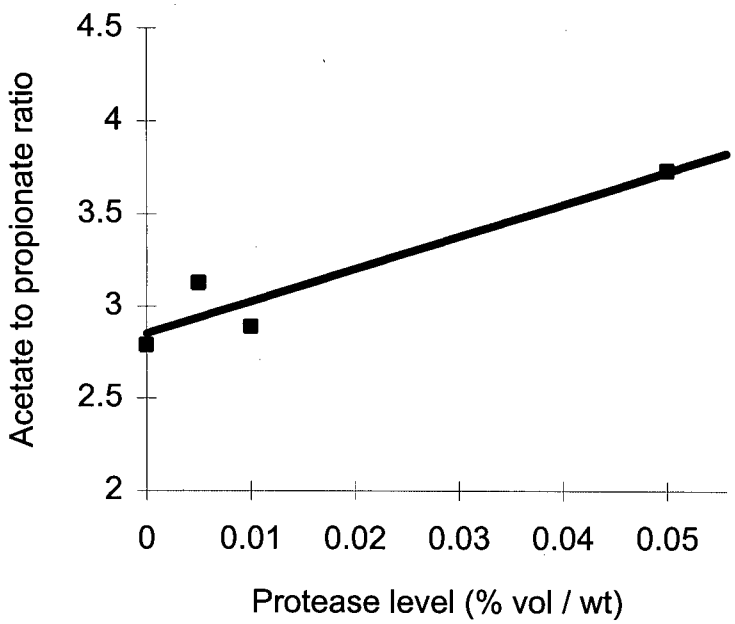


Figure 4.7. Linear relationship between level of protease application and the ratio of acetate to propionate in canola meal following a 6 h *in vitro* incubation with rumen fluid (Y = 17.546 * protease level + 2.851 : n = 4, R^2 = 0.839, S.E. = 0.17).

CHAPTER 5

5.0 GENERAL CONCLUSIONS

In the Lower Fraser Valley region of British Columbia (B.C.), maximizing feed utilization is a primary goal as feed costs increase. Greater awareness of the environmental impact of the intensive dairy and animal production in this area also promotes research into minimizing nutrient wastes. Barley grain is the major energy source and canola meal is the major alternative protein source to soybean meal used in rations for dairy cattle. Canola meal is restricted in dairy cattle diets because of it's high fiber content which lowers it's energy value. Barley grain is relatively high in starch, however research for improving it's nutritional value is also as a consequence of it's relatively high fiber content.

Current processing technology for ruminant feedstuffs entails conventional methods. The possibility of enzymes used in conjunction with processing technology has yet to be realized. The objective of the current study was to investigate the feasibility of applying enzymes and mild hydrothermal treatment to barley and canola meal in order to improve their nutritional value for dairy cattle.

5.1 BARLEY

In vitro analyses were used to evaluate the effects of barley treated with β-glucanase, protease, heat and moisture. Results of *in vitro* digestibilities of barley are presented in Chapter 2, section 2.3. As mentioned in Chapter 2, dairy producers

in the Lower Fraser Valley region of B.C. face a unique challenge to match the high soluble nitrogen content present in grass silages (von Keyserlingk et al., 1996) with a readily available carbohydrate source in order to maximize rumen microbial protein production and optimize performance (Krishnamoorthy et al., 1991; Stokes et al., 1991). The intensive agricultural practices in this environmentally fragile region also require an active role by producers to reduce nutrient wastes by balancing the supply of nitrogen and carbohydrates to the rumen (Huntington, 1995) and the maximizing of feed utilization. Mild heat appeared not to have an effect, whereas the addition of moisture had a quadratic relationship with digestibility. From this study, it appears xylanase alone is detrimental towards the digestibility of barley. However, potential applications exist for protease applied alone at a moderate level (0.01%) or a combination of protease and β-glucanase to improve the feeding quality of barley.

5.2 CANOLA MEAL

Results of the *in vitro* digestibilities of the canola meal treatments may be found in Chapter 3, section 3.3. A second experiment utilized the gas production technique to obtain information of the effects of the enzyme and mild hydrothermal treatments on the fermentation characteristics of canola meal. The results of the gas production experiment may be found in Chapter 4, section 4.3. Results from these two experiments suggested that some treatments may elicit their effects on specific feed components. The gas production study appeared to be more sensitive to the

differences between treatments than the digestibility experiment, although similar conclusions were reached in selecting beneficial treatments.

The principle behind applying heat and moisture treatments was to optimize enzyme activity. The results of the gas production experiment indicated these conditions were not conducive to optimizing enzyme action on the feed substrate. Moisture elicited a quadratic effect on the digestibility of canola meal that was not detected in the evaluation of gas production. Treatment of canola with xylanase was detrimental to digestibility, similar to the results previously observed with barley, whereas, the addition of protease at a moderate level appeared to be successful in increasing the fermentability of canola meal.

5.3 SUMMARY

In conclusion, it appears that treatments which improved the nutritive value of barley and canola meal as determined using *in vitro* analyses, may be primarily explained by the addition of protease. However, it is important that animal performance trials be conducted on selected treatments to clearly define the potential applications of protease and other enzymes in ruminant feeds. Increasing feed costs and a competitive market for both dairy producers and the feed industry alike, will promote research into innovative processing technology such as that investigated in the experiments discussed in this thesis. Although the "economics" of implementing enzymes into ruminant feeds have not been discussed in this thesis, enzymes are an expensive commodity and their practical applications remain

dependent upon the return on investment into most likely, more expensive, "value added" product.

5.4 REFERENCES

Huntington, J. A. 1995. Rumen fermentation dynamics. Pages 17-19 in Feed Compounder, May issue.

Krishnamoorthy, U., Soller, H., Steingass, H. and K. H. Menke. 1991. A comparative study on rumen fermentation of energy supplements *in vitro*. J. Anim. Physiol. Anim. Nutr., 65:28-35.

Stokes, S. R., Hoover, W. H., Miller, T. K. and R. Blauweikel. 1991. Ruminal digestion and microbial utilization of diets varying in type of carbohydrate and protein. J. Dairy Sci., 75:345-357.

von Keyserlingk, M. A. G., Swift, M. L., Puchala, R. and J. A. Shelford. 1996. Degradability characteristics of dry matter and crude protein of forages in ruminants. Anim. Feed Sci. Technol., 57:291-311.

6.0 APPENDICES

APPENDIX 6.1 TEST OF SUBSTRATE ACTIVITY[1] OF THE ENZYMES: β-GLUCANASE, PROTEASE AND XYLANASE SUPPLIED BY FFI (Wiltshire, U. K.).

Table 6.1. Enzyme activity tested on various substrates.

Enzyme[2]	Protein content[3] (%)	Cellulose[4]	CMC	Xylan	Starch	Pectin	β-glucan	Alfalfa hay extracted	Hulless barley grain	Casein[5]
Xylanase	3.0	7.8	26.9	1478.5	nil	13.3	61.0	30.2	387.0	4.0
β-glucanase	21.3	126.1	727.1	210.2	nil	6.2	1042.1	134.7	1808.7	nil
Protease	6.7	nil	nil	6.9	10.1	10.1	26.6	16.4	432.9	34175.0

[1] Analyses conducted by Lethbridge Agricultural Research Station, Lethbridge, Alberta; May 1994.
[2] Enzymes supplied by Finn Feeds International Ltd., Wiltshire, U. K.
[3] N x 6.25
[4] Microcrystalline. For all substrates, activity is expressed as ppm of glucose released by the enzyme (at 0.250 mg / ml) in the conditions of the test.
[5] Protease activity expressed as relative units (RU) per 0.250 mg / mL of enzyme

APPENDIX 6.2 COMPOSITION OF THE CONCENTRATE RATION FED TO DONOR COWS.

Table 6.2. Nutrient composition of the dairy concentrate ration.

Ingredient	Content (% as fed basis)
Barley (11.5% CP)	58.6
Wheat millrun	14.7
Canola meal	11.2
Dairy fines	3.5
Beet pulp	3.0
Molasses/cane/LI	2.7
Limestone	1.75
Corn (8.5%)	1.0
Salt and trace mineral mix	0.88
Distillers grain	0.87
Soya oil	0.75
Raypell binder	0.7
Meat and bone meal	0.35

APPENDIX 6.3. SAS PROGRAM USED TO EVALUATE BARLEY DATA.

```
options nocentre linesize=75;
filename invitro 'filename';
data;
infile invitro;
input feed rep ivdmd ivtd adf ndf heat moist beta prot xyl;
title 'in vitro results and fiber analyses of barley';
proc glm;
class heat moist beta prot xyl;
model ivdmd ivtd adf ndf = heat | moist | beta | prot | xyl / nouni ss3;
random heat*moist*beta*prot*xyl / test;
lsmeans heat moist beta prot xyl heat*moist heat*beta heat*prot heat*xyl
moist*beta moist*prot moist*xyl beta*prot beta*xyl prot*xyl heat*moist*beta
heat*moist*prot heat*moist* xyl heat*prot*xyl heat*beta*xyl heat*beta*prot
moist*beta*prot moist*beta*xyl moist*prot*xyl beta*prot*xyl
heat*moist*beta*prot heat*moist*prot*xyl heat*beta*prot*xyl heat*moist*beta*xyl
moist*beta*prot*xyl / stderr e = heat*moist*beta*prot*xyl;
run;
```

Normally, only relevant interactions are included as terms in the LSMEANS statement. Due to the large number of factors in this experiment, the error term includes the highest order interaction as an indication of experimental error. The

model statement includes the sum of all of terms included in the LSMEANS statement.

APPENDIX 6.4. SAS PROGRAM USED TO EVALUATE CANOLA MEAL DATA.

```
options nocentre linesize=75;
filename invitro 'filename';
data;
infile invitro;
input feed rep ivdmd ivtd adf ndf heat moist prot xyl;
title 'in vitro results and fiber analyses of canola meal;
proc glm;
class heat moist prot xyl;
model ivdmd ivtd adf ndf = heat | moist | prot | xyl / nouni ss3;
random heat*moist *prot*xyl / test;
lsmeans heat  moist  prot  xyl  heat*moist heat*prot  heat*xyl  moist*prot
moist*xyl  prot*xyl  heat*moist*prot  heat*moist* xyl  heat*prot*xyl
moist*prot*xyl  heat*moist*prot*xyl  / stderr e = heat*moist*prot*xyl;
run;
```

Normally, only relevant interactions are included as terms in the LSMEANS statement. Due to the large number of factors in this experiment, the error term includes the highest order interaction as an indication of experimental error. The model statement includes the sum of all of terms included in the LSMEANS statement.

APPENDIX 6.5 CALIBRATION OF THE GAS MEASUREMENT SYSTEM (Schofield and Pell, 1995).

Calibration of the gas production system was required in order to establish the linear relationship between the change in pressure arising from gas production and the subsequent voltage response. A regression equation was developed to describe this relationship and to convert voltage response to the resulting gas volume (Pell and Schofield, 1993). Interpretation of gas production results requires accommodation for highly soluble gases such as CO_2 (Pell and Schofield, 1995). Gas composition is also important because of differing solubilities. Volume changes related to calibration corrections involving methane production were assumed to be insignificant in this experiment (Schofield and Pell, 1995). As fermentation proceeds, an increase in pressure within the flask results in some of the gas to dissolve and remain in solution. Only insoluble gases are available to induce a pressure change. The following equation is Henry's law used to describe the relationship between the molar volume of gas dissolved into a medium in response to it's partial pressure within a fixed volume container (Schofield and Pell, 1995).

$$vol.\ CO_2\ (aqueous) / vol.\ CO_2\ (gas) = 0.0246\ R\ T\ (Va/Vg), \quad \text{(Equation 1)}$$

where R is the universal gas constant (0.082 L*atm/mol*°K), T is the temperature in degrees Kelvin, Va is the liquid volume in the flask and Vg is the available gas volume within the flask.

Calibration of the gas production system was conducted under the same conditions as described in Chapter 4, section 4.3.6. The system, solutions and

equipment were prewarmed to 39°C ± 1 °C and 15 mL of buffer was added to 12 Erlenmeyer flasks (50 mL). The flasks were repeatedly injected with either 5, 10, 15 or 20 mL of CO_2 through the port and allowed to equilibrate for 10 min. The data in Table 6.3 illustrates the effect of pressure on the solubility of CO_2. Sensor response was obtained by subtracting the difference between the voltage recorded at baseline from the voltage recorded at 10 min. A calibration correction factor was obtained for CO_2 solubility in this system using the above equation (Equation 1) for CO_2 at 39°C:

$$[CO_2]\ aq\ /\ [CO_2]\ gas = 0.0246\ R\ T\ *15\ mL\ /\ 41.5\ mL = 0.227$$

The gas production data collected for the present study was calculated using the regression below (Equation 2) ($r^2 = 0.994$) which describes the relationship between gas production and subsequent voltage response:

$$Y = 64.60 \pm 3.58\ (mL\ /\ V)\ X\ -1.27 \pm 0.47\ mL, \quad \text{(Equation 2)}$$

where *Y* represents the gas volume and *X* is the voltage response produced by the sensors.

Table 6.3. The effect of CO_2 solubility of gas on it's ability to induce a pressure change and elucidate a voltage response in the gas production system.

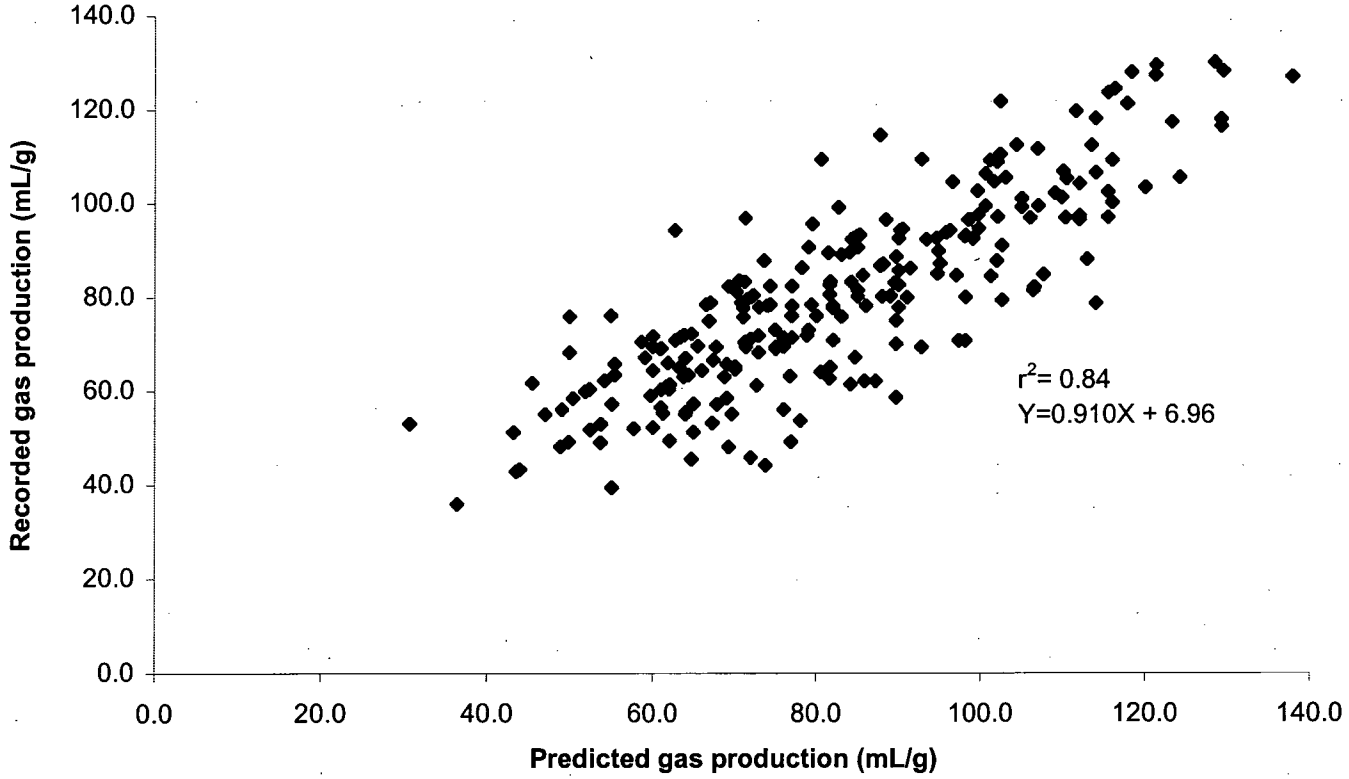
CO_2 Injected	Amount of CO_2 dissolved in buffer	CO_2 available to induce pressure change	Voltage Response (V)
5 mL	1.14 mL	3.86 mL	0.0747
10 mL	2.27 mL	7.73 mL	0.1450
15 mL	3.40 mL	11.60 mL	0.2039
20 mL	4.54 mL	15. 46 mL	0.2534

6.5.1 REFERENCES

Pell, A. N. and P. Schofield. 1993. Computerized monitoring of gas production to measure forage digestion *in vitro*. J. Dairy Sci., 76:1063-1073.

Schofield, P. and A. N. Pell. 1995. Validity of using accumulated gas pressure readings to measure forage digestion *in vitro*: a comparison involving three forages. J. Dairy Sci., 78:2230-2238.

APPENDIX 6.6. RECORDED GAS PRODUCTION VS. STOICHIOMETRICALLY CALCULATED GAS PRODUCTION FROM VFA COMPOSITION AT 6 HOURS OF A RANDOM SELECTION OF CANOLA MEAL SAMPLES.



APPENDIX 6.7. SAS PROGRAM USED TO DETERMINE GAS PRODUCTION PARAMETERS FOR CANOLA MEAL.

```
options nocentre linesize=75;

filename gas production 'filename';

data;

infile gas production;

input time feedrep1 feedrep2 feedrep3;

title ' test of fitting simple exponential with lag' ;

proc nlin method=dud ;

parms a=1 b=0.1 lag=0 ;

model y = a*(1 - exp( -b*(t-lag) ) );

output out=b predicted=yhat residual=yres ;

proc print ;

proc plot ;

plot y*t='o' yhat*t="p" / overlay ;

run ;
```

APPENDIX 6.8. RAW DATA FOR THE ESTIMATED GAS PRODUCTION PARAMETERS FROM CANOLA MEAL: RATE OF GAS PRODUCTION, LAG PERIOD AND CUMULATIVE 18 h GAS PRODUCTION.

Table 6.4. The effect of canola meal treated with enzymes and hydrothermal conditions on rate of gas production, length of the lag phase and cumulative 18h gas production.

Feed ID	Heating time (min)	Moisture[1] (%)	Protease[1] (%)	Xylanase[1] (%)	Mean Rate[2]	S.D.[3]	Mean Lag[2]	S.D.[3]	Cumulative 18 h gas production (mL / g DM)	S.D.[3]
1	0	0	0	0	0.083	0.007	0.255	0.024	201.125	6.9
2	0	0	0.005	0.001	0.074	0.005	0.270	0.029	187.840	7.3
3	0	0	0.01	0	0.117	0.004	0.190	0.024	240.580	5.2
4	0	0	0.05	0.001	0.095	0.001	0.160	0.023	253.480	9.2
5	0	0	0	0.001	0.079	0.002	0.259	0.023	190.574	5.8
6	0	0	0.005	0	0.078	0.002	0.287	0.021	214.456	5.9
7	0	0	0.01	0.001	0.164	0.004	0.120	0.024	221.860	6.4
8	0	0	0.05	0	0.080	0.004	0.261	0.029	166.250	8.5
9	0	10	0	0	0.047	0.004	0.416	0.023	184.000	9.5
10	0	10	0.005	0.001	0.061	0.003	0.275	0.025	180.450	7.2
11	0	10	0.01	0	0.130	0.004	0.180	0.024	235.400	9.1
12	0	10	0.05	0.001	0.073	0.004	0.283	0.017	180.500	8.3

Feed ID	Heating time (min)	Moisture[1] (%)	Protease[1] (%)	Xylanase[1] (%)	Mean Rate[2]	S.D.[3]	Mean Lag[2]	S.D.[3]	Cumulative 18 h gas production (mL / g DM)	S.D.[3]
13	0	10	0	0.001	0.076	0.003	0.278	0.027	195.450	8.6
14	0	10	0.005	0	0.064	0.003	0.299	0.017	170.000	8.1
15	0	10	0.01	0.001	0.069	0.003	0.289	0.019	165.460	5.6
16	0	10	0.05	0	0.102	0.007	0.222	0.024	200.456	7.6
17	0	20	0	0	0.115	0.004	0.456	0.046	180.560	9.4
18	0	20	0.005	0.001	0.102	0.001	0.144	0.016	171.450	9.1
19	0	20	0.01	0	0.091	0.004	0.261	0.022	220.416	8.2
20	0	20	0.05	0.001	0.082	0.002	0.267	0.027	185.456	7.8
21	0	20	0	0.001	0.073	0.002	0.264	0.022	210.486	8.7
22	0	20	0.005	0	0.052	0.002	0.356	0.023	180.546	9.9
23	0	20	0.01	0.001	0.093	0.003	0.008	0.035	230.456	9.5
24	0	20	0.05	0	0.135	0.005	-0.300	0.024	250.456	9.2
25	0	30	0	0	0.032	0.004	0.954	0.147	184.456	8.6
26	0	30	0.005	0.001	0.072	0.003	0.263	0.024	120.456	7.6
27	0	30	0.01	0	0.162	0.005	-0.100	0.024	280.546	7.7
28	0	30	0.05	0.001	0.082	0.006	0.212	0.024	160.854	7.1
29	0	30	0	0.001	0.107	0.006	0.013	0.024	225.560	9.8
30	0	30	0.005	0	0.060	0.006	-0.007	0.024	231.540	8.2

Feed ID	Heating time (min)	Moisture[1] (%)	Protease[1] (%)	Xylanase[1] (%)	Mean Rate[2]	S.D.[3]	Mean Lag[2]	S.D.[3]	Cumulative 18 h gas production (mL / g DM)	S.D.[3]
31	0	30	0.01	0.001	0.050	0.006	-0.123	0.024	251.144	8.4
32	0	30	0.05	0	0.107	0.006	0.200	0.024	200.287	9.1
41	15	0	0	0	0.061	0.006	0.247	0.024	162.415	8.8
42	15	0	0.005	0.001	0.043	0.005	0.584	0.033	160.456	7.1
43	15	0	0.01	0	0.094	0.003	0.321	0.024	273.456	6.3
44	15	0	0.05	0.001	0.054	0.006	0.572	0.024	254.450	9.2
45	15	0	0	0.001	0.048	0.006	0.348	0.024	176.450	5.1
46	15	0	0.005	0	0.094	0.002	0.227	0.024	225.150	6.8
47	15	0	0.01	0.001	0.115	0.010	0.186	0.024	220.500	9.6
48	15	0	0.05	0	0.073	0.005	0.222	0.007	193.553	8.4
49	15	10	0	0	0.070	0.005	0.471	0.091	191.256	4.1
50	15	10	0.005	0.001	0.086	0.003	0.248	0.004	202.560	8.0
51	15	10	0.01	0	0.090	0.005	0.246	0.020	198.540	9.3
52	15	10	0.05	0.001	0.106	0.011	0.263	0.033	192.487	8.7
53	15	10	0	0.001	0.103	0.004	0.061	0.055	225.640	6.4
54	15	10	0.005	0	0.087	0.006	0.269	0.014	214.692	5.5
55	15	10	0.01	0.001	0.093	0.004	0.201	0.011	222.456	9.5
56	15	10	0.05	0	0.100	0.003	0.272	0.009	173.354	8.3

Feed ID	Heating time (min)	Moisture[1] (%)	Protease[1] (%)	Xylanase[1] (%)	Mean Rate[2]	S.D.[3]	Mean Lag[2]	S.D.[3]	Cumulative 18 h gas production (mL / g DM)	S.D.[3]
57	15	20	0	0	0.095	0.003	0.614	0.078	191.450	4.8
58	15	20	0.005	0.001	0.087	0.008	0.281	0.027	202.232	9.8
59	15	20	0.01	0	0.084	0.008	0.398	0.022	195.366	7.2
60	15	20	0.05	0.001	0.102	0.006	-0.164	0.014	200.000	8.7
61	15	20	0	0.001	0.081	0.008	-0.013	0.022	199.378	7.7
62	15	20	0.005	0	0.093	0.009	0.173	0.026	233.490	8.3
63	15	20	0.01	0.001	0.127	0.004	-0.009	0.041	255.399	7.5
64	15	20	0.05	0	0.097	0.009	0.316	0.036	184.697	5.1
65	15	30	0	0	0.076	0.006	0.902	0.794	201.590	8.3
66	15	30	0.005	0.001	0.058	0.009	0.296	0.020	165.687	9.4
67	15	30	0.01	0	0.128	0.011	-0.012	0.043	241.632	8.2
68	15	30	0.05	0.001	0.066	0.004	-0.018	0.145	177.894	9.4
69	15	30	0	0.001	0.090	0.005	0.325	0.011	196.548	5.6
70	15	30	0.005	0	0.063	0.008	0.245	0.011	171.465	4.9
71	15	30	0.01	0.001	0.099	0.007	0.194	0.006	210.400	6.3
72	15	30	0.05	0	0.074	0.007	0.289	0.033	195.778	4.8
73	15	0	0	0	0.078	0.007	0.528	0.026	201.780	11.1
74	30	0	0.005	0.001	0.083	0.009	0.680	0.034	220.485	8.1

Feed ID	Heating time (min)	Moisture[1] (%)	Protease[1] (%)	Xylanase[1] (%)	Mean Rate[2]	S.D.[3]	Mean Lag[2]	S.D.[3]	Cumulative 18 h gas production (mL / g DM)	S.D.[3]
75	30	0	0.01	0	0.071	0.009	0.265	0.030	227.841	8.4
76	30	0	0.05	0.001	0.100	0.006	-0.010	0.060	194.678	6.0
77	30	0	0	0.001	0.099	0.010	0.237	0.024	224.529	9.5
78	30	0	0.005	0	0.097	0.008	0.265	0.025	231.569	8.3
79	30	0	0.01	0.001	0.135	0.006	0.197	0.022	240.450	9.9
80	30	0	0.05	0	0.073	0.004	0.255	0.022	197.840	5.9
81	30	10	0	0	0.057	0.009	0.275	0.021	205.546	8.7
82	30	10	0.005	0.001	0.081	0.011	0.293	0.026	237.870	10.2
83	30	10	0.01	0	0.152	0.004	-0.209	0.151	238.846	8.9
84	30	10	0.05	0.001	0.058	0.009	0.249	0.017	190.458	7.5
85	30	10	0	0.001	0.105	0.011	0.087	0.042	220.301	9.3
86	30	10	0.005	0	0.074	0.013	0.115	0.013	198.799	8.1
87	30	10	0.01	0.001	0.101	0.009	0.280	0.015	240.112	7.4
88	30	10	0.05	0	0.064	0.010	0.385	0.017	200.566	8.2
89	30	20	0	0	0.084	0.012	0.283	0.035	165.650	9.8
90	30	20	0.005	0.001	0.072	0.011	0.040	0.106	174.540	7.8
91	30	20	0.01	0	0.123	0.005	-0.160	0.035	243.540	9.3
92	30	20	0.05	0.001	0.118	0.006	0.274	0.021	249.450	8.2

Feed ID	Heating time (min)	Moisture[1] (%)	Protease[1] (%)	Xylanase[1] (%)	Mean Rate[2]	S.D.[3]	Mean Lag[2]	S.D.[3]	Cumulative 18 h gas production (mL / g DM)	S.D.[3]
93	30	20	0	0.001	0.081	0.009	-0.016	0.012	187.456	8.4
94	30	20	0.005	0	0.067	0.006	0.301	0.004	178.450	7.5
95	30	20	0.01	0.001	0.153	0.017	-0.080	0.076	252.456	8.1
96	30	20	0.05	0	0.122	0.011	0.507	0.065	266.456	7.3
97	30	30	0	0	0.045	0.007	0.220	0.009	122.555	8.2
98	30	30	0.005	0.001	0.035	0.006	0.308	0.008	190.564	8.1
99	30	30	0.01	0	0.092	0.006	0.280	0.018	211.546	9.5
100	30	30	0.05	0.001	0.058	0.004	0.251	0.027	145.550	7.4
101	30	30	0	0.001	0.100	0.004	0.439	0.002	233.000	8.3
102	30	30	0.005	0	0.106	0.010	0.301	0.010	235.455	8.8
103	30	30	0.01	0.001	0.140	0.003	-0.082	0.073	288.448	6.6
104	30	30	0.05	0	0.143	0.003	0.150	0.022	255.444	7.2
105	60	0	0	0	0.075	0.003	0.259	0.017	203.997	8.4
106	60	0	0.005	0.001	0.066	0.006	0.261	0.021	168.942	9.5
107	60	0	0.01	0	0.099	0.003	0.251	0.023	199.455	6.8
108	60	0	0.05	0.001	0.080	0.003	0.224	0.021	175.456	6.5
109	60	0	0	0.001	0.083	0.005	-0.005	0.041	205.459	7.9
110	60	0	0.005	0	0.064	0.006	0.268	0.012	171.440	9.5

Feed ID	Heating time (min)	Moisture[1] (%)	Protease[1] (%)	Xylanase[1] (%)	Mean Rate[2]	S.D.[3]	Mean Lag[2]	S.D.[3]	Cumulative 18 h gas production (mL / g DM)	S.D.[3]
111	60	0	0.01	0.001	0.056	0.004	0.340	0.013	162.000	9.4
112	60	0	0.05	0	0.081	0.004	0.289	0.026	171.850	5.1
113	60	10	0	0	0.047	0.005	0.421	0.004	173.450	7.8
114	60	10	0.005	0.001	0.059	0.004	0.252	0.032	164.460	5.9
115	60	10	0.01	0	0.107	0.002	0.227	0.045	223.568	8.3
116	60	10	0.05	0.001	0.123	0.007	-0.036	0.233	262.440	11.4
117	60	10	0	0.001	0.079	0.010	0.151	0.033	205.456	7.9
118	60	10	0.005	0	0.054	0.004	0.511	0.399	246.550	10.5
119	60	10	0.01	0.001	0.095	0.003	0.301	0.018	265.781	8.2
120	60	10	0.05	0	0.092	0.007	0.541	0.026	231.556	9.4
121	60	20	0	0	0.086	0.002	0.291	0.027	167.415	6.7
122	60	20	0.005	0.001	0.100	0.010	0.301	0.009	189.569	9.7
123	60	20	0.01	0	0.121	0.006	-0.011	0.070	276.357	8.3
124	60	20	0.05	0.001	0.091	0.005	0.259	0.037	168.335	6.8
125	60	20	0	0.001	0.061	0.003	0.246	0.002	194.540	7.1
126	60	20	0.005	0	0.090	0.002	0.354	0.034	187.623	8.4
127	60	20	0.01	0.001	0.154	0.017	0.056	0.007	300.450	9.2
128	60	20	0.05	0	0.089	0.003	0.443	0.032	189.640	8.2

Feed ID	Heating time (min)	Moisture[1] (%)	Protease[1] (%)	Xylanase[1] (%)	Mean Rate[2]	S.D.[3]	Mean Lag[2]	S.D.[3]	Cumulative 18 h gas production (mL / g DM)	S.D.[3]
129	60	30	0	0	0.087	0.003	0.593	0.041	214.336	8.8
130	60	30	0.005	0.001	0.095	0.003	0.301	0.008	218.779	6.4
131	60	30	0.01	0	0.083	0.003	-0.010	0.043	226.450	7.6
132	60	30	0.05	0.001	0.060	0.004	0.205	0.006	163.456	4.8
133	60	30	0	0.001	0.071	0.005	0.324	0.012	186.976	7.2
134	60	30	0.005	0	0.077	0.003	0.289	0.020	225.458	10.1
135	60	30	0.01	0.001	0.098	0.004	0.295	0.049	210.456	5.9
136	60	30	0.05	0	0.077	0.003	0.320	0.025	196.456	6.7

[1] % added.
[2] Gas parameter estimates rate and lag, were calculated using the equation $Y = A\ (\ 1 - e^{(-b(t-l))}\ )$.
[3] S.D., standard deviation, N = 384 (3 replicates).